Experimental Infection of Equine Rotavirus in Foals

Hiroshi Imagawa,* Ryuichi Wada,* Masanobu Kamada,* Takeshi Kumanomido,* Yoshio Fukunaga,* and Kiyoshi Hirasawa

The HO-5 strain of equine rotavirus (ERV) was isolated from the feces of a foal with acute diarrhea in the horse breeding district in Japan. Two neonatal foals were inoculated orally with it of equine rotavirus to examine its pathogenicity. The establishment of infection after the inoculation was proved by virus recovery from the intestinal contents and the detection of specific immunofluorescent antigen from the intestine. One foal showed obvious diarrhea. From these results, it was ascertained that the HO-5 strain of ERV caused diarrhea in neonatal foals and that it was one of the important etiological agents for foal diarrhea which occurred in the horse breeding district. Moreover, it was revealed that the villous epithelial cells and the cells of villous lamina propria of the jejunum were most important for multiplication of ERV in foals.

Key words. diarrhea, equine rotavirus, foal

Introduction

Rotaviruses are established as major pathogens of acute neonatal diarrhea in many animal species, including man,1,2) Rotavirus has been detected from the feces of foals with diarrhea by electron microscopy in the U.K.,3) the U.S.A.,4-6) Australia7,8) and Ireland.9) Conner and Darlington4) detected it by electron microscopy from 26 (30%) of 86 foal diarrhea fecal samples collected in the central area of Kentucky, U.S.A. They proved that rotavirus was a major cause of diarrhea in foals.

In Japan, serological surveys were conducted by the authors10,11) and Goto et al.12) to reveal that rotavirus infection was widespread among horses in the breeding district and racecourses. Recently, the authors13) made an attempt to isolate rotavirus in cell cultures from fecal samples of foals with various types of diarrhea in the Hidaka district of Hokkaido. They reported that the virus had been isolated only from the feces of foals with acute diarrhea.

Kanitz,6) and Conner and Darlington4) carried out experimental infection of foals with fecal filtrates containing rotavirus from foals with diarrhea, and demonstrated that this had caused acute diarrhea in foals. There are no reports, however, about the experimental infection of equine rotavirus (ERV) isolated from foals in Japan. The objectives of this study are to clarify the pathogenicity of isolated ERV for foals and to know the replication site of ERV in the intestine and other tissues of infected foals.

Materials and Methods

Virus. The virus used in this study was
the HO-5 strain. The strain was isolated in MA-104 (rhesus monkey kidney) cells from the feces of a foal with acute diarrhea in a breeding district of Hokkaido in 1982.

**Experimental animals.** Two healthy neonatal foals, Nos. 1 and 2, were employed, as shown in Table 1. At the time of inoculation, they had neutralizing antibody titers of less than 1:2 and 1:8, respectively. Foal No. 1 was detached from its dam before it drank colostrum, and given sterilized evaporated milk for this study. Foal No. 2 was foaled naturally and allowed to be nursed by its dam. Both foals were necropsied on post inoculation day (PID) 2 and 5, respectively.

**Inoculation.** The 2 foals were given an inoculum containing $10^6$ fluorescent focus-forming units (FFU)/20 ml of virus at the 4th cell passage level by the aid of a stomach tube. Such volume of sterile distilled water as could be taken by each foal at a time was administered immediately after the inoculation. The volume of water administer was 50 ml for Foal No. 1 and 300 ml for Foal No. 2.

**Clinical and hematological examinations.** Clinical response was recorded twice every day. Body temperature was taken rectally. Erythrocyte and total leukocyte counts were determined with the hemacytometer.

**Virus recovery.** Fluorescein-conjugated anti-calf rotavirus (Lincoln strain) serum was prepared in such manner as previously described and used to detect viral antigen. For virus isolation, the fecal and intestinal contents were homogenized to make a 10% suspension in modified Eagle's minimum essential medium (MEM) (Flow Laboratories, U.S.A.). The suspension was filtered through a membrane filter of 0.45 μm in pore diameter. The filtrate was treated with 10 μg/ml of acetyl trypsin (bovine origin, type V-S, Sigma, U.S.A.), as described previously. Serial tenfold dilutions of the treated filtrate was made in MEM. Then 0.2 ml of aliquot was inoculated into culture tubes of MA-104 cells, and the tubes were incubated as described previously. Specific immunofluorescent antigen (IFA) in each culture cell was examined 7 day after inoculation by the method described previously.

