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Ovine rotavirus strain LLR-85-based bovine rotavirus candidate vaccines: Construction, characterization and immunogenicity evaluation

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ABSTRACT

Group A bovine rotaviruses (BRVs) are the most important cause of diarrheal diseases in neonatal calves and cause significant morbidity and mortality in the young animals, and epidemiologic surveillance of bovine rotavirus G genotypes conducted in various cattle populations throughout the world has shown that approximately 90% of the bovine rotavirus isolates belong to G6 and G10. Based on the modified Jennerian approach to immunization, we constructed and characterized a reassortant rotavirus stain, which bears a single bovine rotavirus VP7 gene encoding G genotype 6 specificity while the remaining 10 genes are derived from the ovine attenuated rotavirus LLR-85. The reassortant rotavirus strain, named as R191, and its parental virus strain LLR-85 were combined as bivalent vaccine candidates to inoculate the colostrums-deprived neonatal calves for evaluation of the immunogenicity. The calves were orally inoculated with the reassortant R191 (group 1), the parental rotavirus LLR-85 (group 2), or combined the R191 and LLR-85 (group 3), and serum specimens were detected to determine the immune response of IgG and IgA antibodies. Results showed that seroconversion to positivity for IgG and IgA antibodies occurred at postinoculation day (PID) 10 in all of the inoculated calves, and the highest titers of the serum IgG (range 1:800 to 1:6400) and IgA (range 1:800 to 1:3200) antibodies were obtained at PID 21 for all calves. Meanwhile, virus shedding was detected after inoculation, showing that the inoculated virus was positive in 2 of 77 fecal specimens (2.6%) collected from the inoculated calves during the first 7 days of oral inoculation with the rotavirus vaccine candidates. The results suggested that the rotavirus strains R191 and LLR-85 are promising bivalent vaccine candidates for the prevention of bovine G6 and G10 rotavirus infection.

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1. Introduction

The rotaviruses (RVs) compose a genus within the family *Reoviridae*, and comprise seven distinct groups (A–G), of which groups A–C rotaviruses are those currently found in both humans and animals, whereas viruses in groups D–G have been found only in animals to date (Knipe and Peter, 2007). The rotavirus genome is composed of 11 segments of double-stranded RNA and can undergo genetic reassortment during mixed infections, leading to progeny viruses with novel or atypical phenotypes. There were numerous descriptions of rotavirus strains isolated from human and animals that share genetic and antigenic features of viruses from heterologous species (Matthijnssens et al., 2006; Rahman et al., 2005; Santos and Hoshino, 2005). Recently, there were reports of novel rotaviruses that are apparently derived from transmission between human and sheep (Ciarlet et al., 2008; Matthijnssens et al., 2009). The emergence of novel strains derived from interspecies transmission has implications for design and implementation of successful reassortant rotavirus vaccine strategies.

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In prior studies, a dual classification system based on the two major outer capsid proteins defines several G and P genotypes of group A rotaviruses. To date, 23 G and 31 P genotypes have been identified based on the glycosylated VP7 and the protease-sensitive VP4 (Schumann et al., 2009; Ursu et al., 2009). Recently, a novel classification system based on the nucleotide sequences of all rotavirus genome segments has been suggested (Matthijnssens et al., 2008a,b) enabling a comprehensive characterization of rotavirus strains. Based on nucleotide identity cut-off percentages, different genotypes were defined for each genome segment. The calculated cut-off values for the 11 rotavirus RNA segments VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5/6 are 80%, 80%, 85%, 83%, 84%, 81%, 79%, 85%, 85%, 85% and 91%, respectively. To designate the complete genetic makeup of a virus, the schematic nomenclature was proposed: Gx-P[*x*]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, representing the genotypes of respectively the VP7–VP4–VP6–VP1–VP2–VP3–NSP1–NSP2–NSP3–NSP4–NSP5/6 encoding gene segments, with *x* indicating the number of the corresponding genotypes.

