RESEARCH ARTICLE

Histomorphometric Analysis and Role of Immunoglobulins in Small Intestine of Mice Infected with Heterologous Rotavirus A

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Abstract

Homologous rotavirus (RV) causes acute severe enteritis in mammals, whereas only restricted replication occurs for heterologous RV in the intestine leading to mild enteritis in animals. To understand the role of immunoglubulins and related intestinal morphometry, day-old suckling Swiss albino mice pups (n=45) were randomly divided into two groups. The treatment group of pups (n=30) were orally inoculated with 100 µL of heterologous rotavirus A (RVA) containing 5x $10^{6.5}$ $TCID_{50}$ particles/mL per pup. The control group (n=15) received same volume of sterile phosphate buffered saline. The pups (6 each from treated and 3 from control group) were sacrificed at 1, 2, 4, 7, and 14 days-post infection (DPI). RT-PCR amplification of *VP6* gene in rectal contents revealed 227 bp amplicon products of RVA from 1st to 7th DPI in the infected group. The level of sIgA and IgG immunoglobulins in rectal contents showed significantly decreasing trends from 2nd DPI to 7th DPI in the infected group of mice. The morphometry of jejunum and ileum demonstrated shortening of the villi, relative decrease in the villous height, villous width, and villous: crypt ratio, while gradual increase in the crypt depth was noted from 1st to 14th DPI in the intestine segments. The histomorphometry of small intestine of suckling mouse model and level of immunoglobulins suggest that the heterologous RVA strains are less pathogenic to induce pathology in intestine.

Keywords: Immunoglobulin's, Mice, Morphometry, Rotavirus.

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Introduction

The rotaviruses belonging to the family Reoviridae are important cause of neonatal diarrhoea. These are highly infectious and cause severe enteritis and diarrhoea in homologous species (Cicchese *et al.,* 2018), whereas infections with heterologous RVs in suckling mice do not cause clinical diarrhoea due to the regulatory role of interferon (IFN), which restricts viral replication in the intestinal epithelium up to 10^4 folds (Lin *et al*., 2016). Rotaviruses mainly replicate in the mature villous enterocytes. Triple protein coat of virus helps them to escape unaffected from the acidic pH of the stomach and the digestive enzymes in the gut. The mature enterocytes of duodenum villi are the first to become infected to release significant number of virions, to favour more severe attack on enterocytes of mid and distal portion of small intestine (Ramig, 2004). As the virus multiplication progresses, the mature enterocytes from villus surface is slough off and immature cells of the crypts, proliferate to compensates the loss of enterocytes from villus surface. Due to these sudden changes, imbalance between the absorption and secretion occurs which leads to the accumulation of fluid in the intestinal lumen (Ramig, 2004). Numerous studies have assessed the level of protection against rotavirus infection and illness provided by pre-existing serum antibody titre (Premkumar *et al*., 2014). The immunoglobulin A (IgA) participates in containing and clearing a primary infection and in the prevention of a secondary infection (Kushnir *et al*., 2001). In gnotobiotic piglets and monkeys, the immunoglobulin A (IgA) in the gut and IgG mediate protection against rotavirus diarrhoea (Westerman *et al*., 2005). Therefore rotavirus infection induces both innate and adaptive immune responses (Jiang *et al*., 2002). In this paper, the authors have performed experiments on mice infected with heterologus rotavirus A for the analysis of morphometric changes in small intestine and role of IgA and IgG in infection.

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Materials And Methods

All the experimental procedures on Swiss albino suckling mice were carried out after obtaining approval from the Institute Animal Ethics Committee (IAEC). The management of mice, feeding and watering, anaesthesia and other measures were followed as per the guidelines set forth by the CPCSEA.

Animals

Day-old suckling Swiss albino pups (n=45) along with dams, obtained from recognized Laboratory Animal Resource Section of Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India, were screened for the absence of RVs in its feces by targeting *VP6* gene using RT-PCR before the start of experimental inoculation of heterologous rotavirus A (RVA) of bovine origin. Cell culture adapted RVA with titre of $5\times10^{6.5}$ $TCID_{50}$/mL (courtesy by the Division of Biological Standardization, IVRI) was used in the study for inoculation. The test group of 30 pups was gavaged with 100 µL of RVA on day 5, while the control group of pups (n=15) was gavaged with the same quantity of aseptic phosphate buffered saline (PBS). All the pups of both the groups were kept with their respective dams in separate sterilized polypropylene cages fitted with sippers and provided pelleted feed. The control groups of pups with their dams were housed separately. All the pups were observed daily for any clinical signs of diarrhoea with gentle abdominal palpation.

