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Alternative method for the evaluation of monovalent inactivated foot and mouth disease virus vaccine

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Foot and mouth disease is a highly contagious viral disease of cloven-hoofed animals that has a significant economic impact on livestock. A recent outbreak was detected and recorded as exotic strain of foot and mouth disease virus SAT2 (Serotype SAT2, topotype VII, Lib-12 lineage). The emergency vaccine was produced and assessed in vivo and large number of vaccine batches were urgently needed. The present work was aimed to provide a rapid evaluation of inactivated foot and mouth disease SAT2 oily vaccine to exclude the unsatisfactory batches during emergency circumstances and to reduce time, effort and cost. The extraction of foot and mouth disease antigen content from oily adjuvanted vaccine was carried out using isopropyl myristate and benzyl alcohol methods. The extracted viral antigen was identified by foot and mouse disease serotyping ELISA and 146S content was quantified using sucrose density gradient analysis. Evaluations were carried out instantly and at 2h, 6h and 24h. The results indicated the efficiency of benzyl alcohol to breakdown the oil emulsion either MONTANIDE™ ISA 206 VG or MONTANIDE™ ISA 50 V2, while the isopropyl myristate was efficient for MONTANIDE™ ISA 50 V2 only. The identification and quantification of 146S for extracted antigen using benzyl alcohol indicated significant stable records at different time intervals for the vaccine batches, while the extraction using isopropyl myristate indicated unstable records at different time intervals. It was concluded that the evaluation of monovalent foot and mouse disease vaccine could be conducted *in vitro*, using serotyping ELISA and quantification of 146S for the extracted antigen, either using benzyl alcohol or isopropyl myristate (MONTANIDE™ ISA 50 V2 only), with the consideration that 146S content should not less than 4 µg/mL.

Keywords: Foot-and-Mouth Disease; benzyl alcohol; Enzyme-Linked Immunosorbent Assay; *in vitro* techniques; vaccine potency.

Introduction

Foot and mouth disease (FMD) is a highly contagious viral disease that has a significant economic impact on livestock. Foot and mouse disease virus (FMDV) is a positive sense single strand RNA virus of genus *Aphthovirus*, family *Picornaviridae*.[1] The disease affects cloven-hoofed ruminants.[2] Infected animals suffer from fever, appearance of vesicles on feet, in and around the oral cavity and on the mammary glands of females, so mastitis is a common sequel of FMDV infection in dairy cattle. This virus also causes myocarditis in fatal calves leading to Tiger heart.[3] There are seven serotypes of FMDV, namely O, A, C, (South Africa Territory) SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against other.[4]

The outbreaks of FMD still occur all over Egypt although vaccination is obligatory in the country. The SAT2 serotype was not detected in Egypt after the 1950s, but re-invaded the country in 2012 and is endemic untill the present. The FMDV topotype O-EA3 had been isolated recently. It differs from the

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previous topotype Middle East-South Africa (ME-SA) with lineage Panasia2 (O Panasia2) that was prevalent in Egypt from 2010 to 2012.[5] FMD serotype SAT2 outbreaks in Egypt were officially reported to OIE on 14 march 2012,[6] and serotype O outbreaks in 2009,[7] while the recent outbreak was detected and recorded as an exotic strain of FMDV SAT2 (Serotype SAT2, topotype VII, Lib-12 lineage).[8]

Vaccination plays an important role in control the disease. The vaccine must contain multiple serotypes of FMDV to achieve the protection against the current endemic field strains. There are two types of commercial inactivated FMDV vaccine: aluminium hydroxide gel and oily adjuvant. All types of commercial vaccines either local or imported are subjected to evaluation at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbassia Cairo. FMD vaccine evaluation is mainly depending on serum neutralization test (SNT) in tissue culture (*in vitro*) and challenge test (*in vivo*).[2]

Rapid and accurate quality control of emergency FMDV vaccine is essential for effective control of the disease outbreaks.[9] Emergency vaccine can prevent or decrease local virus replication and releasing into the environment.[10] The available regularly used trivalent vaccines (SAT2, topotype VII, Gharbia12 lineage) did not provide protection against recent circulating field isolate, therefore the emergency manufacturing of the monovalent vaccine (Serotype SAT2, topotype VII, Lib-12 lineage) and the vaccination campaign were processed. Thus, we impetus our staff in CLEVB to develop an alternative method for evaluation of newly manufactured monovalent FMD vaccine rather than traditional methods (SNT and challenge test).

