Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Research article

Evaluation of the virucidal efficacy of commercial antiviral drugs against Lasota virus a surrogate for enveloped viruses

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(Received 27/8/2017, Accepted 28/12/2017)

Abstract

Many antivirals are commonly used in Iraq's poultry farms and there is controversy about effective of these commercial antiviral drugs. The aim of this study was tested individually for the effectiveness of these commercial antiviral drugs. Four kinds of commercial antiviral drugs including VIRUX®, TopAMD®, V8® and Licorice® was used in this study. Lasota virus was used as a surrogate for enveloped viruses. The following tests: Spot hemagglutination, Antigen Rapid NDV Ag Test Kit, Electron microscopy, qRTPCR and Egg inoculation in order to determine the effect of these antivirals on hemagglutinating activity, viral protein, viral morphology, virus titer and viral infectivity respectively. All antivirals had no toxic effect on the chicken embryos. All these antivirals had no effect on heamagglutination activity except Licorice. No antivirals changed nucleoprotein antigenicity of Lasota virus. All antivirals had no effect on the morphology of the virus except Licorice destroyed the viral morphology and decreased in viral spikes. Three of the four antiviral reduced the viral titer while Licorice complete degradation of viral RNA and prevent detection it by qRTPCR. The allantoic fluid harvested from inoculated eggs with the treated Lasota virus with antivirals showed a remarkable decrease in viral infectivity as following: TopAMP about 20%, V8 40%, and Virux 60% while Licorice showed a complete reduction of viral infectivity (100%). In conclusion, the Licorice revealed the best antiviral activity.
Keywords: Antiviral activity, Lasota virus.

Introduction

The scourge of viral infections over world are increasing and attempts at providing antiviral agents to effectively control them appear to be highly challenging. The difficulty in controlling viral diseases is attributable to the distinct nature and characteristics of the viruses. It obligatory depend on their host; hence, the antiviral agent must deal with the virus without being inimical to the host (11). The importance of using antiviral drugs as tools for prevention and treatment of viral infection (1). Documentation of the effectiveness of antivirals against viruses is minimal, and there is no information available on mechanism of action (3). The structure of viruses is important and closely associated with intrinsic resistance to antivirals (14,15). Viruses are generally classified into two groups: enveloped viruses are sensitive to most antivirals and non-enveloped viruses having a much higher level of resistance; however, the specific extent of resistance can vary depending on the virus type as well as on the antiviral under investigation (9). Different antivirals have different modes of action for virus inactivation; many studies were done to examine the effect of common antivirals, which used in poultry production on hemagglutinin activity, virus recovery and as the titer well as the mechanism of action and their ability to effect on viral RNA so

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that it could be detected by Real Time -RT-PCR. In vivo testing relies on the H surface glycoprotein to bind to receptors on a variety of mammalian and avian erythrocytes if the virus is active. This produces heamagglutination, or clumping of cells that is visible to the naked eye (19). Lasota virus are used in this study as a surrogate for enveloped viruses because it is nonpathogenic, easily propagate in embryonated egg and have hemagglutinin activity. Lasota virus belonging to the Paramyxoviridae family is enveloped, negative-sense single-stranded RNA virus (2). The antiviral used in this study were composed of four kinds. Firstly, VIRUX® contains following active ingredients: Garlic extract, oregano extract, iodine and acids (citric, orthophosphoric, malic, lactic, and formic, acetic). Secondly, TopAMD® contains active ingredient is Amantadine HCL 100%. Thirdly, V8® contains following active ingredients: Herbs like Isatis, Scutellaria, Forsythia, Dyers and Woad Leaf. Lastly, Licorice® contains dried licorice root, sucrose and dextrin as excipient. These antivirals are commonly used in Iraq's poultry farms and there is controversy about these commercial antiviral drugs because of persistent disease outbreaks in spite of the use of these commercial antiviral drugs. Therefore, these kinds of antivirals were tested individually for effectiveness under laboratory conditions that was the aim of this study in order to recommend suitable antivirals for effective viral inactivation.

