IMPROVED INDIRECT ELISA BASED ON THE 3ABC POLYPROTEIN FOR DIFFERENTIATING INFECTION FROM VACCINATION IN FOOT-AND-MOUTH DISEASE.

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SUMMARY

In an attempt to circumvent the specificity problems founded until now in the current diagnosis test available to differentiate infected from vaccinated animals, and develop a convenient, fast and simple test, we have explored the suitability of an indirect ELISA based on a recombinant 3ABC protein excised from a preparative SDS-polyacrylamide gel. In this communication we report promising preliminary results obtained in a study that includes experimental and field sera from non-infected, vaccinated and infected animals of different species (cattle, pigs and sheep). As a part of this study we analysed field sera from sheep collected in Morocco during the 1999 FMD outbreak, pig sera from China and Philippines and cattle sera from Argentina.

Modifying protocol of purified 3ABC protein allowed to increase the specificity and sensibility of the ELISA to about 98%. So, with this improved test the differentiation between infected and vaccinated animals is more clear, and the confirmation of the results by Immunoblotting test is avoided. Furthermore, the ELISA developed is more simply than the other described before, is less time consuming and it isn’t necesary to use monoclonal antibodies. Therefore this ELISA is a suitable test to large scale samples and could be very advisable to serological surveys.

Our results testing sheep sera collected in Morocco during the outbreaks declared in 1999, indicate the capacity of the ELISA describe here to detect subclinically infection in small ruminants. Therefore, this test could be very advisable too, during serological surveys when any outbreak has been declared.
INTRODUCTION

Identifying animals that have been infected with foot-and-mouth disease virus (FMDV) is of considerable importance because it is well established that infected cattle and sheep frequently become carriers of the virus and consequently may become the source of new outbreaks of the disease (1,2,5). Furthermore in small ruminants, sheep and goat, the course of the disease is characterised by very mild or absent clinical signs, so FMD can spread unnoticed (9). This situation is compromised by the difficulty in distinguishing infected animals from those that have been vaccinated against the disease since both groups contain neutralizing antibodies in their sera. Moreover, asymptomatic carrier animals can be found in vaccinated herds.

Consequently, efforts have been directed to the development of diagnostic tests that can distinguish infected animals from those that have been vaccinated. The approach has been to identify antibodies against virus-specific proteins that are present only in infected animals. As the vaccines use currently consist in viral particles inactivated where the non-structural proteins are not present or in very few concentration, the FMDV non-structural proteins meets this criterion. Their potential use to differentiate infection from vaccination was first report by radioimmunoprecipitation (RIP). As this technique is not well suited to screening large numbers of samples, different strategies were investigated, as Immunoblotting test (IB) and mainly ELISA (4,6,8).

Among the FMDV non-structural proteins, different studies suggested that the polyprotein 3ABC was apparently the most immunogenic in pigs (8) and cattle (6). This virus-specific protein has been expressed in several heterologous systems (i.e. E. coli and insect cells) and used for the development of ELISAs to discriminate the sera from vaccinated and infected animals. These tests present specificity problems that make interpretation of results difficult, thus limiting their efficacy. It has been shown that in many cases the specificity problems are associated with the presence in the sera samples of antibodies against expression vector antigens (proteins from E. coli or insect cells) that co purify with recombinant products.

Therefore our approach has been to improve the purification system of recombinant proteins in an attempt to increase the sensitivity and specificity of the diagnosis test based on the use of this proteins. The results exposed in this communication indicate that the ELISA develop using a purified 3ABC protein allows not only to distinguish clearly between vaccinated and infected animals, also is very useful alternative method to detect subclinically infection, mainly in small ruminants.
MATERIAL AND METHODS

- **Sera**

  In order to evaluate the capacity of the new ELISA developed to differentiate between infected and vaccinated animals to FMDV, sera from the three main hostess of the virus, cattle, pig and sheep, were analyzed.

  Sheep sera were collected in Morocco, from infected regions (Oujda, Khouribga and Beni-Mellal) during the outbreaks declared in 1999 and from the provinces in the north (Tanger) after to start the vaccination campaign. Negative sheep sera to FMDV were collected from different region in Spain, a FMD free country.

  Cattle sera from experimental infected animals with the three main serotypes A, O and C, were obtained in experiments carry on in Argentina. Sera from vaccinated cattle with a trivalen vaccine were collected also in Argentina, as well as the negatives controls from naive cattle.

