New strategies for the differentiation of Foot-and-Mouth disease virus-infected from vaccinated animals: Development of a competitive ELISA and a multiplexed Luminex assay

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Introduction
Available vaccines to foot-and-mouth disease (FMD) stimulate the production of antibodies indistinguishable from those produced by infected animals in response to live virus and because vaccinated animals can be infected and become carriers of FMDV, efforts have been made to develop diagnostic test that can differentiate vaccinated animals from those that are convalescent and from those that have been vaccinated and become carriers following subsequent contact with live virus. Using traditional serological techniques it is not possible to distinguish FMD infected animals from vaccinated animals and control authorities have limited possibilities to monitor virus presence or circulation. Currently the detection of antibodies to non structural proteins (NSPs) is the preferred diagnostic method to distinguish virus infected from vaccinated animals. Considerable effort and attention is now being directed toward the development of new methods and techniques for the rapid and accurate detection of anti-NSP antibodies, harmonization and standardization of current diagnostic techniques, as well as the production of defined reagents.

Two new methodologies were evaluated for the detection of NSPs: A competitive ELISA (cELISA), to detect FMD virus antibodies in cattle, sheep and pigs without any modification in the test procedure and a Luminex-base multiplex immunoassay to measure four NSPs simultaneously from a single sample.

Materials and Methods
Non structural proteins cloning
Viral RNA was isolated using TriPure Isolation Reagent from FMDV infected BHK-21 cells following manufacturer instructions (Boehringer Mannheim, Laval, Quebec). The complete gene encoding for the 3ABC, 3D, 3A and 3B proteins were amplified using reverse transcription-PCR using primers derived from the published sequence of FMDV strain O/Campos/Brazil/58 (Pereda et al., 2002). The sense primer was designed such that part of the FMDV NSP protein was in the same reading frame as the PET 6xHis tag and the PinPointXa-1 vector sequence (for 3ABC) encoding a consensus biotinylation site. In vitro amplification by RT-PCR and post-PCR analyses were as previously described (Clavijo et al., 2003). Molecular cloning was accomplished by standard techniques (Sambrook et al., 1989).

NSP recombinant proteins expression and in vivo biotinylation
Expression of 6xHis-3ABC, 3D, 3A and 3B recombinant protein was carried out as described previously (Clavijo et al., 2001). The expression of the biotinylated 3ABC was carried out as the 6xHis-NSP recombinant protein, except that LB media supplemented with D-biotin (2µM) and carbenicillin (50µg/mL) was used. The protein expression was induced by adding IPTG to a final concentration of 100µM. For extraction of the recombinant proteins 6xHis-NSP and biotin-3ABC, the cell pellet was resuspended at room temperature with BugBuster reagent (Novagen, Madison, WI, USA). Following cell lysis, the suspension was incubated on a shaking platform at a slow setting for 20 min at room temperature the inclusion bodies were collected.

Solubilization and refolding of recombinant proteins
Recombinant proteins were solubilized by the addition of IB solubilization buffer (50 mM CAPS, pH 11; 0.3% N-lauroylsarcosine; 1 mM DTT). The solubilized protein was dialyzed (20 mM Tris-HCl pH 8.5; 0.1 mM DTT). The refolded 6xHis-NSP recombinant proteins and the biotinylated 3ABC recombinant protein (B-3ABC) were diluted 1:1 in glycerol and stored at -20°C until used as antigen in the cELISA or multiplex Luminex assay.

Guinea pig anti-3B hyperimmune sera
A 15 amino acid peptide from the 3B NSP region was used as antigen to immunize 6 Hartley guinea pigs of about 600-700g. For inoculation, 200 µl of antigen were emulsified with an equal volume of Freund-s complete adjuvant and inoculated into two sites subcutaneously. Subsequent inoculations on dpi 28 consisted of antigen combined with an equal volume of Freund-s incomplete adjuvant. Samples were taken at 0, 27 and 43 days post infection (dpi) and the animals were exsanguinated via cardiac puncture under general anaesthetic on dpi 44. Prebleed and titre checks were performed using the saphenous vein.

