Chapter 6
Assessment of in-vivo protective efficacy of anti-human rotavirus egg yolk antibodies (anti-HRVIgY) against rotavirus induced diarrhea in infant mouse model

6.1. Establishment of human rotavirus induced diarrhea in infant mice

To date mouse is the most extensively used animal species to study various aspects of rotavirus infection. Numerous strains of inbred, outbred, immunologically deficient and genetically altered rotavirus naive mice are commercially available. Infection of mice <2 weeks of age with murine rotaviruses often leads to clinical disease and intestinal pathological changes similar to that observed in human and other animal species (Burn et al., 1995). However, the utility of murine rotaviruses in pathophysiology studies is limited by a number of factors such as fastidious nature of these viruses to grow in cell culture, attenuation during cell culture adaptation and lack of availability of wild type strains in sufficient quantities (Burn et al., 1995; Macartney et al., 2000). Oral inoculation of heterologous rotaviruses of simian, bovine and human origin is capable of causing diarrhea and histological changes in the intestine of infant mice identical to those produced by the murine strains (Offit et al., 1984; Gouvia et al., 1986; Raming 1988; Ijaz et al., 1989). HRV strains of genotype 3 (G3) specificity have been demonstrated to be serotypically similar to murine rotaviruses (Greenberg et al., 1986). Limited studies performed using human rotaviruses have consistently reported clinical disease with morphological changes in the villus epithelium of the small intestine of a suckling mouse model (Gouvia et al., 1986; Bell et al.; 1987). However, quantitative assessment of viral replication in the anatomical regions (duodenum, jejunum and ileum) of the small intestine and its relationship with histological changes throughout the course of infection have not been reported. In the present study, a mouse model was developed to analyze clinical, histopathological and virological features of HRV strain YO induced gastroenteritis.
6.1.1. Materials and methods

6.1.1.1. Virus and cells

The HRV strain YO with G3P[8] specificity was propagated in MA104 cell line in the presence of trypsin as described in the preceded Chapter 4, Section No. 4.1.1.. The infected culture harvest was frozen and thawed twice, clarified by centrifugation at 10,000 rpm for 30 min and then pelleted by centrifugation at 35,000 rpm for 2 hr. EEP titer of the stock was determined by an infectivity assay followed by ELISA as described in Chapter 4, Section No. 4.1.3.

6.1.1.2. Animal inoculation and clinical observations

The animal experiments were approved by Institutional Animal Ethical Committee and Institutional Biosafety Committee of National Institute of Virology, Pune, India. Conventionally bred inbred BALB/c mice were obtained from Animal House Division, NIV, Pune. The mice were tested for the presence of rotavirus antibodies by ELISA and the seronegative animals were selected for the study.

Four to five day old mice (n=23) borne to rotavirus antibody free dams were inoculated orally with 4000 EEP of YO strain of rotavirus suspended in 50 µl 0.01 M PBS, pH-7.4. The animals of the control group (n= 20) were fed with 50 µl of plain PBS. Prior to inoculation, the pups from different litters were randomly distributed among the dams, with seven or eight per dam to ensure maternal care and reduce confounding effect of litter size. The mice were examined individually twice daily for rotavirus induced diarrhea by gentle palpation on the abdomen. The animals were given scores from 0 to 5 based on the expression/ no expression of stool (0-no fecal expression, 1-expression of brown/black formed feces, 2-soft brown feces, 3-soft yellow feces, 4-loose yellow feces, 5-liquid yellow feces) (Takahashi et al., 2002; Pant et al., 2006). A score of ≥2 was considered as diarrhea. The outcome was expressed as percent diarrhea (defined by the number of pups with diarrhea on a daily basis), severity (defined as the sum of diarrhea scores of each pup during the course of the experiment) and duration of diarrhea (defined as time in days with diarrhea). The infected animals were also screened for weight loss during the disease period. The stool samples were collected in 100 µl of virus transport medium (VTM) and stored at -70 °C until used.
6.1.1.3. Collection and processing of tissue samples

Intestinal tissue samples were collected at 24 hr intervals up to 144 hpi (hour post inoculation). At each time point three mouse pups were sacrificed from the control and rotavirus infected groups. The three anatomical parts of small intestine, duodenum, jejunum and ileum were collected (Figure 6.1.1) and processed separately for RNA quantification and histopathology.

![Figure 6.1.1](image1.png)

**Figure 6.1.1 (A) HRV-3 inoculated mice at 72 hpi; (B) Anatomical parts of intestine.**

Intestinal tissues intended for RNA quantification were homogenized and suspended in VTM (2 ml/gm) as described earlier (Crawford et al., 2006). For histopathology, the tissue segments were inflated with 10% neutral buffered formalin, treated with ascending and descending grade of alcohol and embedded in paraffin according to the standard protocol (Luna, 1968) to prepare sections of 3-5 µm thickness. Briefly, the procedure for tissue processing was as follows:

