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ANTIVIRAL ACTIVITY OF VIRO CARE GZ-08™ AGAINST NEWCASTLE DISEASE VIRUS IN POULTRY AND ITS IN-VITRO CYTOTOXICITY ASSAY

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ABSTRACT:- Newcastle disease (ND), one of the most important disease of poultry throughout the World is caused by Newcastle Disease Virus (NDV). It is causing huge economic losses in poultry industry of Pakistan. Regardless of vaccination, other prevention and control measures are necessary to prevent ND outbreaks. Natural resources have been exploited to obtain antiviral compounds in several latest studies. In this study, the antiviral activity of Viro Care GZ-08™ was checked up in-vitro, in-ovo and in-vivo. The cytotoxicity assay of the product was performed using Vero cell line. All the trials revealed that the stock solution and 1:2 dilution of GZ-08™ had some antiviral activity as well as were cytotoxic. As the concentration decreased, cytotoxicity as well as antiviral activities were lost. Based on these findings, it was concluded that GZ-08™ sanitizer or spray can be used as antiviral agent to clean or disinfect some non-living surfaces against different viruses in general and NDV in particular. However, in-vivo use of GZ-08™ in poultry against NDV is recommended only as pre-treatment with ND vaccines as it significantly reduced morbidity and mortality as compared to the use of vaccines alone. However, further work is recommended in future on GZ-08™ for its use as post-treatment of ND as well as on other antiviral compounds of natural origin to develop a novel antiviral drug against NDV in poultry.

Key Words: Poultry; Antiviral Activity; Viro Care GZ-08™; Newcastle Disease Virus; Cytotoxicity; Pakistan.

INTRODUCTION

Poultry is the second largest industry in Pakistan after the textile. In 2009-10, the export of live poultry and meat was Rs. 27 million, that was increased up to Rs. 1.08 billion during 2010-11. However it was decreased to Rs. 365 million in 2011-12. Pakistan exports poultry and meat to Vietnam, Iran, Hong Kong and Afghanistan. This sector produces direct and indirect employment for about 1.5 million people. Its contribution in livestock and agriculture is 11.5% and 6.4%, respectively. Out of total annual meat production, 25.8% is poultry meat. A total of Rs. 200 billion is being invested currently in poultry industry. The poultry sector reflects its inherent potential by showing robust growth of 8-10% annually (GoP, 2011-12).

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Poultry industry is mainly threatened by infectious diseases. In Pakistan, poultry industry is affected by various diseases such as Newcastle Disease (ND), Infectious Bronchitis (IB), Infectious Bursal Disease (IBD), Egg Drop Syndrome (EDS), Hydro Pericardium Syndrome (HPS) and Avian Influenza (AI) (Numan et al., 2005). Mortality of chicken due to various infectious and non-infectious diseases is a major constraint for profitable poultry production (Ahmed et al., 2009). Whole world, including Pakistan is suffering from reasonable losses caused by these diseases in terms of high mortality, morbidity, stress, decreased egg productions and hatchability (Alexander, 2000).

Newcastle Disease (ND) is known all over the world as one of the most important disease of poultry. It not only causes more flock mortality but also has a negative economic impact that may arise due to trading restrictions (Sharma, 1999). Avian Paramyxovirus type 1 (APMV-1) serotype of the genus Avulavirus belonging to the sub-family Paramyxovirinae, family Paramyxoviridae is the causative agent of ND. Avian isolates of paramyxoviruses have been classified by serological testing and phylogenetic analysis. There are ten subtypes of virus designated from APMV-1 to APMV-10 (Miller et al., 2010). ND virus (NDV) has been designated APMV-1.

NDV also produces infections in human. The most common infection in human is conjunctivitis, which develops within 24 h of NDV exposure to the eye (Swayne and King, 2003). Infections caused by NDV in human are non-life threatening and normally not remain for more than a day or two (Chang, 1981). Clinical signs in human infections have been eye infections, typically consisting of unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and subconjunctival hemorrhages. Although NDV produces severe infections to eye in human but these infections do not infect cornea. NDV infections do not spread from human-to-human. From lung tissue, urine and faeces of an immunocompromised patient who died of pneumonia a pigeon-like APMV-1 have been isolated (Goebel et al., 2007).

