Evaluation of cytotoxicity and antiviral activity of ivermectin against Newcastle disease virus

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Abstract: Cytotoxic and antiviral potential of ivermectin and ribavirin was evaluated. Cytotoxicity was checked on chick primary fibroblast cell line through MTT assay. Antiviral potential was determined against Newcastle disease virus on 9-day old chicken embryos. Six different concentrations (200, 100, 50, 25, 12.5 and 6.25µg/mL) of both the drugs were evaluated. The 100µg/mL concentration of ivermectin and higher were cytotoxic. The 25µg/mL concentration of ribavirin and higher were cytotoxic. Comparison of ivermectin and ribavirin showed that ivermectin was safe at 50µg/mL and lower concentrations. Ribavirin was protective for cell at 12.5µg/mL and 6.25µg/mL only. Comparison of antiviral activity indicated that ivermectin has strong antiviral potential at 100µg/mL and higher but same concentrations were cytotoxic. Ribavirin showed strong antiviral potential at all concentrations.

Keywords: Ivermectin, Ribavirin, Cytotoxicity, Antiviral activity and MTT assay.

INTRODUCTION

Ivermectin is16-membered macrocyclic lactone ring containing antibiotic, originated in 1975 from species of Actinomyces, *Streptomyces avermectinius* named avermectin A1a-B2b (Takahashi *et al*., 2002). Avermectins yet lack antibacterial or antifungal activities have activity against helminthes and arthropods are the disaccharide derivatives of pentacyclic lactone (Chabala *et al*., 1980).

Ivermectin a neutral molecule, having molecular weight 874g/mol is poorly soluble in water. It is soluble in lipids and most organic solvents (Lo *et al*., 1985; Fisher and Mrozik, 1992).

Ivermectin is endectocide have activity against endoparasites and ectoparasites (Dourmishev *et al*., 1986). Aziz *et al*. (1982) introduced ivermectin against *Onchocerca volvulus* infestation in human. It is used for the treatment of loiasis and bancroftian filariasis and other intestinal nematodes such as strongyloidiasis (Ottesen and Cambell, 1994) and scabies (Kar *et al*., 1994).

Ivermectin is a potent endectocide, causing paralysis of arthropods, nematodes and insects by suppressing the conduction of nervous impulses in the intermediary neurons synapses of nematodes and the nerve-muscle synapses of the arthropods and insects (Chhaiya *et al.,* 2012).

In poultry, Newcastle disease virus (NDV) causes a highly infectious neurological, respiratory, or enteric disease. NDV belongs to the family Paramyxoviridae (Mayo, 2002). NDV genome is a single-stranded negative-sense RNA consisting of 15,186 nucleotides (Berhanu *et al*., 2010).

NDV is an enveloped virus. The envelope of virus is embedded with two different glycoproteins, the haemagglutinin-neuraminidase (HN) and fusion (F) proteins. Viral infectivity or virulence is linked with the moderate interaction between these two complex glycoproteins (Hulslander *et al*., 1997). One of these proteins can stimulate protective immunity (Meulemans *et al.,* 1986; Nagy *et al.,* 1991). There is a layer of relatively hydrophobic non-glycosylated matrix (M) protein beneath lipid membrane, which is linked with the membrane by N-terminal segment of the HN protein located on its inner surface (Sastre *et al.,* 1989).

Cytotoxicity is expressed in terms of toxicity to a cell. MTT assay is widely used for evaluation of cytotoxic potential of a compound. To measure the reducing potential of the cell this assay is used. MTT reagent will be reduced by viable cells to formazan, a colored product.

Chicken embryos are inoculated with mixture of virus and tested compound to evaluate antiviral activity. For the primary isolation and propagation of a mixture of different viruses chicken embryos are used as a laboratory host system. Chick embryos are enclosed by numerous supporting membranes. There is a hard fibrous membrane instantly beneath the shell called shell membrane. To inner side of shell membrane, a highly vascular chorioallantoic membrane (CAM) is present which serves as respiratory organ of the embryo. Routes of virus inoculation in chicken embryos include CAM, allantoic cavity, amniotic cavity and yolk sac (Senne *et al*., 2003).

