Intestinal and extra-intestinal pathogenicity of a bovine reassortant rotavirus in calves and piglets

Hyun-Jeong Kim a, Jun-Gyu Park a, Jelle Matthijnssens b, Ju-Hwan Lee c, You-Chan Bae d, Mia Madel Alfajaro a, Sang-Ik Park a, Mun-II Kang a, Kyoung-Oh Cho a, a

A R T I C L E   I N F O

Article history:
Received 30 November 2010
Received in revised form 20 April 2011
Accepted 11 May 2011

Keywords:
Group A rotaviruses
Reassortant
Cross-species
Pathogenicity
Extra-intestinal spread

A B S T R A C T

Despite the impact of bovine group A rotaviruses (GARVs) as economically important and zoonotic pathogens, there is a scarcity of data on cross-species pathogenicity and extra-intestinal spread of bovine reassortant GARVs. During the course of characterizing the genotypes of all 11 genomic segments of bovine GARVs isolated from diarrheic calves in South Korea, a unique G6P[7] reassortant GARV strain (KJ9-1) was isolated. The strain harbors five bovine-like gene segments (VP7: G6; VP6: I2; VP1: R2; VP3: M2; NSP2: N2, and NSP4: E2), five porcine-like gene segments (VP4: P[7]; NSP1: A1; NSP3: T1, and NSP5: H1), and one human-like gene segment (VP2: C2). To investigate if this reassortant strain possessed cross-species pathogenicity in calves and piglets, and could induce viremia and extra-intestinal spread in calves, colostrum-deprived calves and piglets were experimentally inoculated with the KJ9-1 strain. The KJ9-1 strain caused severe diarrhea in experimentally infected calves with extensive intestinal villous atrophy, but replicated without causing clinical symptoms in experimentally infected piglets. By SYBR Green real-time RT-PCR, viral RNA was detected in sera of the calves at post-inoculation day (PID) 1, reaching a peak at PID3, and then rapidly decreasing from PID4. In addition, viral RNA was detected in the mesenteric lymph node, lungs, liver, choroid plexus, and cerebrospinal fluid. An immunofluorescence assay confirmed viral replication in the extra-intestinal organs and tissues of virus-inoculated calves. The data indicates that the homologous/heterologous origin of the NSP4 gene segment (E2 genotype), may play a key role in the ability to cause diarrhea in calves and piglets. © 2011 Elsevier B.V. Open access under CC BY-NC-ND license.

1. Introduction

Group A rotavirus (GARV), a member of the family Reoviridae, is one of the major pathogens that cause severe, acute dehydrating diarrhea in young children and in a wide variety of animals (Estes and Kapikian, 2007; Martella et al., 2010). The GARV genome consists of 11 segments of double-stranded RNA enclosed in a trilaminar capsid and encodes six structural viral protein (VP1–4, VP6, VP7) and six non-structural proteins (NSP1–6) (Estes and Kapikian, 2007). This segmented nature of the GARV genome facilitates genetic reassortment during mixed infections, leading to progeny viruses with novel or atypical phenotypes (Estes and Kapikian, 2007; Rahman et al., 2007).

0378-1135 © 2011 Elsevier B.V. Open access under CC BY-NC-ND license.
doi:10.1016/j.vetmic.2011.05.017
A new classification system was recently proposed for GARVs, in which nucleotide percentage identity cut-off values define different genotypes for all the 11 genomic RNA segments (Matthijnssens et al., 2008b). Among these genotypes, the G (for glycoprotein) and P (for protease-sensitive) genotypes for the VP7 and VP4 outer capsid proteins are the most important and frequently analyzed for GARV classifications, because they are of high relevance to immune protection and vaccine development (Estes and Kapikian, 2007; Matthijnssens et al., 2010a). In extensive genomic studies, 25 G and 32 P genotypes have been described for GARVs of humans and animals (Collins et al., 2010; Esona et al., 2010; Ursu et al., 2009). Although certain types of GARV strains are found usually in a their specific host species, natural inter-species transmission and the reassortment of GARVs have been described on multiple occasions (Ha et al., 2009; Martella et al., 2010; Matthijnssens et al., 2006a, 2009b, 2010c).

The natural ability of GARV to cause disease in different species is not well-defined at the molecular level. However, the VP3, VP4, VP7, NSP1, NSP2, and NSP4 genes have been implicated in host range restriction and/or virulence (Bridger et al., 1998; Broome et al., 1993; Burke and Desselberger, 1996; El-Attar et al., 2001; Hoshino et al., 1995; Kojima et al., 1996; Mori et al., 2003). Depending on the strains or isolates investigated, the pathogenicity of bovine reassortant GARV strains tended to be different in experimentally infected calves and pigs. For example, the bovine GARV strain PP-1 replicated in experimental calves without causing clinical signs (Bridger and Pocock, 1986), whereas this strain induced diarrhea in experimentally infected pigs with severe intestinal villous atrophy (Bridger and Brown, 1984). In contrast with the PP-1 strain, the bovine CP-1 strain replicated and induced diarrhea in experimentally infected calves but not in pigs (Bridger and Brown, 1984; Bridger and Pocock, 1986; El-Attar et al., 2001). A comparison of the gene segments identified in these two strains, suggested that VP4 and NSP4, but not VP7 and NSP1 were involved in the species-specificity to induce disease, at least in calves and piglets (El-Attar et al., 2001).

