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GENETIC CHARACTERIZATION AND PATHOBIOLOGICAL STUDIES OF
BOVINE GROUP A, B AND C ROTAVIRUSES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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ABSTRACT

Group A rotaviruses cause diarrhea in young children and animals, including calves. Both group B and C rotaviruses occur in sporadic cases of diarrhea in young calves and adult cattle. Characterization of the VP7 (G type) and VP4 (P type) genes of group A bovine rotaviruses (BRV) from field samples was performed using RT-PCR/RFLP analysis. When these samples were analyzed for G and P type specificity, the P[5]G6 type was most prevalent accounting for 46.7% (41/86) of the samples; 12.8% (11/86) were P[11]G10; 7% (6/86) were P[5]G10 and an equal number were P[11]G6 subtype. The VP7 genes of the G6 subtypes C-8336 and MC27 showed high homology to each other (~98%) and with other bovine G6 subtypes and showed lower, but substantial homology (80-87% nucleotide sequence homology) with human G6 subtypes and prototype G6 BRVs. Serologically, the C-8336 strain was neutralized by a G6 monoclonal antibody (IC3) and showed 4- to 16-fold differences in antibody titers with G6 BRV NCDV and IND in 2-way neutralization tests.

Ninety calf diarrhea fecal samples and 81 adult cow diarrhea fecal samples (winter dysentery) were tested for group B rotaviruses. By RT-PCR, five (5.5%) calf samples and 15 (18.5%) adult cow diarrhea samples were group B rotavirus positive. The VP7 genes of WD634tc, ATI and Mebus group B BRVs showed high (over 90%) nucleotide
and deduced amino acid homologies among them, and lower homologies (45 - 57 %) with interspecies group B rotaviruses. The WD653, ATI and Mebus strains caused diarrhea in gnotobiotic calves within 3 days post-inoculation and group B rotaviruses were detected in feces by RT-PCR, PAGE and IEM.

WD534tc group C rotavirus was isolated in monkey kidney (MA104) cells from a diarrheic adult cow fecal sample. Genetically and serologically, the WD534tc/C strain was more closely related to the Cowden porcine group C strain than to the Shintoku bovine strain. When the pathogenesis of WD534tc/C alone or combined with group A BRV was examined in gnotobiotic calves, the coinfection of calves with virulent BRV IND/A enhanced fecal shedding of the bovine group C rotavirus and the extent of histopathological lesions in the intestine. These findings suggest a potential new, novel hypothesis involving dual infection for the adaptation of heterologous rotaviruses to new host species.
Dedicated to My Family
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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Rotaviruses, members of the Family Reoviridae, have a triple layered capsid and a genome that consists of 11 segments of double stranded RNA (dsRNA) (68, 117). Rotaviruses contain 6 structural proteins of which VP1, VP2 and VP3 comprise the core, VP6 is the major inner capsid protein and VP4 and VP7 are the outer capsid proteins (68). Rotaviruses are divided into 7 (A to G) morphologically indistinguishable but antigenically distinct serogroups based on VP6 encoded by genes 5 or 6 (68, 117). The representative prototypes of seven serogroups are follows: group A (A / conventional rotaviruses); group B (B / human ADRV, rat IDIR and porcine NIRD-1); group C (C / human Bristol, bovine Shintoku and porcine Cowden); group D (D / avian 132); group E (porcine DC-9); group F (F / avian A4); and group G (G / avian 555) (23, 197, 200).

Group A rotaviruses are a major cause of diarrhea in humans and animals, including calves. In humans, group A rotaviruses are responsible for the death of about 1 million children per year worldwide (10, 117). In cattle, group A rotaviruses also cause economic
losses of nearly $500 million per year due to neonatal calf diarrhea in the U.S. (97). Unlike group A rotaviruses, the detection of non-group A rotaviruses is still rare (except in poultry and lambs) in humans and animals. However, during this past decade, group B and C rotaviruses have been associated with epidemic diarrhea in older children and adults and possibly with food- and water-borne transmission, which is different epidemiologic pattern from group A rotaviruses whose transmission is by direct fecal-oral contact (23, 197, 200).

Both group B and C rotaviruses have been detected in young calves and adult cattle with diarrhea, and recent studies suggest these nongroup A rotaviruses, especially group B rotaviruses, may be emerging pathogens in cattle (1, 23, 40, 142, 171). Group B bovine rotaviruses do not grow in cell culture, which is a major limitation for their characterization and study. Although serological evidence exists for bovine group C rotaviruses in the U.S., there are no reports of their isolation: to date, the Shintoku group C bovine rotavirus, which was isolated in Japan from an adult cow, is the only bovine isolate of group C rotavirus (232, 234).

1.2 Group A rotaviruses

Structure

Rotaviruses are non-enveloped and triple shelled particles (triple shell: 65-70 nm; double shell: 55-60 nm; and core: 40nm). The outer and inner shells surround a core which contains the viral genome of 11 dsRNA segments (68). When negatively stained, rotaviruses appear to have an outer smooth, circular margin with spokes (117, 183). The outer shell of
rotaviruses is composed of two proteins, VP7 and VP4. Three dimensional structural studies using electron-cryomicroscopy (183, 262) showed an unique feature of the rotavirus structure: the presence of aqueous channels in the outer shell (68). These channels are believed to be involved in importing enzymes for RNA transcription and exporting viral mRNA transcripts during virus infection (182). The inner shell of rotaviruses is composed of the protein VP6 (780 molecules per virion). Three VP6 proteins cluster together to form a trimer which encloses a 20 angstrom-diameter channel. The trimers are shaped like knobs and interact with VP4 and VP7. VP6 trimers are further organized in a hexamer which encloses a larger channel (60 angstrom diameter). These channels in the inner shell coincide with the aqueous channels in the outer shell and provide a link between inner and outer shells (183).

The outer capsid protein VP7 consists of 780 molecules arranged around 132 aqueous channels. The dimeric VP4 consists of 60 spikes (120 molecules) extended from the outer capsid surface and can serve as the viral hamagglutinin (183). The core is composed of VP1, VP2 and VP3, and surrounds the viral genome.

**Genome and Proteins**

The average size of the rotavirus genome (all segments combined) is 18,550 base pairs (58). The genome consists of 11 segments (each segment codes for a single protein) of dsRNA: six of these segments code for viral structural proteins (VPs) and the remaining five code for non-structural proteins (NSPs) (16).

1) Structural proteins
VP1 (gene 1) is a subcore protein present in a few copies and functions as the minor viral RNA-dependent RNA polymerase (16). VP1 has an affinity to positive sense RNA (mRNA), which may initiate viral replication after recruiting VP2 (175).

VP2 (gene 2) is the most abundant protein (approximately 200 molecules per virion) of the central core and has the ability to bind to single stranded (ss) RNA and dsRNA non-specifically (134). VP2 has been identified as a major component of the RNA polymerase complex which is responsible for transcription and replication of rotaviruses (16, 174). Since VP2 has affinity to dsRNA, it was suggested that VP2 is also responsible for packaging of the viral genome (11 dsRNA) into the virion (174).

VP3 (gene 3) is a minor component of the central core and is about one-third as abundant as VP1. During infection, rotaviruses transcribe capped mRNA containing the 7mGpppGmp cap at the 5’end. VP3 has guanylyltransferase activity which is involved in the capping of rotavirus mRNA (16, 68, 177). Also, it is suggested that VP3 may be associated with viral transcription as an associated phosphatase or ATPase activity (174).

VP4 (gene 4) is a multifunctional protein involved in cell penetration, hemagglutination, neutralization and virulence (24, 174). VP4 forms dimers and constitutes the spikes located on the outer surface of the rotavirus virion, and is involved in the attachment of the virus to cells (174). Proteolytic enzymes cleave VP4 into VP5 (C-terminal) and VP8 (N-terminal), which is essential for penetration of rotavirus virions into cells (67, 174). The cleavage site of all group A rotaviruses is conserved (amino acids 241-247 for SA11 rotavirus strain) (174). It is reported that enzymatic cleavage of VP4 exposes a fusion domain to the cell membrane, allowing virus penetration into the cell. The fusion domain (amino acids 384-404
for SA11 strain) of VP4 shares homology with that of the E1 glycoprotein of Sindbis and Semliki Forest viruses which show similar penetration mechanisms during infection (174). VP4 also possibly induces cell-to-cell fusion (77,78). As an outer capsid protein, VP4 induces neutralizing antibodies referred to as P (protease-sensitive) serotypes, and so far, at least 21 P serotypes [genotypes] have been reported among human and animal group A rotaviruses (174).

VP6 (gene 6) forms trimers, it is the major viral structural protein and it comprises the inner capsid. Antigenic variation in VP6 confers subgroup specificity (I and II) to group A rotaviruses (67, 68). Although VP6 does not have any enzymatic function, VP6 is a component of the viral transcriptase and replicase during virus infection (174). Also VP6 plays an important role in viral morphogenesis: VP6 of newly formed double-shelled particles in the cytoplasm binds to NSP4 located on the membrane of the endoplasmic reticulum (ER), which allows the transfer of single-shelled particles into the ER (4, 144). VP6 is highly immunogenic and immunodominant among rotaviral proteins during infection. Although VP6 does not induce neutralizing antibodies, it was suggested that antibodies against VP6 play an important role in protection against rotaviral disease (28). Dharakul et al. (59) reported that VP6 possesses a cross-reactive (among group A rotaviruses) cytotoxic T lymphocyte epitope, and transfer of CD8+ T lymphocytes from mice immunized with recombinant VP6 significantly reduced the rotavirus shedding in severe chronic immunodeficient (SCID) mice challenged with rotaviruses.

VP7 (gene 7, 8 or 9) is the only glycoprotein among the structural proteins and comprises the major component of the rotavirus outer capsid. It is reported that VP7 is involved in virus
attachment to cells, and it induces neutralizing antibodies which determine the G (glycoprotein) serotypes of rotaviruses. So far, at least 14 G serotypes have been reported among human and animal group A rotaviruses (67, 68). VP7 is retained in the ER during infection, and fully mature virions emerge after double-shelled particles combine with VP7 in the ER. (16). Interaction between VP7 and VP4 plays important roles in viral stability and antigenicity (35, 61). Reassortants of different VP7 and VP4 combinations showed differences in viral stability, and monoclonal antibodies (MAbs) against VP7 inhibit viral hemagglutination mediated by VP4 (35, 61). VP7 has a putative Ca\(^{2+}\) binding site, and binding and unbinding (chelating) of Ca\(^{2+}\) plays an important role during virus infection. Calcium chelation-induced conformational change in VP7 mediates uncoating of triple-shelled rotavirus particles after penetration, and late in infection, Ca\(^{2+}\) binding stabilizes new progeny viruses (134). Dormitzer et al.(60, 61) reported VP7 expressed by a herpes simplex virus vector system lacked several neutralizing epitopes, which could be restored by addition of Ca\(^{2+}\) or by coexpression with other rotavirus proteins.

2) Nonstructural proteins.

NSP1 (gene 5) is produced at low levels in the infected cell. The protein can be detected early during infection in single-shelled particles, and is suggested to be involved in viral replication (172, 174). NSP1 has zinc finger motifs, and may play a role either during replication or packaging of the RNA segments into the virion by binding RNA (16, 134).

NSP2 (gene 7, 8 or 9) is associated with replicase complexes with NSP1, NSP3 and NSP5 in infected cells. NSP2 appears to have RNA binding properties, and complexes of
NSP2/NSP5 showed more efficient RNA binding than either alone (134). NSP2 might play a role in replication or the packaging of RNA into subviral particles (16).

NSP3 (gene 7, 8 or 9) has the ability to bind to ssRNA and is found in infected cells in association with replicase complexes (16). Piron et al. (180) reported that the viral protein NSP3 is bound to the viral mRNAs 3' end and recruits cellular eIF4GI (translation initiation factor).

The NSP4 (gene 10) protein of rotavirus is a nonstructural glycoprotein located in the ER membrane. NSP4 plays a unique role in virus morphogenesis in infected host cells by mediating the conversion of double-shelled particles in the cytoplasm to triple-shelled particles in the ER. It was recently reported that NSP4 also acts as a viral enterotoxin in the mouse model by inducing Ca\(^{++}\) influx in the cytoplasm of infected cells and destabilizes lipid membranes, thereby inducing a secretory diarrhea (5, 229, 265). Alteration of plasma membrane permeability or destabilization of the lipid membranes of cells by NSP4 may cause cell lysis and the release of progeny virions (157).

NSP5 (gene 11) undergoes post-translational modification and interacts with protein kinases to become phosphorylated (69). NSP5 is present in low amounts in infected cells and has been localized to the viroplasm (253). It has been hypothesized that NSP5 plays a regulatory role in rotavirus replication (134). NSP5 has been found to be rearranged in rotavirus strains from several species, producing short or supershort electropherotypes (134). The most common rearrangements have involved in tandem duplication of some regions of the gene, leaving the open reading frame and the ends unaffected (134).
Replication and Morphogenesis

Propagation of rotaviruses in cell culture has permitted insight into the replication cycle and morphogenesis of rotaviruses in cells.

1) Attachment

The first step of virus replication in the host cell is attachment of the virus to the cell surface. Rotaviruses have complicated systems for initial virus-cell interaction: both outer capsid proteins, VP4 and VP7 independently interact with cell surface receptors (134). Binding to cells does not require cleaved VP4 or glycosylated VP7 (68). The cell receptor for VP4 is related to sialoglycoconjugates, and the binding is either sialic acid-dependent or sialic acid-independent (68). Lee et al. (127) reported that VP8, the N-terminal trypsin cleavage product of rotavirus VP4, contains the virus neutralizing epitopes in the hemagglutination domain, but is not critical for rotavirus attachment to MA-104 cells.

Recently, Rolsma et al. (188, 189) reported that porcine gangliosides, especially N-glycolylGM3 were required for porcine rotavirus binding to host cells and functioned as an in vitro relevant rotavirus receptor on porcine enterocytes. Superti et al. (214) also investigated the receptors involved in the attachment of a simian rotavirus (SA-11) to a monkey kidney cell line (LLC-MK2), and found bovine brain gangliosides, mainly GM1, strongly reduced virus binding to cell membranes.

2) Penetration

All viruses are internalized by 60-90 min after binding (68). Rotavirus virions can utilize either of two routes of infection: 1) endocytosis or 2) direct penetration. The addition of lysosomotropie agents which stimulate endocytosis proved to have little or no effect on the
yield of virus (74), which indicates that endocytosis is not an important route for viral infection. Konno et al. (122) also reported direct penetration via VP4 cleaved by trypsin is essential for replication of the virus, whereas endocytotic internalization does not give rise to viral replication. Gilbert et al. (77, 78) investigated more detailed mechanisms for virus penetration via VP4 using bovine and simian rotaviruses and virus-like particles. They showed that virus penetration was dependent on the presence of both outer-layer proteins, VP4 and VP7, and that an arginine residue at site 247 in VP4 appeared to be required for activation of VP4 functions, cell-cell fusion and virus penetration.

3) Uncoating

Once the virus is in the cell cytoplasm, the outer shells of the virus are disrupted. Ludert et al. (130) studied the effect of Ca$$^2+$$ concentration on intracellular rotavirus uncoating by treating cells with the calcium ionophore A23187 which increases the intracellular Ca$$^2+$$ concentration. They found that the low Ca$$^2+$$ concentration in the intracellular microenvironment was responsible for rotavirus uncoating. Using expressed SA11 VP7, Dormitzer et al. (60) found that VP7 bound calcium in the absence of other rotavirus proteins, and that the calcium chelation-induced conformational change in VP7 mediated uncoating of triple-shelled rotavirus particles.

4) Transcription

After removal of the outer capsid, the subviral particle-associated transcriptase containing cores and the inner capsid VP6 are activated and produce messenger or positive sense ssRNAs. Newly synthesized ssRNAs are extruded from the transcriptase particles into the cytoplasm (125). The released RNAs are capped by VP3 which is a guanylyltransferase, a
member of the nucleotidyltransferase family by transferring GMP to the phosphate end of nascent RNAs (134, 174).

5) Translation

Viral mRNA is translated with cellular machinery. But unlike most eukaryotic mRNAs containing a 5' cap structure and a 3' poly(A) sequence which synergistically increase the efficiency of translation, rotavirus mRNAs lack poly(A) sequences (67). For increasing the efficiency of viral translation, rotaviruses have unique features. Piron et al. (180) reported that the viral protein NSP3 is bound to the viral mRNAs 3' end and recruits cellular eIF4GI (translation initiation factor). They also found that a physical link between the 5' and the 3' ends of mRNA was necessary for the efficient translation of viral mRNAs. They suggested the closed loop model for the initiation of translation.

6) Replication

RNA replication occurs within particles assembled with newly synthesized viral proteins and positive-sense RNA. Each genome segment is replicated asymmetically with viral positive-strand RNA (mRNA) serving as the template for the synthesis of minus-strand RNA to produce dsRNA (68, 173). Although the expression levels of each protein are different, it is reported that full length positive, minus strand RNA and the eleven segments of viral dsRNA were synthesized in equimolar concentrations in infected cells (173). Rotavirus replicase particles showed a series of replication intermediates (RIs). Early RIs contain VP1, VP3, NSP1, NSP2, NSP3, NSP5 and possible host components, and mature single-shelled RIs contained VP1, VP2, VP3, VP6 and much reduced amounts of NSP2, NSP3 and NSP5 (173). The presence of VP6 in enzymatically active replicase particles suggests that, as for
transcription, this protein is required for rotavirus RNA replication (173). Chen et al. (36) established a template-dependent in vitro replication system. Replicase activity was associated with subviral particles containing VP1, VP2 and VP3, derived from native virions or baculovirus coexpression of rotavirus genes. A cis-acting signal involved in replication was localized within the 26 3'-terminal nucleotides of a reporter template RNA (37, 174). Furthermore, the 3'-terminal consensus sequence of rotavirus mRNA was the minimal promoter of negative-strand RNA synthesis (176, 254). It was suggested that the binding of VP1 to the 3' end of the template mRNA, followed by VP2 interaction, initiated viral replication (176).

7) Assembly, maturation

Rotaviruses have a unique morphogenesis. Newly synthesized individual structural proteins (VP1, 2, 3, 4, 6) and dsRNA are assembled into double-shelled subviral particles in cytoplasm, and they bud through the membrane of the endoplasmic reticulum (4, 68). Subviral particles obtain a transient membrane-envelope and after budding into the ER, which is mediated by the viral nonstructural glycoprotein, NSP4. This membrane is replaced by VP7 which is localized in the ER, and which results in formation of infectious virus (4, 68).

8) Release

EM studies showed that the infectious cycle ends when progeny viruses are released by host cell lysis (68). Extensive cytolysis and drastic alterations in the permeability of the plasma membrane result in release of virus from infected cells. However, there could be other mechanisms of virus release. Musalem et al. (153) observed that virus particles were associated with membranes and the cytoskeleton in the late stage of virus infection. Jourdan
et al. (114) reported that simian rotavirus strain (RRV) was released almost exclusively from the apical pole of Caco-2 cells by ER through a carbonyl cyanide m-chlorophenylhydrazone-sensitive vesicular transport before any cells lysis occurred. Recent studies suggested that NSP4 mediated changes in both ER and cellular membrane permeability, which may trigger viral budding into the lumen of the ER and from infected cells (68).

**Pathogenesis**

Diarrhea occurs when rotaviruses infect the target tissue, the small intestinal epithelium. Rotaviruses infect exclusively matured absorptive enterocytes in tip of villi, which causes villous atrophy and replaced by nonmatured secretory enterocytes. Malabsorption and alterations of fluid balance are the proposed classical pathophysiologic mechanisms of rotavirus diarrhea, but host differences may occur in rotavirus pathogenesis. There is evidence of malabsorption after extensive viral destruction of intestinal epithelium in pigs and calves. Little villous atrophy is evident and the viral infection is age restricted in mice with rotavirus diarrhea. In humans, there is limited information about intestinal lesions after viral infection.

1) Histopathological changes by rotavirus infection

Argenzio et al.(3) summarized that rotaviruses caused diarrhea by virus-induced villous atrophy followed by intestinal hypersecretion, malabsorption, or both in neonatal calves. Mebus et al.(142) studied intestinal lesions caused by rotaviruses using gnotobiotic calves. The calves were euthanatized 6 hours after onset of diarrhea, and they found villi in the cranial, middle and caudal parts of the small intestine were shortened and epithelial cells were immature. Bridger et al.(22) showed that bovine rotaviruses infection (clinical diarrhea) was
dependent on strains in the second week of life, and that older calves (6 weeks and over) were also susceptible to rotavirus infection and disease. Diarrhea by rotavirus infection elevated plasma levels of enteroglucagon and neurotensin and caused xylose malabsorption in calves (89). Woode et al. (256) observed D-xylose malabsorption by intestinal damage after rotavirus infection in calves.

When gnotobiotic piglets were infected with rotaviruses, Hall et al. (88) observed the desquamation of the epithelial cells of the villi resulting in severe stunting, a severe damage to microvilli and accumulation of lipid within the cytoplasm. Virus particles were seen in epithelial cells covering the stunted villi. Ward et al. (246) also studied the pathogenesis of virulent and attenuated Wa human rotavirus (HRV) in gnotobiotic pigs: the 50 % infectious dose (ID₅₀) of virulent and attenuated Wa HRV was significantly different (less than 1 f.f.u. vs 1.3 x 10⁶ f.f.u.), and diarrhea and villous atrophy were developed by only virulent Wa HRV.

When murine rotavirus (EDIM strain) was orally inoculated into seronegative newborn mice, clinical diarrhea was induced at 72 h. Only enterocytes in the upper two-thirds of villus epithelia were infected by fluorescent-antibody analysis and most virus was found in the middle small intestine (213). Villus tips were convoluted, corresponding to the shedding of virus-infected cells and the lower regions of infected villi were shrunken and considerably narrowed compared to tips (213). But mice were susceptible to rotavirus illness for only a short time period (approximately 15 days of age). Ijaz et al. (103) reported that inoculation of newborn mice with rotavirus caused significantly less D-xylose absorption compared with uninoculated control mice, and suggested malabsorption as the pathogenesis of rotaviral
diarrhea in newborn mice.