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Ribavirin activity against a range of RNA viruses including Paramyxoviruses, Flaviviruses, Picornaviruses, Orthomyxoviruses, Reoviruses and Bunyaviruses has been proven (Crotty *et al*., 2002). This drug also shows activity against some DNA viruses in cell culture. Ribavirin shows its antiviral activity by decreasing the intracellular guanidine triphosphate pools that stops the capping of viral transcripts or suppresses humoral and cellular immune responses (Cameron *et al*., 2001).

In present study, cytotoxicity of ivermectin and ribavirin was compared using chick primary fibroblast cell line through MTT assay. Antiviral potential of both drugs was evaluated against NDV using 9-day old chicken embryos at 6.25-200µg/mL concentration-range.

MATERIALS AND METHODS

The research was designed to determine the cytotoxicity of ivermectin and ribavirin by MTT assay and their antiviral potential against Newcastle disease virus (NDV).

Cytotoxicity

Cytotoxic potential of ivermectin and ribavirin was evaluated following the protocol described by Twentyman *et al*. (1987). Six concentrations 200, 100, 50, 25, 12.5 and 6.25µg/mL were evaluated for their cytotoxicity on chicken primary fibroblast cell line. Different concentrations were prepared in cell culture media at the time of MTT assay using stock solution. In cytotoxicity assay monolayer of primary fibroblast cells grown in 96-well cell culture plate were treated with each concentration. In this assay primary fibroblast cells along with cell culture media were kept as negative control whereas primary fibroblast cells, DMSO (4%) and cell culture media were taken as positive control respectively. Viability of cells was checked by using MTT calorimetric assay (Twentyman *et al.,* 1987).

CSP was calculated by following formula:

$$\text{CSP} = \frac{\text{mean OD of test} - \text{mean OD of -ive control}}{\text{mean OD of +ve control}} \times 100$$

Statistical means of both the drugs were compared for cytotoxic potential.

Antiviral activity

Antiviral potential of ivermectin and ribavirin was determined following the protocol described by Chollom *et al*. (2012). Chicken embryos of 9 day old were procured from Hi-tech laboratory Lahore. Haemagglutination (HA) titer of NDV LaSota vaccine was adjusted corresponding to EID_{50} as described by Abbas *et al*. (2006). The virus from LaSota vaccine was mixed in 1:2 with different concentrations of both the drugs to prepare drug/virus suspension. These suspensions were kept at 4°C for 2 hours to allow reaction for *in vitro* activity. For *in vivo* antiviral assay 0.1mL of virus was inoculated in chicken embryos followed by different dilutions of drug after 6 hours.

In vitro evaluation of antiviral activity

Nine-day-old chicken embryos were sterilized with 70% alcohol. Boundary of air sac and head spot were marked using lead pencil. The eggs were divided into 8 groups A, B, C1, C2, C3, C4, C5 and C6 having five each. Drug-virus suspensions (0.1mL) of each concentration were injected through allantoic cavity route into eggs of groups C1 to C6 using separate syringes, in order to evaluate drug's antiviral activity. Group A chicken embryos served as negative control and 0.1mL normal saline solution was injected in each egg. Similarly group B chicken embryos (positive control) were injected with 0.1mL of viral suspension under similar experimental conditions.

In vivo evaluation of antiviral activity

Chicken embryos were divided in groups on similar pattern as was for *in vitro* evaluation. In this experiment viral inoculums were injected through allantoic route in chicken embryos of groups C1 to C6 and incubated at 37°C. Different dilutions of both the drugs were injected into each egg of groups C1-C6 six hours post virus inoculation. Negative and positive control eggs were treated on similar pattern as for in vitro evaluation. Inoculated chicken embryos were placed in incubator already adjusted at 37°C with relative humidity of 60-70% for the time period of 48 hours. Candling of eggs was done with an interval of 12hours to check the viability of embryos for different concentrations of drug. The dead and live embryos were marked with lead pencil. Then after 72h of incubation the dead and live marked eggs were placed in refrigerator for chilling at 4°C for overnight. Amniotic-allantoic fluid was harvested from each of the eggs and tested for the presence of NDV by spot agglutination test, using 1% chicken RBC's suspension (Thayer and Beard, 1998; Murakawa *et al*., 2003).

