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DIFFERENTIATION OF INFECTION FROM VACCINATION BY DETECTION OF ANTIBODIES TO THE NON-STRUCTURAL PROTEIN 3ABC OF FOOT-AND-MOUTH DISEASE VIRUS

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A study was performed in order to evaluate a serological method, the 3ABC-ELISA, for the differentiation of FMDV-infected animals from those that had merely been vaccinated. Sets of sera from naive, vaccinated and experimentally infected cattle as well as from animals that had been vaccinated and subsequently infected, were examined. All sera from naive and from vaccinated, but non-infected animals were found negative for antibodies to 3ABC antigen. FMDV infected cattle that had not been vaccinated before infection was consistently scored positive in the indirect trapping 3ABC ELISA developed at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) as well as in a commercially available test, the CHEKIT FMD-3ABC. Also antibodies to FMD polyprotein 3ABC could be detected in sera from six out of eight cattle that had been challenged after vaccination, The level of 3ABC antibodies declined gradually over time, but in serum of one animal antibodies were detectable for at least 19 weeks post infection. The 3ABC tests also allowed to clarify the status of LPBE/VN positive animals that had been sampled during 1997-2001 as a part of the national serological surveillance program for FMD. It was concluded that the 3ABC-ELISA will be a useful tool in FMD control and eradication programs facilitating the detection of virus circulation in FMD-vaccinated populations, because on a herd basis, vaccinated and infected cattle can be differentiated from those that had merely been vaccinated.

Key words: cattle, foot-and-mouth disease, vaccination, diagnosis, non-structural proteins, 3ABC- ELISA.

Foot-and-mouth disease (FMD) is a severe and highly infectious viral disease of cloven-hoofed animals. The economic impact of the disease is significant due both to the cost of controlling outbreaks and the resulting loss in trade (11). The cornerstone of FMD control in Europe still is the culling of infected and contact animals ("stamping out policy"). However, during the epidemic in spring 2001, massive culling met strong

criticism from the general public, veterinarians and farmers because of environmental, ethical, emotional and economic reasons (1).

The introduction of FMD to Western Europe last year again demonstrated the necessity of rapid and accurate diagnosis. Conventional serological methods for the diagnosis of FMD, i.e. virus neutralization test (VN) (7) and liquid-phase blocking ELISA (LPBE) (9), rely on the detection of antibodies to the structural, capsid VP1-VP4 proteins of the FMD virus. Antibodies to the structural proteins are induced by both vaccination and infection. Vaccines consist of purified preparations of inactivated virions and therefore induce antibody almost exclusively to the structural proteins of the virus. Viral replication during infection results in the production of a number of non-structural (NS) proteins, which are more or less immunogenic (24).

Differentiation of infection from vaccination based on the detection of antibodies to NS proteins has been described using either panels of proteins (2, 3, 4, 14, 23) or the individual proteins 3D (19, 26), 2C (12), 3AB1 (22) or 3ABC (5, 20). The response to 3ABC and its cleavage products (mainly 3AB, 3A, and 3B) and to 2C have been found the most reliable indices of FMDV infection (2, 3, 12, 13, 17, 20). The ability to distinguish FMD infected animals from those that had only been vaccinated is important for import/export serology, for FMD control programs and especially for eradication campaigns employing ring vaccination followed by serological screening.

The first studies on the application of 3ABC-ELISAs were performed at the Department of Foot-and-Mouth Disease of the National Veterinary Research Institute in 1998. Our preliminary results showed that the sera of all LPBE/VN positive cattle from the region around Zduńska Wola, derived from animals intended for export or tested as a part of the national serological surveillance program for FMD, were negative to polypeptide 3ABC. Only 2 out of 82 sera collected from LPBE/VN positive animals imported to Poland during 1985-1991 gave a positive reaction in 3ABC-ELISA test (18). The principal aim of these studies was to asses the diagnostic potential of the 3ABC-ELISA for the detection of FMD infection in cattle.

