Aly *et al.*, *J. of Virol. Sci.*, Vol. 7: 86- 96, 2020
ISSN: 2636-2937

Journal of Virological Sciences

RESEARCH

Ultrasonic dispersion of aluminum hydroxide-adjuvanted Rift Valley Fever vaccine enhances formula characteristics and immune response

Shourok E. Aly [1], Karim Zaki[1], Soad E. Elsayed[2], M. Elgamal[2], Ausama A. Yousef[1], Mohamed A. Shalaby[1]

ABSTRACT

Background: Rift Valley Fever (RVF) is a mosquito-borne viral zoonotic disease that is endemic in Africa and the Arabian Peninsula. Outbreaks of RVF resulted in severe losses to the animal industry and major threats to the public health. Control of RVF in Egypt is dependent on vector control and yearly vaccination of livestock using an aluminum hydroxide gel-adjuvanted inactivated Rift Valley fever virus (RVFV) vaccine.
Objective: Herein, we aimed to enhance the immunogenicity of the conventional vaccine by developing a smaller particle size vaccine formulation through ultrasonic dispersion of aluminum hydroxide adjuvant.
Methods: This was achieved by ultrasonication of aluminum hydroxide gel in the presence of trehalose as dispersant and measuring different physical characters including particle size, Zeta potential, loading capacity and imaging of adjuvant before and after formulation.
Results: Ultrasonication of aluminum hydroxide gel resulted in generation of 120-200 nm gel structures followed by vaccine formulation showed enhancement of vaccine loading capacity. Animals vaccinated using the enhanced formula were completely protected against RVF challenge virus and produced higher RVFV neutralizing antibody titers.
Conclusion: The vaccine developed based on small particle vaccine formulation is an economic solution that can be added inline to existing production platforms to enhance immunogenicity.

Keywords: RVF; Aluminum hydroxide adjuvant; Dispersion; Dispersed aluminum hydroxide.

BACKGROUND

Rift Valley fever (RVF) is a mosquito-borne zoonotic disease caused by Rift valley fever virus (RVFV) which is a member of genus *Phlebovirus* within the family *Phenuivirida* of the order *Bunyavirales*. The virus possesses a single-stranded, negative sense, segmented RNA genome (Plyusnin *et al.*, 2011). Virions are likely to have an icosahedral symmetry with the diameter varying from 101 nm to 106 nm with a highly ordered structure. The surface is covered by a shell of 120–122 glycoprotein capsomers arranged in an icosahedral lattice with T = 12 (Huiskonen *et al.*, 2009).

RVF was first reported in sheep and humans in Kenya in 1931 (Daubney *et al*., 1931). Recurrent epidemics have been reported and now RVF is endemic in many countries on the African continent, in the Arabian Peninsula, and some Indian Ocean Islands (Himeidan *et al*., 2014). The virus can infect wide range of hosts including sheep, cattle, goats, camels, buffaloes, and others. Pregnant ruminants infected with the virus subject to high rate of abortion, fetal malformation and ocular disease, or death usually due to hemorrhagic fever and thrombosis (Ikegami and Makino, 2011). Owing to the massive health and economic losses signified by the number of deaths among humans and the high rates of abortions in previous outbreaks, RVFV is considered as an OIE high-impact transboundary pathogen with potential for bioterrorism and a setback to international livestock trade (OIE, 2014).

Correspondence: sherouk3bdelhady@gmail.com
Full list of author information is available at the end of the article

Despite the wide spread of RVF and its endemicity in many countries in Africa and Arabian Peninsula, nevertheless, the vertical transmission of RVFV from infected mosquitoes into offspring and absence of eradication programs for mosquitoes indicating that regular vaccination may be the only practically effective approach to protect susceptible livestock animals continuously exposed to RVFV infected mosquitoes. Only few countries apply routine vaccination programs (Ikegami, 2017). Currently, the commercially available vaccines are the Smithburn vaccines, Clone 13 vaccine and inactivated RVF vaccines. After 1977 outbreak, vaccination with live attenuated Smithburn vaccine was applied in Egypt at intermittent periods, and killed vaccine was used for pregnant and young animals. As the live attenuated Smithburn held a potential for reversion to virulence in addition to their ability to cause viremia arising the possibility on these animals become infected and maintain the virus in the environment, The Egyptian government announced in 2008, that the live attenuated Smithburn vaccine was no longer produced or used in the control programs (kamal, 2011) and since then only the inactivated RVF vaccine is used in Egypt adjuvanted by aluminum hydroxide gel.