Kohler et al. (121) performed small intestinal mucosal biopsies in 40 children with acute rotavirus diarrhea and found only 2 children (5%) had well defined injuries of intestinal mucosa. They concluded that correlations were not found between clinical findings and morphological results. However, histological and morphometric examinations of the small intestine of two children who died from rotavirus gastroenteritis revealed shortened villi and enhanced mitotic activity of epithelial cells (159).

2) Rotaviral gene(s) related with pathogenesis

Rotaviruses have 11 segments of dsRNA which encode 6 structural and 5 non-structural proteins (67). The role of individual gene segments in rotavirus pathogenesis is unclear, but it is reported that VP3, VP4, VP7, NSP1 and NSP4 genes are involved in pathogenesis. Nonstructural NSP1 and NSP2 are involved in viral replication and morphogenesis and are implicated as virulence factors in mice (25). Broome et al. (25) found that highly virulent reassortants were correlated with NSP1 and NSP2 genes from virulent parental strains in mice. NSP1 was also implicated with determination of growth characteristics, which may related to the virulence of rotavirus (85). Bridger et al. (21, 24) showed VP4 was a viral determinant of rotavirus pathogenicity in pigs using a monoreassortant system: S-F4, which carries RNA segment 4 of the pig-pathogenic variant 4F in the genetic background of the pig-nonpathogenic variant 4S, was found to be pathogenic in gnotobiotic piglets. Hoshino et al. (96) reported that VP3, VP4, VP7 and NSP4 genes, but not a single gene, were important for virulence in gnotobiotic pigs based on studies of human and pig reassortant rotaviruses.

The NSP4 protein of rotavirus is a nonstructural glycoprotein, which plays a unique role
in virus morphogenesis in infected host cells. It was recently reported that the NSP4 protein also acts as a viral enterotoxin in the mouse model by inducing Ca\(^{++}\) influx in the cytoplasm of infected cells and destabilizes lipid membranes, thereby inducing a secretory diarrhea (5, 229, 265). The nucleotide and deduced amino acid sequence of NSP4 from pairs of virulent and attenuated (attenuated strains derived by extensive cell culture passages) rotaviruses have been compared in an attempt to further define the potential role of NSP4 in viral diarrheal mechanisms (2, 251, 265). To date, comparisons of those virus pairs have yielded varying results (2, 251, 265). Zhang et al. (265) reported major changes in the deduced amino acids of the NSP4 of virulent and attenuated pairs of OSU and Gottfriied porcine rotavirus strains with most changes clustered in the 3' end regions. Ward et al. (251) reported only one amino acid difference out of 175 amino acids between the virulent and attenuated pairs of a human group A rotavirus strain 89-12. Angel et al. (2) also failed to find any consistent and significant changes in comparison of amino acid sequences of NSP4 from three sets of virulent and attenuated pairs of murine rotaviruses. Chang et al. (31) sequenced two (Wa and M strains) pairs of NSP4 genes of virulent and attenuated (after 30 to 40 passages in cell culture) human group A rotaviruses and a pair of NSP4 genes of virulent and attenuated porcine group C rotavirus (Cowden strain), and found only 3 (Wa) and 2 (M and Cowden) amino acids differed between the virulent and attenuated strains.

3) Host factors related to pathogenesis

Mammals of all ages are physiologically susceptible to infection by appropriate strains of rotavirus (146). Clinical signs from rotavirus infections occur more frequently and more
severely in the young than in adults. Such age resistance may be due to changes with age in the contents and distribution of body water, renal capacity to regulate water and electrolyte balance, development of the immune system, and specific acquired immunity based on prior clinical or subclinical infections (146). Moon et al. (144) reported that epithelial cell replacement of crypt cells by mature cells and the rate of differentiation of crypt cells increased with age in mammals. The capacity of the large intestine to compensate for maladsorption in the small intestine also increases with age (146). Compensatory capacity of the large intestine may differ among species: mice and rabbits have higher capacity than pigs and calves, which may be a reason for greater age restriction of clinical rotavirus infections in mice and rabbits.

Animal model to study immune responses and protection against rotaviruses.

It is important to understand the immunological reaction of the host to rotaviruses and to individual rotaviral proteins for the development of more efficient vaccines. Animal models using mice, rabbits, pigs and calves have been used to study the pathogenicity and immune mechanisms against rotavirus disease. Because group A rotaviruses have complex antigenic structures (at least 14 different G serotypes and 21 different P types among animals and humans inducing distinct neutralizing antibodies, 134), the question of whether immunization by heterologous (different G or P type) strains can induce protection against homologous or heterologous challenge has yet to be answered. Rotavirus infections are localized to the small intestines of young animals and humans. Therefore, local passive and active immunity are important. For inducing local immune responses to rotaviruses, the inoculation route (peroral
or intranasal inoculation) is important. Systemic immune responses induced by parental immunization have been shown to be less effective in protection against rotavirus infections in some studies (138, 163), but various degree of protection in other studies (44, 47, 137, 145). Since most adult animals and humans are seropositive for antibodies to rotavirus, a variable degree of passive immunity will be transferred to their offspring. Passive immunity provides protection against rotavirus infections for a limited time, before active immunity is acquired by infection or vaccination with rotaviruses. Because passive immunity may interfere with active immune responses to vaccination, the balance of passive immunity and active immunity is important for overall strategies to control rotavirus infections by vaccination. A recently licensed tetravalent attenuated rotavirus vaccine for human use is now available. This vaccine has 4 different G types of human rotavirus strains and is recommended for peroral inoculation (to stimulate active local immunity) of infants at 3 time intervals (2, 4 and 6 months of age).

Current research for the development of a new generation of more effective rotavirus vaccines includes a subunit vaccine using virus-like particles, the use of adjuvants for mucosal stimulation and a DNA vaccine approach. There are several advantages to using subunit vaccines, the most important being safety (49, 50). When four structural rotavirus proteins (VP2, VP4, VP6, VP7) encoded in recombinant baculoviruses are coinoculated into Spodoptera frugiperda (Sf-9) insect cells, the expressed proteins spontaneously assemble into VLPs which are non-infectious, stable and highly immunogenic (49, 50). Also using different VP7 (G type) and VP4 (P type) recombinant baculoviruses, VLPs can be constructed for any combination of G and P types. Newly developed and reported effective mucosal adjuvants
include CT (cholera toxin), LT (heat labile *E.coli* toxin), QS21 (saponin adjuvant) and ISCOM (immune stimulating complex). DNA vaccines have also been tested as a way to induce an immune response and protect against rotavirus disease. DNA vaccines are prepared by inserting cDNA encoding for rotavirus proteins (VP4, VP6 and VP7) into human cytomegalovirus (CMV), simian virus (SV40) or beta-actin (β-actin) promoter based vectors (38,39,41,42). Using a bombardment device (gene gun), DNA can be introduced into target cells where protein(s) will be endogenously expressed. The expressed protein(s) in the body cells induces further immune responses.

1) Studies using mouse models

Since only suckling mice younger than 15 days of age are susceptible to rotavirus diarrhea, Ward et al.(247) developed an adult mouse model to study rotavirus infections using the EDIM murine rotavirus strain and adult BALC/c mice. The EDIM strain caused consistent infection of neonatal (with diarrhea) and adult BALB/c mice (without clinical diarrhea) as determined by viral shedding and seroconversion. When mice were oral immunized and challenged with EDIM strain, homotypic but not heterotypic protection was observed without any correlation with serum neutralizing antibody titers (247). Woode et al.(257) also found that although all mouse, simian and bovine strains induced a serological antibody response in mice, only the homologous immune responses were protective. McNeal et al.(138) reported that homologous protection in the mouse model was related to the intestinal replication properties of the different strains, and the titers of serum rotavirus IgA, but not IgG, following oral immunization was directly related to protection. Intraperitoneal
immunization with homologous and heterologous rotaviruses provided protection against rotavirus infection of mice, which was not related to serum neutralizing antibody titers to the challenge virus (137). Coffin et al. (44) found that intramuscular inoculation with live, wild-type rotavirus (EDIM) induced complete protection from viral shedding after challenge, and this protection correlated with virus-specific IgA, whereas, intramuscular inoculation with inactivated EDIM, cell culture-adapted EDIM, or simian strain RRV was associated with only partial protection. Feng et al. (71) investigated the importance of serotype-specific immunity in a mouse model, and found oral inoculation of rhesus rotavirus-based modified Jennerian vaccines induced both heterotypic and homotypic protection in mice, which correlated with fecal IgA antibody titers to VP6, but not serum IgG antibody responses.

Moser et al. (149) studied the oral vaccination of mice with either free or microencapsulated rotavirus (simian rotavirus strain RRV) and challenged with murine rotavirus strain EDIM. They found that microencapsulated rotaviruses induced enhanced protection against challenge at 16 weeks after immunization, but not at 6 weeks, and that quantities of virus-specific IgA antibody were correlated with the degree of protection. McNeal et al. (140) reported that mice immunized with either live viruses or inactivated triple layered particles (EDIM) with QS-21 were almost fully protected, but double-layered particles with QS-21 provided partial protection against challenge with homologous viruses. They also noted that QS-21 consistently enhanced immune responses. All mice inoculated with 2/6-VLPs mixed with LT or LT-R192G were totally protected from rotavirus challenge, whereas mice inoculated with 2/6-VLPs mixed with CT showed a mean 91% protection from challenge (160, 161).
Plasmid DNA vaccines encoding murine rotaviral proteins VP4, VP6, or VP7 were all effective in protecting mice against infection (reduction in virus excretion) after homotypic rotavirus challenge (EDIM) (38). In addition, one dose of a rotavirus VP6 DNA vaccine encapsulated in poly(lactide-coglycolide) (PLG) microparticles and administered orally elicited both rotavirus-specific serum and intestinal immunoglobulin A (IgA) antibodies and provided protection against homologous rotavirus challenge (39). However, Choi et al. (41, 42) reported that epidermal immunization (using the PowderJet particle delivery device) of the plasmid vector pcDNA1/EDIM6, 4 or 7 encoding EDIM rotavirus VP6, VP4 or VP7 induced high levels of serum IgG antibody against rotaviruses, but failed to protect mice against EDIM infection.

2) Studies using rabbit models

A Rabbit model to study rotavirus infection was developed by Conner et al. (45, 46). Seronegative New Zealand White rabbits (neonatal to 4 months old) were inoculated orally with cultivatable rabbit rotavirus strains Ala, C11, and R2 and with the heterologous simian strain SA11. Although adult rabbits did not develop clinical diarrhea, inoculated animals excreted viruses in feces from 2 to 8 days after the primary inoculation and seroconverted to inoculated strains (45, 46).

Conner et al. (47) reported that parental vaccination of rabbit using two, but not one, dose of SA11 rotavirus in either Freund's incomplete adjuvant or aluminum phosphate induced active protection against challenge by high doses of live, virulent rabbit (Ala) rotavirus. O'Neal et al. (160) reported that oral immunization of VLPs (2/6- or 2/6/7-) induced partial
protection, but intranasal immunization of VLPs (2/6- or 2/6/7-) provide complete protection from homologous lapine rotavirus challenge: intranasal immunization of VLPs (2/6- or 2/6/7-) induced higher serum and intestinal antibody responses than VLPs administered orally. Ciarlet et al. (43) reported that parental administration of two doses of SA11 VLPs (2/6-, G3 2/6/7-, or P[2]G3 2/4/6/7-VLPs) or SA11 rotavirus in Freund's incomplete adjuvants, QS-21, or aluminum phosphate were immunogenic and protective against challenge by live Ala lapine rotavirus. They noted that Freund's incomplete adjuvant was a better adjuvant than QS-21.

3) Studies using gnotobiotic pig models

Yuan et al. (263) reported that neonatal gnotobiotic pigs orally inoculated with virulent Wa human rotavirus developed diarrhea, and were protected (87%) against homologous virulent virus challenge at postinoculation day (PID) 21. However, pigs inoculated with cell culture-attenuated Wa rotavirus developed subclinical infections and seroconverted but were only partially protected against challenge (33% protection rate). They noted systematic and intestinal antibody-secreting cell responses and the magnitude of the LPA responses (245) were correlated with protective immunity to human rotavirus. Yuan et al. (264) also reported that when newborn gnotobiotic pigs were inoculated twice perorally (p.o.), intramuscularly (i.m.) or three times i.m. with inactivated Wa strain human rotavirus, neither p.o. nor i.m. inoculation conferred significant protection against virulent Wa rotavirus challenge, or induced significant anamnestic virus-specific IgG and IgA ASC responses. In this study, they also noted that there was no correlation between protection and neutralizing antibody titers of serum or intestinal contents, but a positive correlation existed between protection and
serum IgA, intestinal IgA and intestinal IgG antibody titres (230). The effects of circulating maternal antibodies on passive protection and active immunity to human rotavirus (HRV) were examined in gnotobiotic pigs by Hodgins et al. (95). Pigs that received high antibody titer serum passively were partially protected against challenge with virulent Wa rotavirus, but these antibodies interfered with active immune responses. They noted that successful immunization in populations with high titers of maternal antibodies may require higher titers of virus, multiple doses, or improved delivery systems to overcome the suppressive effects of maternal antibodies.

4) Studies on passive immunity to protect against rotavirus infections

Many early studies of animals focused on the role of passive immunity for protection against rotaviral diarrhea. Maternal immunization to provide passive immunity to calves was effective in protecting calves against challenge with virulent rotavirus strains and was suggested as a practical vaccine approach in animals (207, 208). Saif et al. (194) noted that the combination of IM and intramammary vaccination with adjuvant of pregnant cows significantly elevated IgG1 rotavirus antibodies in colostrum and milk. Higher concentrations of IgG1 antibodies in colostrum (by IM and intramammary but not by only IM vaccination) was protective when colostrum was fed to calves challenged with virulent rotavirus (194). Besser et al. (11) also studied the effect of circulating passive antibody on immunity to bovine rotavirus infections in neonatal calves. They observed that circulating IgG1 antibodies were transferred to the gastrointestinal tract of neonatal calves, and provided protection against rotavirus challenge. Fernandez et al. (72, 73) used SA11 VLP and inactivated rotaviruses to
vaccinate pregnant cows. They found that although both vaccines induced high rotavirus antibody titers, VLP induced higher antibody responses in serum, colostrum and milk than inactivated viruses. In addition, the colostrum from heterotypic (SA11 P[2]G3) VLP vaccinated cows provided heterologous passive protection, but the colostrum from 2/6 SA11 VLP (CLP) or inactivated SA11 vaccinated cows provided only partial protection against heterologous IND (P[5]G6) bovine rotavirus (BRV) diarrhea or shedding.

Schaller et al. (205) studied the efficacy of passively administered bovine antibody to Wa HRV for preventing HRV-induced diarrhea using a gnotobiotic pig model. They found that both viral shedding and diarrhea were effectively reduced or eliminated in a dose-dependent manner by HRV immune antibody feeding. Ward et al. (244) also observed that circulating maternal antibodies play a significant role in reducing clinical disease following porcine rotavirus infection (OSU strain) challenge of conventional neonatal swine.

Offit et al. (163) reported that newborn mice suckled on dams immunized either orally or parenterally with the primate rotavirus SA-11 were protected against SA-11 virus challenge. Also, they found that only homotypic rotavirus provided protection against challenge, which was closely correlated with the neutralizing activity of maternal serum against the challenge virus. When monoclonal antibodies (MAbs) against capsid proteins, VP4, VP7 and VP6 were tested for their ability to passively protect suckling mice against virulent rotavirus challenge, MAbs with neutralizing activity (VP4 and VP7) to challenge viruses provided protection, but not MAbs against VP6 (132, 164). Burns et al. (28) developed a murine "backpack tumor" transplantation model to determine the protective effect of antibodies against VP4 and VP6. They found that only non-neutralizing IgA antibodies to VP6 were capable for preventing
rotavirus infections, and suggested that in vivo intracellular viral inactivation by secretory IgA during transcytosis is a mechanism of host defense against rotavirus infection.

Milk containing high concentrations of Ig prepared from rotavirus-hyperimmunized cows was evaluated for passive protection of infants. Ebina et al. (62) reported that orally administered colostrum of pregnant cows immunized with HRV (Wa strain) showed significant protection against rotaviral diarrhea in infants. The colostrum or milk containing high antibody titers of Ig against rotavirus were also effective in treating infants suffering from acute rotavirus gastroenteritis (94).

**Human vaccine development**

Cell culture passaged rotaviruses from non-human hosts (simian and bovine strains) were developed as live attenuated oral vaccines for newborn children (Jennerian approach). However, this Jennerian approach proved to be ineffective (118, 250). One major reason for the failure is that such strains were heterologous to human strains (118). In addition, live attenuated heterologous rotavirus vaccines might be ineffective because they replicate poorly in the intestinal epithelium and consequently induce insufficient immune responses (118, 250). A modified Jennerian approach has been developed using reassortant rotavirus vaccines containing genes encoding human VP7 or VP4 in the simian or bovine rotavirus background. Such reassortant vaccines provide more serotype-specific immune responses (homologous) for human rotaviruses. In 1998, a rhesus rotavirus based tetravalent (RRV-TV including G1, G2, G3 and G4) attenuated vaccine was licensed for oral immunization of human infants. Administration of the first dose of this vaccine was associated with a relatively high rate of
febrile responses and virus shedding (about 50%, detected with ELISA) (57, 252). Although, it is approximately 80-90% effective at reducing severe rotavirus disease after 3 oral doses (2, 4 and 6 months of age), the overall efficiency of the vaccine against diarrhea episodes was only 50-60%. Field trials of this vaccine showed it to be effective in reducing rotavirus diarrhea in the USA and Finland (113, 119), but it was less effective in trials in some developing countries (124, 128). The reduced efficacy may reflect differences in titers of maternal antibodies, differences in exposure to rotavirus before and during the vaccination process, or differences in trial design (113, 124, 128). A recent cost-effectiveness analysis of a rotavirus immunization program for the U.S. indicated that $9 per dose is cost-effective, but the current cost is $50-60 per dose.

**Coinfection by group A rotaviruses and other enteropathogens**

The effects of coinfection by group A rotaviruses and other enteropathogens including *Escherichia coli* in calves, lambs, pigs and children have been studied by others: generally coinfection of group A rotavirus with other enteropathogens (*E. coli*) caused more severe diarrhea, lesions and shedding than infection by either alone (9, 81, 231, 236).

Coinfection by group A rotaviruses and *E. coli* in calves were common in nature (191, 145). Moon et al. (145) found a high prevalence of coinfection by rotaviruses and other enteropathogens including coronavirus, *E. coli* and cryptosporidia among naturally occurring diarrhea in newborn calves from 12 herds in Iowa. The coinfections were either both rotaviruses and coronaviruses, one or both rotaviruses and coronaviruses plus cryptosporidia, or rotavirus plus enterotoxigenic *E. coli*. Runnels et al. (191) observed naturally occurring
coinfections by *E. coli* and rotavirus associated with fatal diarrhea of calves about 1 week old. When rotaviruses and *E. coli* were coinoculated into calves, the microscopic lesions observed were similar between coinoculated and rotavirus alone inoculated calves, except there was more severe atrophy of ileal villi of coinoculated calves (191). Also they found intensive colonization of *E. coli* in the small intestine in calves coinfectcd with rotaviruses. Gouet et al. (81) reported that the experimental inoculation of calves with rotavirus, which is not lethal in itself, followed by non lethal inoculation of *E. coli* led to dehydration and death in the newborn colostrum-deprived calves. Tzipori et al. (236) reported that when newborn calves were inoculated with rotavirus, enterotoxigenic *E. coli*, either alone or in combination, calves coinoculated with rotavirus and *E. coli* had the most extensive diarrhea and severe lesions. Torres-Medina et al. (231) also observed similar synergistic effects using rotavirus (NCDV) and enterotoxigenic *E. coli* in newborn gnotobiotic calves. Hess et al. (93) reported that coinfection of calves with rotavirus and *E. coli* resulted in not only more severe histological lesions, but also increased shedding of both pathogens in experimentally inoculated newborn colostrum-deprived calves. Snodgrass et al. (209) also reported that in dual infections, both rotavirus and ETEC shedding was increased, but the severity of diarrhea was not greater than that caused by rotavirus alone. Wray et al. (259) used colostrum-deprived lambs to study coinfections with rotaviruses and *E. coli*. When both of the agents were administered, the mortality rate was higher, although the duration of diarrhea was no greater than that observed when either of the two agents was administered alone.

Benfield et al. (9) studied the effects of coinfection of rotaviruses and enterotoxigenic *E. coli* (ETEC) in gnotobiotic pigs. They found that pigs coinfected with rotaviruses and ETEC
developed more severe diarrhea, compared to pigs inoculated with the single agents. All coinoculated pigs died between 3 and 6 days after inoculation, but there was no difference in the severity of the villus atrophy between the coinoculated pigs and pigs inoculated only with rotavirus. Coinfection of rotaviruses and \textit{E.coli} has been reported in rabbits. Thouless et al.(228) reported that rotavirus infection resulted in only mild diarrhea in weaning rabbits, but when rotaviruses were inoculated with \textit{E.coli}, a synergistic effect between rotavirus and \textit{E. coli} occurred, causing more severe diarrhea.

The severity of group A rotavirus diarrhea was compared with that of mixed infections of rotaviruses with \textit{E.coli} in children by Unicomb et al.(237). They found that the severity of mixed infections of rotaviruses and \textit{E.coli} was the same as that for infections with rotaviruses alone.

\textbf{Bovine group A rotaviruses}

1) Epidemiology, diagnosis and serotyping of bovine group A rotaviruses (BRV)

Group A BRV have been reported worldwide (201). In field studies, BRV has been consistently detected in a high percentage of scouring calves, 1 to 3 weeks old, both on ranches and dairy farms (129, 141, 255). Viruses are transmitted via the fecal-oral route (201, 226). Sequential outbreaks of rotavirus diarrhea are common in some herds and may be due to the presence of repeated infections of calves by BRV strains or recrudescence of viral shedding in calves (226).