Material and Methods

Sera. Sets of 165 sera from naive, 95 sera from vaccinated, 47 sera from infected as well as 66 sera from vaccinated and subsequently infected cattle were examined. Naive sera, negative for antibodies to the structural proteins of FMD virus by the LPBE/VN, were taken from healthy animals that had never been exposed to FMD virus. "Post-vaccination" sera were collected from animals repeatedly vaccinated during 1985-1989 with trivalent anti-FMD vaccine. Reference convalescent sera from animals infected with different strains of FMDV were supplied by the World Reference Laboratory for FMD at Pirbright, U.K. Sixty-six samples of sera from eight cattle vaccinated with a commercial aqueous monovalent A_{24} vaccine and challenged 28 days post vaccination with homologous FMD virus, were collected following a potency trial that, in principle, had been performed according to the method described in the European Pharmacopoeia. The samples of blood from the cattle were taken at regular intervals for up to 19 weeks post infection (w.p.i.). One animal had to be slaughtered 7 w.p.i. for reasons unrelated to the trial. Moreover, 27 samples of archive LPBE/VN positive sera collected during 1997-2001 as a part of the national serological surveillance program for FMD were examined. All sera were kept frozen at -20°C until used.

ELISA methods. The LPBE was performed according to the method described by Hamblin *et al.* (9). Antibody titers were expressed as the final dilution of test serum giving 50% of the mean OD_{492} value recorded in the virus control wells where test serum was absent. Titers greater than log 1.6 were considered as positive. Antibodies to the FMD virus 3ABC polyprotein were detected using the IZSLER indirect trapping 3ABC-ELISA method developed by De Diego *et al.* (5). A positive reaction was scored when the OD_{492} values, obtained after subtraction of the background of wells without antigen, were higher than 0.2 (cut-off level). Reagents were kindly provided by Dr E. Brocchi, IZSLER, Brescia, Italy. Additionally, the presence of 3ABC protein was examined using the commercially available CHEKIT FMD-3ABC bo-ov, Dr Bommeli AG, Switzerland. The test was carried out according to the manufacturer's protocol.

Results

The results of examination of the sera from naive, vaccinated and (non vaccinated) infected cattle using 3ABC-ELISA are presented in Fig. 1.

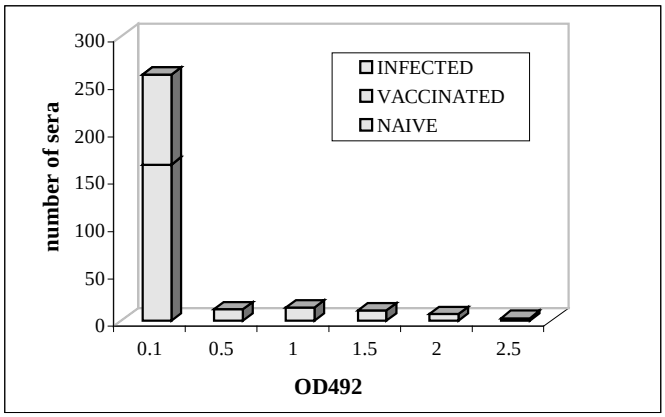

Fig. 1. Frequency distribution of OD_{492} values of sera from naive, vaccinated and infected cattle in the 3ABC-ELISA.

All sera from naive as well as vaccinated LPBE/VN positive animals gave negative results against 3ABC polypeptide. The NS protein antibodies profiles of sera from infected cattle were markedly different from those of sera from naive and vaccinated animals. A frequency distribution of OD_{492} values recorded by 3ABC-ELISA from animals infected with any of the seven serotypes of FMDV revealed that all 47 experimental post-infection sera gave OD_{492} values over threshold value 0.2 and ranging up to 2.5 units.

The level of antibodies to FMD particles (structural proteins) assayed by LPBE and the results of 3ABC serology in sequential sera collected from six cattle after a vaccine potency test are demonstrated in Fig. 2.

