Performance characteristics of virus neutralization test (VNT) and liquid phase blocking ELISA (LPBE) and their relationship in the cattle immunized with trivalent foot and mouth disease vaccine

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Alternative to challenge.

Summary
Virus neutralization test (VNT) and liquid phase blocking ELISA (LPBE) are accepted tests for screening and as *in vitro* alternative to challenge in FMD vaccine potency testing. To replace VNT by LPBE for the screening of cattle, the optimized tests need to be first evaluated for their diagnostic performances. To replace it with LPBE in the absence of protection data, the interrelationship between VNT and LPBE have to be established to find out LPBE cut-off titer corresponding to the currently used VNT titers. Accordingly, VNT and LPBE were carried out using known negative (n = 306) and positive samples [Serotype O (n = 43), A (n = 14) and Asia1 (n = 11)], for the initial screening. The cut-off of < 1.5 $\log_{10}$ LPBE was comparable with that of < 1.2 $\log_{10}$ VNT titer for screening. LPBE was comparable to VNT in terms of specificity, sensitivity as shown by ROC curve and least varying (coefficient of variation 7.73% in LPBE *vs* 24.19% in VNT). Based on linear regression model using 471 bovine sera, the predicted LPBE titers corresponding to the currently used $\log_{10}$ VNT titers of 1.65, 1.5 and 1.5, were 2.24, 1.87 and 2.00 for O, A and Asia1, respectively. These LPBE titers hence can be used as cut-off titers for classifying cattle as protected or not protected until correlation based on *in vivo* challenge between protection and antibody titer is established.

Introduction

Foot and mouth disease (FMD) is one of the highly contagious virus infections of cloven-hoofed animals (Grubman and Baxt 2004). It adversely affects the productivity and profitability of food animal industry and the endemic countries are banned for trading animals or its products. Presently, the disease is globally widespread and highly prevalent in Asia and Africa (King and Henstock 2015). The causative agent of the disease, FMD virus (FMDV), belongs to the genus *Aphthovirus* of the *Picornaviridae* family. It has seven genetically and immunologically distinguishable serotypes named as O, A, Asia1, C and SAT1, 2, and 3. In India, serotype O, A, and Asia1 are prevalent and the current FMD vaccine targets these serotypes (PDFMD Annual Report, 11-16 2016).

Even if FMD in an endemic region could be limited through controlling the movement of animals and animal products from outbreak areas, mass vaccination using inactivated oil-adjuvanted vaccine containing all three representative strains of FMDV, type- O, A and Asia1 is indeed the mainstay tool for reducing the spread of the disease. In India, vaccination is included in the National FMD Control Programme (FMD-CP) (DADF Annual Report 17-18 2018). With the increase in coverage of FMD-CP, the quantity of vaccine required has significantly increased as well as the requirements for quality control of the vaccine. Among the various quality parameters, the potency of FMD vaccine assumes major importance as it has a direct influence on the herd immunity. As per Indian Pharmacopeia, 2018 (IPC 2018), and World Organization for Animal Health (OIE 2018), potency testing of FMD vaccine requires vaccination of FMDV seronegative cattle. Hence, it is essential to have a validated serological test to detect FMD free animals for vaccine testing.

The basis for the serological tests is that the FMDV exposure or vaccination induces detectable levels

of antibody response. With regard to screening of cattle, a positive/negative cut-off of 1.2 log_{10} in virus neutralization test (VNT) is recommended (OIE 2018) for certification of individual cattle. Since the results of serological tests vary between laboratories and strains of the virus, it needs to be established at each laboratory with a set of known positive/negative samples (OIE 2018). To date, the diagnostic performance of VNT has not been evaluated using Indian vaccine strains of FMDV.

In trivalent vaccine potency testing, animals are challenged with one serotype and clinical protection of 75% is set as a cut-off. For the other two serotypes, predicted VNT titers are used to assess the protection status. In India, the mandated VNT antibody titers for O, A and Asia1 are 1.68, 1.50 and 1.50 log_{10}, respectively for use in FMD-CP. Despite the fact that the VNT serves a gold standard for assessing the protection, it has many limitations including live virus handling, time-consuming, less robust, demands cell culture facility and expertise.

