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Original Article

Investigation of the Relationship between Virus Neutralization Test and ELISA with Challenge Test in Evaluation of FMD Vaccine Potency

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Abstract

Background and Aims: Foot-and-mouth disease (FMD), is a highly infectious and contagious disease in livestock. An effective and efficient vaccine is needed to Control FMD and reduces the associated damage. The goal of this study was to find out if the protective dose or challenge (PD50%) could be used instead of the antibody titer method to measure the effectiveness of the FMD vaccine. To achieve this, several calves were selected and divided into four groups. The vaccine was administered in three doses: a full dose, a 1/3 dose and a 1/9 dose. Twenty-one days after vaccination, all animals were challenged with a 10,000 LID50% virus in the tongue epithelium. For 7 days after, the animals were evaluated and monitored for the appearance of FMD symptoms. The PD50% (Protective Dose) for each vaccine against the virus was determined in the experiment. After obtaining the VN results that indicated the antibody titer and the PD50% level, a comparison was made between these two parameters. By examining 5 test cases, a formula was derived that accurately determined the PD50% with a high degree of precision using the VN50% result. This study determined the constants for A and O types using the VN50% test results. By incorporating these values into the derived formula, the PD50% level could be determined.

Keywords: FMD, serum neutralization test, inactivated FMD vaccine, challenge test, determination of protective dose.

Introduction

Foot-and-mouth disease (FMD), poses a significant obstacle to animal health and production causing severe of economic damages. Vaccines for immunizing susceptible animals against FMD have been a crucial and effective tool for many years. A suitable and effective vaccine is essential to Control FMD. The challenge test is the most important and reliable method for evaluating the effectiveness of FMD vaccines (1, 2, 3). In this test, animals are vaccinated with different vaccine dilutions and, after 3 weeks, injected with the FMD virus to assess their resistance to occurrence of disease symptoms. The ultimate goal is to determine the PD50% or the percentage of protection achieved. Another test for FMD vaccine evaluation is the serum neutralization test, which involves taking blood from vaccinated animals, isolating the serum, and testing its ability to neutralize the live virus in vitro (4, 5, 6, 7, 8).

Since the challenge test is performed in an in vivo environment using host animals (cows), it incurs significant costs. Moreover, as the vaccine belongs to the category of inactivated or killed vaccines, it must be evaluated within short intervals, making each test expensive. In this study, we aimed to simultaneously perform the challenge test and VNT and compare the results to determine if the VNT can be used to determine the PD50% of FMD vaccine for A and O virus types instead of the challenge test. We hope to derive a formula that can accur-

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ately calculate the protective dose using the VNT results for the two mentioned virus types, thus avoiding the high costs and the need for animal experimentation and slaughter (9, 10).
To date, no well-established and specific research has been conducted in this regard within the country. VNT and challenge tests have been scattered without a research-based approach to establish a correlation between these two tests.

Methods and Materials

In this study, 5 challenge tests and one repetition for each were used.

Preparation of Different Types of FMD Viruses in BHK Cell Culture
FMD viruses of types O2016, A15, A05, O2010, and Asia were propagaated in BHK cells and used as the virus strains in VN-ELISA, and challenge tests, as well as for determining the PD50%.

Adaptation of Prepared Viruses to Calf and their Titer Determination
For challenge test, it was necessary to adapt the viruses to the calves. This was achieved by injecting the isolated virus into the epithelium of the calf's tongue by Intradermolingual rout. After injection, within 24 to 48 hours, the animal's condition, including body temperature, the extent of lesions in the tongue and gums, and the presence of blisters and lesions in the Interdigital region, was examined and recorded on specific forms.
If three out of four legs show signs of blisters, it indicates a generalized infection with the virus in the animal's body. If these symptoms were not observed, second passage was done. The epithelial layer was harvested by sterile forceps.
The samples were suspended in PBS - Glycerin buffer. After grind in pestle mortar, the suspension was centrifuged at 1000g for 15 minutes. After centrifugation, the supernatant is carefully removed, and the remaining solution was passed through a 0.45-micron filter and store in -20°C.

Determination of the Adapted Virus Titer on the Calf Tongue (Median Lingual Infective Dose, LID50%/ml)
Two healthy calves was selected, and the tongue of each calf was divided into three parts by Indian ink. To determine the approximate boundaries of these parts, a mixture of Indian ink was injected intra dermo lingually into the calf's tongue. Different dilutions of the virus (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) are then injectted into each part. The virus titer was calculated based on the number of blisters formed on the tongue using the Reed and Muench method.

Injection of FMD Aqueous Vaccine with Different Doses
For each challenge test, 17 healthy calves of 9-12 months and free of anti-virus antibodies were needed.
The selected calves were divided into three groups of five and one group of two. The vaccine was injected into the three groups of five calves at the following doses: full dose, 1/3 dose 1/9 dose. The control group did not receive the vaccine but was injected with full dose amount of

Blood Sampling for VNT and ELISA Test
Blood sampling from the different groups of animals on days 7, 14, and 21 after vaccine injection. Injection was achieved VNT and ELISA competitive test was conducted to measure the antibody titer against FMD virus and the level of protection.

