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RESEARCH

Process development of nano emulsion Foot and mouth disease virus vaccine

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ABSTRACT

Background: Foot and mouth disease is a highly contagious disease of livestock that is endemic in Egypt with severe economic impact. The main method of control is through vaccination of the livestock with a potent vaccine.
Objective: Process development of the current formulations and generating new ones is crucial. Currently, Montanide ISA 206 is the adjuvant used in FMDV vaccine produced in Egypt which is w/o/w microemulsion.
Methods: We aimed to increase the efficacy of the conventional formulation by converting the microemulsion to Bio-compatible and stable Nano emulsion. Emulsification was applied through ultrasonication of Montanide ISA 206 in the presence of Tween 80 as a detergent. The physical characteristics were studied and imaged by TEM.
Results: Images showed particle size ranged from 200-400 nm instead of 1 micron of the original. Immunological evaluation by serum neutralization test showed enhanced immunogenicity of developed formula when compared to the conventional one. Challenge test revealed high protection levels that reached 90% of the Guinea pig vaccinated by nano emulsion.
Conclusion: Our findings suggest that we aimed to enhance immunity through nano emulsion of Montanide ISA 206 is well achieved.

Keywords: FMD; ISA206; NE; TEM; VNT.

BACKGROUND

Foot and Mouth Disease Virus (FMDV) is a communicable and highly contagious positive-stranded RNA virus belonging to the family *Picornavirdae* of genus *Aphthusvirus*. The virus is an economically devastating disease of cloven-hoofed livestock animal characterized by vesicular lesions on oro-nasal mucosa, interdigital cleft, coronary band, udder, teats epithelium, and causes death in calves due to myocarditis (OIE, 2018).

Although the cornerstone in controlling and eradication strategy for FMD is vaccination with potent inactivated vaccine during the outbreak, previous failures to elicit effective immune responses by vaccination has been frequently reported (He *et al.*, 2007; Wei, 2007). The need to enhance the immunogenicity of available FMD vaccine to protect the animal from infection is still crucial and countries with endemic status are embarking on new control initiatives may benefit from guidance on how to optimize vaccine-based control program to combat FMD.

Adjuvant is one of the critical components of inactivated vaccine as it plays major role in eliciting immune response. Currently, the Montanide™ ISA 206 of oil-adjuvants (SEPPIC, France) is the adjuvant used for FMD vaccine produced in Egypt and is considered the magic piece for potentiation immune response (Zhao *et al.*, 2014). It is described as water-in-oil-in-water (w/o/w) double emulsion, with particle size average 2 M. Lately; the use of nano adjuvant had been an interest as adjuvant and potential replacer for micro sized emulsion. Nano emulsions

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can exist as oil-in -water (o/w) or water-in-oil (w/o) or double emulsion (w/o/w), where its droplet size can range from 50-600 nm, these droplets carry antigen inside their core for efficient vaccine delivery. They have been proven to enhance both humoral and cellular immune response (Hamouda, 2001).

In this study, Bio-compatible and stable w/o/w emulsion was developed and used as an adjuvant for foot and mouth disease (FMD) vaccine minimizing the particle size of original adjuvant montanide ISA206, the physical characters were assessed after treatment. The developed nano emulsion and the original micro sized emulsion were compared in guinea pig for their ability to induce humoral immunity by viral neutralization test and the ability of vaccinated animals to resist challenge.

MATERIALS AND METHODS

Viruses

Foot and Mouth Disease Virus (FMDV) serotype O_1, strain Pan-Asia 2012 for vaccine formulation (O_1/egy/Pan-Asia/2012) and strain Manisa for challenge test (O_1/EGY/Manisa69) were obtained from Veterinary Serum and Vaccine Research Institute (VSVRI).

Vaccine formulation

Plaques Assay and Plaque purification were performed. FMD virus (O/egy/Pan-Asia/2012) then confirmed by PCR, after that propagated on BHK-21 clone13 Roller bottle at 37 ^{o}C for 16-18 hours, pH around 7.5, Cytopathic effect was Observed microscopically, then undergo three cycles of freezing and thawing, clarify the thawed harvest at 3000 rpm for 20 minutes, 4 ^{o}C for removal of the cell debris.

Titration of the clarified harvest is not less than 10^6 TCID50 /ml (OIE,2017). Then inactivation of the clarified viruses was carried out using binary ethylene-imine 1% of the 0.1 M stock solution (BEI) to reach final concentration of 1mM (Bahnemann, 1975).