The diagnosis of BRV is based on the detection of viral particles, antigens or nucleic acid in fecal specimens, rectal swabs, gut contents or sections (191). A variety of diagnostic tests
have been developed for the detection of BRV, including immune electron microscopy (IEM), immunofluorescent (IF) staining of inoculated cell cultures or infected tissue sections, enzyme-linked immunosorbent assays (ELISA), plaque assays, radioimmune assays (RIA), complement fixation and latex agglutination (LA) tests, polyacrylamide gel electrophoresis (PAGE), nucleic acid hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (6, 8, 92, 142, 169, 190, 192, 201, 225).

Lucchelli et al. (129) reported that the overall prevalence of BRV infections with clinical or non-clinical signs (1 to 30 days of age) from Ohio dairy herds to be 16.4% (74/450) using ELISA. Bellinzoni et al. (7) studied the incidence of rotavirus in beef herds in Argentina and reported that 53% of fecal samples from diarrheic calves contained rotaviruses. The peak age of infection was between 10 to 19 days of age. Other investigators reported prevalence rates from 30% to 83% from beef and dairy calves in various countries (141, 185, 201).

Determination of the serotype specificity is important for development and evaluation of more efficacious BRV vaccines, but this is complicated by the dual serotype specificity of rotavirus (201). Several assays have been developed for the G and P typing of rotaviruses and according to the methods used, there were significant differences in their specificity and sensitivity. These include plaque reduction and fluorescent focus virus neutralization, ELISA using MAbs, nucleic acid probes, and RT-PCR (7, 8, 82, 83, 129, 152, 170, 211, 215, 218).

Previous investigators have studied the prevalence of infection of calves with serotype G6, G8 and G10 rotaviruses (129, 170, 211, 258). Woode et al. (258) used two-way cross-neutralization assays and demonstrated that 89% of the isolates (n=73) were related to G6, and of the remaining isolates, 63% were related to G10. Snodgrass et al. (211) used a MAb-
based ELISA for G typing and reported that 66% of the isolates were G6, 7.4% were G10, and only 1 sample (0.6%) was G8. In the same study, two viruses belonging to a monotype within serotype G6 failed to react with the serotype G6-specific MAb (211). Lucchelli et al. (129) also used a MAb-based ELISA, and demonstrated that among 308 calf fecal samples, 79% tested positive by a broadly reactive VP7 MAb, and of these, 54% were G6, 14% were G10, 4% were both G6 and G10, and 28% were G6 and G10 negative. Parwani et al. (170) used PCR-generated G type specific cDNA probes and reported that 36.3% of BRV field samples tested were G6, 12.8% were G10 and 2.9% were G8; however 23.5% of the field samples tested were not typable using this method.

There is only limited data on the prevalence of P types of BRV field samples. One reason for the difficulty in P typing rotaviruses is that the VP4 protein is much less abundant in the outer capsid than VP7, which means that assays based on VP4 antigen quantitation may be less sensitive for routine P typing (134). Therefore, assays based on the genomic P typing seem to be more consistent and reliable. Suzuki et al. (215) reported that 10 % (4/40) of BRV field isolates were P[1], 55 % (22/40) were P[5], 27.5 % (11/40) were P[11] and 3 samples were not typable using a nested PCR method. Parwani et al. (170) also reported P typing of BRV field samples using cDNA probes: 2.2 % (2/93) were P[1], 20.4 % (19/93) were P[5], 9.3 % (10/93) were P[11] and 40.8 % (38/93) remained untypable using this method.

Suzuki et al. (215) reported the G/P combination of BRV field isolates in Japan after using nested PCR to determine individual G (G6, 10) and P (P[1,5,11]) types. They found that P[5]G6 was most frequent (42.5%) followed by P[11]G6 (17.5%), P[1]G6 (10%), [P5]G10

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(10%) and P[11]G10 (7.5%). All possible combinations were observed, which could be caused by naturally occurring mixed infections and reassortment in the host.

Sequence analysis of VP4 and VP7 genes of BRV strains has been published. The nucleotide and deduced amino acid sequences for the VP4 gene of BRV including C-486, NCDV, B641, B223 and UK are available (90, 91, 181). Kantharidis et al. (116) compared the deduced amino acid sequence of VP4 cleavage regions (a.a. residues 227-260) of BRV UK strain with several other rotaviruses including additional BRV. The homology of this region of the UK (P[5]) strain to NCDV (P[1]) and C-486 (P[11]) strains was 59 to 65 %, consistent with different P types. The nucleotide sequences for the VP7 genes of various BRV strains including NCDV, UK, 61A, B11, B60, T449, Cody and B223 have been reported (12, 30, 217, 239). Xu et al. (258) compared the deduced amino acid sequences of VP7 from serotype G10 and G6 BRV strains, and found that there was less than 5 % divergence among the same G-type strains, but about 20 % differences between different G serotypes.

G6 serotype strains are mainly from cattle (NCDV-Lincoln, UK, RF, IND, OK, ID, KN-4), but were also reported from humans (PA151, PA169) (75, 76, 101, 131, 150). Among G6 strains, genetic variations were reported as G6 subtypes of bovine and human strains (75, 131). Gema et al. (75) reported G6 human strains recognized by a MAb to a BRV G6 showed some genetic variation (about 10 % in amino acid sequences) with typical bovine G6 strains (UK and NCDV). Matsuda et al. (131) reported the isolation of a G6 bovine strain (KN-4) showing about 10 % variation in amino acid sequence with other prototype bovine G6 strains. The KN-4 strain failed to react with G6-specific MAbs but was antigenically related to the NCDV (P[1]G6) and 0510 (P[5]G6) G6 serotypes; and KK-3 (P[11]G10) via the common
P[11] serotype. From the nucleotide and deduced amino acid sequence analysis, G6 subtype strains had 79 – 86 % homology in nucleotide and 87 – 92 % homology in amino acids with prototype G6 serotype rotaviruses. Among different BRV G types (G6, G8 and G10), nucleotide and deduced amino acid sequences homologies were 72-74 % and 80-84 %, respectively; and within typical G6 BRV, the homologies were greater than 95 % in nucleotide and amino acid sequences (75, 131, 150). Interestingly, the reported G6 subtypes were associated with other than P[5], which is the predominant P type among field samples (76, 101). Among group A G10 rotaviruses, a human strain (Mc35) was reported. The nucleotide sequence homologies between Mc35 and the four G10 bovine strains were lower than among the four G10 bovine strains (239).

2) Prevention of BRV infections

The BRV infects mainly epithelial cells in the small intestine leading to villous atrophy followed by malabsorption and diarrhea. Because BRV infections are localized in the small intestine of young calves, local passive and active immunity are important (198, 201). Most adult animals are seropositive for antibodies to BRV, and mothers will transfer a variable degree of passive immunity to their offspring via colostrum and milk (201, 202). Antibodies in colostrum are absorbed by the neonate for a limited period after birth and they continue to provide passive immunity in the gut upon nursing. Passive immunity provides protection against rotavirus infections for a limited time; meanwhile active immunity is acquired by infection or vaccination (201, 202). Because passive immunity provides limited period of protection and can interfere with active immunity, the balance between passive and active
Immunity is important for overall strategies to control BRV using vaccination.

Vaccination approaches to enhance both active and passive immunity have been studied to control BRV infection in calves. Oral immunization using modified live BRV vaccine in newborn calves was reported effective in experimental studies using gnotobiotic calves (201, 202). However, in field trials, the efficacy was questionable probably due to the presence of maternal antibodies (55, 56, 201). Maternal immunization to enhance lactogenic immunity and the transfer of passive protection has been reported to be effective in protecting against rotavirus infection in calves (194). Studies have shown that maternal immunization of cows with live or inactivated BRV vaccines in incomplete Freund’s adjuvant significantly increased colostral antibodies that passively protected calves from diarrhea following experimental or field BRV challenge (201, 207).

The one commercial live rota-coronavirus vaccine presently available in the U.S. for oral administration to calves is frequently not effective in protecting young calves and outbreaks of enteric disease associated with BRV continue in vaccinated herds (129, 201). The commercial vaccine, administered parentally to pregnant cows, consistently failed to significantly enhance colostral BRV antibodies, or to provide passive protection (202).

Because immunization of homologous strains seems to be more effective in protection of challenge of homologous strains, determination of the serotype specificity and characterization of the genetic and antigenic diversity among BRV in field are important to develop more efficacious BRV vaccines. Neonatal animals need to receive adequate levels of passive antibodies for passive protection. Maternal immunization of pregnant cows should induce higher concentration of IgG1 antibodies to rotavirus in colostrum by using effective
immunization routes and/or methods; ie, IM and intramammary immunization or using more immunogenic antigens such as VLPs. For active immunity, immunization may require higher titer of virus, multiple doses or improved delivery systems to elevate sufficient local immunity and/or overcome the suppressive effects of maternal antibodies.
1.3 Group B rotaviruses

Rotaviruses are divided into 7 morphologically indistinguishable but antigenically distinct serogroups (23, 68, 197, 200). The prototype group B rotavirus strains include human ADRV, porcine NIRD and rat IDIR strains. Group B rotaviruses may be emerging pathogens for humans and appear to be associated with diarrhea in adults and older children more frequently than in neonates (23, 197). In calves and adults cows, group B rotaviruses have been reported in association with sporadic cases of diarrhea (1, 23, 171, 197). The group B rotaviruses are difficult to grow in cell culture, and in the host, are shed in low titers in the feces, making it difficult to detect and further characterize these viruses (23, 197).

Morphology

Group B rotaviruses have a more indistinctive morphology compared to group A rotaviruses, and both double- and triple-shelled particles were seen by EM of viruses in fecal samples. Theil et al. (224) reported that complete virions were rarely observed in the intestinal contents of group B rotavirus infected animals; the predominant particle detected by IEM was a corelike particle about 52 nm in diameter.

Genome / Proteins

Group B rotaviruses have 11 dsRNA segments similar in molecular weight ranges to group A rotaviruses. The genomic profile (electropherotype) of group B rotaviruses are not uniform. Although many animal group B rotaviruses showed 4-2-2-3 profiles, the first reported porcine group B rotavirus, NIRD displayed a 4-2-2-2-1 profile (23). The human
group B rotavirus ADRV strain showed 4-2-1-1-1-1-1 profile, whereas rat IDIR strain displayed 4-3-1-1-1-1 profile. In spite of the discrepancy in genomic profiles among these strains, they are considered the same group based on serology or pathologic lesions (syncytia) (23, 200). It has been reported that the polypeptide structure of group B rotaviruses is similar to that of group A rotaviruses (244). Sequence analysis of the VP6 genes of IDIR and ADRV showed less than 20% identity with those of group A rotaviruses (note: identity of VP6 genes between group A and C rotaviruses is about 40%) (134).

Cell culture cultivation

Although four porcine group B rotaviruses caused cytotoxic effects (syncytia) in MA104 cells after inoculation, none were serially propagated (224). Recently a porcine group B rotavirus was isolated in MA104 cells, which is the only report of the isolation of a group B rotavirus in a cell culture system (204).

Pathogenesis of group B rotaviruses

Although the overall mechanism of the pathogenesis of group B rotaviruses is similar to group A rotaviruses, ie villous enterocytes are infected, microvilli become shortered, enterocytes are lost leading to villous atrophy, and crypts became elongated, there is a main difference group B rotaviruses because syncytia formation in infected enterocytes. Enterocyte syncytia were observed with group B rotaviruses of pigs, lambs, rats and calves. Group B rotaviruses caused diarrhea in pigs, rats, sheep and cattle, when homologous viruses were inoculated into animals. When group B rotaviruses caused large outbreaks of diarrhea in
humans in China in the early 1980's, it was reported that human group B rotavirus infections
were more severe than is usually associated with group A rotaviruses: cholera-like illness with
sudden onset of severe watery diarrhea occurred and dehydration was common (23).

Huber et al. (99) studied the pathogenesis of group B rotavirus infections in rats and
observed shortening of small intestinal villi, villous epithelial cell necrosis, and villous
epithelial syncytia. The lesions were most often present in the distal small intestine. By day
3 post-inoculation, epithelial necrosis, and syncytia were no longer present; however, the
villous epithelium was disorganized and irregularly vacuolated, and the intestinal crypt
epithelium was hyperplastic. Salim et al. (203) also studied group B rotavirus infection in rats,
and observed diarrhea by 24-36 hours post inoculation and increased crypt depth followed
by villous shortening. Group B rotavirus infections caused enterocyte loss associated with a
net secretory state and impaired sodium absorption.

Diagnosis

For diagnosis of group B rotaviruses, several factors should be considered: its limited
shedding and instability during storage or IEM preparation (23). Several assays to detect
group B rotaviruses have been described. These include polyacrylamide gel electrophoresis
(PAGE), immune electron microscopy (IEM), enzyme linked immunosorbent assay (ELISA),
c-DNA hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (1, 40,
63, 64, 83, 200, 227, 244).
Epidemiology

**Bovine group B rotaviruses.** Bovine atypical rotavirus (group B rotavirus) was identified in two epizootics of neonatal calf diarrhea, which occurred several hundred kilometers and 8 months apart (241). Fecal preparations from an epizootic of neonatal calf diarrhea inoculated into calves induced the formation of syncytia in small intestinal villous epithelial cells (241). Saif et al.(199) detected group B rotaviruses by IEM associated with adult cattle from dairy herds with classical winter dysentery. Parwani et al.(171) identified group B rotaviruses with short genome electropherotypes from adult cows with diarrhea. Chinsangaram et al.(40) tested 136 fecal samples, collected from 47 dairy calves on a calf ranch and in a dairy herd in California, and found a high number of calves (38 calves, 81%) were positive for group B rotavirus using reverse transcription-polymerase chain reaction (RT-PCR). Brown et al.(26) found a high prevalence of group B rotavirus-specific antibody in animal sera in U.K.: pigs, 97%; cattle, 71%; sheep, 91%; and goats, 91% using ELISA based on human ADRV strain, but Bridger (23) reported a low prevalence (20 %) of cow sera in the U.K. using porcine NIRD-1 strain.

**Porcine group B rotaviruses.** Theil et al.(223) identified rotavirus-like virus (group B rotaviruses) in fecal samples of a diarrheic pig. This virus was antigenically unrelated to group A rotaviruses, or group C rotaviruses, but was antigenically related to a bovine group B rotavirus. When inoculated into MA104 cells, this virus induced syncytium formation after low-speed centrifugation of the inoculum onto the monolayer. In Australia, Nagesha et al.(154) identified group B rotaviruses in 16 out of 237 (5.3%) of diarrheic fecal samples from piglets using PAGE. Theil et al.(223) reported ten of 44 (23%) of Ohio swine sera were
group B rotavirus antibody positive using an indirect immunofluorescence test.

**Lamb group B rotaviruses.** Theil et al. (227) reported a group B rotavirus associated with an outbreak of neonatal lamb diarrhea in Ohio in the U.S. In the U.K. Chasey and Banks (32, 33) reported atypical ovine rotaviruses which cause giant syncytia of epithelial cells when they inoculated into colostrum-deprived lambs. Snodgrass et al. (210) also reported atypical rotaviruses which failed to react group A rotaviruses by ELISA and showed distinct electropherotypes from group A rotaviruses in diarrheic lambs.

**Human group B rotaviruses.** During the early 1980's, there were severe epidemics of diarrhea among both adults and children in China involving several large communities associated with group B rotaviruses and referred to as adult diarrhea rotavirus (ADRV) (34). Dai et al. (53) reported that group B rotaviruses were also associated with an infant outbreak in a nursery in China. In the U.S., Eiden et al. (64) reported diarrhea associated with group B rotavirus which was similar to rat IDIR virus antigenically and by genomic profile. However, a low antibody prevalence has been reported in human sera in U.S. Brown et al. (26) found a prevalence in human sera from the U.K. of 4-10% using ELISA. Nakata et al. (155, 156) also reported a low prevalence (10 of 237 or 4.2%) of group B rotavirus antibodies in human sera in China using ELISA.

**Immunity and Control**

Little information is available on immunity to group B rotaviruses. In humans, because the transmission of viruses may be food- and/or water-borne, the control of group B rotaviruses may be achieved by improvement of public sanitation and public health. The
control of group B rotaviruses in animals may be similar to that for group A rotaviruses, and neonatal animals probably need to receive adequate levels of passive antibodies for passive protection. However little is known about active immunity or protection in adult animals which is a different scenario than to group A rotaviruses.
1.4 Group C rotviruses

Group C rotaviruses were first detected in swine in 1980 (193), and subsequently identified in humans, ferrets, and cattle (24, 200). Previous studies indicated that group C rotavirus infections are widespread in swine and humans in some parts of the world. During the last decade, human group C rotaviruses have been associated with several outbreaks of acute diarrhea among children and adults in Japan (24, 200). They have also been detected in diarrheic human fecal samples worldwide (24, 200) including ones from the U.S. (112), suggesting that group C rotaviruses may be emerging enteric pathogens for humans.

In swine, group C rotaviruses were reported as a cause of a large outbreak of enzootic diarrhea in neonates (148) and in older weaned pigs (120). Prevalence studies of group C rotavirus antibodies in pig sera showed that 100% of adults, 59% of weaning pigs, and 86% of nursing pigs surveyed had antibodies, which suggests that group C rotaviruses are endemic in U.S. swine herds (197, 200, 221, 234).

Group A rotaviruses are endemic and group B rotaviruses cause sporadic cases of diarrhea in calves and cows in the U.S. (23, 197, 200). Although serological evidence exists for bovine group C rotaviruses in the U.S. (234), there are no reports of their detection or isolation. To date, the Shintoku group C bovine rotavirus, which was isolated in Japan from an adult cow, is the only bovine isolate of group C rotavirus (232).

Morphology

The morphology of group C rotaviruses is indistinguishable from group A rotaviruses, and both double- and triple-shelled particles were seen by EM of viruses in fecal samples. Like
group B rotaviruses, group C rotaviruses are more easily disrupted during EM preparation (179).

**Genome / proteins**

The genomic profile of group C rotaviruses is described as 4-3-2-2 (23, 200). The polypeptide structure of group C rotaviruses is similar to that of group A rotaviruses (134). Although group C rotaviruses are antigenically distinct from group A rotaviruses they are genetically and serologically related. The VP6 genes of group C rotaviruses shared 88.4 to 90.6% homology among them (Cowden, Shintoku and Bristol) and 41.3 and 16.3% homology with the VP6 of bovine group A (RF) and human group B (ADRV) rotaviruses, respectively (111). Antigenically, Tsunemitsu et al. (235) reported that five monoclonal antibodies (MAbs) to porcine group C rotaviruses were reactive with both group A and C rotaviruses in cell culture immunofluorescence (CCIF) tests, which suggested that group A and C rotaviruses share a common antigen located on VP6. Also antigenic and genetic variation exists within group C rotaviruses. Tsunemitsu et al. (233, 236) found that there are at least 3 G serotypes based on the VP7 gene within group C rotaviruses using two-way cross-neutralization tests and sequence analysis of VP7 genes (69.9-74.7% identity among the serotypically distinct strains)

**Cell culture cultivation**

A porcine group C rotavirus (Cowden strain) was adapted to serial propagation in roller tube cultures of primary porcine kidney cells using high concentrations of pancreatin, and
consequently was adapted to serial passage in a continuous monkey kidney cell line (MA104). (195, 222). Tsunemitsu et al.(232) isolated a bovine group C virus (designated the Shintoku strain) in MA104 cells from the feces of adult cows with bloody diarrhea in Japan.

Pathogenesis

Like group B rotaviruses, group C rotaviruses are frequently associated with older animals than are the group A rotaviruses. Group C rotaviruses caused profuse, watery, yellow diarrhea and occasional vomiting in pigs (15, 148). In human, clinical symptoms associated with group C rotaviruses were reported to be less severe than those seen with group B rotaviruses: mainly abdominal pain and vomiting were predominant (133). Tsunemitsu et al.(232) found a bovine group C rotavirus associated with bloody diarrhea in adult cows, but its pathogenesis in cows or calves has not been studied.

When group C rotavirus was inoculated into gnotobiotic pigs, viruses were found in villous enterocytes of the small intestines by immunofluorescence, and villous atrophy and diarrhea were evident (15). Mortality was high when gnotobiotic pigs less than 5 days old were infected (15). Morin et al.(148) necropsied piglets from different outbreaks of diarrhea associated with group C rotaviruses and found multifocal villous atrophy in the small intestine, especially in the ileum.

Diagnosis

Several assays to detect group C rotaviruses have been described. These include PAGE, IEM, ELISA, cell culture immunofluorescent test (CCIF), c-DNA hybridization and RT-PCR.
Like group B rotaviruses, it has been reported that group C rotavirus shedding by infected animals or humans may be limited and the virus may be unstable during storage or IEM preparation (23). Jiang et al. (112) noted difficulty in detecting group C rotaviruses in diarrheic samples from children because of the low titers of viruses in fecal samples, and suggested that use of conventional methods such as PAGE may underestimate the true detection rate for group C rotaviruses.

**Epidemiology**

**Bovine group C rotaviruses.** A virus (designated the Shintoku strain) which was morphologically indistinguishable from group A rotaviruses, was detected and identified as a group C rotavirus by PAGE from the feces of adult cows with diarrhea in Japan (232). This is the only report of the detection of group C rotaviruses in cattle. However, Tsunemitsu et al. (234) reported a moderate prevalence of antibodies to group C rotaviruses in sera from adult cattle (47 to 56%) in the United States and Japan using ELISA.

**Porcine group C rotaviruses.** Saif et al. (193) first reported pararotaviruses (group C rotaviruses) from diarrheic pig samples. The porcine group C rotavirus was the same size, morphology as group A rotaviruses, and had a tropism for villous enterocytes of the small intestine. However, it was antigenically and electrophoretically (migration of dsRNA in PAGE) distinct from group A rotaviruses. Morin et al. (148) reported a porcine group C rotavirus associated with outbreaks of enzootic neonatal diarrhea with high morbidity (up to 100%) and low mortality (5-10%): clinical signs were characterized by a profuse yellow diarrhea lasting a few days. Terret et al. (221) determined the prevalence of group C rotavirus
antibodies in pig sera. In pigs from four herds, group C virus antibodies were detected in 100% (68 of 68) of adults and 59% (24 of 41) of weanling pigs, while 86% (24 of 28) of nursing pigs from 12 herds were seropositive.

