

Inhibition of Bovine Rotavirus by Neomycin

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Abstract: The effects of neomycin on field isolates of Bovine Rotavirus (BRV) were studied. Neomycin decreased BRV cytopathogenicity, virus titer and viral RNA concentration in dose dependent manner. The effect of neomycin on the virus was optimum when applied at the time of infection or postinfection while the drug was kept during the time of infection. Pre-treatment of cells with neomycin had no effect on the virus.

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1. Introduction

Rotaviruses, members of the family *Reoviridae*, are a major cause of acute gastroenteritis in humans and other animal species. Several studies pointed to rotavirus A as the agent responsible for 20-60% of cases of gastroenteritis (*Estes 2001*). Newborn calves are susceptible to rotavirus infection during the first weeks of life, thus making it difficult to actively immunize the animals before exposure to the virulent pathogen (*Fernandez et al., 1996; Kim et al., 2002*). A family of aminoglycoside antibiotics including neomycin B, paramomycin, gentamicin and streptomycin has been shown to be capable of binding to RNA molecules (*Schroeder et al., 2000*). These aminoglycosides have proven to be able to interrupt the interaction between a protein and specific regions of the genome of human immunodeficiency viruses (HIV) (*Wang et al., 1996; Hamasaki et al., 1998*). Neomycin B has been shown to inhibit viral replication by several mechanisms, including blockage of viral penetration or inhibition of viral nucleic acid synthesis (*Herold and Spear 1994*). Neomycin B has been determined to inhibit the replication of herpes simplex virus type I by both binding to the virus receptor and by altering the synthesis of virus polypeptides (*Langeland et al., 1987*). Neomycin B inhibits the replication of simian rotavirus (SA-11) and bovine rotavirus (NCDV) in-vitro on Rhesus monkey kidney fetal cells (MA-104) with dose dependent manner (*Manchego and Spencer 2003; Ali et al., 2009*). Rotavirus multiplication was inhibited by neomycin B by affecting the synthesis of the viral genome. Genome replication involves a two-step mechanism, one is plus-strand synthesis or transcription, and the other is minus-strand synthesis or replication. The results indicate that both activities are inhibited by aminoglycoside (*Manchego and Spencer 2003*). Previous work shown that neomycin inhibits the

standard strain of bovine rotavirus cytopathogenicity and virus yield (*Ali et al., 2009*). The aim of this work was to demonstrate the effect of neomycin on locally isolated field Egyptian BRV replication via; 1) identification of the role of neomycin B on virus CPE, 2) identification of the role of neomycin B on reduction of virus plaque number, 3) identification of the role of neomycin B on reduction of virus RNA concentration.

2. Material and Methods

Cells and Viruses and Neomycin B:

Madin Darby Bovine Kidney (MDBK) cells were obtained from American Type Culture Collection (ATCC). Bovine Rotavirus field isolates was obtained from Rinderpest like disease Department, Veterinary Serum and Vaccine Research Institute, (VSVRI), Abbassia, Cairo. The virus was propagated in MDBK cells. Neomycin was purchased from (Sigma, USA) and prepared as a stock solution at 50 mM in MEM pH 7.3, sterilized by filtration and maintained at 4°C.

Neomycin B toxicity on MDBK:

To select the optimum dose for treatment of virus without toxic effect on the cells, various concentrations were tested. MDBK cells were seeded at a density of 5×10^3 cells per well of a 96-well plate in MEM supplemented with 5% FBS. After 24 hr, various concentrations of neomycin were added. Cells were allowed to proliferate for 3 days at 37°C, after which the cell number was determined by means of the MTS/PMS (Promega) method.

Effect of neomycin B on BRV Cytopathic effect:

To test the effect of neomycin on BRV cytopathic effect and to test the optimum time for treatment, MDBK cells (6.5×10^5 cells/well) were seeded in a six-well culture plate and different concentrations of neomycin (2, 4, 6 and 8 mM) were added to MDBK

monolayers as follow: **1-** One hour before BRV infection, then neomycin was removed and cells were inoculated with BRV. **2-** Cells were inoculated with BRV and treated with neomycin at the same time of infection and maintained throughout the experiment. **3-** Neomycin was added after one hour of BRV infection and maintained throughout the experiment. In all cases of neomycin treatment, cells were inoculated with BRV (MOI = 2). Confluent Cells were maintained at 37°C in serum-free MEM containing 2.5 µg/ ml trypsin. The plates were incubated for 72 hours at 37°C with daily observation for the development of specific CPE. Mock-infection was carried out as negative control.

