Appendix 67

DETECTION OF PERSISTENTLY FOOT-AND-MOUTH DISEASE INFECTED CATTLE BY SALIVARY IGA TEST

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Introduction

The new European council Directive 2003/85/EC on FMD has made provision for vaccination and the use of post-vaccination serosurveillance to detect sub-clinical infection. Carrier state can develop following clinical or sub-clinical disease, even in vaccinated ruminants exposed to live virus. Since carriers may be considered a risk for transmitting infection, they must be identified by post-vaccination serosurveillance to substantiate freedom from infection and to regain the FMD-free status for the purpose of international trade. Therefore the main aim of this study was to develop and validate an indirect IgA ELISA for the detection of persistently FMDV infected cattle.

Materials and methods

875 naïve saliva and serial saliva samples collected weekly from five vaccine challenge experimental cattle (n=106) were used to develop and validate the assay. The assay was performed as per Parida et al., (2006) with an addition of a heterologous antigen control to increase the specificity. The individual sample O.D value was normalised with respect to an in-house positive control as percentage of positivity. Standardisation of cut-off value for IgA ELISA was performed by using two-graph receiver operating characteristic (TG-ROC) analysis. Results of different virological and serological assays were used for analysis of IgA results.

Results

Using the standardised cut-off value of 40 percentage of positivity, a specificity of 98.74% and a sensitivity of 89.18% was found for IgA assay. The anti-FMD IgA response was significantly higher (P<0.05) in carrier animals as compared to non-carriers as a result of local replication of virus in the oro-pharynx.

Conclusion

The detection of salivary mucosal IgA has potential to work as screening or confirmatory DIVA test to identify subclinical infection in vaccinated population.

INTRODUCTION

The new European council Directive 2003/85/EC on FMD and the OIE Terrestrial Animal Health Code (OIE, 2004) have made the provision for emergency vaccination and the use of post-vaccination serosurveillance to detect sub-clinical infection. The European Directive on FMD control specifies that serosurveillance should be carried out at least one month after an outbreak has finished or one month after the last use of vaccine, whichever is the later. Further it states that entire vaccinated population should be sampled and tested, or enough should be sampled and tested to give 95% confidence to detect a within-herd prevalence of infection of 5%. Countries using the above approach can regain their FMD-free status after six months of the last reported infection (OIE, 2004). To demonstrate the absence of infection in vaccinated population, serological surveillance need to be based on the detection of antibodies to the non-structural proteins of FMDV as vaccination elicits antibodies against structural proteins whereas infection elicits antibodies against both structural and non-structural proteins. The sensitivities and specificities of currently available commercial and in-house NSP tests were compared at an international workshop in Brescia, Italy (Brocchi et al., 2006). The specificity of the tests ranged between 97 %-98 % whereas the sensitivity to detect the viral carriers in vaccinated and subsequently infected cattle range from 68%-94%, particularly 86.4% in Cedi-NS test (Brocchi et al., 2006). Thus if, NSP serology were to be used in large scale post-outbreak serosurveillance to identify potential carriers, a certain level of inconclusive false positive and false negative results are inevitable. Therefore it is
a requirement to develop new NSP tests or alternative tests to NSP which can be used as screening or confirmatory tests to the existing NSP tests. An indirect IgA-ELISA, using saliva samples, to detect FMD carrier cattle following vaccination and challenge exposure has been developed earlier (Parida et al., 2006). Although this IgA-ELISA has a fairly good sensitivity for detecting carrier cattle in vaccinated populations, the non-specific reaction to naive saliva is a major problem. Here we report, on the further development and validation of IgA-ELISA as confirmatory or screening DIVA test.

MATERIALS AND METHODS

Saliva samples were collected and processed as described before (Parida et al., 2006). In order to determine the cut-off value and specificity of the mucosal IgA detection test, 875 saliva samples had been collected from FMD free cattle, from the Compton dairy farm, UK and from Republic of Ireland. Saliva samples were also collected at different time-points from four vaccine challenge experiments each consisting of 25 Holstein-Friesian cattle (Steers), aged 4-8 month, carried out in biosecurity containment at the world reference laboratory (WRL) for FMD, Pirbright, U.K. Each experiment involved vaccination of cattle with oil adjuvant O Manisa vaccine and subsequent challenge by contact with donor cattle that had been previously inoculated in the tongue with O UKG 2001 (Cox et al., 2007; Parida et al., 2006). Further, to study the effect of multiply vaccination on the salivary anti-FMDV IgA level in cattle, saliva samples were also collected from one multiply vaccinated-challenge experiment in cattle. In this experiment six calves had been inoculated with (1X) O1 Manisa vaccine (18PD$_{50}$) 3 times at 21 day intervals and then challenged on the 35th day after the 3rd vaccination by a contact challenge. Known virological and serological results that had been obtained prior to this study were used for analysis of current IgA-ELISA findings.