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 447

Antiviral activity tests

This study was carried out in College of veterinary medicine/ University of Basrah, during October 2016 to March 2017. The virus used was a Lasota strain of Newcastle disease virus (Vir 116 Biovac, Czech). The virus was propagated and titrated in 9-day-old embryonated chicken eggs and the $100EID_{50}$ was calculated (4). The $100EID_{50}$ concentration of the virus and diluted antiviral were used as positive and negative controls, respectively. Four kinds of commercial antiviral drugs including VIRUX® (Fertizone, Malaysia), TopAMD® (Topsurf, Canada), V8® (UNiPharma, Malaysia) and Licorice® (QILU SANVA, China) were used for the virus inactivation test. All the antivirals were diluted with phosphate buffer saline following the manufacturers' recommendation for each product. The virus/antiviral suspensions were kept at 37°C for 30 minutes to allow reaction to occur (18). In order to determine the effect of these antivirals on hemagglutinating activity, viral protein, viral morphology, virus titer and viral infectivity. We did the following tests:

1-Spot heamagglutination test

A drop of 1% washed chicken red blood cells was used to dispense on a white tile. A pasture pipet was used to put a drop of the virus/antiviral suspension, which was mixed with the drop of blood. The tile was gently rocked and observed for visible heamagglutination, indicating viral activity (16). This was done for every virus/antiviral suspension, positive control and negative control then the observations were recorded.

2-Antigen detection test

Antigen Rapid NDV Ag Test Kit (Bionote, Korea) was used for detection of NDV antigen with a high degree of accuracy. The principle is Immunochromatographic assay. The kit uses a monoclonal antibody against the nucleoprotein of NDV.

3-Electron microscopy

Every virus/antiviral suspension and positive control were examined in negatively stained preparations using scanning electron microscopy FE-SEM (SUPRA 55VP) Zeiss Germany.

4-Quantitative real-time polymerase chain reaction (qRTPCR)

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Viral RNA was extracted by the use of AccuPrep® Viral RNA Extraction Kit (Bioneer, Korea) according to the kit protocol. Virus titers from all groups (experimental and control groups) were analysed by qRTPCR amplification with specific primer sets, as in (10) Table (1). The qRTPCR was performed in one-steps. The KAPA SYBR FAST One-Step qRT-PCR Master Mix (2X) Kit (Kapa Biosystems, South Africa) was used to do qRTPCR according to the kit protocol. The reverse transcription was performed at 45ºC for 30 min and samples were then incubated at 95ºC for 5 min to inactivate reverse transcriptase. The real-time PCR conditions were 45 cycles of 95ºC denaturation for 10 s, 65ºC annealing for 10 s, and 72ºC extension for 10 s. The specificity of amplification was determined by melting curve analysis that consisted of 95ºC denaturation for 1 min, 65ºC annealing for 5 min, and heating to 95ºC at the rate of 0.11ºC/s (10), using SmartCycler® System (Cepheid, USA).

Table (1): Primer sequences and the characteristics of the NDV Lasota amplicon generated by SYBR Green I-based real-time RT-PCR (10).

Primers	Sequences (5'-3')	Genomic region	Amplicon size (bp)
Lasota F	TACAACAGGACATTGACCACTTTGCTCAC	4793-4821 (Fgene)	299
Lasota R	TGCATCTTCCCAACTGCCACTGC	5069-5091 (Fgene)	

5-Egg inoculation

Each egg was inoculated with 0.1 ml of the inoculum and five eggs were inoculated with each of the virus/antiviral suspension, while inoculating the antiviral only and the 100EID_{50} concentration of the virus as negative and positive controls, respectively. Three days after inoculation the allantoic fluids were harvested and tested for the presence of hemagglutinating activity by spot testing as a sign of viral growth in the eggs.

Results

All antivirals had no a negative effect on development of the different organs of the chicken embryos; there was no growth retardation and no mortality was observed in any of the embryos that had been injected with pure antivirals up till 72 hour post inoculation Figure (1).

Figure (1): There were no cytotoxic effects on inoculated embryos with antivirals up until 72-hour post inoculation (normal chicken embryos)

In the present study, four commercial antivirals were evaluated for their antiviral activity against Lasota virus. After incubation period 30 min at 37 C all antiviral/virus suspension groups and positive control group showed positive heamagglutination by spot-testing except Licorice/virus suspension group gave negative result Figure (2), that mean Licorice effect on viral spikes (HA protein) which responsible for heamagglutination. The results of spot heamagglutination test were shown in Table (2).

Table (2). Results of heamagglutination test of antiviral/Lasota virus suspension after incubation period 30 min at 37 C.

