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RESEARCH ARTICLE

Serum Neutralization Titers and Protective Efficacy Induced by Foot and Mouth Disease Virus Inactivated Vaccine with different 146S Particles Concentrations in Cattle

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ABSTRACT

Foot and Mouth Disease (FMD) vaccine evaluation required viral challenge of vaccinated cattle. Difficulties of potency testing include finding animals FMDV antibodies free and the challenge should be conducting in high containments. Alternative approaches, such as Serum Neutralization Test (SNT) and antigen concentration (146S) were developed. In this study, antigen dose, SNT, and animal challenge were evaluated after a single dose. Three vaccine batches with different antigen doses of inactivated trivalent FMD vaccine prepared from Egyptian strains (serotypes A, O, and SAT2) were evaluated. Calves 6-8 month old were vaccinated with 2ml of the vaccine, sera collected at 28 days post vaccination and animals were challenged with 10^4 BID_{50} of homologous FMDV strains via intra-dermolingual route. All the antigen doses induced SN protective titers with the exception of one animal in each of the serotype O batch-2 (3.1µg/dose) and serotype SAT2 batch-1 (2.7µg/dose) which showed 0.9 log10 SN titers. All animals were completely protected against challenge with serotype A in all tested batches. Serotype O batch-1 and 3 (2.5 and 5µg/dose) induced a complete protection; however, batch-2 (3.1µg/dose) showed 80% protection. In serotype SAT2 challenge, the lowest dose of 2.7µg/dose showed 80% protection meanwhile other doses of 3.3 and 5.4µg/dose completely protected the animals. In both serotypes O and SAT2, feet lesions were observed in the calves that showed the lowest SNT levels (0.9log10). In conclusion, the study results indicate that 146S particles concentration (3.3, 2.5 and 1.4 µg) for serotypes SAT2; O and A, respectively can be used for vaccine formulation. Additionally, both SNT and 146S particles concentration could be suggested as an alternative method for vaccine evaluation.

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INTRODUCTION

Foot and Mouth Disease (FMD) is an economically devastating disease of livestock. Although, vaccines are available since 1900s, the FMD inactivated vaccines still used for FMD eradication from parts in the world, the disease still affecting millions of animals around the world. The FMD remains a major economic concern for livestock-health in endemic countries and a continued threat to disease free countries (Boklund *et al*., 2013) and remains the main sanitary barrier to commerce of animals and animal's products (Depa *et al*., 2012; Ashfaq *et al*., 2015; Jamil *et al*., 2015; Zhang *et al*., 2015).

Foot and Mouth Disease virus (FMDV) is a small positive sense ssRNA virus (approx. 8.3kb) which belongs to the aphthovirus genus of the family picornaviridae (Belsham, 1993). There are seven antigenically distinct serotypes of FMDV (A, O, C, Asia 1and South African Territories (SAT) types 1-3) and each serotype has many subtypes. This antigenic variation creates a major problem for the control of FMD as infection or vaccination with one serotype does not protect against other serotypes and may fail to protect fully against other subtypes within the same serotype (Paton *et al*., 2005). There are three types of viral protein in the harvest of FMDV infected BHK_{21}cells: i) the infective 146S virus particles, comprising one molecule of ssRNA and 60 copies of each of four

polypeptides VP1, VP2, VP3 and VP4; ii) the empty 75S particles devoid of RNA and comprising 60 copies of each of VP1, VP3 and VP0 (precursor of VP2 and VP4) and iii) the 12S particles consisting of VP1, VP2 and VP3 but devoid of VP4 (Spitteler *et al*., 2011; Nawaz *et al*., 2014).

The immunogenic antigen in FMDV preparation is the intact virion (146S antigen). Therefore, the immunogenicity of FMDV vaccines depends to large extent on the production of the whole virion (146S particles) in tissue culture and the stability after virus inactivation and vaccines formulation (Crowther *et al*., 1995). However, preparations of some FMDV strains contain, in addition to intact virions, quantities of empty particles (75S antigen), which is also immunogenic. The 12S subunit antigen is of extremely low immunogenic and plays no role in the immunogenicity of FMDV vaccines (Spitteler *et al*., 2011). Therefore, the use of 146S assay only or with some serological tests was suggested to increase the reliance on estimating potency of specific vaccine rather than animal challenge (Alkan *et al*., 2008).

