Characterization of antigenic relationships among bovine rotaviruses

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EXPLANATION OF THESIS FORMAT

The following thesis consists of a general introduction, a review of the literature, a separate manuscript, a general summary, literature cited, and acknowledgments. The master's candidate, Mary Ann Brooks, is the senior author and principal investigator for the manuscript.

GENERAL INTRODUCTION

Serologic surveys indicate rotaviruses are ubiquitous in cattle populations and one of the most commonly detected bovine enteropathogens (Mebus et al., 1969: Hammami et al., 1989; Bellinzoni et al., 1987; Acres et al., 1975; Acres and Babiuk, 1978; Woode, 1978; Woode and Bridger, 1975). Rotavirus infection usually affect calves under two weeks of age and manifests itself as depression, diarrhea and dehydration (Acres and Babiuk, 1978; Tzipori, 1981; Tzipori et al., 1981; Woode and Crouch, 1978).

The most frequently detected rotavirus from rotavirus induced diarrheic calves are the group A rotaviruses (Allen et al., 1989; Theil and McCloskey, 1989; Hammami et al., 1989) although several reports of atypical rotaviruses which do not contain the common group A antigen have also been reported (Vonderfecht et al., 1986; Chasey and Davis, 1984; Brown et al., 1987; Snodgrass et al., 1984a). Group A serotype 6 strain NCDV (bovine rotavirus serotype 1) rotavirus is the only established serotype in cattle (Mebus et al., 1969; Hoshino et al., 1984). At least one and possibly two additional serotypes have been proposed (Woode et al., 1983; Snodgrass et al., 1984b; Brussow et al., 1987; Murakami et al., 1983) suggesting the existence of additional serotypes. The relative prevalence of different serotypes in cattle populations has not been determined. The majority of bovine rotavirus field isolates have been identified as bovine rotavirus serotype 1 by serologic analysis (Snodgrass et al., 1984; Woode et al., 1983); however, antigenic

diversity between field isolates and the established bovine serotype 1 rotavirus NCDV has been shown by differences in in vitro neutralization assays and lack of in vitro cross protection (Gaul et al., 1982; Woode et al., 1983; Paul et al.,1988).

Serotyping of rotaviruses is determined by neutralization specificities encoded by the outer capsid protein VP7 (Greenberg et al., 1983b; Kalica et al., 1981). An additional outer capsid protein VP4 (formerly VP3 Liu et al., 1988) can also induce neutralization specific antibodies (Offit and Blavat 1986). Results from traditional serotyping methods can be ambiguous due to antibodies directed against VP4, (Hoshino et al., 1984; Hoshino et al., 1985). Therefore, emphasis needs to be placed on better understanding the role VP4 plays in in vitro neutralization, virulence and in vivo protection. The existence of two VP4 types within a single serotype of bovine rotavirus has been demonstrated, (Hoshino et al., 1984; Kantharidis et al., 1988) but the prevalence of VP4 types in the cattle population has not been determined.

We recently isolated a bovine rotavirus (VMRI) which demonstrated a unique RNA electrophoretic pattern which was classified as bovine rotavirus serotype 1. Initial studies indicated homologous VMRI neutralization titers were at least 10-fold higher than heterologous NCDV titers which indicated some degree of antigenic diversity existed between NCDV and VMRI (Paul et al., 1988).

The purpose of this study was three fold: First, to further characterize the VMRI bovine rotavirus isolate. Second, to determine the differences at the antigenic and genetic level between NCDV, VMRI, and

B641. Third, to generate information useful in the understanding of the molecular mechanism for lack of cross protection among bovine rotavirus strains NCDV and B641. These rotavirus strains are believed to be members of the same serotype (Woode et al., 1983; Woode et al., 1987).

Antigenic analysis of bovine rotaviruses at the genetic level may offer several advantages: First, it may provide information on the serotypes which are associated with enteric disease in cattle. Second, it will aid in developing new strategies for the prevention and control of rotavirus-induced enteritis in cattle. Third, it may provide information about neutralization proteins which may lead to the development of a serotyping classification based on both outer capsid proteins VP4 and VP7.