To overcome the limitations of VNT, various forms of ELISA (Hamblin *et al.* 1986, McCullough *et al.* 1992) are evaluated. Of these, liquid phase blocking ELISA (LPBE) (Hamblin *et al.* 1986) has been successfully employed and is considered as a reference methodology (OIE 2018). The test uses a constant amount of antigen that binds to FMDV antibodies in serially diluted sera and the leftover antigen is trapped in a sandwich ELISA. For screening of animals, an LPBE cut-off titer of 1.60 log_{10} is recommended by OIE. However, as with VNT, the LPBE cut-off titer for screening needs to be established for individual laboratories for each strain of FMD virus. At present, the LPBE cut-off titer for either screening or potency testing is not available for Indian vaccine strains. Determining the protective titer of LPBE demands a large number of challenge experiments. As an alternative, if a functional relationship between VNT and LPBE titers can be established, the LPBE protective titers can be deduced for the currently used VNT cut-off titers.

In the present report, we first evaluated the diagnostic characteristics of VNT and LPBE for using it as a screening test. Second, the quantitative relationship between the titers of VNT and LPBE was determined by linear regression to find the possibility of replacing VNT with LPBE for prediction of protection in FMD vaccine potency testing.

Materials and methods

Cells and viruses

Baby Hamster Kidney-21 (BHK-21) cells clone #13 maintained in Glasgow Modified Eagle Medium (GMEM) containing 10% each of tryptose phosphate broth and fetal calf serum were used to propagate the vaccine strains of FMDV (O/IND/R2/1975, A/IND/40/2000, and Asia1/IND/63/1972). The virus was harvested about 18 h post-infection when the cytopathic effect was maximum. The virus was clarified by centrifugation at 200 g for 10 min and stored at - 80°C use. For LPBE, inactivation of the virus was done by exposing it to 3 mM binary ethyleneimine for 24 h at 37 °C for two successive cycles. The inactive virus was aliquoted and stored at - 80°C.

Working control serum

High virus neutralizing (VN) titer, medium VN titer and FMDV negative sera having mean antibody titer (Log_{10} VNT titer) of 2.30, 1.88 and 0.9 for O, 2.60, 1.96 and 0.9 for A, 2.60, 2.19 and 0.9 for Asia1, respectively, were simultaneously tested with test samples to monitor the repeatability and authenticate the validity of results of the VNT and LPBE. The high and medium SN titer sera were collected from cattle vaccinated with inactivated trivalent FMD vaccine on day 28 post-vaccination (dpv). FMDV negative sera were collected from an apparently normal Hallikar breed of the bull calf of < 6 months with no history of either vaccination or infection. The FMD free status was confirmed by a negative result in 3ABC ELISA (Hosamani *et al.* 2015).

Reference negative serum

Sera from normal male cattle (n = 306) of Hallikar breed aged 6-9 months with no history of infection or vaccination and tested negative in the 3ABC indirect ELISA served as FMD negative samples to calculate diagnostic specificity.

Reference positive serum

Monovalent sera for the FMDV serotype O (n = 43), A (n = 14) and Asia1 (n = 11) were collected from cattle immunized with inactivated oil adjuvant vaccine containing FMDV O/IND/R2/1975, A/IND/40/2000 and Asia1/IND/63/1972 strains, respectively. The sera were collected between 14-130 dpv to serve as reference reagents for the optimization and monitoring of the diagnostic performance of the VNT and LPBE.

Trivalent vaccinate serum

Sera from the cattle (n = 471) vaccinated with inactivated oil adjuvanted/vectored FMDV vaccine expressing capsid protein of O, A and Asia1 vaccine strains were used as test samples to determine the functional equivalence between VNT and LPBE titers.

Virus neutralization test (VNT)

The virus neutralization test was performed as described (OIE 2018) using BHK-21 cells. Briefly, 100 $TCID_{50}$/50 µL of FMDV O, A and Asia 1, were incubated with 50 µL of 2 fold serially diluted heat inactivated test sera (0.9 to 3.1 log_{10}) in 96 well tissue culture plates (n = 2 wells/dilution) for 1 h at 37 °C. Then, 50 µL of BHK-21 cells (10^6 cells/mL) in GMEM containing 10% fetal calf serum was added to each well. After incubation for 48 h at 37 °C in 5% CO_2 incubator, wells were examined for cytopathic effect. Antibody titers were expressed as Log_{10} of the reciprocal of highest serum dilution required for neutralization of 100 $TCID_{50}$ of the virus in 50% of the wells. When a sample had a VNT value that was beyond the lowest (< 0.9 log_{10}) and highest dilutions (> 3.1 log_{10}), respective limits of detection (0.9 and 3.1 log_{10}) were considered as the VNT value.

