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Immunogenicity of non-structural proteins of foot-and-mouth disease virus: differences between infected and vaccinated swine

Ana Rodríguez*, J. Dopazo, J. C. Sáiz***, and F. Sobrino**

Centro de Investigación en Sanidad Animal, INIA, Valdeolmos, Madrid, Spain

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Summary. Non-structural as well as VP1 recombinant proteins of foot-and-mouth disease virus (FMDV) produced in *E. coli*, have been used to study the specific antibody response of infected or vaccinated swine. An analysis of sera from infected pigs, using a direct ELISA, showed that polypeptide 3ABC (spanning non-structural proteins 3A, 3B and 3C) was the most antigenic among the recombinant proteins studied and allowed specific detection of FMDV infected swine from the second week after the infection. The sensitivity of this assay was comparable to that obtained when the whole FMDV was used as ELISA antigen. Conversely, use of polypeptide 3ABC did not allow detection of significant levels of antibodies in sera from vaccinated animals. This differential pattern of ELISA reactivities offers a promising approach for the distinction of infected from vaccinated pigs. In addition, a highly specific and sensitive method of diagnosis for FMDV replication was achieved using an immunoblotting assay which detected antibodies against the 3ABC polypeptide.

Introduction

Foot-and-mouth disease (FMD) is an economically devastating disease of cloven-hoofed animals, mostly cattle and swine. The causative agent is FMD virus (FMDV), an aphthovirus of the *Picornaviridae* family for which seven serotypes have been described (reviewed in [7, 13]). The FMDV genome consists of a single RNA molecule that encodes four structural proteins (VPs) and, at least, ten non-structural polypeptides induced in infected cells, including a viral RNA replicase 3D, also termed FMDV infection associated antigen (VIAA) [15]. The disease is controlled by vaccination with chemically inactivated virus (reviewed in [2]).

* Present address: Department of Cell Biology, Yale University School of Medicine. New Haven, CT, U.S.A. ** Present address: Centro Nacional de Biotecnología, Canto Blanco, Madrid, Spain. *** Present address: Centro de Biología Molecular, Universidad Autónoma de Madrid, Canto Blanco, Madrid, Spain.

Detection of VIAA by agar gel immunodiffusion test [6] or virus isolation [5] has been used to identify infected animals. To overcome the low sensitivity of these procedures, specific ELISA assays to detect antibodies against the whole virion [8], viral subunits [17] or VIAA [1] have been developed. These methods do not allow a reliable distinction between infected and vaccinated animals; commercial vaccines produced from viral infected cell culture extracts usually contain sufficient 3D protein to be immunogenic [14].

Diagnosis of viral replication is important for cattle, which frequently develop a persistent and inapparent infection [5]. Attempts to use 3D protein expressed in *E. coli* for the detection of FMDV infected cattle has been reported [12, 19]. The new non-vaccination policy in western Europe contemplates ring vaccination in case of outbreaks. Thus, diagnosis of FMDV replication in pigs, in these areas, may contribute to the effectiveness of FMD control.

To assess the immunogenicity of different FMDV proteins in swine, and as an attempt to develop reliable procedures to detect viral infection in susceptible animals, we have analyzed the specificity of anti-FMDV antibodies produced against non-structural proteins in sera from infected or vaccinated pigs. The results obtained indicate that 3ABC is the most immunogenic virus-induced polypeptide and can be used to distinguish between infected and vaccinated pigs.

Materials and methods

Experimental infections and vaccinations

Landrace × large white pigs were infected by inoculation with 10^4 to 10^5 p.f.u. of FMDV isolates O1 Kaufbeuren (O1Kb) or C1 Santa Pau Sp70 (C-S8). Vaccination of pigs was carried out with one dose of oil emulsion commercial vaccine (Laboratorios Sobrino, Olot, Spain), which included ethylenimine inactivated virus (equivalent to 5×10^7 p.f.u.) from each of strains of serotype C (C-S8), A (A5 Fra 1/68) and O (O-Granollers Sp/71).

Sera from animals were obtained between 7 to 30 days post-infection (p.i.), and 21 days post-vaccination (p.v.). Sera from animals that received a second vaccination after 20 days p.v., were collected at 39 days from the first immunization.

