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A novel approach for estimation of anti-FMDV protective immunity generated in multiple vaccinated field animals

Beenu Jain[1], Anuj Tewari[2], Minakshi Prasad[3], Surender Kumar Kadian[1]

[1]Department of Veterinary Microbiology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar, Haryana, India; [2]Department of Veterinary Microbiology, College of Veterinary & Animal Sciences, Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India; [3]Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar, Haryana, India

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Summary. – In this study, forty serum samples from field buffaloes vaccinated with inactivated foot-and-mouth disease (FMD) vaccine were collected. These animals were multiple vaccinated with the above vaccine during previous years. The study was conducted to determine the actual status of the protective antibodies generated after vaccination. Initially, the serum samples were tested by Liquid phase blocking ELISA (LPBE), and only samples with titer more than 1.4 in LPBE were chosen for further analysis. These samples were tested with an in-house Gold Nanoparticle-based test for detection of anti-FMDV structural protein antibodies, in which the antibodies were detected at 10^{-4} dilution; this was suggestive of strong antibody titer generated post-vaccination. To test the binding affinity of these antibodies with the antigen, an avidity ELISA was developed and outcomes were expressed in terms of avidity index (AI). It was found that the avidity was low in some of the animals even after multiple vaccinations. Therefore, multiple vaccinations and strong antibody titer generation may not be the actual indicator of the protective immune response generated. We conclude that avidity ELISA can be a better approach than LPBE to measure the level of protective antibodies generated post-vaccination.

Keywords: avidity ELISA; foot-and-mouth disease; post-vaccination monitoring; herd immunity; PCP-FMD

Introduction

Foot-and-mouth disease (FMD) is an economically important disease of domestic and wild cloven-hoofed animals. It is caused by the foot-and-mouth disease virus (FMDV) of the family *Picornaviridae*. In endemic countries, the annual loss incurred by FMD outbreaks and cost of vaccination amount to US$ 6.5 to 21 billion (Knight-Jones and Rushton, 2013). Additionally, FMD outbreaks in FMD-free countries cause an annual loss of more than US$ 1.5 billion (Knight-Jones and Rushton, 2013). FMD outbreaks in endemic countries are controlled by biannual vaccination. Both pre-vaccinated and post-vaccinated sera are collected and are subjected to Liquid phase blocking ELISA (LPBE) to measure and compare the protective antibody titer generated. Alternatively, the antibody titer can also be measured by using a gold nanoparticle (GNP)-based assay (Jain *et al.,* 2018). The antibodies generated after vaccination are considered to be protective and to prevent future outbreaks, although they do not provide sterile immunity.

In an endemic setting, along with the measurement of antibody titer, it is also necessary to measure the binding affinity of the antibodies to the antigen, particularly in the case of multiple vaccinated animals, where the booster dose is given every six months. This can be achieved by

E-mail: venusjain31@gmail.com; phone: +91-6398475233. #First and second authors have equal contribution.

Abbreviations: AI = avidity index; FMD = foot-and-mouth disease; FMDV = FMD virus; GNP = gold nanoparticle; LPBE = Liquid phase blocking ELISA; OD = optical density

developing an avidity ELISA to determine the strength of binding of antibodies to their corresponding antigen. The avidity of an antibody refers to the strength of its bond to antigen and is a measure of the overall strength of an antibody-antigen complex. In previous studies, for vesicular stomatitis virus and bovine viral diarrhea virus, the avidity of antibody has been found to be positively correlated with the neutralization titers of the antibodies in the virus neutralization test (Bachmann *et al*., 1997; Franco Mahecha *et al*., 2011). Avidity ELISA has also been applied for the assessment of heterologous protection against FMDV in cattle (Lavoria *et al*., 2012). The avidity of antibody responses against FMDV and its relationship with protection has not been investigated, although the idea of its relevance in complementing quantitative assessments has been already proposed in previous reports (McCullough *et al*., 1986; McCullough *et al*., 1992; Mulcahy *et al*., 1992; Scicluna *et al*., 2001).

In this regard, we hereby report the development of an avidity ELISA for measuring the protective immunity generated post-FMDV vaccination in multiple FMDV-vaccinated animals. We also report that avidity ELISA is a better approach than LPBE to measure the protective antibodies generated post-FMDV vaccination in multiple vaccinated field animals.

