

VALIDITY OF ASCORBIC ACID AS VIRAL INACTIVANT FOR INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS AND BOVINE VIRAL DIARRHEA VIRUS

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Abstract

The present study was devoted to assess the potentiality of ascorbic acid (vit.C) catalytic with copper sulphate as inactivator for Infectious Bovine Rhinotracheitis Virus (IBRV) and Bovine Viral Diarrhea Virus (BVDV) compared with the conventional inactivants Binary Ethylene Imine (BEI). Stock viruses suspension of the two viruses were prepared. Virus infectivity titer were $10^{7.5}$ and $10^{6.5}$ respectively. The (BVD) and (IBR) viruses for suspension were inactivated separately with different concentrations (0.5,1.0 and 1.5mg/ml) of ascorbic acid. The treated viruses were incubated at 37°C incubator with continuous stirring for different period of time. It was found that the ascorbic acid induced complete virus inactivation at concentration of (1.0 and 1.5 mg/ml) after (24 and 18) hrs for BVDV and after (more than 24,24 hrs) for IBRV. While 0.001 M-BEI induced complete virus inactivation within 4 and 6 hours post treatment at 37°C respectively. Safety tests were carried out for the inactivated viruses either by using ascorbic acid or BEI, both of them was proved safe when inoculated MDBK tissue culture, in mice and in rabbit revealing complete virus inactivation. This study concluded that the optimum ascorbic acid concentration for IBRV and BVDV is (1.5mg/ml) because it made complete viral inactivation for two virus with 24 hour for each virus. Further study should be focused on IBRV and BVDV ascorbic acid inactivated vaccine to envisage the animal immune response comparing with traditional method (BEI inactivated vaccine) with the presence of ascorbic acid as an immune modulator agent in its composition especially in its nano form.

INTRODUCTION

Bovine Viral Diarrhea Virus belongs to family Flaviviridae, genus pestivirus genome ORF single-stranded, positive sense RNA virus (*Ridpath,2005*), Infectious Bovine Rhinotracheitis belongs to family Herpesviridae, genus alpha herpesvirus its genome is double stranded DNA virus (*Brian et al.,2010*). The two previous viruses are economically important viral pathogen of cattle causing BRDV syndrome. Combined Binary inactivated vaccines containing the two viruses are used extensively as an aid in prevention of Bovine respiratory syndrome (BRD) using BEI as an inactivant. Inactivation of these previous viruses is essential for the preparation of vaccines, diagnostic reagents and research purposes. Currently recommended inactivating

agent for this virus is a very expensive Binary Ethylenimine (BEI) chemical and potentially carcinogenic (*Girard et al., 1977*), other chemicals like formaldehyde and phenol not only inactivate the virus but also adversely affect its antigenicity. There is therefore a need to find alternative inactivating agents which are not expensive and are easily available and doesnot effect in antigenicity and immunogenicity. It was demonstrated by (*Turner, 1964*). That ascorbic acid undergoing auto oxidation catalyzed by cupric ions inactivated vaccinia virus (*White et al., 1986*). Subsequently this was confirmed with other DNA and RNA viruses and it was also shown that the inactivated viruses retained good antigenicity (*Rawal et al., 1995*). Recent studies have shown that this agent could also inactivate HIV7 and bacteriophages (*Murata et al., 1986*) and interest has been renewed for using this chemical as an antiviral agent. Keeping this in mind the effect of ascorbic acid on the viruses was investigated and the study here indicate that this chemical along with cupric ions is capable of causing complete and irreversible inactivation of (IBRV) and (BVDV) without adversely affecting the antigenicity.

MATERIALS AND METHODS

Viruses and Cell Culture:

Bovine Viral Diarrhea virus (BVD), Egyptian cytopathic strain (Iman strain) of a titer $10^{5.5}$ TCID₅₀/ml and Infectious Bovine Rhinotracheitis (IBR) virus, a local Abou Hammad strain of a titer $10^{7.5}$ TCID₅₀/ml.

The two viruses were obtained from RinderPest like Diseases department, Vet. Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt (VSVRI), and propagated and titrated on Madin Darby Bovine Kidney (MDBK) cell line cultures. The MDBK cells were grown and maintained in minimum essential medium with Earle's salts (MEME) supplemented with 1% antibiotic-anti mycotic 100x solution and 10% new borne calf serum (NCS). Both MDBK cells and NCS were proved to be free from Mycoplasma and adventitious BVDV strain.