Liquid phase blocking ELISA (LPBE)

Liquid Phase Blocking ELISA is modified form of sandwich ELISA in which the residual antigen, after blocking by antibody in the liquid phase, is captured and detected by serotype-specific anti-140S FMDV polyclonal serum (Hamblin *et al.* 1986, OIE 2018). Briefly, separate 96-well ELISA plates (Nunc-MaxiSorp, Denmark) were coated with serotype-specific (O, A, and Asia1) polyclonal anti-140S FMDV rabbit serum (50 µL/well) at a predetermined dilution in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. In perspex plates, test sera were serially diluted starting from 1/4 to 1/512. At each test, three working control sera as described in 'Working control serum' section, were included to monitor the performance of the assay. From the perspex plate, serum was transferred to separate FMDV-O, A and Asia1 non-binding deep well plates and equal quantity of respective antigen were added (which lead to final serum dilution as 1/8 to 1/1024), and incubated at 4 °C overnight. Next day morning, the coated ELISA plates were washed three times with washing buffer containing Tween-20 0.05% in phosphate-buffered saline and incubated with the antigen-antibody mixture from deep well plates (50 µL /well in duplicates for each serum dilution). The plates were then incubated for 1 h at 37 °C. The plates were washed as before and detector antibody (anti-140S FMDV guinea-pig antibody) in predetermined dilution in blocking buffer was added to the type-specific plates. After 1 h incubation at 37 °C, plates were washed again and 50 µL/well of rabbit anti-guinea pig immunoglobulin-HRPO conjugate (Dako, Catalogue # P0141, Denmark) were added to the wells. The plates were washed after 1 h of incubation and substrate solution (10 mg of orthophenylene diamine and 8 µL of 0.05% H_2O_2 per 20 mL of 0.1 M citrate-phosphate buffer pH 5.0) was added to each well. After 15 min of incubation at 37 °C, the reaction was stopped by adding 1 M sulphuric acid. The optical density (OD) of each well was read at 492 nm using a spectrophotometer (Tecan Infinite-F50 Absorbance Microplate Reader, Männedorf, Switzerland), and the percent age of inhibition (PI) was calculated. The endpoint was defined as log_{10} of that dilution at which half of the wells showed 50% inhibition compared to the 0% inhibition observed in the antigen control.

Statistical analysis

Receiver operating characteristic (ROC) curve was constructed using the reference sera (positive and negative) in pROC package (Robin *et al.* 2011) and ggplot2 package (Wickham 2009) of R Programming language (Version 3.1.1-"Sock it to Me") (R Development Core Team 2014). The cut-off titer for VNT and LPBE was determined based on the Youden Index (Fluss *et al.* 2005) to find out the diagnostic sensitivity and specificity of VNT and LPBE. The area under the ROC curve was analyzed by the Z test. Significance was set at 95%. A simple linear regression model was fit by regressing the titer of LPBE (Y) on VNT (X) of test sera.

Results

Performance characteristics and repeatability of VNT and LPBE

The performance characteristics of VNT and LPBE for the three serotypes of FMDV are presented in the form of ROC curve (Figure 1A to C). No significant differences were found in the area under the curves between VNT and LPBE for any of the serotypes [serotype O ($p = 0.937$), A ($p = 0.37$) and Asia1 ($p = 0.733$)]. Youden index based cut-off estimation revealed that < 1.2 and < 1.5 log_{10} titer in VNT and LPBE, respectively, was suitable across all 3 tested serotypes with high sensitivity and specificity (Table I). The VNT and LPBE using monovalent vaccinate serum against homologous and two other heterologous vaccine virus serotypes indicated that they were serotype specific and titers against heterologous serotypes were at least 2 fold less than that of the homologous serotype (Table II). The cross-reactivity was least for VNT and minimum for LPBE. The VNT titer results were valid as virus titration and back titration results of FMDV vaccine strains were within 0.5 Log_{10} differences on either side of 2.0 Log_{10} $TCID_{50}$. The repeatability of VNT and LPBE as assessed with high titer, medium titer, and negative working control sera indicated that the inter-day variation did not exceed 2 standard

