Short Communication

DEMONSTRATION OF ANTIVIRAL ACTIVITY OF IVERMECTIN AGAINST R_2B STRAIN OF NEWCASTLE DISEASE VIRUS-INFECTED 9- DAY OLD CHICKEN EGGS

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ABSTRACT

Antiviral activity of ivermectin was examined against R_2B strain of Newcastle disease virus in 9 - day old embryonated chicken eggs. Five distinct concentrations (500, 250, 100, 50, 10 µg/mL) of ivermectin were used in the study. Haemagglutination test was performed to determine the antiviral activity of ivermectin by using chicken RBC. The results revealed that ivermectin can exert a significant antiviral activity against Newcastle disease virus at higher concentrations (500, 250 and 100 µg/ml) but with cytotoxic effects. On the contrary a moderate to weak antiviral activity without cytotoxicity was demonstrated at lower concentrations (50, 10 µg/ml).

Keywords: Ivermectin, R_2B strain, Haemagglutination test

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Ivermectin, a macrocyclic lactone is obtained from *Actinomyces* species and *Streptomyces avermectinius* (Takahashi *et al.,* 2002). It is soluble in lipids as well as in organic solvents but not in water (Fisher and Mrozik, 1992). When used orally, it has an excellent safety profile. Ivermectin is a potent endectocide that paralyses arthropods, nematodes and insects by inhibiting nerve impulse transmission at intermediary neurons synapses and nerve muscle impulses of arthropod and insect (Chhaiya *et al.,* 2012).

The Food and Drug Administration (FDA) has authorized ivermectin as an oral therapy for intestinal stronglyloidiasis and onchocerciasis.

Ivermectin usage other than antiparasitic medication is documented very well. For example, ivermectin interferes with HIV-1 integrase and dengue virus nonstructural protein-5 from getting into the nucleus and significant antiviral activity have been demonstrated against both viruses (Wagstaff *et al.,* 2012). Furthermore, ivermectin has antiviral activity against various flavivirus species like Japanese encephalitis virus, west nile virus (WNV), tick borne encephalitis virus, chikungunya virus, and zika virus (Yang *et al.,* 2020).

Further, the host's immune system is also influenced by ivermectin. Newcastle disease virus (NDV) strain R_2B is mesogenic in nature and highly communicable chicken disease (Berhanu *et al.,* 2010). Newcastle disease virus is a highly infectious and fatal virus that infects almost all species of bird on the planet (OIE. 2009, Samal, 2011). NDV encoded six structural proteins like nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin (HN) and large (L) proteins. NDV belongs to the paramyxoviridae family (Mayo, 2002) and its genome contains single-stranded negative-sense RNA (Berhanu *et al.,* 2010). The viral infectivity is associated with the moderate contact between haemagglutinin-neuraminidase (HN) and fusion (F) proteins (Hulslander and Morrison, 1997) and one of these proteins has the ability to boost protective immunity (Meulemans *et al.,* 1986). The R_2B vaccine strain, which is used in India, has given excellent results in older birds (> 6 to 8 weeks old) with long-lasting protection, but it has been reported to be harmful in young chicks (Chellappa *et al.,* 2012). Keeping these points in view, an attempt has been made to evaluate the antiviral activity of ivermectin against Newcastle disease virus (NDV), a well-known pathogenic virus infecting poultry leading to huge economic loss to the industry.

Ivermectin was obtained from Neospark Pharma Private Limited, 241, B.L. Baghpunjagutta, Hyderabad, Andhra Pradesh - 500 082. Indovax Private Limited provided the live R_2B strain of Newcastle disease virus vaccine. Nine day old embryonated chicken eggs were procured from the Poultry Research Station, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram.

The antiviral activity of ivermectin was evaluated against R_2B strain of Newcastle disease virus through allantoic route by using 9-day old embryonated chicken egg model at 10, 50, 100, 250, 500 µg/mL concentrations. Briefly, the eggs of nine-day-old chicken embryos were grouped into A, B, C1, C2, C3, C4, C5 and C6 groups. Eggs were sterilized with 70% alcohol and candled to ensure viability. A lead pencil was used to outline the air sac's boundary and the location of the head spot. Eggs were drilled slightly above the mark with a tiny hole that penetrated the air-cell but not the part of the egg below the air-cell. With the use of a 1 ml syringe (25 G, 0.5 inch (12 mm) needle), 0.1 ml of normal saline

was injected into group A chicken embryos (negative control). The group B chicken embryos were injected with 0.1 ml of viral suspension (positive control). Similarly, 0.1 ml of di-methyl sulfoxide (DMSO) was injected into the C1 group (DMSO control). The viral inoculums were injected into C2 to C6 groups through allantoic route and incubated at 37°C. Six hours after viral inoculation, different dilutions of ivermectin were administered into each egg of groups C2 to C6. Holes were sealed with wax, and infected chicken embryos were placed in an incubator at 37°C for 48 hours with a relative humidity of 60-70%. The viability of the embryos was determined by candling eggs at a 12 hour interval. After 48 hours of incubation, the eggs were chilled overnight in the refrigerator and allantoic fluid from embryo was collected as per the procedure described by Brauer and Chen, 2015.