In order to increase the specificity of the IgA-ELISA, a heterologous FMDV antigen was taken. This negative antigen control was added during the antigen addition step of indirect IgA-ELISA. The optical density (O.D) values of negative antigen control wells were subtracted from the O.D values of positive antigen wells, in order to calculate the corrected O.D values. Later on individual sample O.D values were normalized with respect to an in-house positive control as percentage of positivity (PP).

The test specificities and sensitivities were plotted against different cut-off values (Fig. 1), and a suitable cut-off value was selected as the intersection point of the two graphs based on the principle of "two-graph receiver operator characteristic" (TG-ROC) analysis (Greiner, 1995). The student’s t-test was carried out to compare the anti-FMDV IgA responses between vaccinated carriers and noncarriers.

RESULTS

Principal outcomes from the four vaccine challenge experiments in cattle including clinical protection and laboratory findings were described else where (Parida et al., 2005, 2006; Cox et al., 2007). From these four vaccine challenge experiments 37 cattle (vaccinated, n=32 and unvaccinated, n=5) had been detected as FMD virus carrier by both virus isolation (VI) and RT-PCR tests beyond 28 days post-challenge. In the current IgA assay 33 out of 37 virologically detected carriers were found positive.

The point of intersection of the two graphs in TG-ROC analysis was found in the range of 30-40 PP (Fig.1), which gave equal weightage to both sensitivity (Se) and specificity (Sp). Keeping the cut-off value as 40 PP, a specificity of 98.74% and sensitivity of 89.18% was obtained in IgA assay.
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![Graph](image)

**Fig. 1:** Two graph curves (specificity and sensitivity) for determination of suitable cut-off values. The point of intersections of the two graphs indicates the suitable cut-off value in the range of 30-40 PP.

From the four vaccine challenge experiments 48 out of 80 vaccinated cattle were detected as non-carriers by virological tests. The mean anti-FMDV IgA antibody response (Fig. 2.A) in the saliva samples of these 48 vaccinated non-carrier animals was below the cut-off value throughout the sampling period whereas the mean anti-FMD IgA response in vaccinated carrier animals was remained above the cut-off value (Fig. 2.B) and a significant difference (P<0.05) was observed.

**Fig. 2:** Mean anti-FMDV IgA antibody response in the saliva samples: (A) Vaccinated non-carrier animals, (B) vaccinated carrier animals, (C) unvaccinated carrier animals and (D) unvaccinated non-carrier animals.

Out of 20 unvaccinated challenged cattle, five were detected as carriers by both virological and by IgA tests. The mean anti-FMDV IgA response in saliva samples of these 5 unvaccinated carriers cattle was found above the cut off value on or after 35 days of post-challenge (Fig.2.C). The mean IgA antibody response against FMDV in the saliva samples of 15 unvaccinated and subsequently challenged non-carrier animals was below the cut-off value (40 PP) throughout the sample collection period (Fig.2.D). All the 20 unvaccinated animals (5 carriers and 15 non-carriers) were scored positive by Cedi NSP test.
Multiply vaccinated animals were clinically protected and none of them scored as carriers after 28 dpc by VI or RT-PCR tests. The IgA antibody responses in saliva samples of five out of six multiply vaccinated animals remained below the threshold value during post-vaccination period (Fig.3.A). However, one animal was seen marginally positive in IgA assay for one day immediately after 2nd vaccination and remained negative even after the 3rd vaccination. Out of six multiply vaccinated cattle, two were scored positive for anti-FMD NSP antibody (Cedi test) after 3rd vaccination.

(Fig.3.B).

![Graphs showing antibody response](image)

Fig.3. Antibody response in multiply vaccinated animals: (A) Anti-FMDV IgA antibody in saliva; (B) Anti-NSP antibody response in serum.