  The validation of the ELISA test in pig was performed testing pig sera collected in Philippines and China from farms genetically controled and vaccinated with a comercial vaccine to FMDV type O. Experimental infection of pigs were performed in our lab, in order to collect sera from infected animals to different days post-infection. These sera allowed to perform a cinetic of antibodies response to 3ABC protein, in those pigs.

- **Antigens**

  Inactivated FMDV, isolate O1-Manisa, supplied by WRCL in Pirbright (7) was used to perform LPBE for detection of antibodies against FMDV structural proteins.

  Recombinant 3ABC polypeptide from FMDV, isolate O1-Kaufbeuren expressed in *E. coli* 537 (10) was used to perform ELISAs for detection of antibodies against FMDV non structural proteins. In this system, the 3ABC polypeptide is expressed as a fusion protein with the N-terminal part of MS2 polymerase, under the control of the inducible lambda PL promoter. The recombinant 3ABC expressing *E. coli* was kindly provided by F. Sobrino. The protein was obtained from heat induced bacterial cultures and semipurified by an adaptation of the method described by Strebel *et al.*, 1987 (10). The specificity of the semipurified material was assayed by immunoblotting using 2C2 monoclonal antibody kindly provided by E. Brocci.
A new batch of protein was further purified excising the protein from a poliacrilamida gel of 12.5% and eluting it in a CO$_3$NH$_4$ buffer.

- **ELISA**

The LPBE was performed as described in the protocol supplied by the World Reference Central Laboratory (WRCL) in Pirbright, UK (7). Samples were tested in two-fold serial dilutions spanning from 1/25 to 1/3200. All the sera were tested by duplicates and the standard deviation were always lower than 5%. The cut off was established following standard procedures (7). The negative controls gave always an OD$_{620}$ above 0.9. The end point dilution titres were expressed as the inverse of the log of the serum dilution that gave the same OD$_{620}$ as the negative control at a 1/25 dilution.

Sera samples were tested to 3ABC recombinant protein in two-fold serial dilutions spanning from 1/25 to 1/3200. All the sera were tested by duplicates and the standard deviation were always lower than 5%. Detection of antibodies bound to 3ABC was performed by addition of anti-sheep, anti-pig IgG or protein-A (for cattle samples) conjugated to horseradish peroxidase (Sigma). 200 µl of 80.6 mM 3-dimethylaminobenzoic acid (Sigma D-1643): 1.56 mM methyl-2-benzothiazolinone hydrazine hydrochloride monohydrate (Sigma M-8005) (1:1) and 0.0075% H$_2$O$_2$ were used as substrate. Reactions were stopped by adding 50 µl of 3N H$_2$SO$_4$ and absorbances were measured at 620 nm (A$_{620}$). The negative controls gave always an OD$_{620}$ below 0.3.

**RESULTS**

**Sheep Samples.**

The presence of antibodies to FMDV structural proteins was analysed in 345 sera collected from areas where FMD outbreaks were declared during the 1999 epizootic in Morocco. 23 out of 299 sera tested were identified as seropositive to FMDV. None of the seropositive sheep had shown clinical signs consistent with FMD, and therefore had not been diagnosed as FMDV-infected.

Figure 1A shows the antibody titres to FMDV structural proteins detected in the 29 sheep sera collected from the FMDV-infected flock in Oujda. The 23 seropositive sera displayed a wide range of antibody titres to FMDV type O structural proteins. The graphic 1B shows the titer of antibodies to 3ABC non-structural protein detected in the
same flock. The differences in the sensibility of the ELISA performed with the semipurified (graphic up) or purified protein (graphic below) are clear. When the purified protein was used all the seronegatives detected to FMDV structural proteins were also negatives to 3ABC protein. The cut-off can be establish clearly in a OD 620 of 0.4.

**Figure 1.** Comparison of the to FMDV and to 3ABC in a flock exposed to FMD infection during the 1999 outbreaks declared in Morocco.

The first graphic shows the antibody titres to FMDV structural proteins. The graphics below compare the OD620 measured in the 3ABC recombinant ELISA using the old purified protein (semi-purified) or the new purified protein (excised from gel).
The clare bars correspond to seronegatives sera to FMDV structural proteins. The line indicate the cut-off of the assay.