Initially, GARV infections were thought to be restricted to the small intestine. However, there is a growing amount of data suggesting that GARV infections in children can cause systemic infections. GARV antigen and RNA was present in the blood of approximately 65% of children with GARV diarrhea (Chiappini et al., 2005; Fischer et al., 2005). Moreover, GARV RNA and proteins have been detected in extra-intestinal tissues such as the liver, heart, lungs, spleen, kidney, and central nervous system of infected children (Cioc and Nuovo, 2002; Lynch et al., 2001; Morrison et al., 2001; Nuovo et al., 2002). These case reports suggest that GARV can escape the intestine and routinely disseminate to systemic sites (Blutt and Conner, 2007; Crawford et al., 2006). In experimental animal models, it has been clearly demonstrated that GARVs cause not only gastrointestinal but systemic infections (Blutt et al., 2003; Ciarlet et al., 2002; Crawford et al., 2006; Fenaux et al., 2006). Since these experiments were performed with either fully homologous or heterologous GARV strains, it is unclear if bovine reassortant GARV can induce viremia and systemic infections in calves.

Bovine GARVs are considered as important animal pathogens due to their economic impact on livestock production and are potential zoonotic sources of human GARV infections (Dhama et al., 2009; Martella et al., 2010; Matthijnssens et al., 2006b). However, there is a scarcity of data on intestinal and extra-intestinal infections caused by bovine reassortant GARV strains (El-Attar et al., 2001, 2002; Hall et al., 1976; Tzipori et al., 1980). The present study was conducted using a bovine reassortant strain designated KJ9-1. The study addressed whether KJ9-1 strain replicates and causes pathological changes in the digestive tract of calves and piglets, viremia in calves, and damage to the systemic organs or tissues of calves.

To address the cross-species pathogenicity in calves and piglets, the digestive tract and feces were sampled fromcolostrum-deprived (Cols-D) calves and piglets inoculated with a reassortant bovine GARV strain KJ9-1. The samples were analyzed for morphological changes, antigen distribution using an immunofluorescent assay, and viral RNA presence by RT-PCR. To evaluate viremia and extra-intestinal infection in experimental calves, blood and extra-intestinal organs and tissues were sampled from experimentally infected calves, and analyzed for morphological changes, antigen distribution, and viral RNA presence by RT-PCR and real-time RT-PCR.

2. Materials and methods

2.1. Virus inoculum

The bovine GARV strain KJ9-1, was originally isolated and identified from a diarrheic fecal sample of a calf (Park et al., 2011). By the full-length nucleotide sequence analyses of the 11 genomic segments, this strain was characterized as a reassortant virus containing five bovine-like gene segments (VP7: G6; VP6: I2; VP1: R2; VP3: M2; NSP2: N2 and NSP4: E2), five porcine-like gene segments (VP4: P7; NSP1: A1; NSP3: T1 and NSP5: H1), and one human-like gene segment (VP2: C2) (Park et al., 2011). The virus titer of this strain was determined by a cell culture immunofluorescence assay with monoclonal antibodies against the VP6 protein of the porcine GARV strain OSU, using a slight modification of a method described previously (Kang et al., 1989). Virus titers were expressed as fluorescent focus units (FFU) per milliliter.

The supernatant from mock-infected TF-104 cell cultures was for the mock oral inoculation of control Cols-D calves and piglets. The possibility that the RT-PCR could detect residual GARV inoculum after inoculation was tested by inoculating another Cols-D calf and piglet with the KJ9-1 strain inactivated by a chloroform treatment (Meng et al., 1987).

2.2. Animals and experiment design

Ten 2-day-old Cols-D Holstein calves were used (Table 1). They were fed commercial calf formula. Eight calves were inoculated orally with 40 ml of the cell culture supernatant from the KJ9-1 strain containing a virus titer
of $1.3 \times 10^7$ FFU/ml. One calf was inoculated orally with 40 ml of mock-infected TF-104 tissue culture supernatant, and the remaining calf was inoculated with 40 ml of the KJ9-1 strain inactivated by chloroform treatment.