Haemagglutination (HA) test: Preparation of 1% chicken red blood cells (RBCs) and haemagglutination test was performed as per the procedure given by (Umar *et al.,* 2014). Initially, 50 µl of PBS was filled in all 12 wells of the V-bottom 96-well plate. Then, 50 µl of allantoic fluid was added into the first well and resuspended to produce a two-fold dilution of the virus, 50 µl from the second well was transferred to the third well and resuspended, and serially diluted until the 11^{th} well. After that, 50 µl of 1 per cent chicken RBC suspension was added to each of the 12 wells and left at room temperature. Red buttons emerged at the bottom of the well after 30 to 60 minutes, and the final well where the red button did not appear was used to determine the HA titre.

The results of HA test are presented in Table (1) and Fig (1). The inoculated R_2B strain of Newcastle disease vaccine virus in allantoic fluid demonstrated agglutination upto the sixth well. Antiviral activity of ivermectin was evident at higher concentrations (500, 250 and 100 µg/ml) in which the drug exhibited the highest (100 %) action against NDV. At lower concentrations (50, 10 µg/ml) ivermectin exerted moderate to poor antiviral activity in the current study. Embryos of virus inoculated group, DMSO treated group and ivermectin treated groups showed haemorrhage (Fig, 2) throughout the body surface compared to saline treated group.

In the present study, the antiviral activity of ivermectin against against R_2B strain of NDV was examined. Ivermectin showed strong antiviral activity at higher concentrations, while at lower concentrations a moderate to poor antiviral activity was observed. Azeem *et al.* (2015) demonstrated that at 100 µg/ml or at higher concentrations ivermectin is found to reduce the viral growth significantly and at lower concentrations ivermectin (50, 10 µg/ml) showed weak antiviral property. Further, they also observed cytotoxicity at 100 µg/ml or greater, but at lesser concentrations (50 or 10 µg/ml) it showes non cytotoxic in MTT assay. Molinari *et al.* (2009) demonstrated that an ivermectin formulation (Ivomec®) exhibited cytotoxicity in Chinese hamster ovary cells

within the concentration of 1 to 250 µg/mL in MTT assay. Wagstaff *et al.* (2012) examined ivermectin's antiviral efficacy against HIV and Dengue virus on the Hela cell line and noticed that ivermectin at 50 µM virtually halted virus growth, while at 25 µM greatly decreased virus production.

Ivermectin inhibits the nuclear import of Bovine herpesvirus 1 DNA polymerase and impedes the viral replication in the Maden darby bovine kidney (MDBK) cell line (Raza *et al.,* 2020). Similarly, *in vitro* studies revealed that ivermectin inhibits replication of the severe acute respiratory syndrome coronavirus- 2 (SARS-CoV-2) (Caly *et al.,* 2020; Lehrer and Rheinstein, 2020). In comparison to the saline group, embryos from eggs injected with DMSO showed haemorrhagic areas on all body surfaces. Our findings are consistent with the previous studies conducted by Wyatt and Howarth, 1976.

In conclusion, it has been observed that ivermectin exhibits strong antiviral activity at higher doses (500, 250 and 100 µg/ml) but at the cost of cytotoxicity. On the contrary at lower concentrations (50, 10 µg/ml), it exhibits moderate to weak antiviral activity without apparent cytotoxicity. However, further *in vivo* studies are warranted to confer antiviral activity of ivermectin and to reposition the day against Newcastle disease in poultry.

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Table 1. Haemagglutination test

Group(s)	Haemagglutination
Negative control group (Saline)	No agglutination
Positive control group (virus)	HA titre- 64 or 2^6 HA units
DMSO treated group	No agglutination
Ivermectin - 500 µg/ml	No agglutination
Ivermectin - 250 µg/ml	No agglutination
Ivermectin - 100 µg/ml	No agglutination
Ivermectin - 50 µg/ml	HA titre- 16 or 2^4 HA units
Ivermectin - 10 µg/ml	HA titre- 32 or 2^5 HA units

