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INACTIVATION OF RIFT VALLEY FEVER VIRUS BY ASCORBIC ACID

By

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ABSTRACT

Ascorbic acid is an important antioxidant. However, in the presence of transition metals such as Copper sulphate, it also has pro-oxidant capabilities. This work investigates the effect of ascorbate Cu^{+2} in the infectivity of Rift Valley Fever virus (RVFV) as an inactivator. RVFV was treated with different concentrations (0.5, 1.0, 1.5 mg/ml) of Ascorbic Acid in the presence of Cu^{+2}Micro-titer technique and mice assay were used to titrate the used virus, toxicity of Ascorbic Acid and also determination of inactivation kinetics of RVFV.The results revealed that RVFV was completely inactivated within 24 hours post treatment with 1.0 mg and 1.5 mg/ml of Ascorbic Acid compared with the control group, and the potency test of the experimental batch of A.A inactivated RVFV vaccine had ED_{50}0.014 in immunized mice, concluded that ascorbic acid can be used as an inactivator agent for preparation of safe and potent RVF inactivated vaccine.

INRODUCTION

Rift Valley Fever is a vector-borne viral disease that infects livestock and human **(Flick and Bouloy, 2005)** in Africa and Arabian Peninsula **(Papin *et al.*, 2011)**.The disease is considered a zoonotic disease for human. Economic losses in endemic regions can be great due to death of the host and/or abortion in livestock, particularly cattle,sheep, camels and goats. The disease is caused by a virus of the genus phlebovirus, family Bunyaviridae **(Ikegami, 2012)**. The virus was firstly isolated in 1931 during an epidemic among sheep in the Rift Valley Region in Kenya **(Daubney *et al.*, 1931)** since then; the disease has been reported in regions of eastern and southern Africa, but also in Egypt and Saudi Arabia. OIE classified the virus as list A, with a highly potential pathogen for international spread **(OIE, 2011)**. Vaccine antigenicity and immunogenicity is related to the virus inactivator used **(Blackburn and Besselaar, 1991 and Mohamed *et al.*, 1997)**. Therefore, it is important to find an inactivator

with rapid inactivation efficacy, higher safety measures and no adverse effects. For a number of years, formalin, beta-propiolactone and gamma radiation have been used for the inactivation of RVFV, although these are satisfactory, a gamma emission source is expensive and often not readily available. Also, the use of beta-propiolactone in production quantity places the producer at a risk in the use of this carcinogenic material. The side effects of formaldehyde are well known and the risks are many. The use of new inactivants must be considered for availability, higher safety measures and better antigenicity.Choosing available, cheap and safer inactivants are of the parameters for improving the quality and immunogenicity of the vaccines **(Madhusudana *et al.,* 2004)**. The use of Ascorbic Acid (Vitamin C) as an antiviral agent has been reported primarily as a prophylactic and therapeutic agent whereas ascorbic acid is a water soluble and pre-eminent scavenger of oxygen radicals that undergo auto oxidation catalyzed by cupric ions to inactivate vaccinal viruses **(Madhusudana *et al.,* 2004)**.Also, ascorbate enhances several function of the immune system as the production of interferon, which helps to prevent cells from being infected **(Karpinska *et al.,* 1982).** The study approach was to determine the effect of ascorbic acid as an inactivator of RVF virus, as measured by the infectivity of cell cultures and mice assay.

MATERIAL AND METHODS

Rift Valley Fever strain (RVFV):

RVFV (ZH_{501}) strain was kindly provided by RVF Department, Vet. Serum and Vaccine Research Inst. Abbasia, Cairo. Its titer was $10^{7.5}TCID_{50}$/ml.

Cell Line Culture:

Monolayer of the African green monkey kidney (Vero) cell line supplied by RVF Dept in (VSVRI) was used for propagation and titration of RVF virus as well as toxicity test of Ascorbic acid,

Thesecellswerepassagedandmaintainedusingminimumessential medium(MEM) supplemented with 10 % newborn calf serum.

Animals:

Adult (21-28 days of age) and baby Swiss albino mice obtained from the animal house unit in Vet. Serum and Vaccine Research Inst. Abbasia, Cairo, were used for titration of RVF virus, toxicity of inactivate, determination of complete virus inactivation and determination of the safety and potency of the prepared inactivated vaccine batch. The animals were maintained under hygienic laboratory conditions.

Inactivant:

Ascorbic acid and Copper Sulphate:

Stock solution containing 0.1M of Copper Sulphate and 0.5M of Ascorbic acid were used and sterilized by membrane filtration. These salts were supplied by Sigma Chemical Company (USA).

Virus Titration:

Titration of stock virus used was performed using the Micro-titer technique. The virus titer was calculated according to **Reed and Menuch, (1938)**. Mice assay was conducted where the same 10 fold serially diluted virus injected in a group of mice. Each mice group (8/group) received 0.1ml/mouse intraperitoneal. Mice groups were observed for detection of signs of illness and mortality for 7 days' post inoculation. The end point induced 50% mice mortality was determined.

