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Original Article

STUDY ON ISOLATION AND EXPERIMENTAL PATHOGENESIS OF BOVINE ROTAVIRUS IN RABBIT MODEL

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Abstract: Bovine rotavirus being one of the major causes of gastroenteritis in calves in intensive and semi-intensive dairy farms in India. The samples were collected from calves with severe diarrhoea from Karnataka, Uttarapradesh, Uttarakand. Rotavirus was isolated from fecal samples by culturing in MA104 permanent cell line with pre-treatment of virus with trypsin, incorporation of trypsin in culture medium, centrifugation during adsorption. Cell culture adopted virus produce characteristic CPE and desquamation from surface, after 9 passage virus titre reached to 10^5 per mL TCID50. The isolation and propagation of virus was confirmed by immunofluorescence test. Further, the isolated bovine rotavirus G6P11 has elicited transient viremia and immune response without shedding in feces and producing clinical signs in rabbit model. To conclude Bovine rotavirus can be effectively isolated and propagated in MA104 cell line. The bovine rotavirus strain G6P[11] is non pathogenic to rabbits or rabbit model does not act as good animal model to study pathogenesis.

Keywords: Rota virus, Bovine rotavirus

Dr. Ranganath G. J., Assistant Professor, UAS, Dharwad perused MVSc and PhD from prestigious Indian Veterinary Research Institute. He has bagged 7th rank in ICAR-JRF examination during 2007. He received several awared like Dr. Patri Rama Rao award for best PhD thesis during 2014; Co- awardee for Ramachandran memorial award for research in molecular oncology and best poster presentation award for anticancer potential of Morinda citrifolia (Noni) paper. Published nine research papers as first and co-author in National and international Journals.

INTRODUCTION

Infectious calf diarrhoea being one of the biggest health challenge to the sustenance of dairy industry worldwide. The major infectious agents responsible for calf diarrhea in calf include *Escherichia coli, Salmonella* species, *Clostridium perferingens, Rotavirus, Coronavirus, Cryptosporidium*. Rotaviruses are non-enveloped icosahedral structures containing a genome of 11 segmented double stranded RNA. Virion are three layered with VP2 protein farming the core, VP6 protein farm inner layer comprise species specific epitopes and VP4 and VP7 proteins farm the outer layer [1]. Serologically Rotaviruses classified into A to H, among these group A Rotaviruses responsible for severe gastroenteritis in young humans and animals, including diarrhoeal disease in calves. Neonatal calf diarrhoea caused by bovine rotavirus (BRV) has been detected in both dairy and beef cattle herds worldwide [2-3].

Initially Group A rotavirus (GARV) infections were thought to be restricted to the small intestine. However, there is a growing amount of data suggesting that GARV infections in children can cause systemic infections. GARV antigen and RNA was present in the blood of approximately 65% of children with GARV diarrhea [4-5]. Moreover, Rotavirus RNA and proteins have been detected in extra-intestinal tissues such as the liver, heart, lungs, spleen, kidneys and central nervous system of the infected children. In experimental animal models, it has been clearly demonstrated that rotavirus cause not only gastrointestinal but systemic infections also [6]. Hence, present study designed to study the isolation of rotavirus from diarrhoea sample of calves and pathogenesis in rabbit model.

MATERIALS AND METHODS

Materials: Faecal samples collected from bovine calves suffering from severe diarrhoea in organized and unorganized farms of Uttar Pradesh and transported in ice or 50% glycerol saline. MA104 cell lines received from IVRI, Mukteswar campus, Kumao Hills, Uttarkhand. Dulbecco's modified Eagle's minimum essential media (DMEM) (Sigma Aldrich, USA), fetal bovine serum (FBS) (Gibco, USA), L-glutamine (Amresco,USA), sodium bicarbonate (Amresco,USA), phosphate buffer saline tablets,100ml (Amresco, USA), trypsin 1:250 (Amresco,USA) HEPES buffer sodium salt (Himedia, India pvt Ltd), amphotericin B (HiMedia , India) gentamycin sulphate (HiMedia , India), penicillin G sodium salt (HiMedia , India) were used to grow the cells.

METHODS:

Screening of faecal samples for rotavirus by sandwich ELISA: Samples were screened for the presence of rotavirus (RVs) by commercially available antigen capture sandwich ELISA kit employing monoclonal anti-rotavirus antibodies, by following the manufacturer's instructions with required modifications (Bio-X Diagnostics, Belgium).