**DISCUSSION:**

In the process of development and validation of the IgA-ELISA, efforts had been made to increase the specificity of the IgA assay. Non-specificity in the old assay format may be due to the high content of detached cells, proteases and tissue particles in the saliva samples, which often contribute to the high back ground reaction by non-specifically binding to the test antigen. The use of tissue cultured inactivated crude antigen or polyclonal FMD specific rabbit hyper immune sera as a trapping antibody may be other reasons for the less specificity obtained in the past assay. Thus, in order to have a good specificity, a heterologous negative antigen control was included in the new test system. Parida et al., (2006), after analysing the saliva samples from 173 naïve cattle, suggested a cut-off optical density (O.D) value of 0.6, resulting in a test specificity of 99%. When more samples were collected from naïve cattle from the Compton farm, IAH, UK and from the Republic of Ireland 10-20% nonspecificity was observed in the old assay format (unpublished results). In the current study, 875 normal saliva samples from Compton farm, IAH, UK and from the Republic of Ireland are analysed in the new format of IgA test and a test specificity of 98.74 % was found at a cut-off value of 40 PP.

In the current IgA assay 33 out of 37 virologically detected carriers were found positive, which resulted in a sensitivity of 89.18 %. However, all the 37 carrier animals were not constantly scored positive either by VI or RT-PCR or by combining the results of both the tests as seen some times by IgA ELISA. The achieved level of sensitivity (89.18%) and specificity (98.74%) are fairly good for the detection of infection in a vaccinated herd as seen in NSP tests. However when screening of individual vaccinated animal is to be considered for surveillance the amount of sensitivity found in the IgA assay may not be sufficient to detect all the carrier animals in which case the IgA test may be used as a confirmatory test to the other established screening tests.

The mean anti-FMDV IgA response in the vaccinated carrier animals was above the cut-off value after 28 days and a significant difference (P <0.05, t-test) was observed between carriers and non-carriers for anti-FMDV IgA response. This IgA response should be viewed as genuine FMD specific...
antibody response sustained by resident antibody secreting B-cells, rather than plasma transudation (Archetti et al., 1995). Therefore, the persisting virus in carrier animals stimulated the local immune response for the production of anti-FMDV IgA antibody which supports the suggestion that high level of anti-FMDV specific IgA is an indicator of oro-pharyngeal replication (Parida et al., 2006).

It has been well established that inactivated FMD vaccine when administered parenterally stimulates very little or no FMDV specific mucosal immune response (Francis et al., 1983; Parida et al., 2006). However, an immediate question arises about the effect of repeated vaccination on the mucosal anti-FMDV IgA response, particularly in the endemic countries where bi-annual prophylactic vaccination is carried out. In order to address this question, the opportunity was taken to test saliva samples for the detection of anti-FMDV specific antibody in multiply vaccinated (3 times emergency vaccination with 21 days intervals) animals. The results indicate that IgA essay should not suffer from non-specificity when multiple vaccinations are in use in endemic countries as seen from our multiple vaccination experiment.

Cedi-NSP was found to be the most suitable DIVA test (Brocchi et al., 2006) as it is commercially available and detects 86.4% NS seropositive animals with 98% specificity. In the four vaccine challenge experiments described in this study, Cedi test and IgA ELISA detected 34 and 33 carriers respectively out of 37 total carriers. However, Cedi test also detected all unvaccinated non-carrier animals (n=15). Thus NSP tests can not entirely be used for the detection of carriers, as NSP antibody response in an animal may be due to replication of virus either in acute or persistent phase of infection and animal found sero-positive in NSP tests may or may not be actual virus carriers. Further, the analysis of serum samples originated from multiply vaccinated animals showed an increased humoral immune response to the anti-FMDV NSP antibody following 3rd vaccination in two animals. The detection of NSP antibody in multiply vaccinated animals prior to challenge may be due to the presence of trace amounts of contaminating NSP proteins in the commercial FMD vaccine and dependent on antigen pay load of the vaccine and the frequency of vaccination.

CONCLUSION

- Levels of FMDV specific IgA become elevated during acute phase of infection and were stronger in FMDV carrier animals, irrespective of vaccination status.
- The IgA essay should not suffer non-specificity from multiple vaccinations in endemic countries.

RECOMMENDATION

- The salivary mucosal IgA detection test may be considered as a potential DIVA test for the detection of persistently infected animals after application of vaccinate-to-live policy as screening or confirmatory test.

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