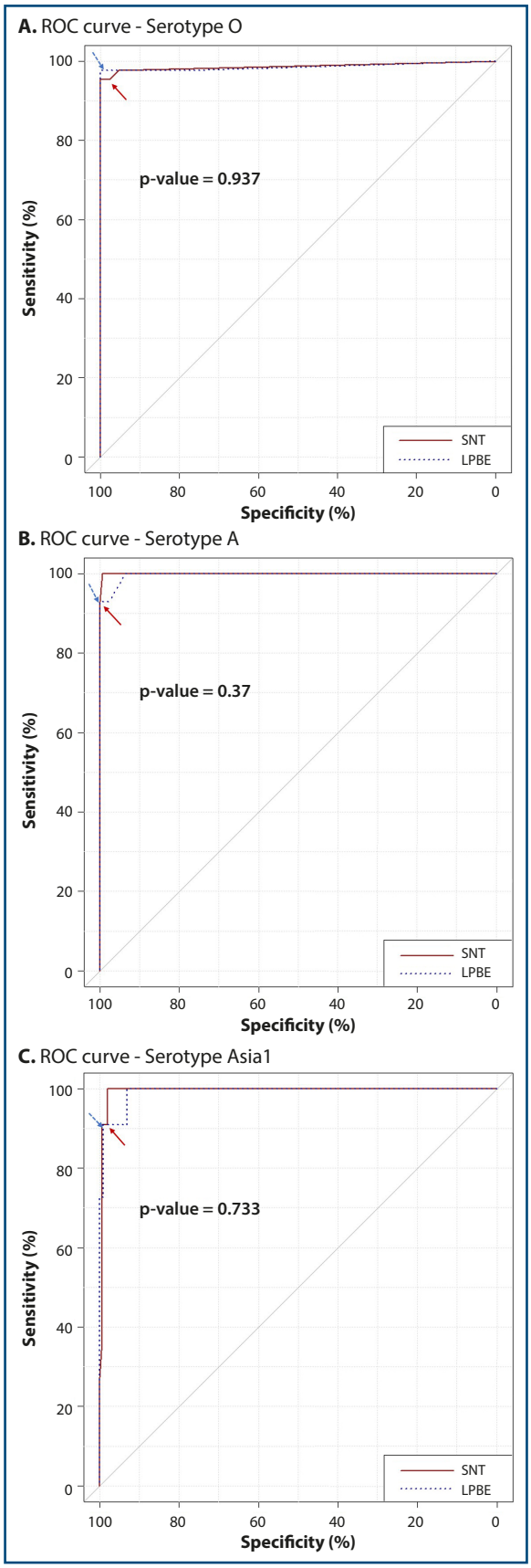

Figure 1. *Receiver operating characteristic (ROC) curves for virus neutralization test and Liquid Phase Blocking ELISA for detecting Foot and mouth disease virus antibodies.* **A.** Serotype O **B.** Serotype A and **C.** Serotype Asia1. X axis indicates the false positive rate while Y axis indicates the true positive rate. The arrow indicates the cut-off titers.

deviations (SD) from the mean titre on 95% of the tested occasions. This extent of less variation around mean titer indicates that the results are reliably reproducible (Table III).

Relationship between VNT and LPBE titers

Comparison of LPBE titers with that of VNT indicated that the LPBE titers were higher with a significant ($p < 0.0001$) positive correlation (Spearman's rho $\rho = 0.71$, 0.76 and 0.76, for FMDV O, A, and Asia1, respectively). A simple linear regression model was fitted using VNT titers as the independent variable and LPBE titers as a dependent variable to predict LPBE titers from VNT titers. Model coefficients indicate that the VNT titers are highly significant ($p < 0.001$) for serotype O, A and Asia1 (Figure 2A, B, and C) as compared to intercept only model. The LPBE titers corresponding to the currently used cut-off VNT titers of 1.65 and 1.51 was predicted to be 2.24, 1.87 and 2.00 and for O and A and Asia1, respectively (Table IV) from the parameter estimates. Though the confidence interval was narrow, the predictive interval (Figure 2A, B, and C) was wider with a coefficient of determination ranging from

Table I. *Diagnostic sensitivity, specificity, and accuracy of virus neutralization test (VNT) and Liquid Phase Blocking ELISA (LPBE) for the three serotypes of Foot and mouth disease virus.*