- **Fixation**: 10% neutral buffered formalin (72 hr)
- **Dehydration**: Alcohol 50%, 60%, 70%, 80%, 90%, Absolute I, Absolute II (1 hr each)
- **Clearing**: Acetone I, Acetone II, Xylene I, Xylene II (45 min each)
- **Impregnation**: Paraffin I, Paraffin II (58°C) (45 min each)
- **Blocking**: L mould or Plastic cassettes
- **Microtomy**: Tissue trimming and sectioning (3-5 µm) (Thermo Fischer Scientific, USA)
- **Adhesion**: of tissue sections on slides coated with egg albumin/poly-L lysin (Sigma, USA)
6.1.1.4. Histopathology and immunohistochemistry (IHC)

(i) Histopathology

Principle

Hematoxylin and eosin (H&E) staining is the standard method used in histology. H&E staining gives an overview of the structure of the tissue enabling differentiation of structure being examined as normal, inflamed or degeneratively changed or pathological. Hemalum a complex formed from aluminium ions and oxidized haematoxylin binds to arginine-rich basic nucleoproteins such as histones and stains the nuclei dark violet to blue/black while an alcoholic solution of eosin stains eosinophilic structures (those having affinity to eosin) such as cytoplasm, collagen, erythrocytes and keratin pink to red. The tissue sections are examined under light microscope for histological changes.

Procedure

Sections from different parts of small intestine were stained with haematoxylin and eosin by standard procedure (Luna, 1968). Brief description of the protocol is mentioned below.

- Deparafinization - Xylene I, Xylene II (5 min each)
- Rehydration - Alcohol 100%, 70%, 50% (2 min each)
- Hematoxylin (25 min)
- Acid alcohol dip (single)
- Blueing in running tap water (30 min)
- Eosin (15- 30 min)
- Dehydration – Alcohol 20%, 50%, 70%, Absolute (single dip each)
- Air drying
- Clearance - Xylene (10 min)
- Mounting in DPX and examination under light microscope (Olympus, USA)

(ii) IHC

Principle

IHC refers to the process of localizing antigens (eg. proteins) in cells of a tissue section exploiting the principle of antibody binding specifically to an antigen in biological tissue. Rotavirus antigens present in the intestinal tissue sections
bind to primary anti-rotavirus antibodies. The antigen-antibody binding is visualized by a secondary antibody conjugated to an enzyme, such as peroxidase, that catalyses a color-producing reaction on a substrate like diaminobenzidine tetrahydrochloride (DAB).

**Procedure**

For immunohistochemical demonstration of rotavirus antigen in the tissue sections of intestine of rotavirus infected mice, the assay was performed using polyclonal anti-rotavirus rabbit antibody at 1:100 dilution, with a Vector horse anti-rabbit secondary antibody (Avidin and Biotinylated horseradish peroxidase [HRP] Complex), the Vector ABC Elite label diaminobenzidine (DAB) as the chromogen (VECTASTAIN Elite ABC system, Vector Laboratories, USA), and hematoxylin as the counterstain. The stepwise description of the protocol is as follows:

- **Deparafinization - Xylene I, Xylene II (5 min each)**
- **Rehydration - Alcohol 100%, 70%, 40%, 20% (2 min each)**
- **Ag retrieval - Boiling Ag retrieval buffer supplied in the kit (15-20 min in pressure cooker or microwave)**
  - (0.01 M PBS pH 7.4 wash, x2)
- **Peroxidase blocking - 5% H$_2$O$_2$ (1/2 hr) (PBS wash, x2)**
- **Protein blocking – 3% SMP (1/2 hr) (PBS wash, x2)**
- **Primary Ab – Anti-rotavirus rabbit Ab (1:100) (1 hr) (PBS wash, x2)**
- **Secondary Ab - anti-rabbit Ab conjugated to HRP (1 hr) (PBS wash, x2)**
- **Post secondary enhancer (1/2 hr) (PBS wash, x2)**
- **Substrate - DAB (2-3 min) (PBS wash, x2)**
- **Counter staining - Hematoxylin (5 min)**
- **Blueing in running tap water (10 min)**
- **Dehydration - Alcohol 20%, 40%, 70%, Absolute (2 min each)**
- **Clearance – Xylene (10 min)**
- **Mounting on DPX and examination under light microscope (Olympus, USA)**
6.1.1.5. ELISA

Ten percent suspension of the diarrheic stool samples were prepared in VTM and subjected to an antigen capture ELISA according to the directions of manufacturer (Rotavirus Antigen, Generic Assay, Germany).

Principle

Rotavirus antigens if present in the specimens bind with anti-VP6 antibodies coated on the solid phase of the microplate and react with anti-VP6IgGHRP simultaneously. Unbound components are removed from the solid phase immune complexes by the following wash step. The HRP converts the colorless solution of TMB added to the wells into a blue product. The enzyme reaction is stopped by acidic solution turning the solution from blue to yellow.