No.	Antiviral/virus	Result of HA
1	V8/Lasota	Positive
2	Virux/Lasota	Positive
3	Top AMP/Lasota	Positive
4	Licorice/Lasota	Negative
5	Positive control	Positive

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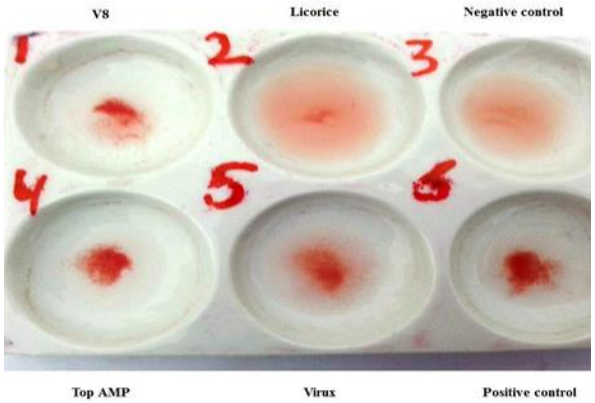

Figure (2). Results of heamagglutination test of virus/antiviral suspension after incubation period 30 min at 37 C; V8, Top AMP and Virux gave positive result as in positive control while Licorice gave negative result as in negative control.

The nucleoprotein of Lasota virus was still recovered from samples treated with antivirals; Top AMP, V8, Virux and Licorice for 30 min time exposure at 37 C, that indicating all these antivirals did not change nucleoprotein antigenicity of Lasota virus Figure (3).

Figure (3): Rapid test to detect the nucleoprotein of Lasota virus, all treated samples with antivirals gave positive results.

3.3.4 Electron microscopy

All antivirals had not effect on the morphology of the virus except Licorice destroied the viral morphology and decreased in viral spikes as showed in Figure (4).

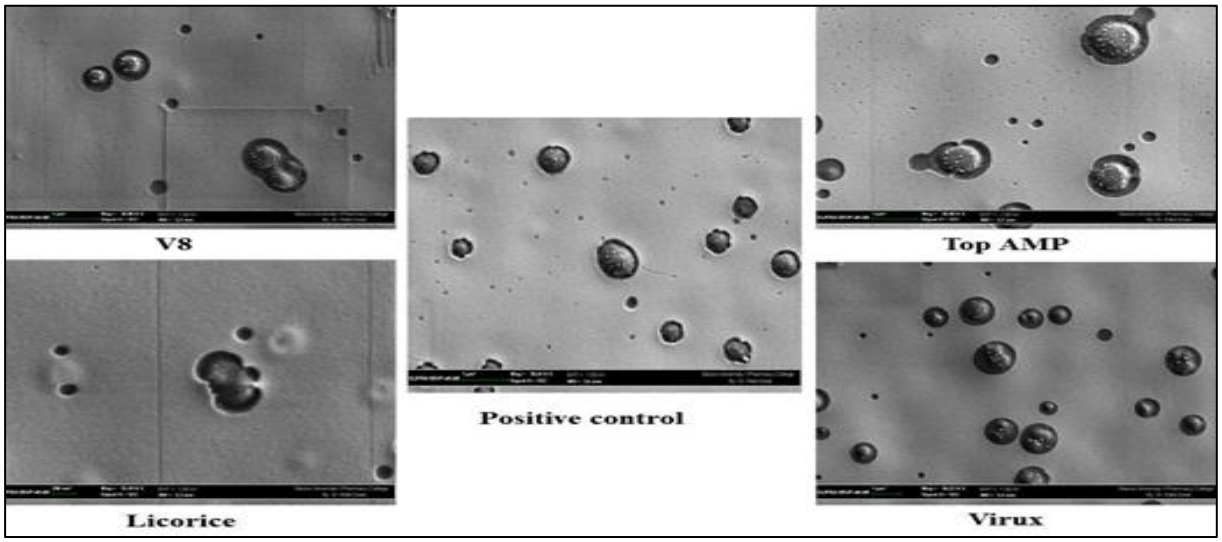

Figure (4): Scanning electron microscopic picture by negative staining, all antivirals have no effect on viral morphology except Licorice destroyed the virus and the viral spikes had been disappear.

When compared with positive control the qRTPCR showed three of the four antiviral reduced the viral titer especially Virux (reduction viral RNA to very low level). The exception was Licorice (complete degradation of viral RNA and prevent detection it by qRTPCR) Figure (5).