Group A rotaviruses, which are transmitted by the fecal–oral route and selectively infect the mature villous absorptive epithelial cells (Holland, 1990), cause disease that varies in severity, including asymptomatic infection, mild and self-limiting diarrhea, or severe diarrhea with excessive fluid loss and severe electrolyte imbalance. Diarrhea is one of the most important diseases of neonatal dairy and beef calves, and substantial economic loss occurs due to increased morbidity and mortality, treatment costs, and reduced growth rates (House, 1978). For example, livestock and poultry production with $70 billion per year industry in the United States is estimated to suffer a 15–20% annual loss in potential productivity from disease and environmental problems (United States Department of Agriculture, 1987), in which, calf loss from diarrhea is an important segment of total loss in cattle industry. It was reported in the late 1970s that enteric pathogens killed up to 25% of calves per year, resulting in more than $250 million in losses (Hunt, 1985). Rotavirus, coronavirus, Escherichia coli strain K99, coccidia and Cryptosporidium parvum are the main infectious agents inducing enteric infections in neonatal calves less than 2 months of age. Among the pathogens described above, rotavirus as a major cause of neonatal bovine diarrhea (Bellinzoni et al., 1990; Bendali et al., 1999; de la Fuente et al., 1998; Maes et al., 2003; Reynolds et al., 1986) was found to be responsible for approximately 46% of the scours cases in dairy calves (Snodgrass et al., 1986). In addition to causing economic losses, diarrhea in livestock is important because of the public health implications. Thus, the availability of a safe and effective bovine rotavirus vaccine capable of preventing this enormous economic and public health burden would represent a global goal.

The advent in 1998 of the first licensed human rotavirus vaccine, a RRV (rhesus rotavirus) -based quadrivalent vaccine (RotaShield^{TM}, Wyeth-Lederle Vaccines and Pediatrics, Philadelphia, PA) (Kapikian, 2001), provided an impetus for the expansion of programs of global human rotavirus strain surveillance and assessment of the rotavirus disease burden. In addition, availability in the early 1990s of a reliable and relatively easy methodology for rotavirus G and P genotyping accelerated this trend (Das et al., 1994; Fischer and Gentsch, 2004; Gentsch et al., 1992; Gouvea et al., 1990). Such information has indeed influenced the approaches to the development of an effective rotavirus vaccine. For example, additional candidate human rotavirus vaccines have been constructed that would give antigenic coverage not only for G1–G4 but also G5, G6 and G8–10 as well as P1A[8], P1B[4] and P2A[6] (Hoshino et al., 2005). However, there was limited research in the field of animal rotavirus vaccine. The bovine rotavirus isolate (the Lincoln strain, G6 genotype) was adapted to serial propagation in cell culture (Fernelius et al., 1972; Mebus et al., 1971), which resulted in an attenuated virus for calves (Mebus et al., 1973). This attenuated strain was incorporated into a vaccine licensed by the US Department of Agriculture (USDA) in 1973 for oral inoculation of newborn calves or intramuscular inoculation of pregnant cows to provide passive protection to their calves. Apart from this, there was no advent of new bovine rotavirus vaccine in the world.

Field surveys have demonstrated that, so far, G genotype G6 and G10 are the epidemiologically most important bovine rotavirus genotypes worldwide, accounting for 90% of the rotavirus-caused diarrhea approximately. Next to the G6 and G10 genotypes, G8 is the third typical bovine rotavirus genotype (Alfieri et al., 2004; Chang et al., 1996; Falcone et al., 1999; Garaicoechea et al., 2006; Monini et al., 2008; Parwani et al., 1993; Reidy et al., 2006; Snodgrass et al., 1990). In China, our epidemiologic surveillance has shown that rotavirus diarrhea is one of the most important diseases of neonatal calves, and that G6 and G10 were also determined to be the most prevalent genotype of the bovine rotaviruses using nested RT-PCR and sequencing (Chang et al., 2008). Since monovalent rotavirus vaccine cannot completely protect against heterologous rotavirus infection (Feng et al., 1994), the multivalent rotavirus vaccine candidates are the most important research goal for the protective immunity of rotaviruses. In addition, the reassortant rotavirus vaccine approaches have been successfully employed to develop various human rotavirus candidate vaccines that include rhesus rotavirus based and bovine rotavirus based multivalent vaccines listed earlier (Hoshino et al., 2002, 2003, 2005; Midthun et al., 1985, 1986). In this study, an ovine rotavirus strain LLR-85-based bovine rotavirus reassortant was constructed and combined with its parental virus LLR-85 as bivalent vaccine candidates for providing an attenuation phenotype of an ovine rotavirus in bovines and antigenic coverage of the main bovine rotavirus genotypes for G6 and G10. Our study showed that the bivalent vaccine candidates can induce immune response of IgG and IgA antibodies in the sera of the inoculated colostrum-deprived neonatal calves, and fecal shedding of rotavirus is uncommon following oral inoculation of calves with the rotavirus vaccine candidates.