Test	Cut-off titer (Log_{10})	Diagnostic sensitivity (%)			Diagnostic specificity (%)			Accuracy (%)		
		O	A	Asia1	O	A	Asia1	O	A	Asia1
VNT	< 1.2	95.4	92.9	90.9	97.7	100.0	99.4	97.4	99.7	99.1
LPBE	< 1.5	93.3	92.9	90.9	99.0	100.0	99.0	98.9	99.7	98.7

Table II. *Serotype specificity of virus neutralization test (VNT) and Liquid Phase Blocking ELISA (LPBE) using monovalent post-vaccinate reference sera raised against Indian Foot and mouth disease vaccine strains.*

Monovalent serum	Animal ID	Test	FMD virus		
			O/IND/ R2/1975	A/IND/ 40/2000	Asia1/IND/ 63/1972
O/IND/ R2/1975	BC 889 21 dpv	VNT	**1.50**	< 0.9	< 0.9
		LPBE	**1.65**	< 0.9	1.34
	BC 927 21 dpv	VNT	**1.95**	< 0.9	< 0.9
		LPBE	**2.26**	1.20	1.34
A/IND/ 40/2000	BC 830 28 dpv	VNT	< 0.9	**1.95**	< 0.9
		LPBE	1.65	**1.95**	1.04
Asia1/ IND/63/1972	BC 809 21 dpv	VNT	< 0.9	< 0.9	**1.50**
		LPBE	< 0.9	< 0.9	**1.95**
	BC 914 21 dpv	VNT	1.04	< 0.9	**1.65**
		LPBE	< 0.9	< 0.9	**1.65**

Figures in bold indicate the serotype specific reactivity.

Table III. *Repeatability of Liquid Phase Blocking ELISA (LPBE) (n = 25) in comparison with virus neutralization test (VNT) (n = 20).*

Serum ID	Test	Serotype O			Serotype A			Serotype Asia1		
		Mean	S.D.	CV (%)	Mean	S.D.	CV (%)	Mean	S.D.	CV (%)
BC # 41*	VNT	2.31	0.35	15.15	2.60	0.20	7.69	2.61	0.34	13.03
	LPBE	2.90	0.08	2.76	2.90	0.08	2.76	2.99	0.05	1.67
BC # 55**	VNT	1.91	0.32	16.75	1.90	0.26	13.68	2.15	0.52	24.19
	LPBE	2.39	0.15	6.28	2.20	0.17	7.73	2.47	0.18	7.29
BC # 66***	VNT	0.90	0.00	0.00	0.90	0.00	0.00	0.90	0.00	0.00
	LPBE	0.92	0.06	6.52	0.93	0.08	8.60	0.96	0.10	10.42

*High VNT titer; ** medium VNT titer; ***negative working control serum; S.D. = Standard deviation; CV (%) = Co-efficient of variation.

