Experimental studies on The Effect of Neomycin B on Rotavirus Replication

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ABSTRACT

Bovine Rotavirus (BRV) is a major cause of acute gastroenteritis in newborn calves throughout the world causing economic losses to beef and dairy industries. Neomycin B, an aminoglycoside antibiotic has been shown to inhibit rotavirus replication *in-vitro*. The current study was carried out to study the availability of neomycin B as preventive and chemotherapeutic mean for rotavirus infection. It was conducted by following up the replication of virus inside the infected and infected treated mice. Moreover the shedding of virus by infected and infected treated mice was determined by immunofluorescence (IF). The group of mice inoculated with BRV and simultaneously treated with neomycin had significant reductions in virus shedding by 1.43 and 1.7 logs at first day and 5th day post BRV inoculation respectively. It was revealed that neomycin has an interfering effect on rotavirus replication *in-vivo* (in mice), when the drug administered before, during and after viral infection. Further studies for field applications of neomycin as therapeutic measure for Bovine Rotavirus infection in newborn calves especially in calves born from non vaccinated animals or born with low titer of antibodies need to be done.

INTRODUCTION

Rotaviruses constitute a genus of the family *Reoviridae*. The virus contains 11 genome segments of double-stranded RNA in a 70 nm, double-shelled, icosahedral capsid. Rotaviruses are common causes of severe gastroenteritis in neonatal calves throughout the world *(1)*. Rotavirus is composed of three layers of structural capsid proteins. The core comprises of three viral proteins VP1, VP2 and VP3. The inner capsid consists of the most abundant protein, VP6, to which a majority of the group specific antibodies is directed *(2)*. The outer capsid layer contains the major surface glycoprotein, VP7 (glycoprotein, G) and VP4 (Protease sensitive, P). Rotaviruses are classified into seven serogroups (A-G) determined by the antigenic properties of VP6. Group A, the most common, is further divided into serotypes determined by VP4 (P type) and VP7 (G type) *(3)*. Although rotavirus vaccines are often administered to pregnant animals to increase levels of rotavirus antibodies in colostrum, neonates frequently exhibit rotaviral diarrhea. The later may be attributed to failure of passive transfer from dam to calf *(4)*. Therefore other therapeutics need to be considered for controlling of Rotavirus infection. Neomycin B has been shown to inhibit viral replication by several mechanisms, including blockage of viral penetration or inhibition of viral nucleic acid synthesis *(5)*. Neomycin B inhibits the replication of BRV *in-vitro* on Rhesus monkey kidney fetal cells (MA-104) with dose dependent manner *(6)*. To enforce the significance of previous *in-vitro* studies and examine the efficacy of field application of neomycin for rotavirus infection, this work was aimed to study the availability of using neomycin as preventive or therapeutic mean for rotavirus infection *in-vivo* (in mice).

MATERIAL AND METHODS

Cells: Rhesus monkey kidney fetal cells (MA-104) were obtained from American Type Culture Collection (ATCC).

Bovine rotavirus (BRV): Nebraska calf diarrhea virus (NCDV) was obtained from Department of Veterinary Sciences, South

Dakota State University (SDSU), USA. Its titer is 10^5 TCID50/ml.

Neomycin B (Sigma, USA) was prepared as a stock solution 30 mg/ ml 1x MEM

Antibody: Anti-NCDV VP6 monoclonal antibody prepared in mice and labeled with fluorescin isothiocyanate (FITC) obtained from Department of Veterinary Sciences, SDSU, USA. It used in IF.

Mice: Swiss albino mice were obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo and used at 9-12 days of age with average body weight 7-13 gm. Mice were housed in microisolation cages and shown to be rotavirus negative before use by analyzing serum samples for the presence of rotavirus antibodies.

Detection of specific BRV antibodies using serum neutralization test (SNT)

Test sera of two fold serial dilution in MEM were distributed by four wells for each dilution. Pretiterated standard diluted BRV of 100 $TCID_{50}$ per 50 µl was then added to each well and incubated at 37°C for one hour. Confluent monolayers of MA-104 grown in 96-well plates were inoculated with 100 µl of virus-antiserum mixtures. Cell and virus controls (1, 10 and 100 $TCID_{50}$) were included .The plates were incubated at 37°C for one hour in 5% CO_2 condition. The excess inoculum was decanted and replaced with 150 µl of maintenance media containing 2.5µg/ml trypsin (BRV needs trypsin in protein cleavage in its replication). Plates were incubated at 37°C for 3 days with daily observation for the development of CPE *(7)*.