A total of eight 3-day-old Cols-D piglets were used to evaluate the pathogenicity of the bovine KJ9-1 strain in piglets. They were fed commercial piglet formula. Six piglets were inoculated orally with 4 ml of cell culture supernatant from the KJ9-1 strain containing a virus titer of $1.3 \times 10^7$ FFU/ml. After inoculation, color and consistency of feces of each calf and piglet were evaluated daily. The consistency of the feces was scored on a scale of 0–4, with 0 representing firm; 1, pasty; 2, semimucoid; 3, liquid; and 4, profuse diarrhea. Fecal samples were collected daily from each calf and piglet before and after inoculation, and nasal and blood samples were collected daily from each calf before and after inoculation, as described previously (El-Kanawati et al., 1996). The inoculated calves were euthanized at post-inoculation day (PID) 1–5, 7, 12, and 14 (Table 1). The calves inoculated with the mock- and inactivated virus were euthanized at PID 2 and 3, respectively. The inoculated piglets were euthanized at PID1–5, and 7 (Table 2). The piglets inoculated with the mock- and inactivated virus were euthanized at PID 2 and 3, respectively.

A necropsy of all the calves and piglets was performed immediately after euthanasia. At the necropsy, the intestinal tracts were removed from the abdominal cavities, and the small and large intestinal contents were collected. Intestinal segments were excised and immediately immersed in 10% buffered formalin for histological examination. In calves, the intestinal segments examined included the duodenum (approximately 15 cm caudal to the pyloric valve), jejunum (mid region of the small intestine), and ileum (approximately 15 cm cranial to the ileocecal junction). The small intestine in the piglet was defined as the portion of the digestive tract between the pylorus and the ileocecal valve, with the first 10-cm segment being duodenum. The jejunum constituted of approximately 40% of the small intestine below the duodenum, with the ileum constituting approximately 60% (Wang et al., 2008). The nasal turbinates, trachea, lungs, livers, spleens, mesenteric lymph nodes, brains, kidneys, hearts, and choroid plexus were also excised from experimental calves, immediately placed in 10% buffered formalin for histological examination. For the detection of viral antigen in each tissue, intestinal, and extra-intestinal organs and tissues were sampled from virus-inoculated and mock-inoculated calves, embedded in the Optimum Cutting Temperature (OCT) compound, immediately snap-frozen in liquid nitrogen, and stored at $-80\,\text{°C}$. Cerebrospinal fluid (CSF) was collected after euthanasia from each experimental calf and stored at $-80\,\text{°C}$. All samples collected for RT-PCR and real-time RT-PCR were immediately snap-frozen in liquid nitrogen, and kept at $-80\,\text{°C}$ until use. All studies were approved by the University Animal Care Committee.

### 2.3. Histological examination

Formalin-fixed and paraffin-embedded sections from each organ and tissue were stained with Mayer’s hematoxylin and eosin, and examined microscopically. The histological evaluation was performed in a blind fashion on coded samples, and a comparison was made with the sections from the mock-infected control.

Atrophy of small intestinal villi was measured from histological sections of the duodenum, jejunum, and ileum. The small intestinal changes were scored according to the average villi/crypt (V/C) ratio plus the grade of epithelial cell desquamation, which was measured as follows: V/C...
ratio, 0 = normal (V/C ≥ 6:1), 1 = mild (V/C = 5.0–5.9:1), 2 = moderate (V/C = 4.0–4.9:1), 3 = marked (V/C = 3.0–3.9:1), 4 = severe; (V/C ≤ 3.0:1); desquamation grade, 0 = normal (no desquamation), 1 = mild (a few desquamated cells of tip villous epithelium), 2 = moderate (desquamation of upper villous epithelium), 3 = marked (desquamation of lower villous epithelium), and 4 = severe (desquamation of crypt epithelium). These mean lesion changes were determined by measuring 10 randomly selected villi and crypts on intestinal histological sections, respectively, similar to methods described previously (Park et al., 2007).

The lesion changes in the respiratory tract were scored as described previously (Park et al., 2007). Lesions in the nasal turbinates and trachea were classified according to the degree of degeneration (cell swelling and loss of cilia) and necrosis (characterized by pyknosis, karyorrhexis, karyolysis and the absence of nuclei associated with cell swelling and loss of cilia) of the epithelium as follows: 0 = normal, 1 = one to two degenerating cells, 2 = three to five degenerating and necrotic cells, and 3 = many degenerating and necrotic cells. The lung lesions were scored according to the extent of interstitial pneumonia caused by the infiltration of macrophages and lymphoid cells in the interstitium, the accumulation of hyperplastic type II pneumocytes, and the degree of the degeneration, necrosis and desquamation of the alveolar epithelial cells. The lesions were classified as follows: 0 = normal, 1 = scattered mild thickening of interstitium, and two or three degeneration and necrosis, and four to six desquamation of alveolar epithelial cells, 2 = multifocal moderate thickening of interstitium, and four to six degeneration and necrosis, and many desquamation of alveolar epithelial cells, and 3 = diffuse severe thickening of the interstitium as well as many degeneration, necrosis and desquamation of alveolar epithelial cells (Park et al., 2007).