Competitive ELISA test procedure
Competitive ELISA (cELISA) was performed by sensitising polystyrene microtiter plates (Nunc-Immunoplate, Roskilde, Denmark) overnight with 100 µl/well of 10 µg/mL of streptavidin (Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania) in coating buffer (0.01 M phosphate buffered saline (PBS))(NaCl
times with 100µl PBS–T (10mM NaH$_2$PO$_4$, 150mM NaCl, 0.02% v/v Tween-20). To each well, 50µl PBS–T/BSA/NMS was added followed by 50µl biotin-SP conjugated goat anti-bovine IgG (H+L) (Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania) diluted to a final concentration of 1:200 in test. The beads were resuspended in 100µl PBS/BSA and transferred to a round bottom plate. Plates were then sealed, wrapped in tinfoil and incubated at 37°C with vigorous agitation for 1 hour. Plates were washed 3 times with 100µl PBS-T (10mM NaH$_2$PO$_4$, 150mM NaCl, 0.02% v/v Tween-20). To each well, 50µl PBS-T/BSA/NMS was added followed by 50µl biotin-SP conjugated goat anti-bovine IgG (H+L) (Jackson Immunoresearch Laboratories Inc.) diluted to a final concentration of 1:300 in test. The beads were resuspended by pipetting up and down. Plates were again sealed, wrapped in tinfoil and incubated at 37°C with vigorous agitation for 1 hour followed by washing. 50µl PBS/BSA was added to each well along with 50µl 3µg/ml Streptavidin-R-PE (Qiagen), beads were resuspended by pipetting. After a final 1 hour incubation and washing step, beads were resuspended in 100µl PBS/BSA and transferred to a round bottom plate. The plate was then read in the LiquiChip (Qiagen) using Luminex 100 IS software, version 2.2. All data was recorded in MFI units.

Results

Cloning and expression of NSP recombinant protein

The recombinant NSP proteins were expressed in *E. coli* with a six histidine tag at the amino and carboxy terminus as indicated by the sequencing of the pET clones. The 6xHis-NSP fusion proteins 3ABC, 3A, 3B and 3D were coupled to Penta-His beads (Qiagen LiquiChip Penta-His Bead Set B) overnight as follows. Each protein was diluted in PBS/BSA (10mM NaH$_2$PO$_4$, 150mM NaCl, 0.1% w/v BSA) for a final dilution of 1:50 to 1:100 in the coupling reaction. The reaction was set up in microfuge tubes which were then wrapped in tinfoil to prevent photobleaching of the beads. Reactions were incubated overnight at 4°C in an Eppendorf Thermomixer R at 750rpm. Reactions were washed with PBS/BSA and then microfuged at 10,000g for two minutes. The wash step was repeated, the supernatant was removed and the beads were resuspended in PBS/BSA. The beads (10µl) were counted and made to a final concentration of 25 beads/µl in PBS/BSA. Prior to starting the test, filter plates (MultiScreen-BV, Millipore) were pre-wet with 100µl PBS-T/BSA/NMS (10mM NaH$_2$PO$_4$, 150mM NaCl, 0.02% v/v Tween-20, 0.1% w/v BSA, 3% Normal Mouse Serum) and the buffer was then removed by vacuum. For tests involving a single recombinant protein-bead coupling, the following was added to each well: 35µl PBS-T/BSA/NMS, 15µl coupled beads at 125 beads/µl, 50µl diluted serum samples (final 1:200 dilution in test). For the multiplex test 20µl PBS-T/BSA/NMS, 15µl each bead (60ul volume in total) and 20µl 1:40 dilution of serum samples (final 1:200 dilution in test) was added to each well. Filter plates were sealed, wrapped in tinfoil and incubated at 37°C with vigorous agitation for 1 hour. Plates were washed 3 times with 100µl PBS-T (10mM NaH$_2$PO$_4$, 150mM NaCl, 0.02% v/v Tween-20). To each well, 50µl PBS-T/BSA/NMS was added followed by 50µl biotin-SP conjugated goat anti-bovine IgG (H+L) (Jackson Immunoresearch Laboratories Inc.) diluted to a final concentration of 1:300 in test. The beads were resuspended by pipetting up and down. Plates were again sealed, wrapped in tinfoil and incubated at 37°C with vigorous agitation for 1 hour followed by washing. 50µl PBS/BSA was added to each well along with 50µl 3µg/ml Streptavidin-R-PE (Qiagen), beads were resuspended by pipetting. After a final 1 hour incubation and washing step, beads were resuspended in 100µl PBS/BSA and transferred to a round bottom plate. The plate was then read in the LiquiChip (Qiagen) using Luminex 100 IS software, version 2.2. All data was recorded in MFI units.
The streptavidin system has been used successfully in developing immunoassays and demonstrated good streptavidin-coated plates as an easy single step purification. The specific binding of a biotinylated 3ABC recombinant fusion protein in a crude bacterial extract to sensitivity and specificity (Clavijo et al., 1998) because of the reduced nonspecific binding and the ability to Silberstein et al., 1997). In this study, we report the development of a rapid competitive ELISA that utilises The majority of available NSP tests use the principle of indirect or blocking ELISA (Shen et al., 1999; polyprotein 3ABC or part of this protein have so far been the most successful (Mackay et al., 1998). Kinetics of the antibody response to NSP antigens The kinetics of the antibody response to 3ABC antigen in experimentally infected animals was evaluated. Positive reactions could be found in all infected animals as early as 6-7 days post infection (dpi) (Figure 5) There was no difference in the antibody response of cattle, sheep or pigs to the 3ABC recombinant protein when different serotypes of the virus were used. Anti-3ABC antibodies were detected in 10 of 13 cattle at 7 dpi. All 13 cattle were seropositive at 10 dpi and remained positive until the end of the experiment on day 28-30 (Figure 5). All 12 sheep were positive against 3ABC at dpi between 7 and 10 (Figure 5). Some pigs seroconverted as early as 7 dpi, but all were positive at 10 dpi (Figure 5). Recombinant proteins 3ABC, 3D, 3A and 3B conjugated to fluorescent microspheres provided the basis for a novel immunoassay using the Luminex technology to detect the immune response to these antigens. To determine whether the individual NSP immunoassay could be multiplexed, we examined the kinetics of the immune response of experimentally infected animals with different strains of FMDV. Results indicated that the immune response can be detected between 7-14 days post infection (Figure 6). There was also considerable variation in both the magnitude of the overall response to NSPs in individual animals, which in turn may reflect the difference in the NSP immunogenicity and extent of viral replication.