Owing to its high safety margin Aluminum hydroxide adjuvant is the most widely used adjuvant. The gel is made of small primary fibers with an average dimension of 4.5 × 2.2 × 10 nm but the average size in aqueous solution is 1–20 μm due to aggregation (Hem *et al*., 2007). Recently, several studies showed that smaller aluminum hydroxide particles were more immunogenic than larger ones (Mumper *et al.,* 2003; Li *et al*., 2011). Similarly, a study showed that 3 μm aluminum phosphate particle had better uptake than 17 μm aluminum hydroxide aggregates owing to the better uptake of smaller particles by antigen presenting cells (Morefield *et al*., 2005).

In the present study, we report the development of an inactivated RVF vaccine with a size reduced aluminum hydroxide adjuvant prepared by dispersion of the original ALhydrogel and formulation of a more immunogenic formula than the locally produced vaccine.

MATERIALS AND METHODS

Rift valley fever virus

Rift Valley Fever (RVF) Zagazig Human 501 (ZH501) virus was supplied by department of RVF vaccine research, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. The Virus was propagated in Vero cell line. The virus titer was $10^{7.4}$ TCID50/ ml.

Virus Inactivation

Inactivation was preformed using Binary Ethyleneimine (BEI) that was added to the viral suspension at final concentration 0.1 mM and placed in the incubator at 37°C for 24 h with continuous stirring. The BEI action was neutralized by adding sodium thiosulphate giving a final concentration of 10% of the BEI volume used. Inactivation was checked using two passages in Vero cell lines.

Aluminum hydroxide gel

Rehydragel® LV a 2% (w/v) aqueous suspension of aluminum hydroxide gel adjuvant, was purchased from CHEMITRADE, United States of America.

Dispersion of aluminum hydroxide

The 2% stock solution of Aluminum hydroxide gel was hand mixed thoroughly and Trehalose 10% which was added by ratio 3:1; respectively before ultrasonication. Samples of 2 ml volumes were treated using a probe ultrasonicator (Misoix Sonicator 3000, USA) with a

microtip probe at intensity setting 3, with 15/5 s on/off time ultrasonication and cooling cycles for 20 minutes.

Particle sizing

Particle sizing was conducted using Nanosizer Zs (Malvern, UK). Ultra pure water was served as the diluent.

Transmission electron microscopy

TEM study was performed at ambient temperature using high resolution transmission electron microscope JEM-2100 JEOL, Japan, at 100 KV. The inactivated virus was fixed with formaldehyde (final concentration, 0.01%) and concentrated through a sucrose cushion (20%, w/v) in TN buffer (50 mM Tris-HCl, 100 mM NaCl) at 100,000 ×g for 1 h. The pellet was resuspended in TN buffer and mixed with either dispersed aluminum hydroxide or untreated one and the vaccine was diluted in TN buffer 1:4. The vaccine was negatively stained by phosphotungstic acid 1%.

Determination of protein binding

The protein-adjuvant mixtures were centrifuged at 3000 ×g for 5 min at room temperature. The supernatants were collected and assessed for protein concentration by measuring the absorbance at 280 nm. The amount of protein adsorbed was calculated by subtracting the remaining free protein in the supernatant from the total protein concentration added initially.

Vaccine formulation

Three formulas were prepared, the first is commercial vaccine the second is vaccine with dispersed aluminum hydroxide as adjuvant, the third is aluminum hydroxide added to it trehalose without treatment as an adjuvant. Commercial vaccine was prepared with RVFV ZH 501 virus with titer of $10^{7.4}$ MLD50 inactivated by Binary-Ethylene-Imine to Aluminum hydroxide gel with mixing volume ratio 50:50 stirred for 30 minutes at 150 rpm. Similarly, the second group was formulated using the dispersed aluminum hydroxide gel, the third group with antigen added to non-treated aluminum hydroxide and trehalose, the fourth group was prepared like the first group using Aluminum hydroxide gel and inactivated virus to be followed by dispersion using ultrasonication.