2. Materials and methods

2.1. Virus strains, cell cultures, and animals

The bovine rotavirus strain NCDV (G6 P[1]), which was originally isolated in the stool of a neonatal calf with

diarrhea in Nebraska, USA, in 1971 (Fernelius et al., 1972), was obtained commercially from China Institute of Veterinary Drug Control, Beijing. The ovine rotavirus strain LLR-85 (G10 P[15]) (Chen et al., 2009; Knipe and Peter, 2007), which was initially derived from a lamb with diarrhea in Qinghai province, China, in 1981 (Zhou et al., 1985), was propagated from monovalent human rotavirus vaccine LLR (Lanzhou Institute of Biological Products, China) (Bai et al., 1994). These virus strains were adapted to growth in MA104 cell line, and passaged 10 times on DMEM medium (GIBCO) before a triple plaque purification. Colostrum-deprived newborn calves without antibodies to rotavirus were divided into three groups to orally inoculate candidate vaccines within 24 h after their birth. The calves were kept separate from each group and fed the substituted milk.

2.2. RT-PCR and sequence analysis

Primers for the amplification of gene segments VP4, VP6, NSP1, NSP2, NSP4 and NSP5 were synthesized (Table 1) based on the previous study (Park et al., 2006). RT-PCR was carried out with an initial reverse transcription step of 60 min at 42 °C, followed by PCR for activation at 94 °C for 5 min, 30 cycles of amplification (45 s at 94 °C, 45 s at 55 °C, and 60S at 72 °C for VP4, VP6, NSP1, and NSP4 and 45 s at 94 °C, 45 s at 50 °C, and 60S at 72 °C for NSP2 and NSP5), with a final extension of 10 min at 72 °C. The PCR amplicons were purified with the Gel Extraction kit (Tiangen Biotech Co., Ltd., China) and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China. The sequences were analyzed with Lasergene software, version 7.2.

2.3. RNA polyacrylamide gel electrophoresis

RNA polyacrylamide gel electrophoresis (PAGE) was done as previously described (Chudzio et al., 1989). Briefly, the supernatant of the culture lysates were mixed with an equal volume of RNA extraction buffer (0.02 M Tris–hydrochloride [pH 7.4], 0.3 M NaCl, 0.01 M $MgCl_2$, 0.1% sodium dodecyl sulfate, 0.005 M EDTA, 4% sucrose and 0.04% bromophenol blue), and each preparation was mixed with an one-tenth volume of phenol–chloroform (1:1) and vortexed for 30 s to yield a homogeneous suspension followed by spinning in a microcentrifuge at 12,000 × g for 10 min. The upper layer (dark blue solution) containing the double-stranded segments of RNA was collected and stored at 4 °C or −20 °C for electrophoretic analysis by the standard 10% polyacrylamide gel electrophoresis. Genomic RNAs were electrophoresed at 10 mA for 16 h and the resulting migration patterns were visualized by staining of gel with silver nitrate.

2.4. Construction of rotavirus reassortant with single VP7 gene substitution

Confluent cell line of fetal rhesus monkey kidney, MA104, was co-infected at a multiplicity of infection (m.o.i.) of one with bovine rotavirus strain NCDV and ovine rotavirus strain LLR-85. When approximately 75% of the infected cells exhibited cytopathic effects, the cultures were frozen and thawed three times and the lysate was plated on MA104 cells in a six-well plate for selection of the desired reassortant. Each desired reassortant was plaque purified three times in MA104 cells and then its genomic RNAs were analyzed by PAGE to confirm the origin of genes of each reassortant. When the parental origin of a gene was questionable by PAGE, the indistinguishable RNA segments were analyzed by sequence analysis for confirmation.

2.5. Plaque assay technique

The plaque assay was performed with techniques similar to those of Matsuno et al. (1977). Briefly, MA-104 cell monolayers were prepared in six-well tissue culture dishes and washed three times with PBS buffer. Serial tenfold dilutions of virus prepared in DMEM (without FBS) were inoculated in 0.2-ml amounts. After 1 h of adsorption at 37 °C, each culture received 2 ml of agar overlay medium, which consisted of 1% purified agar in DMEM containing 5 μg of trypsin per ml. After the agar overlay solidified, the dishes were placed in an inverted position in a CO_2 incubator at 37 °C for 3–5 days. Then an equal amount of a second agar overlay medium, containing

Table 1
Oligonucleotide primers for sequencing of the rotavirus genes.