In Egypt, the disease is endemic and outbreaks have been reported since 1950. FMD serotypes SAT2, A and O were reported in Egypt (Aidaros, 2002). Vaccination in Egypt used to control FMD, the used vaccine in Egypt before 2012 was inactivated bivalent vaccine prepared from the local strain O1/3/1993 and type A/1/EGY/2006. After isolation and molecular characterization of the recent FMD O Pan-Asia (Bazid *et al*., 2014), A Iran/05(OIE, 2012) and SAT2/2012 (Shawky *et al*., 2013) a new local trivalent inactivated vaccine was prepared containing the three serotypes of the virus.

The efficiency of inactivated FMD Vaccine was evaluated by challenge according to OIE, but there are some difficulties to find animals for potency tests in the countries like Egypt where FMD is endemic. In addition, potency tests must be carried out in containments having high biosecurity levels. There are many publications indicating a correlation between protections from virus challenge and neutralizing antibody response. However, up to now, none of the suggested methods has been found valid (Alkan *et al*., 2008).

Knight-Jones *et al*. (2015) reported that monitoring post-vaccination serology is an important component of evaluation for FMD vaccination programs. So, the present study aimed to evaluate the FMDV trivalent inactivated vaccine by determination of the optimal 146S content /dose which gave protective level of neutralizing antibody measured by serum neutralization test on sera collected from vaccinated calves. This might be an aid to avoid the challenge test of vaccinated animals during vaccine evaluation.

MATERIALS AND METHODS

FMD virus strains: All virus work was conducted in biosafety level 3 laboratories at Middle East for Veterinary Vaccines (MEVAC), Egypt. FMDV serotypes (O/EGY/4/2012, A/EGY/1/2010 and SAT-2/ EGY/A/2012) were propagated in BHK_{21} monolayer cultures for preparation of viruses. Aseptically, the harvested FMD viruses were clarified using Millipore filters (Millisak+® Pod Deth filter Cat# MC0HC054H1) to remove cell debris. The titers of the propagated viruses were 8.5, 8.0, and 9.5 $\log_{10}$ for A/EGY/1/2010, O/EGY/4/2012, SAT2/EGY/A/2012, respectively.

Virus inactivation and concentration: FMD viruses were inactivated by two cycles 2mM binary ethylamine (BEI) according to the method described previously (Barteling and Cassim, 2004). The excess of BEI was neutralized using sterile 2mM sodium thiosulphate. The inactivated antigens of the three serotypes were treated by 8% polyethylene glycol 6000 (PEG-6000) solution at 4ºC then concentrated by Millipore filters (Millisak+® Pod Deth filter Cat# MC0HC054H1), the viruses were then eluted with TrisKcl buffer pH 7.6 (Barteling and Meloen, 1974). The 146S particles in the concentrated antigen preparations were estimated by using sucrose density gradient ultracentrifugation by determining the absorbance at 254nm using ISCO 520C Density Gradient system (Doel and Chong, 1982).

Antigen preparation and vaccine formulation: The aqueous phase of the 3 vaccine batches was prepared using different 146S protein concentration. Briefly, vaccine batch 1; contained 2.7, 2.5, and 1.4 µg/dose, vaccine batch 2 contained; 3.3, 3.1, and 1.7 µg/dose, and vaccine batch 3 contained;5.4 , 5, 2.8 µg/dose ofSAT2/ EGY/A/2012, O/EGY/4/2012, and A/EGY/1/2010, respectively. The inactivated vaccine consisted of an equal volume of oil phase (Montanide ISA 50®, Seppic, France) and aqueous phase which were mixed thoroughly.

Safety test: Prepared emulsions were then tested for viral and bacterial sterility according to the OIE guidelines before being used in animal experiments. The vaccine was inoculated into two calves /batch by one dose and four days later four doses of the vaccine were inoculated subcutaneously (S/C). The inoculated calves were observed for ten days after inoculation (OIE, 2012).

Challenge test: Eighteen native calves aged from 6-8 month /batch were used. These calves were clinically healthy and free from antibodies against FMDV as tested by SNT. The evaluated vaccine was inoculated S/C into 15 calves at the dose rate of 2 ml /animal. The other three calves were injected by the same route with the adjuvant only were kept as control. Two samples were taken from all calves, the first one just before vaccination and the second one at 28 days post vaccination. At 28 days post vaccination all calves are challenged with the virulent FMD type O/EGY/4/2012, A/EGY/1/2010 and SAT-2/EGY/A/2012 viruses with titer of ($10^4 BID_{50}$) inoculated intradermolingually (OIE, 2012).