Sample Collection

Six suckling mice (pups) from the infected group and three suckling pups from the control group were sacrificed on 1st, 2nd, 4th, 7th, and 14th days post-inoculation (DPI). The pups were dissected out and intestines were carefully removed from the visceral organs. The intestines were cut open with sterile scissors and forceps and intestinal contents from the jejunum to rectum were carefully collected in sterile cryovials, without disturbing the lining mucosa. The small pieces of the jejunum and the ileum were collected from designated places in 10% neutral buffered formalin (NBF) for histopathological studies.

Preparation of Intestinal Contents Suspensions

Intestinal contents (10% w/v) were prepared by mixing the contents in sterile 1X PBS (pH 7.2). Supernatants were collected after centrifugation at 8000 x g for 10 min, and stored at -20^{o}C for further use (Kamdi *et al*., 2020).

Screening of RVA

Total RNA was extracted from the faecal contents of mice by Trizol (Sigma, USA) method. The eluted RNase in RNase free water (30 µL) were quantified by using Nanodrop ND-1000 Spectrophotometer (Thermo, USA). RNA samples were subsequently used for cDNA synthesis by using High-Capacity cDNA RT Kit (Invitrogen, USA). The cDNAs were screened for the detection of RVA by RT-PCR using previously described RT-PCR primers targeting *VP6* gene (F-TTTGATCACTAATATTCACC, R- GGTCACATCCTCTC ACTA), and having an amplicon size of 227 base pair (Mondal *et al*., 2013).

Histopathology

The formalin fixed tissues were processed by standard paraffin embedding techniques at the Central Histopathology Laboratory of the Institute. The microtome cut tissue paraffin sections were mounted on grease free glass slides and stained with Haematoxylin and Eosin (H & E) (Luna, 1972).

Morphometry

Morphomery of small intestine were done on H&E stained sections. Villous height (VH), crypt depth (CD) and villous width (VW) were measured in well-oriented sections (10 measurements per region per mouse) using micrometer. All scores were averaged per intestinal segments per animal to calculate the mean scores per segment per time-point (±SEM) (Boshuizen *et al*., 2003).

Preparation of Sample and Quantification sIgA and IgG

The 50 mg rectal tissue with lumen contents was homogenized in PBS (pH 7.2) by using homogenizer. Then homogenate was centrifuged for 20 min at 3000 g and supernatant was removed. Sample was kept at -80° C until use. The sIgA and IgG were quantified in the rectal contents by using mouse immunoglobulin A (IgA) Sandwich ELISA kits (Kinesis DX, LOT# MIA0218). Protocol was followed as per the manufacturer's instructions.

A standard curve was made on a graph paper with standard concentrations taken on the horizontal axis and OD values on the vertical axis, and the concentrations in the samples were found out corresponding to the sample OD value, or calculated using the straight line regression equation of the standard curve with the standard concentration and the OD value in the equation and then calculated the sample concentration. The experimental results were analyzed with the help of SPSS software, wherever necessary.

Results and Discussion

Shedding of Virus and sIgA and IgG in Faeces of Mice

The rectal contents collected during the sacrificed mice showed RVA specific RT-PCR products of 227 bp of *VP6* gene till 7th DPI (Fig. 1) and thereafter the RVA presence in the rectal contents was not found in the infected group of mice. The control group of mice did not yield specific amplification. The level of sIgA in rectal contents showed significantly decreasing trends from 2nd to 7th DPI (0.68±0.17 to 0.43±0.19 µg/mL) in the infected group of mice as compared to that of the control group (Table 1). The level of IgG in rectal contents of mice also showed significantly decreasing trends

from 2nd to 7th DPI (0.40±0.02 to 0.16±0.03 µg/mL) in the infected group of mice (Table 1). The level of sIgA and IgG also decreased on 14th DPI, but was not significantly related.