Based on sedimentation coefficients, FMDV can be divided into four specific particles using sucrose gradient centrifugation: intact virions (146S or 140S), empty capsids (75S), virus infection-related peptides (45S) and 12S protein subunits (12S). The efficacy of inactivated vaccines is mainly dependent on the integrity of the FMDV particles (146S).[11]

The goal of this work is to provide a rapid and accurate evaluation of inactivated FMD oily vaccine to exclude the unsatisfactory batches obtained by extraction of FMD antigen content using isopropyl myristate and benzyl alcohol methods, in addition to identification by serotyping ELISA and 146S content quantification. Such aim could be considered a preliminary decision for vaccine batches release.

Materials and Methods

Monovalent inactivated oily FMDV virus vaccine batches

Five batches of monovalent inactivated oily FMDV vaccine, type SAT2, topotype VII, Lib-12 lineage, were evaluated at CLEVB. These batches had been evaluated around three months ago for their sterility, safety and potency.

The safety and potency tests were conducted in vivo through inoculation the calves subcutaneously by 2 ml (one dose) of vaccine batches according to the evaluation protocol.[2] The efficacy of FMDV vaccines were assessed according to SNT and challenge test results. The vaccine batches were locally produced with different two adjuvants (MONTANIDE™ ISA 206 VG oil in 3 batches, while the other 2 batches were adjuvanted with MONTANIDE™ ISA 50 V2 oil.

Extraction of viral antigen content from inactivated oil FMDV vaccines

Different chemical methods (n = 2) were used for viral antigen extraction from monovalent inactivated oil FMDV vaccines. Since organic solvents break the vaccine emulsion and release the antigen in the aqueous phase, we have conducted the following methods for extraction of viral antigen:

1- Isopropyl myristate

Extraction of viral antigen from water in oil in water emulsion monovalent FMDV vaccine:[12] 2 mL from vaccine and 8 mL from isopropyl myristate were mixed and vortexed for 15 min at 4,000 g. After 1 min centrifugation at 14,000 rpm the upper oil phase was removed and the aqueous phase contacting the viral antigen was obtained carefully.

2- Benzyl alcohol

Viral antigen was extracted using benzyl alcohol: 5 mL of vaccine sample was taken in a 50 mL centrifuge tube and one-tenth volume of benzyl alcohol was added slowly through the wall and vortexed for 5 min. After breaking the emulsion, the samples were centrifuged at 12,000 g for 5 min. The aqueous phase containing viral antigen was collected carefully.[13]

Identification of extracted viral antigen by FMDV serotyping ELISA

This test was carried out by using FMDV serotyping ELISA Kit (FMD O, A, SAT 1, SAT 2, Asia 1) IZSLER:

Brescia, Italy, The Pirbright Institute, UK–Lot. 01-2019 190301a. The test procedures were performed according to the instructions of ELISA kit insert.

Quantification for 146S content of extracted viral antigen

The test was carried out as the following: 2.2 mL sucrose (25%) was added with a pipette in Phosphate Buffer to a 5 mL centrifuge tube, and then 2.2 mL sucrose (10%) was added with a long syringe needle in Phosphate Buffer under the 25% sucrose layer. Then, 0.2 mL of extracted viral antigen was added to the top of the gradient. The tube was centrifuged with gradient in the ultracentrifuge (Kontron Instrument, Model -Centrikon T-1080 with swinging rotor) for 40 min at 45,000 rpm at 4°C. Concentration of 146S particles in the sample was calculated as:

Peak area (mm^2) x 0.0116 = µg/mL[14]

In general, payloads vary from 1 to 10 µg of 146S per strain per vaccine dose to achieve an equivalent potency. Because the relationship between 146S concentration and potency does not appear to be a simple linear function, payloads higher than approximately 10 µg of 146S of a given strain do not necessarily give proportionately higher potencies.[15]

Keeping quality of extracted viral antigen

The extracted viral antigens from oil emulsion monovalent FMD vaccine batches were tested for identification and 146S content, at different time intervals (instantly, 2h, 6h and 24h) post extraction process.

Results

Five batches of monovalent inactivated oily FMDV vaccine type SAT2 topotype VII, Lib-12 lineage, which were evaluated at CLEVB for their potency by using traditional methods (SNT and challenge test) indicated satisfactory results in four batches, while one batch was unsatisfactory as shown in Table 1. These results were considered for assessment of the alternative method.

The extraction of viral antigens through oil adjuvant breakdown using benzyl alcohol was efficient for either MONTANIDE™ ISA 206 VG or MONTANIDE™ ISA 50 V2 adjuvants, while isopropyl myristate was efficient for MONTANIDE™ ISA 50 V2 adjuvant only (Table 2).

The identification for the extracted viral antigen of five vaccine batches, which were tested instantly using serotyping ELISA indicated positive to FMDV

Table 1. Evaluation of humoral immune response and protection level of vaccinated calves with inactivated FMDV vaccine batches using SNT and challenge test.

