RESEARCH ARTICLE

Experimental Infection of Avian Rotavirus by Using Chicken Embryo

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Abstract

Objective—To determine the infectivity of pigeon rotavirus PO-13 by using chicken embryo as animal model.

Materials and Methods—PO-13 was passaged four times in MA 104 cells before inoculation of eggs. Specific pathogen free (SPF) chicken eggs were obtained from SPF flock which maintained in Nisseiken, Japan. PO-13 was inoculated into seven-day-old commercial embryonated and SPF eggs. SPF eggs were dead with PO-13 but commercial embryonated eggs were not dead. Seven-day-old embryonated SPF eggs were inoculated with 200 µl of serially diluted PO-13 virus (3.1 x 108 FFU/ml) via yolk sac route to evaluate ELD50. 1000FFU (3.1 x 105 FFU/ml) of PO-13 virus was inoculated into SPF eggs to determine growth of virus in embryo. At various time of post inoculation, inoculated eggs were harvested, and samples were collected to evaluate virus titer and histopathological changes in small intestine. Virus titer was determined by a fluorescent focus assay in MA104 cells. Dead embryos were diagnosed by RT-PCR to confirm the presence of virus in all samples.

Results—Commercial eggs and laying hens possessed antibodies against PO-13 but SPF eggs do not possessed. The ELD50 of PO-13 in SPF 7day-old eggs was 8.2 ~ 8.5logFFU/ml. Virus replication in allantoic fluid, yolk sac fluid and embryos increased from four to five days post inoculation, and reached peak at six to seven days post inoculation. Rotavirus genes could be amplified successfully by RT-PCR method. Lamina propria of intestine was infiltrated with neutrophils, lymphocytes and mononuclear cells. Rotavirus antigens were observed as granular fluorescences in the intestinal epithelial cells of embryo. PO-13 could be replicated in SPF embryonated eggs.

Conclusion—Chicken embryo model would be useful as an infective model to study the infectivity, pathogenesis and pathogenicity of rotaviruses.

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Keywords: MA104 cells; SPF eggs; Rotavirus PO-13; RT-PCR

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Introduction

Group A rotaviruses are recognized as a major etiological agent of severe viral gastroenteritis not only infants and young animals of many mammalian species [1], but also in avian species [2]. Turkey and pheasant viruses are the important causes of diarrheal disease and increase of mortality in both poultries in Europe and U.S.A [3]. In Japan, avian rotaviruses also invade in Japanese chicken flocks [2]. The use of animal models of rotavirus infection has provided key insights in our understanding of the pathogenesis of both human and animal rotaviruses. Five models in three large (cow, pig, and sheep) and two small laboratory (rabbit and mouse) animal species have been used to define parameters of rotavirus infection [4]. Yolken *et al* [5] showed that a wide range of naturally occurring glycoproteins, including ovalbumins and ovomucoids from chicken and turkey eggs, and mucin derived from bovine submaxillary glands are capable of inhibiting the in vitro replication of rotaviruses and preventing the development of rotavirus gastroenteritis in experimentally infected animals. Minamoto and coworkers [2] made an effort to evaluate distribution of pigeon rotavirus PO-13 by a serological survey in man and animals using haemagglutination inhibition (HI) test. The results of the survey indicated that antibody reacting with the pigeon rotavirus was widespread in human and animal populations in Japan. The bovine rotavirus strain 993/83 was isolated from a calf with diarrhea in Germany and its serological and biochemical properties were very similar to pigeon rotavirus PO-13, which has been isolated from the feces of pigeon in Japan. It has been suggested that interspecies transmission from birds to cattle have occurred in nature [6]. Experimental infection of rotaviruses isolated from chicken (Ch-1) had been reported in conventional and specific-pathogen-free (SPF) turkey poults [7]. But infectivity of pigeon rotavirus in avian is still not clear. In our laboratory, an effort has also been made to prove that avian rotavirus (PO-13) is transmissible to mammalian species [8]. However, the exact transmission of pigeon rotavirus PO-13 infection to chicken is still not obvious. Because of knowing the importance of pathogenicity of avian rotavirus PO-13 infection in poultry, we attempted in this study to examine the infectivity of rotavirus PO-13 by using embryonating chicken eggs.