Target gene	Primer name	Sequence (5′–3′)	Region (nt)	Size (bp)	Reference
VP4	Con3	TGGCTTCGCTCATTTATAGACA	11–32	880	Gentsch et al. (1992)
	Con2	ACTTCGGACCATTTATATCC	890–871		
VP6	VP6-F	GACGGAGCGACTACATGGT	747–766	380	Iturriza Gomara et al. (2002)
	VP6-R	GTCCAATTCATACCTGGTGG	1126–1106		
NSP1	NSP1-F	AAGCCATGGCTACTTTTAAGG	27–47	595	
	NSP1-R	AAGAATGACATTCTAGTGAAA	621–601		
NSP2	VF3F	GGCTTTTAAAGCGTCTCAGTC	1–21	1058	Varghese et al. (2004)
	VF3R	GGTCACATAAGCGCTTTCTATTC	1058–1036		
NSP4	10BEG.16	TGTTCCGAGAGAGCGCGTG	16–34	725	Lee et al. (2000)
	10END.722c	GACCATTCCTTCCATTAAC	740–722		
NSP5	VF1F	GGCTTTTAAAGCGCTACAGTG	1–21	664	Varghese et al. (2004)
	VF1R	GGTCACAAAACGGGAGTGGG	664–645		

Table 2
Inoculation of colostrums-deprived calves with rotavirus vaccine candidates and detection of rotavirus in the fecal specimens.

Group	Calf no.	Inoculum	Postinoculation day on which rotavirus was detected						
			1	2	3	4	5	6	7
1	1	R191	–	–	–	–	–	–	–
	7		–	–	–	–	–	–	–
2	8	LLR-85	–	–	–	–	–	–	–
	11		–	–	–	–	–	–	–
3	2	LLR-85 + R191	–	–	–	–	–	–	–
	3		–	–	–	–	–	–	–
	4		+	–	+	–	–	–	–
	5		–	–	–	–	–	–	–
	6		–	–	–	–	–	–	–
	9		–	–	–	–	–	–	–
	10		–	–	–	–	–	–	–

"–" refers to being negative for rotavirus by RT-PCR.
"+" refers to being positive for rotavirus by RT-PCR.

1% purified agar in DMEM and 0.003% neutral red was added, and plaques were counted the next day.

2.6. Inoculation of neonatal calves and sample collection

Two calves were orally inoculated with 3.6×10^6 $TCID_{50}$ of the attenuated LLR-85, two calves were orally inoculated with $3.2 \times 10^{6.5}$ $TCID_{50}$ of the reassortant R191, and seven calves were orally inoculated simultaneously with 1.8×10^6 $TCID_{50}$ of the attenuated LLR-85 and $1.6 \times 10^{6.5}$ $TCID_{50}$ of the reassortant R191 (Table 2). Rectal swab specimens were collected each day over the 7-day experimental period postinoculation to detect rotavirus. Blood samples were collected at postinoculation days (PIDs) 0, 10, 21 and 28, processed for serum, and kept at −20 °C until they were tested.

2.7. ELISA for detection of serum IgG and IgA antibodies

Plates were coated overnight at 4 °C with semipurified BRV or mock-infected cell culture supernatant in 0.1 M carbonate–bicarbonate buffer (pH 9.6), followed by a blocking step of 1 h at 37 °C with 5% nonfat dry milk. The plates were washed three times with 0.5% Tween 20 in PBS, pH 7.4. Serial twofold dilutions, starting at 1:100, of each test sample were added to duplicate wells and incubated for 1 h at 37 °C. After washing, the plates were incubated with horseradish peroxidase-conjugated monoclonal antibodies against bovine IgG or IgA (with dilution of 1:5000 and 1:2000, respectively) for 1 h at 37 °C. After washing, the plates were incubated with 1.8% TMB (3,3′,5,5′-tetramethylbenzidine) substrate for 20 min at room temperature. The reaction was stopped with 2 M H_2SO_4. The A_{450} was read by using an ELISA plate-reader (Zenyth 3100, HVD Anthos). The ELISA antibody titer of each sample was expressed as the reciprocal of the highest dilution that had a corrected A_{450} value (sample absorbance in the virus-coated well minus sample absorbance in the mock antigen-coated well) greater than the cut-off value (mean corrected A_{450} of negative controls + 3SD) (Parreno et al., 1999; To et al., 1998).