2.4. RT-PCR

To detect the viral RNA in the fecal, nasal swab, and serum specimens sampled from each experimental animal, RT-PCR with a primer pair specific to the VP6 gene of GARVs was employed, as described previously (Schwarz et al., 2002). As negative controls, fecal, nasal swab, and serum specimens sampled from mock-inoculated animals were used. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

2.5. Real time RT-PCR using SYBR Green chemistry

Based on SYBR Green detection, a one-step real-time RT-PCR assay with a primer pair specific to the VP6 gene of GARVs was performed to quantify the RNA of GARV in the samples, as described previously (Hosmillo et al., 2010; Park et al., 2011; Schwarz et al., 2002). Briefly, total RNA was extracted from the feces, nasal swab, serum, CSF fluid, mesenteric lymph node (MLN), liver, lungs, and choroid plexus. Each was individually weighed. All tissue samples from the calves and piglets were homogenized or vortexed at a 1:10 dilution in 0.01 M phosphate-buffered saline, pH 7.2, and centrifuged (tissues 13,000 × g for 3 min; fecal samples 5000 × g for 10 min). The supernatants, along with the remaining bulk samples, were collected and stored at −80 °C until used. All reactions were performed using a Corbett Research Rotor-Gene Real-Time Amplification system (Corbett Research, Mortlake, Australia) and SensiMix one-step RT-PCR kit with SYBR Green (Quantace, London, UK), as described previously (Hosmillo et al., 2010). Real-time RT-PCR was performed in a final volume of 25 μl containing 5 μl of RNA template, 12.5 μl SensiMix one-step mixture, 1 μl each of 0.5 M forward and reverse primers (final concentration of each primer: 20 nM), 0.5 μl of 50× SYBR Green solution (final concentration: 1×), 0.5 μl of RNase inhibitor (final concentration: 10 units), 0.5 μl of MgCl₂ (final concentration: 4.0 mM), and 4 μl of RNase free water. Reverse transcription was carried out at 50 °C for 30 min, followed by the activation of the hot-start DNA polymerase at 95 °C for 15 min and 40 three-step cycles: 95 °C for 15 s, 51 °C for 30 s, and 72 °C for 1 min. Quantitation was carried out using a standard curve derived from serial 10-fold dilutions of the in vitro transcription of complementary RNA (cRNA) amplified in separate PCR tubes. The Rotorgene 2000™ software was used for the calculation of the amount of GARV-specific RNA in the samples. The threshold was defined automatically in the initial exponential phase, reflecting the highest amplification rate. With regard to the crossing points resulting from the amplification curves and this threshold, a direct relation between the cycle number and the log concentration of RNA molecules initially present in the RT-PCR reaction was evident. By linear regression analysis of these data, Rotorgene 2000 software set up a standard curve which allowed the determination of the concentration of RNA present in the samples.

2.6. Immunofluorescence assay (IFA)

The immunofluorescence assay was performed for the detection of GARV antigen in each tissue and organ sampled from experimental animals as described elsewhere (Ciarel et al., 2002). Briefly, cut frozen sections from experimental animals were fixed in 100% cold acetone for 10 min and allowed to completely air dry. Slides were washed twice with PBS (pH 7.2), and incubated for 2 h at room temperature (RT) with a 1:100 dilution of monoclonal anti-VP6 antibody diluted with PBS (pH 7.2). Slides were washed twice with PBS (pH 7.2), and incubated with goat anti-mouse Ig conjugated to fluorescein isothiocyanate (Jackson Immunoresearch Labs, Baltimore, MD, USA) diluted 1:100 in PBS (pH 7.2) for 1 h at RT. Following incubation, the slides were washed twice with PBS (pH 7.2). Slides were incubated with propidium iodide diluted in 500 mM PBS (pH 7.2) for 10 min at RT as a nucleic acid stain. Slides were washed twice with PBS (pH 8.0), and covered with 60% glycerin in PBS (pH 8.0) and glass cover slips. Fluorescence was examined under the UV light illumination with a Leica microscope (Leica Microsystems, Wetzlar, Germany).

To calculate the number of antigen–positive cells in the organs or tissues, 10 fields per section were analyzed, using a 40× objective and a 10× eyepiece, yielding a final magnification of 400×.
3. Results

3.1. Comparison of clinical sign and fecal virus shedding caused by bovine reassortant GARV strain in calves and piglets

The KJ9-1 strain caused diarrhea in all inoculated calves by PID 1, persisting until the termination of the experiment (Table 1), but did not cause any diarrhea in the inoculated piglets (Table 2). Diarrhea was not present in either the inactivated KJ9-1 inoculated or the mock-inoculated calves and piglets. None of the calves and piglets inoculated with the KJ9-1 strain showed any other signs except for diarrhea following inoculation. Using RT-PCR, fecal virus shedding was detected at PID1 and persisted for 7–8 days in virus-inoculated calves, whereas virus-inoculated piglets shed virus via feces at PID1 and shedding persisted for 3 days. Fecal virus shedding was not detected in the inactivated KJ9-1 inoculated or mock-inoculated calves and piglets by RT-PCR.