Vaccine Batches No.	1	2	3	4	5
*SNT Antibody titer (Log_{10} $TCID_{50}$)	2.1	2.1	1.95	2.4	0.9
**Protection level (Percentage %)	100	100	80	100	20

*the protective serum neutralizing antibody titer ≥ 1.5 (Log10 TCID50)
**the protection level (%) of challenge test ≥ 75%

Table 2. Identification of the extracted FMDV type SAT2 antigens using FMDV serotyping ELISA.

Vaccine batches	Serotyping ELISA result for SAT2 serotype							
	Isopropyl myristate				Benzyl alcohol			
	Inst	2h	6h	24h	Inst	2h	6h	24h
Batch (1) (ISA50)	++++	++	+	Negative	++++	++++	++++	+++
Batch (2) (ISA50)	++++	+++	++	Negative	++++	++++	++++	+++
Batch (3) (ISA206)	Negative	Negative	Negative	Negative	++++	++++	++++	+++
Batch (4) (ISA206)	Negative	Negative	Negative	Negative	++++	++++	++++	+++
Batch (5) (ISA206)	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Negative: < 1000 +: 1000-1500 ++:1500-2000 +++:2000-2500 ++++: > 2500

type SAT2 for four vaccine batches (1, 2, 3 and 4) and negative to FMDV type SAT2 for one batch (5) for extracted viral antigens using benzyl alcohol. The extracted antigen using isopropyl myristate indicated positive results to FMDV type SAT2 for two vaccine batches (1 and 2) only when MONTANIDE™ ISA 50 V2 was used (Table 2).

The identification test was conducted for viral extracted antigens at different time intervals post extraction (2h, 6h and 24h). It was found that the viral extracted antigens using benzyl alcohol indicated positive to FMDV type SAT2 for four vaccine batches (1, 2, 3 and 4) at different time intervals post extraction with slight decrease in ELISA readings, while the viral extracted antigens using isopropyl myristate indicated positive to FMDV type SAT2 for two vaccine batches only (1 and 2) at 2h and 6h post extraction with significant variation in ELISA readings, as shown in Table 2.

All the positive identified antigens using serotyping ELISA were tested to quantify the 146S content. Records for the 146S content for extracted antigens using isopropyl myristate, at different time intervals, indicated instability of 146S particles and a rapid decrease, showing potent vaccine batches (1 and 2) instantly and at 2h post extraction. The 146S content records in case of benzyl alcohol indicated significant stability at different time intervals, showing potent vaccine batches (1, 2, 3 and 4), the 146S particles in the tested vaccine were assessed on margin not less than 4 µg/mL to be considered a potent vaccine as shown in Table 3.

Discussion

The present study considered the results of five batches of monovalent inactivated oily FMDV vaccine type SAT2 that were evaluated at CLEVB for their potency by using traditional methods: SNT and challenge test. Four vaccine batches indicated satisfactory results with protection level more than 75% and protective serum neutralizing antibody more than 1.5 $\log_{10}$ $TCID_{50}$. The alternative method was conducted on the same vaccine batches to evaluate the vaccine batches using extraction of FMD viral antigen, identification of FMD viral antigen and quantification of 146S content as it has significant correlation with the vaccine potency.

The extraction of viral antigen carried out using isopropyl myristate and benzyl alcohol for five vaccine batches, indicated the efficient of benzyl alcohol to breakdown the oil emulsion either MONTANIDE™ ISA 206 VG or MONTANIDE™ ISA 50 V2 adjuvants. These agreed with a study where the benzyl alcohol method was efficient in extracting 146S from the monovalent and trivalent fresh and stored FMD vaccines.[16] While the isopropyl myristate was efficient to breakdown the oil emulsion of MONTANIDE™ ISA 50 V2 adjuvant only, and failed with MONTANIDE™ ISA 206 VG adjuvant.

The identification of the extracted viral antigen of five vaccine batches carried out instantly, using serotyping ELISA, indicated positive to FMDV type SAT2 for four vaccine batches and negative to FMDV for one batch for extracted viral antigens using benzyl alcohol, while the extracted antigen using isopropyl myristate showed positive to FMDV type SAT2 for two vaccine batches only when MONTANIDE™ ISA 50 V2 was used. This result came parallel to a study conducted in Uganda where a similar picture was reported,[17] and other results obtained of the analysis performed using FMDV serotype-specific antigen capture ELISA that revealed the co-circulation of four serotypes, A, O, SAT 1, and SAT 2 during 2011-2014, which confirmed that FMD is endemic in Nigeria.[18]

Table 3. Estimation of 146S content in the extracted FMDV type SAT2 antigens at different time intervals.