Sterility

The vaccine was tested for sterility according to OIE regulations. Samples were tested in thioglycolate and soybean casein digest medium. Each sample was inoculated into thioglycolate and soybean casein digest medium. The thioglycolate cultures were incubated at 37 °C for 7 days and the soybean casein digest medium cultures were incubated at 20 °C for 14 days**.**

Mouse Care

All work including animals was conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the Cairo University. Mice were housed 5 to a cage with food and water.

Safety

Safety was determined by intracerebral injection of 10 adult mice and two litters of 10 infant mice per litter. The mice were observed for a period of 14 days.

Immunization studies

Adult young mice (21 days age) were each vaccinated intraperitoneal with 0.2 ml doses of vaccine on days 0 ,7 and 14. The mice were bled Fourteen days after the second dose of vaccine and serum was obtained to measure their serologic response. Unimmunized negative control group was included.

RVFV antibody detection by VNT

Test sera were inactivated for 30 minutes in a water bath at 56 °C. Twofold serial dilutions of the sera were added to duplicate wells of microtiter plate starting from 1:5 to 1:80 and 50 µL of 100 TCID50 ZH501 RVFV were added to each well. Thereafter, the mixtures were incubated at 37 °C for 1 hr, and 10^5 Vero cells were added to each well then, the plates were incubated at 37 ºC with 5% CO_2 for 5–6 days. Neutralization index was determined.

Challenge test in mice

To establish the potency of the vaccine adult mice (21 days age) were each vaccinated intraperitoneal with 0.2 ml doses of vaccine on days 0 and 7 and 14. Fourteen days after the second dose of vaccine, mice were challenged by I/P 0.1 ml / mice with 1000 LD_{50} of ZH 501 RVFV. The challenge dose was previously standardized by LD50 determination of virulent virus in mice. Parallel positive control and negative control groups were included.

RESULTS

Particle sizing and potential

The particle size and potential were measured for the aluminum hydroxide dispersed by ultrasonication in the presence of 1.5% trehalose for 20 min the first trial showed a visually noticed difference in the physical characters between the treated sample compared to the control one as shown in fig (1) the treated sample was more clear than the untreated. The treated sample was of an average of 1494, 1499 and 1251 nm for the records of the first sample fig (2). The Zeta potential was 0.378, -0.216 and -0.726 mV (fig (3). The second sample was ultrasonicated for also 30 min but with final 3% trehalose concentration the average size was 315,346 and 380 nm fig (4). Zeta potential was 28.5, 31 and 32.5 mV fig (5).

Fig. 1: Aluminum hydroxide gel sample with 1.5% trehalose after treatment with ultrasonication for 30 min with setting intensity 3 in the presence of ice the sample becomes clearer by time and the viscosity changes (1), control sample without treatment (2).

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 1494	Peak 1:	249.4	71.4	60.30
PdI: 1.000	Peak 2:	28.74	20.6	8.084
Intercept: 0.333	Peak 3:	5.051	5.9	1.293

Result quality : Refer to quality report

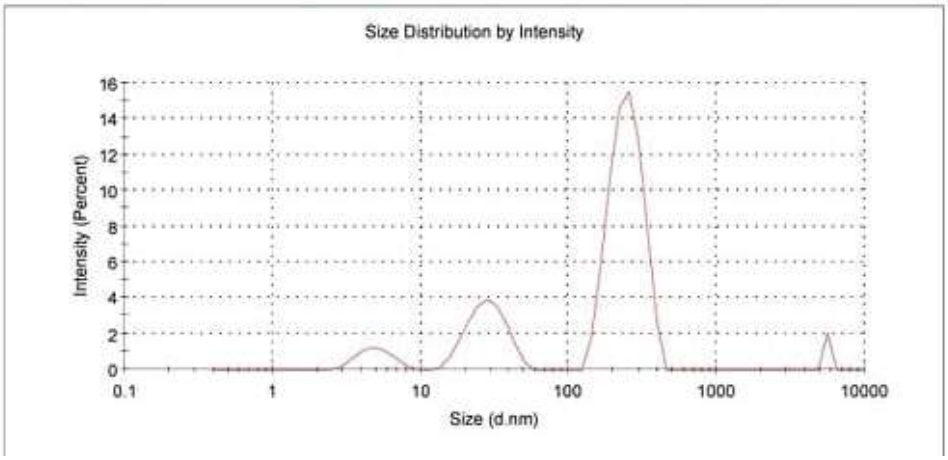

Fig. 2: Average size of the treated aluminum hydroxide gel sample with 1.5% Trehalose using Zeta analyzer.