Chemicals:

Ascorbic Acid and Copper Sulphate:

These salts were supplied by Sigma Chemical Company (USA). Fresh Stock solutions containing 0.01 M of copper sulphate and 0.5 M of ascorbic acid were prepared and sterilized by membrane filtration.

Binary Ethylenimine (BEI):

(2 Bromo Ethylamine Hydrobromide 95% d.w. 204.90 HV) was supplied by Aldrich chemical Co. Ltd. Grillingham Dorest. England. It was prepared according to *Girard et al., (1977)*.

Sodium Thiosulphate Solution:

It was used at a final concentration of 2% to stop the reaction of the (BEI) inactivate.

Inactivation experiment:

1- Using Binary Ethylene Amine:

BEI was added to the both (BVD&IBR) viral suspension with a final concentration 0.01 of 0.01M. The treated viruses were incubated at 37°C with continuous stirring. The inactivation process was stopped by addition of sodium thiosulphate with final concentration 2% (*Girard et al., 1977*).

2- Using Ascorbic Acid and Copper Sulphate:

Stock solutions of 0.5M of ascorbic acid and 0.01M of copper sulphate were prepared in sterile distilled water and sterilized by Millipore filtration. The stock solutions were added to both viruses to obtain final concentration of 5µg/ml of copper sulphate and 0.5, 1.0 and 1.5 mg/ml ascorbic acid.

The treated viruses were incubated at 37°C with continuous stirring (*Madhusudana et al., 2004, Gabriel et al., 2004*)

Complete viruses inactivation was confirmed by virus titration in cell culture as 1 ml of treated viruses was collected at predetermined time interval. Virus samples were 10 fold serially diluted 10^1 - 10^8 in maintenance media (MEM). The Prepared dilutions were dispensed onto MDBK pre cultured cell line in 96 well plates. Infected cultured plates were incubated in CO₂ incubator and daily examined for detection of specific Cytopathic Effect (CPE).

Sterility test:

It was performed in accordance with USA Code of Federal Regulation, *CFR (1987)*.

Safety test:

It was performed in accordance with USA Code of Federal Regulation, *CFR (1987)*.

1- Mice: 10 young mature mice each weighting 10-15gm.

2- Rabbit (As a model for cattle): 6 young mature rabbit.

RESULT AND DISCUSSION

Bovine Respiratory Diseases (BRD) have crucial and serious impact on the beef and dairy cattle industry, both for stocker and feedlot entities. Economic losses result from death, decreased performance of diseased cattle. Pneumo enteritis problem in cattle and calves in Egypt is caused by viral agents include Bovine Viral Diarrhea Virus (BVDV), Infectious Bovine Rhinotracheitis Virus (IBRV), Parainfluenza-3 Virus (PI-3V) and Bovine Respiratory Syncytial Virus (BRSV) (*Samira et al., 2001*). Optimization of the conventionally produced vaccine is absolutely necessary especially in terms of the inactivating material so as to modulate production recipe of superior quality vaccine. BEI has been used satisfactorily but its use in mass production loads put the producer at a risk because it is carcinogenic material, also effort for getting rid of precipitating salt which lead to losing cell associated antigen as concluded by (*Nietert, et al. 1974*).

there is a need to find an alternative inactivating agent which is not expensive and always available as ascorbic acid which is undergoing auto oxidation results in the formation of OH groups that could induce the virus inactivation (*Murata et al., 1986*).

The use of ascorbic acid (vitamin C) as an antiviral agent has been reported primarily as a prophylactic and therapeutic agent. 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 product. *Turner (1964)* reported the inactivation of vaccinia virus by ascorbic acid undergoing auto-oxidation catalyzed by copper. Similar reactions with bacterial, fungal and viral agents were described by *Ericsson and Ludbeck (1955)*. While examining the survival of enteroviruses in food, *Salo and Cliver (1978)* also investigated the effect of ascorbic acid on polio-, coxsackie-, and echoviruses inactivation.

Our approach was to determine the effect of ascorbic acid on IBR and BVD viruses, as measured by the infectivity in cell cultures.