Preparation of therapeutic doses of Neomycin B

The therapeutic dose of neomycin used in the experiment was 30 mg/ kg body weight *(8)*. Stock solution of neomycin B was prepared by mixing 30 mg neomycin in 3ml 1x MEM, sterilized by filtration and maintained at 4°C. Each mouse was treated with neomycin according to its body weight.

Preparation of infectious dose of BRV

The mice were orally inoculated with 1000 infective dose-50 (ID50) of BRV strain NCDV. BRV was diluted to 10^4 ID50 per ml in 1x MEM and each mouse was inoculated with 100 µl of virus suspension. The stock virus titer is 10^5 TCID50/ml.

The experiment

The mice used in the experiment were assigned randomly into 3 major groups. Microisolation cages were used to house the mice.

Group I: Simultaneous treatment assay

Fifteen mice were inoculated with BRV and neomycin at the same time.

Group II: Pre-infection treatment assay

Thirty mice in this group were divided into two subgroups;

Subgroup II-1: The mice were treated for one hour with neomycin and then infected with BRV.

Subgroup II-2: The mice were treated for 24 hours with neomycin, and then infected with the virus. Fifteen mice were used at each time.

Group III: Post-infection treatment assay

Thirty mice in this group were divided into two subgroups;

Subgroup III-1: The mice were inoculated with BRV, then after one hour they treated with neomycin.

Subgroup III-2: The mice were inoculated with BRV for 24 hours and then treated with neomycin. Fifteen mice were used at each time.

Positive and negative controls were maintained as the followings; fifteen mice treated and non-infected (Neomycin control group), fifteen mice non-treated and infected (Virus control group) and fifteen mice non-treated and non-infected.

The mice were observed daily for diarrhea and/or deaths. The results were recorded.

Collection and Preparation of fecal samples

Fecal samples were collected from each mouse following rotavirus inoculation by 1, 3, 5, 7, and 10 days. They were prepared as either a 10% (wt/vol) suspension of solid or

semisolid feces in Earl's balanced salt solution or as a 20% (vol/vol) suspension of liquid feces in Earl's balanced salt solution containing penicillin 1000IU/ml, streptomycin 1000 µg/ml, homogenized and centrifuged at1500 xg for 10 min, 4◦C to remove debris. The supernatants were stored in sterile vials at -80°C *(9)*.

Detection and Titration of rotavirus in fecal samples using Immunofluorescence (IF)

The quantities of rotavirus shed in the fecal samples were determined by titration of rotavirus by immunofluorescence. Serial ten fold dilutions of the fecal samples were prepared in test tubes containing 1x MEM supplemented with 2.5 µg/ml trypsin. Confluent MA-104 monolayers were inoculated with sample dilutions 100 µl from each dilution in 4 wells. The plates were incubated at 37°C for one hour in 5% CO_2 condition. The excess inoculum was decanted and replaced with 150 µl of maintenance media containing 2.5µg/ml trypsin. The plates were incubated for 3 days at 37°C in 5% CO2 condition. IF was performed according to standard procedures previously described *(10)*. Briefly after infection period, MA-104 cells were fixed with 80% acetone for 20 minutes. The cells were incubated for one hour at 37°C with rotavirus VP6 monoclonal antibodies labeled with FITC, diluted 1/100 in PBS. The plates were washed 3 times with PBS then examined for specific positive intracytoplasmic greenish yellow fluorescence using Fluorescent microscope. The virus end point was the highest dilution of virus that infects 50% of the inoculated cells $TCID_{50}$. The virus titer was expressed as Log_{10} tissue culture infective dose 50 per ml using the formula of *(11)*.

Data analysis

Analysis of variance (ANOVA) ($p < 0.05$) was done *(12)*. All statistics were run on the computer using the SPSS program.

RESULTS

Clinical score of diarrhea in mice post BRV inoculation

Diarrhea was observed daily in the animals after opening of intestinal tract. Diarrhea measured daily in the animals was assessed as mild or moderate. Mild diarrhea was characterized by soft yellow feces with the consistency of paste. Moderate diarrhea was characterized by bright yellow feces with a much softer consistency more appropriately described as a semiliquid gel **(Figure 1).** Mild and moderate diarrhea was observed only in virus control group of mice. There were significant differences existed between different groups of mice. No diarrhea, however mild was observed in simultaneous treatment group of mice.