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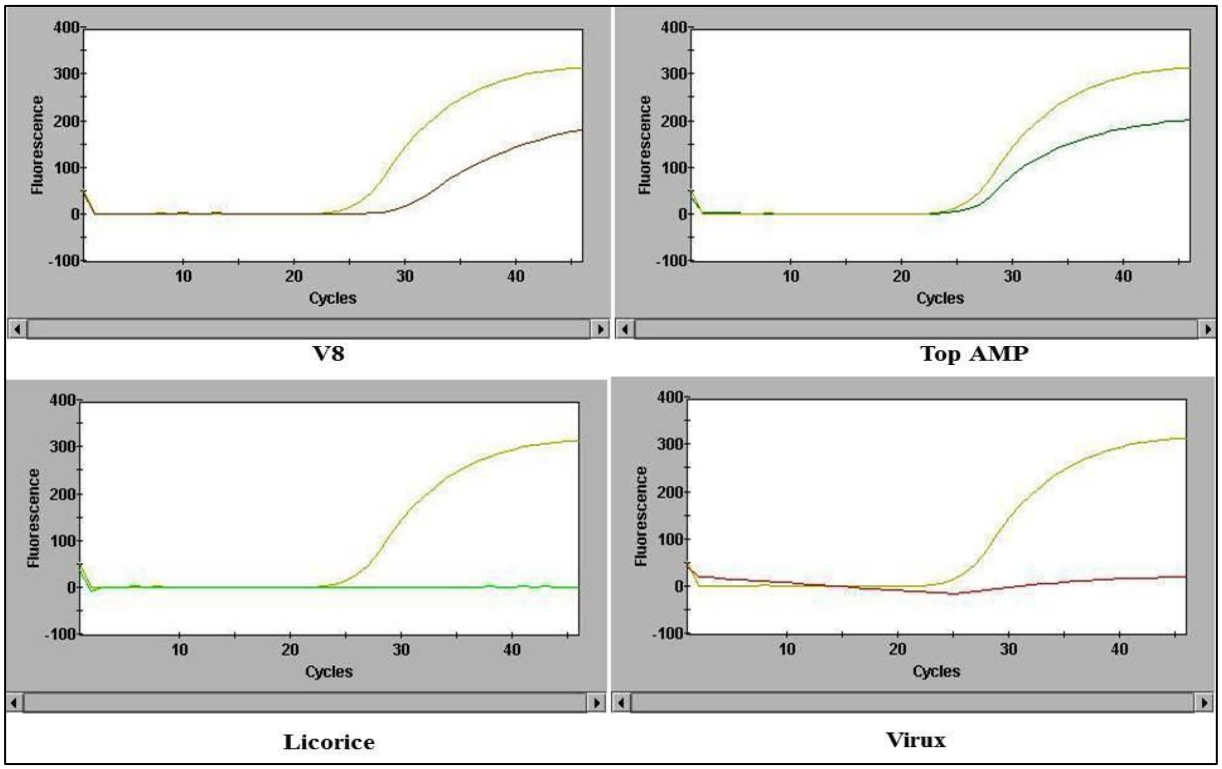

Figure (5): Results of qRTPCR were shown all antivirals reduced the viral titer especially Virux while Licorice destroyed the virus completely when compared with positive control.

The allantoic fluid harvested from inoculated eggs with the treated Lasota virus with antivirals showed remarkable decrease in viral infectivity as following: Top AMP about 20%, V8 40%, and Virux 60% while Licorice showed complete reduction of viral infectivity (100%), as shown in Table (3) and Figure (6).

Table (3): Antiviral effect on infectivity of Lasota virus after 30 min exposure at 37 C. [Infectivity = (infected embryos) / (total embryos inoculated; n = 5)]

No.	Antiviral	Virus infectivity
1	Top AMP	4/5 (80%)
2	V8	3/5 (60%)
3	Virux	2/5 (40%)
4	Licorice	0/5 (0%)
5	Positive control	5/5 (100%)