Human group C rotaviruses. Nicolas et al.(158) reported the first detection of a group C rotavirus from a human diarrheic sample in U.K. Since 1988, several diarrhea outbreaks associated with group C rotaviruses have been reported in Japan (107, 166, 206). In April, 1988, there was a large outbreak (almost 60,000 people) characterized acute gastroenteritis, mild, predominantly abdominal pains and vomiting with diarrhea, associated with group C rotavirus, which occurred among schoolchildren and their teachers simultaneously at seven elementary schools in Japan (23). Sekine et al.(206) reported an outbreak of acute gastroenteritis due to group C rotavirus at an elementary school in Tokyo: fifty-one (13%) of 393 students became ill, and the main clinical symptoms were diarrhea (100%), abdominal pain (68%) and vomiting (56%). Oishi et al.(166) also reported a severe diarrhea outbreak by group C rotaviruses via food-borne transmission among college students who joined a group trip. Recently, an outbreak of gastroenteritis associated with group C rotaviruses in adults and children in Brazil was reported by Souza et al.(212). Group C rotaviruses associated with family outbreaks of gastroenteritis have been reported in England and Finland (29, 135). Also group C rotaviruses have been detected in diarrheic fecal samples from Thailand, Nepal and the U.S. (112, 135).

Ishimaru et al.(107) studied the epidemiology human group C rotavirus infections in Matsuyama, Japan from January to May, 1988. Group C rotavirus gastroenteritis occurred following an epidemic of group A rotavirus infections, primarily in children aged 4-7 years,
but rarely in those aged 0-1 years. Antibody was seldom found in the sera of patients under 3 years of age, but it was found in 20-40% of those who were 4 years or older. Riepenhoff-Talty et al.(187) reported 30% of umbilical cord serum samples were group C rotavirus positive during 1990 to 1992 by an indirect immunofluorescence assay. James et al.(109) used baculovirus expressed VP6 of human group C rotavirus to detect group C rotavirus serum antibodies and reported that an average of 43% of samples had antibodies to human group C rotavirus with the highest proportion (66%) in the 71-75 year-old age group in the U.K. Cox et al.(52) also reported that the serum antibody prevalence to group C rotavirus was low in children under 5 years of age and increased slowly with age to 36% seropositivity in adults in Brazil.

Immunity and Control

Little information is available on immunity to group C rotaviruses. In humans, because the transmission of viruses may be food- and/or water- borne, the control of group C rotaviruses may be achieved by improvement in sanitation and public health. The control of group C rotaviruses in animals may be similar to methods based for group A rotaviruses; ie, animals need to receive adequate levels of passive antibodies to prevent neonatal diarrhea and may require active immunization to prevent diarrheas in weaning or adult animals.
REFERENCES


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CHAPTER 2

THE CHARACTERIZATION OF VP7 (G TYPE) AND VP4 (P TYPE) GENES OF BOVINE GROUP A ROTAVIRUS FROM FIELD SAMPLES USING RT-PCR AND RFLP ANALYSIS

2.1 SUMMARY

Characterization of the VP7 (G type) and VP4 (P type) genes of bovine group A rotaviruses (BRV) from field samples was performed using RT-PCR and RFLP analysis. After RT-PCR amplification of the full length VP7 genes and partial length VP4 genes (nucleotides 1 to 1096), four enzymes, EcoRV, Nla IV, BamHI and Hpa II were used for digestion analysis. For VP7, four RFLP profiles were observed after analysis of the digests: they were designated as G6, G6s (subtype, showed about 86% nucleotide and 90% amino acid identity to reference G6 strains), G8 and G10. For VP4, three RFLP profiles were observed: designated as P[1], P[5] and P[11].

The G typing analysis of 86 BRV fecal samples from 5 states, representing at least 11 different herds revealed that 60.5% (52/86) were G6, which included G6s (9/52); 19.8% (17/86) were G10; 7% (6/86) were G8; 10.4% (9/86) were G6 and G10 mixtures including two G6s samples; and 2.3% (2/86) were G6 and G6s mixtures. The P typing analysis of the
same 86 fecal samples revealed that 64% (55/86) were P[5]; 28% (24/86) were P[11]; 1.2% (1/86) were P[1] and 6 samples (7%) were mixtures of either P[11] with P[1] or P[5]. When the same samples were analyzed according to G and P type specificity, all possible combinations of G and P types existed in the field. The G6P[5] type was most prevalent and accounted for 46.7% (41/86) of the samples; 12.8% (11/86) were G10P[11]; 7% (6/86) were G10P[5] and an equal number were G6sP[11]. The G6P[11] (n=2), G8P[1] (n=1), G8P[5] (n=1) and G8P[11] (n=3) combinations were also observed. The following mixed BRV infections were observed in the field samples; G6sP[5+11] (n=1), G8P[5+11] (n=1), G6+G10P[5] (n=1), G6+G10P[5+11] (n=2), G6+G6sP[11] (n=1), G6+G6sP[1+11] (n=1), G6s+G10P[11] (n=1) and G6s+G10P[5+11] (n=1). Information on the G and P types and G/P combinations in the field samples should be useful for understanding the epidemiology of BRV and designing vaccination strategies to control BRV in the field.

2.2 INTRODUCTION

Group A rotaviruses, members of the Reoviridae family, are important viral diarrheal agents in young animals and humans, including young calves worldwide (23). Rotaviruses possess two outer capsid proteins, VP4 (encoded by gene segment 4) and VP7 (encoded by gene segment 7, 8 or 9 depending on the strain), each of which independently induce neutralizing antibodies (6, 16). The neutralization specificity related to VP7 is referred to as the G serotype (for glycoprotein), and that associated with VP4 is referred to as the P serotype (for protease-sensitive protein) (6, 16). On the basis of G types (VP7), at least 14 serotypes of group A rotaviruses have been described in humans and animals (16). At present
at least 7 G types have been reported among bovine rotavirus (BRV) strains (G1-G3,G6,G8,G10,G11) with G6 and G10 predominating (3, 18, 24). The established G6 strains include NCDV-Lincoln and UK, and the B223 strain serves as a prototype for G10 (23, 26). At least three P types (VP4) of BRV exist, P[1], P[5] and P[11] represented by NCDV-Lincoln, UK and B223, respectively (23).

Determination of the serotype specificity is important for development and evaluation of more efficacious BRV vaccines, but this is complicated by the dual serotype specificity of rotavirus (23). Several assays have been developed for the G and P typing of rotaviruses and according to the methods used, there were significant differences in their specificity and sensitivity. These include plaque reduction and fluorescent focus virus neutralization, enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (MAbs), nucleic acid probes, and RT-PCR (1, 2, 8, 15, 17, 18, 24, 25, 27). There is a need for additional reagents and methods to examine human and animal rotavirus specimens, because some of the currently available methods may fail to detect " subtypes " or " monotypes " that occur within rotavirus (24) and few MAbs are available for analysis of P serotypes especially for animal rotaviruses. In this study, G and P typing of field samples of group A BRV were performed using RT-PCR and endonuclease digestion of amplified VP7 (full length) and VP4 (partial length including VP8 region) genes. In addition, the combination of G and P types was assessed for each sample tested to determine their distribution in the field.

2.3 MATERIALS AND METHODS

Viruses and cells.
The reference group A BRV strains used for RFLP analysis have been described previously and included: NCDV-Lincoln (G6P[1]), NCDV-Cody (G8P[1]), IND (G6P[5]), OK (G6P[5]), ID (G6P[5]), B223 (G10P[11]), 2292B (G10P[11]) and Cr (G10P[11]) strains (19). Also for G typing, Wa (G1, human), DS-1 (G2, human), SA11 (G3, simian) and 69M (G8, human) strains were included. The reference viruses were grown as described previously in Rhesus monkey kidney (MA104) cells in the presence of pancreatin (50ug/ml) (22).

Field strains of BRV.

Eighty-six fecal samples positive for group A BRV by ELISA, cell culture immune fluorescence (CCIF) or immune electron microscopy were obtained from diarrheic calves in Ohio (n=28, at least 4 different herds), South Dakota (n=27, at least 3 different herds), Nebraska (n=13, two herds), California (n=4, one herd), Wyoming (n=3, one herd) and from field samples passaged in gnotobiotic calves (n=11) during 1990 to 1994. As negative controls, group A BRV negative field samples, fecal samples from an uninfected gnotobiotic calf and samples from gnotobiotic calves infected with bovine group B and C rotaviruses were also included (28). Before RFLP analysis, all samples were tested for the presence of group A BRV dsRNA by polyacrylamide gel electrophoresis (PAGE) (20).

Extraction of dsRNA and electrophoresis.

Rotavirus dsRNA was extracted from cell culture-propagated virus and fecal viruses using previously described procedures (20). Rotavirus dsRNA in extracted samples was analyzed by polyacrylamide gel electrophoresis (PAGE) to confirm the presence of dsRNA and to
examine the genomic electropherotypes as previously described (9). The discontinuous buffer system of Laemmli was utilized and dsRNA was resolved in 10% polyacrylamide slab gels (15). Electrophoresis was conducted at 12mA for 14 to 16 h. The dsRNA bands were visualized by silver or ethidium bromide staining (21).

RT-PCR of full length VP7 and partial length VP4 genes

RT-PCR was used to produce full length BRV VP7 and partial length VP4 gene fragments. cDNA was synthesized and amplified in an RT-PCR mixture containing 10X buffer (200 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, KCl, 0.05% gelatin), dNTP (10mM, each), 200ng of primer A (For VP7 sense, 5' end: 5'-GGCCGGATTTAAAAGCGACAA TTT-3' and for VP4, 5'-GGCTATAAAAATGGCTT CGCT-3', nt 1-20 of VP4, P[5]), 200ng of primer B (For VP7 antisense, 3' end: 5'-GGTCACATCATACAACTCTA-3' and for VP4, antisense 5'-AATGCCTGTGAATCGTCCCA-3', nt 1075-1094 of VP4, P[5]) (19), 5U RNAsin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 10U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) and 2.5 U of Taq polymerase (Boehringer Mannheim Biochemicals). First strand cDNA synthesis was accomplished by incubating the above mixture for 90 min at 42 C. Thirty amplification cycles were conducted, with each cycle consisting of 94 C for 1 min (denaturation), 48 C for 1 min (annealing) and 72 C for 2 min (extension), followed by a 7 min extension at 72 C. The full-length PCR products were analyzed on 1% agarose gels using standard procedures (21).

Enzyme digestion of amplified VP4 and VP7 genes
Three enzymes, *Eco RV, Bam HI* (Boehringer Mannheim Biochemicals) and *Nla IV* (New England Biolab) for VP7 and *EcoRV, Hpa II* (Boehringer Mannheim Biochemicals) and *Nla IV* for VP4 were used for digesting the amplified gene products. These enzymes were chosen because they produced distinct digestion patterns on the basis of published sequence data and empirical investigation. For each digestion, 10 ul of amplified DNA and 10X buffer and enzyme were mixed and incubated at 37°C for 2 hours and analyzed on 1% agarose gels using standard procedures (21).

**Cloning and sequencing of the VP4 and VP7 genes**

To verify the G and P types of selected BRV field strains, amplified cDNA fragments representing each BRV RFLP type and the samples which showed unusual patterns were cloned into the pCRII plasmid (Invitrogen) using TA cloning and sequenced using the primer extension method (21). The sequence data were analyzed using a DNASTAR program.

### 2.4 RESULTS

**RT-PCR**

The VP7 and VP4 genes of all cell culture-passaged and 86 field BRV fecal samples were successfully amplified using RT-PCR. The amplified genes were of the expected size (1,062 bp for full length VP7 and 1,096 bp for partial length VP4) (Figs. 2.1 and 2.2). No reactions were observed with BRV negative control samples or group B and C BRV positive samples.

**Enzyme digestion patterns (RFLP) of reference rotavirus strains**

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Digestion of the VP7 gene of G1, G2, G3, G6, G8 and G10 rotavirus strains with both Eco RV and Nla IV resulted in 6 RFLP digestion profiles (Fig. 2.1A,B); whereas, with Bam HI, 4 digestion profiles were observed (Fig. 2.1C). Overall the RFLP patterns of the three enzyme digests could discriminate G6, G8 and G10 types from other types (G1, G2, G3) (Fig. 2.1A and B). For the G8 strains, 69M and NCDV-Cody (I801), different patterns were evident after digesting with Nla IV and Bam HI due to the low nucleotide sequence homologies between the two G8 strains (4). For VP4, digestion of reference BRV strains with both EcoRV and Hpa II resulted in 3 digestion profiles, whereas with Nla IV, 2 digestion profiles were observed (Fig 2). These RFLP patterns of the three enzyme digests could discriminate P[1], P[5] and P[11]. The results of G and P type analysis of reference BRV strains were as follows: NCDV-Lincoln (G6P[1]), NCDV-Cody (G8P[1]), IND, ID, OK (G6P[5]) and B223, 2292B and Cr (G10P[11]) (Fig 2).

Digestion patterns of field BRV

After digestion of the VP7 genes with Eco RV, Nla IV and Bam HI, BRV field strains could be classified into four RFLP (I-IV) types (Table 1, Fig. 1-A,B, and C). RFLP type I represented strains with NCDV-like (G6) profiles, and RFLP type II represented strains with B223-like (G10) profiles. Additional BRV strains were classified as RFLP types III (G6s) and IV (G8). RFLP type III strains had restriction enzyme profiles similar to NCDV-Lincoln strain for Eco RV and Bam HI digests but not for the Nla IV digest. RFLP type IV was similar to NCDV-Cody (I801)(G8) in Bam HI digestion profiles and with 69M (G8) in Nla IV digestion profiles. Therefore the RFLP types III and IV were further characterized and the
genotypes were confirmed by nucleotide sequencing. After nucleotide sequence analysis, RFLP types IV exemplified by C-8008 showed high homology (86-89%) with G8 strains and RFLP III exemplified by C-8663 showed high homology (97%) with the KN-4 strain (23) which is a G6 subtype (G6s) based on genetic and serological analysis.

After the digestion of VP4 gene products with *EcoRV*, *Hpa II* and *NlaIV*, the BRV field strains could be classified into RFLP types I-III (Table 1, Fig 2). RFLP type I represented strains with NCDV-Lincoln-like profiles (P[1]), RFLP type II represented strains with IND-like profiles (P[5]), and type III was classified as B223-like profiles (P[11]). Some strains (n=5) showed atypical profiles with one or two enzymes, and after additional analysis (nucleotide sequencing), they could be classified as P[5] or P[11] (data not shown).

*Prevalence of G, P types and G/P combinations of BRV strains analyzed by RFLP.*

The results of the G typing analysis of 86 BRV field samples using RT-PCR, RFLP and sequence analysis are presented in Table 2. The G typing analysis revealed that 60.4% (52/86) were G6, which included G6s with pattern III (9/52); 19.8% (17/86) were G10; 6.9% (6/86) were G8; 10.4% (9/86) were G6 and G10 mixtures including two G6s samples; and 2.3% (2/86) were G6 and G6s mixtures (Table 2). The results of the P typing analysis of 86 BRV field samples using RT-PCR, RFLP and sequence analysis are presented (Table 3). The P typing analysis of 86 fecal samples revealed that 1.2% (1/86) were P[1], 64% (55/86) were P[5], 28% (24/86) were P[11] and 6 samples (7%) were mixtures of either P[1] and P[11] or P[5] and P[11].

All possible combinations of G and P types existed in the field (Table 4). The G6P[5] type

2.5 DISCUSSION

In the present study, we examined the prevalence of G and P types of group A BRV field strains using RT-PCR, RFLP and sequence analysis. Overall four restriction enzymes, Eco RV, Nla IV, Hpa II and Bam HI could discriminate G and P types of BRV strains. In addition, these methods allowed us to identify BRV G6 subtypes among the field samples.

Previous investigators have studied the prevalence of the infection of calves with serotype G6, G8 and G10 rotaviruses (15, 19, 24, 29). Woode et al (29) used two-way cross-neutralization assays and demonstrated that 89% of the isolates (n=73) were related to G6, and of the remaining isolates, 63% were related to G10. Snodgrass et al (24) used a MAb-based ELISA for G typing and reported that 66% of the isolates were G6, 7.4% were G10, and only 1 sample (0.6%) was G8. In the same study, two viruses belonging to a monotype within serotype G6 failed to react with the serotype G6-specific Mab (24). Lucchelli et al. (15) also used a Mab-based ELISA, and demonstrated that among 308 fecal samples, 79% (244/308) tested positive by a broadly reactive VP7 MAb, and of these, 54% were G6, 14% were G10, 4% were both G6 and G10, and 28% were G6 and G10 negative. Parwani et al
used PCR-generated G type specific cDNA probes and reported that 36.3% of BRV field samples tested were G6, 12.8% were G10 and 2.9% were G8; however, 23.5% of the field samples tested were not typable using this method. In the present study, all strains were typable and 60.5% of the BRV fecal samples tested belonged to G6, 19.8% were G10 and 7% were G8. Mixed samples containing G6 and G10, G6 and G6s, and G6s and G10 accounted for 8.1%, 2.3% and 2.3% of the samples, respectively (Table 2). The G6s strains showed up to 18% differences in nucleotide sequence homology with established G6 BRV and human strains (NCDV-Lincoln, UK, PA151 and PA169) (data not shown). The G6s strains showed high conservation in amino acid sequences within several serotype-specific antigenic regions (A,B,C) (5). However, some the G6s strains showed low reactivity with the IC3 MAb reactive with the G6 UK strain and other G6 strains (15) in ELISA (data not shown). In this study, G6 subtypes occurred in cattle in relatively high prevalence (over 15% of field samples) in different geographical regions suggesting that this is not a random occurrence. These G6 subtype strains have not been previously recognized possibly due to a lack of suitable reagents and/or use of sensitive procedures. By using conventional typing methods (serotyping ELISA, cDNA probes), these G6 subtype strains may appear to be non-G6 and therefore misclassified.

There is only limited data on the prevalence of P types of BRV field samples. One reason for the difficulty in P typing rotaviruses is that the VP4 protein is much less abundant in the outer capsid than VP7, which means that assays based on VP4 antigen quantitation may be less sensitive for routine P typing (16). Therefore, assays based on the genomic P typing seem to be more consistent and reliable. Suzuki et al. (25) reported that 10% (4/40) of BRV field
isolates were P[1], 55% (22/40) were P[5], 27.5% (11/40) were P[11] and 3 samples were not typable using a nested PCR method. Parwani et al. (19) also reported P typing of BRV field samples using cDNA probes: 2.2% (2/93) were P[1], 20.4% (19/93) were P[5], 9.3% (10/93) were P[11] and 40.8% (38/93) remained untypable using this method. In our study, the overall prevalence of P types was as follows: P[5] (64%) was the most common followed by P[11] (28%) and P[1] (1.2%). It is interesting that only two samples including mixed infections were identified as P[1] in the field samples because the P[1] strains (NCDV-Lincoln and Cody) were first isolated in the U.S. and NCDV-Lincoln is the BRV vaccine strain used in the U.S. This rarity in the U.S. compared to a higher prevalence in another country (25) could be due to the widespread use of the BRV (G6P[1]) vaccine in the U.S. The VP4 genes (VP8 region) showed more diversity than VP7 genes among BRV: 5 samples had atypical RFLP profiles with one or two enzymes, which were confirmed as P[5] or P[11] by sequence analysis (data not shown).

Suzuki et al. (25) reported the G/P combination of BRV field isolates in Japan after using nested PCR to determine individual G (G6, 10) and P (P[1,5,11]) types. They found that G6P[5] was most frequent (42.5%) followed by G6P[11] (17.5%), G6P[1] (10%), G10P[5] (10%) and G10P[11] (7.5%). In the present study, the G6P[5] type was also most frequent (46.7%) followed by G10P[11] (12.8%), G10P[5] (7%), G6sP[11] (7%), G8P[11] (3.5%), G6sP[5] (2.3%), G6P[11] (2.3%), G8P[1] (1.2%), G8P[5] (1.2%) and the combinations of mixed infections. Although the G6P[5] (represented by reference strains UK, IND, OK and ID) and G10P[11] (represented by reference strains B223, 2292B and Cr) types were dominant in the field samples, overall, all possible combinations occurred in the field. This
could be caused by naturally occurring mixed infections (as demonstrated in this study) and reassortment in the host. The pathogenicity of these different G/P type combinations seemed similar after passage of these samples in gnotobiotic calves (Saif and Chang, unpublished).

We demonstrated in the present study that RT-PCR and RFLP is a reliable method for the G and P typing of BRV based upon initial analysis of a panel of reference BRV strains. The accuracy of G and P type analysis of selected field samples determined by this method was further confirmed by sequence analysis. Although this method may not be suitable for every diagnostic laboratory, information on the G and P types and G/P combinations in the field should be useful for understanding the epidemiology of BRV and designing vaccination strategies to control BRV in the field.

2.6 ACKNOWLEDGEMENT

The synthetic primers used in this study were kindly provided by Dr. Baoming Jiang at Lederle-Praxis Biologicals, Rochester, NY. Various rotavirus strains were kindly provided by Drs. Ram Mohan, Ohio Department of Agriculture; Fernando Osorio, University of Nebraska; Hana Van Campen, University of Wyoming; Barbara Daft, University of California; and David Zeman, South Dakota State University. Salaries and research support provided by State and Federal Funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Approved as Journal Article No. 95-3. This study was supported in part by the NRI Competitive Grants Program, USDA, CSREES, Award No. 93-37204-9201.
2.7 REFERENCES


the VP7 genes in bovine rataviruses as confirmed by VP4 sequence analysis of G8 and G10

S (1992) Identification of human and bovine rotavirus serotypes by polymerase chain

Hirai T (1991) Isolation, characterization and serial propagation of a bovine group C

29. Woode GN, Kelso NE, Simpson TF, Gaul SK, Evans LE and Babiuk L (1983) Antigenic
relationships among some bovine rotaviruses: serum neutralization and cross-protection
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<th>Pattern</th>
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<th>P typing</th>
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<tr>
<td></td>
<td>G type</td>
<td>P type</td>
</tr>
<tr>
<td></td>
<td>Enzyme</td>
<td>Size of band</td>
</tr>
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</tr>
<tr>
<td></td>
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<td>1062</td>
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<td></td>
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<td>795, 267</td>
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*The size of the bands (bp) obtained after enzyme digestion were calculated on basis of nucleotide sequence data.