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -0.216	Peak 1:	-3.85	89.4	5.11
Zeta Deviation (mV): 13.9	Peak 2:	34.5	10.0	2.31
Conductivity (mS/cm): 1.70	Peak 3:	-23.6	0.6	1.92

Result quality : See result quality report

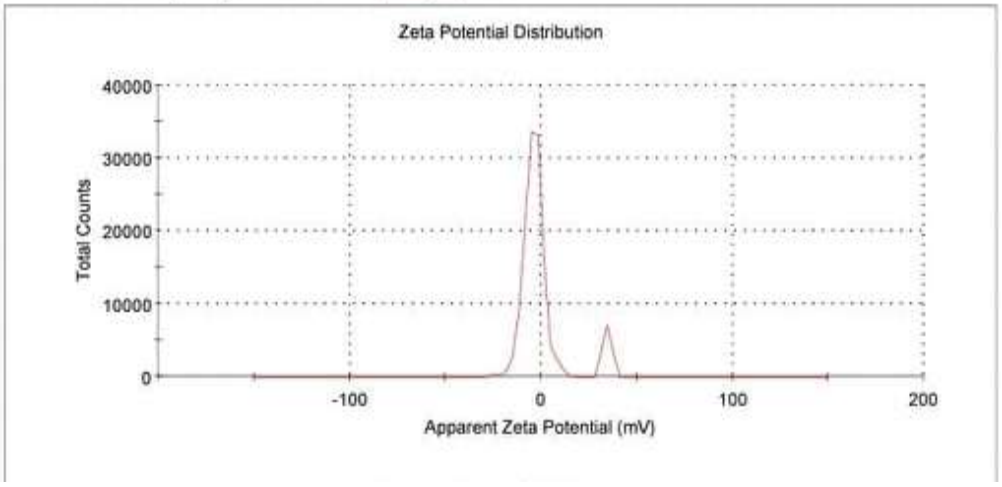

Fig. 3: Zeta potential for the treated aluminum hydroxide gel sample with 1.5% Trehalose using Zeta analyzer.

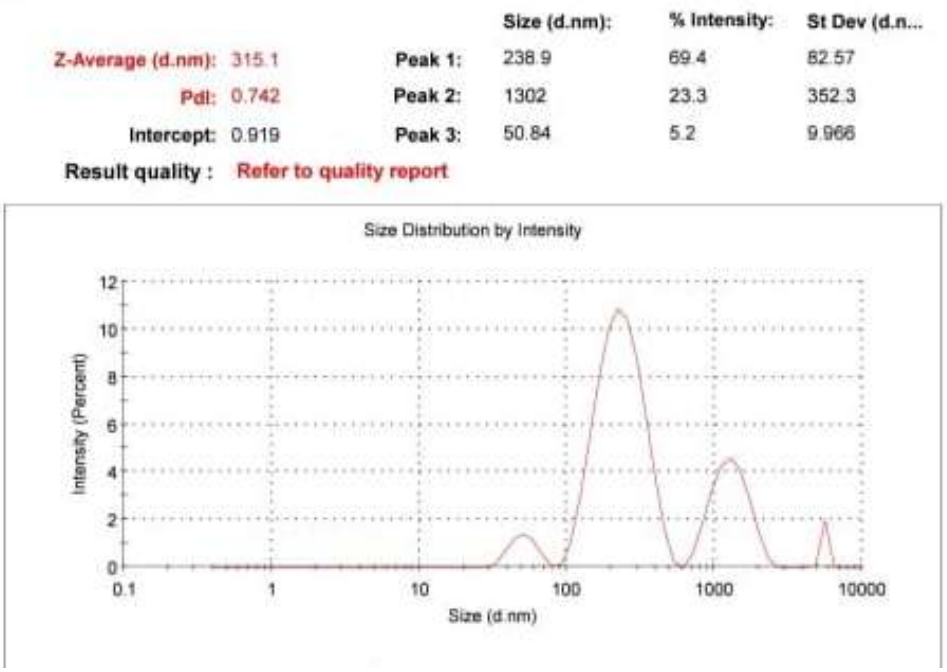

Fig. 4: Average size of the treated aluminum hydroxide gel sample with 3% Trehalose using Zeta analyzer.

