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Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle

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Abstract

For the first time, a multiplex bead immunoassay was used to test simultaneously, with a single sample, the immune response to foot-and-mouth disease non-structural proteins 3ABC, 3A, 3B and 3D from experimentally infected and vaccinated cattle. We cloned and expressed these non-structural proteins (NSPs) as recombinant antigens. The purified proteins were coupled to microspheres labeled with anti-His monoclonal antibody with different proportions of red and orange fluorescent dyes and reacted against serum specimens. Antibody reacting against different NSPs, and thus, the different colored beads, was detected by use of the Luminex system. This multiplex bead immunoassay can detect the immune response to NSPs in cattle as early as 7 days post-infection. In general antibodies to the protein 3D appeared early after infection and anti-3ABC antibodies were detected at higher levels than the other NSPs. A clear differentiation was established between infected and vaccinated or uninfected cattle. The multiplex bead immunoassay was compared to individual indirect enzyme-linked immunosorbent assays (iELISAs) for the same NSP's responses. Results indicated that this new assay had a high positive correlation with those generated by iELISA. The Luminex-based technology promises to be a sensitive and efficient method that permits multiplexed NSP antibody detection from a single sample and would therefore provide both a time and cost saving to the laboratory.

Keywords: Foot-and-mouth disease; Multiplexed Luminex assay; Vaccinated cattle

1. Introduction

Foot-and-mouth disease virus (FMDV) causes a highly contagious vesicular disease in cloven-hoofed animals, and the recent outbreak of foot-and-mouth disease (FMD) in the United Kingdom is a clear reminder of the economic devastation that this disease can produce [1]. When an outbreak of this disease occurs, quarantine measures are applied and the animals on the infected farm are culled and their carcasses destroyed to break the chain of infection as quickly as possible. When considered necessary, preventive culling of animals in suspect farms may also be applied [2]. Routine vaccination is used widely and successfully to control FMD in countries where the virus is endemic or poses recurrent threats of virus incursions from neighboring countries. Intensive vaccination of livestock over decades eventually allows such countries to reduce the incidence of FMD to the point at which they are able to eradicate the infection, allowing them to acquire disease-free status [3]. In turn this brings considerable economic gains and entry into world markets for animal and animal products.

During FMDV replication, antibodies are produced against both viral capsid proteins and non-structural proteins (NSPs). The latter proteins are involved in the replication of the virus. Most FMDV vaccines that are used globally in routine vaccination are inactivated whole-virus vaccines grown in cell culture and therefore animals vaccinated against FMD will develop antibodies to structural proteins only. Whist all FMD vaccines require a concentration process in their production, manufacturers are encouraged to include a purification process. Purification of vaccine antigens serves two

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purposes; the elimination of proteins that can induce allergic reactions and secondly, NSPs are removed or their concentration considerably reduced. Therefore, it is expected that vaccines prepared from purified antigen will not induce antibodies against NSPs [4].

This has allowed for the development of tests for the detection of antibodies against NSPs having the potential to differentiate vaccinated from infected animals with the added advantage of detecting antibodies independent of virus serotype [5]. Tests to detect antibodies to the polyprotein 3ABC have so far been the most successful in distinguishing infected animals from those that have been vaccinated. This virus specific NSP has been produced either in recombinant *Escherichia coli* [6] or in insect cells infected with the appropriate recombinant baculovirus [7]. In order to reduce the number of false positives, an enzyme-linked immunoelectrotransfer blot assay (EITB) has been used as a confirmatory test [8]. This test uses several NSPs (2C, 3A, 3B, 3ABC and 3D) which are subjected to Western blotting. The membranes carrying the proteins are cut in strips, each strip is then incubated with a bovine serum, and the immune reaction detected by enzymatic amplification [9]. Using five NSPs in a single test considerably increases the test specificity. However, the EITB assay is highly subjective and prone to human errors in its interpretation, limiting its use as a reliable confirmatory test.