Procedure

1. The reagents were brought to the room temperature (RT) (20-25ºC) and mixed gently without causing foams before use.
2. Two drops of conjugate (Kit solution D) were dispensed into the respective wells.
3. Two drops of negative (N) and positive (P) controls and 50 µl samples were dispensed into the identified wells.
4. The plate was sealed and incubated at RT for 60 min.
5. The plate was washed (x5) using wash solution (Kit solution B).
6. Two drops of substrate (Kit solution E) were added to each well and incubated at RT for 10 min in dark.
7. Two drops of Kit stop solution (F) were added to each well and mixed well gently.
8. The OD values of the wells at 450 nm were calculated by an automated ELISA reader (BioTek Instruments Inc., USA).
9. The CO value was determined as OD of negative control + 0.2 OD units. The test sample showing $OD_{450}$ value > CO was considered positive and $OD_{450}$ value $\leq$ CO was negative.
6.1.1.6. Real time PCR

**Principle**

Real time PCR is based on the detection of the fluorescence produced by a reporter molecule, the concentration of which increases with each cycle of amplification as the reaction proceeds. These fluorescent reporter molecules constitute dyes (i.e. SYBR® Green) that bind to the double-stranded DNA or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates monitoring of the progress of reaction and quantification of the end product that contains amplified DNA.

**Procedure**

The mouse stool and intestinal tissue samples were extracted with TRIzol®LS and TRIzol® respectively (Invitrogen Life Technologies, USA) as per the manufacturer’s protocol for isolation of rotavirus RNA. Complementary DNA (cDNA) was prepared from 10 µl RNA by reverse transcription using M-MuLV reverse transcriptase (Roche, USA) in the presence of VP6 gene specific primers-VP6F-5’GACGGVGCRACATCATG3’ and VP6R 5’GTCCAATTCATNCCTGGTG3’ (where R=A or G; V=A, C or G; N= A,T, C or G). The resulting cDNA was used as template for real time PCR. Quantitect SYBR green PCR mastermix kit (Qiagen, USA) was used for real time PCR quantification of rotavirus VP6 RNA as described earlier (Kang et al. 2004). Eight serial dilutions of the plasmid containing $10^8$ to $10^1$ copies of rotavirus VP6 gene fragment were included in each reaction to serve as positive controls and to construct the standard curve as well as to quantitate the rotavirus in the experimental specimens. The optimal viral load cut off that can be associated with infectious intestinal disease in mice was equivalent to the detection limit of ELISA as described earlier (Phillip et al, 2009).

6.1.1.7. In-vitro neutralization assay

To detect the anti-rotavirus NAbs in the infected animals, the sera samples collected from the infected mice at 10 and 20 days post inoculation were tested by the MA104 cell monolayer based in-vitro neutralization assay combined with a rotavirus antigen detection ELISA. The assay included constant virus concentration (100 EEP) with varying serum dilutions. The procedure for the assay and estimation of neutralization by the test serum samples were performed as described in Section 5.1 of Chapter 5.
6.1.2. Results

6.1.2.1. Clinical status of the mice inoculated with rotavirus

Mice inoculated with rotavirus developed diarrhea over a period of 5 days (Figure 6.1.2 and 6.1.3). The onset of diarrhea occurred as early as 24 hpi and the highest percent diarrhea (95%) was observed at 72 hpi with increased severity of infection (mean diarrhea score 4.4). Age matched PBS inoculated control mice did not develop diarrhea at any time point. The duration of diarrhea ranged from 2 to 4 days with mean duration of 2.8 days. The attack rate of diarrheal illness among the infected animals was 100%. The animals were lethargic and had abdominal bloating with unhealthy body coats indicating dehydration. Weight loss in the infected animals during the peak disease period was minimal (p > 0.01, p = 0.144), however, the weight gain was significantly reduced as compared to those inoculated with PBS (p < 0.01) (Student t-test). Once diarrhea ceased by 144 hpi, the normal growth rate of the infected animals was resumed and became similar to that of PBS inoculated group. None of the animals died following infection.

Figure 6.1.2 (A) HRV-3 inoculated mouse presenting with diarrhea and (B) PBS inoculated mouse (control).
6.1.2.2. Pathological changes in different parts of the small intestine

There were no marked gross pathological lesions except ballooning of the intestines of infected mice (Figure 6.1.4A). The histopathological changes in the small intestine of rotavirus infected mice appeared at 24 hpi and persisted through 144 hpi. The major changes included i) hydropic degeneration of the distal one third of the villi characterized by swelling of enterocytes with accumulation of large vacuoles in the cytoplasm causing displacement of nuclei and loss of polarity; and ii) diffuse necrosis of the villi tips resulting in architectural loss and villus blunting/shortening (Figure 6.1.4B). Morphologically the affected villi became spatulated with swollen tips, tapering bases and infiltration of inflammatory cells in the villus stroma. In addition, the intestinal lumen contained mucus, while there was edematous deposition in the submucosa along with hemorrhages in a few sections. A few infected animals exhibited reduced cell density in peyer’s patches at 72 hpi as compared to PBS inoculated control.
animals. The histological changes were most pronounced in the villi epithelium of terminal jejunum and ileum of small intestine. Pathologically, the extent of damage of intestinal epithelium at various time points was graded as mild (25-50%), moderate (50-75%) and minimal (<25%).