The concentration of the FMD antigen was performed using Polyethylene glycol 8000(PEG) 7.5% (Kaaden *et al.*, 1971). The amount of 146S antigen was quantified by the sucrose density gradient ultra-centrifugation (SDG) technique described by Barteling and Meloen (1974). According to the manufacturer's guidelines for Montanide ISA 206 formula, the antigen-adjuvant ratio was determined. Safety and Sterility Test were performed according to OIE (2009).

Nano emulsion preparation

Montanide ISA-206 was provided by SEPPIC (pharmaceutical division, Paris, France) Specialty Chemicals Co., Ltd. FMDV strains O Egypt vaccine, addition of Tween 80 at 2% to the aqueous part then mixing well then add o/w emulsion immune stimulant at concentration of 0.1% to the montanide oil ISA206, then was subjected to ultrasonic emulsification by 750-watt Ultrasonic processor (Vibra-cellTM ultrasonic liquid processor -VC750, USA), on ice, at 20KHz for 20 minutes.

Characterization of the Emulsion

Measurement of particle size, size distribution (Polydispersity index, PDI), the zeta potential of particles was measured by (Zetasizer Nano ZSP, Malvern Instruments, Malvern, UK) based on dynamic light scattering (DLS). Morphology of the particle using High -

Resolution Transmission Electron Microscope (EM-H. R2100, Japan). The viscosity of the emulsions was measured with Brookfield RVDVE230 Medium-range viscometer) at room temperature.

Nano emulsion -antigen loading

Dispersion of the antigen (water phase) into ultrasonicated oil phase Montanide™ (ISA206), mixing was applied using Magnetic stirrer at 4 ^{o}C for 30 minutes.

Stability of nano emulsion

Heating and cooling, the formulated emulsion was kept at incubator (45^{o}C), other samples were kept at refrigerator (4 ^{o}C) For 72 hours. Freezing-thawing stress, nano emulsion freezing to -20 ^{o}C for 72 hours, thawed at room temperature 30 ^{o}C, this cycle repeated three times. Centrifugation, formulated nano emulsion was centrifugated at 5000 rpm for 20 minutes and observed for phase separation. Thermodynamic stability, the formulated emulsion was kept at different temperatures for 2 months (4 , 25 and 37 ^{o}C), Keeping at room temperature for 6 months.

Sterility Test

The samples of the vaccine suspension, buffered saline, serum, adjuvant, and cell culture medium was cultured by dropping 1.0 ml of the samples on each of the bacteriological media (nutrient broth, brain heart infusion broth), mycotic medium (Sabouraud dextrose agar). The plates and medium were incubated for 72 hrs. at 37 ^{o}C and observed each day for microbial growth (OIE ,2017), Mycotic medium at 25 ^{o}C.

Safety Test

The group 1 set of 7 guinea pigs was used for safety test. The animals were subcutaneously inoculated with 0.5ml of the vaccine per guinea pig at the left thigh of the hind leg. The test and control animals were observed for any pathological moribund or death for 14 days with adequate supply of feed and water. They were bled after 21 days and the sera were separated and stored at -20 ^{o}C ready for use.

Animals

Guinea Pigs: twenty (30) guinea pigs were used in this study and they were supplied by the laboratory animal unit of the Veterinary Serum &Vaccine Research Institute, the animals were certified fit by a veterinarian. The guinea pig weighed 470 to 520 g. Animal housing and all procedures related to using animals throughout this study conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the Cairo University, cairo, Egypt.

Immunization of the animal

Animals used were classified into 3 groups, the first group 10 Guinea pig were immunized subcutaneously S/C (400µL) at days 1 and 14 by vaccine with the size reduced ISA 206 adjuvant, the second group 10 Guinea pig were immunized by the prepared vaccine with ISA 206 as adjuvant to represent the commercially available vaccine, 10 Guinea pig were kept as control negative group. The animals were observed for local reactions within 24 hrs. post vaccination. Sera were collected 3 weeks after the first immunizations and stored at -20 ^{o}C before further analysis.

Immunological evaluation

The gold standard specificity test is virus neutralization test: The micro serum neutralization test method was used in accordance with Rweyemamu and Pereira (1978) method. The test was carried out in a 96 well flat bottom sterile disposal micro titer plates, serial diluted serum is incubated with set amount of infectious virus and incubated at 37 ^{o}C for 1 hr, then susceptible cells are added to virus-serum mix, incubation at 37 ^{o}C for 2-3 day, after that wash and stain plates. The neutralization titers were expressed as the reciprocal of the final dilution of the serum that neutralized the virus.