Materials and Methods

Serum samples. In this study, forty serum samples from multiple FMDV vaccinated animals were collected randomly from the field and tested for their avidity. The animals were already vaccinated by a trivalent inactivated FMDV vaccine (comprising of O, A and Asia 1 antigens) by the field veterinarians. The serum samples were collected after consent from the animal owners. Proper history of FMD vaccination was taken for the multiple FMDV vaccinated animals. Only samples showing LPBE titer more than 1.4, suggestive of strong antibody titer, were tested for their avidity. These serum samples were also tested by the GNP test for the antibody titer. One hundred negative samples were also tested to determine the cut-off value of the assay.

Gold nanoparticle test. The details of the GNP test developed have been given in our earlier publication Jain *et al.* (2018). In this study, the GNPs were synthesized by the chemical reduction method (Turkevich *et al*., 1951). First, a dot-blot assay was standardized and then, a dot-blot assay using GNP was standardized.

Antigen titration for avidity ELISA. FMDV antigen was titrated in two-fold dilutions from 500 ng/ml to 3.9 ng/ml. Both, antigen concentration and serum dilution were determined by the chequerboard titration method.

Serum dilution for avidity ELISA. The serum sample was diluted from 1:5 to 1:320. Serum dilution was determined by the chequerboard titration method.

Avidity ELISA. The ELISA plates (Nunc MaxiSorp™ flat-bottom) were coated with 50 µl of FMDV O antigen at the concentration of 250 ng/ml and incubated at 4 °C overnight. The next day, plates were washed three times with blocking buffer [Phosphate buffered saline (PBS) + 1% Normal Rabbit Serum (Sigma) + 0.2% Tween 20 (Sigma)]. Fifty microliters of serum sample diluted in blocking buffer at the 1:10 were added. Plates were kept at 37°C for one hour followed by three washings with blocking buffer. The antigen-and-antibody complex was treated with 50µl/well of a chaotropic agent (7 M Urea) in PBS for 20 minutes. The samples were tested in two rows, one treated with a chaotropic agent and the other one non-treated. The plates were incubated for one hour at 37°C and then washed three times with PBS. Subsequently, anti-bovine IgG conjugated with HRP (Sigma) at a dilution of 1:15000 (as per the manufacturer's recommended concentration) was added to the plates and kept for one hour at 37°C followed by three washings with PBS + 1% Normal Rabbit Serum + 0.2% Tween 20. At the end, chromogen/substrate mixture (50 µl/well) containing 5.05 mM ortho-phenylene-diamine dihydrochloride (Sigma) and 30% (w/w) hydrogen peroxide (Sigma) diluted at 1:2,000 was added and the color reaction was read at 490 nm in an ELISA plate reader (Tecan, Austria). The test was interpreted as AI.

$$AI = \frac{\text{Optical density (OD) of Urea treated samples x 100}}{\text{OD of Urea untreated samples}}$$

Cut-off value of avidity ELISA. The cut-off value of avidity ELISA was calculated by measuring the average AI of the hundred negative samples. The average value + 2SD was taken as the cut-off value. Samples having AI more than the cut-off value were considered as having high avidity antibodies and less than the cut-off were considered as having low avidity antibodies.

Results

Standardization of dot-blot assay using GNP conjugation

The detailed results of GNP test has been previously published in our paper Jain *et al.* (2018).

Avidity ELISA

Both antigen concentration and serum dilution were finalized by the chequerboard titration method.

Antigen titration: The optimization of antigen concentration for avidity ELISA using three negatives and three positive samples is shown in Fig. 1. The horizontal line shows the cut-off value of 0.54 for the assay. The X-axis depicts antigen concentration in ng/ml and Y-axis depicts optical density (absorbance). FMDV antigen was titrated in two-fold dilutions starting from 500 ng/ml

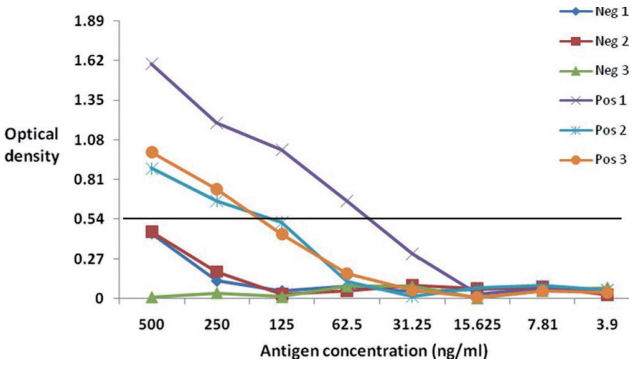

Fig. 1

Antigen titration for testing anti-FMDV antibodies in an indirect ELISA

Pos: Positive sample for anti FMDV antibody; Neg: Negative sample for anti-FMDV antibody.

