Comparison of ELISAs for the 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 validate ELISAs for the differentiation of FMDV-infected cattle from those that had merely been vaccinated. Sets of sera from naive and from vaccinated animals as well as from cattle that had been vaccinated and subsequently infected were tested for antibodies to nonstructural proteins (NSPs) of FMDV in a commercial test, the Chekit FMD-3ABC bo-ov (Bommeli/Intervet) and a competitive ELISA developed at the Danish Veterinary Institute. Probang samples from infected animals were examined by plaque test and by RT-nPCR in order to determine whether the animals had become virus carriers.

With sera from naive or vaccinated, but non-infected animals the specificity of both tests exceeded 99%.

In 21 out of 22 bovines, that had been challenged after vaccination, antibodies to FMD polyprotein 3ABC could be detected. The percentage of vaccinated and infected cattle scored positive by NSP serology depended on the test used and the time after infection. The competitive ELISA showed a higher sensitivity than the Chekit ELISA. However, when the cut off value of the Chekit ELISA was set at 10% instead of 20%, the sensitivity of this test could be increased significantly with only a slight loss of specificity. It became evident, that the seroconversion to NSPs in some animals was delayed significantly in comparison to seroconversion to structural proteins as well as in comparison to seroconversion to NSPs in non-vaccinated, infected animals. In both ELISAs the sensitivity exceeded 80%, but had to be seen in relation to time after infection. In contrast to that of the competitive NSP ELISA, the sensitivity of the Chekit test decreased after about four months p.inf.. It can be concluded that the 3ABC-ELISA will be a useful tool in FMD control and eradication programmes 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.

20 out of 22 vaccinated and infected animals became carriers, proving once again that even high potency vaccines can not prevent the development of a carrier state after massive challenge.

Keywords: foot-and-mouth disease, cattle, vaccination, diagnosis, non-structural proteins, 3ABC-ELISA
Material and Methods

Sera. In the frame of a validation trial sera of naive German cattle were tested in the CHEKIT ELISA by German regional laboratories; results of 593 sera were reported to the FRCVA until early August 2002 and included into this study. 414 sera of cattle vaccinated experimentally with a commercial vaccine (Bayer AG) were tested at the FRCVDA. 990 sera of naive Danish cattle as well as sera of 960 sheep and 1056 pigs were tested in the competitive ELISA at the Danish Veterinary Institute.

134 samples of sera from cattle vaccinated with commercial FMD vaccines and challenged 21 or, in case of the A24 trial, 28 days post vaccination, with homologous FMD virus, were collected following potency trials that, in principle, had been performed according to the method described in the European Pharmacopoeia. Only one vaccinated animal used for the study had not been protected. The cattle were sequentially bled for up to 22 weeks post infection (w.p.i.). The sera were tested in the Chekit FMD-3ABC bo-ov at the FRCVDA and in the competitive ELISA at the Danish Veterinary Institute.

Detection of the carrier state
A cell suspension plaque Test (PT) was used to test probang samples for FMD virus. BHK21-CT cells, a special clone of BHK21 cells selected for optimal sensitivity to FMDV, were used for the cell suspension plaque test as previously described (Moss and Haas, 1999). Probang samples were kept at –70°C until testing and were treated with a mixture of 1,1,2-trichlor-trifluoroethane («Arcton») and chloroform (3:1) by shaking for 15 minutes at 4°C. After centrifugation, the aqueous phase was examined.

A reverse transcription nested polymerase chain reaction (RT-nPCR) was used as an additional tool to determine whether an animal was a virus carrier. The PCR was carried out as previously described (Moss and Haas, 1999) with some modifications. Probang aliquots examined by RT-nPCR were mixed with extraction buffer immediately after thawing (Trizol, GibcoBRL). Primers were derived from the genome of strain O1 Kaufbeuren, spanning from N-terminus of polymerase 3D to C-terminus of 3A. The PCR resulted in a product of approximately 900 bp. One µl of this product was amplified in a nested PCR. All samples producing a DNA band of the expected size of about 900bp or 580bp, respectively, were considered positive.

ELISAs The presence of 3ABC protein was examined using two ELISA - the commercially available CHEKIT FMD-3ABC bo-ov, Dr. Bommieli AG, Switzerland, and a competitive ELISA developed at the Danish Veterinary Institute, Lindholm.