Figure 6.1.4 (A) Photograph of mouse intestine, (I) uninfected control and (II) infected with HRV-3 indicating ballooning appearance of the gut loops. (B) Photomicrograph of H & E stained sections of mouse ileal villi, (I) uninfected control and (II) infected with HRV-3, arrows indicate different histological changes such as vacuolar degeneration and necrosis (→) of enterocytes, shedding of villus tip (→), villus shortening (→), spatulated shape of villus (→), inflammatory cells in the villus stroma (→), mucus in the lumen (→). Original magnification 20X and 10X.
Accordingly, histological changes were i) minimal to mild at 24-72 hpi and minimal to no abnormality detected (NAD) at 96 -144 hpi in duodenum; ii) mild to moderate at 24-72 hpi and mild to minimal to NAD at 96 to 144 hpi in jejunum; iii) mild to moderate at 24-96 hpi and mild to minimal at 120-144 hpi in ileum (Table 6.1.1). Intensity of the pathological changes reduced 96 hr onwards with decreased enterocyte vacuolation, clear lumen and increased vasculature in the villi. The regeneration of the mucosa was obvious at the villi bases with appearance of clumps of epithelial cells and the process was well advanced by 120 hpi and virtually completed by 144 hpi in the proximal and mid small intestine leaving minimal focal lesions in the ileum. Such observations were not made in the control mice at any time point wherein villi were well preserved with variable length and fingerlike shape histologically (Figure 6.1.4B). Enterocytes were polarized with localized nuclei at the base.

**Table 6.1.1: Extent of damage of intestinal epithelium in relation to vacuolation of enterocytes and loss of villi architecture**

<table>
<thead>
<tr>
<th>Parts of small intestine</th>
<th>Hours post inoculation (hpi)</th>
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<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Duodenum</td>
<td>+ to ++</td>
</tr>
<tr>
<td>Jejunum</td>
<td>++</td>
</tr>
<tr>
<td>Ileum</td>
<td>+++</td>
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Pathological grade: + (<25%) - Minimal  
++ (25-50%) - Mild  
+++ (50-75%) - Moderate  
NAD – No abnormality detected

**6.1.2.3. Viral shedding in diarrheic stool**

The highest shedding of rotaviral antigen and RNA in diarrheic stools that was noted at 24 hpi persisted up to 120 hpi with gradual reduction. (Figure 6.1.5).
Figure 6.1.5 Shedding of rotavirus antigens/RNA in the diarrheic stool expressed in the 4-5 day old mice inoculated with HRV-3.

6.1.2.4. Distribution of rotaviral RNA in the small intestine

Rotaviral RNA was detected in all three parts, duodenum, jejunum and ileum of the small intestine at 24 hpi, while it could be detected continuously only in the ileum up to 120 hpi with maximum viral RNA load at 48 hpi (Figure 6.1.6). However, the viral load in any part of the intestine did not exceed the load in the inoculum. Rotavirus RNA was not detected in any segment of the small intestine of control mice.
Figure 6.1.6 Distribution and persistence of rotavirus RNA in different parts of small intestine in HRV-3 inoculated mice. Each bar represents mean Ct value for six animals.

6.1.2.5. Immunolocalization of rotavirus antigens in the ileum

Based on the comparative analyses of histopathology and rotaviral RNA distribution in different parts of the small intestine of human rotavirus infected mice, ileum was found to be the most affected part, and hence, immunohistochemistry staining was performed on the tissue sections of ileum collected at 72 hpi. The localization of rotavirus antigens was demonstrated in both enterocytes and crypt cells of the infected villi of ileum. The ileum of control mice showed lack of peroxidase staining indicating absence of rotavirus antigen (Figure 6.1.7).
Figure 6.1.7 Photomicrograph of immunoperoxidase stained sections of mouse ileum, (A) uninfected control, (B) infected with HRV-3 indicating the presence of rotavirus antigens (→) at 72 hpi. Original magnification 20X.

6.1.2.6. Detection and titration of anti-rotavirus neutralizing antibodies in the infected mice

All mice (n = 23) inoculated with rotavirus showed seropositivity to NAbs. The titers ranged from 1:200 to 1:400 at 10 day post infection and increased up to 1:1600 to 1:3200 after 20 day post infection while the uninfected control animals remained free of rotavirus antibody.

6.1.3. Discussion

This part of the present study reports characterization of human rotavirus strain, YO induced gastroenteritis in a mouse model. In heterologous rotavirus infections, the initial load of viral inoculum, age of the animals and host genetics are of particular importance for induction of viral replication, intestinal damage and diarrhea (Feng et al., 1994; Ciarlet et al., 2000; Blutt et al., 2006). In view of this, 4/5 day old rotavirus antibody free BALB/c mice and a higher dose of viral inoculum were employed in the study. The high susceptibility to oral infection with HRV-3 YO strain with an attack rate of 100% was evidenced by acute enteric disease manifesting diarrhea, dehydration and transient growth retardation in the
pups. Diarrhea prevailed over a period of 120 hpi, starting after 24 h, mounting to peak at 72 hr followed by decreased severity and final resolution of the symptoms in the succeeding period. The reduced weight gain at the peak phase of clinical disease was rapidly overcome by the animals once the diarrhea ended. These findings are similar to those described as self limiting for natural rotavirus infections in human and experimental infections in different species of animals (Shephered et al., 1975; Snodgrass et al., 1977; Theil et al., 1978; Coehlo et al., 1981; Maki et al., 1981; Gouvia et al., 1986; Kovacs et al., 1987).

