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Serological and genotypic characterization of group A rotavirus reassortants from diarrheic calves born to dams vaccinated against rotavirus¹

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Abstract

Two strains of bovine rotavirus (BRV), designated strain Nebraska Scottsbluff-1 (NS-1) and NS-2, were isolated from 2 neighboring cow-calf beef cattle ranches where dams had been vaccinated with a commercial vaccine containing group A BRV strain Neonatal Calf Diarrhea Virus (NCDV)-Lincoln (P1:G6). Northern blot hybridizations using whole genomic RNA probes indicated that strains NS-1 and NS-2 had identical group A RNA electrophoretic patterns and were homologous at all gene segments. Strain NS-1 was compared with reference group A BRV strains using serological and genotypic methods. In vitro virus neutralization assays indicated that strain NS-1 was neutralized by a G6-specific neutralizing monoclonal antibody (mAb) and guinea pig hyperimmune serum (GPHS) raised against BRV strain B641 (P5:G6), but not by G10-specific neutralizing mAb or GPHS raised against BRV strain B223 (P11:G10). Nucleic acid hybridization experiments using whole-genomic RNA probes revealed that gene segment 4 of strain NS-1 differed from BRV strains NCDV-Lincoln and B223, but hybridized with strain B641. Conversely, gene segment 5 of strain NS-1 hybridized with BRV strain B223, but not with BRV strains NCDV-Lincoln and B641. A G-specific cDNA probe produced by reverse transcription polymerase chain reaction (RT-PCR) amplification of strain NS-1 hybridized specifically only with G6 strains NCDV-Lincoln and B641, but not with G10 strain B223. Co-electrophoresis experiments using strains NS-1, B641, and B223 further confirmed these results, suggesting that strain NS-1 was a naturally-occurring reassortant BRV between

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strains B641 and B223. Taken together these results indicated that a naturally-occurring group A BRV reassortant with a P gene different from the vaccine virus was responsible for the diarrheal syndrome observed on both ranches. Results from this study also indicate the existence of at least 2 different gene segments 5 among group A BRV infecting cattle.

Keywords: Rotavirus; Cattle, rotavirus; Diagnosis, rotavirus; Diarrhoea; Vaccination

1. Introduction

Bovine rotavirus is an important etiological agent of neonatal calf diarrhea worldwide. Economic losses due to BRV infection in the cattle industry include losses associated with retarded growth, medication costs and animal death. Rotavirus is a member of the *Reoviridae* family and the viral genome is composed of 11 segments of double-stranded RNA which are surrounded by a double-shelled capsid (Estes and Cohen, 1989). Currently, rotaviruses are classified into 7 groups (A through G) based on antigenic differences on the inner capsid protein (VP6). Group A rotaviruses which are commonly associated with neonatal calf diarrhea (Theil, 1989), contain 2 major outer capsid proteins, P or VP4 and G or VP7 encoded by gene segments 4 and 8 or 9, respectively (Estes and Cohen, 1989). The P and G proteins contain antigenic epitopes which are involved in neutralization of viral infectivity (Hoshino et al., 1985; Offit et al., 1986). Based on antigenic differences in the P and G proteins, group A rotaviruses have been classified into 11 different P-types and 14 different G-types (Estes and Cohen 1989; Isegawa et al., 1992; Hussein et al. 1993; Parwani et al. 1993).

At least 8 different BRV G-types have been associated with neonatal calf diarrhea (Mebus et al. 1971; Woode et al., 1983; Matsuda et al., 1990, Snodgrass et al., 1984, Snodgrass et al., 1990; Hussein et al. 1993; Parwani et al., 1993). In the United States, type G6 appears to be most widespread, with type G10 accounting for a lower percentage of infections (Parwani et al., 1993). Limited information is available on the prevalence of BRV P types in cattle. However, one study indicates 3 different BRV P-types in the United States, with P5 being the most prevalent (Parwani et al., 1993). Reference strains for each P- and G-types known to infect cattle include strains NCDV-Lincoln (P1:G6), United Kingdom (UK) and B641 (P5:G6), and B223 (P11:G10) (Woode et al., 1983; Zheng et al., 1989; Matsuda et al., 1990; Hardy et al., 1991, Hardy et al., 1992).