Preparation of 10% faecal homogenates: Fecal homogenates (10%, w/v) were prepared in phosphate buffer saline (PBS, pH 7.4), the homogenate then centrifuged at 8000g for 1 minute to remove large particulate matters and fibrous materials. The supernatant was tested for rotavirus by sandwich ELISA, and positive samples stored at – 80° C until used for isolation.

Maintenance of MA104 cells: MA104 cells are maintained in 25 cm^2 cell culture flasks in DMEM with L-glutamine, supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), flasks were incubated at 37° C with 5% CO_2 air-atmosphere. Cells were sub cultured to maintain confluent cell layer as per the standard procedures fallowed earlier [7].

Virus inoculation and propagation: The sample supernatant was filtered through 0.2 µM syringe filters (Nunc, USA). Rotavirus was trypsin activated by adding 200 µl of filtrate to equal volume of DMEM antibiotic solution containing 20µg/ml trypsin and incubated at 37° C for 30 minutes. The growth medium from monolayer was replaced with serum free DMEM 24 hours before inoculation. The serum free media was removed and 200 µl trypsin activated rotavirus suspension was added and incubated at 37° C with atmosphere of 5% CO_2 for 60 minutes with rotation to facilitate adsorption. The inoculum was replaced by DMEM containing 0.04 U/ml trypsin and incubated at 37° C for up to 7 days. Cells were observed for appearance of cytopathic and morphological changes using inverted light microscope. The virus inoculated cell culture were

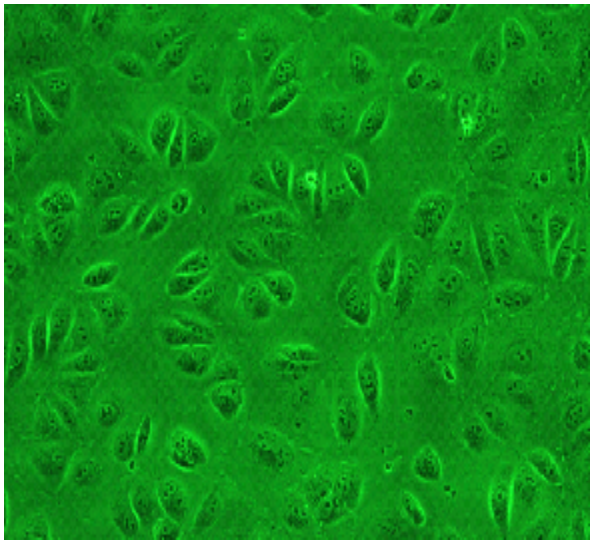

Fig. 1: Normal MA 104 cells, 200X

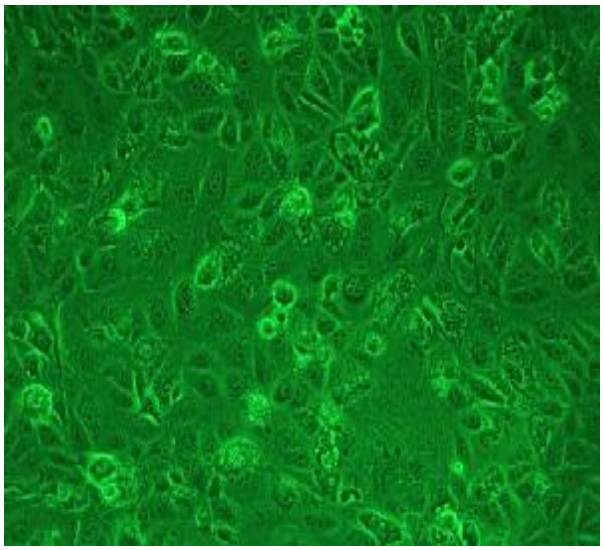

Fig.2: 24 hour p.i, cells showing cytoplasmic vacuolation, hyper granulations, 200X