Concerning the preparation of stock virus for vaccin production, the present work optimized some conditions for yielding a high virus titer to be suitable for preparation of a potent inactivated viral antigen. The result revealed that propagation of BVDV and IBRV viruses in MDBK cells culture which was being carried out at 24 hours post seeding of the cells where the cells did not reach a complete confluence resulted in characterized by cytopathic effect represented by Granulation of cytoplasm, bunches of grapes formation in case of IBRV with infectivity titer $10^{7.5}$ TCID₅₀/ml and cell rounding, Shrinkage of the cell wall, Granulation of the cytoplasm, Vacuation, Cell elongation in case of BVDV with infectivity titer infectivity titer $10^{5.5}$ TCID₅₀/ml as shown in Table (1).

The experimental results comparing the effectiveness of BEI and different concentration of ascorbic acid and Cu²⁺ on BVD and IBR viruses represented in (table 2-3) revealed that 0.001M of BEI made Complete inactivated IBR and BVD viruses occurred after 6&4 hours post treatment respectively after cyclization of 2-bromoethylamine hydro bromide in alkaline solution (sodium hydroxide) (*El-Sabbagh et al., 2001*), while complete inactivated with different ascorbic acid solution (1.0 And 1.5mg of ascorbic acid /ml & 5µ/ml of copper sulphate) occurred at (more than 24 hours post treatment (h.p.t), 24 h.p.t) for IBR and (24,18 h.p.t.)for BVD viruses respectively at 37°C while 0.5mg/ml & 5µ/ml induced complete inactivation in both viruses at 48hpt and more than 24h.p.t respectively at 37°C , these findings are coincidence with those obtain by (*White et al., 1986*) Who emphasized that the auto-oxidation of ascorbic acid catalyzed with cupric ions liberate (OH) group that inactivated vaccinia virus as well as inactivated HIV7 and bacteriophages (*Murata et al., 1986*).

The results clarified that the different inactivated viruses had no cytopathic effect on inoculated in MDBK cell culture and also the different inactivated viruses had no adverse effect on inoculated mice and rabbit (as a model of cattle) that was improved safety of virus .The potency of antigen measured through current study should be completed with further immunization with the two types of vaccines incorporated with either ascorbic acid or BEI and monitoring the cellular and humeral immunity.

Table 1. Cytopathic effect of IBR and BVD viruses on MDBK cells previously seeded within 24hrs:

Viruses	CPE		Virus Titer
	h.p.i	Signs	
BVD virus	72	<ul style="list-style-type: none"> Granulation of the cytoplasm Vaculation Cell elongation 	10 ^{5.5} TCID ₅₀ /ml.
IBR virus	24-36	<ul style="list-style-type: none"> Cell rounding Shrinkage of the cell wall Granulation of cytoplasm Bunch grapes formation 	10 ^{7.5} TCID ₅₀ /ml

MDBK: Madein Derby Bovine Kidney. **CPE:** Cytopathic Effect.
BVDV: Bovine Viral Diarrhea Virus. **IBRV:** Infectious Bovine Rhinotachiitis.
h.p.i: hour post inoculation.

Table 2. Inhibition of IBRV infection in MDBK cells after treatment with BEI and different concentration of ascorbic acid and cu²⁺ at 37°C:

Conc.of inactivator	Virus infectivity titer (Log ₁₀ TCID ₅₀ /ml) on HPI**									
	1/2	1	2	4	6	8	12	16	18	24
BEI(0.001Mol)	+	+	+	+	-	-	-	-	-	-
Ascorbicacid(0.5mg/ml)	+	+	+	+	+	+	+	+	+	+
Ascorbic acid(1.0mg/ml)	+	+	+	+	+	+	+	+	+	+
Ascorbic acid(1.5mg/ml)	+	+	+	+	+	+	+	+	+	-

IBRV: Infectious Bovine Rhinotacheitis. **HPI** :hours post inoculation.
BEI: binary ethylenamine.

Table 3. Inhibition of BVDV infection in MDBK cells after treatment with BEI and different concentration of ascorbic acid and cu²⁺ at 37°C:

Conc. of inactivator	Virus infectivity titer (log ₁₀ TCID ₅₀ /ml) on HPI									
	½	1	2	4	6	8	12	16	18	24
BEI(0.001Mol)	+	+	+	-	-	-	-	-	-	-
Ascorbic acid(0.5mg/ml)	+	+	+	+	+	+	+	+	+	+
Ascorbic acid(1mg/ml)	+	+	+	+	+	+	+	+	+	-
Ascorbic acid(1.5mg/ml)	+	+	+	+	+	+	+	+	-	-