Materials and Methods

Cells, virus and antibodies

Fetal African green monkey kidney MA 104 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum, tryptose phosphate broth and antibiotics. Group A pigeon rotavirus strain PO-13 which was originally isolated from pigeon in Japan, was passaged four times in MA 104 cells in presence of trypsin (bovine pancreas, Sigma, USA) before inoculation of eggs. To prepare the stock virus PO-13 with a high titer, a culture fluid of MA 104 cells infected with PO-13 was harvested by freezing and thawing 3 times after 3 days inoculation, and then centrifuged at 3000x g for 10min at room temperature. The resulting supernatant was stored at -80°C as stock virus. All antibodies used in this study; anti-PO-13 rabbit serum, fluorescein-conjugated anti-rabbit IgG goat serum

(Miles Lab., USA), biotinylated monoclonal antibody against VP6 of PO-13 (P3-29) and fluorescein-conjugated streptavidin (Zymed, U.S.A) were obtained from our zoonotic disease laboratory [9].Virus titer was determined by a fluorescence focus assay MA 104 cells as described previously [8]. Commercial embryonated eggs used in this experiment were obtained from Goto farm, Japan. Specific pathogen free (SPF) White Leghorn chicken eggs were obtained from SPF flock which maintained in Nisseiken, Japan. Eggs were incubated at 37°C incubator with roller apparatus.

Three 7-day-old embryonated eggs or SPF eggs for each dilution were inoculated 200µl of serially diluted virus with 1ml syringe and 23 gauge needle via the yolk sac route. Inoculated eggs were observed daily for death by candling. Base on the results obtained from a series of virus dilutions, the 50% egg-lethal dose (ELD_{50}) was calculated by the method of Reed and Muench [10]. To compare age resistant of chicken eggs to rotavirus, 200µl of serially diluted virus was inoculated to seven, nine and eleven-day- old SPF eggs by yolk sac route. 1000FFU ($3.1x10^5$ FFU/ml) of PO-13 virus was inoculated to seven-day-old SPF eggs to determine growth of virus in embryo.

Sample collection and histopathological examination

All the dead eggs after inoculation were harvested and samples were collected. Any eggs dead at one day post inoculation were discarded. At various times of post inoculation, two eggs that had been inoculated with 1000FFU of PO-13 and one egg for control were harvested, and samples were collected. The eggs containing embryos were chilled at 4°C overnight before harvesting. The harvested allantoic fluids and yolk sac fluids were centrifuged at 3000xg for 5 minutes at room temperature to remove blood and cells. The supernatant were collected and stored at -80°C until use. One part of embryos were weighed and stored at -80°C for further use. The embryos were homogenized by grinding in a sterile mortar and pestle. The homogenate was diluted 1:5 in a balanced salt solution containing antibiotics and centrifuged for10mins at 750xg at 4°C. The resulting aqueous layers were collected as an inoculum for MA104 cells. Virus titers were determined by the fluorescent focus assay and expressed as FFU per gram. Seven-day-old SPF eggs were inoculated with 1000FFU of PO-13, and their intestines were collected at various times post inoculation. After the intestines had been fixed in 4% buffered paraformaldehyde for 1 day and equilibrated with 30% sucrose in phosphate-buffered saline (PBS), they were embedded with the Tissue-Tek O.C.T compound (Sakura, Japan) and frozen. Cryosections of 10µm in thickness were prepared and were used for indirect fluorescent (IF) staining. Staining was performed as described previously (8).

Reverse transcription and polymerase chain reaction (RT-PCR)

Dead embryos were diagnosed by RT-PCR. Genomic dsRNA was extracted with phenol/chloroform by using Isogen. RT-PCR were performed using two synthetic oligonucleotide primers which correspond to 5' and 3' ends of the known sequence of group A gene 6. Primer RT-11 (CTTACCAATTAGAAATTGGAA) correspond to the 5' end, and primer RT-9 (TTCACAAACTGCAGATTCAA) was complementary to the 3' end of gene6 (Mori *et al*., 2001). The complementary DNA (cDNA) of rotavirus genome was

synthesized by using Ready- To-Go You-Prime First-Strand Beads (Amersham Pharmacia, NJ, USA). The PCR was performed in a TaKaRa PCR Thermal Cycler MP (Takara, Japan) in 25µl reaction mixture. The profiles of first and second PCRs were composed of denaturation for 5min 95°C, followed by 30 cycles of denaturation at 95°C for 50sec, reannealing at 48°C for 30sec and elongation 72°C for 1min 30sec, and a final incubation for 5min at 72°C. The amplified cDNA of approximately 840 base pairs was determined by electerophoresis.