Challenge with FMD Virus Inoculation into the Calves Tongues Epithelium
After 21 days from vaccine injection, adapted viruses with a titer of 105 /ml are injected in tongue Epithelium Intradermolingually rout to all calves at a volume of 100 microliters separated by type, using a 23-gauge needle. Isolation challenge was performed for each virus type.

Monitoring of Challenged Calves
After injecting the virus into vaccinated and control group, they were monitored during 7 days for FMD symptoms.

The symptoms are recorded in the corresponding form every day. According to the number of protected and unprotected animals in different groups, PD50% was calculated from the following formula.

Antilog [(Protected Animal /100) - 0.5 x Log of vaccine dilution] = PD50%

In order to calculate the protective dose through the amount of antibody in the VN test, the following formula defined by the Foot and Mouth Reference laboratory in Pirbright, England were used.

Log (VN90) = 0.923 log (PD50) + PA50

PA50 = 0.70 for O type

PA50 = 0.54 for A and Asia types

PD50: Serum titre corresponding to 50% protection

VN90: mean serum titre of calculated antibody response to undiluted vaccine

According to the calculation of 50% in the routin VN test, the following formula is suggested by Present research.

Log (VN50)= 0.923 log (PD50) + PA50

PA50= 1.3 for type O

PA50 = 0.95 for types (A) and (Asia)

Using the results obtained from challenge tests and VNT, the PD50 value and serum titers are compared.

Results

Titers of adapted viruses (O2016, A15, A05, O2010 ,Asia) on calf tongue are showed in Table 1 .

Table 1: the titers of the adapted viruses on calf tongue

Virus	A15	O2016	O2010	Asia	A05
Lingual infective Dose (LID50)/100ul	$10^{6.1}$	$10^{5.84}$	$10^{6.17}$	$10^{6.1}$	$10^{5.51}$

Results of Injecting the Virus into Vaccinated Calf Tongues

The results of injecting the virus into vaccinated calf tongues were evaluated up to one week after the intradermolingual injection.

The examination included assessing symptoms, potential lesions on the tongue, gums and legs, temperature, and the overall condition of the animals. The evaluation began with the full dose group, the 1/3 and 1/9 dose groups, and finally the control group. In the control group, it was ensured that at least three out of four legs were affected; showing signs of blisters and wounds, and the temperature was above 40 degrees Celsius .

After 48 hours, the animals exhibited complete generalization of the disease symptoms, with 3 feet out of four showing vesicles and a recorded body temperature of 40 degrees Celsius. An example of data for challenge test and determination of PD50% (Protective Dose) Have been shown in Table 2 .

Table 2: Percentage of protection against the O 2016 virus at different vaccine dilutions.

Grouping	Animal No.	Protect	Protect Total	Protect %
Full Dose Vaccinated	423	+	4/4	100
	412	+		
	411	+		
	414	+		
1/3 Dose Vaccinated	416	+	3/4	75
	413	+		
	422	+		
	419	_		
1/9 Dose Vaccinated	418	_	2/4	50
	421	+		
	420	+		
	424	_		

Calculation of PD50/Dose based on the method of the World Reference Center for FMD (Pirbright) Against virus type O2016 according to data from table 2:

Total % Protected = 225/100 = 2.25

2.25 - 0.5 = 1.75

Proportional distance = 1.75 × Log interval = 1.75 × 0.47 = 0.822

Antilog 0.822 = 6.63

PD50/Dose = 6.63

the amount of protective dose against virus types based on challenge test have been shown in Table 3 .

Table 3: PD50 for Vaccine against different FMD virus types.

Virus used for challenge	A15	O2016	O2010	Asia	A05
PD50 / Dose	6.29	6.63	1.38	5.07	7.76

Serum Neutralization assay results (antibody titer) are presented in Table 4 to 8.

Table 4: Mean serum titers of animals against type A15 challenge after 21 Days post vaccination.

TIME*	Full Dose	1/3 Dose	1/9 Dose	Control
0	0.55	0.74	0.56	0.6
7	1.24	1.02	0.93	0.6
14	1.27	1.15	0.91	0.8
21	1.67	1.8	1.635	0.85

*Days after vaccine injection

Table 5: Mean serum titers of animals against type O2016 challenge after 21 Days post vaccination.

TIME*	Full Dose	1/3 Dose	1/9 Dose	Control
0	0.5	0.84	0.8	0.7
7	1.35	1.12	1.15	0.95
14	1.41	1.2	095	0.8
21	2	1.95	1.65	0.95

*Days after vaccine injection

Table 6: Mean serum titers of animals against type O2010 challenge after 21 Days post vaccination.