3. Results

3.1. Generation and characterization of rotavirus reassortant

In view of rotavirus genotypes G6 and G10 predominating in cattle worldwide, and the Jennerian and modified Jennerian approaches being successfully employed to develop various rotavirus candidate vaccines, we constructed G10-based bovine rotavirus reassortant followed by molecular and biological characterization. MA104 cells were co-infected with bovine rotavirus strain NCDV and ovine rotavirus strain LLR-85 at an m.o.i. of one, and the progeny viruses grew without selective pressure for generating resssortant. 450 plaques were picked to screen for LLR-85-based reassortant with single NCDV-VP7 gene substitution. All plaques were routinely picked for initial screening by RNA PAGE analysis, and the indistinguishable RNA segments were further analyzed by sequence analysis for confirmation. As indicated in Fig. 1, electrophoretic migration patterns of genomic RNAs of the parental viruses

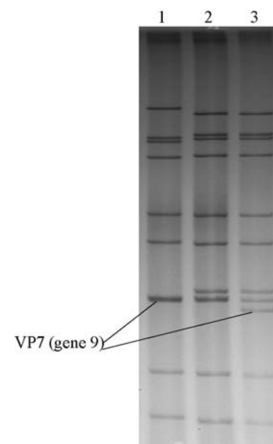

Fig. 1. Electrophoretic migration patterns of genomic RNAs of bovine rotavirus NCDV strain (lane 1), reassortant R191 (lane 2), and ovine rotavirus LLR-85 strain (lane 3) in 10% polyacrylamide gel. Genomic RNAs were electrophoresed at 10 mA for 16 h and the resulting migration patterns were visualized by staining of gel with silver nitrate.

Table 3
Comparison of nucleotide sequences between the desired reassortant and its parental viruses.

RNA genome segment no. (encoded protein)	Virus strain	Nucleotide identity (%)	
		LLR-85	NCDV
4(VP4)	R191	99.8	71.1
	LLR-85	–	71.8
5(NSP1)	R191	99.9	76.0
	LLR-85	–	76.7
6(VP6)	R191	99.7	84.4
	LLR-85	–	85.5
8(NSP2)	R191	99.9	87.6
	LLR-85	–	86.8
10(NSP4)	R191	100	81.2
	LLR-85	–	82.1
11(NSP5)	R191	100	92.7
	LLR-85	–	93.0

and the desired reassortant showed that the desired reassortant derived VP1, VP2, VP3 and NSP3 gene segments from the LLR-85 strain, and derived VP7 gene segment from the NCDV strain. The remaining gene segments could not be confirmed by RNA PAGE, and were further confirmed by sequence analysis. A comparison of VP4, NSP1, VP6, NSP2, NSP4 and NSP5 gene segments of the desired reassortant with that of the parental viruses was shown in Table 3. For the VP4, NSP1, VP6, NSP2, NSP4 and NSP5 genes, nucleotide sequence identities between the reassortant and LLR-85 were 99.8%, 99.9%, 99.7%, 99.9%, 100% and 100%, respectively, whereas nucleotide sequence identities between the reassortant and NCDV were 71.1%, 76.0%, 84.4%, 87.6%, 81.2% and 92.7%, respectively, indicating that the desired reassortant derived VP4, NSP1, VP6, NSP2, NSP4 and NSP5 gene segments from the LLR-85 strain. Thus, the ovine rotavirus LLR-85-based reassortant with a single VP7 gene of bovine rotavirus strain NCDV, named as R191 here, was confirmed by RNA PAGE (Fig. 1) and sequence analysis (Table 3). R191 as well as its parental strains NCDV and LLR-85 were propagated on confluent monolayers of MA104 cells at the equal m.o.i.