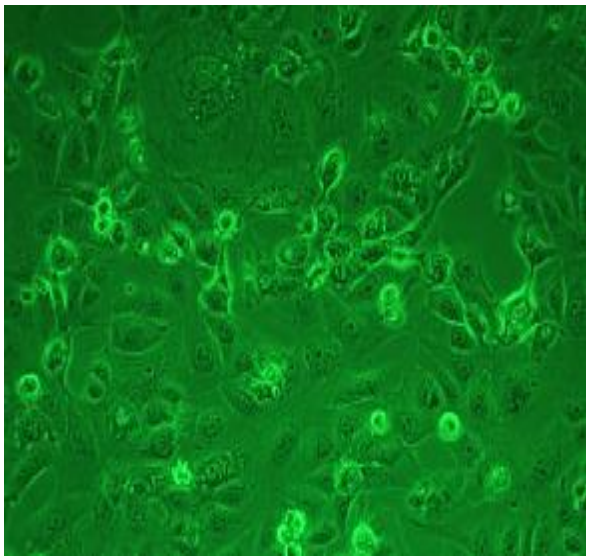

Fig.3: 48 hours p.i., spindle shaped cells, rounding, syncitia formation and detachment from surface, 200X

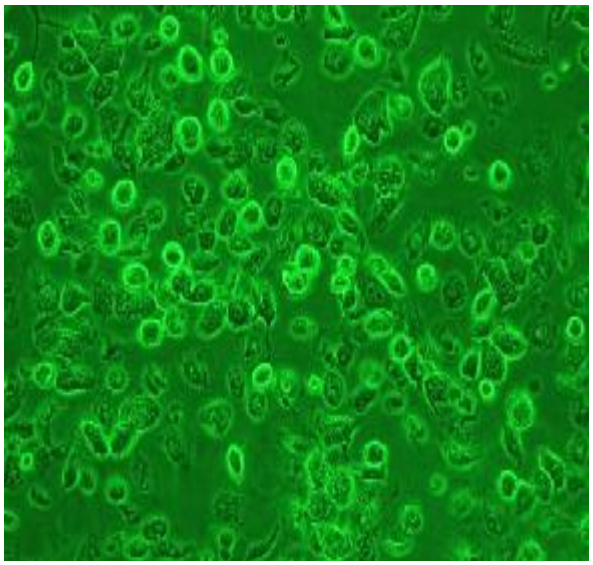

Fig.4: 72 hour p.i., more than 50% of cells rounded and detached from surface, 200X

harvested by using two freeze thaw cycles (-80° C) on appearance of 70% CPE, if not on 7th day. The subsequent virus passages were performed after trypsin activation of the inoculums as detailed above. From the 2nd to 4th passage, cells were incubated for 4-5 days and fifth to 9th passage given in 150 cm^2 flasks and infected with 1ml of activated virus suspension as described above and harvested at stage of 70% CPE by two freeze (-80° C) thaw cycle.

Virus quantification: Virus infectivity was assessed by inoculating four cell culture wells per $\log_{10}$ dilution using standard procedure. Final reading of CPE carried out after an incubation period of 5 days. The infectious titre was expressed 50% tissue culture infective dose (TCID50) per ml according to the method Spearman and Kaerber [8].

Immunofluorescence assay to detect rotavirus in cell culture: The direct immonofluorescence was performed on confluent monolayer of MA104 cells in 24 well cell culture plate after inoculated with 20 µl of trypsin activated virus suspension. The cells were incubated for 24 hours 37° C with 5% CO_2 air-atmosphere, washed with PBS for 5 minutes and fixed with ice cold 80% acetone for 10 minutes. Each well was stained with 1:20 diluted FITC conjugated anti-rotavirus mouse monoclonal antibodies (Bio-X-diagnostics, Belgium) for 1 hour at 21°C±3° C. After washing with PBS cell monolayer overlaid with glycerol and observed under inverted fluorescent microscope (Olympus optical). Infected cell monolayer stained with Haematoxylin and Eosin to demonstrate intracytoplasmic inclusions and syncitia formation as per standard procedure.

Experimental pathogenesis of rotavirus in rabbit model: Thirty, 6 week old New Zealand white rabbits tested negative for rotavirus were used in this study and are procured from the Laboratory Animal Research Facility, IVRI. After acclimatization period of 1week animals were divided in to 3 groups as detailed below and maintained in separate cages.