3.2. Comparison of intestinal histopathology caused by bovine reassortant GARV strain in calves and piglets

In GARV-infected calves, histological evaluation of multiple cross-sections of the small intestine revealed mild to severe villous atrophy, widespread villous fusion and increased crypt depth caused by the degeneration or necrosis of cells lining the villi (Fig. 1A). Some mononuclear lymphoid cells infiltrated the lamina propria of the small intestines. Sequential histological lesion changes in the small intestines of virus-inoculated calves are summarized in Table 3. At PID 1, mild villous atrophy caused by degeneration or necrosis of villi lining cells was confined to the duodenum, jejunum and ileum. As time elapsed, duodenal lesions became more aggravated and other parts of intestine revealed milder epithelial change. Eventually the duodenum displayed severe villous atrophy and the other parts of the intestines including the jejunum and ileum had moderate to marked lesions in their villi.

In contrast to GARV-infected calves, GARV-infected piglets showed mild villous atrophy and increased crypt depth in the small intestine (Fig. 1B). Some mononuclear lymphoid cells infiltrated in the lamina propria of the small intestines. Sequential histological lesion changes in the small intestines of virus-inoculated calves are summarized in Table 4. Lesion scores tended to increase sequentially, but comparatively much milder than those in virus-inoculated calves. No lesions were observed in small intestines sampled from inactivated KJ9-1 strain- and mock-inoculated calves and piglets (Fig. 1C and D).

Fig. 1. Histopathological changes in the small intestine of bovine reassortant GARV-infected calves and piglets. (A) Duodenum sampled from virus-infected calf at PID3 shows severe mucosal changes including the widespread villous atrophy, increased crypt depth, and villous epithelial desquamation. (B) Duodenum sampled from virus-infected piglet at PID4 has mild villous atrophy, mild hyperplasia of crypt epithelium, and intact villous epithelium. (C and D) Duodenum from mock-inoculated calf (C) and piglet (D) has normal structure of mucosal membrane. Hematoxylin and eosin stained. Bars A–D = 200 μm.
Table 3
Summary of the histopathological findings in the small intestine of the colostrums-deprived calves after inoculation with a reassortant KJ9-1 strain.

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>PID at euthanasia</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lesion scorea</td>
<td>GARV Ag distribution (%)</td>
<td>Lesion scorea</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1.2</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>3.4</td>
<td>2.1</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3.5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>3.7</td>
<td>1.5</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>3.6</td>
<td>0</td>
<td>3.5</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>3.5</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The small intestinal changes were scored according to the average villi/crypt (V/C) ratio plus the grade of epithelial cell desquamation, which was measured as follows: V/C ratio, 0 = normal (V/C ≥ 6:1), 1 = mild (V/C = 5.0 to 5.9:1), 2 = moderate (V/C = 4.0 to 4.9:1), 3 = marked (V/C = 3.0 to 3.9:1), and 4 = severe (V/C ≤ 3.0:1); desquamation grade, 0 = normal (no desquamation), 1 = mild (cuboidal attenuation of tip villous epithelium), 2 = moderate (desquamation of upper villous epithelium), 3 = marked (desquamation of lower villous epithelium), and 4 = severe (desquamation of crypt epithelium).

3.3. Comparison of antigen localization of bovine reassortant GARV strain in small intestines of calves and piglets

Rotavirus antigen-positive cells specific to VP6 protein was detected in selected tissue section from duodenum (Fig. 2A and B). Sequential changes of antigen detection in the small intestines of calves and piglets are summarized in Tables 3 and 4, respectively. GARV-infected calves showed that GARV antigen tended to increase sequentially from PID1 to PID3 or 4, and to decrease sequentially from PID4 or 5. GARV-antigen was no longer detected after PID7. In contrast, only a few antigen positive cells were detected at PID1 to PID3 in GARV-infected piglets. No antigen-positive cells were observed in small intestines sampled from inactivated KJ9-1 strain- and mock-inoculated calves and piglets (Fig. 2C and D).

3.4. Histological changes in extra-intestinal organs and tissues of GARV-infected calves

Bovine reassortant GARV strain caused lymphoid cell depletions in the cortex of MLN of calves by PID3 and this lesion persisted until PID14 (Fig. 3A). Some macrophages and neutrophils infiltrated in the cortex. No histopathological changes were observed in MLN sampled from inactivated KJ9-1 strain- and mock-inoculated calves and piglets (Fig. 3B). Histological sections of lungs sampled sequentially from virus-infected calves revealed several changes. At PID 1, no lesions were observed in the lung tissues. As time elapsed, however, the width of the interstitium increased. As a consequence, the lung tissues sampled at PID 4 revealed marked interstitial pneumonia (Fig. 3C). The increase in cellularity appeared to be due to

Table 4
Summary of the histopathological findings and antigen distribution in the small intestine of the colostrums-deprived piglets after inoculation with a reassortant KJ9-1 strain.