Detection and Titration of rotavirus in fecal samples using IF

This approach was utilized to estimate the amount of infectious virus present in feces. To estimate the level of decrease in viral titer, the duration and the magnitude of virus shedding were determined by titration of rotavirus in fecal samples by IF. The curve of virus shedding against the number of days post-inoculation of NCDV for each individual mice was done. The level of decrease in viral titer in virus infected and neomycin treated groups were then calculated. Mice treated with neomycin before and after virus inoculation shed significantly lower quantities of rotavirus in their feces than those shed by infected not treated mice **(Table 1 and Figure 2-4).**

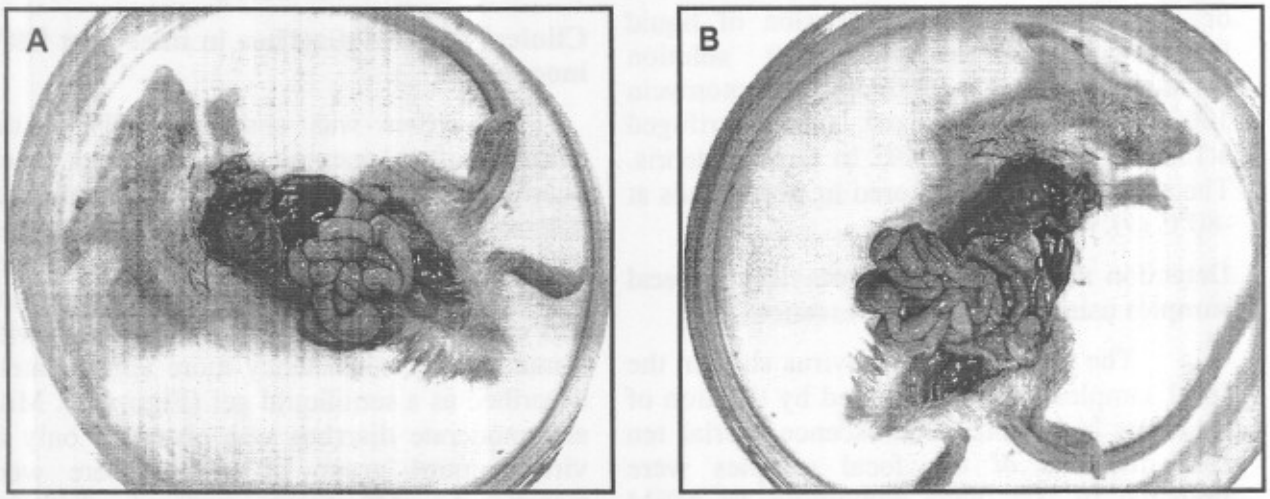

Figure 1. Intestinal tract of mice post BRV inoculation.
A- Virus infected mouse showing moderate mucoid diarrhea.
B- Infected and treated mouse with neomycin showing almost normal intestine

Table 1. Detection and Titration of rotavirus in fecal samples using IF.

Group	Days post virus inoculation (BRV titer Log 10 TCID50/ml)				
	1st	3rd	5th	7th	10th
I	2.03 ± 0.08 b	2.3 ± 0.05 c	3.1 ± 0.05 c	2.1 ± 0.21 a	1.1 ± 0.1 a
Subgroup II-1	3.13 ± 0.08 a	2.9 ± 0.08 b	4.06 ± 0.12 b	1.3 ± 0.14 b	-
Subgroup II-2	3.13 ± 0.12 a	2.5 ± 0.06 c	5.3 ± 0.12 a	2.03 ± 0.03 a	-
Subgroup III-1	3.26 ± 0.06 a	3.1± 0.05 b	2.4 ± 0.14 c	2.06 ± 0.15 a	-
Subgroup III-2	3.15 ± 0.14 a	3.6 ± 0.08 a	4.1 ± 0.25 b	2.1 ± 0.06 a	1.1 ± 0.09 a
Virus control	3.46 ± 0.11 a	2.8 ± 0.05 a	4.8 ± 0.11 ab	2.2 ± 0.09 a	1.2 ± 0.11 a

The Table displays the average of viral titer in fecal samples collected from 3 mice per each group ± standard errors.