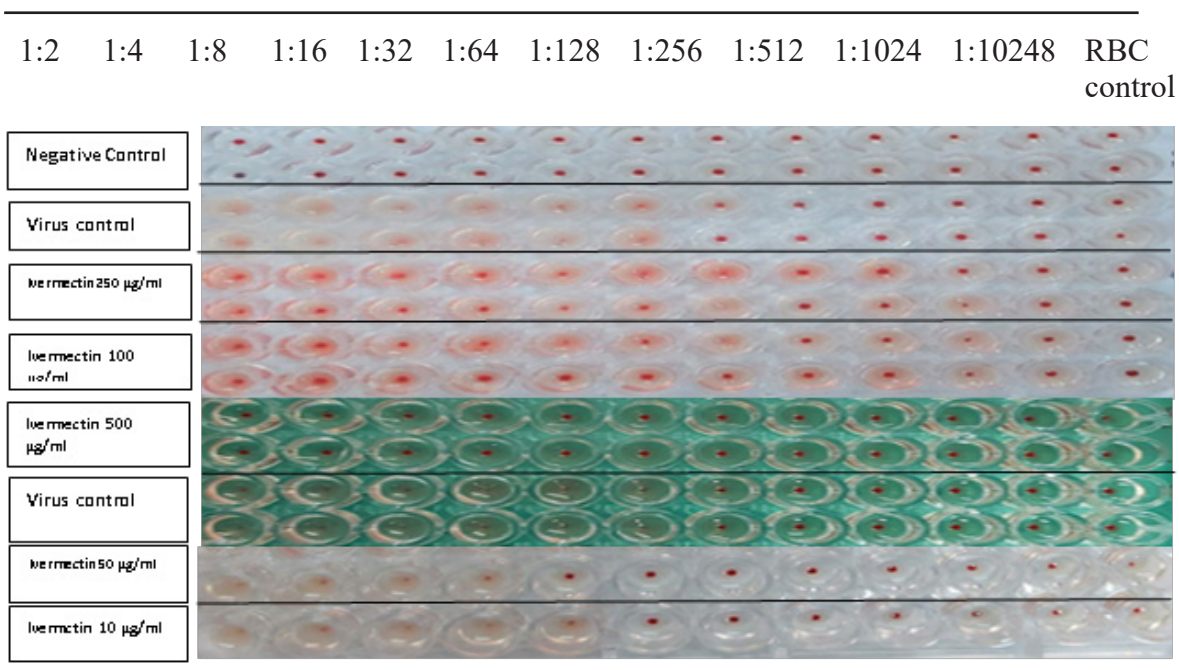

Fig. 1. Haemagglutination test

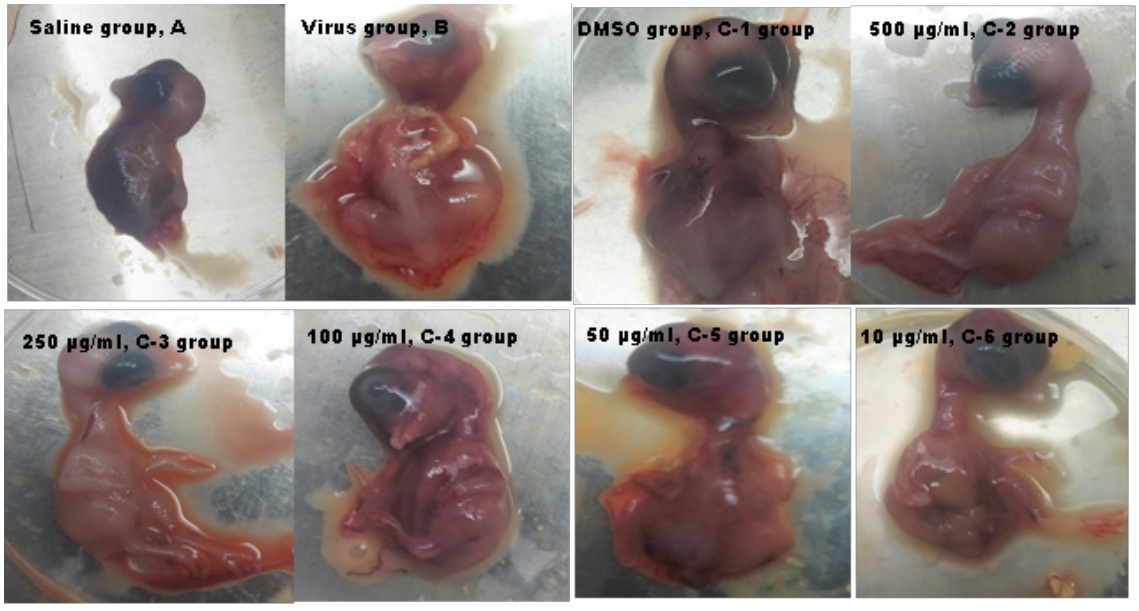

Fig. 2. Chicken embryos showing haemorrhage

DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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