The Chekit FMD-3ABC bo-ov was performed according to the manufactures instructions. Briefly, dilutions of the sera were incubated in microtiter plates pre-coated with recombinant FMDV 3ABC viral antigens. Antibody specific for 3ABC bound to the antigen and is recognized by adding an anti-IgG-peroxidase conjugate. After washing and adding of a chromogen, the degree of colour which develops in the well was proportional to the amount of antibody specific for 3ABC present in the sample. Results were expressed as % by comparing the optical density (OD) of samples to the OD recorded for the positive control. The manufacturer considers values below 20% as negative, values between 20 and 30 % as ambiguous and values above 30% as positive. However, also the feasibility of a lower cut off value for the doubtful range of 10% was investigated.
The competitive NSP ELISA was performed as described previously (Sorensen et al., 1998a) with modifications. Briefly, microtiter plates were prepared by capturing 3ABC protein produced in the Baculovirus expression system with a monoclonal antibody (MabD5) coated on the plates. Dilutions of the sera were incubated and competing antibody bound to 3ABC was recognised by adding the peroxidase conjugated MabD5 directed against the protein. After washing and addition of chromogen (TMB), the colour development was measured and the results expressed as ODp values (percentage of average negative control value).

Results

Detection of the Carrier state
In case of the A24 and O BFS experiments (9 animals), samples were taken weekly and examined in both the plaque test and RT-nPCR. Detailed results are shown in table 1. In all 9 animals virus could be recovered beyond day 28 p. inf. Most virus titres were quite low. Only in two animals, No 9437 and 9259 occasionally virus titres of more than 50 PFU/100µl were recorded. The intermittent nature of virus excretion could be seen especially in case of animals 22129 and 83 793. While PCR results in general were in good agreement with virus isolation results, there were contracting results on some samples of low virus content.

The carrier state for all animals included in the study is indicated in tables 3 and 4 in the column 3. 20 out of 22 vaccinated and infected animals became carriers.

Cut off and specificity
Not all German regional laboratories involved in a validation trial for the Chekit FMD-3ABC bo-ov test had reported their results until early August 2002, but on the basis of available data on 593 cattle sampled for screening purposes unrelated to FMD, the high specificity claimed by the manufacturer could be confirmed. For the calculation of specificity, results in the “doubtful” range were counted as positive, because all doubtful or positive sera would be retested by EITB and true positives can be expected to be confirmed. Using a cut off of 20% only 1 out of 593 sera reacted positively (99,8% specificity) and at a cut off of 10% 3 further sera were scored as “doubtful” (99,3% specificity). Also of 414 sera from animals vaccinated with a commercial vaccine, three gave doubtful result (99,3% specificity).

The specificity of the competitive NSP ELISA was determined with sera collected at slaughterhouses in Denmark. A normal distribution of ODp values was obtained with serum samples from 990 cattle, 960 sheep and 1056 pigs (figure 1). The mean ODp values, SD and specificity at a cut off value of 50 (Positive result with ODp value < 50) are shown in table 2.

<p>| Table 2. Results obtained with sera from naive animals |
|----------------------------------------|------|</p>
<table>
<thead>
<tr>
<th>Cattle</th>
<th>Sheep</th>
<th>Pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of sera (n)</td>
<td>990</td>
<td>960</td>
</tr>
<tr>
<td>Mean ODp values</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Specificity % at cut off 50</td>
<td>99.4</td>
<td>99.6</td>
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Sensitivity
In table 3 the results of testing sera from 22 vaccinated and subsequently challenged animals in the Chekit FMD-3ABC bo-ov are shown. The ELISA results at different times after infection are given
as percentage of net OD of the positive control. Light shading of the boxes indicate a doubtful result of between 10 and 20%. Dark shading indicates a positive result.

In table 4 the results of testing sera from 22 vaccinated and subsequently challenged animals in the competitive NSP ELISA are shown. The ELISA results at different times after infection are given as Odp values. Dark shading of the boxes indicates positive result.