Different subscripts in the same column mean significant differences ($P < 0.05$)

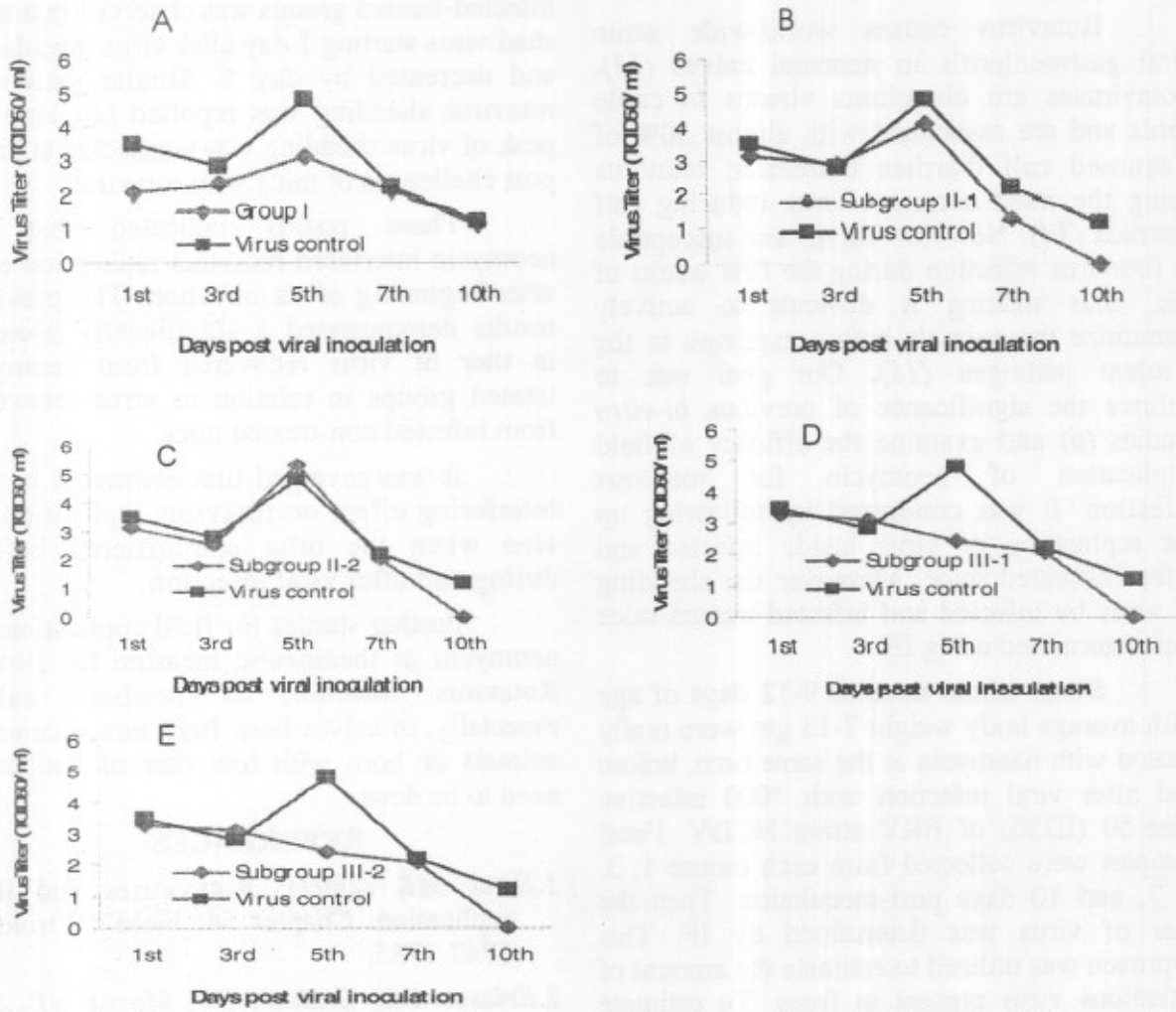

Figure 2. Detection and Titration of rotavirus in fecal samples using IF. **A-** Difference in viral titer between virus control and Group-I. **B-** Difference in viral titer between virus control and subgroup-II-1. **C-** Difference in viral titer between virus control and subgroup-II-2. **D-** Difference in viral titer between virus control and subgroup-III-1. **E-** Difference in viral titer between virus control and subgroup-III-2.