Neutralization test, latex agglutination test and confirmation test by using antibody

Virus neutralization titers against the PO-13 strain in the yolk sac of embryonated eggs and SPF eggs were determined by fluorescent focus neutralization (FFN) test with MA 104 cells as described previously (2). To evaluate distribution PO-13 in commercial laying hens and acquisition of passive immunity from hens to eggs, hens' serums form Goto poultry farm were detected by Latex Agglutination test.. To confirm whether embryos were dead due to PO-13 virus infection, we determined the survival of embryo by using anti-rotavirus antibody. Briefly, anti-PO-14 rabbit serum (:50) and Normal rabbit serum (:50) were inactivated at 56°C for 30min. An equal amounts of anti-PO-13 rabbit serum (:50) and PO-13 virus (100 FFU and 1000FFU) were incubated at 37°C for 90min, prior to inoculate into 7-day-old SPF eggs via yolk sac. Normal rabbit serum (:50) was use as a control.

Results

Pigeon rotavirus PO-13 was inoculated via yolk sac into seven-day-old commercial embryonated chicken eggs and SPF eggs. SPF eggs were dead with PO-13 infection but commercial embryonated eggs were not dead. Commercial embryonated eggs and laying hens possessed antibodies against PO-13 but SPF eggs do not possessed.

Infection of rotavirus in commercial laying eggs: All of inoculated embryo survived throughout observation period and virus was undetectable from any rotavirus –inoculated eggs and control eggs.

Detection of maternal antibody in eggs by FFN test: Antibody against PO-13 rotavirus strains was detected within range of 8 to 32 in titer in the allantoic fluid and yolk sac of all commercial eggs but not detected in those of all SPF eggs.

Detection of antibody against PO-13 rotavirus in commercial laying hens' serum: Latex agglutination titers of 8 or higher were detected in White Leghorn hens' sera. The geometric mean of antibody titer in hens' sera was 11.5 when they were tested at dilutions of 1:8 or higher (**Table1**).

Table 1. Results of Antibody Titer of Goto Hens' Serum Examined by Latex Agglutination Test

No. of samples	Titer								Positive rate	GM[a]
	:<8	:8	:16	32	:64	:128	:256	:512		
83	28	7	17	10	8	5	5	3	55/83(66.3%)	11.5

[a]GM= geometric mean of antibody titer

ELD_{50} value of PO-13 rotavirus in SPF embryonated eggs

Since commercial laying eggs have antibody to rotavirus, the degree of infection of pigeon rotavirus in SPF eggs was determined. The ELD_{50} of PO-13 in SPF seven-day-old eggs was 8.2- 8.5 log FFU/ml. Infections virus was detected from all dead rotavirus-inoculated eggs with 1.0-4.0 log FFU/ml of titers but not form control eggs.

Infection of rotavirus in different age group of SPF eggs

The 7, 9 and 11-day-old eggs were inoculated with 10-fold serially diluted PO-13 virus. The ELD_{50} in 7, 9 and 11-day-old eggs were 7.0, 6.0 and <5.0log FFU/ml respectively. Virus was detected from allantoic fluid, yolk sac fluid and embryo in all dead rotavirus inoculated eggs but not in control eggs. The infectivity of PO-13 virus was gradually decreased in older eggs.

Growth curve of PO-13 in SPF embryonated eggs

To confirm the replication of rotavirus in SPF eggs, the virus titers in yolk sac, allantoic fluid, and embryos of the eggs inoculated with PO-13(1000FFU) were measured. The results are shown in **Figure 1** and **Table 2**. Virus titers of yolk sac were higher than that of embryo and allantoic fluid. Virus replication in allantoic fluid increased gradually from four to five days post inoculation. Dead of embryo were found three to ten days post inoculation. Virus titer in each part increased gradually and reached peak at six to seven days post inoculation. Virus growth gradually declined after seven days post inoculation in yolk sac and allantoic fluid. From six to ten days post inoculation, PO-13 showed constant titers of about 2.0log FFU in embryo.

Table 2. Dead of SPF Embryonated Eggs After Inoculation with 1000FFU of PO-13

	dpi									
	1	2	3	4	5	6	7	8	9	10
Virus-inoculated eggs	0/2*	0/2	1/2	0/2	1/2	2/2	2/2	2/2	1/2	1/2
Control eggs	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1

dpi =day post inoculation

*number of dead/number of inoculated egg

RT-PCR

Rotavirus genes from all samples of dead virus- inoculated eggs were amplified successfully by RT-PCR method, but not from not- dead virus-inoculated samples due to small amount of virus (**Figure 2**).