BVDV: Bovine Viral Diarrhea Virus. **HPI:** Hours Post Inoculation.
BEI: Binary Ethylenamine

Table 4. Inactivation rate of IBRV & BVDV by Different Concentration of Ascorbic Acid:

Additive	Virus Titers after Hrs. of Incubation at 37°C							
	IBR				BVD			
	0	8	18	24	0	8	16	18
None Treated Virus	7.5	7.5	6.5	6.3	6.5	5.5	5.5	5.4
Ascorbic 1 mg/ml + Cu ²⁺ 5µg/ml	7.5	5.3	2.0	0.7	6.5	2.8	0.5	0
Ascorbic 1.5 mg/ml + Cu ²⁺ 5µg/ml	7.5	4.8	1.2	0	6.5	1.5	0	0

Table 5. Ascertainness of full inactivation of IBR and BVD viruses inactivated with different inactivators using laboratory animal as safety parameter:

IBRV	h.p.t	Mice	Rabbit
BEI(0.001Mol)	6	-ve	-ve
ascorbic acid(1mg/ml)	24	-ve	-ve
ascorbic acid(1.5mg/ml)	18	-ve	-ve
BVDV			
BEI(0.001Mol)	4	-ve	-ve
ascorbic acid(1mg/ml)	18	-ve	-ve
ascorbic acid(1.5mg/ml)	16	-ve	-ve

IBRV:Infectious Bovine Rhinotachiitis virus.

BVDV:Bovine Viral Diarrhoea virus.

h.p.i:hour post treatment.

BEI: Binary Ethylenamine.

CONCLUSION

From the present obtained results, it could be concluded that ascorbic acid has a potential for being used as safe inactivating agent without marked affection the antigenicity of the BVDV and IBRV viruses. The recommended concentration is ascorbic acid (1.5mg/ml) because it made complete viral inactivation for two viruses.

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فاعلية حمض الأسكوربيك كمادة مثبطة لفيروس التهاب القصبة الهوائية الرغامى و فيروس الأسهال البقرى المعدى

مها رأفت عبد الفضيل ، رشا ابراهيم الهوارى ، عفت لطفى السيد

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في هذه الدراسة نناقش تأثير حمض الاسكوربيك (فيتامين ج) المحفز بسلفات النحاس كمتبط لفيروسي التهاب القصبة الهوائية الرغامى و فيروس الإسهال البقرى المعدى بالمقارنة بالبينارى كطريقة معتادة للتنشيط. معلقين من الفيروسين قد حضر بعبارية $10^{6.5}$, $10^{7.5}$. كل معلق قد تمت معالجته علي حدا بكل من المادتين المثبتتين بتركيزات (0.5، 1، و 1.5 مليجرام/ملي) حمض الاسكوربيك(فيتامين ج) المحفز بسلفات النحاس و البينارى بتركيز 0.001 مول وتم تحضين الخليط عند 37 درجة مئوية مع التدوير للمحتويات بالمغناطيس الدوار. تبين ان البينارى بتركيز 0.001 مول قد ثبت كل من التهاب القصبة الهوائية الرغامى البقرى و فيروس الإسهال البقرى المعدى فى غضون 6 ، 4 ساعات على التوالي أما حمض الاسكوربيك عند درجة حرارة 37° م بتركيزات (1 و 1.5 مليجرام/ملي) قد ثبتت خلال (أكثر من 24،24)،(18و24)على التوالي.اختبارات الامان قد تمت علي خلايا الزرع النسيجي (MDBK)،الفئران والأرانب . مما سبق نستوضح أن حمض الاسكوربيك مادة مثبطة فعالة لكل من التهاب القصبة الهوائية الرغامى البقرى و فيروس الإسهال البقرى المعدى المنمى على خلايا الزرع النسيجي(MDBK) . أوضحت الدراسة انسب تركيز مثبط من حمض الاسكوربيك المثبط لفيروسي التهاب القصبة الهوائية الرغامى و فيروس الأسهال البقرى المعدى هو (1.5 مليجرام/ملي) لأنه ثبت كليتا كل من الفيروسين خلال 24 ساعة. هذه الدراسة توصى بعمل دراسات أخرى لمعرفة مدى فاعلية هذا الفيروس المثبط بحمض الاسكوربيك فى عمل اللقاح ومدى فاعلية حمض الاسكوربيك كمنشط مناعي موجود فى تركيبه خاصة عند استخدامه فى صورته النانوية.