TIME*	Full Dose	1/3 Dose	1/9 Dose	Control
0	0.6	0.8	0.8	0.7
7	0.9	0.85	0.93	0.9
14	1.19	0.9	0.91	0.8
21	1.35	1.15	0.935	0.75

*Days after vaccine injection

Table 7: Mean serum titers of animals against type A05 challenge after 21 Days post vaccination.

TIME*	Full Dose	1/3 Dose	1/9 Dose	Control
0	0.8	0.9	1.01	0.6
7	1.35	1.25	1.05	0.8
14	1.41	1.05	1.15	0.8
21	1.78	1.95	1.65	0.9

*Days after vaccine injection

Table 8: Mean serum titers of animals against Asia type challenge after 21 Days post vaccination.

TIME*	Full Dose	1/3 Dose	1/9 Dose	Control
0	0.55	0.74	0.85	0.6
7	1.15	1.05	0.95	0.75
14	1.25	.105	0.95	0.9
21	1.6	1.35	1.25	0.85

*Days after vaccine injection

The level of serum protection at Full Dose groups demonstrated by vaccinated animals against the target viruses in the ELISA test are presented in Table 9.

Table 9: Competitive ELISA test results for antibody titer in vaccinated animals in Full Dose groups.

% Inhibition in competitive ELISA in full dose vaccine					
A15	A05	Asia	O2016	O2010	Time
18	14	12	17	15	**Before vaccination**
58	68	35	67	21	**Day 7 post-vaccination**
67	72	65	73	54	**Day 14 post-vaccination**
89	98	91	97	68	**Day 21 post-vaccination**

Correlation between Challenge Test and Serum Titer Based on VNT

In Pirbright's formula, VN 90% was used, but in this study, VN 50% was used, and according to this change, the amount of PA constant for VN 50% is as follows:

Log (VN50) = 0.923 log (PD50) + PA50

PA50 = 0.95 for types (A) and (Asia)

PA50 = 1.3 for type O

Comparison of the PD50% according the challenge tests and calculated with VNT90% and VNT50% have been shown in Table 10. The numbers used in this formula were obtain from Full Dose groups on day 21.

Table 10.

Challenge No. (V. type)	Based on challange	Based on VN 90%	Based on VN 50%
1 (A15)	6.29	6.17	6.02
2 (2016)	6.63	6.12	5.75
3 (O2010)	1.38	1.25	1.13
4 (A05)	7.76	7.54	7.76
5 (Asia)	5.07	4.93	5.01

Discussion & Conclusion

In fact, the measurement of the amount of antibody from vaccinated animals indicates the quality of the vaccine, which in the case of foot and mouth virus should be examined against types and sub types separately. This means that the presence of sufficient antibodies against one type or subtype cannot indicate the

presence of sufficient antibodies against subtypes of the same type. For example, the presence of sufficient antibody against A13 virus does not necessarily confirm sufficient antibody against A15 (3).

Currently two methods used for measure the number of antibodies against foot-and-mouth disease virus., serum neutralization test and ELISA. VN test methods have a direct relationship with the quality of the vaccine. This means that the increase in the amount of antibody obtained from the vaccine indicates the quality of the vaccine. In most of the articles the VN test is one of the most important and effective methods, followed by the ELISA test (11). The protection level of the FMD vaccine will be determined by challenge test. The amount of the dose by which the animals show their resistance against the disease. In any case, the level of immunity is related to different factors, including Race, individual differences in terms of immune system and maternal antibody (12).

In this research, the challenge test and VN have been examined and compared, and answer the question is it possible to use the challenge test instead of VN for determining the PD50%, so decreasing the cost of providing animals, and also to observe the ethics of working with animals .The protective titer of foot-and-mouth disease vaccine against Field virus in terms of VN test is 1.2 based on the logarithm of 2 in references (13). According to the opinion of McCullough and colleagues in 1992, the antibody titer below 1/16 serum dilution relative but incomplete immunity and above 1/16 titer is complete Immunity.

The World Reference of Foot and Mouth Disease, located in Pirbright, England, has developed a relationship between VN 90%, protective percentage and PD50, according to which the PD50 or % Protective dose 50 of the vaccine can be determined from the antibody titer in the serum (14).

In this formula, there is a fixed number that is different for each type that each vaccination center must obtain for itself. This formula will be obtained based on experience and doing many challenges.

In this way, one can calculate the PD50 for all serotypes with a VN test and measure the antibody titer against all serotypes, and there is no need to perform a challenge test with that high cost and time and risks of working with animals

In Pirbright's formula, VN 90% was used, but in this study, VN 50% was used, and according to this change, the amount of PA constant for VN 50% is as follows:

Log (VN50) = 0.923 log (PD50) + PA50

PA50= 0.95 for types (A) and (Asia)

PA50 = 1.3 for type O

Results of comparison of the challenge PD50% with Formula (VN50%) PD50% (Table 10), shows the value are almost in the same range and the number calculated by the formula is a little lower than the number obtained in the challenge test, which indicates the strictness of this formula.

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Conflict of Interest

No conflict of interest is declared.

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Ethics Approval and Consent to Participate

Not applicable.

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