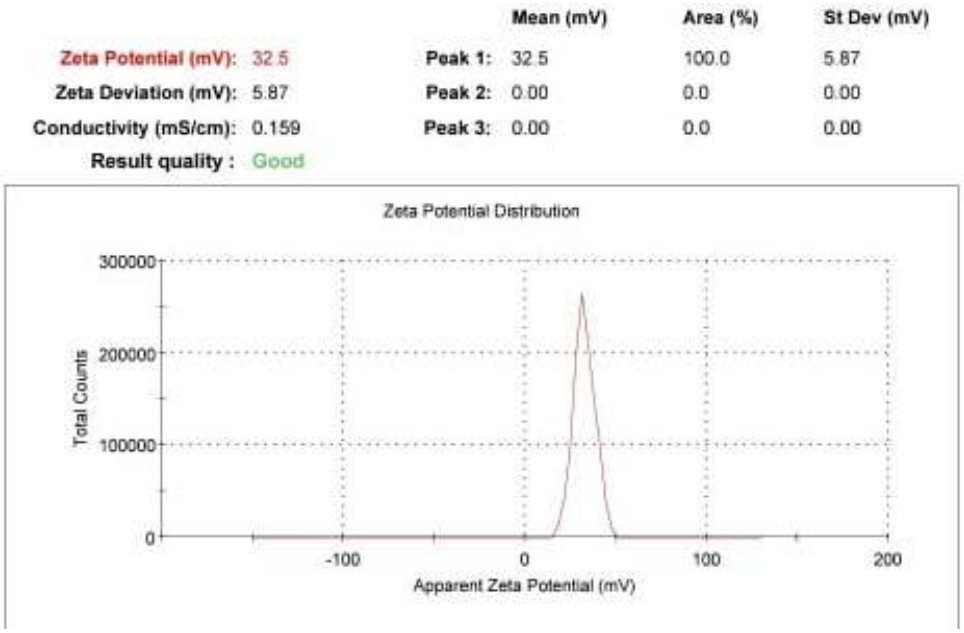

Fig. 5: Zeta potential for the treated aluminum hydroxide gel sample with 3% Trehalose using Zeta analyzer.

Transmission electron microscopy

The high-resolution transmission electron microscopy of the untreated samples of aluminum hydroxide gel showed dense clusters of size range about 1-12 µm while the dispersed sample with 3% trehalose showed a much smaller size clusters with the majority of 100 -200 nm, also less dense clusters of the primary fine aluminum hydroxide crystalline structure indicates

better dispersion and distribution. The vaccine with untreated aluminum hydroxide showed non uniform aggregates in micro scale similar to that of the control before loading inactivated virus to it. The dispersed ones showed single virus particles coated with aluminum hydroxide with uniform shape and size of 300 nanometer.