Challenge test

Post vaccination challenge method: Vaccine challenge method of Nicholls *et al.,* (1990) was adopted. The foot pad of the hind leg of the guinea pig was dipped into phosphate buffered saline (PBS), pH 7.2 for 60 seconds and the challenge virus ($O_{manisa/egy}$) suspension was carefully inoculated (0.02 ml) subcutaneously at the foot pad using tuberculin syringe and needle. The infected foot pad was wetted the second day and then observed for 14 days.

Effect of ultra-sonication on FMD antigen loading

The ultrasonicated montanide oil ISA 206^{TM} formulated with FMDV antigen was centrifuged at 12,000 rpm for 15 min to get phase separation. FMDV antigen content in the aqueous phase recovered from the emulsion was quantitatively determined by Sucrose density gradient (Barteling and Meloen, 1974) developed. The antigen content in aqueous phase before being emulsified with oil phase was also determined for comparison.

RESULTS

Sterility test

Table 1 shows the results of the sterility tests of the vaccine and other vaccine inputs cultured on bacterial and fungal culture the results obtained showed that the materials were sterile.

Table 1: Sterility tests of the vaccine and other vaccine inputs cultured on bacterial and fungal media.

Samples	NB	BHIB	SDA
Vaccines	-	-	-
Serum	-	-	-
Cell culture medium	-	-	-
Cell culture harvest	-	-	-
Emulsified ISA206	-	-	-
Inoculation medium	-	-	-

Key: NB= nutrient broth, BHIB=Brain Heart Infusion Broth, SDA= Sabouraud dextrose agar, - = no growth.

Characterization of particle

The average diameter particle size (d.nm)of sonicated ISA 206 montanide oil measured by Zetasizer reach 427 nm , average poly dispersion index (PdI) reach 0.630. Morphology of the particle and size by Electron microscopy (HR -TEM).

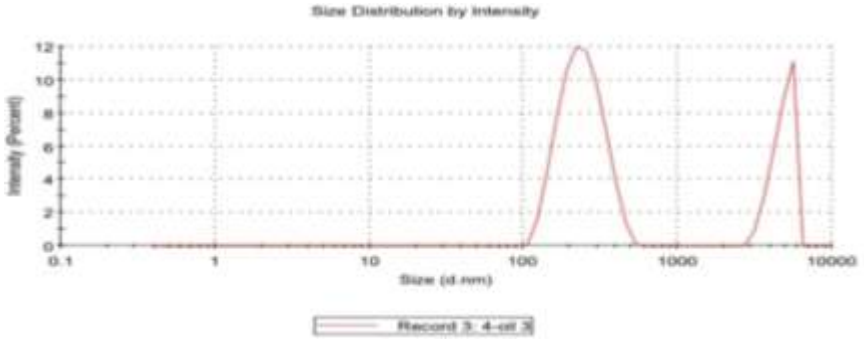


Fig.1: Show that the particle of oil ISA206exposed to ultra-sonication already becomes less than the crude ISA 206

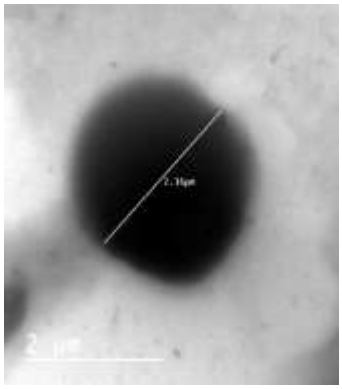


Fig. 2: Particle size and morphology of ISA206 before ultrasonication.

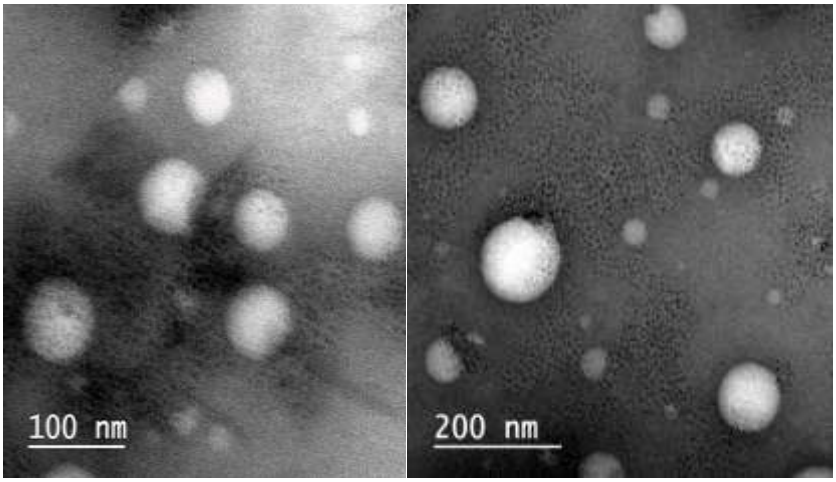


Fig. 3: Particle size and morphology of exposed ISA 206 to ultra – sonication by H-R TEM