Recombinant proteins

Different FMDV O1Kb non-structural polypeptides as well as VP1 capsid protein were expressed as fusions to the N-terminal part of MS2 polymerase under the control of the inducible lambda PL promoter, from pEX derived plasmids transformed into *E. coli* 537 bacteria [18]. Proteins were obtained from heat induced bacterial cultures by sonication and purification in urea 7 M, and the antigenic specificity of the materials was confirmed by western blotting using specific antisera to each of the non-structural polypeptides, as described [18]. The location of the proteins used in the viral genome is shown in Fig. 1.

ELISA procedure

Purified FMDV fusion proteins (0.3 μg/ml) in coating buffer (0.1 M carbonate/bicarbonate pH 9.6) were bound to 96 well plates (Nunc Immuno II). The efficiency of coating for each of the polypeptides was confirmed by an ELISA using rabbit specific antisera (kindly provided by Dr. E. Beck, Giessen, Federal Republic of Germany) and protein-A-peroxidase

as reagents. After saturation with 10% calf serum in PBS, two-fold dilutions of sera (starting from 1/25) from immunized animals were added. Plates were incubated with a mixture of three biotin-labelled murine Mab's anti swine immunoglobulins IgG_1, IgG_2 (kindly provided by Dr. Stokes, Bristol, U.K.) and IgM (Mengeling, 1985), at 2 µg/ml respectively. Finally, horseradish-peroxidase-avidin D (Vector), diluted 1/2 000 in PBS, was added to the well. As a substrate, 200 µl of 80.6 mM 3-dimethylaminobenzoic acid (Sigma D-1643): 1.56 mM methyl-2-benzothiazolinone hydracine hydrochloride monohydrate (Sigma M-8006) (1:1) and 0.0075% H_2O_2 were used. Reactions were stopped by adding 25 µl of 3N sulfuric acid. All incubations were performed in 50 µl volume at 37 °C for 1 h, followed by extensive washes with 0.02% tween-20 in water. Sera from non immunized pigs were used as control. Absorbance at 620 nm (A_{620}) was measured in a Titertek Multiskan plus mark 2 reader. End point titers were expressed as the log of the serum dilution that gave the same value as the maximum A_{620} for control sera (always lower than 0.3 at dilution 1/25).

When using whole FMDV particles as antigen, the assay was performed as described [16]. Briefly, plates were coated with SD6, a Mab that recognizes a continuous epitope in VP1 [10]. Plates were incubated with supernatant of BHK-21 cells infected with FMDV (strain C-S8), prior to the addition of sera.

Immunoblot

FMDV O1Kb fusion proteins, resolved on a 12.5% SDS-PAGE, were electrophoretically blotted onto nitrocellulose filters (approximately 1 µg/mm). The efficiency of this procedure was shown to be high for all the polypeptides used, as judged by staining with ponceau red. Nitrocelluloses were incubated with 1:100 dilutions of sera from immunized animals followed by an incubation with protein A-peroxidase conjugate (Sigma) diluted 1:1 000. Both incubations were performed for 1 h at 37 °C followed by two washing cycles with 0.02% tween-20 in PBS. The reacting polypeptides were revealed by incubation with 4-chloro-1-naphthol in methanol and hydrogen peroxide 0.05% in PBS.

Results

Detection of FMDV antibodies by an ELISA assay based on recombinant polypeptides

Figure 1 shows location of the nine different FMDV antigens used in the indirect ELISA. Results obtained with 17 sera from 9 pigs experimentally