A vaccine for prevention of rotavirus-associated neonatal calf diarrhea has been commercially-available in the United States for over 20 years (Sharpee et al., 1988; Saif and Jackwood, 1989). However, BRV continues to be routinely identified in feces of calves with diarrhea, often from herds where vaccination of the dams with BRV vaccines is current (G. Duhamel, personal observation). There has been no detailed studies on the genetic background of BRV causing calf diarrhea on cattle ranches where breeding stock are vaccinated with the commercial vaccine. In this paper, we present serological and genotypic characterizations of BRV strains isolated from newborn calves with diarrhea that were born to dams vaccinated with commercially-available BRV vaccines in beef cattle ranches in Nebraska, United States.

2. Materials and methods

Case history. Outbreaks of neonatal calf diarrhea occurred in the spring of 1991 on 2 separate but adjacent beef cow-calf ranches in western Nebraska. Clinical aspects of these outbreaks including data on the vaccination schedule with commercially-available vaccines containing BRV strain NCDV-Lincoln have been described elsewhere (Grotelueschen et al., 1992). The age of the calves at the onset of diarrhea ranged from 3 days to 15 days on ranch 1, and morbidity associated with diarrhea was approximately 80% in calves from heifers and 25% in calves from cows. Mortality records were not available from ranch 1. On ranch 2, 11.6% of the calf crop developed diarrhea at approximately 21 days of age and 25% of the affected calves died as a result. Fecal samples obtained from calves with diarrhea on each ranch were submitted to the Nebraska Veterinary Diagnostic Laboratory System for routine laboratory examinations. Rotavirus was the only significant pathogen identified.

Reference virus strains. Strain NCDV-Lincoln was obtained from American Type Tissue Culture Collection, Rockville, Maryland. Strains B641 and B223 were kindly provided by Dr. Gerald Woode (College of Veterinary Medicine, Texas A and M University). All the reference viruses were grown onto Rhesus monkey kidney (MA-104) cell monolayers.

Virus isolation and identification. Fecal samples positive for the presence of rotavirus particles by transmission electron microscopy were diluted in Minimum Essential Medium (MEM), centrifuged at 1000 *g* for 20 min at room temperature, and the supernatant was ultracentrifuged at 25 000 rpm (Beckman SW 28 rotor) for 2 h at 10°C. The pellets were mixed with an equal volume of phosphate buffered saline (PBS, pH 7.4) containing 1 mg/ml gentamicin sulfate and 25 µg/ml amphotericin B, centrifuged at 1000 *g* for 30 min at room temperature, and the supernatant was kept overnight at 4°C. After treating each sample with trypsin (10 µg/ml; Type III, Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C, the samples were inoculated in duplicate onto washed MA-104 cell monolayers grown in roller tubes (16×25 mm; Costar, Cambridge, MA). After adsorption for 1 h at 37°C, the inoculum was removed and the cell monolayers were washed twice with MEM before adding serum free MEM containing 1 µg/ml of trypsin, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The inoculated cultures were maintained at 37°C and examined daily for cytopathic effect. After 6 days, the cells were frozen and thawed 3 times, and the lysate was passaged onto fresh monolayers 2 more times. The presence of rotavirus was confirmed by staining infected monolayers with fluorescein-conjugated calf anti-BRV antibodies (FITC-Calf anti BRV; National Veterinary Service Laboratory, Ames, IA). Cytopathic BRV isolates, designated Nebraska Scottsbluff 1 (NS-1) and NS-2 from ranch 1 and ranch 2 respectively, were plaque purified after the third passage, as previously described (Hoshino et al., 1984). Virus stock was kept at –20°C until needed. The RNA from each field BRV strain was extracted with phenol-chloroform and their RNA electrophoretic patterns were compared with reference BRV strains after separation on a discontinuous, vertical sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (10% resolving, 5% stacking) run in Tris buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS [pH 8.3]) at 4°C using a constant current of 30 mA (Biorad Protean II electrophoresis unit, Biorad, Richmond, CA). The electropherotype of each virus individually and after co-electrophoresis with reference BRV strains B641 and B223 was visualized after staining with ethidium bromide.