Effect of neomycin B on BRV plaque formation:

MDBK cells were maintained at 37°C in serum-free MEM containing 2.5 µg/ ml trypsin and were inoculated with BRV (MOI 4000 PFU/10⁶ cells). After 1 hr adsorption, the inoculum was removed and the cells were washed and overlaid with media containing 0.8% seaplaque agarose and various concentration of neomycin B. Agarose overlay was allowed to solidification and the plates were incubated for 5 days and then examined by fixation with 10% formol saline and staining with crystal violet 0.5% (*Hasenack et al., 2002*).

Effect of neomycin B on BRV titer:

This assay was done further investigate the effect of neomycin on BRV growth. Confluent Cells were maintained at 37°C in serum-free MEM containing 2.5 µg/ ml trypsin and the cultures were inoculated with BRV (MOI = 2). After 1 hr, the inoculum was removed and the cells were replenished with serum-free MEM containing various concentrations of neomycin. The cells were incubated for 72 hours at 37°C. The virus titer was determined 72 h post infection after three cycles of freeze-thawing of infected cultures by TCID₅₀.

Effect of neomycin on BRV RNA by quantitative Real-time PCR (qRT-PCR):

To understand the mechanism underlying neomycin inhibition of BRV-induced CPE and virus yield reduction, we investigated the effects of neomycin on viral RNA synthesis. Confluent Cells were maintained at 37°C in serum-free MEM containing 2.5 µg/ ml trypsin and the cultures were inoculated with BRV (MOI = 2). After adsorption, the inoculum was removed and the plates were incubated for 72 hours at 37°C. Total RNA was extracted from BRV infected MDBK cells using QIAamp^R viral RNA Mini kit (Qiagen) according to the manufacturer's instruction. The extracted dsRNA of BRV were denatured by heating at 95°C for 5 min and then chilled

on ice for 5 min. The cDNA was generated by reverse transcription using random hexamers with SuperScript III RT (Invitrogen). Real-time PCRs were carried out using SYBR green PCR master mix (Roche, Molecular Biochemicals) and primers VP6-Fw1: 5' GGATGTCCTGTACTCCTTGTCAAAA 3' and rev1: 5' TCCAGTTTGGAACCTCATTTC 3'. Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The specificity of the reactions was determined by melting curve analysis of the amplicons. Real-time fluorescence measurements were taken and a threshold cycle (C_T) value for each sample was calculated by determining the end point at which the fluorescence exceeds a threshold limit (*Logan et al., 2006*).

3. Results and Discussion:

Neomycin B toxicity on MDBK: The 50% cytostatic concentration (CC₅₀) was defined as the concentration that inhibited the proliferation of exponentially growing cells by 50% and was calculated using logarithmic interpolation. Neomycin concentrations of 2 to 8 mM did not demonstrate any change in the cells that could be considered toxic effect. However, from 10 mM to 50 mM cell rounding and clumping were observed with increasing intensity at higher concentrations of the neomycin. So, these concentrations (2, 4, 6 and 8 mM) were used for this study.

Effect of neomycin B on BRV Cytopathic effect, BRV plaque formation and on BRV titer:

The results showed that neomycin protect the cells against BRV CPE in dose dependent manner and the optimum time for treatment was shown in MDBK treated at the same time of infection or 1 hr post infection while the drug was kept during the whole time of infection. There was no effect of neomycin on the CPE of BRV in the cells treated before infection. Two mM concentration of neomycin had no effect on the rate of CPE of BRV, while 4, 6 and 8 mM of neomycin were able to inhibit the CPE of BRV by 50%, 83.33% and 91.67% respectively (**Table 1 and Figure 1**). Similar results were obtained in previous studies (*Ali et al., 2009*) where they mentioned that neomycin B inhibits the replication of BRV in-vitro on Rhesus monkey kidney fetal cells (MA-104) with dose dependent manner and neomycin must be present at the time of infection to exert maximal effect, whereas the addition of the antibiotic 1 h post-infection without keeping the drug all the time had no effect. *Langeland et al., 1987* revealed that neomycin affects early stages of HSV-1. Thus, when the first steps of infection have occurred, the presence of neomycin does not interfere with the further, normal

course of infection. In contrast, our results suggest that the drug affects a step of viral replication other than penetration possibly a step related to the synthesis of the viral genome and the difference could be attributed the nature of the viruses, where HSV and HIV are enveloped while BRV is non enveloped.