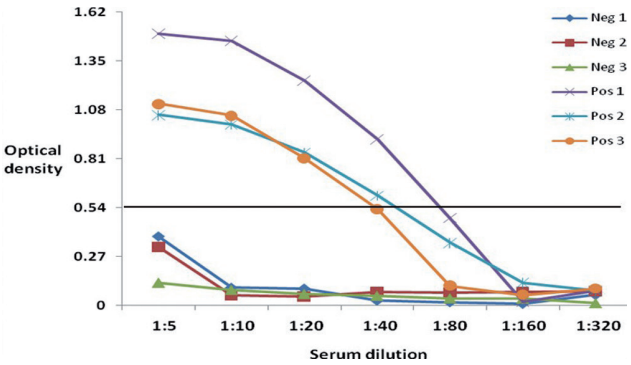

Fig. 2

Serum dilution of three positive and three negative samples for testing anti-FMDV antibodies in an indirect ELISA

Pos: Positive sample for anti FMDV antibody; Neg: Negative sample for anti-FMDV antibody.

to 3.9 ng/ml. The optimal concentration of antigen was chosen as the concentration showing the least optical density (OD) value in the negative samples without any significant drop in the OD of the positive samples (Fig. 1). The optimal concentration of antigen used for coating wells was 250 ng/ml.

Serum dilution. The optimization of serum dilution for avidity ELISA using three negatives and three positive samples is depicted in Fig. 2. The horizontal line shows the cut-off value of 0.54 for the assay. Serum dilution was chosen based on dilution of serum, in which the negative sample showed the least background OD and the positive sample was still detectable as positive (Fig. 2). The optimal serum dilution was 1:10 because, at dilution more than 1:10 the negative samples showed constant OD value.

Forty serum samples were collected from buffaloes that had been vaccinated several times against the FMD virus. The cut-off value was fixed at OD = 0.54 (mean OD value of 100 negative samples + 2SD). Only untreated samples (PBS wash) with an OD value over 0.54 (corresponding to LPB-ELISA titer over 1.4) were considered to calculate the AI.

Table 1. Avidity index and number of samples falling in the range of depicted avidity index

Avidity index	Number of samples	Percentage of total samples
<10	1	2.5
10-20	3	7.5
20-30	2	5.0
30-40	10	25
40-50	13	32.5
50-60	5	12.5
60-70	3	7.5
70-80	1	2.5
80-90	1	2.5
90-100	1	2.5

Cut-off value of avidity ELISA

One hundred negative samples were subjected to avidity ELISA and their AI was calculated. The mean AI was 25.96 with a standard deviation of 6. 40. The cut-off value calculated was mean + 2SD, which turned out as 38. 77. Therefore, samples showing AI less than 38.77 were considered as having low avidity and equal to or more than 38.77 as high AI.

AI of multiple vaccinated animals

Sixteen animals had a low AI and the remaining twenty-four animals had high AI (Supplementary data). One sample had AI less than 10, three samples had AI in the range of 10–20, two samples had AI in the range of 20-30, ten samples had AI in the range of 30–40, thirteen samples showed AI in the range of 40-50, five samples had AI in the range of 50-60 (Table 1). This was followed by three samples having AI in the range of 60–70. The remaining three samples had an AI of more than 70 (Table 1).

Discussion

Serology forms an important part of post-vaccination monitoring and post-outbreak sero-surveillance. Detection of pathogen-specific antibodies in the serum con-

firms present or past infection. Similarly, a strong titer of antibodies elicited post-vaccination is suggestive of a successful vaccination regime. Further evidence of the protective nature of elicited antibodies can be confirmed by virus neutralization test, which is time-consuming. A rapid means of measuring the generated immune response can be an avidity ELISA, which has been used for other diseases in the past (Bachmann *et al.,* 1997; Franco Mahecha *et al*., 2011; Lavoria *et al*., 2012).

The avidity of an antibody refers to the strength of its bonding with antigen and is related to the overall strength of bond between antigen-antibody sites. The avidity of antibodies can be measured by using bio-specific interaction analysis called Biacore SPR biosensors and this is considered to be the most accurate method. However, this method is costly, hence, needs sophisticated labs for conducting the test. Another method is "avidity ELISA", which involves the binding of the antigen-antibody complex followed by breaking of this bond using a chaotropic agent. Here, we have described the application of "avidity ELISA" to measure the protective action of antibodies generated after FMDV vaccination.