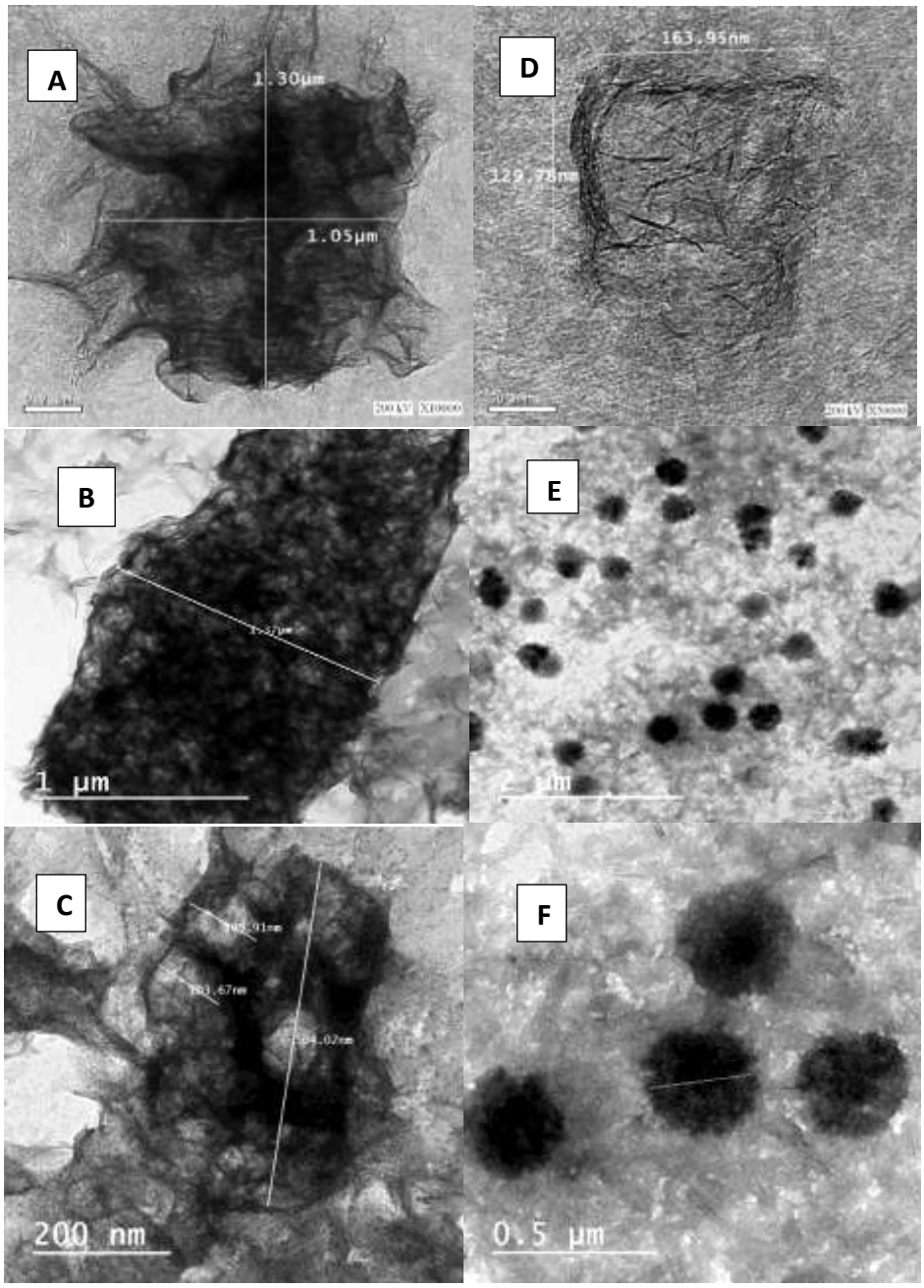

Fig. 6): TEM images for aluminum hydroxide gel. A: Aluminum hydroxide gel before treatment D: Dispersed aluminum hydroxide gel after treatment by ultrasonication for 30 min with setting intensity 3 in the presence of 3% trehalose. (A) and (D) considered control as they represent the gel before loading. B: The loaded inactivated RVFV on aluminum hydroxide showing non-uniform aggregates where the virus particles are imbedded in it as shown more clearing with lower field scale shown in (C). D: The loaded inactivated RVFV on dispersed aluminum showed uniform particles of 300 nm diameter as also shown in (F).

The TEM images of the formulated vaccine with dispersed aluminum hydroxide gel showed the mechanism by which the uniform spherical particles are formed as one of the images in the studied sample (figure 7) showed a virus particle partially surrounded by aluminum hydroxide which is normal as not all the virus particles are normally completely loaded. The image also viewed that the inactivated virus preserved its morphology during formulation.

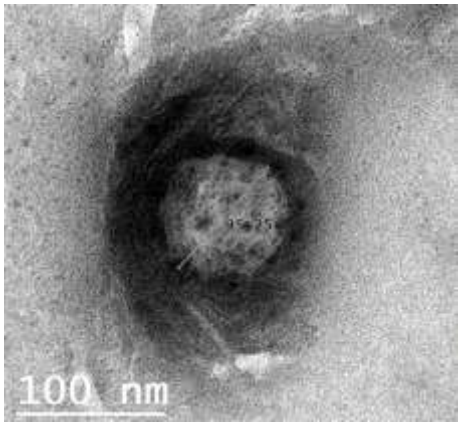

Fig. 7: Showing the layers between the adjuvant and the antigen as one of Rift Valley fever virus particles attracts dispersed aluminum hydroxide fibers after treatment of aluminum hydroxide by ultrasonication in the presence of trehalose 3%.

Determination of protein binding

Table 1: Adsorbed protein values.

Adjuvant	mg protein/ml
Aluminum hydroxide gel	1.3
Treated Aluminum hydroxide gel with trehalose	1.87
Aluminum hydroxide gel with trehalose untreated	1.1

Sterility

All samples for different vaccine formulations showed no turbidity on the thioglycolate cultures throughout 7 days of incubation at 37°C or the soybean casein digest medium cultures throughout 14 days.