Discussion
Identifying animals that have been infected with FMDV is important for the control of FMD as recovered cattle and sheep frequently remain carriers of the virus and consequently may become the source of new outbreaks of the disease (Alexandersen et al., 2003). The diagnostic challenge is to distinguish infected animals that have been vaccinated or unvaccinated from those that have been only vaccinated against FMDV since both groups have neutralizing antibodies in their sera. Several ELISAs have been developed to distinguish infected animals from those that have been vaccinated, all based on the detection of antibodies to the NSPs of FMD virus (Bermann et al., 2000; Shen et al., 1999; Sorensen et al., 1998). Tests to detect antibodies to the polyprotein 3ABC or part of this protein have so far been the most successful (Mackay et al., 1998). The majority of available NSP tests use the principle of indirect or blocking ELISA (Shen et al., 1999; Silberstein et al., 1997). In this study, we report the development of a rapid competitive ELISA that utilises the specific binding of a biotinylated 3ABC recombinant fusion protein in a crude bacterial extract to streptavidin-coated plates as a easy single step purification. The streptavidin system has been used successfully in developing immunoassays and demonstrated good sensitivity and specificity (Clavijo et al., 1998) because of the reduced nonspecific binding and the ability to bind biotin in an interaction that is almost irreversible (Stayton et al., 1999). Streptavidin-biotin interactions are so strong that elution of biotin-tagged proteins from streptavidin-conjugated resins usually requires denaturing conditions. For the evaluation of the specificity of the 3ABC cELISA a broad spectrum of negative sera from across Canada was analyzed. The antibody response to 3ABC NSP in cattle, sheep and pigs was detected as early as 7 dpi. Although infection of susceptible cattle with FMDV results in a rapid rise (4-5 dpi) of serum-neutralizing antibody which will protect against infection with homologous and antigenically related viruses, the immune response to NSPs is usually delayed (Mackay et al., 1998). Rodriguez et al., 1994 assessed the immunogenicity of different FMDV proteins in swine. After analyzing the specificity of the anti-FMDV antibodies produced against NSPs in sera from infected or vaccinated pigs, they showed that the NSP 3ABC antibodies were detectable from 2 weeks post infection. There is also considerable variation in both the magnitude of the overall response to NSPs in individual animals, which in turn reflected the difference in the NSP immunogenicity and extent of viral replication. Mackay et al., 1998, using a profiling ELISA showed that the antibody response to 3ABC appeared early after infection and antibody to 3ABC could be detected for longer than antibody to any other NSP. The response to 3A, 3B, 3D and 3ABC could be detected in cattle as early as 7-10 dpi and subsequently decreased gradually (Sorensen et al., 1998). However, antibodies to NSPs 3AB and 3ABC in sheep were detected not until 14 dpi as shown by Sorensen et al, 1998.
results were comparable with those obtained by others, an increased sensitivity was seen in early detection of the antibody response to 3ABC in all three species. However, as seen with other NSP tests its use for detection of infection in non-vaccinated animals is limited compared with tests that used structural proteins as antigens due to its low relative sensitivity to detect positive animals early after infection. We did not see any variation in the detection level when sera from animals infected with different serotypes of FMDV were analyzed. Foster et. al., 1998, performed a series of longitudinal studies of the humoral and cellular immune responses to NSPs in animals infected with different serotypes of FMDV. They observed a highly variable immune responses to all NSPs in short duration. Although we expect to use this test for the discrimination between infected and vaccinated animals, the 3ABC cELISA will have the same limitations as other NSP tests. Positive diagnosis may be only possible at the herd level because of the great variability in the initiation, specificity and duration of the immune response to the NSPs. This also indicates that the likelihood of detecting or confirming an infected animal is greatly increased if multiple antigens are used as diagnostic reagents (Clavijo et al., 2004).