Collected data were analyzed using statistic package for social sciences (SPSS, windows version 13, SPSS inc., Chicago, IL, USA). Data were analyzed using One-way ANOVA followed by Duncan's multiple range tests. Differences were considered significant at $P<0.05$.

RESULTS

Cytotoxic and antiviral potential of ivermectin and ribavirin determined both *in vitro* and *in vivo*. Results of cytotoxicity and antiviral activity of ivermectin are presented at table (1). Higher concentrations of ivermectin 200 and 100µg/mL were toxic for the cells. At lower concentrations ivermectin showed cell survival percentage greater than 50%, which indicated its safety

Table 1: Cytotoxic and antiviral potential of ivermectin

Conc. of drug (µg/mL)	Antiviral activity of ivermectin		Cytotoxicity assay of ivermectin	
	In vitro log2 reduction mean ± S.D	*In vivo* log2 reduction mean ± S.D	Mean OD	Cell survival %
200	9.00±0.00^{e}	9.00±0.00^{f}	1.173	20
100	9.00±0.00^{e}	9.00±0.00^{f}	1.314	29
50	4.40±0.54^{d}	5.40±0.54^{e}	1.992	51
25	3.40±0.54^{c}	4.40±0.54^{d}	2.401	67
12.5	1.80±0.83^{b}	2.6±0.54^{c}	2.668	75
6.25	1.60±0.54^{b}	1.8±0.44^{b}	2.743	78

*Statistical means carrying same superscripts differ non-significantly

Table 2: Cytotoxic and antiviral potential of ribavirin

Conc. of drug (µg/mL)	Antiviral activity of ribavirin		Cytotoxicity assay of ribavirin	
	In vitro log2 reduction mean±S.D	*In vitro* log2 reduction mean±S.D	Mean OD	Cell survival %
200	9.00	9.00	1.273	24
100	9.00	9.00	1.499	32
50	9.00	9.00	1.649	38
25	9.00	9.00	1.960	49
12.5	9.00	9.00	2.096	54
6.25	9.00	9.00	2.499	69

for cell. The cytotoxicity potential of ivermectin was compared with ribavirin. Cell survival percentage showed that ribavirin was non-toxic and safe for the cells only at lower concentrations (table 2) which were 12.5 and 6.25µg/mL. Comparison of ivermectin and ribavirin showed that ivermectin was safe at 50µg/mL and lower concentrations (Fig. 1).

In present study antiviral potential of ivermectin against Newcastle disease virus (NDV) was determined and compared with ribavirin. The results of antiviral assay of ivermectin showed that at higher concentrations (200 and 100µg/mL) drug had maximum (100%) activity against NDV. However, these doses were cytotoxic. Lower concentration of ivermectin showed moderate to weak antiviral activity. At concentration of 50µg/mL, ivermectin showed agglutination up to 5th well by HA test that indicated moderate antiviral activity. At concentration of 25µg/mL ivermectin showed lesser antiviral activity. At further lower concentrations (12.5 and 6.25µg/mL), ivermectin showed weaker antiviral by showing viral growth till 7th well. Ribavirin showed strong antiviral activity against NDV as it reduced the viral titer completely and stopped the viral replication indicated by haemagglutination test. Antiviral activity comparison indicated that ivermectin has strong antiviral activity at higher concentration but same concentrations were cytotoxic while ribavirin showed strong antiviral activity at all concentrations.