Group	Inoculum (P/o)	No. of animals	Dose	Sacrifice (PID)/2 animals
GP –I	Field strain of rotavirus	18	$TCID_{50}$	1-5, 7, 12, 14
GP-II	Chloroform inactivated	4	--	3, 7
GP-III	Cell culture media	6	--	3, 7, 14

Group-I animals were administered orally with 1 ml of 10mM sodium bicarbonate solution followed by 4 ml of 5.88 X 10^5 $TCID_{50}$ /ml virus suspension. Similarly, group –II animals were first gave 1 ml of 10mM sodium bicarbonate solution followed by 4 ml of chloroform inactivated virus suspension, while Group-III animals were administered 4ml of cell culture media using catheter. Animals were observed for development of clinical signs like diarrhoea, faecal staining on abdomen, tail and legs, nasal discharge, lacrimation, anorexia and lethargy. Faecal samples collected from rectum using swabs and blood from ear vein daily. At the designated point of sacrifice animals were anaesthetized by intramuscular administration of ketamine and bled by heart puncture. The entire content of the intestine, tissues like small intestine, caecum, colon, mesenteric lymphnodes, liver, kidney, adrenal, urinary bladder, spleen, lung, heart and brain were collected during necropsy for histopathological studies.

Histopathological examination: Buffered formalin fixed tissue sections were subjected to histopathological processing and staining as per the standard procedures. Haematoxylin and Eosin (HE) stained individual sections were microscopically examined and the histological alterations were recorded and digitally photomicrographed (Olympus, BX-41 using ProGres software).

Immunohistochemical staining of tissues: Immunostaining was performed on neutral buffered formalin fixed tissues after antigen retrieval, using monoclonal anti-rotavirus antibodies raised in mouse (Bio-X-diagnostics, Belgium). Sections were incubated with 100-150 µl of 1:20 diluted (diluted in PBS-NaN3 solution) monoclonal primary antibodies overnight at 4° C in humidified chamber, then sections were washed by rinsing in PBS thrice. The sections were incubated with biotinylated Goat Anti-Mouse IgGs (Sigma Aldrich,USA, dilution 1:15) in humidified chamber at room temperature for 30 minutes and sections were washed thrice in PBS. Extravidin peroxidase (Sigma Aldrich, USA) was diluted similar to secondary Ab's and applied sufficiently (100-150 µl) to cover the moist sections and incubated for 30 minutes at room temperature. Sections were stained with 3-Amino-9-ethylcarbazol (AEC) chromogen (Sigma Aldrich, USA) and counter stained with Mayer's haematoxylin. Immunostaining was observ-ed under

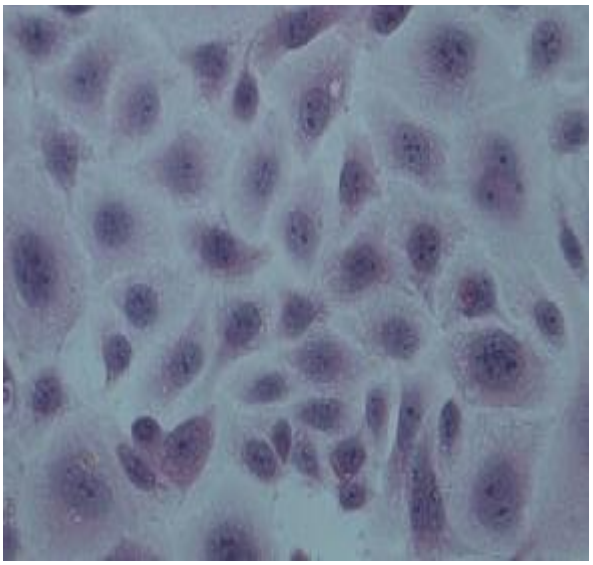

Fig.5: Normal MA 104 cells, HE stain, 400X

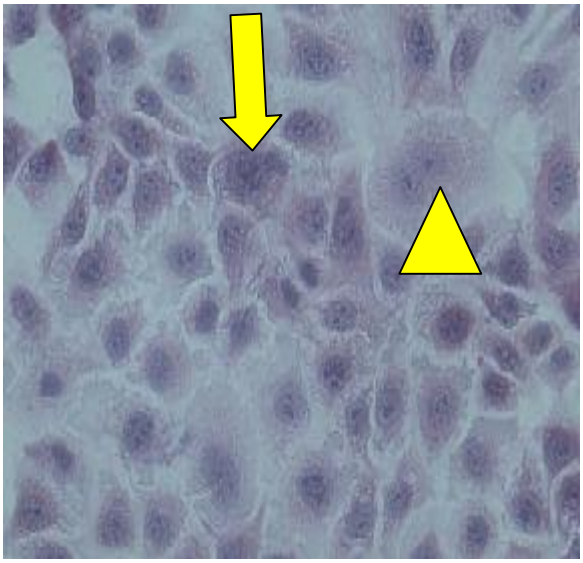

Fig.6: MA 104 cells, syncitial cell formation (arrow head), Intracytoplasmic inclusion bodies (arrow), HE stain, 400X