Monoclonal and polyclonal antibodies. Eight- to 10-week old BALB/c ANn mice were immunized intraperitoneally with 10^6 fluorescent focus units of BRV strain NCDV-Lincoln in equal volumes of Freund's complete adjuvant. Identical booster injections were given 5 weeks later. Three weeks after the last booster injection and 2 days prior to harvest of splenocytes, the mice were immunized intravenously with NCDV-Lincoln diluted in PBS. Mouse splenocytes were fused with NS-1 myeloma cells, and the culture supernatants from hybridomas were screened for the presence of neutralizing antibody against the homologous BRV (Coulson et al., 1985). After subcloning twice by limiting dilution, ascites tumors were produced as previously described (Galfre et al., 1977). The VP7 specificity of mAb 142/36 was confirmed by radioimmunoprecipitation (Harlow and Lane, 1988). mAb B223-N7, specific for VP7 of BRV strain B223 was provided by Dr. Gerald N. Woode (Zheng et al., 1989). Guinea pig hyperimmune sera (GPHS) were produced against BRV strains B641 and B223 by repeated immunization of rotavirus-seronegative guinea pigs with gradient purified virus mixed in equal volumes of Freund's complete (first immunization) or incomplete (subsequent boosters) adjuvant.

Serological characterization of field BRV. The serological relationship between the BRV field strain NS-1, and reference strains B641, NCDV-Lincoln and B223 was determined using fluorescent focus neutralization assays (FFN; Knowlton et al. 1991) with modifications. Briefly, 100 μ l of 2-fold dilutions of mAb or heat-inactivated (30 min at 56°C) GPHS was mixed with 100 μ l of virus (200 TCID₅₀) in 96-well culture plates (6 wells per dilution) and incubated for 1 h at 37°C. The virus-antibody mixture then was transferred to fresh MA-104 cell monolayers grown in 96-well culture plates. The plates were centrifuged at 1000 *g* for 1 h at room temperature. After 20 h of incubation at 37°C in a CO₂ atmosphere, the cell monolayers were fixed with 80% acetone in water and stained with FITC-Calf anti BRV (NVSL). The neutralization titer was expressed as the reciprocal of the highest dilution of antibody which completely inhibited fluorescence.

Northern blot with whole genomic RNA probes. Whole genomic RNA probes of field and reference BRV strains were synthesized and labelled with α -³²P-GTP (3000 Ci/mmol, Amersham Co., Arlington Heights, IL) by in vitro transcription from purified single-shelled viral cores (Flores et al., 1982). RNA from each virus was separated by SDS-PAGE, blotted onto nylon membranes (Amersham), and fixed by irradiation with ultraviolet light (312 nm) for 10 min. Northern blot hybridizations were performed as previously described (Hesse et al., 1993), except that the membranes were prehybridized for 4 h and hybridized for 18 h.

G-type classification of field BRV. Two primers, complementary to nucleotides 51 to 71 of the minus strand and nucleotides 376 to 391 of the plus strand of the VP7 gene of group A rotavirus (Flores et al., 1991) were synthesized (National Sciences, Plymouth, MN). After denaturation at 80°C for 5 min, purified viral RNA from strains NS-1, B641, NCDV-Lincoln and B223 was used as RNA template and reverse transcribed (RT) into single stranded cDNA by polymerase chain reaction (PCR) as described by the manufacturer (GeneAmp RNA PCR kit, Perkin Elmer Cetus, Norwalk, CT) using a thermal cycler (GeneAmp™ 9600, Perkin Elmer Cetus). The final volume of the RT mixture was 20 μ l [2.5 U/ μ l reverse transcriptase, 5 mM MgCl₂, 1 × PCR buffer, 1 mM dNTPs, 1 U/ μ l RNase inhibitor and 50 pmol of each primer]. Incubation time and temperature for RT were 25 min at 42°C, 5 min at 99°C and 5 min at 4°C. The resulting cDNA was amplified by