H-R TEM image of FMDV + nano emulsion by negative stain

By negative staining of the nano emulsion FMDV particle, the size was 200 nm as predicted by zeta analysis. Nano emulsion is formed of several size droplets, larger containing small size droplets. Medium size droplet measures 70-90 nm and are shown containing electron dense structure.

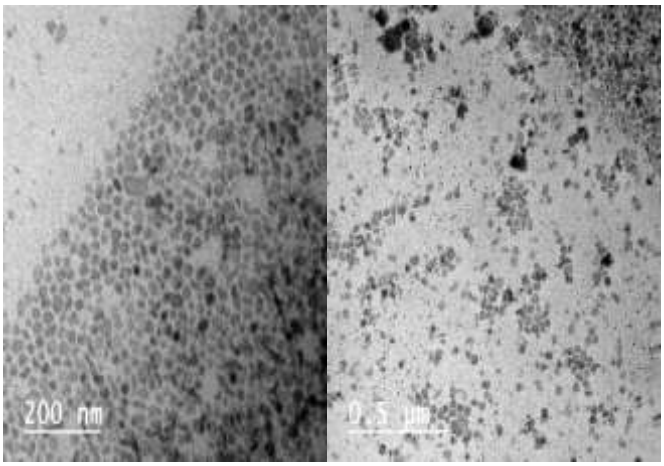


Fig. 4: foot and mouth disease (FMDV) and emulsified with ISA206 ultrasonicated, negative stained H-R TEM.

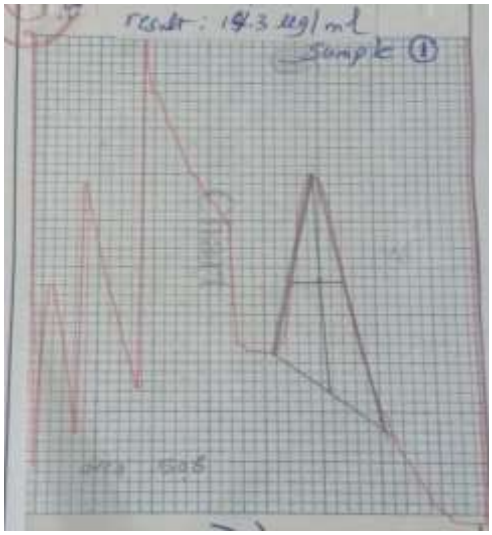
Stability of Nano emulsion with antigen

The nano-emulsion formulated with FMDV antigen showed good stability. Over 60 days storage, the particle size is in nanoscale (below 400 nm), while the untreated ISA 206 (control) showed creaming or phase separation, though gradual increase in particle size was detected due to particle movement flocculation.

After three cycles of freezing and thawing no changes were observed visually, on the other hand the control showed aggregation. After centrifugation at5000 rpm, phase separation occurred at control oil, but the nano sized ISA 206 showed no phase separation.

The effect of emulsification on antigen

The amount of 146S of FMDV antigen is 14.3 µg/ml before formulation using the Nano scale ISA 206, after emulsification and centrifugation 12000 rpm the amount of free 146S is measured is 2 µg/ml.



Loading efficiency

By comparing the total amount of FMDV (146S) found in aqueous phase separated from the emulsion through centrifugation with its initial amount in the aqueous phase before being emulsified with the oil phase, it reaches 86%.

Immunological assay

The data obtained from the guinea pigs inoculated with single and repeated does of the vaccine were presented in Table 2. No antibody to FMD virus was detected in their pre-vaccination sera the booster dose gave the expected immunological reaction with increase in antibody production. Both the single and repeated SN titers were above the required 1.51 minimum neutralization titer (Table 2).

Table 2: Virus Neutralization antibody titers in G. pigs (1) FMDV vaccine formulated with sonicated ISA 206 and G.pig (2) FMDV vaccine formulated with ISA 206 without sonication process, FMD vaccine.