Vaccine batches	146S (µg/mL)							
	Isopropyl myristate				Benzyl alcohol			
	Inst	2h	6h	24h	Inst	2h	6h	24h
Batch (1) (ISA50)	6.5	4.5	2	0.11	6.5	6.4	6.1	5.8
Batch (2) (ISA50)	6.3	5.1	2.9	0.26	6.5	6.5	6.2	5.9
Batch (3) (ISA206)	*N/A	N/A	N/A	N/A	5.9	5.7	5.5	5.3
Batch (4) (ISA206)	N/A	N/A	N/A	N/A	6.4	6.3	6.3	6.1
Batch (5) (ISA206)	N/A	N/A	N/A	N/A	1.1	1	1	0.8

*N/A: Not Applicable

The identification test was conducted for the viral extracted antigens at different time intervals post extraction (2h, 6h and 24h). It was found that the viral extracted antigens using benzyl alcohol indicated positive to FMDV type SAT2 for four vaccine batches (1, 2, 3 and 4) at different time intervals post extraction with slight decrease in ELISA readings, while the viral extracted antigens using isopropyl myristate indicated positive to FMDV type SAT2 for two vaccine batches only (1 and 2) at 2h and 6h post extraction with significant variation in ELISA readings.

All the positive identified antigens using serotyping ELISA were tested to quantify the 146S content. Records for the 146S content for extracted antigens using isopropyl myristate, at different time intervals, indicated instability of 146S particles and a rapid decrease, showing potent vaccine batches (1 and 2) instantly and at 2h post extraction. The 146S content records in case of benzyl alcohol indicated significant stability at different time intervals, showing potent vaccine batches (1, 2, 3 and 4).

Regarding the 146S antigen amount records, the vaccine potency could be evaluated and assessed. It was reported that FMD vaccine (O, A, SAT2) should contain at least 3 µg/2mL (cattle and buffaloes dose) or 1.5 µg/2mL (small ruminant dose) from each serotype of FMDV 146S particles which gave in vivo protective immune response against FMDV.[19] Studies revealed that the useful operational limits of the antigen payload were between 1.5 and 9.2 µg of 146 S,[20] while it was reported that the vaccines having a payload of 3.5 µg were able to elicit a robust SN titer.[21] All these reports could assist to detect the margin of 146S particles which should not less than 4 µg/mL to estimate the vaccine potency. Thus, the revealed results here indicated the efficacy for four batches of the tested vaccines (benzyl alcohol) and two batches (isopropyl myristate).

Conclusion

The evaluation of monovalent FMDV vaccine could be conducted in vitro using serotyping ELISA and quantification of 146S particles content for the extracted antigen either by benzyl alcohol or isopropyl myristate (MONTANIDE™ ISA 50 V2 only), with consideration the 146S content should not be less than 4 µg/mL, to release the vaccine batch during the emergency circumstances.

Conflict of interest

The authors whose names are listed certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Contributors

Abousenna M.S. and Heba A Khafagy conceived and designed the study, wrote the manuscript and provided the critical revisions that are important for the intellectual content.

Mahmoud M. Abotaleb and Darwish D M provided the support to conduct the research study and collection the data.

Barghooth W.M and Nermeen G.Shafik analyzed and interpreted the results.

All authors approved the final version of the manuscript.

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Método alternativo para la evaluación de la vacuna monovalente inactivada contra el virus de la fiebre aftosa

Resumen

La fiebre aftosa es una enfermedad viral altamente contagiosa de los animales de pezuña hendida que tiene un impacto económico significativo en el ganado. Se detectó un brote reciente que se registró como causado por una cepa exótica del virus de la fiebre aftosa (serotipo SAT2, topotipo VII, linaje Lib-12). La vacuna de emergencia se elaboró y evaluó *in vivo*, existiendo una urgente necesidad de contar con un gran número de lotes de la misma. El presente trabajo tuvo como objetivo proporcionar una evaluación rápida de la vacuna oleosa inactivada (SAT2) contra la fiebre aftosa, para excluir los lotes insatisfactorios durante circunstancias de emergencia, reduciendo tiempo, esfuerzo y costo. La extracción del contenido de antígeno de fiebre aftosa, de la vacuna oleosa adyuvada, se llevó a cabo utilizando miristato de isopropilo y alcohol bencílico. El antígeno viral extraído se identificó utilizando un ELISA de serotipificación y se cuantificó el contenido de 146S mediante análisis de gradiente de densidad de sacarosa. Las evaluaciones se realizaron de forma instantánea y a las 2h, 6h y 24h. Los resultados indicaron la eficacia del alcohol bencílico para separar la emulsión de aceite para MONTANIDE ™ ISA 206 VG o MONTANIDE ™ ISA 50 V2, mientras que el miristato de isopropilo fue eficaz para MONTANIDE ™ ISA 50 V2 únicamente.

Palabras clave: Fiebre aftosa; alcohol bencílico; ELISA; técnicas *in vitro*; potencia de la vacuna.

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