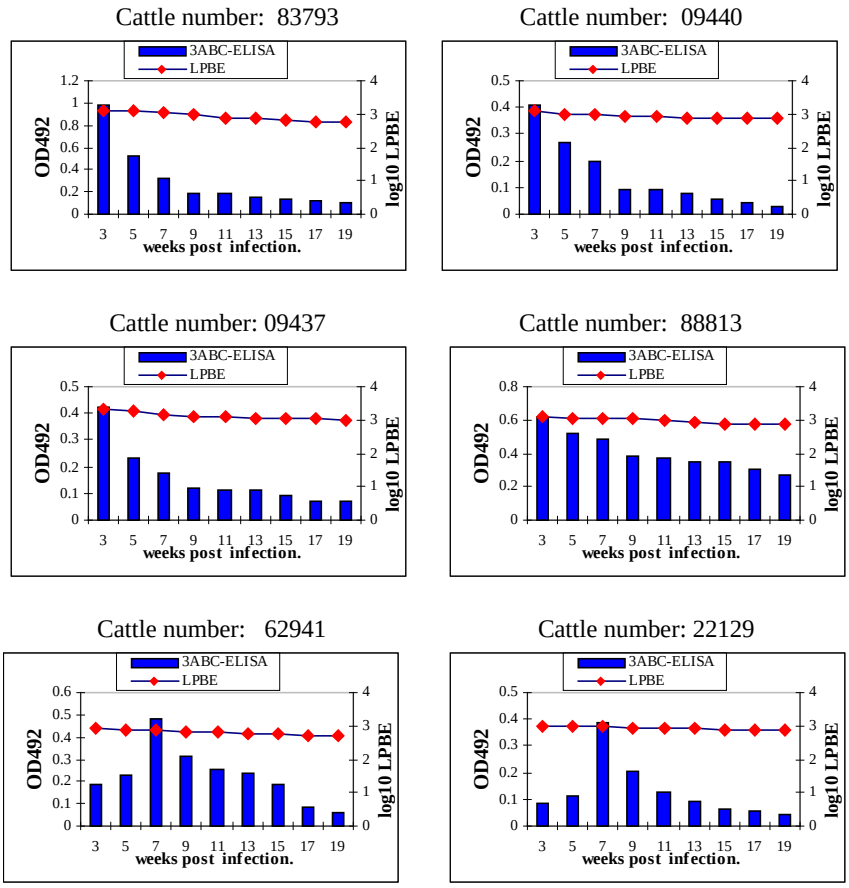

Fig. 2. Comparative development of antibody to FMD virus capsid proteins and NS polyprotein 3ABC in sera of six vaccinated cattle following challenge with FMD virus. Sera were collected from animals that had been infected 4 weeks after vaccination with a monovalent A_{24} commercial vaccine.

Six out of eight cattle that had been vaccinated and subsequently infected showed seroconversion to 3ABC antigen (OD_{492} values over threshold value 0.2). One animal (88813) remained 3ABC-ELISA positive throughout the period of the experiment. The development of antibodies against 3ABC protein was delayed in cattle number 22129 and 62941. Sera from cattle number 99440 and 83793 showed positive or borderline reactions up to 7 w.p.i. and from cattle number 62941 until 13 w.p.i. Sera from two animals, nos 80835 and 09430 (data not shown) reacted negatively (< 0.2 OD_{492}). Antibodies to 3ABC declined more rapidly than antibodies to structural proteins which remained at a high level in sera of all animals until the end of the experiment.

Also in the commercial CHEKIT FMD-3ABC test six out of 8 vaccinated and subsequently infected animals were scored positive (Table 1). According to the manufactures protocol, a value >30% of the net OD of the positive control was taken as cut off level. Values between 20 and 30% were considered inconclusive. Both animals negative in the IZSLER 3ABC-ELISA were also found negative or inconclusive, respectively, in the CHEKIT FMD-3ABC test.

Table 1
Development of antibody to FMD NS protein 3ABC measured
in the CHEKIT FMD 3ABC-ELISA.
Results are given in % net OD of the positive control.
For comparison, sera reacting positively in the IZSLER 3ABC ELISA
were marked by shading

Weeks post infection (w.p.i.)			3	5	7	9	11	13	15	17	19
Cattle No.	Protection	Dose									
22129	Yes	1	14	17	45	24	20	14	11	4.5	2.4
9430	Yes	0.25	0	2.2	2.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9437	Yes	1	26	15	14	8.5	7.5	9.2	8.2	5.3	3.8
9440	Yes	0.25	59	47	40	18	19	14	9.2	12	14
83793	No	0.0625	52	42	27	15	13	14	16	11	6.2
88813	Yes	1	37	35	34	34	33	34	30	29	28
80835	Yes	1	6.5	9.2	18	17	19	25	14	13	12
62941	Yes	0.25	6.2	29	43	37	31	30	20	16	15

n.d.- not done

The methods of 3ABC antibody detection allowed to clarify the status of LPBE/VN positive animals that had been sampled during the national serological surveillance program for FMD (Table 2). It was found that all sera positive for antibodies to the capsid protein were 3ABC antibody negative.