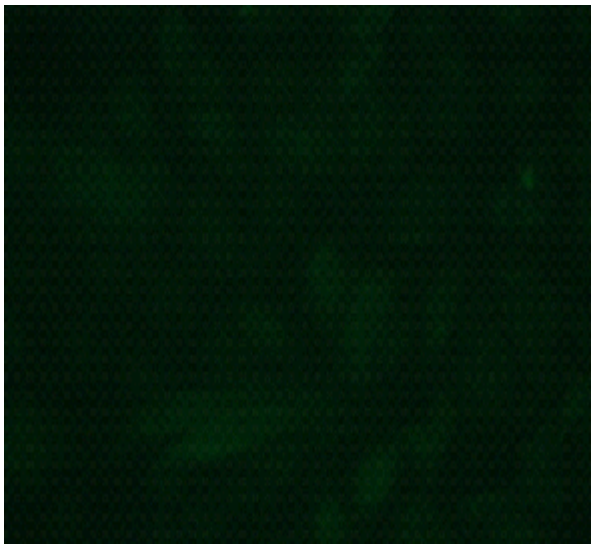

Fig.7: MA 104 cells, direct FAT, Negative control, 400X

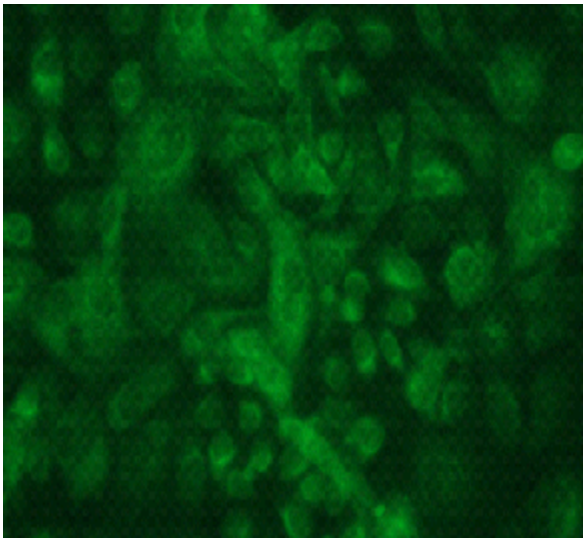

Fig.8: Rotavirus antigen detection in MA104 cells by direct FAT, cytoplasm showing granular apple green fluorescence, 400X

Table.1: Depicting the values of TLC and DLC of experimentally infected rabbits

Parameters	TLC laks/ml			Lymphocyte count laks/ml			% Lymphocyte count			% Neutrophils count		
	Gp-I	GP-II	GP-III	Gp-I	GP-II	GP-III	Gp-I	GP-II	GP-III	Gp-I	GP-II	GP-III
Mean	11.92	6.96	6.74	5.36	2.62	2.773	55.53	39.05	40.20	39.17	53.70	52.97
Sd	1.242	0.620	0.292	0.530	0.232	0.240	3.166	1.456	2.851	4.518	3.081	2.967
SE of mean	0.717	0.358	0.1690	0.306	0.134	0.138	1.828	0.873	1.646	2.609	1.779	1.733

microscope and photomicrographed using olympus microscope fitted with ProGres camera and software.

RESULTS AND DISCUSSION

Neonatal calf diarrhoea is an important clinical condition resulting in higher morbidity and mortality rate, the diarrhoea associated mortality rate estimated to be more than 50% of total calf mortality in dairy farms, which inturn affects the herd health, farm profitability and as a whole the economy of the country. In neonatal calves, the rotavirus infection associated mortality rate may go up to 80%, but majority of reports suggest it is around 5–20% [9]. The present study was conducted to isolate, propagate bovine rotavirus in MA104 cell line and pathogenesis study in rabbit model.