Since its recognition in 1926, ND is counted as being endemic in many countries. Regardless of vaccination, other prevention and control measures are necessary to prevent ND outbreaks. Every year, ND causes destruction of poultry industry in Pakistan and in this way badly affects the economy of country. Due to aerosol transmission and the resist-ance of the virus in the environment, NDV causes infection in other birds. Besides, immune birds develop as carrier (Allan et al., 1978).

Natural resources are being exploited to obtain antiviral compounds in several recent studies. Particularly various in-vitro and in-vivo studies have been conducted to check the antiviral activity of different compounds containing zinc. In recent times, zinc oxide (ZnO) is an extensively used compound that has gained attention by researchers because of its substantial antimicrobial properties (Leung et al., 2012). Numerous Zn materials have significant medical importance showing anti-infective activity, enhanced wound healing, and privileged epithelialization rates. ZnO is being used as an active component in various commercial materials, such as bandages, stock-

ings, and occlusive adhesive dressings (Lansdown et al., 2007).

Viro Care GZ-08™ is an aqueous antimicrobial solution made by a Japanese Company; Shagdery with Earth Pvt. Ltd. It contains zinc oxide (ZnO), citric acid and kathon. It is claimed to have antibacterial and antiviral activity against different pathogens. Hence, the product has not yet been evaluated against any viral infection of poultry. Keeping in view the importance of ND and its high vaccination cost, the present study was conducted to evaluate the product for its antiviral activity against NDV *i*n-vitro, in-ovo and in-vivo. The cytotoxicity was also determined in-vitro using Vero cell line.

MATERIALS AND METHOD

Virus, Cell Culture and Product

The virulent New Castle Disease Virus (NDV) was isolated and characterized from field outbreaks according to the standard scheme of isolation as described in OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2004). The Viro Care GZ-08™ was procured from Shigadry with Earth® Ltd. a Japanese company through its distribution in Al-Qusais Dubai, UAE. The Vero cell line was obtained in the form of Semi-confluent monolayer from Veterinary Research Institute (VRI), Lahore, Pakistan.

Antiviral Activity

In-vitro antiviral activity was checked up, incubating the NDV with different concentrations of GZ-08™ in eppendrof tubes. Spot haemag-glutination test with 25% RBCs suspension was conducted post-incubation along with negative control (NDV only). The reduction in HA activity of virus was used as indicator of *in-vitro* antiviral activity of GZ-08™. Similarly, micro haemag-glutination test was conducted using 96-well micro titration plates with 1% RBCs suspension (Chen et al., 2013).

In-ovo antiviral activity was evaluated in 9-11 days old embryonated chicken eggs (ECEs). For this purpose, two types of inoculums were prepared. The first inoculum was prepared by mixing 0.5ml of virus with 0.5 ml of different concentrations of GZ-08™. Similarly, the second inoculum was prepared by mixing 0.1ml of virus and 0.9ml of different concentrations of GZ-08™. After preparation of inoculum 0.2ml of each concentration was inoculated in group of ten ECEs through allantoic cavity route. The inoculated ECEs were incubated for three days. After incubation the ECEs were removed from the incubator, chilled at 4°C and allantoic fluid was harvested. The antiviral activity was determined through reduction in HA titer in harvested fluids, survival of embryo and percentage of mean embryo weight (Mabiki et al., 2013).

The in-vivo antiviral activity of GZ-08™ was ascertained in 100 day old 'A' grade broiler chicks, purchased from Olympia Chicks Pvt. Ltd. The feed was purchased from Shareef Feeds Pvt. Ltd. The chicks were housed under standard conditions of management. The feeding and watering was done as per normal routine. All the recommended vaccination was done as per standard immunization schedule in broilers. The chicks were divided into two groups i.e., 1 and 2 of 50 birds each. Each group was further subdivided into four sub-groups i.e., A, B, C and D having 12

birds in each (Table 1).