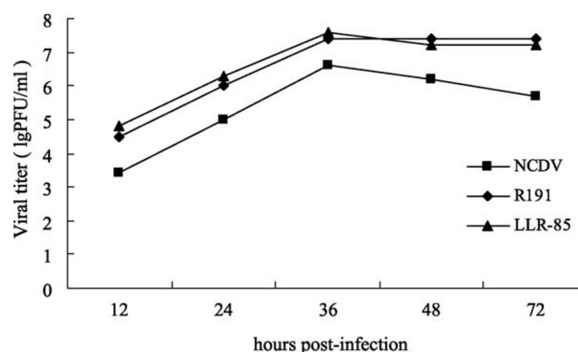

Fig. 2. One step growth curve of the reassortant R191 and its parental viruses.

of 0.1 to determine the growth property of the reassortant. Infection was terminated at postinoculation hours 12, 24, 36, 48 and 72, and the titers of the harvested viruses were determined as plaque-forming units (PFU) per ml by plaque assay technique. The results showed that the titers of the R191 and LLR-85 on peak days of virus growth were very similar ($10^{7.4}$ and $10^{7.2}$ PFU/ml, respectively) (Fig. 2), indicating that the replication efficiency of the reassortant R191 in MA104 cells was not influenced by substitution of the VP7 gene.

3.2. Serum antibody response of the inoculated calves

To evaluate the humoral immune responses induced by the oral administrations of the reassortant rotavirus R191 and one of its parental viruses LLR-85, the colostrums-deprived newborn calves without antibodies to rotavirus were orally inoculated with the reassortant R191 and/or the parental virus LLR-85 within 24 h after their birth as described in Table 2. Two calves in the group 1 were inoculated with the reassortant R191, another two calves in the group 2 were inoculated with the parental rotavirus LLR-85, and seven calves in the group 3 were simultaneously inoculated with the reassortant R191 and the parental rotavirus LLR-85. Blood samples were collected at PIDs 0, 10, 21 and 28 to prepare serum. All sera were

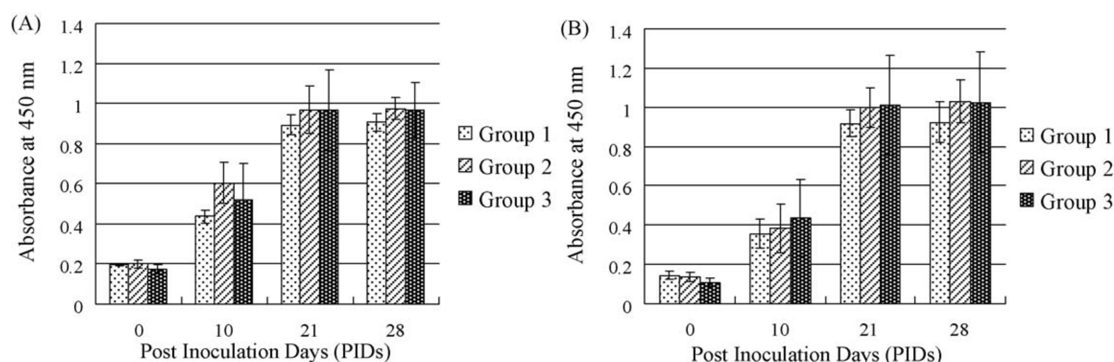

Fig. 3. Kinetics of the IgG (A) and IgA (B) antibodies to BRV in sera of the inoculated calves. The calves in the group 1 were inoculated with the R191, the calves in the group 2 were inoculated with the LLR-85 and the calves in the group 3 were simultaneously inoculated with the R191 and LLR-85. Bars indicate standard deviations.

Table 4
Titers of serum anti-rotavirus IgG and IgA antibodies in the inoculated calves at PID 21.

Anti-rotavirus antibodies	Reciprocal of serum IgG and IgA antibody titers of the inoculated calves at PID 21										
	Group 1[a]		Group 2[a]		Group 3[a]						
	1	7	8	11	2	3	4	5	6	9	10
IgG	1600	1600	800	800	3200	6400	3200	1600	3200	800	3200
IgA	800	800	800	800	1600	3200	1600	1600	1600	800	1600

[a] The calves in the group 1 were inoculated with the R191, the calves in the group 2 were inoculated with the LLR-85 and the calves in the group 3 were simultaneously inoculated with the R191 and LLR-85.

diluted hundredfold to detect IgG and IgA antibodies to group A rotavirus by indirect ELISA. The kinetics of rotavirus-specific IgG and IgA antibodies in sera of the calves inoculated with the rotavirus vaccine candidates were depicted in Fig. 3. Results showed that seroconversion of IgG and IgA antibodies to the rotavirus occurred at PID 10 in all of the inoculated calves, and increased to the peak at PID 21. Serum samples at PID 21 in all of the calves were serially twofold diluted starting at a dilution of 1:100 followed by detection of the titers of rotavirus-specific IgG and IgA antibodies to group A rotavirus by indirect ELISA. The results showed that the titers of serum IgG antibodies were between 1:800 and 1:6400 and the titers of serum IgA antibodies were between 1:800 and 1:3200 (Table 4).