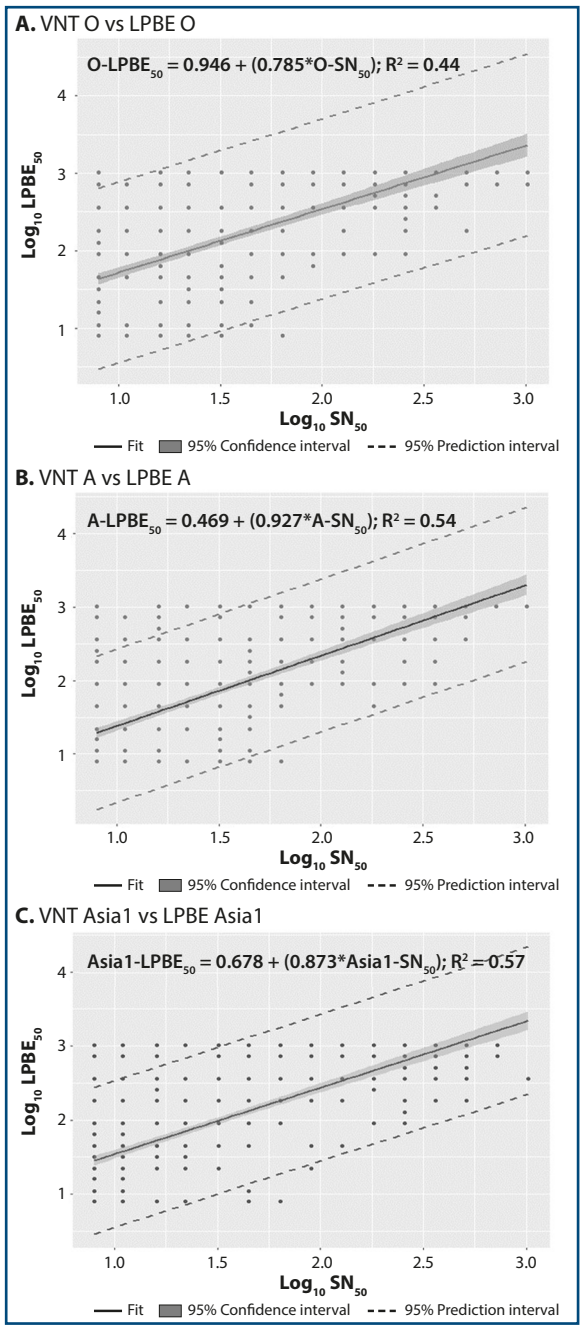

Figure 2. *Regression of LPBE titer on virus neutralization test (VNT) titers for the Foot and mouth disease virus serotypes O, A and Asia 1 (**A-C**): model co-efficient and coefficient of determination (R2) are given for each serotype.*

0.44 to 0.57 indicating moderate precision of the regression model.

Discussion

To assess the immune response to FMD, a robust reproducible test with high accuracy is essential. Hence, before regressing LPBE titers on VNT titers, we investigated the diagnostic performance characteristics of the test. The cut-off of < 1.5 $\log_{10}$ titer in LPBE was comparable with that of < 1.2 $\log_{10}$ titer of VNT for detecting animals with FMDV antibodies (Figure 1A to C). The accuracy (%) of LPBE was 98.9, 99.7 and 98.7 for O, A and Asia1, respectively (Table I). Similar cut-off, sensitivity, and specificity values were obtained for diagnostic LPBE, when the test was standardized for estimating the herd immunity in large scale surveillance programme in South America and Europe (Periolo *et al.* 1993, Smitsaart *et al.* 1998, Van Maanen and Terpstra 1989). The optimized LPBE was more sensitive in detecting FMD positive animals as the test also measures non-neutralizing antibodies. Repeatability of LPBE was superior to that of VNT as indicated by the coefficient of variation (max. 7.73% in LPBE *vs* 24.19% in VNT; Table III), which is below the acceptable limit of 10% (Jacobson 1998). This confirms the previous findings that LPBE is reproducible (Hamblin *et al.* 1986, Maradei *et al.* 2008) and can be used for screening.

A significant moderate positive correlation [Spearman correlation coefficient ρ = 0.71, 0.76 and 0.76, ($p < 0.0001$)] was found between LPBE and VNT titers for FMDV serotypes O, A and Asia1, respectively. The results agree with the findings on lower correlation coefficient (0.75, 0.78, 0.67 and 0.81) between LPBE and VNT titers, when serum from cattle vaccinated with oil-adjuvanted polyvalent vaccine containing O1/Caseros, A/Arg/79, A/Arg/87, C3/Arg/85 (Smitsaart *et al.* 1998) viruses was used. In contrast, higher Pearson's correlation coefficients were reported by two different laboratories for monovalent O1/BFS, A10/Holland (0.91) (Van Maanen and Terpstra 1989) and

Table IV. *Predicted $Log_{10}LPBE_{50}$ $Titer_{50}$ (95% Confidence Interval) for each Log_{10} SN_{50} Titers based on linear regression*[$].