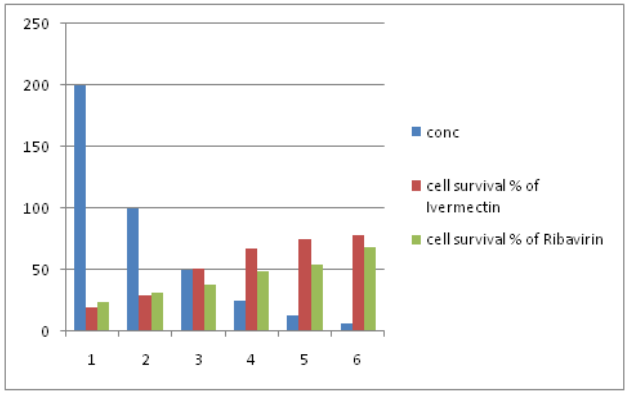

Fig. l: Comparison of cytotoxicity between ivermectin and ribavirin

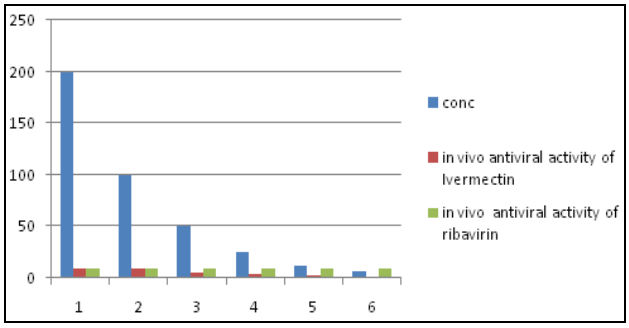

Fig. 2: Comparison of *in vitro* antiviral activity of ivermectin and ribavirin.

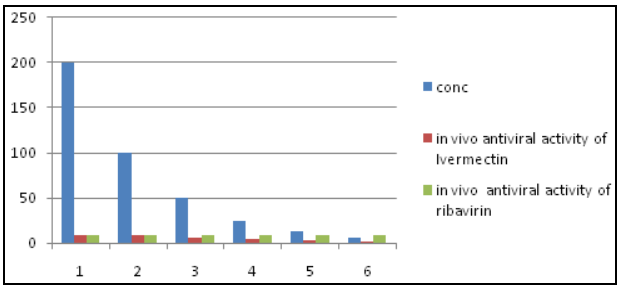

Fig. 3: Comparison of *in vivo* antiviral activity of ivermectin and ribavirin.

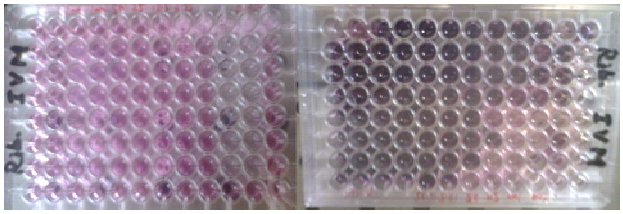

Fig. 4: Cytotoxicity comparison of ivermectin and ribavirin by MTT assay.

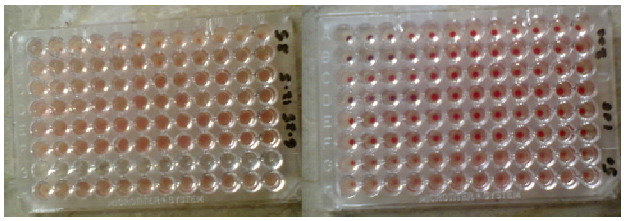

Fig. 5: Haemagglutination test for *in vitro* antiviral activity of ivermectin.

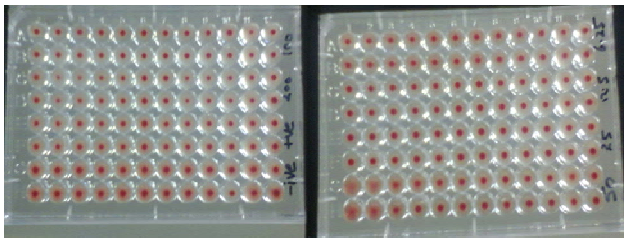

Fig. 6: Haemagglutination test for *in vivo* antiviral activity of ivermectin

Results indicated that ribavirin had antiviral activity higher than 90% at concentration of 250µg/mL. Ribavirin at the concentration of 125µg/mL significantly inhibited the virus titer in each experiment.

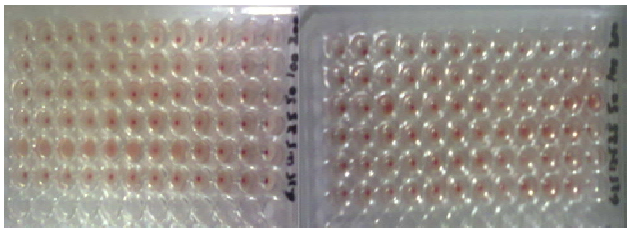

Fig. 7(a): Haemagglutination test for *in vitro* antiviral activity of ribavirin **(b)**: Haemagglutination test for *in vivo* antiviral activity of ribavirin.