Toxicity of Ascorbic acid:

Toxicity of mixture of ascorbic acid (0.5, 1.0 &1.5 mg/ml) and 5µg/ml of copper sulphate for each concentration was determined in cell culture and mice assay according to **Ecobichon, (1997).**

Inactivation of RVFV using Ascorbic acid:

Aliquot of RVFV was inactivated using ascorbic acid prepared as stock solutions 0.1M of copper sulphate and 0.5M of Ascorbic acid in sterile distilled water and sterilized by membrane filtration. The stock solutions were added to the virus suspension to obtain a final concentration of 5µg/ml of copper sulphate and 0.5, 1.0 &1.5mg/ml of ascorbic acid. The treated virus was incubated at 37^0C with continuous stirring. One ml of treated virus was collected at time interval to determine complete virus inactivation, **(El-Karamany, 1987 and Heba *et al*., 2010)**.

Determination of inactivation kinetics of RVFV:

Virus sample (10 fold serially diluted in MEM) was prepared and the treated virus collected at time interval was dispensed on Vero cell line tissue culture plates. The infected and control culture plates were incubated and examined daily for detection of any cytopathic effect. Also, the same treated and diluted virus inoculated intraperitoneally in adult mice and observed daily for signs of illness and/or mortality for 10 days.Another group of mice was left as negative uninoculated control.

Preparation of Experimentally Inactivated Vaccine Batches:

Two portion of inactivatedRVFV infected fluid were prepared,one with Binary Ethelenaimine (BEI)and other portion was inactivated with 1.5mg ascorbic acid/ml &5µg copper sulphate/ml (The best concentration allowing complete virus inactivation). Complete inactivation was confirmed by virus titration in cell culture and mice inoculation that revealed zero virus titer and safety for mice. Twenty percent (20%) of aluminum hydroxide gel (AlOH) was added to the inactivated virus as adjuvant **(El-Nimr, 1980 and El-Karamany *et al.,* 1981)** and subjected to quality control tests as recommended by **OIE (2012)**.

Quality Control Tests of the prepared bathes of vaccines:

Quality control tests including the freedom of foreign contaminants sterility, safety, and potency tests were conducted on the prepared experimental batches according to **OIE (2012)**.

Potency of the inactivated RVFV vaccine:

(Effective Dose end point-ED_{50}).

Potency of the two batches of inactivated RVF viral vaccine prepared using ascorbic acid and copper sulphate (1.5mg/ml&5µg/ml) and BEI as chemical inactivator were evaluated using mice immunization assay (MIA)**,** where the experimental vaccinal batches was 5 fold serially diluted (1/1-1/625) using sterile hank's balanced salt solution (HBSS),pH 7.2±2.Each dilution was inoculated intraperitoneally (I/P) as 0.2ml in 21-day old weaning mice, (8 mice/dilution), another set of 8 mice were left as negative control. One week later the 2nd dose of the vaccine; diluted as previous was inoculated.14 days post the 1st shoot, a challenge doseof0.1ml containing1000-10,000MIPLD$_{50}$wasintraperitoneallyinoculated. Four weaning mice of the negative control group were challenged in the same way. The rest of control mice group was left as negative control. Mortality was recorded throughout 14 days and the potency of inactivated RVF virus vaccines was determined.

RESULTS AND DISCUSSION

The choices of available, cheap and safer inactivants are one of the parameters for improving the quality and immunogenicity of the vaccines **(Madhusudana *et al,* 2004)**. During investigations of various chemical agents as preservatives and inactivators of viral diagnostic antigens, reference was found to the use of ascorbic acid as an antiviral agent and has been reported primarily as a prophylactic and therapeutic agent against the common cold. Also the inactivation of vaccinia virus by ascorbic acid undergoing auto-oxidation catalyzed was reported **(Turner, 1964).** Similar reactions with bacterial, fungal and viral agents were