Safety

No deaths or signs of clinical manifestations and all vaccinated animals were healthy over an observation period of 14 days as stated by OIE guidelines.

RVFV antibody detection by VNT

Table 2: Neutralizing index log mean values and challenge results.

Vaccine	Doses	Neutralizing index log mean values	Protection % (number of mice survived / total number)
Aluminum hydroxide adjuvanted	3 i.p.	2.6	4/5 (80%)
Dispersed aluminum hydroxide	3 i.p.	3.2	5/5 (100%)
Aluminum hydroxide with trehalose without treatment	3 i.p.	2.1	4/5 (80%)
Control negative	Placebo	0	0/5 (0%)

DISCUSSION

Currently, Inactivated Rift valley fever vaccine is the only vaccine incorporated in the control program of RVF in Egypt (kamal, 2011). As RVF is one of the most the most important zoonotic diseases which possesses high risk on livestock and public health, it is always a necessity to work on enhancement of the currently produced vaccine formula and to innovate new ones aiming to obtain more efficacious vaccines. The locally produced vaccine in Egypt is a Binary ethylenimine inactivated Rift valley fever vaccine with aluminum hydroxide gel as an adjuvant.

Aluminum hydroxide adjuvant is composed of small primary fibers with an average calculated dimension of 4.5 × 2.2 × 10 nm (Romero *et al*., 2007). In aqueous form they form porous aggregates with irregular shapes of size 1-20 µm (Hem *et al.,* 2007). This aggregation is attributed to bridging between particle surfaces (liquid and solid bridges) and interlocking (by macromolecular and particle shape effects) caused by surface and field forces (van der Waals, electrostatic and magnetic forces (Tomas, 2007). Once large clusters either aggregates (condensed structures of primary particles, which are held together by solid bridges) or agglomerates (looser and more open structures) are generated in the synthesis process. Although production of a homogenous of uniform size crystallites by disaggregation the clusters has not been achieved, dispersion of the clusters by ultrasonication was shown to be reproducible and to be intensity and time dependent (Nguyen *et al.,* 2011).

During our attempts to disperse the ready to use aluminum hydroxide gel without diluting the sample to a very low concentration no observed change in the physicochemical characters of the samples occurred even with increasing the intensity (750 watt) and time (up to 60 min). To avoid the necessity of diluting the aluminum hydroxide as this dilution was going to impair its following use as an adjuvant, we used trehalose as a dispersant, the choice of trehalose was attributed to efficiency in preventing aggregation while freezing in lyophilized vaccines containing aluminum salts. Initially we started with a 5% trehalose solution added to aluminum hydroxide gel (Rehydragel LV® 2%) to obtain a final concentration of 1.5% to 1.2% respectively. The samples were dispersed by ultrasonication for 30 min using pulse intensity 3 in the presence of ice to avoid rise in temperature. The size analysis showed a reduction in size to ~1400 nm instead of the known average size of 3 µm. The zeta analysis report showed -0.2 mV which indicated not only low stability but also a change in the net charge from being positive (11 mV) to negative value this results indicated that concentration of the trehalose used was limiting and insufficient to induce adequate decrease in the attraction between aluminum particles in aqueous solution without affecting the stability. In the following attempts where trehalose 10% was added to a final concentration of 3% and the gel concentration as aforementioned, the Zeta potential was positive for the three given readings with an average 30 mV which is considered stable as the zeta potential of > 30 mV have more stability than of that < 30 mV. The treated sample was more positive than the original one, this increase in the positive charge may be explained by the increase of the total surface area as a result of the size reduction occurred, thus more metallic hydroxyl groups on their surface were exposed. Indeed, the size was significantly decreased to reach average 300 nm. Further analysis of the sample by imaging via high resolution transmission electron microscope showed a high level of disaggregation when compared to the untreated samples with more diffuse nature and much smaller size.

The loading capacity expressed by the amount of total protein adsorbed in each of the tested samples showed that although the concentration of aluminum hydroxide was less by one third in the sample where trehalose was added to aluminum hydroxide before treatment by sonication followed by adding the inactivated virus, the amount of the adsorbed protein was

more than the untreated sample. The high binding capacity of the treated sample may be attributed to the expected increase in total surface area so increase the binding sites after disaggregation and the increase of the positive charge of the treated aluminum hydroxide in comparison to the untreated (Jones *et al.,* 2005).