DISCUSSION

Newcastle disease is highly pathogenic contagious infection of poultry caused by single stranded RNA virus (Berhanu *et al*., 2010). Newcastle disease virus (NDV) spreads amongst the commercially raised chickens rapidly. Ivermectin belongs to macrocyclic lactones. It is used against black river blindness and scabies in human (Kar *et al.,* 1994).

Present research was planned to evaluate cytotoxic potential of ivermectin and ribavirin using different concentrations. Ivermectin was cytotoxic at 100µg/mL and higher concentrations and safe at lower doses. However, lower concentrations of ribavirin were found to be cytotoxic as determined by MTT assay. In accord, MTT assay had been used by Molinari *et al.* (2009) for cytotoxic evaluation of ivermectin and its formulation ivomec® on Chinese hamster ovary (CHOK1) cells within 1-250µg/mL concentration range. Results of this study concluded that cytotoxicity was observed at higher concentration than 50.0µg/mL.

In another study, Molinari *et al.* (2010) evaluated *in vitro* cytotoxicity of ivermectin and its formulation ivomec® on *Aedes albopictus* larvae (CCL-126TM) cells within 1-250µg/mL concentration range. Conclusion of this study showed that Cytotoxicity was observed at concentrations higher than 25mg/ml. A review summarized the results published so far for estimating the cytotoxicity exerted by both abamectin and ivermectin compounds in several cellular systems. The reports indicating that cell growth inhibition was minimal by both anthelmintic agents either *in vitro* or in *in vivo* bioassays (Molinari *et al*. 2010). Cyotoxic results of present study are consistent with the study of Molinari *et al*. (2009). The result of cytotoxicity is in contrast with the study of Molinari *et al.* (2010). Result may vary due to use of different cell lines and different experimental conditions.

In the present study the antiviral potential of ivermectin against NDV was evaluated and compared with ribavirin. Ivermectin had maximum antiviral activity at higher doses which were cytotoxic. However, at lower concentrations moderate to weak antiviral activity and concentrations were not cytotoxic. Ribavirin exhibited strong antiviral activity against NDV at all of the tested concentrations. However, all of the concentrations were cytotoxic as well. In accord, in ova antiviral potential against NDV had been evaluated by Chollom *et al*. (2012) on aqueous extract of *Moringa oleifera* seed.

In another study, Amagon *et al.* (2010) investigated the antiviral property of flavonoids from *Cucumis metuliferus* fruit pulp in chicken embryo fibroblast (CEF) cells and embryonated chicken eggs (ECE). Similarly, Elizondo - Gonzalez *et al.* (2012) evaluated *in vitro* antiviral

potential of fucoidan against NDV from *Cladosiphon okamuranus* and cytotoxicity by the MTT assay. Conclusion of study showed that ribavirin, used as an antiviral control, exhibited lower antiviral activity than fucoidan and high toxicity at active doses. Crance *et al.* (2003) investigated antiviral potential of ribavirin on vero cell line against pathogenic Flaviviruses.

Similarly, Wagstaff *et al.* (2012) determined the antiviral potential of ivermectin against HIV and Dengue virus on Hela cell line. Ivermectin at concentration 50µM completely stopped and at 25µM significantly reduced the virus production. In another study, Chollom *et al.* (2012) investigated in ovo antiviral potential of aqueous leaf extract of *Psidium guajava a*gainst NDV. The results of Wagstaff *et al*. (2012) supported the present study which indicated that at 25µg/mL ivermectin reduced the viral growth significantly. Results Crance *et al.* (2003) are in agreement with present study indicating that ribavirin completely stopped the virus growth at 125µg/mL concentration.

CONCLUSION

Comparison of ivermectin and ribavirin showed that ivermectin was safe at 50µg/mL and lower concentration while ribavirin was protective for cell at 12.5µg/mL and 6.25µg/mL only. Antiviral activity comparison indicated that ivermectin had strong antiviral activity at higher concentrations but same concentrations were cytotoxic while ribavirin showed strong antiviral activity at all concentrations.

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