**Specific IFA in tissues.** Tissues collected at necropsy were frozen in hexane at $-80^\circ$C and sectioned with a cryostat. The sections were fixed in acetone at $-20^\circ$C for 15 min and stained with fluorescein-conjugated anti-calf rotavirus serum prepared in such manner as previously described.

**Neutralization test.** The neutralization test was conducted with tube method as described in the previous report. The virus used was the HO-5 strain of ERV.

**Bacteriological examination.** Ten kinds of commercial media were used for isolation of bacteria from feces and intestinal con-

### Table 1. History of foals used for experiment

<table>
<thead>
<tr>
<th>Foal No.</th>
<th>Breed</th>
<th>Sex</th>
<th>Age in after birth</th>
<th>Nursing</th>
<th>Neutralizing antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cross-bred</td>
<td>Male</td>
<td>5 hours</td>
<td>Colostrum-deprived</td>
<td>&lt;2</td>
</tr>
<tr>
<td>2</td>
<td>Cross-bred</td>
<td>Female</td>
<td>10 days</td>
<td>Natural</td>
<td>8</td>
</tr>
</tbody>
</table>
Experimental Equine Rotavirus Infection

tents. Identification of bacteria was carried out by the description of Cowan.\textsuperscript{15)}

\section*{Results}

\textit{Clinical and hematological observations}. The results of clinical and hematological observations in experimentally infected 2 foals are shown in Fig. 1. In Foal No. 1, an apparent febrile response (38.9 to 39.8°C) was revealed PID 1 and 2. Marked leukopenia was confirmed as a hematological finding. The foal was necropsied PID 2. In Foal No. 2, a febrile response (38.8 to 39.0°C) was revealed PID 2, 3 and 5. On PID 2, the feces changed into a loose passage. Watery diarrhea was developed PID 3 and continued till the foal was necropsied. The foal became anorexic and depressed. Mild leukopenia was shown. The foal was necropsied PID 5.

\textit{Virus recovery}. The virus recovery from

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Sample} & \textbf{Foal No.} & \\
\hline
Feces (0–2 days)* & -- & -- \\
\text{(3–5 days)} & NT*** & \text{10}^4**** \\
\hline
\text{Digestive tract contents} & & \\
\text{Stomach} & -- & -- \\
\text{Duodenum} & \text{10}^4 & -- \\
\text{Jejunum} & \text{10}^4 & \text{10}^4 \\
\text{Ileum} & \text{10}^4 & \text{10}^4 \\
\text{Cecum} & \text{10}^4 & \text{10}^4 \\
\text{Large colon} & \text{10}^4 & \text{10}^4 \\
\text{Small colon} & -- & \text{10}^4 \\
\hline
\end{tabular}
\caption{Recovery of equine rotavirus from feces and digestive tract contents of foals}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Specimen} & \textbf{Foal No.} & \\
\hline
\text{Stomach} & -- & -- \\
\text{Duodenum} & -- & -- \\
\text{Jejunum} & \# & \# \\
\text{Ileum} & \# & \# \\
\text{Cecum} & + & + \\
\text{Large colon} & -- & -- \\
\text{Small colon} & -- & -- \\
\text{Mesenteric Lymph node} & -- & + \\
\text{Lung} & -- & -- \\
\text{Liver} & -- & -- \\
\text{Kidney} & -- & -- \\
\text{Spleen} & -- & -- \\
\text{Pancreas} & -- & -- \\
\hline
\end{tabular}
\caption{Detection of specific immunofluorescent antigen (IFA) from tissues in foals}
\end{table}

Remarks. *: Time in days after inoculation. **: No virus was recovered. ***: Not tested. ****: Fluorescent focus-forming units/g.