Serum neutralizing antibody assay: SNT has been carried out for quantitative estimation of neutralizing antibodies against FMDV on the sera collected just before vaccination and at 28 days post vaccination. SNT was performed with BHK_{21} in flat-bottomed tissue culture grade microtitre plates. The SNT was performed against FMDV (serotypes A/EGY/1/2010, O/EGY/4/2012 and SAT2/EGY/A/2012). The test was performed as described in OIE manual 2012 (OIE, 2012) briefly the collected sera are inactivated at 56ºC for 30 minutes before testing. The collected samples were diluted starting from 1/4 to 1/64 and tested against 100 $TCID_{50}$ (50% tissue culture infective dose) of FMDV previously titrated. The titers were calculated and expressed as $\log_{10}$.

RESULTS

Viruses' titration and 146S particles quantification: To develop FMDV inactivated vaccine using three different serotypes of FMDV, the viruses were propagated on BHK_{21} cells till reach the maximum titer and maximum yield of complete virus particle (146S). The antigens were concentrated using PEG 6000 and the 146S particles content were 32.5, 37.5, and 35µg/ml for A/EGY/1/2010, O/EGY/4/2012, SAT2/EGY/A/2012, respectively.

Safety of formulated vaccine batches in calves: The three batches (1, 2 and 3) of FMDV oil emulsion inactivated trivalent vaccine (A/EGY/1/2010, O/EGY/4/2012 and SAT2/EGY/A/2012) were sent to the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) for the safety testing, where there is no FMD lesions or raise in rectal temperature except a small ball like swelling appear in calves inoculated with the vaccine and subside within eight days post inoculation which not affect the results of the safety test so the tested vaccine considered to be safe. The results of safety are summarized in Table 1.

Protection of vaccinated cattle in relation to the 146S particles concentrations: The three batches (1, 2 and 3) of FMDV oil emulsion inactivated trivalent vaccine (A/EGY/1/2010, O/EGY/4/2012 and SAT2/EGY/A/2012) were sent to the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) for the challenge test and SNT by homologous FMDV serotypes A/EGY/1/2010, O/EGY/4/2012 and SAT2/EGY/A/2012. The results of potency, efficacy (SNT and challenge) are summarized in Table 2.

DISCUSSION

FMD is still a problem in livestock in many countries resulted in huge economic loses especially in developing countries. Regarding Egypt several outbreaks attack the country due to the infection with either serotypes A, O, and SAT2 (Aidaros, 2002).

Through the last years monovalent FMD vaccine serotype O was used where it was the only serotype recorded in Egypt (Parida, 2009) after isolation of type A, bivalent vaccine was prepared containing both serotypes A and O ((Knowles *et al*., 2007). More recently, FMDV serotype SAT2 was recorded in Egypt (Shawky *et al*., 2013) and this required the preparation of a trivalent vaccine containing the three present serotypes (A, O, SAT2). It is well established that the major immunogenic component of the FMDV is the intact 146S antigen (Brown, 1992). The concentration of antigen per dose indirectly determines the quantity of antibody induced and the duration of immunity.

So, the present study was planned as a preliminary work to establish a trivalent vaccine through the determination of the appropriate 146S antigen content per dose for each serotype inducing protective level of neutralizing antibodies as an indicator for complete protection in the challenge test.

The obtained results revealed that the virus titers of the FMD serotypes (O/EGY/4/2012, A/EGY/1/2010 and SAT2/EGY/A/2012) were 8, 8.5, and 9.5 $\log_{10}$ $TCID_{50}$ and 146S antigenic content37.5µg, 32.5µg and 35µg/ml after concentration with PEG 6000. The antigenicity of samples were studied by estimation of 146S antigen content in relation with the level of serum neutralizing antibodies for each serotype and the degree of protection in the challenge test shown in Table 2.

The results of the safety test in calves (Table 1) showed that there is no FMD lesions or raise in rectal temperature except a small ball like swelling appear in calves inoculated with the vaccine and subside within eight days post inoculation which not affect the results of the safety test so the tested vaccine considered to be safe.

