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Protocols

Development of a dot immunoblot method for differentiation of animals infected with foot-and-mouth disease virus from vaccinated animals using non-structural proteins expressed prokaryotically

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A B S T R A C T

Five non-structural proteins (NSPs) of foot-and-mouth disease virus (FMDV) were expressed in *E. coli* to develop a dot immunoblot (dot blot) assay for the differentiation of FMDV infected animals from vaccinated animals (DIVA). The five NSPs were 3A (24 kDa), 3B (15 kDa), major B-cell epitope regions of 2C (23 kDa), partial 3D (44 kDa) and 3ABC (59 kDa). The criteria for the dot blot were determined and are described as follows: a test sample is considered positive if four or more NSPs demonstrate staining densities equal to or higher than those of their appropriate controls; a sample is considered negative if two or more antigens demonstrate densities below their negative control. A specificity of 100% was observed based on testing of sera from clinical healthy animals with or without vaccination; the sensitivity of the dot blot was 96.1% and 65.8% for testing of samples from infected cattle and swine, respectively, at an early stage of the infection. Meanwhile, high rates of concordance were observed between the dot blot and the PrioCHECK® FMDV-NS test. The dot blot has the potential to act as a confirmatory method for DIVA by 3ABC-ELISA.

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1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of mammals that may cause severe economic loss in susceptible cloven-hoofed animals. The causative agent is FMD virus (FMDV), which belongs to the *Aphthovirus* genus of the family Picornaviridae. The viral RNA genome contains a single long open reading frame (ORF), and the encoded polyprotein can be processed into structural proteins (SPs) and non-structural proteins (NSPs). The SPs (VP0, VP3, and VP1) form the virus capsid after encapsidation of the RNA genome, while the NSPs (including 2A, 2B, 2C, 3A, 3B, 3C, and 3D) participate mainly in RNA replication and folding and assembly of the SPs. Most of the NSPs are responsible for cell membrane association; for example, both the 2B and 2C NSPs localize to the endoplasmic reticulum (ER)-derived outer surface vesicles, which are sites of genome replication in picornaviruses (Gosert et al., 2000; Jecht et al., 1998). In cells infected with FMDV, NSP 2C was detected at the periphery of host cells (Tesar et al., 1989; Lubroth and Brown, 1995) have demonstrated that 2C is absent from the purified virus stocks used for vaccine preparation. The 3A NSP of FMDV has been shown to co-localize with the intracellular membrane system, and this membrane binding activity can also be predicted based on its hydrophobic property. The 3A NSP is incorporated in the stable intermediates that include 3AB and 3ABB (O'Donnell et al., 2001). The above findings indicate that most of the NSP components can be removed after the collection of cell debris during production of FMD inactivated vaccines because of their close binding to cellular membranes. This forms a theoretical foundation for the development of diagnostic assays to differentiate infected animals from vaccinated animals (DIVA) by detection of antibodies against the NSPs of FMDV.

Recently, significant progress has been made in the development of diagnostic methods for the detection of antibodies against FMDV NSPs. Many ELISAs have been shown to be reliable methods for large serological surveys after vaccination, regardless of the serotype (Brocchi et al., 2006; Bronsvoort et al., 2004; Inoue et al., 2006; Yang et al., 2007). Most of these assays are based on the detection of antibodies against NSP 3ABC or 3AB, which are considered to be the most reliable indicators of infection (Bruderer et al., 2004; Yakovleva et al., 2006). However, these studies showed that a single NSP assay could lead to false positive or false negative results. It is necessary, therefore, to find better diagnostic and field applicable procedures. An international workshop on the validation of NSP assays held in Brescia, Italy, proposed the use of at least two assays to attain ideal sensitivity and specificity (Brocchi et al., 2006; Paton et al., 2006). In South America, a combined system of an indi-

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rect 3ABC-ELISA with an enzyme-linked immunoelectrotransfer blot (EITB) assay was implemented to improve the serodiagnostic strategy for FMDV surveillance in cattle under systematic vaccination (Bergmann et al., 2000). Liquid array technology has also been reported to be a promising platform for the development of a multiplexed NSP FMD DIVA assay (Clavijo et al., 2006; Perkins et al., 2006). These tests allow the simultaneous evaluation of multiple signatures and provide more confidence in obtaining a conclusive result. Currently, the World Organisation for Animal Health (formerly the OIE) has recommended the performance of an initial screening test using an indirect ELISA for antibodies against 3ABC, followed by a confirmatory EITB assay. This has been recognized as the most accurate method for DIVA of FMD. However, these methods are used rarely owing to their cumbersome protocols and the expensive facilities required.

Previously, an indirect 3ABC-ELISA has been developed and evaluated for DIVA (Lu et al., 2007), and some suspicious serum samples were judged to be positive after repeated tests with the same method. In order to improve the accuracy of DIVA further, a dot-blot assay was developed to detect simultaneously the antibodies against five NSPs and it was taken as a confirmatory method for the 3ABC-ELISA. The criteria used with the method were slightly different from the OIE published standard (OIE Terrestrial Manual, 2009) for confirmatory determination of one sample.