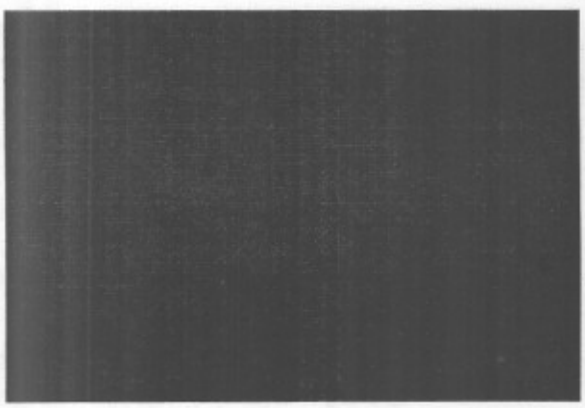

Figure 3. Normal MA-104 cells showing no fluorescence. X 100

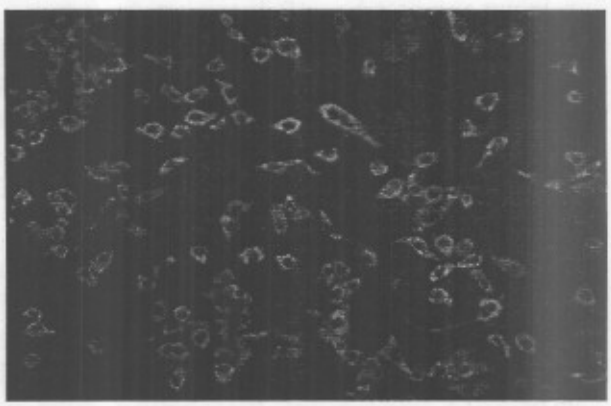

Figure 4. Virus infected cell stained with NCDV MAb against VP6 labeled with FITC, showing intracytoplasmic greenish yellow fluorescence. X 100

DISCUSSION

Rotavirus causes world-wide acute viral gastroenteritis in neonatal calves ***(13).*** Rotaviruses are ubiquitous viruses in cattle herds and are associated with almost 50% of diagnosed calf diarrhea outbreaks; rotavirus being the most common virus inducing calf diarrhea ***(14)***. 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 ***(15).*** Our goal was to enforce the significance of previous *in-vitro* studies ***(6)*** and examine the efficacy of field application of neomycin for rotavirus infection. It was conducted by following up the replication of virus inside infected and infected-treated mice. Moreover the shedding of virus by infected and infected-treated mice was determined using IF.

Swiss albino mice of 9-12 days of age with average body weight 7-13 gm were orally treated with neomycin at the same time, before and after viral infection with 1000 infective dose-50 (ID50) of BRV strain NCDV. Fecal samples were collected from each mouse 1, 3, 5, 7, and 10 days post-inoculation. Then the titer of virus was determined by IF. This approach was utilized to estimate the amount of infectious virus present in feces. To estimate the level of decrease in viral titer, the duration and the magnitude of virus shedding were determined by titration of rotavirus in fecal samples using IF. The curve of virus shedding against the number of days post-inoculation of NCDV for each individual mice was done. The group of mice inoculated with BRV and neomycin at the same time, had significant reductions in virus shedding by 1.43 and 1.7 logs at first day and 5th day post BRV inoculation respectively **(Table 1)**.

To determine if neomycin would protect mice from rotavirus shedding after BRV inoculation, groups of mice were inoculated with BRV then after one and 24 hrs post virus inoculation, they were treated with neomycin. It was observed that mice shed virus starting 1 day after virus inoculation with a peak on day 5 and decreased by day 7 in virus infected not treated group. While in virus infected-treated groups was observed that mice shed virus starting 1 day after virus inoculation and decreased by day 5. Similar pattern of rotavirus shedding was reported ***(16)***and the peak of virus shedding was conducted at day 5 post challenges of mice with rotavirus.

These results indicated that the neomycin interfered rotavirus replication even after beginning of its infection. The previous results demonstrated a significantly decrease in titer of virus recovered from neomycin treated groups in relation to virus recovered from infected non-treated mice.

It was revealed that neomycin has an interfering effect on rotavirus replication *in-vivo* when the drug administered before, during and after viral infection.

Further studies for field applications of neomycin as therapeutic measure for Bovine Rotavirus infection in newborn calves especially, in calves born from non vaccinated animals or born with low titer of antibodies need to be done.

REFERENCES

1.Estes MK (2001): Rotaviruses and their replication. Chapter 54: Field's Virology, 1747-1785.

2.Ericson BL, Graham DY, Mason BB, and Estes MK (1982): Identification, synthesis and modifications of simian rotavirus SA11 polypeptides in infected cells. J. Virol., 42:825-839.

3.Estes MK and Cohen J (1989): Rotavirus gene structure and function. Microbiol. Rev. 53:410- 449.

4.Hassan AA, El-Bakry M, Salama AA, Zeidan SM and Galal SM (2007): Isolation, identification and classification of some viruses induced neonatal calf diarrhea. 5th Int. Sci. Conf., Mansoura, 10-11 April: 1463-1479.