In figure 2 the results from tables 3 and 4 are summarized by grouping all sera taken during a two-weeks period into one group (only one serum per animal was included into the same group) and calculating the percentage of animals recognized as positive by the ELISAs in that group. The competitive ELISA showed a higher sensitivity than the Chekit ELISA. With a cut of 10% instead of 20%, the sensitivity of the later test could be increased significantly. Seroconversion to NSPs in some animals (6845, 62438, 9430, 36259) was delayed significantly in comparison to seroconversion to structural proteins as well as in comparison to seroconversion to NSPs in non-vaccinated, infected animals, which can be expected during the first (Hamblin 1986) or the second week (Sorenson 1998, Mackay, 1998) after infection, respectively. In both ELISAs the sensitivity exceeded 80%, but had to be seen in relation to time after infection.

**Discussion**

In both the competitive ELISA and the Chekit ELISA with the modified cut off value, the percentage of seropositive animals rose from about 65% one month after infection to more than 80% within a few weeks.

In contrast to that of the competitive NSP ELISA, the sensitivity of the Chekit ELISA showed a clear decrease after about four months p.inf.. Unfortunately, the competitive NSP ELISA is currently not available as a commercial test kit for large scale usage. With the original cut off, the sensitivity of the Chekit ELISA reached only about 60% after 3 months and quickly declined. However, the specificity of the Chekit FMD-3ABC bo-ov test for cattle was only slightly reduced to 99.3% by applying a lower cut off for the doubtful range of 10% instead of 20%, while the sensitivity was markedly improved.

One carrier animal (91368) showed only a very brief and weak seroreaction to NSPs in the competitive ELISA. In the Chekit test, even at a cut off of 10%, four animals, of which 3 were carriers, showed no or almost no reactivity. The specificity of the competitive ELISA with sera from naive animals clearly exceeded 99% for all species tested. Similar results were obtained with vaccinated animals (data not shown); however, experiments are still ongoing.

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 (Berger et al., 1990, Diego et al., 1997, Haas, 1997, Mackay et al. 1998, a, b, c). Brocchi et al. (1998) 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 21 out of 22 cattle that had been vaccinated and subsequently infected reacted positively in 3ABC serology. Both Mackay et al. (1998b) and Sorensen et al. (1998b) examined sets of sera collected from vaccinated cattle following challenge in which the carrier status of the animals was known. 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.
On the basis of our results and that of others (Bergmann et al. 1998, Brocchi et al. 1998, Mackay et al. 1998a, b and c, Sorensen et al. 1998a and b) 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 herds, in future hopefully it will no longer be necessary to slaughter all vaccinated animals as it was practiced in the Netherlands during the FMD eradication campaign in 2001. Whereas our results showed again that absolute differentiation of infection from vaccination is not possible by serological means alone, it has to be considered that also virus isolation and PCR do not solve this problem under field conditions. Whilst the isolation of FMDV in oroghappyneal 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 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. In order to gain acceptance for “protective” emergency vaccinations the question has to be answered what restrictions will be imposed on the vaccinated animals and their products and on the non-vaccinated part of the national herd. This will certainly depend on the level of confidence with which vaccinated herds can be classified as “non infected”, which depends on the size of infected clusters and the sensitivity of the NSP tests. The size of infected clusters after emergency vaccination is not known. “Vaccinating into an outbreak” or disease introduction briefly after vaccination would probably lead to a relatively high prevalence of infected animals and several animals would seroconvert to NSPs. However, if in a well protected herd one or a few animals get into contact with FMDV, e.g. by a person violating biosecurity rules, this would create different situation. It is currently impossible to say how many of such animals would eventually become virus carriers. 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 (day 21 or 28 p.vacc.) into the tongue instead by contact and the prevalence of carriers of about 90% recorded here may appear higher than would be expected in the field. However, since there are few well documented post outbreak sera from the field in Europe, an estimate of the sensitivity of NSP tests in vaccinated European herds currently has to be based mainly on sera from vaccine trials. To change that, some well controlled “experimental outbreaks” in high security facilities will have to be funded in the future. In the meantime we should try to gain support for emergency vaccinations complemented by NSP serology and restrictions for the vaccinated animals that, while facilitating to keep them alive and use their products, still allow to quickly restore confidence of trading partners in the FMD-free status of the non-vaccinated part of the animal population.

References

10. Moss, A. and Haas, B.: Comparison of the plaque test and reverse transcription nested PCR for the detection of FMDV in nasal swabs and probang samples; J. of virological Methods, 1999, 80, 59 - 67