Table 2
FMD antibodies detected by 3ABC-ELISA/CHEKIT FMD-3ABC and LPBE in archive bovine sera collected during 1997-2001

Test No	Serum No	3ABC-ELISA (OD_{492}) / CHEKIT FMD-3ABC (%)	LPBE titre (log10)		
			A_{24}	O_1	C_2
T/79/97	1	< 0.2/2.50	1.65	1.95	1.90
T/95/97	2	< 0.2/0.55	1.39	1.84	1.69
T/134/97	13	< 0.2/3.70	2.47	3.01	2.90
T/173/97	114	< 0.2/0.60	1.39	1.84	1.69
T/175/97	47	< 0.2/2.35	1.47	1.80	1.77
	112	< 0.2/0.85	1.39	1.65	1.80
T/13/98	2	< 0.2/7.20	1.54	2.25	2.04
	6	< 0.2/4.80	1.54	2.25	2.04
	18	< 0.2/5.80	1.54	2.25	2.04
	34	< 0.2/0.06	1.54	2.25	2.04
	35	< 0.2/3.75	1.54	2.25	2.04
	47	< 0.2/7.25	1.54	2.25	2.04
T/117/98	1	< 0.2/2.80	1.39	1.80	1.65
T/118/98	44	< 0.2/6.85	1.30	1.92	1.80
	70	< 0.2/0.95	1.39	1.81	1.60
T/222/98	28	< 0.2/3.20	1.74	2.10	2.10
T/224/98	2	< 0.2/0.88	1.69	1.87	2.00
	6	< 0.2/7.25	1.80	2.21	1.93
	24	< 0.2/7.20	1.74	2.14	2.04
T/61/99	7	< 0.2/2.16	1.30	1.84	1.65
T/84/99	9	< 0.2/8.85	2.10	2.34	2.36
T/196/00	6	< 0.2/3.50	1.60	1.95	1.84
T/294/00	72	< 0.2/2.11	1.25	1.80	1.60
T/420/00	134	< 0.2/0.68	1.39	1.92	1.90
T/320/01	4	< 0.2/4.35	1.50	2.10	1.80
T/328/01	6	< 0.2/2.16	1.47	2.04	1.77
T/350/01	98	< 0.2/1.85	1.30	1.92	1.65

Discussion

Foot and mouth disease (FMD) is a devastating disease of livestock, caused by a member of the *Picornaviridae* family. FMD is the most contagious viral disease of animals and is endemic in many regions of the world, including most parts of Africa, Asia and some parts of South America. In FMD-endemic regions the major cost of the disease is associated with reduced livestock productivity, regular mass vaccination and reduced access to international markets for livestock and livestock products. Therefore the achievement and maintenance of an FMD-free status has major benefits for international trade. In many situations, regular vaccination is an essential part of the disease control strategy. However, due to the high number of virus strains, vaccination provides only limited protection. In regions free of FMD, control is based upon prevention of the virus introduction through import regulations and, in case of an outbreak, a combination of movement controls and stamping out. These measures may have to be supported by emergency vaccination in order to limit the spread of the disease. This was emphasized during the 2001 epidemic in Europe, when massive culling met strong criticism (1). However, current vaccines do not prevent the development of a carrier state in infected animals. Infected cattle can excrete virus for years, regardless whether they had been vaccinated before infection. Antibody to structural proteins is produced following either infection or vaccination - severely impairing the recognition of infection in a vaccinated population. Therefore, areas or countries where emergency vaccination was performed and where vaccinated animals were not slaughtered, will suffer from severe trade restrictions.

Recently, some tests have become available, which make possible to distinguish between vaccinated or infected herds. Serological differentiation of post-vaccinal and convalescent animals is based upon the humoral immune response to the non-structural proteins of the FMDV (NSPs). Over the last years several methods to detect antibodies to NSPs have been developed. The potential use of measuring antibody against NSP of FMD virus to differentiate infection from vaccination was first demonstrated by radioimmunoprecipitation (3). Neitzart *et al*. (17) described an electroimmunotransfer-blot (EITB) assay in which sera are examined for the presence of antibodies to several NSPs simultaneously by immunoblotting. However, as ELISA is more suitable than immunoblotting for screening large numbers of sera, considerable effort has been focused on developing sensitive, specific and reproducible ELISA's for the detection of antibodies to NSPs. A number of such assays were described (4, 5, 14, 22, 23). In animals seropositive for antibody to structural proteins, the detection of antibody to the polyprotein 3ABC is the most reliable single index of infection (2, 3, 12, 17, 20). Using sets of sera from naive and vaccinated cattle we could confirm the high sensitivity of this assay previously described (4, 5). In our study 100% naive and non-infected and vaccinated animals were negative for antibodies to 3ABC protein. All animals that had been infected without previous vaccination reacted positively in the 3ABC tests.