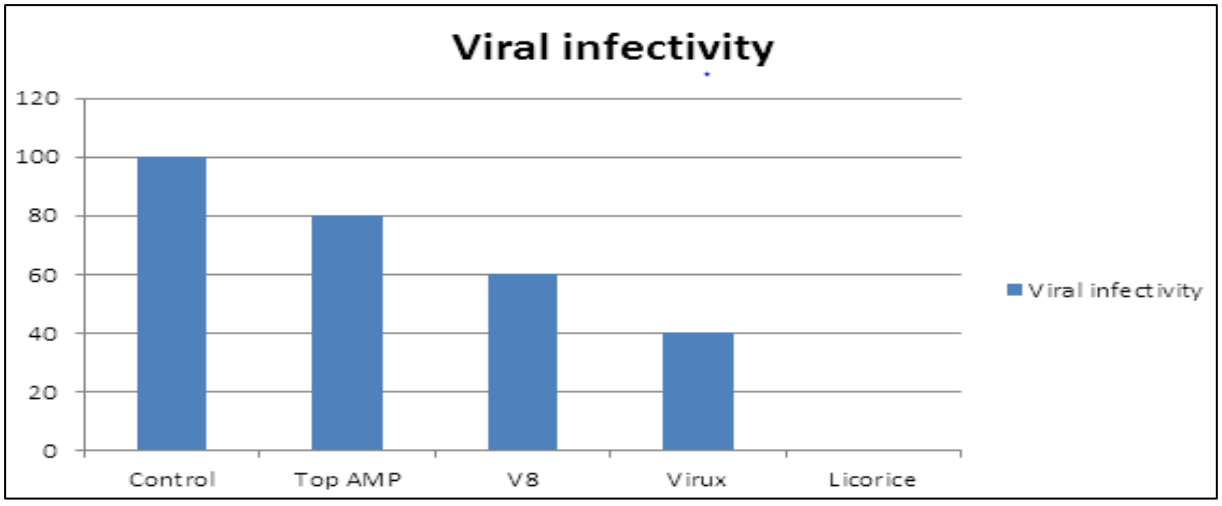

Figure (6): Effect of antivirals on the infectivity of Lasota virus.

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Discussion

The aim of our study was testing of the efficacy of four kinds of commercial antivirals including Top AMP, V8, Virux and Licorice against enveloped viruses (Newcastle disease virus) using multiplication of the Newcastle disease virus in embryonated egg. We used a concentration of antivirals according to the recommendation of the manufacturers. The results of the virus propagation showed that Licorice completely inhibited the growth of Newcastle disease virus in embryonated chicken eggs. The antiviral property observed might be due to the presence of various potentially bioactive ingredients (5), including triterpenoids, flavonoids and phenolic compounds, but the antiviral activity observed in this study could not be attributable to specific compounds. Licorice showed complete reduction of hemagglutinating activity, as seen in Table (2) and Figure (2). Although there is information about the viral target to antiviral agents, there is little information on the mechanism of action of viral biocide. The structure of Newcastle disease virus that offer target sites for antivirals can be separated into the envelope, glycoprotein receptors, the capsid and the viral genome. The viral envelop is derived from cytoplasmic membrane and highly lipophilic in nature therefore highly susceptible to a wide range of membrane-active agents, although it is not known how much damage must be done to the virus envelope before virus infection is prevented. Two viral envelope proteins (hemagglutinin-neuraminidase (HA) and the fusion protein) on Newcastle disease virus play essential roles in viral infection (7), it is likely that interruption of the function of any viral surface protein would have a significant effect on virus infectivity. To date, antiviral treatments for Newcastle disease virus infection are not available because of their cost and toxicity. Although the potent chemotherapeutic antiviral drug ribavirin inhibited many paramyxoviruses in vitro, it showed very poor anti-Newcastle disease virus activity and high toxicity (8). The Licorice shows potential for the control of Newcastle disease virus infection in poultry. No antiviral desaturated the nucleoprotein of Newcastle disease virus, theses antivirals may be do not contain hydroxide ion because the presence of hydroxide ion (OH-) in alkalis make the basis for their disinfectant activity as protein denaturation occurs (12). Results of the in vitro evaluation of antivirals showed that two of the four antivirals were unable to damage the RNA effectively to prevent detection by RRT-PCR. These results indicated that, Licorice followed by Virux are the most effective antivirals for inactivation of Newcastle disease viruses based on degradation of viral RNA. The previous papers have demonstrated the ability of sodium hypochlorite or free chlorine to prevent RT-PCR detection in AI virus (H5N1), poliovirus, hepatitis C virus and rotavirus, respectively (3, 6, 13, 17). The results presented here suggest that Top AMP and V8 are effective for decreasing Newcastle disease virus titer, but they do not adversely affect the viral RNA to the point where it prevents detection by qRT-PCR. The RNA genome degradation could lead to lose the ability of virus to propagate therefore Licorice and Virux can inactivate other viruses containing RNA genome even it were non-enveloped viruses. Cytotoxic controls were collected for each antiviral. All cytotoxic control eggs were survive and no visible lesions were noticed on the embryos also gave negative for HA activity. The Licorice is effective, and safe therefore can used as disinfectants to sterilize the contaminated surfaces and equipment instead of aldehydes, hypochlorites, and stronger inorganic acids, which can be corrosive to many surfaces. Compounds that are able to inactivate viruses and have low toxic can be used for both, treatment of the disease and vaccine preparation. In conclusion, this work

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has shown that the Licorice revealed the best antiviral activity against enveloped model viruses and could therefore be useful in the control of the disease in poultry birds. However, more studies are necessary to confirm its antiviral activity in field experiments and characterize of their active compounds.

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