Table 2.1. Restriction Fragment Length Polymorphism analysis of full VP7 (G typing) and partial VP4 gene (P typing) of BRV strains with three restriction enzymes.
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<th>Sample origin</th>
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<td>G8(^b)</td>
</tr>
<tr>
<td>Field strains</td>
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<td></td>
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<tr>
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<tr>
<td>Wyoming</td>
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<td>2 (0)</td>
</tr>
<tr>
<td>Calf passaged</td>
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<td>6 (1)</td>
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<tr>
<td>Total</td>
<td>86</td>
<td>52 (9)</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>60.4 (10.4)</td>
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</tbody>
</table>

\(^a\) Samples were typed as G6 or G6s if they exhibited RFLP patterns I or III respectively.
\(^b\) Samples were typed as G8 if they exhibited RFLP pattern IV.
\(^c\) Samples were typed as G10 if they exhibited RFLP pattern II.
\(^d\) Samples were typed as mixed if they exhibited both RFLP patterns.

Table 2.2. Identification of bovine rotavirus G types from fecal samples by RT-PCR and RFLP
Table 2.3. Identification of bovine rotavirus P types from fecal samples by RT-PCR and RFLP

<table>
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<td>-</td>
</tr>
<tr>
<td>Calf passaged</td>
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<td>-</td>
<td>5</td>
<td>6</td>
<td>-</td>
<td>-</td>
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<td>28.0</td>
<td>1.2</td>
<td>5.8</td>
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</table>

<sup>a</sup> Samples were typed as P[1] if they exhibited RFLP pattern I.

<sup>b</sup> Samples were typed as P[5] if they exhibited RFLP pattern II.

<sup>c</sup> Samples were typed as P[11] if they exhibited RFLP pattern III.

<sup>d</sup> Samples were typed as P[1+11] or P[5+11] if they exhibited both RFLP patterns.
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<td>G8</td>
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<td>1</td>
<td>3</td>
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<tr>
<td>G10</td>
<td></td>
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<td>-</td>
<td>6</td>
<td>11</td>
<td>-</td>
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<tr>
<td>G6+G10</td>
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<td>-</td>
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<tr>
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<td>55 (64.0)</td>
<td>24 (28.0)</td>
<td>1 (1.2)</td>
<td>5 (5.8)</td>
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Table 2.4. G and P type combination of BRV field samples by RT-PCR and RFLP
Figure 2.1. A, RFLP profiles of rotavirus VP7 genes digested with Eco RV after separation on a 1.5% agarose gel. The gel was stained with ethidium bromide and visualized using UV light illumination. Lanes A: Wa (G1), B: DS-1 (G2), C: SA11 (G3), D: NCDV-Lincoln (G6), E: NCDV-Cody (G8), F: 69M (G8), G: B223 (G10), H: 2292B (G10), I-M: BRV field samples. Digestion of G1, G2, G3, G6, G8 and G10 rotavirus strains with Eco RV resulted in 6 RFLP patterns. Wa and SA-11 shared the same pattern (lane A,C) and other representative G strains had their own patterns unique to each G type. For field BRV strains, 2 patterns were observed: lanes I,J,K (NCDV-Lincoln); and lanes L,M (B223).

B, RFLP profiles of rotavirus VP7 genes digested with Nla IV. Lanes and condition, same as Fig. 1A. Digestion of G1, G2, G3, G6, G8 and G10 rotavirus strains with Nla IV resulted in 6 RFLP patterns. Wa and DS-1 shared the same pattern and other strains had their own patterns. Cody I-801 and 69M (lanes E, F) were the same G serotype (G8) but the RFLP patterns differed. For field BRV strains, sample in lane I was the same as NCDV-Lincoln, samples in lanes J,K displayed unique patterns and samples in lanes L,M were B223-like.

C, RFLP profiles of rotavirus VP7 genes digested with Bam HI. Lanes and condition, same as Fig. 1A. 4 RFLP patterns were observed. Wa, DS-1, NCDV-Lincoln and 69M strains (lanes A,B,D,F, respectively) were not digested by Bam HI and SA-11, NCDV-Cody and B223 strains (lanes C,E,G, respectively) had unique patterns. For field BRV strains, sample in lane I was not digested, samples in lanes J,K displayed the same profiles as Cody I-801 and samples in lanes L,M were similar to the B223 strain.
Figure 2.2. A, RFLP profiles of rotavirus partial VP4 genes digested with Eco RV after separation on a 1.5% agarose gel. The gel was stained with ethidium bromide and visualized using UV light illumination. After digestion with EcoRV, 3 RFLP patterns were observed. Lane A: undigested partial length VP4, B: NCDV-Lincoln (P[1]), C: NCDV-Cody (P[1]), D: IND (p[5]), E: OK (p[5]), F-G: field BRV samples which were designated as P[5], H: B223 (P[11]), I: Cr (P[11]), J-K: field BRV samples which were designated as P[11]. Lane G: the field sample showed an atypical pattern after EcoRV digestion, which was confirmed as P[5] by partial sequence analysis.

B, RFLP profiles of rotavirus partial length VP4 genes digested with NlaIV. Lanes and conditions, same as Fig.2A. Digestion with NlaIV produced 2 patterns: P[1] and P[5] BRV strains (lanes B-G) shared the same pattern.

C, RFLP profiles of rotavirus partial length VP4 genes digested with HpaII. Lanes and conditions, same as Fig.2A. Three distinct patterns were observed for P[1] (lanes B-C), P[5] (lanes D-G) and P[11] (lanes H-K). Lane K: the field sample showed an atypical pattern in HpaII digestion, which was confirmed as P[11] by partial sequence analysis.
CHAPTER 3

COMPARATIVE SEQUENCE ANALYSIS OF THE VP7 GENES OF G6, G8 AND G10 BOVINE GROUP A ROTAVIRUSES AND FURTHER CHARACTERIZATION OF G6 SUBTYPES

3.1 SUMMARY

We previously reported the relatively high prevalence (15 %) of bovine G6 subtypes (G6s) in the field using RT-PCR and restriction fragment length polymorphysm (RFLP) analysis (Chang et al., Arch. Virol. 141:1727-39). In the present study, we report the nucleotide and antigenic characterization of a G6s strain (C-8336). We also sequenced the VP7 genes of four additional bovine rotavirus (BRV) strains: another G6s (MC27), G6 (IND), G8 (C-8008) and G10 (2292B) and compared these with other bovine and human rotavirus strains. The C-8336 and MC27 strains were confirmed as P[11]G6s by RT-PCR and RFLP analysis. The VP7 genes of the C-8336 and MC27 strains showed high homology to each other (~98%) and with other bovine G6s strains (greater than 95 % homology in nucleotide and amino acid sequence with KN-4{P[11]G6s}) and also showed lower, but substantial sequence homology with human G6s strains and prototype G6 BRV (79 - 87 %
in nucleotide and 88 – 91% in amino acid). Serologic analysis of the cell culture isolated C-8336 strain showed that it was neutralized by a G6 monoclonal antibody (MAb, IC3) to similar titers as the reference NCDV and IND G6 strains. In two-way neutralization tests, strain C-8336 showed 4- to 16-fold differences in antibody titers with NCDV and IND G6 BRV. Moreover polyclonal antiserum against strain C-8336 neutralized the NCDV and IND strains weakly. Genetic variability was also observed among G8 and G10 bovine and human group A rotaviruses: the VP7 genes of C-8008 (P[5]G8) and 2292B (P[11]G10) strains showed from 10 to 17 % nucleotide divergence with those of Cody I801 (P[1]G8, bovine), A5 (P[1]G8, bovine), 69M (P[10]G8, human) and Hal 1166 (P[14]G8, human), and I321(P[11]G10, human) and MC35 (P[14]G10, human) rotaviruses, respectively. The divergence of VP7 genes among bovine and human G6, G8 and G10 strains appears related to host species origin and their combination with VP4 (P type). The data presented in this report confirms the genetic variability among homotypic bovine and human strains and highlights the importance of continued monitoring of BRV G and P types circulating in the field for the future development and monitoring of effective vaccines.

3.2 INTRODUCTION

Group A rotaviruses, members of the Reoviridae family, are important viral diarrheal agents in young animals and humans, including calves worldwide (22,33). Several studies have shown the existence of significant genetic diversity amongst rotaviruses of different host species, particularly among the outer capsid proteins VP4 and VP7 (VP4 [encoded by gene
segment 4] and VP7 [encoded by gene segment 7, 8 or 9 depending on the strain] which independently induce neutralizing antibodies, 8,26). This diversity may be partly due to independent reassortment of the eleven segmented dsRNAs comprising the virus genome (9,22). Also, since the host range of group A rotaviruses is very wide, possible cross-species transmission likely occurs in nature, which may further influence variability (22,33). The neutralization specificity related to VP7 is referred to as the G serotype (for glycoprotein), and that associated with VP4 is referred to as the P serotype (for protease-sensitive protein) (8,26). On the basis of G types (VP7), at least 14 G and 20 P serotypes of group A rotaviruses have been described in humans and animals (26,33). At present at least 7 G and 3 P types have been reported among bovine rotavirus (BRV) strains (G1-G3,G6,G8,G10,G11 for G types and P1,P5,P11 for P types) with G6, G10, P5 and P11 predominating (2,4,19,24,29,35). The established BRV strains include NCDV-Lincoln (P[1]G6), UK (P[5]G6), B641 (P[5]G6), IND (P[5]G6) and B223 (P[11]G10) strains (4,7,16,28). Although all possible combinations of G and P types are reported in the field, certain combinations such as P[5]G6 and P[11]G10 are dominant (>70%) among field strains (4,20,36). It has been suggested on the basis of in vitro reassortant studies that bovine rotaviruses (G6, G10) genes may confer replication advantages to reassortant viruses, possibly by mediating VP4 to enhance cell receptor interaction (39). Such an enhancement may occur in vivo under natural conditions with certain G and P type combinations providing "selective advantage in terms of efficient-replication cycles within their respective hosts. This provides one possible explanation why certain G and P combinations occur more frequently among human and animal rotaviruses from diverse geographic regions.
Determination of the serotype specificity and characterization of the genetic and antigenic diversity among BRV in the field is important to develop more efficacious BRV vaccines. The genetic variation among G6 serotypes (G6 subtypes, G6s) for human and bovine rotavirus strains has been described (11,13,25,27,28), but little information is available on antigenic variability. Furthermore, there is little or no information on the importance of VP4 in the neutralization specificities of G6 strains versus G6 subtypes. We previously reported a relatively high prevalence (>15%) of G6s in the field using RT-PCR and RFLP analysis (4). Majority of these G6s strains (75%, n=8) had P[11] combination, irrespective of their geographic origin. In the present study, we describe comparative sequence analysis of the VP7 genes of five group A BRV including two field G6s strains with P 11 types. We also describe the antigenic characterization of one of G6s strain (C-8336) using MAb (G6) and polyclonal antisera against reference BRV.

3.3 MATERIALS AND METHODS

Viruses and Cells.

or from previously published reports.

**Extraction of dsRNA and electrophoresis.**

Rotavirus dsRNA was extracted from cell culture-propagated virus and field specimens
using previously described procedures (4). Rotavirus dsRNA in extracted samples was
analyzed by polyacrylamide gel electrophoresis (PAGE) to confirm the presence of dsRNA
and to examine the genomic electropherotypes as previously described (17). The
discontinuous buffer system of Laemmli was utilized and dsRNA was resolved in 10%
polyacrylamide slab gels (23). Electrophoresis was conducted at 12mA for 14 to 16 h. The
dsRNA bands were visualized by silver or ethidium bromide staining (34).

**G and P typing by RT-PCR/RFLP and sequence analysis of BRV VP7 genes.**

Extracted dsRNA was further purified using RNaid kit (Bio101, California, CA), and RT-
PCR was used to produce full length BRV VP7 and partial length VP4 gene fragments for
G and P typing (4). cDNA was synthesized and amplified in an RT-PCR mixture containing
10X buffer (200 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2, KCl, 0.05% gelatin), dNTP (10mM,
each), 200ng of primer A ( For VP7 sense, 5' end: 5'-GGCCGGATTTAAAA
GCGACAATTT-3' and for VP4, 5'-GGCTATAAAAATGGCTTCGCT-3', nt 1-20 of VP4,
P[5]), 200ng of primer B (For VP7 antisense, 3' end: 5'-GGTCACATCATACAACTCA-3'
and for VP4, antisense 5'-AATGCCTGTGAATCGTCCCA-3', nt 1075-1094 of VP4, P[5])
(4), 5U RNAsin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 10U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) and 2.5 U of Taq polymerase (Boehringer Mannheim Biochemicals). First strand cDNA synthesis was accomplished by incubating the above mixture for 90 min at 42 C. Thirty amplification cycles were conducted, with each cycle consisting of 94 C for 1 min (denaturation), 48 C for 1 min (annealing) and 72 C for 2 min (extension), followed by a 7 min extension at 72 C. The full-length PCR products were analyzed on 1% agarose gels using standard procedures (34).

Three enzymes, Eco RV, Bam HI (Boehringer Mannheim Biochemicals) and Nla IV (New England Biolab) for VP7 and EcoRV, Hpa II (Boehringer Mannheim Biochemicals) and Nla IV for VP4 were used for digesting the amplified gene products for G and P typing of BRV strains (4). For each digestion, 10 ul of amplified DNA and 10X buffer and enzyme were mixed and incubated at 37 C for 2 hours and analyzed on 1% agarose gels using standard procedures (34).

Sequence analysis of the VP7 genes of IND, 2292B, C-8008, C-8336 and MC27 was performed. RT-PCR products of VP7 genes were either sequenced directly or sequenced after cloning into the pCRII plasmid (Invitrogen) using the primer extension method (34). The sequence data were analyzed using DNASTAR software (DNASTAR Inc., Madison, WI).

Virus isolation from a field strain of G6s BRV and production of antisera in guinea pigs.

A field sample identified as P[11]G6s (C-8336) was isolated in MA104 cells using roller tubes (32). The isolated virus was plaque-purified three times. After the propagation of virus to achieve high titers, further purification using CsCl gradient ultracentrifugation was done.
The purified (C-8336) as well as reference virus strains were injected into guinea pigs with Freund's incomplete adjuvant for producing antisera against them.

**Antigenic analysis of G6s viruses using fluorescent focus neutralization (FFN) test.**

MAb (IC3, provided by Dr. H Greenberg, Stanford University, Palo Alto, CA) which has neutralizing activity against G6 serotypes (UK) was used to assess the antigenic reactivity of isolated G6s strains using an FFN test as described previously (21). The neutralizing activity of BRVs including a G6s strain (C-8336) was determined by two-way FFN tests as described previously (21). Briefly, the serum samples were serially diluted with MEM from 1:50 to 1:12,500 and were mixed with viruses containing $1 \times 10^4$ FFU/ml, and reacted at 37 C for 1 hr. The mixtures (100 ul) were inoculated onto monolayers of MA104 cells grown in 96-well plates. After adsorption at 37C for 1 hr, the inoculum was removed and 200 ul (per well) of MEM containing 50 ug/ml pancreatin were added to the cells and incubated at 37 C for 20 hours. The infected cells were fixed with 80% acetone and stained with FITC-conjugated anti-rotavirus serum. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that resulted in a 90% or greater reduction in numbers of FFUs. Each test was repeated at least three times and the neutralization titers averaged from the 3 tests.

**Experiment inoculation of gnotobiotic calves with G6 and G6s strains**

The G6s strain C-8336 (P[11]G6s) and IND (P[5]G6) G6 reference strain were inoculated into gnotobiotic calves (calf A and B, respectively) for comparison of the host responses between a field G6s and a reference G6 strain. Gnotobiotic calves were derived,
fed, inoculated and maintained as previously described (30). The calves were oronasally inoculated with diluted (1:10) fecal filtrates (about 10^7 FFU). After inoculation, calves were examined twice daily for development of diarrhea, and the fecal consistency was scored (0=Normal, 1=pasty, 2=semi-liquid, 3=liquid). Each sample was tested for groups A rotavirus using PAGE, IEM and ELISA as described (4,23,31). Two weeks after inoculation of calf A with C-8336 strain, it was challenged with the virulent IND BRV strain to study cross-protection between the two viruses.

3.4 RESULTS

Virus isolation and confirmation of G and P serotypes.

The C-8336 strain showed cytopathic effects after 3-4 blind passages in MA104 cells. The virus was plaque-purified and showed typical group A rotavirus electropherotypes (4-2-3-2) (data not shown). After RT-PCR and RFLP, the cell culture isolated C-8336 and a field strain MC27 were confirmed as P[11]G6s.

Nucleotide and amino acid sequence analysis.

The nucleotide and deduced amino acid sequences of the complete VP7 genes of five strains including IND, 2292B, C-8336, MC27 and C-8008 were obtained. The G6s strains C-8336 and MC27 shared >97 % homology in nucleotide and amino acid sequences and they showed the highest homology with another bovine G6 subtype strain with the same P[11] type (greater than 95 % homology in nucleotide and amino acid sequence with KN-
4{P[11]G6s}), and they also showed lower, but substantial homology with human G6 subtype strains with different P types (80 - 87% in nucleotide and 90 - 91% in amino acid with PA169 {P[14]G6s} and PA151 {P[9]G6s}) and prototype G6 BRV having different P types (79 - 81% in nucleotide and 88 - 90% in amino acid with NCDV {P[1]G6} and IND {P[5]G6}) (Table 1). For G8 and G10 serotypes of BRV, the nucleotide and deduced amino acid sequence of VP7 genes of C-8336 and MC27 shared 71 - 73% in nucleotide, and about 80% in amino acid homology with Cody I801 (G8) and 2292B (G10) strains. Between the VP7 genes of bovine (IND, UK, B641, NCDV, C-8336, MC27 and KN4) and human (PA151 and PA169) G6 strains, there was 12-20% and 7-12% divergence in nucleotide and amino acid sequences, respectively (Table 1).

The complete VP7 nucleotide sequence of the field G8 strain (C-8008) shared 83-86% nucleotide and 93-96% deduced amino acid sequence homology with the bovine and human G8 strains including Cody I801, A5, 69M and Hall166, respectively (Table 2). For the VP7 gene of the 2292B strain, the nucleotide and deduced amino acid sequences were highly conserved with those of bovine G10 strains (B223 and KK3) having the same P[11] types, but showed significant divergence with those of human G10 strains (I321 and MC35) with different P types (Table 3).

Antigenic relationship of G6s strain to other BRV strains.

Serologic analysis of the C-8336 strain revealed that it was neutralized by a G6 MAb (IC3) to similar titers as reference G6 strains (NCDV and IND; titers ranged from 32,000 to 64,000, Table 4). However, in two-way FFN tests, strain C-8336 showed 4- to 16-fold
differences in polyclonal antibody titers with NCDV and IND. Polyclonal antiserum against the C-8336 strain had low neutralizing antibody titers (8-16-fold lower than homologous titers) response to the NCDV and IND strains (Table 4). The G6 strains NCDV and IND which have different P types showed 2-fold differences in homologous antibody titers, whereas C-8336 (G6s) and 2292B (G10) which shared the same P type, P[11] showed 16- to 32-fold differences in homologous antibody titers (Table 4). The titers of 2292B (P[11]G10) strain against the IC3 MAb and antisera against NCDV and IND G6 BRV were less than 100 or less than 10, respectively.

*Virus inoculation of gnotobiotic calves.*

When C-8336 (calf A) or IND (calf B) strains were inoculated into gnotobiotic calves, diarrhea developed in both calves, 2-3 days after exposure, with virus shedding detected in fecal samples by PAGE, IEM and ELISA. The G and P types of fecal viruses from calf A and B were confirmed as P[11]G6s and P[5]G6, respectively, by RT-PCR and RFLP analysis. Two weeks after inoculation of C-8336, when calf A was re-challenged using virulent IND, calf A showed neither diarrhea nor virus shedding, thus providing preliminary evidence for cross-protection between G6 and G6s strains.

### 3.5 DISCUSSION

Possible inter-species transmission of group A rotaviruses has been suggested, especially between humans and cattle. Gerna et al. (11) reported G6 serotype strains from humans
(PA151, PA169) (11,13,19), which were recognized by G6 MAbs (bovine G6) and showed some genetic variation (about 10 % in amino acid homology) with typical bovine G6 strains (UK and NCDV strains). By RNA-RNA hybridization, most of the gene segments of a human PA151 G6 strains were closely related to AU-1 (P[9]G3) human or NCDV (P[1]G6) bovine strains suggesting that this strain might be a reassortant between AU-1 and NCDV (19). Urasawa et al. (39) reported that a human G10 group A rotavirus may have originated from a bovine host. Blackhall et al. (2) confirmed a bovine G1 strain in Argentina analogous to human G1 serotypes by serologic assays and sequence analysis of the VP7 genes. In addition, rotavirus strain 116E, isolated from the fecal specimen of a newborn asymptomatic infant from India was identified as a P[11] type which was previously reported only in cattle (10).