In order to validate the utility of the ELISA described to differentiate vaccinated and infected sheep, 170 sera from sheep vaccinated in the North of Morocco during the vaccination campaing starting last year were analyzed by the 3ABC-ELISA. The Figure 2 show the distribution of OD620 values obtained testing 50 infected, 170 vaccinated and 50 negatives sheep sera.

Figure 2: Frequency distribution of OD620 values obtained testing infected, vaccinated and naive sheep sera.

The sera were analyzed by duplicates in a dilution of 1:25 and using as antigen the 3ABC protein excised from gel.
**Pig Samples.**

Two pigs Landrance White Large were inoculated with $10^5$ pfu of FMDV type C-S8 developing characteristic clinical signs of the disease. Serum samples were collected from time 0 (before inoculation) until 96 days post infection, bleeding the animal every day in the first week and once a week in the others. The antibodies to 3ABC are detected as soon as 4 days post infection and the maximum titer are reached between 9 and 10 days post-infection (Figure 3). 63 days post-infection the pigs were reinjected with the heterologous FMDV type O-BFS and the animals developed again clinical signs.

After the reinfection the Humoral immune response to 3ABC protein increase, being the maximum titer of antibodies 69 days post-infection, higher that those obtained in the maximum peak after infection.

![Graph showing antibody response to 3ABC in pigs](image)

**Figure 3.-** Kinetics and duration of the antibody response to 3ABC in pigs experimentally infected and reinjected with FMDV. The arrows indicate the time of infection and reinfection with the viruses.
165 vaccinated pig sera collected in Philippines and China were analyzed to 3ABC (Figure 4). The OD620 values obtained in these sera were very similar to those obtained testing 50 sera from naive pigs located in Spain.

**Figure 4.-** Frequency distribution of OD values obtained analyzing pig sera from vaccinated and naive animal.

**Cattle Samples.**

Cattle sera from animals inoculated experimentally in Argentina with the FMDV types 01-Campos, A24-cruzeiro and C3-Argentina 85, were used as positives controls in the 3ABC-ELISA. These sera were collected at 7 days post infection or 1 year post-infection (Figure 5). All of them were positives to 3ABC showing a wide range of titers.

Sera from cattle vaccinated with a trivalent vaccine, prepared with the three serotypes related before were also tested by the 3ABC-ELISA. The vaccinated sera analyzed were classified in two groups: one with animal vaccinated with one dose and a boost, and another one multivaccinated more than twice. All these sera from vaccinated cattle displayed a OD620 values to 3ABC less than 0,4 (Figure 5).
DISCUSSION

The results exposed in this communication shows that the use of a suitable purified 3ABC-protein allow to develop a symple diagnosis test as ELISA capable to discriminated between infected and vaccinated animal. The test described here improved the diagnosis methods described until now increasing the sensibility and specificity. Furthermore, the interpretation of the results with this ELISA is easier, and suggest that it application to routine diagnosis could be facilitate.

The study performed studying sera from infected areas in Morocco has allowed to validate this ELISA to applicate it in field conditions. Since the sheep sera samples collected in this infected areas were collected before to start the vaccination campaing, and that they present antibodies to FMDV structural proteins without be present clinical signs of the disease, the results to 3ABC consitute a confirmation of the existance of subclinically infections in the small ruminants population. Thus, these results indicate
the high potential of the 3ABC-ELISA to be used even more in absence of clinical symptomatology.

The cinetics of antibody response to 3ABC in pigs infected with FMD seems to be shorter than those found in other hostess (as cattle). Further work is in progress to confirm this pattern of reactivity to 3ABC protein in sera from infected pigs, in order to check how long is possible to detect antibodies to this protein. The detection of antibodies to 3ABC even more after a heterologous infection confirm the capacity of this protein highly conserved among the 7 serotypes of FMD to be recognized by animal infected with different serotypes.

In spite of the number of cattle sera analyzed is little, these preliminary results obtained testing sera from multivaccinated animal indicate that the improving of the protein used as antigen allow to improve also the sensibility of the ELISA to distinguish between infected and multivaccinated cattle. This fact can be very advisory to adapted the ELISA to control and serosurveillance of the countries where an outbreak has been declared and have to star a vaccination programm.

Further work would have to be perform in order to determine the possible application of the test also to detect the carrier state in the susceptible animal to FMD.

REFERENCES