During the experiment, the chicks of group 1 (sub-group A and B) were given pre-treatment with stock solution GZ-08™ up to 14 days. While the chicks of sub-group C were prepared for post-treatment of GZ-08™. On the other hand, the chicks of group 2 were grown up without ND vaccine and treatment of GZ-08™. On 15th day, the chicks of group 1 were challenged with Velogenic NDV having titer of 105.0 ELD50 ml^{-1} (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004; Bakari et al., 2013). After challenge, the treatment of GZ-08™ for each group was continued. The group 2 was challenged with Velogenic NDV, having titer of 105.0 ELD50 ml^{-1}, pre-treated with GZ-08™ (different concentrations). The percentage morbidity and mortality was recorded in each group at 24, 48 and 72 hours post-challenge.

Cytotoxicity Assay

The MTT (3-4, 5-dimethylthiazole -2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used to determine the cytotoxicity of GZ-08™ in 96

Table 1. Experimental design for in-vivo antiviral activity of GZ-08™ in broiler chicks

Groups	Sub-groups	Treatment	Duration
1	A	Pre-treatment with GZ-08™ @ 0.67g/bird/day (ND, IB and IBD vaccines)	15 days pre-treatment, Challenge with NDV on 15th day
	B	Pre-treatment with GZ-08™ @ 0.67g/bird/day (IB and IBD vaccines)	15 days pre-treatment, Challenge with NDV on 15th day
	C	Post-treatment with GZ-08™ @ 0.67g/bird/day (IB and IBD vaccines)	Challenge with NDV on 15th day, Post-treatment for 15 days
	D	Vaccine control group (ND, IB and IBD vaccines)	No pre-treatment, Challenge with NDV on 15th day
2	A	Chicks procured without ND vaccine and pre-treatment of GZ-08™ (IB and IBD vaccines)	Challenged with Velogenic virus treated with Stock solution of GZ-08™ after overnight incubation on 15th day
	B	Chicks procured without ND vaccine and pre-treatment of GZ-08™ (IB and IBD vaccines)	Challenged with Velogenic virus treated with 1:2 dilution of GZ-08™ after overnight incubation on 15th day
	C	Chicks procured without ND vaccine and pre-treatment of GZ-08™ (IB and IBD vaccines)	Challenged with Velogenic virus treated with 1:4 dilution of GZ-08™ after overnight incubation on 15th day
	D	Chicks procured without ND vaccine and pre-treatment of GZ-08™ (IB and IBD vaccines)	Challenge with NDV on 15th day

well microtitration plates on Vero cell line. The assay was conducted in Quality Operation Lab (QOL) of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. An adherent 48 h old monolayer of intact Vero cells was used along with RPMI-1640 as cell culture medium. A 100µl of GZ-08™ was added and diluted two fold with a positive control (20% dimethly sulfo oxide) and negative control. After addition of MTT dye, the change in colour was noted. The percentage of live cells was determined by reading the plate in ELISA reader at 460 nm wavelength (Wang et al., 2009; Elizondo-Gonzalez et al., 2012).

Statistical Analysis

The data was analyzed by calculating means ± SE, geometric mean titers (GMTs) and analysis of variance (ANOVA) followed by Tukey's test.

RESULTS AND DISCUSSION

Antiviral Activity of GZ-08™ against NDV

In-vitro

In spot assay, GZ-08™ stock solution of 7500ppm density and 1:2 dilutions inhibited the visible clumping or agglutination of chicken RBCs, which shows the antiviral activity. However, the higher dilutions i.e, 1:4 and above did not show antiviral activity (Table 2). These two dilutions also changed the color of the blood indicating its cytotoxicity to RBCs. The results of micro haemagglutination test were similar to that of spot haemagglutination test. The first two wells of microtitration plate containing NDV treated with stock solution and 1:2 of GZ-08™, respectively inhibited the haemagglutination property of virus and thus showed antiviral activity with no button formation or agglutination which may be the indication of cytotoxicity.