Generally nucleotide and amino acid sequence homologies are 72-74 % and 80-84 %, respectively among different G types of BRV (G6, G8 and G10) (3,25). Within the same G serotypes, the nucleotide and amino acid sequence homologies are greater than 95% (14,16). Interestingly, the rotavirus strains belonging to the atypical G serotypes (G1, G6, and G10) from different host species show a higher degree of genetic variation: 80-85 % nucleotide and 88-92% deduced amino acid sequence homologies to the prototype G serotypes (11,13,25,27,28) Among BRV G6 strains, Matsuda et al.(25) reported the isolation of a G6 bovine strain (KN-4) with P[11] which showed genetic variation (about 10 % in amino acid) from the reference G6 BRV. The KN-4 strain failed to be recognized by a G6-specific MAb but was antignically related to NCDV (P[1]G6) and 0510 (P[5]G6) via the common G6 type; and to KK-3 (P[11]G10) via the common P11 type in two-way neutralization tests (25). Recently, Mummidi et al.(27) also reported a bovine G6 subtype in the U.S., which was
genetically similar to the KN-4 strain. However, none of these investigators described the antigenic characterization and/or host responses to these newly discovered G6 subtypes. We previously reported BRV field strains showing similar genetic variation in the U.S., and after characterization of their VP7 (G type) and VP4 (P type) genes using RT-PCR and RFLP analysis, we found a relatively high prevalence of G6 subtype BRV among the field samples that we tested (4). Interestingly, the majority of these G6s BRV strains (75%, n=8) showed the same P types, P[11]. Although the genetic relationship between G6s and other BRV has been established by sequence analysis, the antigenic relationship of G6s with reference prototype G6 or other BRV serotypes has not been characterized. Also, there was little or no information on their occurrence with certain P types. Therefore, in the present study, we further characterized the genetic diversity of G6, G8 and G10 BRV and the antigenic characterization of a G6s BRV strain (C-8336).

The VP7 genes of the C-8336 and MC27 strains showed high homology to each other (~98%) and with other bovine G6 subtype strains (greater than 95 % homology in nucleotide and amino acid sequence) and also showed lower, but substantial homology with human G6 subtype strains and prototype G6 BRVs (79-87 % in nucleotide and 88-91% in amino acid). In spite of geographic differences in their isolation, G6s BRV strains appear to be highly conserved. However, they show similar divergence from G6s human strains and prototype G6 BRVs.

Originally, G8 strains were isolated in humans and subsequently from cattle (1,15,38). Although G8 BRV strains are rarely reported in the U.S.(around 5 % of field samples, 4,29,35), they are reported as the predominant G type in some regions among humans (5).
The G8 strains, bovine A5, 678 and Cody I801 and human B37 and 69M have been characterized genetically and serologically (1,3,15,38). Interestingly, there was up to 18% nucleotide sequence divergence among A5, B37, 69M and Cody I-801 strains but the amino acid sequence was highly conserved (silent mutations) (3,15,38). A similar trend was observed for the G8 strain (C-8008) which showed lower nucleotide sequence homology with reference G8 strains, but higher amino acid sequence homology (Table 2). Among the G10 rotavirus strains, the 2292B strain showed high conservation in the VP7 gene with bovine G10 strains (B223 and K33) having the same P[11] types, whereas, divergence was seen with the human G10 strains (I321 and MC35) having different P types (Table 3).

Serologic analysis of the C-8336 strain showed that this strain was neutralized by the IC3 G6 MAb (UK BRV strain) which has high neutralizing antibody titers against reference G6 NCDV and IND strains. In two-way FFN tests, C-8336 showed differences from the reference NCDV and IND strains: 2- to 32-fold differences were observed in polyclonal antibody titers with NCDV and IND (especially, polyclonal antibody against the C-8336 strain neutralized the NCDV and IND strains poorly). The differences in antibody titers may be partially due to the VP4 (P[11]) specificity of C-8336 compared to the P[1] and P[5] specificity of NCDV and IND, respectively. Although NCDV (P[1]) and IND (P[5]) have different P types, the antibody titers differed only 2-fold compared to homologous titers. In addition, the neutralization titers associated with common P types (VP4) were minimal between 2292B (G10P[11]) and C-8336 (G6sP[11]) strains (20 and 40) in this study.

The C-8336 G6s and IND G6 strains caused diarrhea when inoculated into gnotobiotic calves, and the expected G and P types of each viral inoculum were recovered in viruses from
the diarrheic fecal samples. Although the antiserum against C-8336 neutralized the IND strain poorly in vitro, the calf infected with C-8336 did not develop any clinical signs or virus shedding after challenge with the IND strain. These initial studies suggest the G6 and G6s are closely related and may provide cross-protection against one another. However, additional studies including the inoculation of more experimental animals and cross-protection studies are needed to confirm this observation.

There is significant genetic diversity among the VP7 genes of G1, G6, G8 and G10 bovine and human strains when they occur and circulate in an atypical host (11,13,25,27,28). Within the same host, the genetic diversity is variable and may be dependent on the combination of G with P types. The typical G6 strains are usually associated with the P[5] VP4 serotype and the P[5]G6 combination is dominant (~50%) in BRV strains from several studies (4,20,36). The reported G6s strains of BRV, KN-4 as well as the G6s strains described here, have the P[11] type. Also we have previously described the existence of P[11] type among 75% (n=8) of G6s strains we tested. It is interesting to speculate that bovine G6s field strains may have a preference for the P[11] VP4 combination and may have a selective advantage over P[5]G6 strains under certain conditions. Among G8 BRV, different P types are associated with similar genetic diversity in the VP7 genes: (Cody I801, P[1]G8 versus C-8008, P[5]G8). Therefore, variation in the VP7 genes of G6, G8 and G10 may occur by cross-species transmission and/or by reassortment with different genotypes (especially VP4 in BRVs) following natural selection by immune pressure during infection, and they may eventually evolve into new G serotypes: the "G serotype" based on antigenic analysis may be the result of a continuum of several "subtypes" existing within a serotype. This may result in constant evolution of the
VP7 genes circulating under natural conditions. Studies such as the one described here are needed to monitor the molecular epidemiology of rotavirus infections in human and the animal populations in order to design and implement improved vaccination strategies. Our future work will focus on further analysis of the G and P combinations of BRV to determine: 1) if certain combinations like P[5]G6 and P[11]G10 are more frequent; and 2) if new combinations resulted from reassortment and natural selection may lead to genetic variations in the VP7 gene such as the P[11]G6s.

3.6 REFERENCES


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Deduced amino acid

GenBank accession numbers of above genes are: IND (U15000); UK (K00037); B641 (H63266); NCDV (M12394); C-8336 (U14997); MC27 (XXXX); KN4 (D12710); PA151(L20881); PA169 (L20880).

Table 3.1. Nucleotide and deduced amino acid sequence analysis of bovine and human G6 and G6s (subtype) strains

116
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### Deduced amino acid

GenBank accession numbers of the above genes: Cody (U14999); A5 (D01054); C-8008 (U14998); 69M (14); B37 (J04334); Hal1166 (L20882).

**Table 3.2.** Nucleotide and deduced amino acid sequence analysis of bovine and human G8 strains
### Table 3.3. Nucleotide and deduced amino acid sequence analysis of bovine and human G10 strains

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Deduced amino acid

GenBank accession numbers of above genes: 2292B (U14996); B223 (X57852); KK3 (D01056); I321 (L07658); MC35 (D14033).
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* Specificity against the UK BRV strain (provided by Dr. H Greenberg, Stanford University, Palo Alto, CA).

<sup>b</sup>Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that resulted in a 90% or greater reduction in the numbers of FFU.

Table 3.4. Virus neutralization titers of G6s (subtype) and conventional BRV strains using two-way FFN tests.
**Figure 3.1.** Phylogenetic tree based on deduced amino acid sequences of the VP7 genes of bovine and human G6, G8 and G10 group A rotavirus strains.
CHAPTER 4

DETECTION OF GROUP B ROTAVIRUSES FROM CALF AND ADULT COW DIARRHEIC FECAL SAMPLES AND CHARACTERIZATION OF THEIR VP7 GENES

4.1 SUMMARY

Groups A, B and C rotaviruses have been identified in cattle. Group B rotaviruses are associated with sporadic cases of diarrhea in calves and adult cows. From diagnostic submissions to our laboratory, 90 fecal samples from cases of calf diarrhea, 81 samples from adult cow diarrhea (winter dysentery) and 20 case control normal adult cow fecal samples were tested for group B rotaviruses by polyacrylamide gel electrophoresis (PAGE), RT-PCR (targeting 279 bp of the VP7 gene), and 53 adult cow diarrheic samples were tested for group B rotaviruses by immune electron microscopy (IEM). By RT-PCR, five samples from calves were group B rotavirus positive (5.5 %). Fifteen samples from adult cows with diarrhea were group B rotavirus positive (18.5 %) and none of the control fecal samples from normal cows were positive for group B rotaviruses. By PAGE, one calf sample (RT-PCR positive) was group B rotavirus positive (short electropherotype), but none of the adult cow samples were positive for groups B rotaviruses. By IEM, five (9.4 %) of 53 adult cow diarrheic samples
were group B positive (all also RT-PCR positive). The VP7 genes of three strains (WD653 from an adult cow; ATI and Mebus calf strains) were sequenced. The VP7 genes showed high (over 90%) nucleotide and deduced amino acid homologies among the three bovine strains, and lower homologies (45 - 57 %) with IDIR (rodent) and ADRV (human) interspecies group B rotaviruses. Although there were some differences of degree, all inoculated calves showed abnormal feces between 1-3 days after inoculation and group B rotaviruses were detected in the feces for up to 2 weeks by RT-PCR but for shorter periods by PAGE or IEM.

4.2 INTRODUCTION

Rotaviruses, members of the *Reoviridae* family, have a triple layered capsid and a genome that consists of 11 segments of double stranded RNA (dsRNA) (12, 13). Rotaviruses are divided into 7 morphologically indistinguishable but antigenically distinct serogroups (12, 13). Group A rotaviruses are a common cause of diarrhea in calves (23, 26). Although nongroup A (group B and C) rotaviruses have been identified in cattle, there is little information on their prevalence (23, 26).

Group B rotaviruses may be emerging pathogens for humans and appear to be associated with diarrhea in adults and older children more than in neonates (3, 23). In calves and adults cows, group B rotaviruses have been reported in association with sporadic cases of diarrhea (1, 2, 18, 19). The group B rotaviruses are difficult to grow in cell culture, and in the host, are shed in low titers in the feces, making it difficult to detect and further characterize these viruses (3, 23).

Several assays to detect group B rotaviruses have been described. These include
polyacrylamide gel electrophoresis (PAGE), immune electron microscopy (IEM), enzyme linked immunosorbent assay (ELISA), c-DNA hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (10, 11, 14, 30). Because of the limited shedding or instability of group B rotaviruses in the feces of infected animals, there is an increased need for highly sensitive and specific assays.

In this study, we describe the analysis of calf diarrheic (n=90), adult cow diarrheic (n=81) and normal (n=20) fecal samples from the field for group A and B rotaviruses by using PAGE, IEM and RT-PCR. For analyzing the VP7 gene diversity of group B rotavirus, the sequence analysis of the full length VP7 genes of three field group B bovine rotaviruses (ATI, Mebus from calves and WD653 from adult cow with diarrhea) was performed and compared with other reference group B rotaviruses. In addition, these three field strains were inoculated into gnotobiotic calves to study the pathogenicity and virus shedding patterns of bovine group B rotaviruses.

4.3 MATERIALS AND METHODS

Viruses and cells. The reference group A BRV strains have been described previously and included: NCDV-Lincoln (G6P[1]), IND (G6P[5]), B223 (G10P[11]) and 2292B (G10P[11]) strains (4). The group B BRV strains included: ATI and the Mebus strains (from calves), KD and WD653 strains (from adult cows) and the group C BRV, Shintoku strain (19, 23, 25, 29). All group A strains and the Shintoku group C strain were grown in Rhesus monkey kidney cells in the presence of pancreatin (50ug/ml) (22, 29). The group B
rotaviruses, which were not cultivable in cell culture, were amplified by passage in gnotobiotic calves and infected intestinal contents were collected as a source of virus. As negative controls, mock-infected cell culture samples and mock-infected gnotobiotic calf feces were used.

**Field samples.** Analysis for group A and B rotaviruses was performed on 90 diarrheic calf fecal samples from Ohio, California, Wyoming, South Dakota and Nebraska; 81 adult cow diarrheic fecal samples (winter dysentery cases) from Ohio, New York and California; and 20 normal adult cow fecal samples (from 2 Ohio herds).

**Extraction and electrophoresis of dsRNA, and IEM.** Rotavirus dsRNA was extracted from cell culture-propagated viruses and fecal viruses using previously described procedures (15). Rotavirus dsRNA in extracted samples was analyzed by PAGE to confirm the presence of dsRNA and to examine the genomic electropherotypes as previously described (4, 15). The discontinuous buffer system of Laemmli was utilized and dsRNA was resolved in 10% polyacrylamide slab gels. Electrophoresis was conducted at 12mA for 14-16 hrs. The dsRNA bands were visualized by silver staining (15). Fifty-three adult cow diarrheic fecal samples were processed for IEM as previously described (19). The processed samples were incubated separately with group A-, B-, C- or bovine coronavirus-specific hyperimmune antisera, then the mixtures were pelleted, negatively stained with phosphotungstic acid, applied to grids, and examined by electron microscopy (19, 24).
Reverse-transcription-PCR assay. RT-PCR was used to produce full length BRV VP7 (group A) and partial length VP7 (group B) cDNA fragments. cDNA was synthesized and amplified in an RT-PCR mixture containing 10X buffer (200 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, KCl, 0.05% gelatin), dNTP (10mM, each), 200ng of primer A (For group A, 5'-GGCCGGATTTAAAA GCGACAATT-3' and for group B, 9B3, 5'-CAGTAACTCTATCCTTTTACC-3'), 200ng of primer B (for group A VP7 antisense, 5'-GGTCACCATCATAACTCTA-3' and for group B, antisense 5'-CGTATCGCAATACAATCCG-3'), (4, 7, 8), 5U RNAsin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 10U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) and 2.5 U of Taq polymerase (Boehringer Mannheim Biochemicals). First strand cDNA synthesis was accomplished by incubating the above mixture for 90 min at 42 C. Thirty amplification cycles were conducted, with each cycle consisting of 94 C for 1 min (denaturation), 48 C for 1 min (annealing) and 72 C for 2 min (extension), followed by a 7 min extension at 72 C. The PCR products were analyzed on 1% agarose gels using standard procedures (21).

Cloning and sequence analysis of the full length VP7 gene of group B bovine rotaviruses. After determining the 5' and 3' end sequences by single primer amplification (16), the full length VP7 genes of three group B rotaviruses [ATI and Mebus (calf) and WD653 (adult cow strains] were produced by RT-PCR with 5' and 3' end primers, and cloned in the pCRII vector (Invitrogen, San Diego, CA). The sequence analysis was performed using the primer extension method (Sequenase v2.0, Amersham, Arlington Height, IL) and the data
were analyzed using the DNASTAR program (DNASTAR Inc. Madison, WI).

**Inoculation of gnotobiotic calves with group B rotaviruses.** Three samples containing only bovine group B rotaviruses (by IEM and RT-PCR) were passaged in newborn (1- to 5-day-old) gnotobiotic calves, derived, fed, inoculated and maintained as previously described (19). All calves were oronasally inoculated with diluted (1:10) fecal filtrates of live virulent bovine group B rotavirus strains (calf, ATI and Mebus; and adult cow, WD653). After inoculation, calves were examined twice daily for development of diarrhea, and the fecal consistency was scored (0=Normal, 1=pasty, 2=semi-liquid, 3=liquid). Each sample was tested for groups A and B rotaviruses using PAGE, IEM and RT-PCR as described. GenBank accession numbers for the VP7 genes of WD653, ATI and Mebus are U84141, U84472 and U84473, respectively.

### 4.4 RESULTS

**Electropherotypes.** The group A and group B rotaviruses displayed typical electropherotypes (for group A, 4/2/3/2 dsRNA distribution patterns and for group B, 4/2/2/3 dsRNA patterns) (25, Fig 1). Like group A rotaviruses, group B rotaviruses showed short and long electropherotypes: the ATI and Mebus calf strains showed long patterns and the two adult strains, KD and WD653, showed short patterns (Fig.1).

**RT-PCR.** As expected, for group A rotaviruses, the full length VP7 gene (1062 bp) was generated, and a partial length VP7 gene was generated for group B rotaviruses (279 bp,
The RT-PCR assay was rotavirus group-specific because there was no PCR product when the RT-PCR assay was applied to the heterologous group rotaviruses and negative control samples.

Prevalence of group B BRV from field samples. Based on our RT-PCR assay, five of 90 samples from diarrheic calves were group B BRV positive (5.5 %); three of these samples were also positive for group A BRV (Table 3). Fifteen samples from a total of 81 adult cows with diarrhea were group B rotavirus positive (18.5 %) (Table 3). None of the fecal samples from normal case-matched control cows were positive for group A or group B BRV. By PAGE, only one calf sample (also RT-PCR positive, short electropherotype) was group B BRV positive, none of the adult cow samples were positive for B rotaviruses and one adult cow sample (also RT-PCR positive) was positive for group A BRV by PAGE analysis (Table 3). By IEM, twenty-nine (54.7 %) of 53 samples from adult cows with diarrhea were coronavirus positive; five (9.4 %) samples were group B positive (all were also RT-PCR group B positive), two of which were also coronavirus positive and one sample was group A positive.

Sequence analysis of the full length VP7 gene of group B BRVs. The full length VP7 genes of group B bovine rotaviruses (calf ATI, Mebus and cow WD653 strains) were PCR-amplified, cloned and sequenced. The full length VP7 genes of the bovine group B rotaviruses were 811 bp, which were a little difference from than those of IDIR (804 bp, GenBank accession number: D00911) and ADRV (814 bp, GenBank accession number: M33872).
VP7 nucleotide similarities showed high homologies (over 90\%) among three bovine strains, and greater diversities (52.5\% and 51.0\%, respectively) with IDIR and ADRV interspecies group B rotaviruses (Table 3). Also the deduced amino acid identities were high among bovine strains, but with the IDIR and ADRV inter-species group B rotaviruses were lower (56.5\% and 45.7\%, respectively, Table 3).

**Pathogenicity and viral shedding patterns of group B BRV in gnotobiotic calves.** Although there were some differences of degree, all inoculated calves showed abnormal feces between 1-3 days after inoculation. The viruses were detected in the feces by IEM and PAGE for 1 to 6 days post-inoculation and for up to 2 weeks post-inoculation by RT-PCR (Table 4).

**4.5 DISCUSSION**

The group B rotaviruses have been regarded as an emerging pathogen for humans and appear to be associated more with older ages rather than infants (3, 23). The group B rotaviruses have also been reported among mice, pigs, sheep and cattle (2, 3, 19, 28). Although there is one report of adapting a porcine group B rotavirus to cell culture (27), the growth of group B rotaviruses in a continuous cell line has not been accomplished. Bovine group B rotaviruses are fastidious in their growth requirements and, this coupled with the fact that these viruses are shed in low quantities in the feces of infected animals, has hampered their further characterization.
The group B rotaviruses have been identified by serology and dsRNA electrophoresis (3, 23, 25). In addition, the production of enterocyte syncytia was characteristic for group B rotaviruses (3, 18, 23, 25). Group B bovine rotaviruses have been identified in the U.S and U.K. (2, 3, 7, 8, 18, 19, 23, 25) but information on the prevalence of bovine group B rotaviruses is very limited. In a serological study in the U.K., 71% of adult cow sera were positive to human ADRV strain (2), and in another study, 20% of cow sera were positive to porcine NIRD-1 strain (2). In the U.S., Chinsangaram et al. (7) reported that 87% (38/47) of dairy calves (1-14 days old) in one calf ranch and in one dairy herd were group B positive by RT-PCR, but were not positive by PAGE. However, in our study, using identical primers, the prevalence of group B rotaviruses in diarrheic calf feces from 5 states was only 5.5% (5/90), and interestingly, for adult cow diarrheic feces (winter dysentery samples), the prevalence was 18.5% (15/81). From the calf diarrheic samples, three samples (out of 5) were both group A and B positive by RT-PCR, which suggests that mixed group A and B rotavirus infections in calves might be common in the field. By PAGE, only one sample (1/171) from calves was group B positive (short electropherotype), which supports the hypothesis that group B rotavirus shedding is low in the infected hosts. The discrepancy in our results with the study by Chinsamgaram (7) could be attributed to geographical differences, sporadic outbreak of group B infections on farms, or differences in timing of sample collection. Our results indicate that group B rotaviruses may play a role in sporadic cases of calf and adult cow diarrhea in the field and may be more associated with adult cattle diarrhea. In addition, although bovine coronavirus may play a major role in winter dysentery in cows (24), other enteric pathogens including bovine group B rotaviruses may also be
involved in this syndrome.

The genetic variation which exists among group B rotaviruses from different host species is poorly characterized, but based on analysis of nucleotide and deduced amino acid sequence data, group B rotaviruses show greater diversity than group A or group C rotaviruses (6, 9, 20). For molecular comparisons of intra-species and inter-species group B rotaviruses, we sequenced the full length VP7 genes of three field strains of bovine group B rotaviruses (ATI, Mebus and WD653), and compared them to the VP7 gene sequences of two heterologous group B rotaviruses, ADRV from humans and IDIR from rats. The similarity of the VP7 gene nucleotide sequence between bovine strains and the heterologous group B rotaviruses was about 50 %, and the amino acid homologies ranged from 45-60 %. For group A rotaviruses, the homology between different G types (VP7 seortypes) is about 70 - 80 % at the nucleotide level and about 80 % at the amino acid sequence level (17). Regardless of their origins, adult cows or calves, and of their electropherotypes, short or long, all three bovine strains showed high homologies in their VP7 genes : over 90 % in nucleotide and amino acid sequences. However, compared with group B rotavirus strains from heterologous species, humans and rats, the bovine VP7 genes showed high diversity. The reason for this high diversity among the VP7 genes of group B rotaviruses from different hosts is not clear and requires further study.

After inoculating three bovine group B strains (ATI, Mebus and WD653) into gnotobiotic calves, all three strains induced at least mild diarrhea, but the clinical signs and the duration of virus shedding varied among calves even with the same strain. Such variability might be due to the timing of inoculation (age-dependent), virus instability or to the different viral
concentrations of each inoculum which were low in comparison with that of group A rotavirus inocula. Additional studies are underway to confirm the pathogenicity and to further characterize additional group B bovine rotavirus strains.

4.6 ACKNOWLEDGMENT

We thank Paul R Nielsen and Douglas C Hodgins for help with gnotobiotic calf management. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center (OARDC), the Ohio State University. Approved as OARDC manuscript #5-97. This work was supported by USDA NRICGP Competitive grant No. 93-37204-9201.

4.7 REFERENCES


diarrheas of man and animals. CRC Press, Boca Raton, FL.