The assay of archive LPBE/VN positive sera, collected from cattle held on the territory of Poland, by 3ABC-ELISA and CHEKIT FMD-3ABC bo-ov tests revealed that the FMDV antibodies detected were induced merely by the immunization of animals with inactivated FMD virus. This confirmed our earlier studies, from which we had concluded that most seroreagents were the animals kept on the farms situated around Zduńska Wola, which were vaccinated annually during 1985-1989 with trivalent A/O/C FMD vaccine (18). The rest of LPBE/VN positive animals were

imported to Poland during 1989-1991 from neighboring countries which employed prophylactic vaccination, e.g. the former Soviet Union, East Germany or Czechoslovakia (10). The assay can therefore provide valuable information when assessing the risk posed by animals which were found seropositive for antibodies to structural proteins during serological survey for FMD or during routine import/export serology.

Several authors studied NSP antibody response in vaccinated cattle exposed to infection to determine whether or not animals which are protected by vaccination seroconvert to NSPs following exposure to FMD virus. Brocchi *et al.* (4) showed that the great majority of cattle which were vaccinated as part of vaccine potency trials seroconverted to 3ABC following challenge. Since well documented sera from field outbreaks are difficult to obtain, also we used sera from vaccine potency trials. In our experiment six out of 8 cattle that had been vaccinated and subsequently infected reacted positively in 3ABC serological test. Two animals reacted negatively or doubtfully to 3ABC antibodies. Antibodies to 3ABC declined gradually over time but in some cases were still detectable until the end of experiment, 19 w.p.i. The identification of 3ABC-positive cattle confirmed their infection with FMD virus and indicated that these animals could constitute a potential source of infection. All eight animals became virus carriers (Haas, unpublished data). Both Mackay (16) and Sorensen (23) examined sets of sera collected from vaccinated cattle following challenge in which the carrier status of the animals was known (6, 21). In both of the studies some vaccinated animals which became persistently infected following challenge did not seroconvert to NS proteins. Although the majority of vaccinated animals responded to NSPs, the response in some animals was delayed, weak or absent. A delay in seroconversion could also be seen in two of the six seropositive animals in our study and in an animal that had been vaccinated against FMD O_{BSF} before homologous challenge (unpublished data).

On the basis of our results and those of others (4, 16, 23) we conclude that with NSP serology virus replication involving more than a few animals would be detected by whole herd testing with 3ABC serology. Because infection can be traced in vaccinated animals, in future it may no longer be necessary to slaughter all vaccinated animals as it was practiced in the Netherlands during the FMD eradication campaign in 2001 (1) once the rules for international trade have been amended. It was suggested that if vaccinated, but non-infected animals could be accurately identified, trade restrictions could be reduced (25). The results showed again that absolute differentiation of infection from vaccination is not possible by serological means alone. Also virus isolation and PCR do not solve this problem under field conditions. Whilst the isolation of FMDV in orogharyngeal scrapings collected from convalescent cattle („probang test“) is reliable for the detection of FMDV in clinical samples collected during the acute stage of FMD, the low virus titre and the intermittent nature of virus recovery at later stages of disease and the low sample throughput considerably reduce its value for the detection of carriers in the field. Whereas transmission from carriers to susceptible animals seems to be a very rare event, this hazard is responsible for the long duration of trade restrictions after an outbreak. Allowing some animals that had been used for vaccine potency trials to survive for some time in high security stables probably does not mimic the field situation after an outbreak perfectly. In vaccine trials animals are usually infected into the tongue instead by contact. In order to win acceptance for reduced trade restrictions after emergency vaccination, further efforts to determine the sensitivity of NSP serology in vaccinated populations will have to be undertaken.

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