described by **Ericsson and Ludbeck (1955)**. In addition to its effect on the released interferon level accordingly it was interestingly tried for inactivation of Rift Valley fever virus as an economic matter of importance **(Mohamed *et al.,* 1997).** The current study amid to determine the effect of ascorbic acid on RVFV as chemical inactivator using the infectivity of cell cultures and mice. The presented results revealed that all concentrations (0.5, 1.0 &1.5mg/ml, and 5µg/ml) of Ascorbic acid and copper sulphate (C^{+2}) were completely safe to tested mice where no signs of illness and no mortality were detected after ten days' post- injection also toxicity in cell culture was not determined, (Table1). Concerning inhibition of RVFV using different concentrations of ascorbic acid with 5µg/ml copper sulphate, the results revealed a significant effect, where RVF virus was completely inactivated within twenty-four hours post treatment by (1mg/ml and 1.5mg/ml) ascorbic acid, where the virus infectivity titer dropped about 1.7 $\log_{10}$ & 2.5$\log_{10}$ value by 4 hrs. to reach complete inactivation by 24 hrs. & 16 hrs. respectively. While 0.5mg/ml had no appreciable reduction in the infectivity titer as it remained at high titer over 24 hrs. (Tables 2, 3). A result that agrees with that obtained by **AbdEl-Razek *et al.,* (2011).** Similar findings were reported by **White *et al.,* (1986)** who used ascorbic acid to inactivate parainfluenza type 2 within 24 hrs. at 37ºC with 1 mg ascorbic acid/ml. The loss of infectivity did not alter either the hemagglutination or complement fixation qualities of the antigen. ***Madhusudana et al., (2004)*** inactivated rabies virus CVS strain on VERO cell culture using ascorbic acid at concentration of 0.5 mg/ml after 72 hours at 4ºC and 1mg/ml after 48 hrs. The same results were obtained using mice inoculation where the groups were inoculated with virus treated with 1.0 &1.5 mg/ml of ascorbic acid and 5µg/ml CuSO4 at 16 hrs. and 24 hrs. Post-treatment remained alive and did not show illness or mortality compared with the positive and negative control groups, proving complete inactivation of RVFV after those periods, while the inoculated group by 0.5mg/ml of ascorbic acid and 5 µg/ml CuSO4 after 24 hrs. Caused death of all mice as well as positive control group (group inoculated by untreated virus) (Table 4). Regarding the quality control results of the prepared batches represented in (Table 5) revealed that, the prepared RVF batches of vaccine was free from aerobic, anaerobic bacteria and fungi. It was also safe and gave satisfactory results represented by the absence of cytopathic effect on tissue culture and absence of deaths in inoculated mice.The immune response against the prepared experimental batches of ascorbic acid and BEI inactivated RVFV vaccine (Vaccine potency, ED_{50}) was evaluated **(Mohamed *et al* 1997)**. The results

revealed that, the ED_{50} was 0.014 while the ED_{50} was 0.018 for the other batch (inactivated with BEI) used. These values were satisfactory as the permissible limit should not exceed 0.02 according to **Randall *et al.*, (1964).** It was concluded that, the treatment of RVFV with ascorbic acid accompanied by Cu^{+2} suggested that ascorbic acid can be used as an inactivated agent for preparation of safe and potent inactivated vaccine.

Table (1): Toxicity effect of different concentration of ascorbic acid and copper sulphate on vero cell and mice.

Concentration of AA + 5 µg/ml Cu^{+2}	**CPE on Vero Cell**	**% of sign of illness or mortality in mice**
0.5 mg/ml AA	**No degeneration**	**0 %**
1.0mg/ml AA	**No degeneration**	**0 %**
1.5 mg/ml AA	**No degeneration**	**0 %**
Control Cell Mice Control	**No degeneration**	**0%**

AA = ascorbic acid.

CPE = cytopathic effect.

Table (2): The effect of different concentrations of ascorbic acid on RVFV at 37ºc on tissue culture.

Concentration of AA+ 5 µg/ml Cu^{2+}	**Infectivity (CPE)/ hours post treatment**						
	0	**4**	**8**	**12**	**16**	**20**	**24**
0.5 mg/ml AA	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
1.0 mg/ml AA	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
1.5 mg/ml AA	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**-ve**

+ve = cytopathic effect.

-ve = no cytopathic effect.

Table (3): Inactivation rate of ascorbic acid treated virus on vero cell.

Concentrationof AA + 5 µg/ml Cu^{+2}	Infectivity Titre (log_{10} $TCID_{50}$/ml)/ hrs.						
	0h	4h	8h	12h	16h	20h	24h
0.5mg/ml AA	7.5	6.5	5.5	5	4.5	4	3.5
1.0mg/ml AA	7.5	5.8	4.1	2.9	1.7	0.5	0.0
1.5mg/ml AA	7.5	5.0	3.5	2.0	0.0	0.0	0.0

A.A. = Ascorbic Acid.

Hrs. = hours after treatment.

Table (4): Inactivation time of RVFV treated by ascorbic acid in mice.

Concentration of AA + 5 µg/ml Cu^{2+}	No of mice death	Time for Complete Inactivation
0.5 mg/ml AA	8/8	>24 hrs
1.0 mg/ml AA	0/8	24 hrs
1.5 mg/ml AA	0/8	16hrs
+ve Control	8/8	_
-ve Control	0/8	_

+ve =mice infected with RVFV

-ve =mice uninfected with RVFV

Table (5): Quality Control Tests of the prepared bathe of vaccines.

Type of RVF vaccine	Sterility	Safety		Potency (ED_{50})
		T.C.	Mice	
Inactivated by BEI vaccine	Sterile	No CPE	No deaths	0.018
Inactivated by A.A. vaccine	Sterile	No CPE	No deaths	0.014

T.C. = Tissue Culture.

CPE= Cytopathic Effect.

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