As in case of effect of neomycin on BRV CPE, 2 mM concentration of neomycin didn't reduce the

number of BRV plaques; while 4, 6 and 8 mM were able to reduce the number of plaques induced by BRV (**Figure 2**). Furthermore, neomycin decreased BRV yields and the virus inhibition was dose dependent manner. There was decrease in virus titer by 1.5, 2.3 and 3 logs in virus infected and treated cells with 4, 6 and 8 mM neomycin respectively.

Table 1: Effect of neomycin B on cytopathic effect of BRV

Variable		Number of wells with CPE	% of CPE of BRV	% of reduction in CPE of BRV
Virus control		12/12	100	0
Neomycin conc	2 mM	12/12	100	0
	4 mM	6/12	50	50
	6 mM	2/12	16.67	83.33
	8 mM	1/12	8.33	91.67

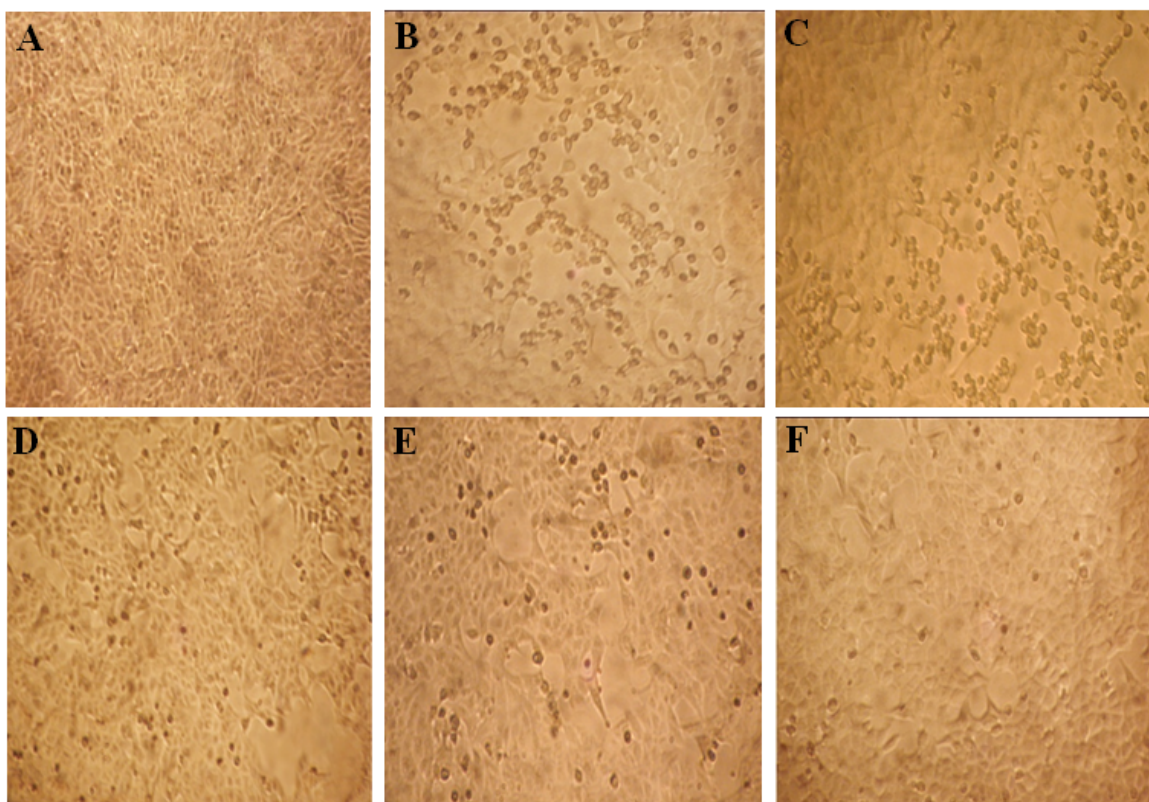


Figure 1: Effect of neomycin on CPE of BRV. **A-** Normal non-infected. **B-** Virus infected cells. **C-** Virus infected treated cells with 2 mM neomycin. **D-** Virus infected treated cells with 4 mM neomycin. **E-** Virus infected treated cells with 6 mM neomycin. **F-** Virus infected treated cells with 8 mM neomycin.