Gross and histopathological lesions

In all experiments, control eggs remained healthy and exhibited no abnormal gross lesion. At post mortem examination, the infected embryos revealed severe generalized haemorrhagic and stunted

Figure 1. Growth of Virus in Yolk Sac Fluid, Allantoic Fluid and Embryo

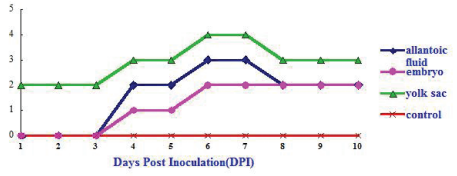
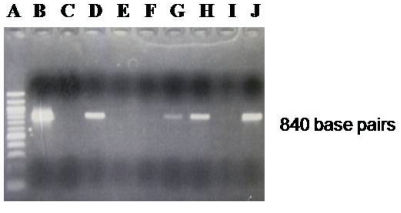


Figure 2. Detection of Rotavirus VP6 Gene from Yolk Sac Fluid



Lane assignments: lane A, marker(100bp); lane B, positive control (stock virus); lane C, distilled water; lanes D, G, H and J, samples from dead eggs; lanes E and F, samples from not dead eggs; lane I, sample from control egg.

growth. The high of infected intestinal villi were shorter than that of control intestinal villi. Histopathological lesions were gradually increased from 7 to 10 days post inoculation (dpi). Lamina propria was infiltrated with neutrophils, lymphocytes and mononuclear cells (**Figure 3**). Rotavirus antigens were observed as granular fluorescences in the intestinal epithelial cells of embryos at 8 dpi. Typical antigen was seen in degenerated epithelial cells of embryos (**Figure 4**).

Protective activity of rotavirus antibody in SPF embryonated eggs

The embryonated SPF eggs were inoculated with 1000FFU of virus pretreated by anti-rotavirus rabbit serum (:8192 neutralization titer) or normal rabbit serum. All normal rabbit serum-inoculated eggs were dead between 3 to 6 days and all anti-PO-13 rabbit serum-inoculated eggs were not dead. Anti-PO-13 serum stopped infectivity of virus when treated with virus before inoculation into the eggs and the embryos survived throughout the observation period.

Discussion

Our results firmly established clinical and virological parameters of group A pigeon rotavirus infection in SPF embryonated eggs by showing that (1) viral replication and disease of rotavirus PO-13 strain occurred in SPF eggs as measured by virus: (2) disease was age dependent: (3) histopathological changes found in the intestine of chicken embryo: (4) commercial embryonated eggs possessed maternal immunity and were resistant to rotavirus infection: (5) anti-rotavirus antibody inhibited to infection and (6) rotavirus infection of eggs resulted in reduced growth of embryo. Analysis of yolk sac, allantoic fluid and embryo homogenates of SPF eggs demonstrated that a complete virus replication occurred in PO-13 infected SPF eggs. The eggs model is being used to define parameters of infection, pathology and disease, therefore, rotavirus infection in older eggs was compared with that of young eggs to warrant use of the egg model for long-term studies. According to the results of virus-inoculated seven, nine and eleven-days-old

Figure 3. Histopathological Lesion in Intestinal Epithelial Cells of Infected Embryo

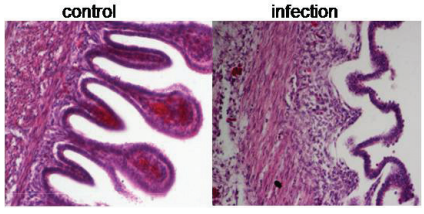
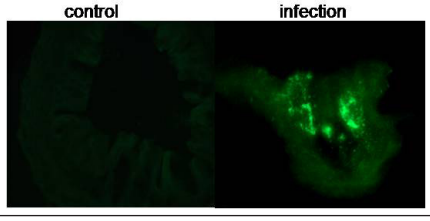


Figure 4. Viral Antigens Observed in the Intestinal Epithelial Cells



SPF eggs, we consented that group A pigeon rotavirus infections were age-dependent as well as mouse model. Even though the small size of the intestinal tract of neonatal mice does not allow pathophysiological study, another study group demonstrated that the intestine of PO-13 inoculated suckling mice showed distinct pathological changes. Our recent work did not provide for vacuolation in intestinal villi. Since we used SPF eggs in this study and others [8] have used mice, it is possible that the different model may differ in their susceptibility to group A rotavirus infection. The histopathological findings in the intestinal tract were in agreement with the clinical observations. The most striking lesion was the hypercellularity of the lamina propria. Cells infiltrating the lamina propria are composed mostly of hetreophils and some eosinophils and mononuclear cells. A similar infiltration was noted by Meulemans *et al* [11] in experimentally avian rotavirus infected chickens.

Minamoto *et al* [2] reported that 51% of chick and 32.4% of pigeon in Japan had antibody against PO-13 strain isolated from pigeon faeces. Our Latex agglutination results reported here agreed that 66.3% of commercial laying hen possessed antibody against VP8 of PO-13 strain. In conclusion, chick embryo model that we described here will be very useful as an infective model to the study of infectivity test of avian rotavirus and mechanism of rotavirus gastrointestinal pathogenesis.

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