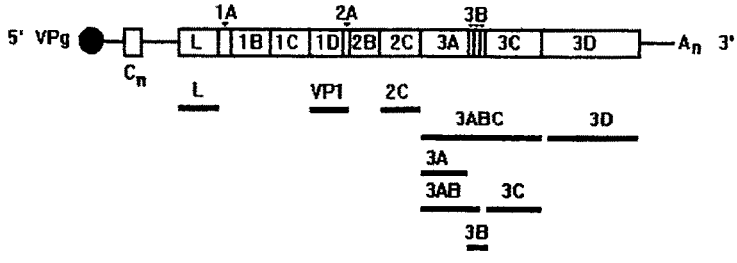

Fig. 1. Polypeptides of the FMDV O1Kb strain used in this study. Solid bars indicate the regions of the FMDV polyprotein expressed by each construction [18]. The limits of genomic FMDV regions expressed in *E. coli* were: *L* 892 to 1812; *VP1* 3001 to 3849; *2C* 4465 to 5151; *3A* 5149 to 5481; *3AB* 5149 to 5730; *3C* 5812 to 6297; *3B* 5482 to 5730; *3ABC* 5149 to 6297; *3D* 6451 to 7800. *L*, *2A* and *3C* proteases; *1A–1D* structural proteins (VPs); *2B*, *2C* and *3A* whose function remains to be established; *3B* three copies of the protein linked to the 5' end of FMDV RNA (VPg); *3D* viral RNA polymerase