<table>
<thead>
<tr>
<th>Piglet no.</th>
<th>PID at euthanasia</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lesion scorea</td>
<td>GARV Ag distribution (%)</td>
<td>Lesion scorea</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.6</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1.2</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>1.2</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>1.8</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The small intestinal changes were scored according to the average villi/crypt (V/C) ratio plus the grade of epithelial cell desquamation, which was measured as follows: V/C ratio, 0 = normal (V/C ≥ 6:1), 1 = mild (V/C = 5.0 to 5.9:1), 2 = moderate (V/C = 4.0 to 4.9:1), 3 = marked (V/C = 3.0 to 3.9:1), and 4 = severe (V/C ≤ 3.0:1); desquamation grade, 0 = normal (no desquamation), 1 = mild (cuboidal attenuation of tip villous epithelium), 2 = moderate (desquamation of upper villous epithelium), 3 = marked (desquamation of lower villous epithelium), and 4 = severe (desquamation of crypt epithelium).

* The antigen distribution in the small intestine was evaluated based on the number of antigen-positive cells in the villi, and was measured as follows: 0 = no positive cells, 1 = one to two positive cells in the villi, 2 = three to five positive cells scattered in the villi, 3 = many positive cells in the villi, and 4 = positive in almost all epithelial cells in the tip and upper part of the villi.
infiltration of macrophages with some lymphocytes and neutrophils into the alveolar interstitium, and hyperplasia of type II pneumocytes. By PID5, these lesions tended to become mild and eventually disappear by PID12. No lesions were observed in the lungs of inactivated KJ9-1 strain- and mock-inoculated calves (Fig. 3D). Histological changes were noted in all livers from the 8 GARV-inoculated calves (Fig. 3E) but in none of the livers from 2 mock-inoculated calves (Fig. 3F). Hepatocyte necrosis scattered around the portal triads was observed by PID3 (Fig. 3E) and persisted until PID7. Inflammatory cells including macrophages and lymphocytes infiltrated in the portal triads of the liver. No lesions were observed in the brains sampled from either GARV-inoculated or mock-inoculated calves (Fig. 3H). However, epithelial degeneration and necrosis of the choroid plexus were observed only at PID3 in virus-inoculated calf (Fig. 3G). Some lymphocytes infiltrated in the tela choroidea (Fig. 3G).

3.5. Extra-intestinal antigen localization of GARV in calves

GARV antigen was detected in the lymphoid cells in the MLN (Fig. 4A), hepatocytes and lymphoid cells in the livers (Fig. 4B), pneumocytes and lymphoid cells in the lungs (Fig. 4C), and epithelial cells and lymphoid cells in the choroid plexus (Fig. 4D). As shown in Table 5, few GARV antigen-positive cells were detected by PID2, an increase was noted at PID3, followed by a decreased by PID4. No antigen was observed in these organs and tissues sampled from mock-inoculated calves.

3.6. Quantification of GARV RNA in the feces, nasal swabs, blood, and extra-intestinal organs and tissues

Fig. 5 shows the viral copy numbers in these samples. In the fecal samples, high viral loads, \(2.1 \times 10^6/mg\) feces, were detected at PID1, reaching a peak at PID3, and then gradually decreased from PID4 to 14. Viral RNAs were detected in sera, MLN, liver, lungs, nasal fluid secretions, and choroid plexus at PID1, attaining a peak at PID3, and then rapidly decreasing from PID4 onward. Low levels of viral copy numbers were detected in CSF at PID1. However, viral copy numbers increased and reached a peak at PID3. Viral RNA was not detected in the feces, sera, nasal swabs, and extra-intestinal organs and tissues sampled from the mock-inoculated calves.

4. Discussion

When GARVs cross the host species barrier, this is usually a dead-end infection since the virus is not able to properly spread in the new host species. However, if the interspecies transmitted virus is able to reassort with GARVs inherent in that host species, the resultant reassortant strains may have a higher probability to efficiently infect and spread among the population of...
Fig. 3. Extra-intestinal histopathological changes in the calves and piglets inoculated with bovine reassortant GARV strain. (A) Mesenteric lymph node sampled from virus-inoculated calf at PID4 manifests marked lymphoid cell depletion in the cortex. (B) Normal mesenteric lymph node sampled from a mock-inoculated calf shows intact cortex with densely concentrated lymphocytes. (C) Lung tissue sampled from virus-inoculated calf at PID4 shows interstitial pneumonia due to infiltration of macrophage with some lymphocytes and neutrophils into the alveolar interstitium, and type II pneumocytes. (D) Normal lung tissue sampled from mock-inoculated calf has thin alveolar walls. (E) Liver sampled from virus-inoculated calf at PID4 has multiple scattered necrotic hepatocytes. (F) No lesions are observed in the liver sampled from a mock-inoculated calf. (G) Choroid plexus sampled from a virus-
the new host (El-Attar et al., 2001; Matthijnssens et al., 2008a, 2009a, 2010b). Depending on the origin of the certain gene segments, the pathogenicity of bovine-porcine reassortant strains in calves and piglets differs (Bridger and Brown, 1984; Bridger and Pocock, 1986; El-Attar et al., 2001; Hall et al., 1976). The GARV KJ9-1 strain was originally isolated from a diarrhea sample of a calf. This strain contains six gene segments of bovine origin (VP1, VP3, VP6, VP7, NSP2, and NSP4) and a porcine origin of four gene segments (VP4, NSP1, NSP3, and NSP5). Therefore, we evaluated the pathogenicity of this strain in calves and piglets. In the present study, KJ9-1 caused diarrhea in experimental calves with extensive intestinal villous atrophy, but replicated in experimental piglets without causing clinical signs.