Table 2. Spot Haemagglutination test for in-vitro antiviral activity of GZ-08™

Sr. No.	Reactants	Haemag-glutination property	Antiviral activity
1	NDV + stock solution of GZ	-ve	+ve
2	NDV + 1:2 dilution of GZ-08™ +RBCs	-ve	+ve
3	NDV + 1:4 dilution of GZ-08™ +RBCs	+ve	-ve
4	NDV + 1:8 dilution of GZ-08™ +RBCs	+ve	-ve
5	Positive control (NDV + RBCs)	+ve	–
6	Negative control (RBCs)	-ve	–

The present results are in agreement with Chen et al. (2010) who performed the similar work by using Curcumin, a natural compound and an ingredient in curry that has antiviral properties against the enveloped viruses. They observed that when the 4HAU of Influenza virus (another virus belonging to the family Paramyxoviridae that also causes the agglutination of RBCs) was pre-incubated with the different dilutions of Curcumin and then checked for agglutination activity of virus by performing the haemagglutination test, the virus did not agglutinate the RBCs, which was taken as the antiviral activity of the tested compound.

In-ovo

In the experiment, stock solution of GZ-08™ showed minute antiviral activity against NDV as it showed relatively less percentage mortality i.e., 80% as compared to control with

severe lesions on embryo but mortality was increased to 90% as the dilution of GZ-08™ increased with moderate to mild embryo lesions. The overall percentage mean embryo weight in this experiment was 102.63%.While the reduction in HA titer for stock solution, 1:2, 1:4 and 1:8 dilutions was 100.00%, 93.75%, 75.00% and 0%, respectively (Table 3). Statistically the results of percentage mortality were non-significant (P> 0.05) whereas there was significant difference (P < 0.05) in percentage mean embryo weight for group A and B when compared with virus control group.

The results of percentage embryo mortality showed that when the quantity of GZ-08™ was increased and the quantity of virus was decreased (9:1), significant antiviral activity was found with stock solution (40%) and 1st dilution (50%), and moderate activity with 2nd dilution (70%) for percentage mortality in comparison to positive control (90%). However, mild to severe lesions were found on embryo. The overall percentage mean embryo weight in this experiment was 103.11%. In comparison to the 1st experiment the reduction in HA titer for stock solution, 1:2, 1:4 and 1:8 dilutions was 100%, 100%, 93.75% and 75%, respectively (Table 4). Statistically the results of percentage mortality in experiment 2 were significant (P< 0.05) when compared to the positive control group for virus whereas a significant difference (P< 0.05) in percentage mean embryo weight for group A and B when compared to virus control group was noticed.

Mabiki et al. (2013) while ascertaining the *i*n-ovo antiviral potential of *Synadenium glaucescens* up against NDV found that survival as well as mean embryo weights were significantly higher in groups treated

Table 3. Percentage mortality in embryonated chicken eggs post-inoculation with NDV and different concentrations of GZ-08™ (Experiment 1 and 2) in-ovo

Group	Treatment	24 h		48 h		72 h	
		Exp 1 (%)	Exp 2 (%)	Exp 1 (%)	Exp 2 (%)	Exp 1 (%)	Exp 2 (%)
A	Stock solution of GZ-08	30	0	70	20	80	40
B	1:2 dilution	40	10	80	30	90	50
C	1:4 dilution	50	20	80	40	90	70
D	1:8 dilution	50	30	90	60	90	90
E	Positive control for virus	50	50	80	80	100	90
F	Positive control for GZ-08	30	40	70	60	80	80
G	Negative control	0	0	0	0	0	10

Table 4. Percentage mean embryo weight in embryonated chicken eggs and reduction in HA titer of harvested allantoic fluid post-inoculation with NDV and different concentrations of GZ-08™ (Experiment 1 and 2) in-ovo

		Experiment 1		Experiment 2	
Sr. No	Treatment	Mean embryo weight ± S.E	Reduction in HA titer	Mean embryo weight ± S.E	Reduction in HA titer
1	Stock solution of GZ-08™	39.74^{bc} ± 0.49	100.00	39.545^{bc} ± 0.47	100.00
2	1:2 dilution	38.575^{cd} ± 0.47	93.75	38.475^{cd} ± 0.51	100.00
3	1:4 dilution	38.08^{de} ± 0.43	75.00	37.88^{de} ± 0.30	93.75
4	1:8 dilution	37.72^{de} ± 0.40	0.00	37.52^{de} ± 0.37	75.00
5	Positive control	37.185^{e} ± 0.34	---	37.185^{e} ± 0.34	---

with extracts of *S. glaucescens* as compared to positive control group. Similarly, Liu and Yan (2009) checked up the in-vivo efficacy of crude extracts from *Artemisia annul* L against NDV in 9-11 days old ECEs. Significant effects of extracts were found on the proliferation of NDV in terms of decrease in embryo mortality and HA titer of virus.