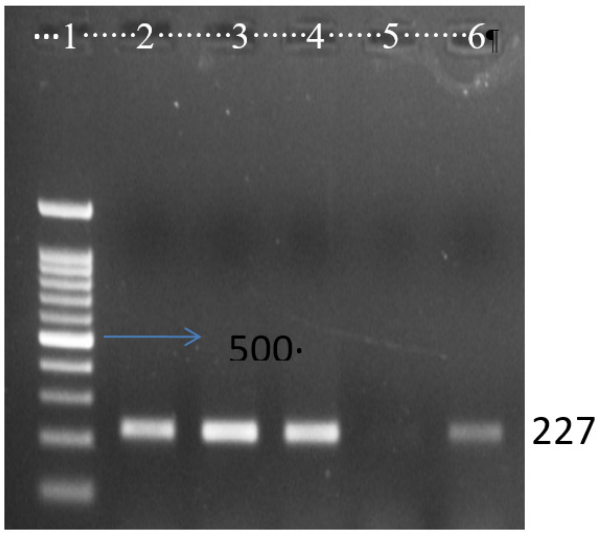


Fig. 1: Ethidium bromide stained 1.5% agrose gel showing amplification of 227 bp for targeted *VP6* gene. Lane I: 1kb plus DNA ladder, Lane 2 (positive sample) at 1 dpi, Lane 3 (positive sample) at 2 dpi, Lane 4 (positive sample) at 4 dpi, Lane 6 (positive sample) at 7 dpi Lane 5: Negative control.

The level of sIgA and IgG in rectal contents showed significantly decreasing trends from 2nd DPI to 7th DPI in the infected group of mice as compared to the control group of mice, which is similar to the results reported by others (Blutt *et al*., 2002) and contradictory to the results of Du *et al.* (2017), who showed sIgA response did not develop up to 7th DPI, after that it was slowly increasing. In preset study we used heterologus virus (bovine strain). In the intestine of the homologous host, homologous rotaviruses (RV) are often more virulent and multiply more effectively than heterologous RV (Feng *et al*., 2013), also the capacity of heterologous and homologous strains to induce secretary IgA differs because, it is dependent on the strain and the inoculating dose of virus (Feng *et al*., 1994). These lower IgA responses may be related to a reduced replication of heterologus virus compared with homologues virus, resulting in a lower amount of antigen presentation in effectors sites. Replication is an important determinant of mucosal IgA responses to rotavirus (Fromantin *et al*., 1998). Although the heterologus strain could replicate to some extent in newborn mice but induces a very weak IgA response as previously reported for bovine strain (Reimerink *et al*., 2007).

Histomorphometry of Small Intestine

Morphometry of the jejunum and the ileum of pups from the infected group revealed significant variations in the VH, VW, CD, and VCR. The VH was significantly lesser in the jejunum on 2nd, 4th, 7th, and 14th DPI as compared to the control group, except on 1st DPI (Table 2). Similarly, the VH in the ileum was lesser on 1st DPI as compared to the control and decreased significantly from 2nd to 14th DPI (Table 2). The CD of the jejunum was more in the infected group, which increased gradually from 1st to 14th DPI as compared to the control. Increase in the CD in the jejunum was significant on 2nd, 4th and 7th DPI. Similarly, the CD of the ileum was also higher in the infected group as compared to the control group, and increased significantly on 2nd, 4th, and 7th DPI. The VCRs of the jejunum were significantly lower on 2nd, 4th, and 7th DPI than those of the control pups (Table 2). Similarly, the VCRs in the ileum were also significantly lower in the infected group on 2nd, 4th, 7th, and 14th DPI as compared to the control. The VWs of the jejunum and the ileum in the infected group were lower than that of the control till 14th DPI, but it differed significantly on 4th and 7th DPI in the jejunum and at 2nd, 4th, and 7th DPI in the ileum.

Histomorphometrically, the jejunum and the ileum revealed a relative decrease in the dimensions of VH, VW and VCR, while that of the CD increased gradually from 1st to 14th DPI. Similar observations have been reported previously in mice (Boshuizen *et al*., 2003), calves and piglets (Kim *et al*., 2011). Shortening of the villi might be associated with infection of myofibroblasts within villi with RV, increase in the smooth muscular contraction and enteric nervous system (Kawagishi *et al*., 2020). Increase in the CD in present study was associated with abundance of immature enterocytes in the villi. The RV infection of the enterocytes is associated with shedding of mature enterocytes from the villi on the apical portion, which induces a negative feedback resulting in enhanced proliferation and upward movement of immature enterocytes in the villi (Boshuizen *et al*., 2003; Feng *et al*., 2008). The deviation in morphometrical indices causes decreased absorption of nutrients from villi, resulting diarrhoea, dehydration and death of animals.