3.3. Virus excretion

In order to determine the profile of virus shedding after inoculation of the rotavirus vaccine candidates, fecal rotavirus shedding was investigated each day over the 7-day experimental period postinocuation. Low-speed centrifugation supernatants of suspensions of rectal swab specimens were detected and typed by RT-PCR (Gouvea et al., 1990, 1994), and results are described in Table 2. Results showed that in the three inoculated groups during the first 7 days of oral inoculation with the rotavirus vaccine candidates, 2 of 77 fecal specimens (2.6%) collected from the 11 inoculated calves contained the inoculated rotavirus. The two rotavirus-positive specimens were collected from the same calf (no. 4) in the group 3 at the first and third day postinoculation. No inoculated rotavirus was detected in other fecal specimens collected from the inoculated calves between 1 and 7 days postinoculation. In prior study (Theil and McCloskey, 1995), rotavirus was detected in 1 of 41 daily fecal specimens collected from the calves following oral inoculation with a USDA-licensed modified live bovine rotavirus-coronavirus vaccine. In our study, the low shedding rate (2/77, 2.6%) was similar to the result (1/41, 2.4%) of previous study, indicating that our vaccine candidates are safe and well tolerated in the colostrums-deprived neonatal calves.

4. Discussion

In 1969, a previously unrecognized virus recovered from diarrheic calves in Nebraska, USA, was described and shown to induce diarrhea in experimentally inoculated, hysterectomy-derived, colostrum-deprived calves (Mebus et al., 1969). This virus was initially referred to as neonatal calf diarrhea virus, Nebraska calf diarrhea virus, reo-like virus, or reovirus-like agent but is now known as bovine rotavirus; the name rotavirus was ultimately chosen because of the wheel-like appearance of the virus on transmission electron microscopy (TEM). Today rotaviral infections are proven to be a common, economically important cause of calf diarrhea throughout the world. Genotypic analyses of a major neutralization protein VP7 of a bovine rotavirus in diarrheal stools collected in different cattle populations throughout the world have shown that the majority of typeable rotavirus isolates belong to G genotypes 6 and 10 (Alfieri et al., 2004; Chang et al., 1996; Falcone et al., 1999; Garaicoechea et al., 2006; Monini et al., 2008; Parwani et al., 1993; Reidy et al., 2006; Snodgrass et al., 1990). Since the first licensed bovine rotavirus vaccine was a monovalent one, monovalent rotavirus vaccine cannot completely protect against heterologous rotavirus infection (Feng et al., 1994). We constructed a reassortant rotavirus strain R191 that contains a single VP7 gene encoding genotype G6 specificity of bovine rotavirus NCDV strain and the remaining 10 genes of ovine rotavirus strain LLR-85. The reassortant R191 and its parental rotavirus strain LLR-85 were used as bivalent vaccine candidates to provide: (i) an attenuation phenotype of an ovine rotavirus in bovines; and (ii) antigenic coverage for G6 and G10 of the main bovine rotavirus genotypes. The attenuated virus strains R191 and LLR-85 were shown experimentally in colostrum-deprived neonatal calves to be able to induce high levels of serum IgG and IgA antibodies to rotavirus, whereas fecal shedding of the rotaviruses was uncommon in the inoculated calves, and therefore are promising bivalent vaccine candidates for prevention of bovine G6 and G10 rotavirus infection.

The benefits for using the LLR-85 strain instead of an attenuated bovine rotavirus strain in production of the reassortant for vaccine purposes are that the former is attenuated in calves due to host range restriction, but the latter is often unstable and may develop back mutation, thus the LLR-85 is more safe to calves; secondly, the passage history of the LLR-85 is clear, it is an attenuated strain to lambs and children (Bai et al., 1994) therefore should be safe to people and there is no public health problem; thirdly, the RNA segments of the LLR-85 are easily distinguished on the PAGE electrophoretic migration pattern, which is useful to identify the reassortant; lastly and importantly, the LLR-85 strain is highly immunogenic to neonatal calves.