FMD vaccine is considered potent if it induced not less than 75% protection and SNT 1.5 $\log_{10}$ (OIE, 2012). For serotype A it was found that using 1.4, 1.7 and 2.8 µg/dose of 146S per dose were potent enough to induce complete protection in challenge test and the mean of SNT of the challenged calves was 1.5 $\log_{10}$ after 28 days post vaccination. For serotype O it was found the using 2.5 and 5µg/dose of 146S were potent to induce complete protection in the challenge test in batch-1 and 3 but by using 3.1µg/dose the vaccine gave 80% protection in batch-2. The mean SNT of the challenged calves was 1.5log10 in batch-1 and3 but in Batch 2 it was 1.3 $\log_{10}$ at 28 days post vaccination as shown in Table 2 where one calf of batch-2 showed feet lesions. The unexpected results of the batch-2 in the serotype O reflect the major drawback of the challenge test where the condition of the tested calves played important role in the results of the test.

Using 2.7µg of 146S per dose from serotype SAT2 in Batch-1 was not potent enough to give complete protection (80%) as shown in the result of challenge where one calf of the challenged group showed feet lesion and the mean of the SNT of the challenged calves was 1.3 $\log_{10}$ (Table 2). The batch 2 and 3 were potent enough to induce complete protection in the challenged calves where the protein was 3.3µg and 5.4µg per dose respectively. The mean SNT of the challenged calves was 1.5 in batch-2 and3 but in Batch 1 it was 1.3 $\log_{10}$ at 28 days post vaccination (Table 2) where one calf of batch-1 showed feet lesions. These results confirm that by increasing the protein content (146S) per dose the level of neutralizing antibodies as well as the degree of protection in the challenge test will subsequently improve.

In field experiment carried out by Hind *et al*. (2013) they found that the amount of (146S) 2.2µg/dose for each serotype (A, O and SA2) is sufficient to protect cattle from FMDV. These results agree with the results of this study but we used different concentration of antigens as well as our results indicated that using of 2.7µg of SAT2 is not sufficient to give complete protection. El-Sayed *et al*. (2012) reported that vaccination of calves with Montanide™ ISA206 induced higher antibody titers than the recommended protective level (1.5 $\log_{10}$ for SNT and 1.9 $\log_{10}$ for ELISA) for both types A and O as estimated by SNT and ELISA on the 4thweek post vaccination. These results confirm the results of our study where vaccination of calves with Montanide™ ISA50 (Triaphthovac®) induced a protective level of neutralizing antibodies (1.5 $\log_{10}$) at 28 days after vaccination.

Table 1: Safety of the formulated 3 vaccine batches in 6-8 month old calves

Days post injection	Batch 1				Batch 2				Batch 3			
	Calf 1		Calf 2		Calf 1		Calf 2		Calf 1		Calf2	
	Temp. °C[1]	Injection site	Temp. °C	Injection site	Temp. °C	Injection site	Temp. °C	Injection site	Temp. °C	Injection site	Temp. °C	Injection site
0	38.2	-	38.5	-	38.0	-	38.5	-	38.2	-	38.0	-
1	38.5	-	38.6	-	38.5	-	38.6	-	38.5	-	38.6	-
2	38.3	-	38.4	-	38.3	-	38.4	-	38.0	-	38.4	-
3	38.4	-	38.5	-	38.0	-	38.5	-	38.4	-	38.5	-
4	38.3	-	38.4	-	38.3	-	38.4	-	38.3	-	38.4	-
5	38.6	-	38.8	-	38.6	-	38.8	-	38.6	-	38.8	-
6	38.6	S[2]	38.7	S	38.6	S	38.0	S	38.6	S	38.7	S
7	38.4	S	38.5	S	38.4	S	38.5		38.4	S	38.5	S
8	38.3	S	38.5	S	38.3		38.5	S	38.0		38.5	S
9	38.2	-	38.4	-	38.2	-	38.4	-	38.2	-	38.4	-
10	38.3	-	38.5	-	38.3	-	38.0	-	38.3	-	38.0	-
11	38.3	-	38.4	-	38.0	-	38.4	-	38.3	-	38.4	-
12	38.3	-	38.3	-	38.3	-	38.3	-	38.3	-	38.0	-

[1]Rectal temperature °C; [2]S; swelling at site of inoculation.