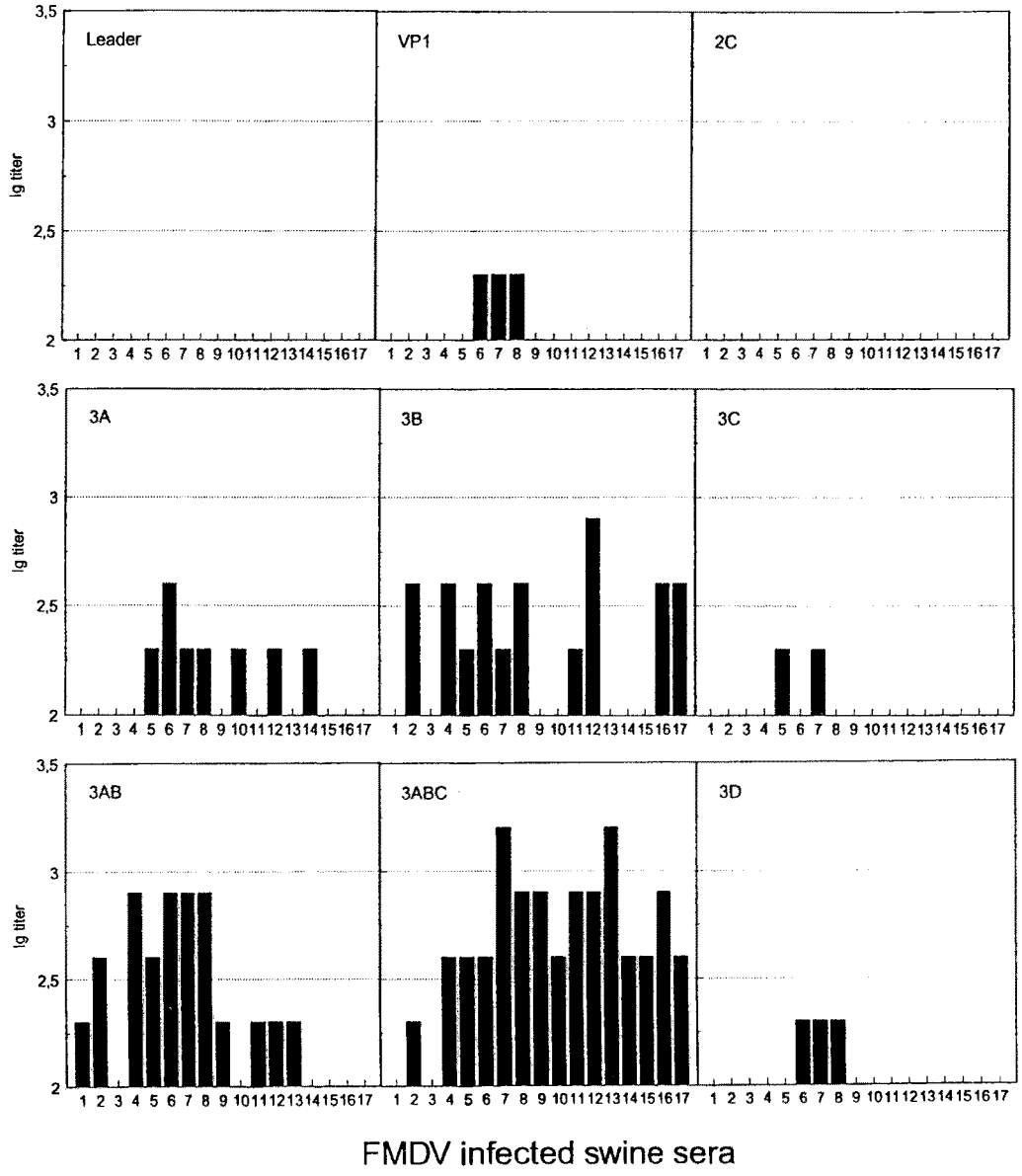

Fig. 2. ELISA reactivity of sera from infected pigs against different FMDV antigens. The following sera were used (in all cases two samples from each animal, which are numbered consecutively, were analyzed): *1–2*, *3–4* infected with O1Kb, 7 and 14 days p.i. respectively; *5–6*, *7–8*, *9–10*, *11–12*, *13–14*, *15–16* infected with C-S8, 23 and 30 days p.i. respectively; *17* infected with C-S8, 30 days p.i.

infected with either O1Kb or C-S8 FMDV strains are shown in Fig. 2. As expected, specific FMDV antibodies were not detected during the first week p.i. From the second week p.i., only 3ABC gave similar titers as those obtained when whole FMDV C-S8 was used as ELISA antigen (Fig. 3a). Both VIAA (3D protein) and VP1 gave lower titers which, in twelve of the sera tested,

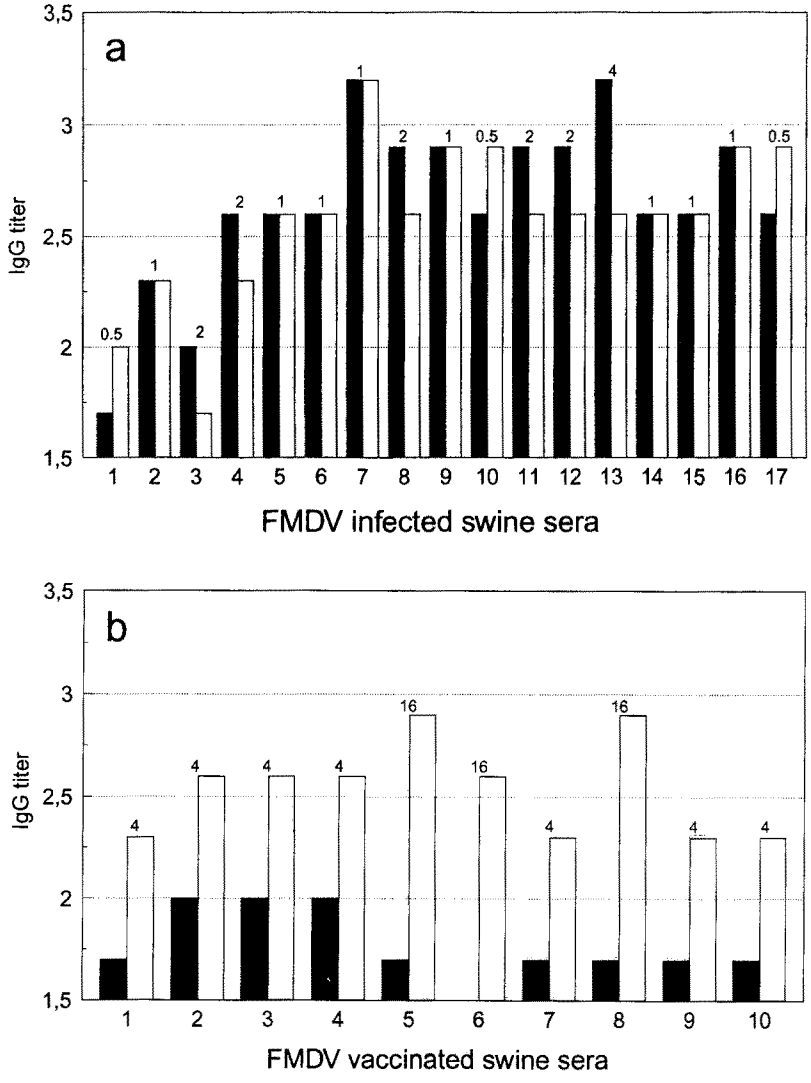

Fig. 3. Comparison of ELISA sensitivity using 3ABC (solid bars) or complete FMDV particles (open bars). **a** Infected swine sera corresponding to the same animals as in Fig. 2; **b** *1–10* Vaccinated swine sera (21 days post-vaccination). Ratios of virus/3ABC reaction are indicated for each sera