The histopathological changes observed in the small intestine of mice examined in the present study appeared mild to moderate following human rotavirus strain, YO infection. Consistent with the findings reported previously (Gouvia et al., 1986; Starkey et al., 1986; Bell et al.; 1987; Osborne et al., 1988; Boshuizen et al., 2003), vacuolar degeneration of the enterocytes leading to necrosis of villi’s tips and blunting of villi were observed as the most conspicuous and constant pathological features in the small intestine during the course of infection in mice. Necrosis, proposed as one of the causes of enterocyte death and blunting of villi in rotavirus infection have been demonstrated possibly to play a role in intestinal malabsorption causing diarrhea and weight loss (Katyal et al., 1999; Ciarlet et al., 2002; Castilho et al., 2004).

In addition to vacuolation of enterocytes and villus blunting, there were number of other pathological changes such as the constriction of villus bases with swollen tips, mucus in the intestinal lumen, edema and occasional hemorrhages in the submucosa, inflammatory infiltration in the villus stroma and depletion of cell density in peyer’s patches that have not been related with enterocyte infection so far. The systemic reactions that are induced by alterations in the intestinal microenvironment due to rotavirus infection might account for these changes. Snodgrass et al. (1977) have described swollen and spatulated appearance of villi together with plugging of submucosal capillaries by neutrophils in lambs following rotavirus infection. Similar evidence favoring such systemic responses has been also provided in localized calicivirus infection in calves (Hall et al., 1984).

It is to be noted that most of the injured villi gradually returned to normal 96 hpi onwards, with reduction in vacuolization, disappearance of systemic reactions and increased circulation of blood in villi epithelium. Regeneration of the intestinal mucosa was nearly complete by 144 hpi when diarrhea was also
not detected in the infected animals. Thus, the recovery from rotavirus disease occurred in parallel with regeneration of damaged intestinal mucosa. These observations corroborate earlier reports describing marked but reversible structural changes in natural and experimental rotavirus infections (Davidson and Barnes 1979; Kovacs et al., 1987; Osbourne et al., 1988; Salim et al., 1995).

The relationship between histopathology and the presence or extent of diarrhea has been described occasionally. Studies performed on intestinal biopsies from children with rotavirus gastroenteritis have shown least changes in the intestinal histology while those carried out in rotavirus infected rabbits described typical histological changes in the intestine in the absence of diarrhea (Holmes et al., 1975; Ciarlet et al., 1998). In several animal species, profuse diarrhea was reported to occur prior to the detection of histological changes in the intestine (Collins et al., 1989; Mebus 1976; Ball et al., 1996). In the present study, infected animals showed intense intestinal lesions along with marked diarrhea at 48-72 hpi indicating a positive relation between the parameters. Thus, the differences in species under study, virus strains, inoculated dose and other experimental conditions may be important in variable presentation of histological changes in the intestine and its relationship with diarrhea.

Stool shedding of viral antigen/RNA reflects the replication efficacies of the rotavirus strains tested in a particular host species. As evidenced by the lack of virus excretion above input titers, non murine rotaviruses have been shown to undergo limited cycles of replication in mice (Offit et al., 1984; Raming 1988; Feng et al., 1994). In the present study, the viral load in the stool and intestinal tissues of mice did not exceed the inoculated dose of virus. However, a rate of rotavirus replication appeared to be maintained to enable the virus/its RNA shedding in the stool and persistence in the intestinal tissues during the clinical disease period i.e. from 24 through 120 hpi. Thus, the viral replication in the intestine coincided with the onset of diarrhea and its perpetuation. This observation is in agreement with an earlier study reporting shedding of human rotavirus antigen and infectious virus for 5-8 days in 5 day old rats (Ciarlet et al., 2002), however, was in contrast with detection of human rotavirus strain (MET) of G3 specificity only up to 2 days post inoculation in whole intestinal tissue of the 7 day old Swiss mice (Gouvia et al., 1986). The discrepancy between the findings may be related to differential replication efficiencies of rotavirus strains in different hosts utilized in the studies. It may also be noted that rotavirus replication in the
present study was supported by immunolocalization of virus specific antigens in the infected villi epithelium at 72 hpi and elevated anti-rotavirus NAb response.

Various parts of the small intestine have been described to possess differential susceptibility to rotavirus infection (Heyman et al., 1987; Kubelka et al., 1994; Hall et al., 1993; Varshney et al., 1995). This phenomenon has been reported to depend on host species and rotavirus strains (Kanwar et al., 1994; Danan et al., 1998; Estes and Atmar, 2003). The histopathological and quantitative PCR analyses of small intestine carried out in the present study affirm that rotavirus infects all of the three parts of the small intestine. The intensity of the histological lesions and viral RNA load in different parts of small intestine varied during the course of infection, however, preferential colonization of both was evident in the ileum. Similar observations have been reported in gnotobiotic dogs, pigs, mice and rats infected with homologous or heterologous rotaviruses (Medina and Underdahl 1980; Johnson et al., 1986; Raming 1988; Ciarlet et al.; 2002; Kordasti et al., 2006). The higher susceptibility of the ileum to rotavirus infection could be due to the higher concentration of rotavirus specific receptors and longer exposure time to infection.