Six rotaviral gene segments (VP4, VP7, VP3, NSP1, NSP2, and NSP4) have been implicated in host range restriction and/or virulence (Bridger et al., 1998; Broome et al., 1993; Burke and Desselberger, 1996; El-Attar et al., 2001; Hoshino et al., 1995; Kojima et al., 1996; Mori et al., 2003). The bovine reassortant PP-1 strain carrying a bovine NSP1 gene and porcine VP4, VP7, and NSP4 genes is pathogenic to piglets, but not to calves. In contrast, the bovine reassortant CP-1 strain bearing bovine VP4, NSP1, and NSP4 genes, and a porcine VP7 gene is pathogenic to calves but not to piglets. These data suggest that VP4 and NSP4, but not VP7 and NSP1, are involved in the species-specificity to induce disease, at least in calves and piglets (El-Attar et al., 2001). The KJ9-1 strain, which caused symptomatic disease in calves, but not in piglets, harbored VP4 and NSP1 genes of porcine origin, and VP7 and NSP4 genes of bovine origin. Taken together, it can be concluded that, of NSP1, NSP4, VP4 and VP7, NSP4 is the gene most associated with host range restriction (at least in calves and piglets). It should be noted that the VP3 and NSP2 gene segments of KJ9-1 are of bovine origin, but, unfortunately, no data on the origin of the VP3 and NSP2 genes are available for strains PP-1 and CP-1 for comparison. In order to clarify the molecular and biological properties of each genomic segment associated with host range restriction and/or virulence, further studies using the other reassortant bovine GARVs with different reassortant gene constellations should be performed.

Recent evidence clearly demonstrates that GARV infection is not limited to intestinal epithelial cells in animals and humans. Since these experimental studies used homologous or heterologous GARVs in experimental animals (Blutt et al., 2003; Ciarlet et al., 2002; Crawford et al., 2006; Fenaux et al., 2006), it remains unclear whether reassortant GARVs cause viremia and extra-intestinal infections. The present results demonstrate that the bovine reassortant GARV causes viremia in inoculated calf at PID3 shows epithelial degeneration and necrosis. (H) Intact epithelium is observed in the choroid plexus sample from a mock-inoculated calf. Hematoxylin and eosin stained. Bars A–D, and G–H = 50 μm. Bars E and F = 100 μm.
experimentally infected calves. The onset of viremia in the GARV-inoculated calves occurred at PID 1, and the amounts of virus RNA peaked at PID3, and lasted until the termination of the experiments at PID14. However, it is unclear how GARV reaches the bloodstream from intestinal lesions after oral inoculation. One possible path is the cell-free GARV penetration of the gut barrier from the luminal side after the destruction of enterocytes in the villi, which would expose the basal membrane, followed by transit into the circulatory system, which can spread to the MLN and other extra-intestinal organs (Azevedo et al., 2005; Fenaux et al., 2006). This hypothesis is plausible because destruction of enterocytes, resulting in exposure of the basal membrane, and villi atrophy were observed at PID1. Moreover, this hypothesis is consistent with the present observation that GARV RNA was present in the sera of the experimentally infected calves. Another possible path for reassortant GARV to reach the bloodstream is that GARV may be taken up by macrophages or other antigen-presenting cells in the gut-associated lymphoid tissue and then enter the bloodstream (Brown and Offit, 1998; Dharakul et al., 1988). However, cell-free rather than cell-associated GARV transmission via the bloodstream seems to be major path in a mouse model inoculated with GARV (Fenaux et al., 2006). Further studies will be needed to determine the pathway that is the major cause of viremia in reassortant GARV inoculated calves.

Interestingly, GARV antigen and RNA was also detected in the extra-intestinal organs or fluids including the MLN, livers, lungs, choroid plexus, and CSF indicating that the bovine reassortant GARV replicates in the extra-intestinal organs. These findings confirm other recent studies documenting the ability of GARV virions to spread to the bloodstream and to substantially extend to the extra-intestinal organs (Blutt et al., 2003; Carlet et al., 2002; Crawford et al., 2006; Fenaux et al., 2006). The above hypothesis suggests that viral RNA detected in the extra-intestinal organs might be from virus particles present in the serum and/or from virus particles that replicate in extra-intestinal organs. Since viral RNA in the serum and viral antigen in the extra-intestinal organs were presently detected in experimentally infected calves, this hypothesis is plausible. In addition, viral RNA and antigen was also detected in the choroid plexus, which produces CSF. These findings could explain why viral RNA was detected in the CSF of virus-infected children with encephalitis and/or seizure (Liu et al., 2009; Nakagomi and Nakagomi, 2005; Nishimura et al., 1993; Pager et al., 2000; Ushijima et al., 1994).