In earlier studies, an ELISA referred to as "bind and break" ELISA has been used (Narita *et al*., 1998; Motrán *et al*., 1999; Raviprakash *et al*., 2000; Yasodhara *et al.,* 2001; Dziemian *et al*., 2008). It uses a chaotropic reagent to break the bonds between antigens and their corresponding antibodies. The chaotropic reagent is used to break the bonds between the antigen and low-avidity antibodies. Thus the antigen and antibodies complex with strong bonding is quantified. The avid antibodies are measured and compared with the untreated control wells, which were not exposed to the chaotropic agent. The remaining more avid antibodies in the well are then measured and compared with the number of antibodies detected in a control well that was not exposed to the chaotropic reagent. The avidity ELISA correlates well with other measurements of avidity like Biospecific interaction analysis (Ward *et al*., 1994) and equilibrium dialysis (MacDonald *et al*., 1988).

In previous studies of some diseases caused by *Haemophilus influenzae* and *Streptococcus pneumoniae*, it has been documented that high avidity antibodies have been related to protective immunity (Schlesinger and Granoff 1992; Usinger and Lucas, 1999). Moreover, a positive correlation has been found between early memory T cell responses and the development of higher-avidity antibodies (Alam *et al*., 2013). Therefore, avidity ELISA can be a true indicator of protective immunity developed in multiple vaccinated animals.

In the present study, when the qualitative analysis of antibodies generated after multiple vaccinations was done by using avidity ELISA, few serum samples were found to have antibodies with low avidity (AI <38.77). Out of 40 samples tested, 16 serum samples showed low avidity with an AI ranging from 8.95 to 38.75 (Supplementarty data). Out of these sixteen samples, one sample showed an AI less than 10, three samples showed AI between 10 to 20, two samples showed AI between 20 to 30, and ten samples showed AI between 30 to 40. All these 16 serum samples showed high titer (>1.4) in LPBE. Despite a high LPBE titer, the AI of these samples was found to be low (<38.77%) (Supplementarty data). The remaining 24 serum samples showed an AI higher than 40. Out of these 40 serum samples, 13 samples showed AI ranging from 40 to 50, five serum samples showed AI between 50 to 60, three serum samples showed AI between 60 to 70, one sample showed AI between 70 to 80, one sample between 80 to 90, and last one sample between 90 to 100. In an earlier study it has been found that IgG class and avidity correlate better with protection than total FMDV antibody (LPBE) or *in-vitro* neutralizing antibody (Lavoria *et al*., 2012). In this work, 60% of samples showing high LPBE titer showed high avidity (>38.77%), whereas 40% of samples with high LPBE titer showed low avidity (AI <38.77%). Thus, no correlation was found between LPBE and AI.

The avidity of antibodies tested in ELISA has serious implications in the estimation of generated immune response and hence predicting the susceptibility of that population for getting FMDV infection. It may be helpful in predicting outbreaks when virus circulation is present and the population has low AI. Zones or areas having low avidity post-vaccination can be marked as having low herd immunity and continuous sero-monitoring may be done. The sero-surveillance using avidity ELISA may particularly be useful in the declaration of FMD-free status post-vaccination and may have a crucial role in Progressive Control Pathway for Foot and Mouth Disease (PCP-FMD) Stage 3 and 4.

Stage 3 involves a control strategy using vaccination and increasing the herd immunity against FMD. Measuring protective immune response after regular time intervals of FMD vaccination can impose proper monitoring of the immune status and preventing any anticipated FMD outbreak. Similarly, attaining zero incursion with vaccination also involves continuous monitoring of the immune status of vaccinated animals. In both cases, avidity ELISA can play a crucial role in monitoring the immune status of the vaccinated animals and designing disease-free zones.

FMD-free status with vaccination where animals have strong herd immunity is an important parameter to rule out the possibility of disease outbreak. In such a scenario, doing sero-surveillance by avidity ELISA will hold high promises to predict that herd immunity is not compromised. The avidity ELISA was developed as a more rapid test than virus neutralization test and LPBE to estimate

the protective immune response post FMD vaccination. We conclude that avidity ELISA can be used as a better approach for measuring the protection developed against FMD post-vaccination.

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Supplementary information is available in the online version of paper.

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