The results of sandwich ELISA confirmed the presence of rotavirus in diarrhoea samples. The clear CPE was appeared on 3rd passage, titer increased substantially in the subsequent passages. In the initial CPE cellular cytoplasm became vacuolated, hyper granular, cells became round or spindle shaped and detached from vessel wall over time and nearly 70% cells detached by 3-4 day depending on the number of passages. The characteristic syncitial cell formation (arrow head), intracytoplasmic inclusion bodies noticed in cell monolayer (Figs. 2-6). The similar pattern of CPE was reported by earlier researchers [7,10]. Direct fluorescent stained infected cells showed characteristic diffuse, granular, apple green fluorescence in cytoplasm (Figs. 7-8). These result as indicating the effective adoption, replication and propagation of bovine rotavirus in MA104 cell line as reported earlier researchers [7,10]. The infectivity of the isolated rotavirus reached the titre to 10^5 /mL within 3-5 days.

Experimental infection of bovine rotavirus to rabbits did not produced clinical signs, and disease. There was a leukocytosis and lymphocytosis in group-I rabbits (Table-1). The histopathological changes were limited to the jejunum of group-I rabbits sacrificed on day 1 and day 2 and they included mild to moderate mucosal congestion and desquamation of villus epithelium. However, the viral antigen could not be demonstrated in intestinal epithelium by immunohistochemical staining (Figs. 9-14). This report suggest that bovine rotavirus strain G6P [11] is non pathogenic to rabbits and it may not be a good laboratory animal model for studying pathogenesis of rotavirus. Previous reports also suggest that human and simian rotavirus did not produce productive infection in rabbits but they elicited immune responses [11].

The probable reasons could be, host range restriction of rotavirus replication as determined by VP7 gene product, strain dependent variation in contribution of gene products of 4, NSP3, NSP2 and NSP4 in pathogenesis were attributed in the ability of rotavirus strain to produce productive infection in homologous and heterologous host species or attenuation of virus strains through tissue culture adaptation [12,13].

CONCLUSION

The outcome of the present study depicts that the Bovine rotavirus was successfully isolated from the faecal sample in MA104 cell line by pretreatment of sample with trypsin and incorporation of trypsin in growth medium. The isolation in MA104 cell line confirmed by FAT technique. The viral antigen was detected in cytoplasm as diffuse, granular, apple green fluorescence. The virus was cytocidal and was characterized by rounding, clumping, syncytia formation and detachment. Experimental infection of bovine rotavirus to rabbits failed to produce clinical signs and disease. This report suggest that bovine rotavirus strain G6P [11] is non pathogenic to rabbits.

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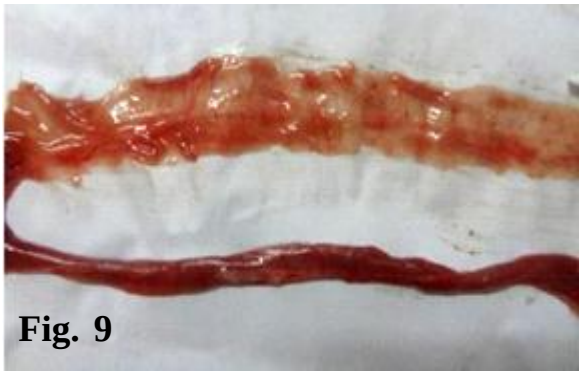