5.Herold BC and Spear PG (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

6.Ali AAH, Salama AA, Zeidan SM and Mansour SMG (2009): Virological and Molecular Studies on Rotavirus. Zag. Vet. J. 37(4): 70-79.

7.Wassel MS (1996): Isolation and preparation of bovine rotavirus (BRV) vaccine in Minnesota, USA. Vet. Med. J. Giza, 44 (3): 583-591.

8.Paget GE and Barnes JM (1964): Evaluation of Drug Activities: Pharmacometrics, Laurence and Bacharach, Vol 1, Academic Press, New York 133-166.

9.Choi AHC, Smiley K, Basu M, McNeal MM, Shao M, Bean JA, Clements JD, Stout RR and Ward RL (2007): Protection of mice against rotavirus challenge following intradermal DNA immunization by Biojector needle-free injection Vaccine 25: 3215–3218

10.Bosch A, Lucena F, Diez JM, Gajardo R, Blasi M and Jofre J (1991): Human enteric viruses and indicator microorganisms in a water supply associated with an outbreak of infectious hepatitis. J. Am. Water Works Assoc. 83:80–83

11.Reed LM and Muench N (1938): A simple method for estimating fifty percent end point. Am. J. Hyg. 27: 493-497.

12.SAS, (1987): SAS user's guide: statistics, version 6 edition. SAS Institute Inc. Cary, NC, USA.

13.Adah MI, Nagashima S, Wakuda M and Taniguchi K (2003): Close Relationship between G8-Serotype Bovine and Human Rotaviruses Isolated in Nigeria. J. Clin. Microbiol. 41 (8): 3945–3950

14.Reynolds DJ, Morgan JH, Chanter N, Jones PW, Bridger JC, Debney TG and Bunch KJ (1986): Microbiology of calf diarrhea in southern Britain. Vet. Rec., 119: 34-39.

15.Kim Y, Nielsen PR, Hodgins D, Chang KO and Saif LJ (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

16.McNeal MM, Stone SC, Basu M, Bean JA, Clements JD, Hendrickson BA, Choi AH-C and Ward RL (2006): Protection against rotavirus shedding after intranasal immunization of mice with a chimeric VP6 protein does not require intestinal IgA. Virology 346: 338 – 347

الملخص العربي

دراسات تجريبية على تأثير النيوميسين-بى على تكاثر فيروس الروتا

على عبد الرشيد على سلامة- احمد عبدالسميع حسن علي - سيد محمد زيدان*
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***قسم الأمراض المشابهه للطاعون البقري معهد بحوث الأمصال واللقاحات البيطرية العباسية – القاهرة**

يعتبر فيروس الروتا البقرى من أهم العوامل المسببة لالتهاب المعدة والأمعاء الحاد فى العجول فى جميع أنحاء العالم مسببا خسائر فادحة فى انتاج اللحوم والالبان. النيوميسين-بى مضاد حيوي من مجموعة الأمينوجليكوسيد وقد اثبتت الدراسات السابقة أنه مثبط لتكاثر فيروس الروتا فى المعمل. ولقد أجريت هذه الدراسة الحالية لتقييم تأثير النيوميسين-بى على تكاثر فيروس الروتا فى الحقل (الفئران)، وبقياس عيارية الفيروس فى عينات البراز باستخدام اختبار الفلوروسنت المناعى لوحظ انخفاض فى عيارية الفيروس بـ ١,٤٣ و ١,٧ لوغاريتم فى عينات البراز المجمعة فى اليوم الأول والخامس على التوالي وذلك فى مجموعة الفئران المحقنونة بفيروس الروتا والنيوميسين في نفس الوقت ، كما لوحظ وجود انخفاض في عيارية الفيروس فى عينات البراز المجمعة من المجموعات المعالجة بالنيوميسين مقارنة بعيارية الفيروس فى الفئران المصابة بالفيروس والغير معالجة بالنيوميسين. مما سبق اتضح ان النيوميسين له تأثير مثبط على تكاثر فيروس الروتا فى الحقل (الفئران) عند اضافته قبل وأثناء وبعد العدوى بالفيروس. وهذا يدعونا الى عمل دراسات مستقبلية على امكانية استخدام النيوميسين للحد من المرض فى العجول حديثة الولادة التى تحتوى على عيارية منخفضة من الأجسام المضادة لفيروس الروتا والمنقولة من الأمهات أو العجول المولودة من أمهات غير محصنة.