Remarks. *: Killed 2 days after inoculation. **: Killed 5 days after inoculation. --: No specific IFA was detected. +: Specific IFA-positive cell was detected very rarely. #: Small number of specific IFA-positive cells were detected. #: Large number of specific IFA-positive cells were detected.
Fig. 2. Specific immunofluorescent antigen in villous epithelial cells and cells of villous lamina propria of jejunum in Foal No. 2

Fig. 3. Specific immunofluorescent antigen in mesenteric lymph node in Foal No. 2

Table 4. Changes in bacterial count of principal species in feces of Foal No. 2

<table>
<thead>
<tr>
<th>Species or genus</th>
<th>0-2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>$10^8$-$10^{10}$</td>
<td>$10^8$-$10^9$</td>
<td>$10^8$-$10^9$</td>
<td>$10^7$-$10^8$</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>$10^8$-$10^9$</td>
<td>$10^8$-$10^9$</td>
<td>$10^7$-$10^8$</td>
<td>$10^7$-$10^8$</td>
</tr>
<tr>
<td><em>Lactobacillus spp.</em></td>
<td>$10^6$-$10^9$</td>
<td>$10^6$-$10^9$</td>
<td>$10^7$-$10^8$</td>
<td>$10^7$-$10^8$</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>$&lt;10^6$</td>
<td>$&lt;10^6$</td>
<td>$10^5$-$10^8$</td>
<td>$10^7$-$10^8$</td>
</tr>
<tr>
<td><em>Staphylococcus spp.</em></td>
<td>$&lt;10^6$</td>
<td>$&lt;10^6$</td>
<td>$10^4$-$10^7$</td>
<td>$10^7$-$10^8$</td>
</tr>
<tr>
<td>Hemolytic streptococcus</td>
<td>$&lt;10^6$</td>
<td>$&lt;10^6$</td>
<td>$10^4$-$10^9$</td>
<td>$&lt;10^9$</td>
</tr>
<tr>
<td><em>Bacillus spp.</em></td>
<td>$10^7$-$10^8$</td>
<td>$10^7$-$10^8$</td>
<td>$10^4$-$10^7$</td>
<td>$10^6$-$10^7$</td>
</tr>
</tbody>
</table>

Remarks. *: Bacterial count per g or ml

Table 5. Distribution of principal bacterial species in intestinal contents of Foal No. 2

<table>
<thead>
<tr>
<th>Species or genus</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>$&lt;10^8$</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>$&lt;10^8$</td>
</tr>
<tr>
<td><em>Lactobacillus spp.</em></td>
<td>$10^8$-$10^9$</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>$10^8$-$10^9$</td>
</tr>
<tr>
<td><em>Staphylococcus spp.</em></td>
<td>$10^4$-$10^6$</td>
</tr>
<tr>
<td>Hemolytic streptococcus</td>
<td>$&lt;10^8$</td>
</tr>
<tr>
<td><em>Bacillus spp.</em></td>
<td>$10^7$-$10^9$</td>
</tr>
</tbody>
</table>

Remarks. *: Bacterial count per g or ml.
Experimental Equine Rotavirus Infection

feces and intestinal contents of the 2 foals is summarized in Table 2. Virus of $10^4$ FFU/g was recovered from the feces of Foal No. 2 which revealed watery diarrhea PID 3–5. No viruses were recovered from the feces of Foal No. 1 which had suffered from no diarrhea. Virus ranging from $10^2$ to $10^4$ FFU/g was recovered from the intestinal contents of both foals.

**Detection of specific IFA in tissues.** The results of specific IFA detection by immunofluorescence are presented in Table 3. A large number of specific IFA-positive cells were detected in the villous epithelial cells and in the cells of villous lamina propria of the jejunum in Foal No. 2 (Fig. 2). A small number of specific IFA-positive cells were detected in the villous epithelial cells of the jejunum in Foal No. 1, the villous epithelial cells of the ileum in both foals and the mesenteric lymph node (Fig. 3) in Foal No. 2. Specific IFA-positive cells were detected, though very rarely, in the epithelial cells of the cecum in both foals and the small colon in Foal No. 2. No specific IFA-positive cells were detected from any other tissue tested.