Overall, the results presented in this study illustrated the clinical, pathological and virological events of human rotavirus strain, YO induced gastroenteritis in infant BALB/c mice. The time course of clinical disease paralleled the viral replication and histopathological lesions in the small intestine. The ileum appeared to be the most supportive part of small intestine for perpetuation of rotavirus infection in mice. This in-vivo model may be useful to study different aspects of rotavirus infection including evaluation of attenuation of candidate vaccines of G3 origin and antivirals.
6.2. Effect of pre and post infection administration of anti-human rotavirus egg yolk antibodies (anti-HRV IgY) on rotavirus induced diarrhea in mice

6.2.1. Materials and methods

6.2.1.1. Anti-HRV-3 IgY treatment of mice infected with rotavirus

All animal experiments were approved by Institutional Animal Ethical Committee and Institutional Biosafety Committee of National Institute of Virology, Pune, India. A previously characterized infant BALB/c mouse model of human rotavirus infection as described in Section 6.1 was used to evaluate pre and post exposure treatment efficacy of anti-HRV-3 IgY.

The infections were induced orally in 4/5 day old mice using 4000 EEP of HRV-3 (strain YO), a dose that proved to cause 100% attack rate manifesting diarrhea and intestinal histopathological changes in the of inoculated mice (Buragohain et al., 2011). Post exposure treatment was commenced at 12 hr post infection via oral route with 50 µl anti-HRV-3 IgY per animal that continued as twice daily till day 3 post infection. Three different doses 1.25, 0.625, 0.3125 mg/ml of anti-HRV-3 IgY were tested respectively in n=21, 21 and 20 mice. Two groups of mice, (i) HRV-3 uninoculated and IgY untreated (n=16) and (ii) inoculated with HRV-3 but not treated with IgY (n=23) were included as controls. The animals were examined daily for the signs of diarrhea during the experimental period of 120 hpi and the outcome was measured as prevalence of diarrhea (defined as the percentage of pups with diarrhea on a daily basis), severity (defined as the sum of diarrhea scores of each pup during the course of experiment) and duration of diarrhea (defined as time in days with diarrhea) (Takahashi et al., 2002; Pant et al., 2006). At the end of the course of treatment (96 hpi), six pups from each group including those from the control groups were necropsied and the tissue samples from lower intestines were processed for quantification of viral load and pathological analysis.

For pre exposure treatment, 19 and 20 animals were fed respectively with 50 µl of 250 and 125 µg/ml of anti-HRV-3 IgY per mouse 2 hr prior to oral challenge with 2000 EEP of HRV-3. Two groups of animals, one without inoculation or treatment (n=10) and the other infected with HRV-3 but not treated with anti-HRV-3 IgY (n=18) served as controls. The occurrence of diarrhea in the animals was recorded during the experimental period. Real time PCR and
histopathological analyses were carried out on the intestinal tissues collected from experimental and control mice groups at 72 hpi.

6.2. 1.2. Real time PCR

The principle and protocol of the test were similar as described in Section 6.1.1.6. Detection of <10 copies (Mean Ct ≥30) of rotaviral RNA in the intestine was considered as clearance of the infection.

6.2. 1.3. Pathology

The intestinal tissue segments were inflated with 10% neutral buffered formalin, treated with ascending and descending grade of alcohol and embedded in paraffin to prepare sections of 3-5 µm thickness and stain with haematoxylin and eosin by standard procedure (Luna, 1968). The principle and protocol of tissue processing and staining were as described in the Sections 6.1.1.3. and 6.1.1.4. Histology of tissue sections from mice treated with different modalities were analyzed for histopathological changes associated with rotavirus infection.

6.2. 1.4. Statistical analyses

Comparison of IgY treated versus untreated mice for the occurrence of diarrhea in day wise manner was done by Fisher’s exact test (Fisher 1954). Severity of infection and duration of diarrhea were analyzed by Kruskal-Wallis test (Kruskal and Wallis 1952). Differences in the intestinal viral load as monitored by real-time PCR were assessed using Mann-Whitney test (Wilcoxon, 1945).

6.2. 2. Results

6.2. 2.1. Effect of post infection administration of anti-HRV-3IgY on the course of rotavirus infection in mice

6.2. 2.1.1. Diarrhea

Oral inoculation of 1:4000 EEP of YO strain of HRV-3 in 4/5 day old BALB/c mice typically induced diarrhea at 24 hpi with a disease peak at day 2 to 3. Administration of antiHRV-3IgY in these mice produced a clear dose dependent positive effect on reduction of percentage of diarrhea and severity and duration of disease (Figure 6.2.1, Table 6.2.1). Feeding of the animals twice daily with 50 µl of 1.25 mg/ml of antiHRV-3IgY resulted in the decrease of diarrhea by 58% on day 2 and 81% on day 3 as compared to the percent diarrhea noted in
infected but untreated mice (p<0.001 for day 2 and p<0.0001 for day 3). The beneficial effect was also pronounced in pups receiving 50 µl of 0.625 mg/ml of antiHRV-3IgY with 53% and 62% reduction in diarrhea on day 2 and 3 respectively as against the untreated group (p<0.001 for both). The lower dose, 0.3125 mg/ml imparted low but significant (p<0.05) protection by reducing diarrhea by 26% on day 2 and 35% on day 3 as compared to untreated control group. IgY antibody treatment also shortened the diarrhea duration by 0.78 to 1.36 days and alleviated severity by 23.19 to 48.45% as compared to that noted in the untreated group of mice (Table 6.2.1).