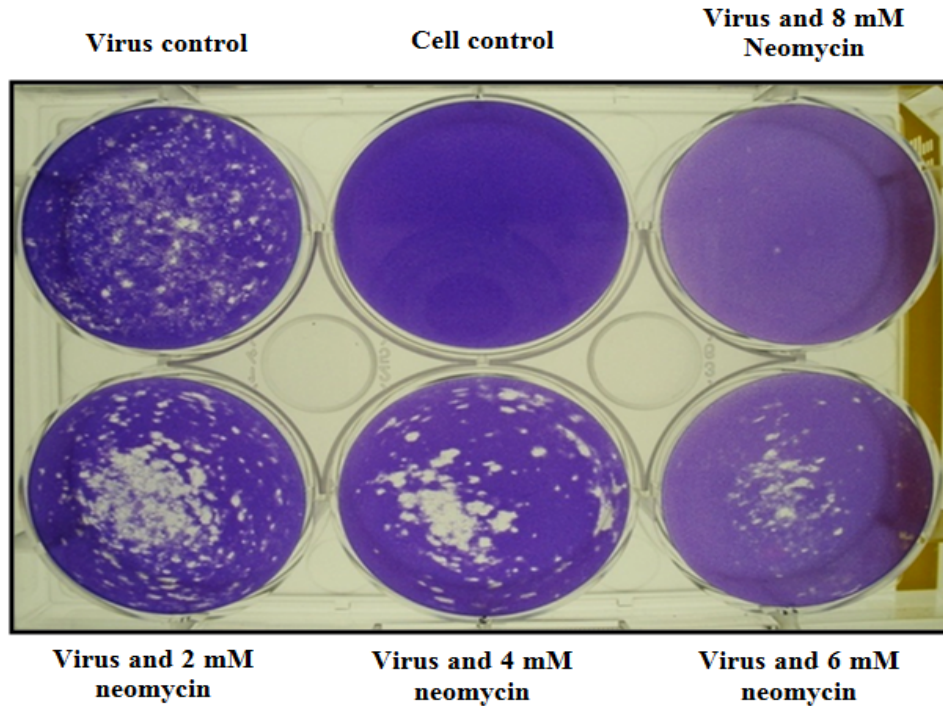


Figure 2: Effect of neomycin B on rotavirus replications: there is difference in number of plaques with dose-dependent decrease.

Effect of neomycin on BRV RNA:

The results showed that neomycin decreased the viral RNA yield. The maximum effect was shown with higher concentration of neomycin, 6 and 8 mM. While, little difference was shown in amount of RNA

in virus infected not treated and virus infected treated cells with 2 and 4 mM of neomycin (**Figure 3**). Previous results indicated that neomycin inhibit RNA and inhibit BRV RNA (*Clouet-d’Orval et al., 1995; Ali et al., 2009*).