Conclusions

In the present study, the potential role of heterologous RVA in inducing immune-globulins and morphometric changes in suckling Swiss albino mice pups was established. The study demonstrated deviation in morphometrical indices

Table 1: Mean ± SE of IgA and IgG in rectal contents of suckling mice of the control and the infected groups at different time points

Immunoglobulin	Group	1DPI	2 DPI	4 DPI	7 DPI	14DPI
IgA (µg/mL)	Control	1.09±0.26	0.96±0.00	1.17±0.16	1.27±0.21	1.15±0.20
	Infected	0.76±0.11	0.68±0.17	0.90±0.15	0.43±0.19	0.85±0.22
IgG (µg/mL)	Control	0.93±0.03	0.95±0.02	1.05±0.04	0.95±0.02	0.85±0.00
	Infected	0.78±0.02	0.40±0.02	0.67±0.02	0.16±0.03	0.60±0.11

Table 2: Morphometric analysis of Jejunum and Ileum in control group and infected groups of suckling mice at different time intervals post-infection

Measurements	1 dpi			2 dpi			4 dpi			7 dpi			14 dpi		
	Control group	Infected group	P value	Control group	Infected group	P value	Control group	Infected group	P value	Control group	Infected group	P value	Control group	Infected group	P value
Jejunum															
VH (µm)	266.60 ±9.39	256.17 ±5.70	0.355	264.11 ±21.80	214.22 ±4.04	0.037*	261.86 ±19.73	190.51 ±10.39	0.005**	268.39 ±12.20	197.97 ±6.67	0.001**	330.67 ±22.32	248.19 ±10.84	0.004**
CD(µm)	21.97 ±1.38	23.69 ±1.04	0.334	22.27 ±1.28	33.22 ±1.47	0.001**	26.163 ±1.28	38.91 ±3.37	0.002**	28.97 ±1.46	46.723 ±1.65	0.001**	34.93 ±1.04	37.13 ±1.42	0.232
VCR(µm)	12.74 ±1.11	11.02 ±0.57	0.19	12.26 ±1.27	6.58 ±0.35	0.001**	10.08 ±0.70	5.29 ±0.63	0.001**	9.57 ±0.78	4.27 ±0.19	0.001**	9.61 ±0.77	6.75 ±0.35	0.004**
VW(µm)	48.40 ±0.91	45.25 ±0.81	0.018*	47.19 ±1.39	40.05 ±4.69	0.162	52.12 ±2.07	44.19 ±1.95	0.012*	54.06 ±2.39	43.35 ±1.48	0.001**	54.17 ±1.63	50.17 ±1.71	0.109
Ileum															
VH (µm)	241.58 ±10.15	218.67 ±6.19	0.07	242.05 ±6.62	181.03 ±18.17	0.005**	236.57 ±14.45	163.76 ±12.36	0.001**	253.33 ±15.14	171.58 ±11.72	0.001**	251.5 ±5.05	204.81 ±12.99	0.004**
CD(µm)	27.59 ±1.32	29.98 ±1.10	0.181	26.21 ±0.93	41.93 ±4.55	0.001**	29.98 ±1.34	45.71 ±2.72	0.001**	29.46 ±2.83	39.08 ±0.85	0.004**	34.2 ±2.59	39.22 ±1.84	0.133
VCR(µm)	8.96 ±0.62	7.37 ±0.29	0.032*	9.35 ±0.43	4.55 ±0.52	0.001**	7.98 ±0.52	3.68 ±0.32	0.001**	9.09 ±0.82	4.41 ±0.32	0.001**	7.77 ±0.64	5.31 ±0.39	0.004**
VW(µm)	46.22 ±1.46	45.71 ±1.91	0.834	45.93 ±1.50	31.83 ±1.66	0.001**	43.35 ±1.08	32.73 ±1.30	0.001**	45.10 ±2.80	35.33 ±1.31	0.005**	49.49 ±1.04	48.28 ±3.25	0.73

*p<0.05 significant, **p<0.001 highly significant.

and decreasing trends of sIgA and IgG in rectal contents of the infected group of mice. Further studies are required to investigate complex interplay of inflammatory and immune cells, and the cytokines in causing intestinal pathology during the course of heterologus rotavirus infection.

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