Figure 6.2.1 Percent diarrhea in mice following IgY treatment. Anti-HRV-3IgY caused significant reduction in diarrhea prevalence in treated groups as compared with the untreated group on day 2 (1.25 and 0.625 mg/ml groups, p<0.001; 0.3125 mg/ml group, p<0.05) and 3 (1.25 mg/ml group, p<0.0001; 0.625 mg/ml group, p<0.001 and 0.3125 mg/ml group, p<0.05) (Fischer’s exact test).
Table 6.2.1: Duration and severity of diarrhea in different groups of mice treated with anti-HRV-3IgY.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice with diarrhea (%)</th>
<th>Duration of diarrhea (mean ± SE, days)</th>
<th>Severity score (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated with antiHRV-3IgY</td>
<td>23/23 (100%)</td>
<td>2.78 ± 0.67</td>
<td>3.88 ± 0.74</td>
</tr>
<tr>
<td>Treated with antiHRV-3IgY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25 mg/ml</td>
<td>7/21 (33%)</td>
<td>1.42 ± 0.53**</td>
<td>2.00 ± 0.47**</td>
</tr>
<tr>
<td>0.625 mg/ml</td>
<td>11/21 (52%)</td>
<td>1.75 ± 0.67*</td>
<td>2.62 ± 0.46*</td>
</tr>
<tr>
<td>0.3125 mg/ml</td>
<td>17/20 (85%)</td>
<td>2.00 ± 0.84*</td>
<td>2.98 ± 0.57*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs Untreated group (Kruskal-wallis test)

6.2. 2.1.2. Histopathology of intestine

Formalin fixed intestinal tissue sections from mice treated with different doses of antiHRV-3IgY were analyzed for the major histopathological changes described to be associated with human rotavirus infection (Boshuizen et al., 2003; Buragohain et al., 2011). The intestinal sections of HRV-3 infected mice untreated with IgY presented typical histopathology with vacuolization in enterocytes at the distal one third of villi and blunting of villi at 96 hpi. The tips of the infected villi were unviable due to epithelial cell death. On the other hand, the intestinal sections from the groups of mice treated with different doses of IgY showed reduced vacuolization and cell death at villus tips at 96 hpi (Figure 6.2.2). Treatment with 1.25 mg/ml of IgY prevented marked visible lesions while the lower doses allowed minimal to mild vacuolization in the villi epithelium of intestine. The uninfected negative control mice showed a normal histology.
Figure 6.2.2 Photomicrograph of H & E stained tissue sections of lower intestine from (A) uninfected control mouse; (B) mouse treated with (I) 1.25 mg/ml anti-HRV-3IgY (resolved histopathology to normal), (II) 0.625 mg/ml and (III) 0.3125 mg/ml anti-HRV-3IgY (reduced vacuolization and moderately resolved histopathology); (C) infected mouse but untreated with IgY (massive vacuolar degeneration at the distal part of the villi). Original magnification 20X.
6.2. 2.1. 3. Rotaviral RNA load in intestine and stool

At the end of the course of antiHRV-3IgY treatment, the group of mice treated with 1.25 mg/ml cleared (GMCt ≥30, 31.88) the virus from intestine while in the groups treated with 0.625 and 0.3125 mg/ml, the reduction in the intestinal virus (respectively GMCt 27.87 and 25.62) (p<0.01/p<0.05) was well below the level (GMCt 24) that could produce infectious intestinal disease (Figure 6.2.3). On the other hand, the viral load in the intestines of infected but untreated mice was higher (GMCt 20.2) than all of the IgY treated groups.

Figure 6.2.3 Effect of post rotavirus infection treatment with anti-HRV-3IgY on rotavirus VP6 RNA load in small intestine of mice as determined by real time PCR. Dashed (-----) and dotted (…..) lines indicate the viral load cut off values for induction of infectious intestinal disease and clearance of virus from intestine. *p<0.05, **p<0.01 (Mann-Whitney U test).
Treatment with anti-HRV-3IgY also resulted in diminished shedding of rotaviral RNA in the diarrheic stool by 8.8-21.4% on day 2 (GMCt of 23.2, 21.2 and 20.0 respectively in 1.25 mg/ml, 0.3125 mg/ml and 0.625 mg/ml IgY treated animals) and 14.65-28.03% on day 3 (GMCt of 25.9, 24.1 and 21.9 respectively in 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg/ml IgY treated groups) as compared to infected but untreated control group (GMCt of 18.2 and 18.7 on day 2 and 3 respectively) (p<0.05).