The immune response induced by dispersed aluminum hydroxide was higher than the untreated formula, this may be a result of increasing the loading capacity of the treated aluminum hydroxide sample and/or the lesser size and more uniform shape of the used formulation. Reducing the particle size of vaccine from micro sized aggregates (1-20 µm) to 300 nm may have influenced the better immune response by increasing the uptake of antigen by antigen presenting cells (Wendorf *et al.,* 2006). This was also reflected by the challenge results as the protection among the vaccinated animals with the conventional vaccine and the group vaccinated with aluminum hydroxide and trehalose was 80% in both groups while there were no mortalities among the group vaccinated with the developed formulation.

CONCLUSION

Dispersion of aluminum hydroxide by ultrasonication lead to increase the loading capacity of the adjuvant and changed the shape of the vaccine from irregular aggregates of aluminum hydroxide diffused in it the antigen to a more uniform single virus particles coated with aluminum hydroxide enhanced the specific antibody responses. Lower aluminum hydroxide concentration in the developed formula was still able to induce higher immune response, this finding may be useful to future use in other formulas in both veterinary and human vaccines to decrease the aluminum hydroxide concentration in vaccine after more extensive studies on its safety profile.

ACKNOWLEDGEMENT

The authors declare that they have no competing interests.

AUTHOR DETAILS

[1]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt, P.O. Box 12211.
[2]Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Egypt.

RECEIVED: Oct.2019; **ACCEPTED**: Dec. 2019; **PUBLISHED:** Jan. 2020

REFERENCES

Daubney, R., Hudson, J.R. and Garnham, P.C., 1931. Enzootic hepatitis or rift valley fever: An undescribed virus disease of sheep cattle and man from East Africa. The Journal of Pathology and Bacteriology.

Hem, S. L. and Hogenesch, H., 2007. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. Expert Review of Vaccines,

Himeidan, Y.E. *et al*., 2014. Recent outbreaks of Rift Valley fever in East Africa and the Middle East. Frontiers in Public Health,

Huiskonen, J.T. *et al*., 2009. Electron cryo-microscopy and single-particle averaging of Rift Valley Fever Virus: Evidence for GN-GC Glycoprotein Heterodimers. Journal of Virology,

Ikegami, T., 2017. Rift Valley fever vaccines: an overview of the safety and efficacy of the live-attenuated MP-12 vaccine candidate. Expert Review of Vaccines,

Ikegami, T. and Makino, S., 2011. The pathogenesis of Rift Valley Fever. Viruses,

Jones, L.S., Laura, J.P., Jonathan, P., Aaron, M., Brian, Y., and Middaugh C.R., 2005. Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. Journal of Biological Chemistry,

Li, X. *et al.*, 2011. Relationship between the size of nanoparticles and their adjuvant activity: Data from a study with an improved experimental design. European Journal of Pharmaceutics and Biopharmaceutics,

Morefield, G.L. *et al.*, 2005. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells *in vitro*. Vaccine,

Mumper, R.J., Cui, Z. and Oyewumi, M.O., 2003. Nanotemplate engineering of cell specific nanoparticles. Journal of Dispersion Science and Technology,

Nguyen, V.S. *et al.*, 2011. Effect of ultrasonication and dispersion stability on the cluster size of alumina nanoscale particles in aqueous solutions. Ultrasonics Sonochemistry,

Romero Méndez, I. Z. *et al.*, 2007. Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants. Vaccine,

Tomas, J., 2007. Adhesion of ultrafine particles-A micromechanical approach. Chemical Engineering Science,

OIE, 2014. Terrestrial Code-Rift Valley fever. Vol. 2. Paris, France: OIE; pp. 1-20.

Wendorf, J., Manmohan S., James C., Jina K., Elawati S., Mildred U. and Derek O., 2006. A practical approach to the use of nanoparticles for vaccine delivery. Journal of Pharmaceutical Sciences,

Cite this article as:
Aly *et al.,* (2020): Ultrasonic dispersion of aluminum hydroxide-adjuvanted Rift Valley Fever vaccine enhances formula characteristics and immune response. *Journal of Virological Sciences*, Vol. 7: 86- 96.