**Bacteriological examination.** The counts of bacteria in the feces of Foal No. 2 with acute diarrhea are shown in Table 4. *Streptococcus faecalis*, *Escherichia coli*, *Corynebacterium* spp, and *Bacillus* spp. decreased slightly in count with the manifestation of diarrhea. On the other hand, *Klebsiella oxytoca* and *Staphylococcus* spp. increased in count with the manifestation of diarrhea. Hemolytic streptococci increased temporarily in count with the manifestation of diarrhea. A normal flora did not change in intestinal contents of Foal No. 2, as shown in Table 5.

**Discussion**

Diarrhea in foals has frequently occurred in Hidaka area of the breeding district in Hokkaido over a period from April to August every year. The authors reported that 23 of 173 foal diarrheal fecal samples examined (13%) were positive for rotavirus isolation in the Hidaka area in 1982 and 1983.

In the present study, 2 newborn foals, Nos. 1 and 2, were inoculated orally with the HO-5 strain of ERV isolated from a foal with diarrhea. Foal No. 1 showed a febrile response and Foal No. 2 a febrile response and acute diarrhea. In both foals inoculated, the establishment of infection was proved by virus recovery from intestinal contents and feces and the detection of specific IFA from intestines and lymph nodes. Since Foal No. 1 contracted infection with the virus, it would have been affected with diarrhea, if it had been subjected to necropsy little later. Kanitz reported that a foal inoculated orally with ERV, suffered from severe diarrhea and was unable to rise. In Foal No. 2, diarrhea was not so severe as reported by Kanitz, probably because the foal possessed an neutralizing antibody titer of 1:8 when it was inoculated ERV.

By immunofluorescence in tissues, a large number of specific IFA-positive cells were detected in the villous epithelial cells of the jejunum and in the cells of villous lamina propria of the jejunum, and a small number of specific IFA-positive cells were detected in the villous epithelial cells of the ileum. This result revealed that the villous epithelial cells and the cells of villous lamina propria of the jejunum were most important for...
multiplication of ERV in foals. Kanitz\(^6\) reported that specific IFA was detected only from villous epithelial cells throughout the small intestine in infected foals. In the present study, however, specific IFA was also detected, in the mesenteric lymph node and the epithelial cells of cecum and small colon. This suggested that ERV might have replicated even in tissues, except those of the small intestine, in infected foals.

In the bacterial examination of Foal No. 2, no pathogenic bacteria were detected in feces or intestinal contents, although some species increased or decreased in number in the normal flora with the manifestation of diarrhea. Therefore, it was indicated that no pathogens were associated with diarrhea in the affected foal.

From these results, it was ascertained that the HO-5 strain of ERV caused diarrhea in neonatal foals, and that it was one of the important etiological agents for foal diarrhea which occurred in the horse breeding district.

Acknowledgments

The authors wish to thank Dr. S. Konishi, of the Division of Veterinary Science, Faculty of Agriculture, Tokyo University, for advice and guidance of this study. Thanks are also due to Mr. T. Yamakawa and Mrs. K. Ito, of the authors’ station, for technical assistance.

Literature Cited

子馬における馬ロタウイルスの感染実験：今川 浩*・和田隆一*・鶴田正信*・細塚駿直 慎*・福永昌夫*・平澤 澄*（*日本中央競馬会研究部総合研究部研所事務所）——我が国の生産地において急性下痢を呈した子馬の糞から分離された馬ロタウイルスの病原性を調べるため，2頭の新生子馬に馬ロタウイルス HO-5 株を経口接種した。腸管内容物からのウイルス回収および腸管における特異蛍光抗原の検出によって2頭の接種子馬の感染が証明された。接種子馬のうちの1頭は明瞭な下痢を起こした。このことから，分離馬ロタウイルス HO-5 株が新生子馬に下痢を起こし，生産地で発生している子馬の下痢の重要な原因の一つであることが確かめられた。さらに，空腸の絨毛上皮細胞および絨毛固有層の細網細胞が子馬における馬ロタウイルスの増殖のために最も重要な組織であることが分かった。