adding *Taq* polymerase and the volume of the PCR reaction was adjusted to 100 μ l (final concentration: 2 mM MgCl₂, 1 \times PCR buffer and 2.5 U/100 μ l *Taq* polymerase). Amplification consisted of 30 cycles with 40 sec at 94°C for denaturing; 1 min at 42°C for annealing and 2 min at 72°C for extension in each cycle. The PCR products were separated by electrophoresis in 1% agarose gels (Seaplaque GTG, FMC Rockland, ME) and transferred to nylon membranes by Southern blotting. The PCR amplified cDNA fragments from strains NS-1 and B223 were purified from agarose gel (BIO 101, LA Jolla, CA), and labelled with α -³²P-dCTP (3000 Ci/mmol, Amersham) using an oligo-labelling kit (Pharmacia LKB Biotechnology, Piscataway, NJ). Each radioisotope labelled cDNA probe was hybridized with a Southern blot membrane using the same conditions as for Northern blot hybridizations described above.

3. Results

Virus isolation and identification. Cytopathic BRVs were isolated from fecal samples obtained from calves with diarrhea on both ranches. Presence of BRV was confirmed based on the ultrastructural appearance of the virus particles and positive fluorescence by direct fluorescent antibody staining. SDS-PAGE indicated that strains NS-1 and NS-2 had identical RNA electropherotypes consisting of a 4-2-3-2 RNA migration pattern characteristic of group A rotaviruses (Fig. 1). Comparison of electropherotypes with reference BRV strains revealed that gene segment 4 of strain NCDV-Lincoln had a lower molecular weight than

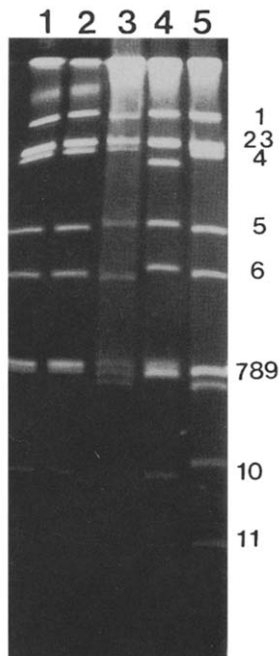


Fig. 1. RNA electrophoretic pattern of reference and field BRV strains. Lane: 1, NS-1; 2, NS-2; 3, B641; 4, NCDV-Lincoln; 5, B223.

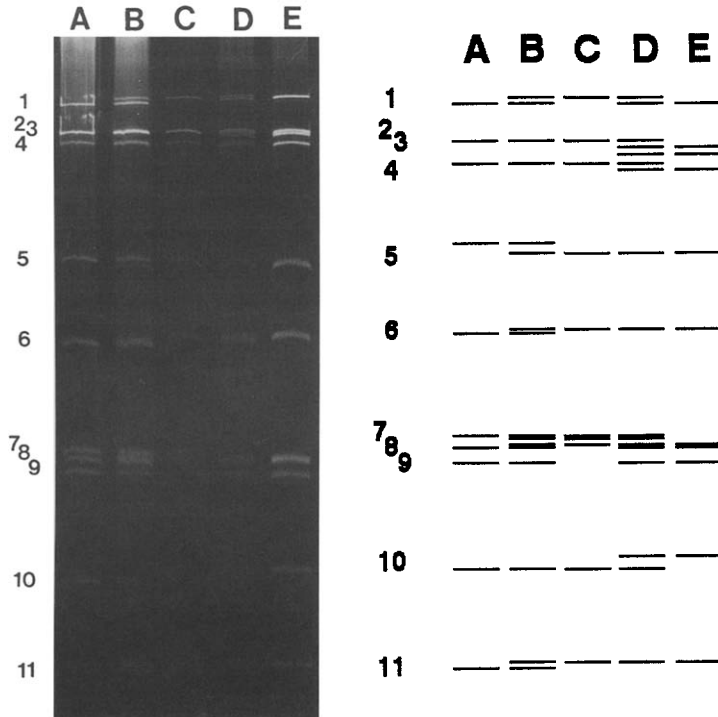


Fig. 2. Co-electrophoresis of reference and field BRV strains. Lanes: A, B641; B, B641/NS-1; C, NS-1; D, NS-1/B223; E, B223.

other BRVs and gene segment 10 of strain B223 moved slower than other BRVs (Fig. 1). By co-electrophoresis gene segments 2, 3, 4, 7, and 10 of strain NS-1 migrated similarly to strain B641, whereas gene segments 5, 6, and 11 had migration patterns identical to strain B223 (Fig. 2). In contrast, gene segments 1 and 9 of strain NS-1 migrated differently from either reference strain B641 and B223, and gene segment 8 could not be resolved.