<table>
<thead>
<tr>
<th>State of sample origin (USA)</th>
<th>Total No.</th>
<th>Group A positive</th>
<th>Group A negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>RT-PCR</td>
<td>PAGE</td>
</tr>
<tr>
<td>Ohio</td>
<td>2 / 35</td>
<td>1 / 23</td>
<td>1 / 12</td>
</tr>
<tr>
<td>California</td>
<td>1 / 6</td>
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<td>0 / 1</td>
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<tr>
<td>South Dakota</td>
<td>2 / 31</td>
<td>1 / 27</td>
<td>1 / 4</td>
</tr>
<tr>
<td>Wyoming</td>
<td>0 / 3</td>
<td>0 / 2</td>
<td>0 / 4</td>
</tr>
<tr>
<td>Nebraska</td>
<td>0 / 15</td>
<td>0 / 9</td>
<td>0 / 6</td>
</tr>
<tr>
<td>Total (%)</td>
<td>5 / 90 (5.5)</td>
<td>3 / 66</td>
<td>2 / 24</td>
</tr>
</tbody>
</table>

* Group A rotavirus positive or negative by RT-PCR

Table 4.1. The prevalence of group B bovine rotaviruses in calf diarrheic fecal samples assayed by RT-PCR and PAGE.
The prevalence of group B bovine rotaviruses in adult cow fecal samples assayed by RT-PCR and PAGE.

<table>
<thead>
<tr>
<th>State of sample origin (USA)</th>
<th>Group B rotavirus positive</th>
<th>Total No.</th>
<th>RT-PCR</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ohio</td>
<td></td>
<td>71</td>
<td>12</td>
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<tr>
<td>New York</td>
<td></td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>California</td>
<td></td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td>81</td>
<td>15 (18.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Controls (no diarrhea)</td>
<td></td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* One sample was group A rotavirus positive by PAGE and RT-PCR.

Table 4.2. The prevalence of group B bovine rotaviruses in adult cow fecal samples assayed by RT-PCR and PAGE.
### Nucleotide sequence similarity (%)

<table>
<thead>
<tr>
<th>Strain</th>
<th>ATI^b</th>
<th>Mebus^c</th>
<th>WD653^d</th>
<th>ADRV^e</th>
<th>IDIR^f</th>
</tr>
</thead>
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<tr>
<td>Amino acid</td>
<td>ATI</td>
<td>-</td>
<td>95.2</td>
<td>91.3</td>
<td>56.8</td>
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<tr>
<td></td>
<td>Mebus</td>
<td>96.8</td>
<td>-</td>
<td>89.5</td>
<td>56.5</td>
</tr>
<tr>
<td>Sequence similarity</td>
<td>WD653</td>
<td>92.7</td>
<td>92.3</td>
<td>-</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
<td>ADRV</td>
<td>59.7</td>
<td>59.3</td>
<td>61.3</td>
<td>-</td>
</tr>
<tr>
<td>(%)</td>
<td>IDIR</td>
<td>48.6</td>
<td>48.2</td>
<td>48.6</td>
<td>48.6</td>
</tr>
</tbody>
</table>

^a Analysis was performed by using the DNASTAR program.

^b GenBank accession number: U84141

^c GenBank accession number: U84472

^d GenBank accession number: U84473

^e GenBank accession number: M3 3 872

^f GenBank accession number: D00911

---

**Table 4.3.** The nucleotide and deduced amino acid sequence comparisons of the VP7 genes of bovine (ATI, Mebus and WD653 strains) with human (ADRV) and rat (IDIR) group B rotaviruses.
<table>
<thead>
<tr>
<th>PID b</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
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<td>Mebus</td>
<td>-/+</td>
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<td>+/+</td>
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</tr>
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<td>WD653</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>A) e</td>
<td>-/-</td>
<td>-/+</td>
<td>-/+</td>
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<td>-/+</td>
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<tr>
<td>B)</td>
<td>-/-</td>
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<td>+/+</td>
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<td>+/+</td>
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</tr>
<tr>
<td>C)</td>
<td>+/-</td>
<td>+/+</td>
<td>+/+</td>
<td>-/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
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<td>ND</td>
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<tr>
<td>D)</td>
<td>-/-</td>
<td>-/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Calves were 1- to 5- days old at the time of inoculation
b PID : post-inoculation days
c -/- : No diarrhea or group BRV shedding
   +/+ : Diarrhea (fecal consistency scored of 1 to 3) and group BRV shedding
d ND : not done
e Four calves (A, B, C and D) were inoculated with WD653 strain

Table 4.4. Pathogenicity of the ATI, Mebus and WD653 strains of group B bovine rotaviruses in gnotobiotic calves.

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Figure 4.1. The genomic electropherotypes of group A, B and C BRV strains. After PAGE, the gel was stained with silver stain. Lanes A-B: group A BRV strains, lane A: IND, lane B: 2292B; lanes C-F: group B BRV strains, lane C: ATI, lane D: Mebus, lane E: KD, lane F: WD653; lane G: group C BRV, Shinhtoku strain and lane H: group C porcine rotavirus, Cowden strain. Lane B, 2292B strain shows short electropherotype for a group A BRV, and lanes E (KD) and F (WD653) show short electropherotypes for group B BRV.
Figure 4.2. The RT-PCR products of reference group A and B strains and field samples. Lane M: molecular weight marker, lanes A-B: reference group A strains (IND and 2292B), lanes C-D: field group A BRV samples, lanes D-E: reference group B strain (ATI), lane E: nested PCR product, lanes G-H: field group B samples.
CHAPTER 5

DUAL INFECTION OF GNOTOBIOTIC CALVES WITH BOVINE STRAINS OF GROUP A AND PORCINE-LIKE GROUP C ROTAVIRUSES INFLUENCES PATHOGENESIS OF THE GROUP C ROTAVIRUS

5.1 SUMMARY

Serological evidence exists for bovine group C rotaviruses in the U.S., but there are no reports of their isolation. Ninety fecal samples from calf diarrhea cases, 81 samples from adult cow diarrhea (winter dysentery) and 20 normal adult cow fecal samples were tested for group C rotaviruses by polyacrylamide gel electrophoresis, immune electron microscopy and RT-PCR. Three samples from adult cow diarrhea cases were positive only by RT-PCR, and a group C rotavirus was isolated from a positive sample in monkey kidney (MA104) cells (WD534tc). Genetically and serologically, the WD534tc/C strain was more closely related to the Cowden porcine group C strain than to the Shintoku bovine strain. Because the original cow feces also contained a group A rotavirus (detected after passage in cell culture), we hypothesized that such dual rotavirus infections might play a role in the pathogenesis and host adaptation of rotaviruses. Thus, we examined the pathogenesis of WD534tc/C alone or combined with virulent (IND/A) or attenuated (NCDV/A) bovine group A rotaviruses in gnotobiotic calves. WD534tc/C alone induced diarrhea without virus shedding in inoculated
calves (n=3). In contrast all calves (n=3) coinfected with WD534tc/C and IND/A (n=3) developed diarrhea and shed both viruses, whereas calves (n=3) coinfected with WD534tc/C and NCDV/A developed diarrhea, but no shedding of either virus. Infection with WD534tc/C or NCDV/A alone caused only mild villous atrophy (jejunum and/or ileum), whereas, dual infection with both viruses induced lesions throughout the small intestine. Although IND/A alone caused villous atrophy, more severe small intestinal lesions occurred in calves coinfected with WD534tc/C and IND/A. In conclusion, coinfection of calves with group A rotaviruses enhanced fecal shedding of a bovine group C rotavirus and the extent of histopathological lesions in the small intestines. Thus our findings suggest a potential new, novel hypothesis involving dual infection for the adaptation of heterologous rotaviruses to new host species.

### 5.2 INTRODUCTION

Rotaviruses are a major cause of diarrhea in young children and animals including cattle (21, 35) and belong to seven distinct antigenic groups (A to G) (7, 21, 24). Although group C rotaviruses are antigenically distinct from group A rotaviruses in serologic tests, they are genetically related and they share a minor non-neutralizing epitope on VP6 detected using monoclonal antibodies (Mabs) (47). The VP6 genes of group C rotaviruses share 88.4 to 90.6% homology (porcine Cowden, bovine Shintoku and human Bristol) and 41.3 and 16.3% homology with the VP6 gene of group A (bovine RF) and group B (human ADRV) rotaviruses, respectively (19). Within group C rotaviruses, antigenic and genetic variation exists. There are at least 3 G types (VP7) identified using two-way cross-neutralization tests.
and sequence analysis of the VP7 genes (70-75% homology among the serotypically distinct strains), and the Cowden porcine and Shintoku bovine strains are different serotypes (45, 46).

Group A rotaviruses are endemic and group B rotaviruses cause sporadic cases of diarrhea in calves and cows in the U.S. (5, 31, 34, 35). However, although a moderate prevalence of antibodies to group C rotaviruses in sera of cattle (47 to 56%) in the U.S. (44) was reported, to date, the Shintoku group C bovine rotavirus, which was isolated from an adult cow in Japan, is the only bovine isolate of group C rotavirus (31, 48). The first group C rotaviruses of any species was isolated in 1980 from nursing pigs (33) and more recently identified as a cause of enzootic diarrhea in neonates (27) and older finishing pigs (22). Serologic studies suggest that group C rotaviruses are endemic in U.S. swine herds (40, 44). In humans, group C rotaviruses are potential emerging enteric pathogens for all ages including adults (4, 17, 31). Since their first detection in humans in 1983 (28), group C rotaviruses have been associated with large outbreaks of diarrhea in Japan (4, 16), and with family or sporadic cases of diarrhea in children and adults worldwide including the U.S. (4, 17).

Assays to detect group C rotaviruses have been described, including polyacrylamide gel electrophoresis (PAGE), immune electron microscopy (IEM), enzyme linked immunosorbent assay (ELISA) and cell culture immunofluorescent test (CCIF) (3, 12, 18, 40, 44). However, because of the limited shedding or instability of group C rotaviruses in feces (4, 17, 31), highly sensitive assays are needed for their efficient detection (17). In this study, we surveyed fecal samples from calf diarrhea and adult cow diarrhea cases for the presence of group C rotaviruses using RT-PCR. A group C rotavirus (WD534tc/C strain) was isolated from an diarrheic adult.
cow fecal sample, which also contained group A rotavirus. The WD534tc/C strain showed a
greater relatedness to Cowden porcine group C rotavirus by serologic and genetic analysis than
to the Shintoku bovine group C rotavirus and was pathogenic in gnotobiotic pigs. During our
preliminary study of the pathogenicity of the WD534tc/C infection, gnotobiotic calves
inoculated with this strain developed diarrhea without or with limited (1 day) virus shedding.
Because the original field sample was a mixed infection with group A rotavirus, and the isolate
was potentially a porcine group C strain, we hypothesized that such dual rotavirus infections
might play a role in the pathogenesis and adaptation of heterologous group C rotaviruses to
cattle. Thus we investigated the coinfection of gnotobiotic calves with group A bovine
rotaviruses and the WD534tc/C bovine group C rotavirus isolate to determine clinical signs,
virus shedding patterns and the extent of histopathological lesions in the intestines.

5.3 MATERIALS AND METHODS

Viruses and cells. Reference group C rotaviruses included the Shintoku bovine strain (48)
and the Cowden porcine strain (41). Group A rotaviruses included a virulent serotype G6
IND(P[5]G6) strain (6) and an attenuated vaccine NCDV (P[1]G6) strain from the American
Tissue Culture Collection (ATCC, Atlanta, GA). The Shintoku/C, attenuated Cowden/C and
NCDV/A strains were grown in monkey kidney cells (MA104 cells) in the presence of
pancreatin (50 μg/ml) (36, 41, 48). The virulent IND/A and Cowden/C strains were prepared
by virus passage in gnotobiotic calves or piglets, respectively, and infected feces or intestinal
contents were collected as a source of virus. As negative controls, mock-infected cell culture
samples and mock-infected gnotobiotic calf feces or pig intestinal contents were used.

**Field samples.** Analysis for bovine group C rotaviruses was performed on 90 diarrheic calf fecal samples from Ohio, California, Wyoming, South Dakota and Nebraska; 81 adult cow diarrheic fecal samples (winter dysentery cases) from Ohio, New York and California; and 20 normal adult cow fecal samples (from 2 Ohio herds that were matched case controls for the winter dysentery cases) (39).

**Extraction and electrophoresis of rotavirus dsRNA.** Rotavirus ds RNA was extracted from cell culture-propagated viruses and fecal or intestinal viruses using previously described procedures (13). Rotavirus dsRNA in extracted samples was analyzed by PAGE to confirm the presence of dsRNA and to examine the genomic electropherotypes as previously described (6, 13, 31). The discontinuous buffer system of Laemmli was utilized and dsRNA was resolved in 10% polyacrylamide slab gels (23). Electrophoresis was conducted at 12mA for 14-16 hrs. The dsRNA bands were visualized by silver staining (13).

**IEM and CCIF assay.** Fifty-three diarrheic adult cow fecal field samples, fecal samples after single or dual inoculation of gnotobiotic calves and pigs and cell culture isolates were processed for IEM as previously described (32). The processed samples were incubated separately with group A-, B-, C- or bovine coronavirus-specific hyperimmune antisera, then the mixtures were pelleted, negatively stained with phosphotungstic acid, applied to grids, and examined by electron microscopy (29, 32). A CCIF assay was used to confirm if group A or
C rotaviruses were present in virus isolates and to detect virus shedding after challenge of
gnotobiotic calves and pigs with group A and/or C rotaviruses as described previously (20).
Briefly, the fecal or cell-passaged rotavirus samples were serially diluted with MEM from
1:50 to 1:12,500 and were inoculated onto monolayers of MA104 cells grown in 96-well
plates. After adsorption at 37 °C for 1 hr, the mixtures were discarded and 200 µl (per well)
of MEM containing 50 µg/ml pancreatin were added to the cells and incubated at 37 °C for
20 hours. The media was discarded, the cells were fixed with 80 % acetone and stained with
FITC-conjugated anti-OSU porcine group A or anti-Cowden porcine group C rotavirus
serum (20, 40). The numbers of fluorescent focus units (FFU) were counted in each well and
titers expressed as FFU/ml.

Reverse-transcription-PCR assay. Field fecal samples and fecal samples collected after
virus challenge of gnotobiotic calves and pigs were tested for group A and C rotaviruses by
RT-PCR (6). The RT-PCR primers were designed to produce partial length group C rotavirus
VP6 based on Cowden and Shintoku VP6 genes and a full-length group A rotavirus VP7
gene. cDNA was synthesized and amplified in an RT-PCR mixture containing 10X buffer
(200 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, KCl, 0.05% gelatin), dNTP (10 mM, each), 200
ng of primer A (sense, for group C VP6, 5'-GAAGCTGTATGTGATGATGA-3' and for
group A VP7, 5'-GGCCGGATTAAAAAGCGACAATTT-3'), 200 ng of primer B (antisense,
for group C VP6, 5'- CATAGCAGCTGGTCTAATCA-3' and for group A VP7, 5'
GGTCACATCATACAACTCTA-3'), (6), 5 U RNAsin (Boehringer Mannheim Biochemicals,
Indianapolis, Ind.), 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer
Mannheim Biochemicals) and 2.5 U of Taq polymerase (Boehringer Mannheim Biochemicals). First strand cDNA synthesis was accomplished by incubating the above mixture for 90 min at 42 °C. Thirty amplification cycles were conducted, with each cycle consisting of 94 °C for 1 min (denaturation), 52 °C for 1 min (annealing) and 72 °C for 2 min (extension), followed by a 7 min extension at 72 °C. The PCR products were analyzed on 1% agarose gels using standard procedures (38).

Isolation of a group C rotavirus from fecal samples. Two adult cow diarrheic fecal samples (from the same Ohio farm) which were group C positive by RT-PCR were inoculated into MA104 cells in roller tubes for virus isolation (36). They were blind passaged until cytopathic effects were seen and monitored for group A and C rotaviruses by CCIF (20, 40).

Cloning and sequence analysis of the full length VP6 gene of WD534tc. The full length VP6 gene of WD534tc was produced by RT-PCR with 5' and 3' end primers, and cloned in the pCRII vector (Invitrogen, San Diego, CA). The sequence analysis was performed using the primer extension method (Sequenase v2.0, Amersham, Arlington Height, IL) and the data were analyzed using the DNASTAR program (DNASTAR Inc. Madison, WI).

Antigenic analysis of WD534tc, Cowden, Shintoku group C rotaviruses and IND group A rotavirus using antiserum to WD534tc in flourescent focus neutralization tests (FFN).

The isolate WD534tc, Cowden, Shintoku group C rotaviruses and IND group A rotavirus were tested in FFN tests using antiserum to WD534tc/C, which was prepared by
hyperimmunization of a gnotobiotic piglet with WD534tc/C, as described previously (20, 40). Briefly, the antiserum was serially diluted with MEM from 1:50 to 1:12,500 and mixed with WD534tc/C or the homologous reference rotaviruses \((1 \times 10^4 \text{FFU/ml})\), and reacted at 37°C for 1 hr. The mixtures (100 μl) were inoculated onto monolayers of MA104 cells grown in 96-well plates. After adsorption at 37 °C for 1 hr, the mixtures were discarded and 200 μl (per well) of MEM containing 50 μg/ml pancreatin were added to the cells and incubated at 37 °C for 20 hours. The medium was discarded, the cells were fixed with 80 % acetone and stained with FITC-conjugated pig anti-OSU group A or anti-Cowden group C rotavirus serum. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that resulted in 80 % or greater reduction in numbers of FFU. Normal sera from unexposed gnotobiotic pigs were used for negative controls.

**Virus challenge of gnotobiotic calves and piglets.** The challenge was either single or dual inoculation simultaneous of group A and group C rotavirus strains into 16 gnotobiotic calves and 6 gnotobiotic piglets (Table 2). All calves and piglets were from 1- to 24-days-old and were derived, fed, inoculated and maintained as previously described (25, 29). Briefly, the gnotobiotic calves and piglets were oronasally inoculated with diluted (1:10) fecal filtrates or virus-infected cell culture lysates. Viruses were inoculated at similar FFU doses (WD534/C: \(10^7\) FFU for calves and \(10^6\) for piglets; IND/A: \(10^7\) FFU; NCDV/A: \(10^5\) FFU for single inoculation and \(10^5\) or \(10^7\) FFU for dual inoculation; virulent and attenuated Cowden/C: \(10^7\) FFU for calf, \(10^6\) for piglets) in a total of 50 ml for calves and 10 ml for pigs. Dual inoculation (WD534tc/C+IND/A, WD534tc/C+NCDV/A or virulent Cowden/C+IND/A) of
gnotobiotic calves was done with the same virus titers (except WD534tc/C+NCDV/A, either $10^5$ or $10^7$ FFU for NCDV/A) and volumes as single inoculations. A titer of $10^5$ FFU for single inoculation of calves with NCDV/A was used because it is the recommended dose of commercial rotavirus vaccines for use in calves (42). After inoculation, calves and piglets were examined twice daily for clinical signs and development of diarrhea, and the fecal consistency was scored (0=normal, 1=pasty, 2=semi-liquid, 3=liquid). Feces and serum samples were collected daily and weekly, respectively, and stored at $-20\,^\circ\text{C}$ until tested. Each fecal sample was tested for presence of groups A and C rotaviruses using CCIF, IEM and RT-PCR, and each serum sample was tested for antibodies to group A and C rotaviruses using the FFN test as described (20, 40).

**Histopathological evaluation.** Single or dual inoculated calves were euthanized within 12 hrs of diarrhea onset (2–4 PID) and duodenum, jejunum and ileum of the small intestines and mesenteric lymphnode (MLN) were collected and placed in fixative (Prefer, Anatech LTD., Battle Creek, MI). The tissues were processed, paraffin-embedded and stained with haematoxylin and eosin (50). Histological evaluation was done on coded samples and a comparison was made with tissues from age-matched controls.

### 5.4 RESULTS

**IEM, PAGE and RT-PCR.** None of 53 diarrheic adult cow fecal field samples tested were group C rotavirus positive by IEM. By PAGE, none of the diarrheic calf, adult cow and
normal adult cow fecal samples were group C rotavirus positive. Fifty-four calf fecal samples (54/90), one adult cow diarrheic fecal sample (1/81) and none of the normal adult cow fecal samples (0/20) were group A rotavirus positive by PAGE. The partial length VP6 gene (expected 610 bp) and full length VP7 gene (expected 1062 bp) were generated for reference group C and group A rotavirus strains using the selected primer pairs (Fig 5.1). The RT-PCR assay was rotavirus group-specific and no PCR product was seen when the RT-PCR assay was applied to the heterologous rotaviruses and negative control samples. No group C rotaviruses were detected in 90 diarrheic calf samples, whereas, 66 samples were group A positive by RT-PCR. For diarrheic adult cow fecal samples, 3 samples (3/81, 2 of them from the same farm) and one sample were group C and group A rotavirus positive by RT-PCR, respectively.

Isolation of a group C rotavirus positive sample into MA104 cells. One adult cow diarrheic fecal sample showed cytopathic effects after 6 blind passages in MA104 cells (WD534tc/C). However, both group C and A rotaviruses were subsequently detected in the inoculated cell culture lysates by CCIF. After the G and P types of the group A rotavirus was determined, the group A rotavirus (P[5]G6) was eliminated by using hyperimmune antiserum to group A rotavirus (polyclonal and monoclonal antibodies to the G6 serotype) to neutralize the group A rotavirus. The group C rotavirus was cloned by plaque isolation twice to further eliminate the group A rotaviruses.

Characterization of the WD534tc/C. 1) IEM. The WD534tc/C isolate showed typical
rotavirus morphology and was aggregated by anti-group C rotavirus (Cowden) serum in IEM.

2) PAGE. The WD534tc/C had a typical group C rotavirus electrophoretype in PAGE (4-3-2-2) (Fig 5.1). 3) Sequence analysis of the full length VP6 gene of WD534tc. The full length VP6 genes of the WD534tc isolate showed higher homologies (over 98%) with the Cowden porcine group C rotavirus than with the Shintoku bovine group C rotavirus (82.5%). Compared to the group A IND rotavirus, the similarity of the VP6 gene was 55.8%.