Table 2: Serum neutralization titers and protective efficacy of the formulated vaccine batches against challenge at 28 days post vaccination

Serotype	Vaccine batch-1							Vaccine batch-2						Vaccine batch-3					
	146S Cont.	No. of calves	SNT titer[2]		Lesion[3]		Prot. (%)	146S Cont.	SNT titer		Lesion		Prot. (%)	146S Cont.	SNT titer		Lesion		Prot. (%)
			0 DPV[4]	28 DPV	Tong.	Feet			0 DPV	28 DPV	Tong.	Feet			0 DPV	28 DPV	Tong.	Feet	
A/EGY/1/2010	1.4	5	0.0	1.5±0.0	2/5	0/5	100	1.7	0.0	1.5±0.0	1/5	0/5	100	2.8	0.0	1.5±0.0	1/5	0/5	100
O/EGY/4/2012	2.5	5	0.0	1.5±0.0	1/5	0/5	100	3.1	0.0	1.38±0.27	4/5	1/5	80	5	0.0	1.5±0.0	2/5	0/5	100
SAT2/EGY/A/2012	2.7	5	0.0	1.38±0.27	4/5	1/5	80	3.3	0.0	1.5±0.0	1/5	0/5	100	5.4	0.0	1.5±0.0	2/5	0/5	100
Serotype A control	NA[5]	1	0.0	0.0	1/1	1/1	0	NA	0.0	0.0	1/3	1/1	0	NA	0.0	0.0	1/1	1/1	0
Serotype O control	NA	1	0.0	0.0	1/1	1/1	0	NA	0.0	0.0	1/3	1/1	0	NA	0.0	0.0	1/1	1/1	0
SAT2 control	NA	1	0.0	0.0	1/1	1/1	0	NA	0.0	0.0	1/1	1/1	0	NA	0.0	0.0	1/1	1/1	0

[1]146S protein content in µg/dose; [2]Serum neutralizing titers (SNT) from 5 vaccinated calves tested 28 days post vaccination and day before vaccination (mean log10 ± standard deviation); [3]Lesion observed after challenge of vaccinated calves with 10^4 Bovine infective dose 50 (BID_{50}); [4]DPV; days post vaccination; [5]NA; not applicable.

The antigen dose and the protection level shown in Table 2, suggested that the minimal effective 146S concentration/dose of the used strains were 1.4µg for serotype A; 2.5µg for serotype O and for SAT2 was 3.3µg to induce complete protection of calves in challenge test. The more required antigen payload of SAT2 is attributed to either SAT2 antigen ability to provoke immune response is poor or relatively higher level of immunity being necessary to confer protection to cattle against the SAT2 challenge virus.

It appears to be a common experience among FMD vaccine producers that it is more difficult to produce vaccines of high potency from serotype O virus strain than from most serotypes A and C virus strains. From the presented results the production of serotype O require more antigen content than serotype A as well as the production of serotype SAT2 is more difficult because it requires more antigen contents than serotype A and serotype O.

Although, the 146S antigen used in FMDV vaccine formulation are generally ranged from 1 to 10 µg/dose, larger amounts of the O serotype antigens are required to obtain the same potency compared to other serotypes (Doel, 2003) these results agreed with the results of this study where the tested trivalent vaccine contain three antigens with different concentrations per dose. The correlation between 146S antigen and potency cannot be easily determined when concentrations of 146S antigen are greater than 10µg/dose as antigen concentrations do not perfectly coincide with potency (Park *et al*., 2014). This agreed with the results of serotype O where the 146S concentration was 3.1µg/dose in batch 2and 2.5µg/dose in batch 1; however, the protection level in batch 2 was 80%.

The *in vivo* potency assessment usually involves cattle, which makes the test very costly and involves the examination of virus challenged animals to establish the protective ability of the vaccines. The extremely infectious nature of FMDV means that high level containment facilities are required to perform such tests, which further limits accessibility and adds to overall cost, especially if the FMD vaccines prepared as multivalent vaccine containing three different strains of virus as the tested vaccine and it is not possible to undertake a challenge test with more than a single strain of virus at any one time.

The most important area of investigation in FMDV inactivated vaccines is the evaluation of the correlation between protection and serological parameters such as neutralizing antibody titers (Pay and Hingley, 1992) and 146S particles content. This study results further confirmed that SNT levels together with 146S protein concentration are good indicator of the protective efficacy of FMD inactivated vaccine.

Conclusions: From the above results it is clear that the Triaphthovac® vaccine with minimal antigenic content (146S) per dose for serotype A was 1.4µg; for serotype O was 2.5µg and for serotype SAT2 was 3.3µg. were good enough to give complete protection in challenge test as well as gave the protective neutralizing antibody titers (1.5log10) in sera collected from the tested calves after 28 days post vaccination So we recommend using the same

concentration of antigens for serotypes (A/EGY/1/2010, OEGY/4/2012 and SAT2/EGY/A/2012)

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