were undistinguishable from the background level (Fig. 2). The specificity of this assay was confirmed by the lack of reactivity found with sera from twelve farm pigs, as well as from ten animals infected with swine vesicular disease virus (data not shown).

When the ELISA was used to study sera from 10 pigs immunized with a commercial FMD vaccine containing strains of each of serotypes A, O and C, none of the polypeptides allowed detection of values significantly different from background titers (data not shown). However, sera from vaccinated animals gave a significant response when whole FMDV was used as antigen. A comparison of the ELISA titers obtained with whole FMDV with those obtained

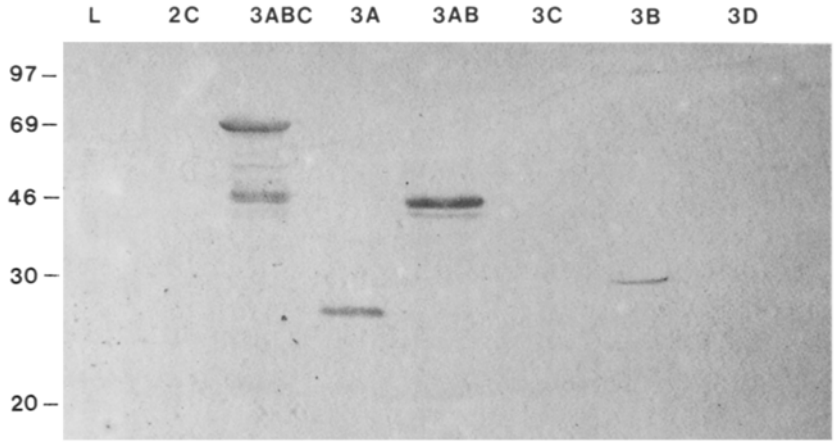

Fig. 4. Immunoblot of viral non-structural proteins with serum no. 4. The two additional bands of lower molecular weight, visible in *3ABC* probably correspond to self-cleavage of this polypeptide

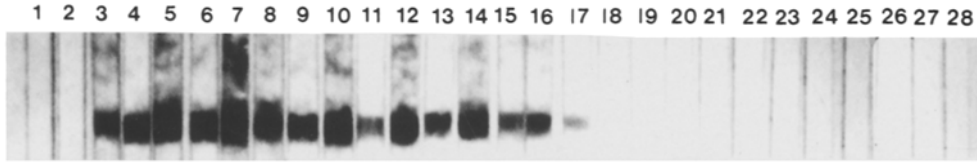

Fig. 5. Immunoblot reaction of 3ABC polypeptide with sera from infected and vaccinated swine (*1–2* infected with O1Kb, 7 days p.i.; *3–4* infected with O1Kb, 14 days p.i.; *5–11* infected with C-S8, 23 days p.i.; *12–17* infected with C-S8, 30 days p.i.; *18–27* correspond to *1–10* in Fig. 3; *28* corresponds to a negative control serum)

using 3ABC is shown in Fig. 3b. An analysis of the ratio of virus titers/3ABC titers revealed values $\geqslant 4$ for vaccinated animals while, except in one case, ratios $\leqslant 2$ were found among infected animals. In addition, sera from two pigs that had received two vaccinations did not react with 3ABC polypeptide (data not shown). Therefore a differential pattern of ELISA reactivity between complete virions and 3ABC has been observed for infected versus vaccinated pigs.