In conclusion, the use of the streptavidin-biotinylated antigen in the cELISA provides not only a single step purification of the recombinant antigen, but also a surface with a high binding capacity sufficient to make available an excess of reagent and a negligible nonspecific binding. This allows for the highest possible assay sensitivity and specificity. Currently no NSP test has been fully validated and before any can be used for mass screening, it will need to be characterized against a set of standards which should reflect different epidemiological situations. The complexity of FMD and its wide range of hosts make this standardization difficult to achieve, but the availability of a competitive ELISA that can be used in all species may facilitate this process.

Luminex-based technology promises to be a highly specific and efficient method that permits multiplexed antibody characterization to NSPs from a single serum aliquot. Currently this methodology is not designed for high throughput screening and perhaps its major use may be as an alternative to current confirmatory tests for the differentiation of infection from vaccination after a positive or suspicious ELISA result.

References


Figure 1. Expression of NSP 3ABC and 3D
Figure 2. Expression of NSP 3B and 3A

- BL21(DE)pLysS cell control
- PET30-3B Uninduced
- PET30-3B Induced (soluble)
- PET30-3B Induced (insoluble)
- BL21(DE)pLysS cell control
- PET30-3A Uninduced
- PET30-3A Induced (soluble)
- PET30-3A Induced (insoluble)
Figure 3. Protein analysis of the pPoint3ABC expression clone. Bacterial cell lysates were resolved using 10% SDS-PAGE and stained with coomassie blue; 1. Soluble extract from JM109 cells; 2. Soluble fraction from induced JM109 cells containing pPoint3ABC expression vector, 3. Insoluble fraction; M. Molecular mass standard in kilodaltons.

Figure 4. Frequency distribution of negative sera from cattle, sheep and pigs. Sera were tested at a final dilution of 1:10 in the 3ABC competitive ELISA. The vertical line indicates the threshold of the test at 50% inhibition.
Figure 5. Kinetics of the 3ABC antibody response from experimentally infected cattle as detected by the 3ABC cELISA. A. Bovine; B. Sheep; C. Pig.
Figure 6. Kinetics of the NSP 3D, 3ABC, 3A and 3B antibody response from experimentally infected cattle as detected by the Luminex-based multiplex immunoassay.