In this study, the development of a microsphere-based multiplexed assay for the detection and quantification of serum antibodies against FMDV NSPs is described. This assay has the potential to replace other tests that use several NSPs as antigens with the benefit that it is not subject to operator interpretation, has the potential to be a useful high-throughput assay and it can aid in the differentiation of FMD-infected from vaccinated animals.

2. Materials and methods

2.1. Antigens

Recombinant proteins to the NSPs 3ABC, 3D, 3A and 3B were produced by cloning PCR amplified DNA fragments obtained from cDNA of the FMDV strain O_1/Campos/Brazil/58 [10] using specific primers to amplify the selected genes (Table 1). Primers targeting the complete gene coding sequence for the NSPs 3A, 3B and 3ABC were designed to include *BamH*I and *Hind*III restriction sites at the forward and reverse primers respectively. The 3D primers included an *Eco*RI and *Not*I restriction sites. The sense primers were designed such that part of the FMDV NSP protein was in the same reading frame as the (six histidine) 6×His tag of the pET30c vector (Novagen, Madison, WI, USA) for 3A, 3D and 3ABC or the pET41c vector (Novagen, Madison, WI, USA) for 3B sequences.

Competent *E. coli* cells BL21(DE3)pLysS or Rosetta (DE3)pLysS (Novagen, Madison, WI, USA) were transformed using the calcium chloride method. Transformants were screened and the positive clones were further analysed by restriction enzyme digestion to determine the presence of the inserted sequences. The identity and reading frame of the selected recombinant clones were confirmed by sequencing.

Protein expression was essentially as described previously [11]. Briefly, overnight cultures of recombinant plasmids in (Luria broth) LB medium were diluted 100-fold and cultured with vigorous shaking at 37 °C in 250 mL of LB medium containing 30 μg/ml of Kanamycin until OD_{600} reached 0.6–0.8. Recombinant protein expression was induced with 1 mM isopropyl β-D-thio-galactopyranoside (IPTG). After induction for 3 h at 37 °C, cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C and the cell pellet stored at −70 °C until purification.

BugBuster reagent plus benzonase nuclease (Novagen, Madison, WI, USA) was used for extraction of the recombinant proteins as recommended by the manufacturer. Following cell lysis, the suspension was incubated on a shaking platform at a slow setting for 20 min at room temperature and insoluble material removed by centrifugation at 10,000 × *g* for 15 min at 4 °C. After washing three times, the insoluble material was processed to produce a soluble recombinant protein using a protein refolding kit (Novagen, Madison, WI, USA) as described previously [11]. The soluble 6×His-nonstructural recombinant proteins were stored in aliquots at −70 °C.

Recombinant proteins were partially purified by polyacrylamide gel electophoresis (PAGE) and the gel was then stained with 0.3 M copper chloride as described elsewhere [12]. The recombinant protein was excised from the gel by referring to pre-stained molecular weight standards. Slices of acrylamide containing the recombinant protein

Table 1
Primers used for the construction of the expression clones

Protein	PCR primers				
	Forward primer	Position[a]	Reverse primer	Position[a]	Size[b]
3ABC	5′-CAATTCCTTCCCAAAAATCT-3′	5361	5′-GTGGTGTGGTTCGGGGTCCAA-3′	6664	1316
3D	5′-TTGATTGTGGACACCAGAGA-3′	6671	5′-CGTTCACCCAACGCAGGT-3′	8060	1404
3A	5′-CAATTCCTTCCCAAAAATCT-3′	5361	5′-CAGCTTGTGGTTGCTCCTCA-3′	5813	465
3B	5′-GACCCTACGCCGGACCACTC-3′	5817	5′-CTCAGTGACAATCAGGTTCT-3′	6028	224

[a] Position in the FMDV genome from the published sequence of FMDV strain O_1/Campos/Brazil/58 (Pereda et al. [10]).
[b] Size of the PCR product in base pairs (bp).