Animal host	Vaccine dose	Pre vaccine titer range	Post vaccine titer range	Mean
G.pig (1)	Single, booster (0.4ml)	0.2	2.1-2.44	2.3
G.pig (2)	Single, booster (0.4ml)	0.2	1.3-1.65	1.5
Control	No vaccination	0.2	<0.2	<0.2

Challenge test

Observation of FMDV lesion on G.pig which inoculated with challenge virus, in the first group one animal showed aphthous lesion in the hind pad so protection reach 90%, on the other hand group (2) protection reached 70%, negative control group showed aphthous and ulceration on hind pad.

DISCUSSION

Foot and mouth disease(FMDV)is a devastating contagious economic disease of cloven-hoofed animals, vaccination is the realistic way to control the disease in endemic developing countries so the efficacy of vaccine used is very critical. Among the most important factors of inactivated vaccine to induce proper immune response is the use of a potent adjuvant thus the development of a safe and efficacious adjuvant (Zenghui *et al.*, 2016).

Recently, applications of nanotechnology are the center of attention in many fields due to the unique functional characters of nano-sized particles. As many studies reported the advantage of the nano-emulsion over micro-sized oil (Zimmer *et al.*, 2016). We aimed to reduce the size of ISA206 from 1-2 µ to nanoscale and study the effect of this process on different parameters. The thermodynamic and high kinetic stability, besides the minute droplet size of nano emulsions, have spurred their rapid development as a system for the delivery of bioactive substances in pharmaceutical Reagent (Che Marzuki, 2019).

After size-reduction of ISA206 to nano-sized using ultrasonication and characterization of the treated sample occurred through zeta sizer to measure the particle size (400nm), poly dispersion index (PDI) was 0.6, and zeta potential unit is positive charge, stability was measured by thermodynamic stability through freezing (-40 ^{o}C) then thawing, stability at hot temperature up to 52 ^{o} C . stability at 4 ^{o} C over 2 months this showed no change in particle size, this is also confirmed by formulation with FMDV antigen and measure its stability over 2-month period to make sure that its size is still in the nano-size range, no apparent changes in the characteristics of ISA206 .The small size of the droplets allow them to deposit uniformly on antigen, also it prevents any flocculation, by the way low surface tension and interfacial tension enhance wetting, spreading and penetration .The very small droplet size causes a large reduction in the gravity force and the Brownian motion may be sufficient for overcoming gravity, so no creaming or sedimentation occurs on storage. Finally, nano emulsion is thermodynamically stable system.

By the way The nanoscale size and positive ζ-potential of the emulsion enables the penetration of the mucous layer, the binding to cell membranes and the cellular uptake that

together mediate the induction of the innate and the adaptive immune responses (Makidon *et al.*, 2008).

Addition of TWEEN 80 due to Nano emulsions are made from pharmaceutical surfactants that are generally regarded as safe (GRAS). The surfactant type and concentration in the aqueous phase are added to provide good stability against coalescence, aggregation and Ostwald ripening, unlike microemulsion which require higher surfactant concentration (usually 20 % and higher), nano emulsion can be prepared using reasonable surfactant concentration (5%-10%).

Formulation of nano-sized ISA 206 with FMDV antigen and measuring its loading efficiency depending on the amount of 146S of FMDV antigen, as amount of 146S recovered from nano emulsion is 2µg/ml, so the recovery ratio was calculated to be about 86 % which is very high loading capacity, so that FMDV antigen is well-retained during sonication, in the nano emulsion.

After formulation, injection into G.pig, The choice of guinea pigs for the test was due to their harmless nature, cheap, easy to handle and maintain. They are as sensitive and susceptible to clinical signs as the target hosts (cattle, sheep, goats, and pigs) (Rweyemamu and Pereira, 1978; Abegunde *et al.*, 1987). Evaluation of neutralizing antibody titer was performed using VNT which is the gold standard for FMDV (OIE, 2017) and the best potential screening test to access the level of immunity produced by FMDV vaccinated or infected animals against the virus, group (1) of the animal had higher antibody titer than group (2), this indicates that the size reduced ISA206 showed better immunogenicity than the micro-scaled non treated ISA206, this was confirmed by challenge test as it gave 90% protection.

CONCLUSION

We developed and converted ISA206 from micron size to the nano size to elicit longer and better immune response as Nano emulsion adjuvant reveals humeral and cell-mediated and mucosal immunity, mucosal immunity is very important in FMDV , as infection occur through mucous membranes of nostrils , mouth , in the further studies will be carried out to evaluate mucosal immunity , cell mediated immunity to study cytokine secretion , kinetics of antibody to the nano emulsion adjuvant.

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The authors declare that they have no competing interests.

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