Some nucleotide mutations of the VP4, NSP1, VP6 and NSP2 gene segments of the R191 occurred in the process of

generation and purification of the reassortant. There existed the insignificant differences of 2 nucleotides (nt), 1nt, 1nt and 1nt between the R191 and the parental LLR-85 for the VP4, NSP1, VP6 and NSP2 gene segments, respectively. Obviously, the possible reasons to induce these nucleotide mutations were serial tissue culture passage for the six-time plaque purifications of the reassortant R191 on MA104 cells.

The mechanisms responsible for immunity to rotavirus infections and illness are not completely understood in animals (Knipe and Peter, 2007). Animal models have been particularly instructive in elucidating the role of antibodies and in dissecting the relative importance of systemic and local immunity. Initially, the observations of some related studies suggested that antibodies in the lumen of the small intestine were the primary determinant of resistance to rotavirus illness, whereas circulating serum antibodies failed to protect (Bridger and Woode, 1975; Offit and Clark, 1985; Snodgrass and Wells, 1976; Woode et al., 1975). But the subsequent study showed that systemic rotavirus antibodies are present in the lumen of the gastrointestinal tract of neonatal calves if the level of circulating antibodies is sufficiently high. Such serum-derived mucosal antibodies can provide protection against experimentally induced infection and diarrhea (Besser et al., 1988). The association between the titers of serum anti-rotavirus IgG and IgA antibodies and protection against infection and illness was investigated in 100 children under 18 months of age attending day care centers in the United States for one or two rotavirus seasons (O'Ryan et al., 1994). These studies showed that preexisting serum anti-rotavirus IgA titer of greater than 1:200 or IgG titer of greater than 1:800 was associated with protection against infection. A similar study of Mexican infants also demonstrated that both serum IgA and IgG were protective, but the titers needed to achieve significant protection were higher than those found in the US study (Velazquez et al., 2000). These previous studies about the association between serum anti-rotavirus antibodies and protection against infection and illness suggested that serum antibodies, if present at critical levels, are either protective themselves or are an important and powerful correlate of protection against rotavirus disease, even though other host effectors may play an important role as well. In this study, we want to evaluate the immunogenicity of the rotavirus vaccine candidates, so we used the major structural protein in virus particles of the semipurified BRV as antigen to detect the IgG and IgA antibodies against the group A rotavirus in serum of the inoculated calves. And the results showed that the highest titers of serum IgG and IgA antibodies were obtained at PID 21, the calves in all inoculated groups developed high levels of serum anti-rotavirus IgG antibodies (range 1:800 to 1:6400) and serum anti-rotavirus IgA antibodies (range 1:800 to 1:3200). These results suggested that the animals inoculated with LLR-85-based vaccine candidates would develop antibodies to the inoculated viruses, and indicated that the attenuated virus strains LLR-85 and R191 were well immunogenic to neonatal calves, and preexisting serum anti-rotavirus IgA titers (range 1:800 to 1:3200) reached to level of protective immunity. However, further evaluation is needed for a protective immune response of the LLR-85-based candidate vaccines in future trials.

To date, there is no criterion for fecal shedding rate of the rotavirus vaccine strains. In prior study, vaccine rotavirus was detected in only 1 of 41 daily fecal specimens collected from the calves following oral inoculation with a USDA-licensed modified live bovine rotavirus–coronavirus vaccine by using negative stain electron microscopy. In contrast, rotavirus was demonstrable by the same negative stain electron microscopic examination procedure in 17 of 19 fecal specimens collected from the calves after inoculation with virulent bovine rotavirus field strains (Theil and McCloskey, 1995). In our study, vaccine rotavirus was detected in only 2 of 77 daily rectal swab specimens collected from 11 neonatal calves following oral inoculation with the rotavirus vaccine candidates. This low shedding rate (2/77, 2.6%) was similar to the result of previous study (1/41, 2.4%), suggesting that the R191 and LLR-85 are safe to calves based on the uncommon fecal shedding of the rotavirus.

The procedure that we have used in the present study for the successful construction and selection of the single VP7 gene substitution reassortant rotavirus vaccine is relatively easy to perform. In recent years, rare genotypes of bovine rotavirus (such as G5 and G15.) were also isolated in calves (Ghosh et al., 2008; Park et al., 2006). So with this procedure, additional LLR-85-based reassortant rotavirus vaccine candidates that carry VP7 gene encoding a desired bovine rotavirus G genotype specificity can be generated readily in the future when the need for such G genotype(s) is warranted from epidemiologic studies.

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