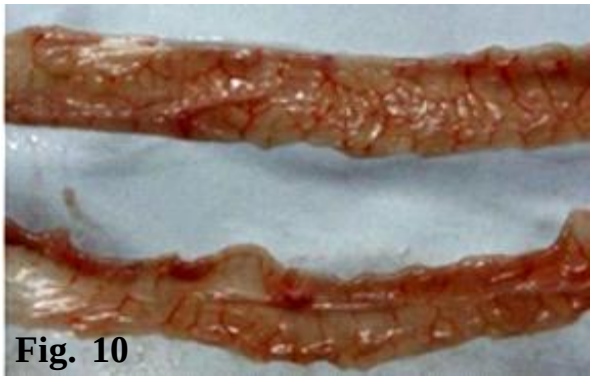

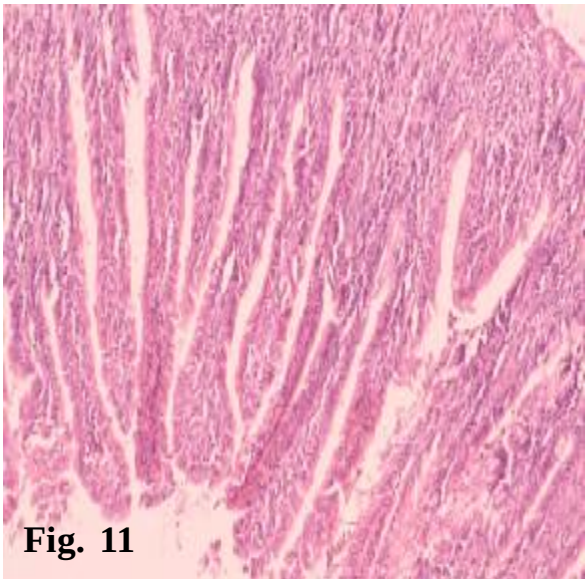

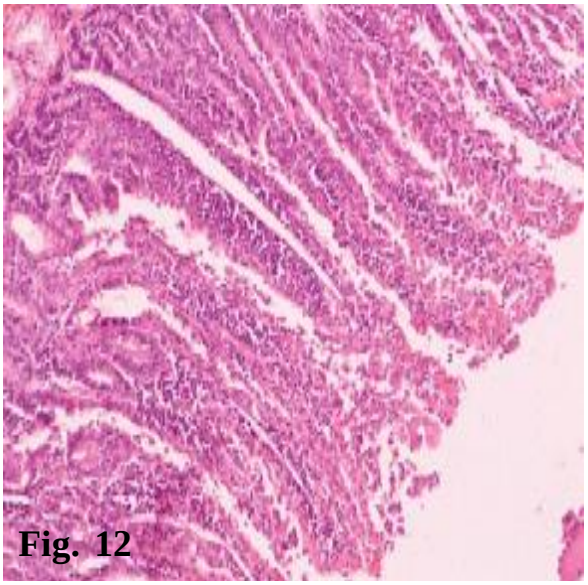

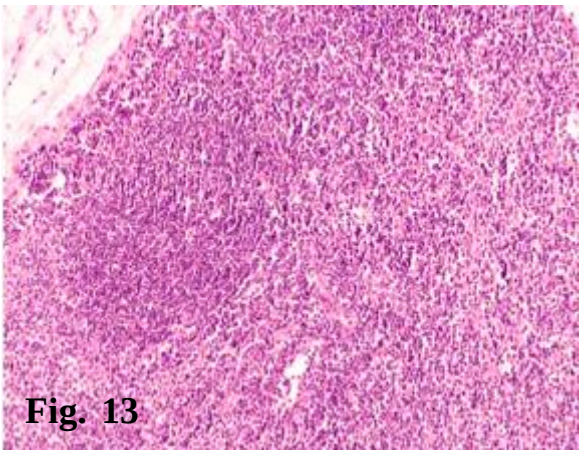

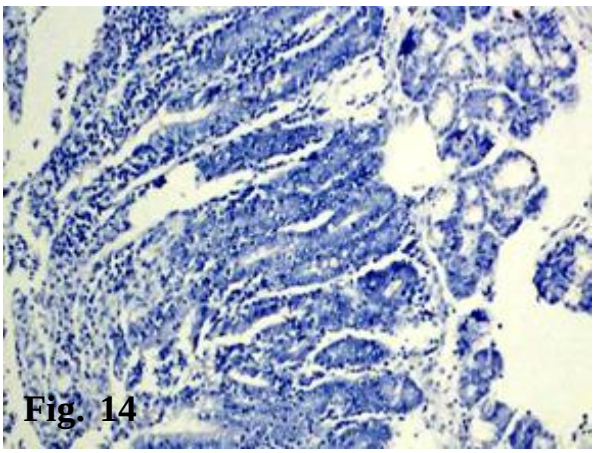

Fig.9: Jejunum of group-I rabbits showing congestion of mucosa and mild oedema

Fig. 10. Jejunum of Gp-III rabbits normal mucosa

Fig.11: Jejunum of Group-III rabbits showing normal intestinal villi, HE stain, 100X

Fig.12: Jejunum of Group-I rabbits showing mild congestion of mucosa and desquamation of villi epithelium, HE stain, 100X

Fig.13: Mesenteric lymph node of Group-I rabbits showing normal cellularity and architecture, HE stain, 100X

Fig.14: Jejunum of grop-I rabbit negative for rotavirus antigen by immunohistochemistry, AEC chromogen, Mayer's haematoxylin counter stain, 200X

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