Table 1
Antigenic relationships of group A bovine rotaviruses

Rotavirus strain	FFN antibody titer with antibodies to: ^a			
	G10-MAb ^b	B223-GPHS ^c	G6-MAb ^d	B641-GPHS ^e
B223	5120	5120	< 80	< 40
NS-1	< 80	< 40	5120	5120
B641	< 80	< 40	10 240	5120
NCDV-Lincoln	< 80	< 40	10 240	5120

^aReciprocal of the highest dilution of the test antibody that completely inhibited fluorescence.

^bG10-MAb: G10-specific monoclonal antibody B223-N7.

^cB223-GPHS: Guinea pig hyperimmune serum to BRV strain B223.

^dG6-MAb: G6-specific monoclonal antibody 143/36.

^eB641-GPHS: Guinea pig hyperimmune serum to BRV strain B641.

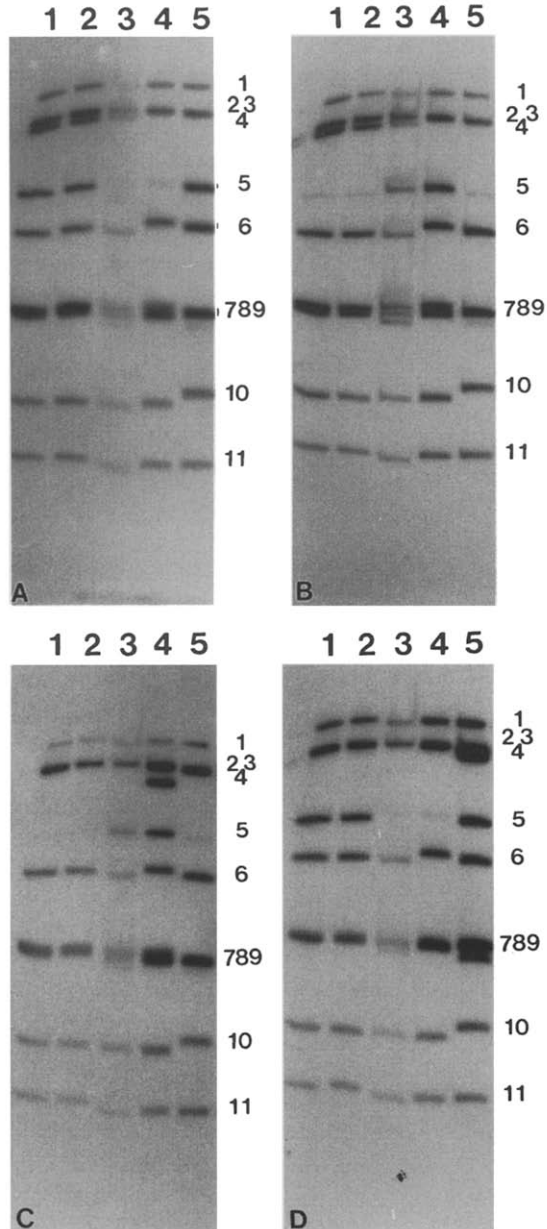


Fig. 3. Northern blot hybridization between reference and field BRV strains using whole genomic RNA probes prepared from strains NS-1 (A); B641 (B); NCDV-Lincoln (C) and B223 (D). Lanes: 1, NS-1; 2, NS-2; 3, B641; 4, NCDV-Lincoln; 5, B223.

Serological characterization of field BRV. Results of FFN assays of the field and reference BRV strains are summarized in Table 1. Strain NS-1 was neutralized by the mAb 143/36 and the GPHS raised against BRV strain B641, but not by the mAb B223-N7 or the GPHS raised against BRV strain B223.