Immunoblotting detection of FMDV antibodies using recombinant 3ABC polypeptide

An immunoblot assay using the recombinant FMDV polypeptides was used to analyze the serum from an animal at 14 days post-infection with O1Kb FMDV (pig no. 4 in Fig. 2). The results obtained, (shown in Fig. 4) were similar to those found with the ELISA. The serum mostly recognized 3ABC, 3A, 3B and 3AB polypeptides. Therefore, and according to our previous results, we explored the ability of an immunoblot assay based on 3ABC polypeptide to detect viral replication in swine. The results obtained (Fig. 5) showed a specific detection of antibodies against polypeptide 3ABC in all the sera from pigs

after two weeks post-infection, while, under the conditions of the assay (sera diluted to 1/100), no detectable recognition was observed for those of vaccinated animals (21 days p.v.). The specificity of the assay did not depend on a dilution effect; all sera from vaccinated animals (including two double vaccinated pigs) diluted to 1/50 did not show any reaction with 3ABC polypeptide. Likewise, all sera from infected animals diluted to 1/200 showed similar results to those obtained at 1/100 dilution (data not shown).

Thus, the immunoblot assay developed allows differentiation between the infected and vaccinated pigs studied.

Discussion

The antigenicity of FMDV non-structural polypeptides in infected or vaccinated pigs has been studied using different viral recombinant fusion proteins.

Polypeptide 3ABC gave the highest ELISA titers and was the only antigen assayed which allowed detection, after two weeks p.i., of all the 15 FMDV infected pigs analyzed. The high crossreactivity found between sera against C-S8 virus and non-structural polypeptides of serotype O is not surprising since the variability displayed by these proteins is relatively low when compared with the structural ones [9]. The low reactivity observed for structural protein VP1 (the most immunogenic when included in the viral particle; [4]) or VIAA could be explained by the lack of native conformation in these constructions. Likewise, the lack of reactivity to 3AB and 3C polypeptides in sera 10 and 14–17 which react with 3ABC, may be due to an altered conformation of these fusions, being 3ABC polypeptide the one showing a more similar structure to that of the native protein.

The sensitivity of the ELISA based on 3ABC polypeptide is comparable to that obtained using complete FMDV particles. Conversely, sera from vaccinated swine gave responses that were indistinguishable from background to all non-structural polypeptides including 3ABC, but showed a comparable reactivity to complete virions. This differential reaction pattern allows distinction of infected versus vaccinated pigs among the animals studied. To exclude possible nonspecific reactions interfering with the ELISA results, an immunoblot assay using the FMDV fusion proteins was used to analyze the available sera. The results confirmed the potential of 3ABC polypeptide to identify and distinguish sera from infected or vaccinated pigs. Under the reaction conditions, none of the sera from vaccinated animals gave a positive reaction with polypeptide 3ABC.

Thus, according to our results a single 3ABC ELISA assay allows distinction between infected (from 21 d.p.i) and vaccinated pigs. Those titers $\leqslant 2$ correspond to vaccinated animals, while infected animals (from 21 d.p.i) present values $\geqslant 2.6$. Distinction of sera with intermediate values, which correspond to those obtained at short times p.i, required an immunoblot assay.

Radioimmunoprecipitation [3], as well as western blotting detection [12] of non-structural polypeptides other than 3D have been proposed as a diagnostic

for FMDV replication in cattle. Our results extend to swine the observation that certain non-structural polypeptides are more immunogenic and/or antigenic than others, especially those corresponding to the 3ABC region.

A high specificity is required for the detection of FMDV infection in animals. The ability of 3ABC to be specifically recognized by sera from infected pigs was confirmed by the lack of reactivity, in both ELISA and immunoblot, with non-vaccinated farm animals, as well as with pigs that had received one or two doses of vaccine. Even when the amounts of non-structural proteins may largely depend on the procedure followed for the preparation of vaccines, and consequently further analyses are required, detection of anti-3ABC antibodies by means of a simple ELISA procedure, constitutes a promising strategy for identification of animals infected with FMDV. In addition, production of 3ABC polypeptide is quick, low cost and does not require manipulation of infective virus.

In conclusion, the development of new sensitive techniques for the detection of antibodies against 3ABC FMDV polypeptide has allowed differentiation between infected and vaccinated pigs.

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Authors' address: Dr. F. Sobrìno, Centro de Investigación en Sanidad Animal, INIA, Valdeolmos, 28130 Madrid, Spain.

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