GARV diarrhea in calves presents as an acute disease having a very short incubation period of 12–24 h presently and 18–96 h in other studies (Chauhan and Singh, 1996; Steele et al., 2004). These findings can explain why high viral titers were detected in the diarrhea of virus-inoculated calves starting from PID1. Compared to the pattern of fecal viral shedding, the replication pattern of GARV RNA in extra-intestinal organs and fluids was slightly different. Virus replication in extra-intestinal

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>PID at euthanasia</th>
<th>Distribution of viral antigen in extraintestinal organs*</th>
<th>Mesenteric lymph node</th>
<th>Livers</th>
<th>Lungs</th>
<th>Choroid plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The antigen distribution in the extraintestinal organs was evaluated based on the number of antigen-positive, and was measured as follows: 0 = no positive cells, 1 = one to two positive cells, 2 = three to five positive cells scattered in tissue, 3 = many positive cells in tissues, and 4 = positive in almost tissue.

**Fig. 5.** Quantification of GARV RNA by SYBR Green real-time RT-PCR in the feces, serum, mesenteric lymph node, livers, lungs, nasal swabs, choroid plexus, and cerebrospinal fluids sampled from GARV-inoculated calves. The geometric means of virus RNA copy number per mg of tissue are presented.
organs and fluids was also detected at PID1, but reached a peak at PID3, and declined more rapidly than in feces. This can be explained by the findings that, in the mouse model, intestinal immunoglobulin A titers peak 1 month after infection and then steadily decline, whereas immunoglobulin G titers continuously increase over time (Ishida et al., 1996; McNeal and Ward, 1995). This could help explain our observations that virus titers were higher in the feces than those of extra-intestinal organs and tissues. Since blood in the organs and tissues could not be removed perfectly from calves by the perfusion, we did not perform perfusion from the calves. Therefore, viruses in the blood of organs and tissues sampled from infected calves could be detected by real-time RT-PCR. Because a small amount of blood is present in the organs and tissues, a respectable number of viruses could be in the cells of each organ and tissue sampled from virus-infected calves. Indeed, the IFA results demonstrated virus antigen in the cells of these organs and tissues.

In this study, higher amounts of GARV antigen and RNA were detected in the MLN than in other extra-intestinal organs in experimentally infected calves. Viral RNA in the MLN was detected at PID1, peaked at PID3, and then drastically declined. Viral RNA detected in the MLN might have originated from virus particles present in the plasma coming from the gut, or/and from virus particles infecting the MLN cells. Possibly, the MLN might play a critical role in RV escape from the small intestine by providing a site for substantial and prolonged secondary viral replication. This would agree with previous work which suggests that rhesus GARV spread from the small intestine passes through the MLN before extending to peripheral tissues (Fenaux et al., 2006).

Our results confirm the possible role of GARVs as an etiological cause for the clinical manifestations reported to be associated with GARV infection and highlight the need for specific studies to determine the involvement of GARV as a cause of non-gastrointestinal disease symptoms. In particular, these findings could provide a new perspective on the pathogenesis of bovine respiratory disease complex (Dyer, 1982; Straub, 1991). In the present study, GARV antigen and RNA were detected in the lungs and nasal swabs of experimental calves. Many respiratory viruses are known to play a role as a predisposing factor for secondary bacterial pathogens such as Mannheimia spp., resulting in bovine respiratory disease complex (Dungworth, 1993; Straub, 1991). Therefore, it is plausible that GARV may also be a predisposing factor for secondary bacterial pneumonia resulting in bovine respiratory disease complex. Additional studies are in progress to delineate if lung infections by reassortant GARV exacerbate secondary bacterial pneumonia.

In conclusion, the NSP4 gene of GARVs most likely plays an important role in host range restriction at least in calves and piglets. In order to clarify the molecular and biological properties of each genomic segment associated with host range restriction and/or virulence, further studies using the other reassortant bovine GARVs with different reassortant genomic allele should be performed. Like homologous and heterologous GARVs, bovine reassortant GARV can cause not only intestinal but also extra-intestinal pathology in calves.

Acknowledgments

This study was supported by the National Veterinary Research and Quarantine Service (NVQRS) and Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, the Regional Technology Innovation Program (RT105-01-01) of the Ministry of Commerce, Industry and Energy (MOCIE), and the Korea Research Institute of Bioscience and Biotechnology (KIRIB), Republic of Korea. The authors would like to acknowledge a graduate fellowship provided by the Korean Ministry of Education and Human Resources Development through the Brain Korea 21 project. JM was supported by an FWO (‘Fonds voor Wetenschappelijk Onderzoek’) postdoctoral fellowship.

References