In-vivo

The results of in-vivo trials of GZ-08™ against NDV showed that it was effective only and showed antiviral activity for protection against challenge when used in combination with normal ND vaccination as it showed 0% mobidity and mortality up to 72 h post-challenge with NDV (Group A). Without ND vaccine alone it did not show significant results (Group B). GZ-08™ did not protect the birds when used as treatment post challenge (Group C). Whereas in all subgroups of Group 2 when birds were given stock solution and various dilutions of GZ-08™ after pre-incubation of 1 hour with NDV, the results of antiviral activity were similar as in in-vitro and in-ovo trials (Table 5, 6). Statistically data was analyzed and it was observed that when chicks were challenged on 15th day with live Velogenic NDV, only the chicks pre-treated with GZ-08™ and with all scheduled vaccination showed significant results ($P < 0.05$) when compared with the vaccinated control group. Moreover, a positive correlation was found between morbidity and mortality.

Bakari et al. (2013) conducted a similar study to check in-vivo antiviral potential of *Commiphora swynnertonii* against Newcastle Disease Virus (NDV) using five months old chickens. By the results, it was examined that extracts possess significant activity in both treatments: pre-infection and post-infection. However, the prophylactic treatment possessed the best antiviral activity as compared to post-infection treatment results.

Cytotoxicity Assay

The cytotoxicity of GZ-08™ was tested through MTT assay using adherent 48 h monolayer of Vero cell

Table 5. Percentage morbidity in broiler chicks of group 1 and 2 during in-vivo trials of GZ-08™

Sub-Group	Morbidity (n=12)					
	After 24 h		After 48 h		After 72 h	
	Group1	Group 2	Group1	Group 2	Group1	Group 2
A	0	0	0	16.67	0	33.33
B	33.33	16.67	66.66	33.33	100	58.33
C	66.66	83.33	100	100	-	-
D	0	100	33.33	100	50	-

Table 6. Percentage mortality in broiler chicks of group 1 and 2 during in-vivo trials of GZ-08™

Sub-Group	Morbidity (n=12)					
	After 24 h		After 48 h		After 72 h	
	Group1	Group 2	Group1	Group 2	Group1	Group 2
A	0	0	0	16.67	0	33.33
B	33.33	16.67	41.67	33.33	58.33	58.33
C	50	83.33	83.33	100	100	-
D	0	100	0	100	0	-

line. The results of test performed in triplicate were averaged. The results showed that stock solution of GZ-08™ having density of 7500 ppm, 1:2, and 1:4 dilutions showed cytotoxicity to Vero cells in-vitro whereas no toxicity was observed at higher dilutions.

It was concluded that Viro Care GZ-08™ although showed significant antiviral activity for pure stock solution and 1:2 dilution during in-vitro, in-ovo and in-vivo trials but at the same time both of these concentrations were cytotoxic as well as shown in MTT assay in Vero cell line. As the concentration decreased, cytotoxicity lost but with the loss of antiviral activity as well. The in-vivo use of GZ-08™ against NDV in poultry is recommended only as pre-treatment with ND vaccines as it significantly reduced morbidity and mortality. However, further work is recommended in future on GZ-08™ for its use as post-treatment of ND as well as on other antiviral compounds of natural origin to develop a novel antiviral drug against NDV in poultry.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

S. No	Author Name	Contribution to the paper
1.	Dr. Muhammad Hidayat Rasool	Conceived the idea, Overall management of article.

S. No	Author Name	Contribution to the paper
2.	Mr. Abuzar Muhammad Afzal	Wrote article
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