2. Materials and methods

2.1. Serum samples

2.1.1. Serum samples from animals infected experimentally with FMDV

50 sera from cattle 10 days post experimental infection (DPI) with Asia 1/JS/05 FMDV, 38 sera from swine infected with O/CHA/99 or Asia 1/JS/05 FMDV at 11 DPI, 70 serum samples from 7 cattle infected experimentally with A/WH/CHA/2009 at 0–229 DPI, and 16 serum samples from one pig infected with O/CHA/99 at 0–192 DPI.

2.1.2. Serum samples from healthy animals

30 sera from clinically healthy swine in a non-vaccinated herd that were determined to be antibody negative against O and Asia 1 FMDV, together with 34 serum samples from swine vaccinated twice, 30 sera from healthy cattle that were determined to be antibody negative against O and Asia 1 FMDV by liquid phase blocking ELISA (LPBE), and 12 sera from cattle vaccinated once with O and Asia 1 bivalent inactivated vaccine at 28 days post vaccination.

2.1.3. Field serum samples

A total of 180 sheep sera were obtained from sheep herds suspected of FMD after an outbreak of Asia 1 in Linxia province in 2006.

2.1.4. Reference serum samples

Twelve reference cattle sera were obtained from the FMD World Reference Laboratory (WRL) in Pirbright, UK for a phase XIX collaboration exercise in September 2006. For details of the background of these sera and the test results see Section 3.3.

2.1.5. Standard sera used for development of the ELISA and dot blot

Positive control sera from cattle, sheep and pigs were collected from animals infected with the O/China/99 strain of FMDV at 90 days for cattle and 30 days for sheep and pigs after the development of typical clinical signs; weak positive sera were derived from animals that developed a low level antibody titer against both SP and NSP 3ABC at 15 days post challenge with Asia 1/JS/05 FMDV.

2.2. Preparation of antigens

The recombinant 3ABC protein was produced according to a method described previously (Lu et al., 2007). The complete 3A and 3B coding regions were inserted into the pET28a plasmid (Merck, Darmstadt, Germany) via *Bam*H I and *Sal* I restriction sites. A ligated fragment, including 174 bp of the 5′-end and 279 bp of the 3′-end of the 2C gene, which encodes an abundance of known B-cell epitopes of the 2C protein (Höhlich et al., 2003), was inserted into the pET30a plasmid (Merck) via *Nco* I and *Sal* I restriction sites. Partial 3D, corresponding to 97–1204 nt of the 3D coding region, was inserted into pET28a via *EcoR* I and *Hind* III restriction sites, and these 3D-expressing vectors were donated kindly by Professor Liu Xiangtao in our Laboratory. The recombinant vectors were then transformed into competent *Escherichia coli* (*E. coli*) cells BL21 (DE3) pLysS or Rosetta (DE3) pLacI (Merck) for protein expression.

The procedure for expression and purification of the five recombinant NSPs (3A, 3B, 2C, 3D, and 3ABC) were performed essentially as described previously (Lu et al., 2007), with modifications. Briefly, the overnight cultures of recombinants in LB medium were diluted 100-fold and cultured with vigorous shaking at 37 °C in 250 mL of LB medium containing 100 μg/mL of antibiotic (3A, 3B, 2C and 3D with kanamycin, 3ABC with carbenicillin) until the OD_{600} reached 0.6–1.0. Expression of the recombinant proteins was induced with 1 mM isopropyl β-D-thio-galactopyranoside (IPTG). After induction for 5 h at 37 °C, the cells were harvested by centrifugation at 4 °C and 8000 r/min for 10 min. The cell pellets were suspended in 0.1× culture volume of inclusion body (IB) wash buffer (20 mM Tris–HCl, pH 7.5; 10 mM EDTA; 1% Triton X-100), and lysozyme was added to a final concentration of 100 μg/ml. The samples were incubated on ice for 30 min and then sonicated on ice for 5 min. Following cell disruption, the cell supernatant containing soluble 3A, 3B, and 3D was collected for subsequent purification with Ni-NTA His binding resin (Invitrogen, California, USA) according to the manufacturer's instructions. Recombinant proteins 2C and 3ABC were expressed to form IB, and were purified sequentially by washing with a second wash buffer (20 mM Tris/HCl, pH 7.5; 2 M urea; 1% Triton X-100) and solubilized by the addition of IB solubilization buffer (50 mM CAPS, pH 11; 0.3% N-lauroylsarcosine; 1 mM DTT). The supernatants containing 2C and 3ABC were purified further by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The slices of acrylamide that contained the target protein were excised from the gel by referring to prestained molecular weight standards, and the target proteins were electroeluted as described previously (Neizert et al., 1991). The five purified proteins were identified by SDS-PAGE, and stored at −70 °C for use as diagnostic antigens in the following assays. The concentration of NSPs was measured by the Bradford method.

2.3. Preliminary characterization of the purified NSPs

Five NSPs were used individually as antigens in an indirect ELISA to determine its reactivity with sera derived from healthy and FMDV infected cattle, sheep and pigs. Briefly, for each antigen the optimal concentration was pre-titrated by a checkerboard titration, and then the 96-well ELISA plates were coated with each recombinant protein at their optimal concentration (3A 40.5 ng/well, 3B 237.5 ng/well, 2C 185 ng/well, 3D 170 ng/well, and 3ABC 95.5 ng/well) to detect antibodies in the positive and negative control sera from different species; the procedure was same as reported previously (Lu et al., 2007). In addition, the rates of concordance for 3A, 3B, 2C and 3D

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