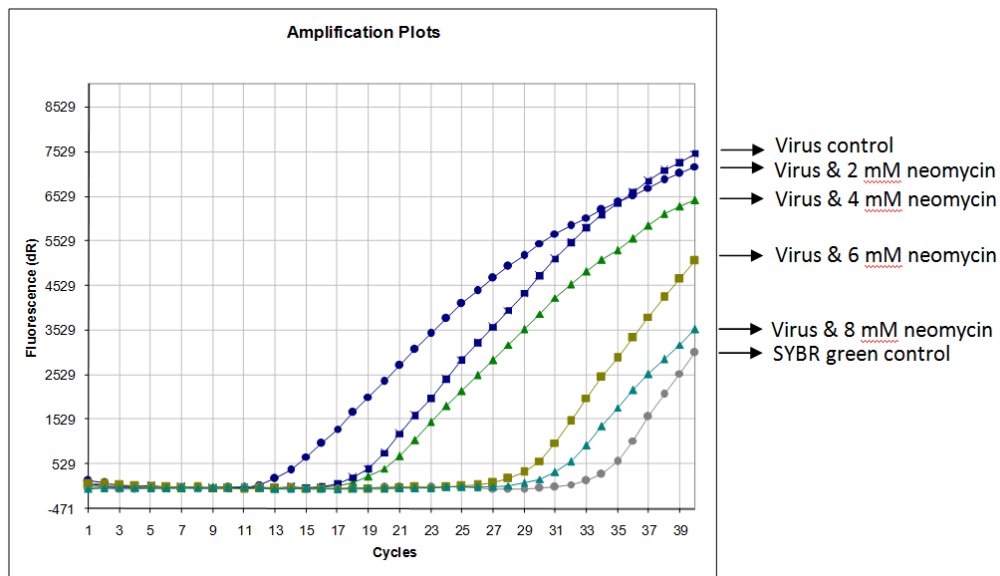


Figure 3: Effect of neomycin on Rotavirus RNA concentration

Our results indicated that 8 mM neomycin concentration was within the range of therapeutic dose of neomycin. In this study, we demonstrated that neomycin inhibited BRV induced cytopathology, plaque formation, viral titers, and viral RNA replication. Further studies are required to clarify the mechanism of neomycin inhibition to rotavirus replication.

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References:

- Ali, A.A.H., A.A. Salama, S.M. Zeidan and S.M.G. Mansour, 2009. Virological and Molecular Studies on Rotavirus. *Zag. Vet. J.* 37(4): 70-79.
- Clouet-d'Orval, B., T.K. Stage, O.C. Uhlenbeck, 1995. Neomycin inhibition of the Hammerhead ribozyme involves ionic interactions. *Biochemistry* 34: 11186–11190
- Estes, M.K., 2001. Rotaviruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds), *Fields virology* 4th edn. LippincottWilliams &Wilkins, Alters Kluwer Company, pp 1747–1785
- Fernandez, F.M., M.E. Conner, A.L., Parwani, D. Todhunter, K.L. Smith, S.E. Crawford, M.K. Estes and L.J. Saif 1996. Isotype-specific antibody responses to rotavirus and virus proteins in cows inoculated with subunit vaccines composed of recombinant SA11 core-like particles (CLP) or virus like particles (VLP) *Vaccine* 14, 1303–1312.
- Hamasaki, K., J. Killian, J. Cho and R.R. Rando, 1998. Minimal RNA constructs that specifically bind aminoglycoside antibiotics with high affinities. *Biochemistry* 37: 656–663
- Hasenack, B.S., M. Valéria, J. Botelho, F.L. Lauretti, F. de Melo, J.M. Orlandi, R.E.C. Linhares, M. Ueda and C.M. Nozawa, 2002. The Effect of Concanavalin A on the Replication of Rotavirus (SA-11) in Cell Culture. *Brazilian Archives of Biology and Technology.* 45 (2): 125-135.
- Herold, B.C. and P.G. Spear, 1994. Neomycin inhibits glycoprotein C (gC)-dependent binding of herpes simplex virus type 1 to cells and also inhibits postbinding events in entry. *Virology* 203: 166–171
- Kim, Y., P.R. Nielsen, D. Hodgins, K.O. Chang and L.J. Saif, 2002. Lactogenic antibody responses in cows vaccinated with recombinant bovine rotavirus-like particles (VLPs) of two serotypes or inactivated bovine rotavirus vaccines. *Vaccine*, 20: 1248-1258.
- Langeland, N., H. Holmsen, J.R. Lillehaug, L. Haarr, 1987. Evidence that neomycin inhibits binding of Herpes simplex virus type 1 to the cellular receptor. *J Virol.*, 61: 3388–3393
- Logan, C., J.J. O'Leary and N. O'Sullivan, 2006. Real-Time Reverse Transcription-PCR for Detection of Rotavirus and Adenovirus as Causative Agents of Acute Viral Gastroenteritis in Children. *J. Clin. Microbiol.* 44 (9): 3189–3195.
- Manchego, A. and E. Spencer, 2003. Effect of neomycin B on rotavirus plus- and minus-strand RNA synthesis. *Arch Virol.*, 148: 1071–1084
- Schroeder, R., C. Waldsich, and H. Wank, 2000. Modulation of RNA function by aminoglycoside antibiotics. *EMBO J* 19: 1–9
- Wang, Y., J. Killian, K. Hamasaki, and R.R. Rando, 1996. RNA molecules that specifically and stoichiometrically bind aminoglycoside antibiotics with high affinities. *Biochemistry* 35: 12338–12346.

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