6.2. 2. Effect of preinfection administration of anti-HRV-3IgY in mice

Preinfection anti-HRV-3IgY treatment of the mouse pups provided 95-100% protection from diarrhea in a dose dependent manner. Only 1 of 20 animals from the group treated with 125 µg/ml of anti-HRV-3IgY developed mild diarrhea on day 1 of HRV inoculation which subsided on day 2. All other animals including those from the group treated with 250 µg/ml were free from the signs of diarrhea on day 2 and day 3, the time points that mark peak clinical disease (Figure 6.2.2.A). Histologically the lower intestines appeared normal and comparable to that of uninfected healthy control (Figure 6.2.2.B). The rotaviral RNA in the intestines was undetectable as per set the criterion (RNA<10 copies, GMCt ≥30) at 72 hpi in both 250 µg/ml and 125 µg/ml IgY treated groups of mice (GMCt 31.8 and 30.5 respectively) (Figure 6.2.2.C). In contrast, 39% (7/18) and 50% (9/18) of HRV inoculated but untreated animals developed diarrhea at day 2 and 3 respectively with an overall diarrhea prevalence of 55%, mean severity score 2.7 and duration 2.2 days over the experimental period of 120 hpi. The intestinal tissue sections collected at 72 hpi showed histological lesions specific to rotavirus infection and the presence of rotavirus RNA (GMCt 19.9) (Figure 6.2.2.A, BIV, C).
Figure 6.2.4 Effect of anti-HRV-3 IgY treatment prior to rotavirus infection on: (A) percent diarrhea in mice; intestinal histology from (B-I) 250 µg/ml and (B-II) 125 µg/ml IgY treated mouse (displaying absence of pathological lesions); tissue sections of intestine from (C-I) uninfected control mouse, (C-II) infected but IgY untreated mouse presenting with rotavirus infection associated lesions (original magnification 20X); (D) rotavirus RNA load in the intestines of treated and untreated mice at 72 hpi. Each bar represents mean Ct value for six animals, *p<0.01 (Mann-Whitney U test).
6.2.2.3. Discussion

This part of the study describes evaluation of anti-HRV-3IgY for oral immunotherapy in a mouse model of homologous human rotavirus infection. The mouse model used in the study has been reported to have a course of rotavirus infection of 5 days with highest rate of virus replication within 24 hpi (Buragohain et al., 2011). Hence, the post exposure treatment of the infected animals with homologous (anti-HRV-3IgY) polyclonal antibodies in the present study was initiated at 12 hpi. Protection achieved by passive oral immunotherapy against enteric infections is known to reflect solely the effect of externally delivered immunoglobulins. A high content of neutralizing activity in the immunoglobulin at the mucosal site is expected to control the infection process (Sarkar et al., 2007). In the present study, the dose of IgY for in-vivo testing was decided predominantly on the basis of neutralizing antibody titer determined in the in-vitro assay followed by its protein concentration. The 12 hourly regimen of post infection treatment with anti-HRV-3IgY antibodies successfully blunted the disease by reducing viral load, normalizing pathological features and mitigating diarrhea in the mouse model used in the present study. The higher doses produced a stronger effect corroborating the dose dependent response of oral immunoglobulin treatment observed earlier against various enteric diseases in different animal models (Sarkar et al., 2007; Pant et al, 2007).

Since most of the naturally occurring rotavirus infections are caused by low doses of virus (Graham et al., 1987), the mice treated with ant-HRV-3IgY prior to infection were challenged with a low dose of HRV-3 that was found to cause diarrhea in 55% of the animals. The animals did not develop diarrhea and were devoid of rotavirus infection with clear intestinal histology and rotaviral RNA at 72 hpi (Figure 6.2.4) as reported earlier (Ebina et al., 1996).

It is to be noted that though in-vitro neutralization of five major HRV serotypes in cell culture and in-vivo protection against HRV-3 induced gastroenteritis in mice could be monitored efficiently by anti-HRV-3IgY treatment, the in-vivo protective efficacy of the same preparation (anti-HRV-3IgY) against HRV serotypes 1, 2, 4 and 9 could not be assessed in the present study due to the absence of a mouse model of gastroenteritis for these serotypes. The HRV serotypes other than 3 have been reported to replicate inefficiently to produce diarrhea in mice (Bell et al., 1987). Nevertheless, establishment of multiserotypic
in-vivo neutralizing potency of anti-HRV-3IgY would greatly simplify the commercial production of rotavirus specific IgY.

Thus, the post rotavirus exposure administration of anti-HRV-3IgY in mice of the present study did demonstrate the negative effect on virus replication in the gut and intestinal histopathology, thereby influencing the main outcome of disease i.e. severity and duration of diarrhea in the animals. On the other hand pre exposure administration of anti-HRV-3IgY presented an ideal situation, wherein IgY imparted immediate effect implying better protection from the disease. The findings ascertain that oral administration of anti-HRV-IgY decreases morbidity and disease incidences in mice infected with human rotavirus. Such preparations need to be explored as oral immunotherapy in human to reduce the rotavirus disease burden. However, implementation of this approach would require development of a formulation containing anti-HRV IgYs and its clinical trial in different settings of the rotavirus endemic region.