4) Antigenic analysis of WD534tc by the FFN test. The WD534tc/C rotavirus was antigenically more closely related to the Cowden (FFN antibody titer of 25,600) porcine group C rotavirus than to the Shintoku (FFN titer of 1,600) bovine group C rotavirus in one-way FFN tests using antiserum to the WD534tc/C strain (homologous FFN titer of 25,600). The WD534tc/C rotavirus was antigenically unrelated to the IND group A rotavirus in the FFN test (FFN titer < 100).

Single inoculation of gnotobiotic calves with IND/A, NCDV/A, WD534tc/C or Cowden/C rotaviruses. The virulent IND/A rotavirus caused diarrhea (from 2 or 3 PID) and virus shedding (from 2 PID, n=2) detected by RT-PCR, IEM and CCIF (Table 5.3). With the attenuated NCDV/A, all calves (n=3, but 2 of them had mild diarrhea before inoculation) developed mild diarrhea without virus shedding and seroconverted to the homologous virus (n=2, No.3 calf was euthanized at 3 PID for histopathology) 3 weeks after inoculation. All calves (n=4) inoculated with WD534tc/C developed diarrhea from 1 to 3 PID with limited virus shedding detected by only RT-PCR or RT-PCR and IEM (1 PID only, for 2 calves, No.7 and 9) or without virus shedding (n=2) and seroconverted to WD534tc/C (n=3, a calf
[No.9] was euthanized at 3 PID for histopathology) 3 weeks after inoculation (Table 5.3). The calf inoculated with virulent Cowden/C porcine rotavirus had no diarrhea and limited virus shedding detected by only IEM (1 PID only) and seroconverted to homologous Cowden/C virus 3 weeks after inoculation (Table 5.3). The control calf (n=1) inoculated with diluent showed no diarrhea or virus shedding (data not shown).

**Dual inoculation of calves with WD534tc/C or Cowden/C and virulent or attenuated group A rotavirus.** Dual inoculation of calves with WD534tc/C and IND/A caused diarrhea (from 1 or 3 PID) with both group A and C virus shedding detected by CCIF, IEM and RT-PCR (n=2, Table 5.4). Cowden/C and IND/A dual inoculation of one calf caused diarrhea after 2 PID with both group A and C rotavirus shedding detected by RT-PCR, IEM and CCIF (calf No.13, Table 5.4).

Dual inoculation with WD534tc/C and attenuated NCDV/A rotavirus induced diarrhea in all calves (n=3) with variable, transient virus shedding depending on the inoculation titer of attenuated NCDV/A rotavirus. Using low titer (10^5 FFU) attenuated NCDV/A (calves No. 14, 15), the effects of dual inoculation were similar to those of single inoculation of calves with WD534tc/C: only group C shedding was detected by only RT-PCR (No.15) or there was no virus shedding (No.14) (Table 5.4). However, the use of higher titer (10^7 FFU) NCDV/A rotavirus for dual inoculation induced both group A and group C virus shedding of limited amounts (detected by only RT-PCR and IEM for NCDV/A and by only RT-PCR for WD534tc/C) and for a limited period (1 and 2 PID, Table 5.4, No.16).
**Inoculation of gnotobiotic piglets with WD534tc/C, virulent or attenuated Cowden/C rotavirus.** WD534tc/C and virulent Cowden/C caused diarrhea and virus shedding in gnotobiotic piglets (n=3 and n=1, respectively), whereas the attenuated Cowden/C strain induced no diarrhea, and virus shedding detected only by RT-PCR (n=2) (Table 5.5).

**Histopathology studies.** The morphologic changes observed in the intestinal tissues of calves euthanized at 3 to 4 PID following single or dual rotavirus inoculation are summarized (Table 5.6, Figure 5.2). Virulent IND/A induced lesions typical of group A rotavirus infection characterized by loss of normal absorptive cells, detachment of absorptive cells from their basement membranes (especially over villi tips), blunting and fusion of villi, crypt hyperplasia, and lymphoreticular hyperplasia within villous tip stroma, small intestinal submucosal lymphoid aggregates, Peyer’s patches, and mesenteric lymph nodes. Inoculation with IND/A alone caused mild to moderate intestinal lymphoid hyperplasia and scattered foci of atrophied villi throughout all regions of the small intestine examined. Inoculation with NCDV/A alone elicited mild intestinal lymphoid hyperplasia and sparse villous atrophy in the jejunum and ileum. Inoculation with WD534tc/C alone elicited little to no lymphoid changes and only slight villous changes in the jejunum. Mild to moderate intestinal lymphoid hyperplasia was observed in the intestinal tissues in calves coinfected with WD534tc/C and either IND/A or NCDV/A, and villous atrophy was more pronounced and more frequently observed throughout all regions of the small intestine (duodenum, jejunum, ileum) at 2 PID (data not shown) and 4 PID (Table 5.6, Fig 5.2).
5.4 DISCUSSION

Group C rotaviruses have been reported in pigs, humans and cattle. In humans, unlike group A rotaviruses, group C rotaviruses are associated with diarrhea in all ages (infants, children and adults) (4, 16, 17, 28, 31). They have been reported worldwide since the early 1980's and are regarded as a potential emerging pathogen (4, 31). Although there is serological evidence for group C rotaviruses in cattle, so far there are no reports of their detection or isolation from cattle in the U.S. (31, 44). When we screened adult cow diarrheal samples from winter dysentery outbreaks for group C rotaviruses using RT-PCR (n=81), we found only three positive samples which were negative by IEM and PAGE. These results suggest that the prevalence of bovine group C rotaviruses in the field may be low, the viruses are unstable in feces, or virus shedding from infected animals may be limited. Therefore, to survey for bovine group C rotaviruses in the field, highly sensitive assays like RT-PCR are required as used in our study. Jiang et al. (17) also reported the difficulty in detecting group C rotaviruses in samples from diarrheic children because of the low titers of virus in fecal samples, and noted that use of conventional methods such as PAGE may underestimate the true detection rate of group C rotaviruses.

From one diarrheic adult cow fecal sample, we isolated a group C rotavirus (WD534tc/C strain) in MA104 cells. Interestingly genetically and serologically, the WD534tc/C strain was more closely related to the Cowden/C porcine strain than to the Shintoku/C bovine strain. Challenge of 3 gnotobiotic calves with the WD534tc/C strain induced diarrhea without virus shedding or with limited virus shedding, and calves seroconverted to this virus, which indicates that the WD534tc/C strain is capable of limited replication in the intestine of the
calves. The more extensive diarrhea and shedding of WD534tc/C in piglets, comparable to that seen with virulent Cowden porcine group C rotavirus suggests that the natural host for the WD534tc/C strain is more likely pigs, rather than cattle.

Because the original sample contained both group A and C rotaviruses, we were interested in the effects of their dual infection on gnotobiotic calves. Single inoculation of the virulent IND/A strain caused diarrhea and virus shedding in the inoculated calves. Theil et al. (42) reported that a titer of $10^5$ FFU of NCDV/A in a commercial modified live bovine rotavirus-coronavirus vaccine induced mild diarrhea at 3-6 PID without virus shedding (except for 1 rotavirus positive sample out of 41 fecal samples examined) by IEM in all 3 gnotobiotic calves, and all calves seroconverted to NCDV/A rotavirus. In this study, although single inoculation of calves with $10^5$ FFU the attenuated NCDV/A strain caused mild diarrhea in one calf (the other 2 calves had mild diarrhea pre-exposure) and seroconversion to homologous virus in all calves tested 3 weeks after inoculation, no virus shedding was detected in fecal samples, suggesting the limited replication of this attenuated virus in the intestine of calves.

Using the virulent IND/A strain, dual infection with WD534tc/C consistently enhanced shedding of the WD534tc/C strain, as was seen also for porcine Cowden/C after dual inoculation of a calf with Cowden/C and IND/A. Using low titer ($10^5$ FFU) attenuated NCDV/A, the effect of dual inoculation was similar to that seen after single inoculation with WD534tc (Table 5.4, No.14, 15): no synergistic effect was seen by co-infection with both viruses. However, the use of higher titer ($10^7$ FFU) NCDV/A for dual infection induced limited amounts of both group A and group C virus shedding (detected by RT-PCR and IEM
for NCDV/A and by RT-PCR for WD534tc/C) for a limited period (1 and 2 PID, Table 5.4, No. 16). Differences between the virulent and attenuated group A rotaviruses and different titers of attenuated group A rotavirus in their effects on dual infection could be due to differences in their replication ability in the intestine and/or the extent of cytopathology (villous atrophy) induced.

In the histopathological study, the intestinal lesions (Fig 5.2, Table 5.6) were generally more severe with dual infection than with single infection of group A and C rotaviruses. Infection with WD534tc/C or NCDV/A alone caused only mild villous atrophy in the jejunum or jejunum and ileum, respectively, whereas, dual infection with both viruses induced lesions throughout the small intestine. Although IND/A alone caused villous atrophy throughout the small intestine, more severe small intestinal lesions occurred in calves coinfected with WD534tc/C and IND/A.

Inter-species transmission of group A rotaviruses has been suggested, especially between humans and cattle. Gerna et al. (9) reported G6 serotype strains from humans (PA151, PA169) (9, 10, 15), which were recognized by G6 MAbs (bovine G6) and showed some genetic variation (about 10% in amino acid identity) with typical bovine G6 strains (UK and NCDV strains). By RNA-RNA hybridization, most of the gene segments of PA151 G6 strains were closely related to AU-1 human or NCDV bovine strains (15). Urasawa et al. (49) reported human G10 group A rotaviruses and suggested this strain originated from a bovine host. Blackhall et al. (2) confirmed a bovine G1 strain analogous to human G1 serotypes in Argentina by serologic assays and sequence analysis of the VP7 genes. In addition, rotavirus strain 116E, isolated from the fecal specimen of a newborn asymptomatic infant from India
was identified as a P[11] type which was previously reported only in cattle (8).

Coinfection with group C and group A rotaviruses were detected in diarrheic fecal samples from children and finishing pigs (17, 22). Kim et al (22) reported a diarrhea outbreak associated with group C rotaviruses in finishing pigs and found these fecal samples also had group A rotaviruses detectable only by a highly sensitive second round PCR assay. Ishimaru et al. (16) studied the epidemiology of a gastroenteritis outbreak associated with group C rotaviruses in the Matsuyama district of Japan and noted that group C rotavirus gastroenteritis occurred following an epidemic of group A rotavirus infections, primarily in children aged 4-7 years but rarely in those aged 0-1 years.

Coinfections by group A rotaviruses and other enteropathogens including *Escherichia coli* in calves are common in nature (26, 30). The effects of coinfection of calves with group A rotavirus and *E.Coli* have been studied (11, 14, 30). Generally coinfected calves had more severe diarrhea, lesions and shedding of both agents than infection by either agent alone, but little information is available regarding the detailed mechanisms for these synergistic effects. When rotaviruses and *E.coli* were coinoculated into calves, Runnels et al. (30) observed that there was more severe villous atrophy in the ileum of dually inoculated calves than for rotavirus singly inoculated calves. They also found more intensive small intestinal colonization by *E.coli* after coinfection with rotavirus. Gouet et al. (11) reported that experimental inoculation of calves with rotavirus, although not lethal in itself, followed by inoculation with a non lethal dose of *E. coli*, led to dehydration and death in newborn colostrum-deprived calves. Hess et al. (14) reported that coinfection of newborn colostrum-deprived calves with rotavirus and *E.Coli* resulted in not only more severe histological small intestinal lesions but
also increased shedding of both pathogens.

The mechanism for the enhancement of virus shedding of the WD534tc/C strain by coinfection with the virulent group A rotavirus is unclear. We hypothesize that the WD534tc/C strain is likely highly cell-associated in the calf intestine as seen in tissue culture (40), and following coinfection by a cytolytic group A rotavirus, the group C virus may be more readily released by cytolysis from the infected cells. Alternatively structural or non-structural proteins of group A rotavirus could assist in the replication or release of the WD534tc/C strain in co-infected cells or could have an indirect bystander effect on group C rotavirus infected cells. A potential candidate is the nonstructural group A rotavirus protein, NSP4, which has recently been shown to function as a viral enterotoxin in mouse studies and has membrane destabilization activity (1, 43).

Based on our results, which showed only limited or no virus shedding of the WD534tc/C strain in singly inoculated calves and the genetic and antigenic similarity of this strain to porcine group C rotavirus, we propose that the WD534tc/C strain may have originated from a porcine host and thus replicates only to a limited extent in the calf intestine. However, if dual infections with both group A and C rotaviruses occur in the bovine host, synergic effects may cause the release of the WD534tc/C strain into the feces, leading to intra (bovine)-species transmission and further host adaptation after serial passage in the new host. Interestingly calves dually infected with WD534tc/C and attenuated group A rotavirus (analogous to currently used oral attenuated vaccine strain) also briefly shed both viruses. Thus our findings suggest a potential new, novel hypothesis for the adaptation of heterologous rotaviruses to new host species.
To explore potential mechanisms for the observed enhanced shedding of WD534tc/C rotavirus by coinfection with virulent group A rotavirus, in future studies, we plan to examine the effects of group A and C rotavirus coinfection and/or co-transfection/infection of a group A rotavirus NSP4 gene and group C rotavirus in an vitro cell culture system.

5.6 ACKNOWLEDGMENT

We thank Dr. D. C. Hodgins for help with gnotobiotic calf work and C. Nielsen for help with histopathological studies. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center (OARDC), the Ohio State University. This work was partially supported by USDA NRICGP Competitive grant No. 97-35204-4682 and grant No. RO1 AI 3356-04 from the National Institute of Allergy and Infectious Diseases, NIH.
5.7 REFERENCE


160


161


163


Production and characterization of monoclonal antibodies to porcine group C rotaviruses cross-reactive with group A rotaviruses. Virology. 91:272-281.


<table>
<thead>
<tr>
<th>Rotavirus strain</th>
<th>VP6 nucleotide identity (%)</th>
<th>Virus neutralization titer(^a) of hyper immune antiserum to WD534tc/C</th>
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<tbody>
<tr>
<td>Group C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shintoku (bovine)</td>
<td>82.5</td>
<td>1,600</td>
</tr>
<tr>
<td>Cowden (porcine)</td>
<td>98.7</td>
<td>25,600</td>
</tr>
<tr>
<td>WD534tc/C (bovine)</td>
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<td>25,600</td>
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<tr>
<td>Group A</td>
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<td></td>
</tr>
<tr>
<td>IND (bovine)</td>
<td>55.8</td>
<td>&lt;100</td>
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Table 5.1. Characterization of the WD534tc strain of bovine group C rotavirus in comparison with other group C rotaviruses
### Table 5.2. Experimental design for single and dual inoculation of gnotobiotic calves and piglets with group C and group A rotaviruses

<table>
<thead>
<tr>
<th>Virus inoculum</th>
<th>Calf groups</th>
<th>Piglet groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IND/A NCDV/A</td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td>virulent</td>
</tr>
<tr>
<td></td>
<td>+ IND/A</td>
<td>+ NCDV/A</td>
</tr>
</tbody>
</table>

| No. of animals | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 1 | 3 | 1 | 2 |

*Calves were challenged at 3 to 8 days of age, except one older calf in group 6 (24-day-old)

*Piglets were challenged at 4 days of age, except one piglet in group 11 (24-day-old)

*Viruses were inoculated at the following doses (WD534tc/C: $10^7$ fluorescent focus unit (ffu) for calves, $10^6$ ffu for piglets; IND/A: $10^7$ ffu; NCDV/A: $10^5$ or $10^7$ ffu; virulent and attenuated Cowden: $10^7$ ffu for calf, $10^6$ ffu of piglets)
<table>
<thead>
<tr>
<th>Calf No.</th>
<th>Age (days) at inoculation</th>
<th>Clinical signs</th>
<th>Diarrhea / virus shedding</th>
<th>Seroconversion</th>
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<td>Virus shedding</td>
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<tr>
<td></td>
<td></td>
<td>Virus shedding</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

* PID=post-inoculation day, b seroconversion to group C rotavirus after 21 PID determined by FFN test, c virus shedding determined by RT-PCR/IEM/CCIF, +/- = no virus detected by any of 3 tests, d euthanized for histopathology, e ND=not done, f all calves were inoculated with 10^5, g calf had mild diarrhea before challenge.

Table 5.3. Pathogenesis of virulent IND/A, attenuated NCDV/A, WD534tc/C and Cowden/C bovine rotavirus in gnotobiotic calves
<table>
<thead>
<tr>
<th>Calf No.</th>
<th>Age (days)</th>
<th>Clinical signs</th>
<th>Diarrhea / virus shedding</th>
<th>Seroconversion</th>
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<td>gpC shedding</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>Diarrhea</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
<tr>
<td></td>
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<td>gpA shedding</td>
<td>+/+/+</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>gpC shedding</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
<tr>
<td>14&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5</td>
<td>Diarrhea</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gpA shedding</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gpC shedding</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>15&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6</td>
<td>Diarrhea</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gpA shedding</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gpC shedding</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>16&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6</td>
<td>Diarrhea</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gpA shedding</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gpC shedding</td>
<td>-/-</td>
<td>-/-</td>
</tr>
</tbody>
</table>

<sup>a</sup> PID=post-inoculation day,  
<sup>b</sup> seroconversion to group C rotavirus after 21 PID determined by FFN test,  
<sup>c</sup> virus shedding = RT-PCR/IEM/CCIF,  
<sup>d</sup> no virus detected by any of 3 tests,  
<sup>e</sup> euthanized for histopathology,  
<sup>f</sup> challenge dose of NCDV/A was 10⁵ ffu,  
<sup>g</sup> challenge dose of NCDV/A was 10⁷ ffu

Table 5.4. Pathogenesis of WD534tc/C in gnotobiotic calves coinfected with either virulent IND/A or attenuated NCDV/A bovine rotavirus
<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Age (days)</th>
<th>Clinical signs</th>
<th>Diarrhea / virus shedding</th>
<th>Seroconverion</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1 PID*</td>
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<td></td>
<td></td>
<td></td>
<td>2 PID</td>
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<td>3 PID</td>
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<td>4 PID</td>
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<td>6 PID</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 PID</td>
<td></td>
</tr>
</tbody>
</table>

**WD534tc/C**

- 1: Diarrhea
- Virus shedding:
  - 1 PID: -
  - 2 PID: +/+
  - 3 PID: +/+
  - 4 PID: +/+
  - 5 PID: +/+
  - 6 PID: +/+
  - 7 PID: +/+

**Cowden/C-virulent**

- 2: Diarrhea
- Virus shedding:
  - 1 PID: -
  - 2 PID: +/+
  - 3 PID: +/+
  - 4 PID: Euthanize

**Cowden/C-attenuated**

- 3: Diarrhea
- Virus shedding:
  - 1 PID: -
  - 2 PID: +/+
  - 3 PID: Euthanize

- 4: Diarrhea
- Virus shedding:
  - 1 PID: +/+
  - 2 PID: +/+
  - 3 PID: +/+
  - 4 PID: +/+

- 5: Diarrhea
- Virus shedding:
  - 1 PID: +/+
  - 2 PID: +/+
  - 3 PID: +/+
  - 4 PID: +/+
  - 5 PID: +/+

- 6: Diarrhea
- Virus shedding:
  - 1 PID: +/+
  - 2 PID: +/+
  - 3 PID: +/+
  - 4 PID: +/+

* PID=post-inoculation day, b seroconversion to group C rotavirus after 21 PID determined by FFN test, c virus shedding = RT-PCR/IEM/CCIF, = no virus detected by any of 3 tests, d euthanized for histopathology, e

ND=not done

**Table 5.5. Pathogenesis of WD534tc/C in gnotobiotic piglets compared to the virulent or attenuated Cowden/C porcine strains**

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Calves were inoculated at 6 to 8 days of age.

Post-inoculation day (PID) of euthanasia.

Sign in parentheses denoted degree of histologic change (lymphoid hyperplasia and villous atrophy from age-matched control: (-) none, same as control; (-/+ minimal, focal; (+) mild, scattered; (++) moderate, widespread.

Small intestinal lamina propria, submucosa and Peyer’s patch lymphoid tissue evaluated.

Table 5.6. Intestinal morphologic changes in individual calves following single or dual inoculation with group A and C bovine rotaviruses.
Figure 5.1. A. The RT-PCR products of reference and field samples detected using primers specific for the partial VP6 gene of group C rotavirus. Lane 1, molecular weight markers; lane 2–5, field fecal samples; lane 6, IND/A; lane 7, NCDV/A; lane 8, Cowden/C; lane 9, Shintoku/C; lane 10, WD534tc/C. B. DsRNA electropherotypes of the reference and WD534tc strains. Lane 1, IND/A; lane 2, ATI/B; lane 3, WD653/B; lane 4, Shintoku/C; lane 6, Cowden/C; lane 7, WD534tc/C.
Fig. 5.1
Figure 5.2. Intestinal lesions (A-D, jejunum) in gnotobiotic calves following oronasal inoculation with A) Mock (2 DPE); B) WD534tc/C (4DPE); C) IND/A (3DPE) and D) coinfection with IND/A and WD534tc/C (2 DPE). Haematoxylin and eosin stain. 50X. Note WD534tc alone caused minimal changes (B), whereas, IND/A and coinfection with IND/A and WD534tc/C caused villous atrophy (C, D), vacuolation of absorptive cells (D) and hyperplasia of cryptic cells (C,D) in the jejunum.
BIBLIOGRAPHY


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60. Das M, Dunn SJ, Woode GN, Greenberg HB, Rao CD. 1993. Both surface proteins (VP4 and VP7) of an asymptomatic neonatal rotavirus strain (I321) have high levels of sequence identity with the homologous proteins of a serotype 10 bovine rotavirus. Virology. 194:374-379.


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205
IMAGE EVALUATION
TEST TARGET (QA-3)

1.0
1.1
1.25
1.4
1.6

1.25
1.4
1.6

1.1
1.25
1.4
1.6

1.0
2.2
2.0
1.8

2.5
2.2
2.0
1.8

150mm
6"