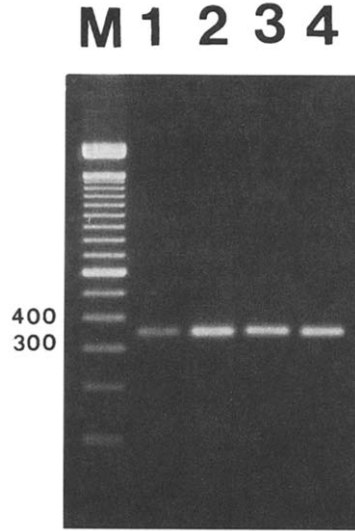


Fig. 4. RT-PCR products obtained with reference and field BRV strains. Lanes: M: 100 bp DNA ladder; 1, NS-1; 2, B641; 3, NCDV-Lincoln; 4, B223.

Northern blot with whole genomic RNA probes. Northern blot studies with whole genomic RNA probes indicated that each virus probe hybridized to all gene segments of its homologous virus (Fig. 3). Additionally, strain NS-2 hybridized at all gene segments with the NS-1 whole genomic RNA probe. Although, the field and reference strains of BRV had a high degree of homology at gene segments 1,2,3,6,10 and 11, gene segment 4 of strain NS-1 hybridized only with gene segment 4 of strain B641, but not with strains NCDV-Lincoln and B223. Additionally, gene segment 5 of strain NS-1 hybridized with strain B223, but not with strains NCDV-Lincoln and B641. Gene segments 7, 8 and 9 migrated in close proximity, making it impossible to determine their origin.

G-type classification of field BRV. The PCR products obtained from BRV strains NS-1, B641, NCDV-Lincoln, and B223 consisted of a cDNA fragment of approximately 340 nucleotides (Fig. 4). Cross-hybridization experiments using α -³²P-dCTP labelled G-spe-

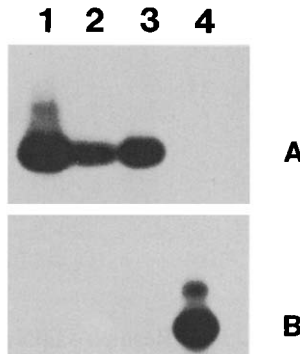


Fig. 5. Southern blot hybridization between reference and field BRV strains using G-type specific cDNA probes from strains NS-1 (A) and B223 (B). Lanes: 1, NS-1; 2, B641; 3, NCDV-Lincoln; 4, B223.

cific cDNA probes showed that the G-specific cDNA probe made from strain NS-1 hybridized with G6 strains B641 and NCDV-Lincoln, but not with G10 strain B223. Conversely, the G10-specific probe made from strain B223 hybridized only to itself, and not to strains NS-1, B641 and NCDV-Lincoln (Fig. 5).

4. Discussion

Two strains of BRV, designated NS-1 and NS-2, obtained from diarrheic calves from 2 cow-calf beef cattle ranches where severe BRV-associated calf diarrhea occurred despite vaccination of the dams with BRV strain NCDV-Lincoln (P1:G6) were characterized. PAGE and Northern blot analyses revealed that the field strains NS-1 and NS-2 had identical group A rotavirus electropherotypes and cross-hybridized completely at all gene segments, suggesting that a single strain of BRV was responsible for the diarrheal syndrome observed on both ranches.

Group A rotaviruses have been assigned to different G-types on the basis of serological methods including neutralization, plaque reduction, and enzyme-linked immunosorbent assays using serotype-specific mAbs and monospecific polyclonal antisera (Estes and Cohen, 1989). More recently, nucleotide sequence analyses has shown that rotaviruses from the same G-type have a high degree of sequence homology in their variable regions, and this is the basis for production of G-type specific differential probes (Flores et al., 1991). The oligonucleotide primer pair used in this study generated a 340 nucleotide cDNA fragment representative of the variable region of each of the field and reference BRV strains examined. Specific hybridization of the cDNA probe obtained from strain NS-1 with G6 BRV strains NCDV-Lincoln and B641, but not with G10 BRV strain B223 indicated that strain NS-1 belonged to the same G-type as the vaccine strain used on these ranches. Serological studies confirmed this result.