$Log_{10}SN_{50}$	$Log_{10}LPBE_{50}$ Titer		
	O	A	Asia1
0.90	1.65 (1.58-1.72)	1.30 (1.24-1.37)	1.46 (1.40-1.53)
1.04	1.76 (1.70-1.83)	1.43 (1.37-1.49)	1.59 (1.53-1.64)
1.20	1.89 (1.83-1.95)	1.58 (1.53-1.64)	1.73 (1.68-1.78)
1.34	2.00 (1.94-2.05)	1.71 (1.66-1.76)	1.85 (1.80-1.90)
1.51	2.13 (2.08-2.18)	**1.87 (1.82-1.92)**	**2.00 (1.95-2.04)**
1.65	**2.24 (2.19-2.30)**	2.00 (1.95-2.05)	2.12 (2.07-2.17)
1.81	2.37 (2.31-2.43)	2.15 (2.09-2.20)	2.26 (2.21-2.31)
1.95	2.48 (2.41-2.54)	2.28 (2.22-2.33)	2.38 (2.33-2.44)
2.11	2.60 (2.53-2.67)	2.43 (2.36-2.49)	2.52 (2.46-2.58)
2.26	2.72 (2.64-2.80)	2.56 (2.49-2.64)	2.65 (2.58-2.72)
2.41	2.84 (2.75-2.93)	2.70 (2.62-2.77)	2.78 (2.71-2.86)
2.56	2.96 (2.86-3.06)	2.84 (2.75-2.94)	2.91 (2.83-3.00)
2.71	3.07 (2.96-3.18)	2.98 (2.88-3.08)	3.05 (2.95-3.14)
2.86	3.19 (3.07-3.31)	3.12 (3.01-3.23)	3.18 (3.07-3.28)
3.01	3.31 (3.18-3.44)	3.26 (3.14-3.38)	3.31 (3.20-3.42)

[$]Regression model: $Y = a+bX$; where, $Y = Log_{10}LPBE_{50}$ Titer; a = intercept; b = slope; $X = Log_{10}SN_{50}$.

O1/BFS, A5(FRA1/68) (> 0.83) (Hamblin *et al.* 1986). Despite the differences between the laboratories, it is clear that higher the VNT titers, higher the value of observed LPBE titers. A possible reason behind the moderate correlation in our study might be due to the presence of more samples in lower VNT titer classes (0.9-1.34), which showed increased variation in LPBE.

Examination of predicted LPBE titers against serotype O for different classes of VNT titer showed that the initial LPBE titers were 5 times higher than VNT titers that ranged from 0.9-1.04. For the VNT titer classes 1.2-1.65 and 1.81-2.56, LPBE titers were 4 and 3 times higher, respectively (Table IV). In the case of serotype Asia1, the predicted LPBE titers are 3 times higher in lower and medium VNT titers. However, in the case of serotype A, all predicted LPBE titers were within 2 times of VNT titers. Predicted LPBE titers were within the limit of 2 times of VNT titer above 2.71, irrespective of serotype analyzed, which may be due to saturation of antigen by antibodies. Though exact reasons for differences in fold increase in LPBE titers are not known, the relatively high antigenic mass of serotype O virus in FMDV trivalent vaccines and/or differences in the relative abundance of neutralizing *vs* non-neutralizing antibodies between serotypes and individual variation in immune response might be hypothesised. Our results indicate that it is not possible to set a common cut-off for all the three serotypes of FMDV to replace the challenge based potency testing and assessing the herd immunity.

For serotype O, the predicted LPBE titers for a mandated cut-off of 1.65 Log_{10} VNT titer was 2.24 Log_{10} LPBE. However, a lower LPBE titer of 1.65 and 1.95 was set as the cut-off for serotype O in FGBI-ARRIAH and CODA-CERVA, respectively (Willems *et al.* 2012). In our study, the LPBE cut-off was deduced to be 1.87 and 2.00 for serotype A and Asia 1, respectively. An LPBE cut-off titer of 2.20 (CEVAN) and 2.11 (VAR) has been reported for serotype A (Mattion *et al.* 2009). This variation could be attributed to the methodological differences between the laboratories while optimizing the LPBE and statistical models to determine the cut-off. Setting a higher cut-off in LPBE especially for FMDV serotype O and A is also favorable to maximize the probability of protection. These viruses, in fact, give rise to frequent generation of antigenically and phylogenetically different variants as a result of continuous circulation in endemic countries like India.

It is concluded that VNT and LPBE with a cut-off of < 1.2 and < 1.5, respectively, can be used for screening of the cattle for FMD antibodies. Further, a positive relationship exists between the titers of VNT and LPBE for the FMD serotypes O, A and Asia1. A cut-off in the range of 2.24,1.87 and 2.00 log_{10} LPBE titer corresponds to the protective VNT titer of 1.65 and 1.51 Log_{10} for O and A and Asia1, respectively, despite the fact that the predictive precision is moderate. This cut-off could be used for classifying the cattle as protected and not-protected till the logistic regression based correlation of protection with antibody titer is established and validated.

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