Northern blot hybridization studies with whole genomic RNA probes revealed that gene segment 4 of strains NS-1 hybridized with strain B641, but not with the vaccine strain NCDV-Lincoln, suggesting that strains NS-1 and B641 belonged to the same P-type which is different from strain NCDV-Lincoln. This result is in agreement with the sequence analysis data of Hardy and co-workers (1991). The results from our study also support recent reports indicating the existence of at least 3 distinct gene segment 4 among BRV in the United States (Hardy et al., 1992; Parwani et al., 1993). Since gene segment 4 of strain B641 has been shown to have a high degree of sequence homology with that of strain UK (Hardy et al., 1991), BRV strains UK, B641 and NS-1 most likely belong to the same P-type.

Although strains B641 and NS-1 had the same P- and G-genes, co-electrophoresis and whole genomic RNA hybridization studies revealed that they had different RNA electropherotypes and gene segment 5, respectively. Gene segment 5 of strain NCDV-Lincoln cross-hybridized with that of strain B641, but not with strains B223 and NS-1. Conversely, gene segment 5 of strain B223 hybridized with that of strain NS-1. Our results thus indicate the existence of at least 2 different gene segment 5 among group A BRV. Gene segment 5 encodes the nonstructural protein NS35 (Estes and Cohen, 1989), but the exact function of this protein in the biology of rotavirus infection is unknown. However, it has been noted

that gene segment 5 displays non-random segregation during rotavirus mixed infections in vivo and in vitro (Gombold and Ramig, 1986). It is possible that strain NS-1 gained a selective advantage over other strains of BRV carrying NCDV-Lincoln gene segment 5.

The segmented nature of the rotavirus genome allows reassortment of individual viral gene segment between 2 or more different viruses during mixed infection (Estes and Cohen 1989). Rotavirus reassortants have been made experimentally using in vitro and in vivo methods (Gombold and Ramig, 1986; Urasawa et al., 1986; Hesse et al., 1993). Based on this evidence, a rotavirus strain potentially can contain any combinations of genes in nature. Naturally-occurring rotavirus reassortants with gene segments derived from different human rotaviruses have been reported (Matsuno et al. 1988; Nakagomi and Nakagomi, 1991). Results from our study suggest that strain NS-1 is a naturally-occurring BRV reassortant with gene segments derived from BRV strains B641 and B223.

Currently, prevention of rotavirus-associated neonatal calf diarrhea is based on passive protection by maternal antibodies following immunization of the dam (Sharpee et al., 1988; Saif and Jackwood, 1989). Calves are protected from rotavirus infection when receiving colostrum containing high titer of rotavirus-specific neutralizing antibodies. It is well established that the P and G proteins have antigenic epitopes involved in neutralization of viral infectivity in vitro and protection in vivo and can segregate and elicit neutralization antibodies independently (Hoshino et al. 1985; Offit et al., 1986). Although the information on homotypic and heterotypic immunity and protection is incomplete, it is likely that passive protection with monovalent vaccine is effective only against challenge with BRV homologous to the vaccine strain at the P and G genes. In the United States, commercially-available rotavirus vaccines for cattle contain only strain NCDV-Lincoln. However, it is now becoming clear that in contrast to natural recovery from infection which results in high titers of P-specific neutralizing antibodies, parenteral immunization primarily elicits G-specific neutralizing antibodies (Shaw et al., 1988; Richardson et al., 1993; Ward et al., 1993). Differences in P proteins has been proposed as a possible mechanism responsible for a lack of cross-protection in vivo between G6 BRV strains NCDV-Lincoln and B641 (Zheng et al., 1989). We have shown that strains NS-1 and NCDV-Lincoln belong to the same G6, but differ at gene segment 4 encoding the P protein, and gene segment 5 encoding protein NS35. Our results are in agreement with the assumption made by Zheng and coworkers (1989), and further suggest that failure of passive protection provided by monovalent vaccine could result from infection of susceptible calves with a BRV containing a P gene different from the vaccine strain. The benefits from vaccination with monovalent vaccine for prevention of BRV-associated diarrhea in neonatal calves may be less than optimal due to diversity of P- and G-types occurring in nature.

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