LITERATURE REVIEW

Rotavirus

Rotaviruses, belonging to the family Reoviridae, include a variety of antigenically and morphologically similar viruses. Rotaviruses are responsible for gastrointestinal diseases of clinical and economical importance (reviewed Estes et al., 1983). The isolation of a virus (NCDV) associated with diarrhea in calves by Mebus et al. (1969) was a major advance in determining the cause of acute infectious nonbacterial gastroenteritis. This discovery led to isolation of several morphologically similar viruses from a variety of species including man and most animals (Bishop et al., 1973)

The earliest reports of a virus resembling rotavirus was in 1943 by Light and Hodes (1943) when they induced diarrhea in calves from the stools of an infected infant. In 1963, Adams and Kraft (1963) isolated Epizootic Diarrhea of Infant Mice virus (EDIM) which resembled rotavirus-like particles. The SA11 virus, now a much characterized rotavirus, was isolated from a healthy monkey by Malherbe and Harwin (1963). The "O" agent from cattle and sheep intestinal washings was described in 1967 by Malherbe and Strickland-Cholmley (1968). All of these viruses have since been identified as rotaviruses. The genus rotavirus was established in the family Reoviridae by the International Committee on The Nomenclature of Viruses (Mathews, 1979).

Rotaviruses are now considered major pathogens of both human and veterinary importance.

The Rotavirion

Morphology and Physicochemical properties

Rotaviruses are composed of an icosahedral core approximately 38nm in diameter which contains the 11 dsRNA segments and an RNA-dependent RNA polymerase (Estes et al., 1983; Cohen, 1977). The core is enclosed by an inner capsid (single shelled particles) and then surrounded by the outer capsid (double shelled particles)(Rodger et al., 1975) Rotaviruses have a characteristic appearance when viewed by negative stain electron microscopy. The morphology of a complete particle is wheel shaped with a sharply defined outer layer representing the outer capsid. The name 'rotavirus', derived from the Latin word rota, meaning wheel, was first used by Flewett et al. (1974), due to its wheel-like appearance of the virus. Double shelled particles have a well defined circular shape approximately 70nm in diameter, an RNA content of 12% and a buoyant density of 1.36 g/ml in cesium chloride (CsCl). Single shelled particles lack the outer capsid, which results in a rougher appearance (Prasad et al., 1988). Single shelled particles measure approximately 55nm in diameter, have an RNA content of 16% and a buoyant density of 1.38 g/ml in CsCl (Bridger and Woode 1976; Welch 1971; Novo and Esparza 1981; Rodger et al., 1975).

Rotaviruses are quite stable to several chemical and physical treatments. The infectivity and morphology of rotaviruses are not

affected by fluorocarbon extraction, freeze-thawing, sonication or treatment of pH 3.0 (Estes et al., 1979; Welch 1971). Treatment of rotavirus with proteolytic enzymes, such as trypsin and pancreatin, results in enhanced viral infectivity of bovine and simian rotaviruses and is necessary for the infectivity of human rotaviruses in tissue culture (Clark et al., 1979; Graham and Estes, 1980; Kitaoka et al., 1986; Kitaoka et al., 1984; Theil et al., 1977; Theil et al. 1978; Theil and Bohl 1980). Treatment of rotaviruses with a chelating agent such as EDTA converts double shelled particles to single shells which results in the activation of endogenous RNA polymerase and loss of infectivity (Estes et al., 1979). Rotaviruses are inactivated by 95% alcohol which has been found to be the most effective disinfectant tested so far (Bishai et al., 1978; Estes et al., 1983).

Genetics

The genome of rotavirus consists of 11 segments of nonpolyadenylated dsRNA ranging in size from 0.2 to 2 X 10^6 daltons with a total molecular weight of 11-12 X 10^6 daltons (Kalica et al., 1978b; McNulty et al. 1981a; Rodger et al., 1975). The dsRNA segments can be divided into four size classes as follows: Class I segments 1-4 = 1.5 - 2.2 kd, Class II segments 5 and 6 = 0.8-1 kd, Class III segments 7-9 = 0.5 kd and Class IV segments 10 and 11 = 0.2-0.3 kd. It has been estimated that rotavirus genome segments range in size from approximately 700 to 3600 base pairs, assuming that the average molecular weight per base pair was 600 daltons. The number of base pairs per segment was estimated for the UK strain of bovine rotavirus and were as follows; segment 1 = 3,300; segment 2 =

2,600; segment 3 = 2,550; segment 4 = 2,370; segment 5 = 1,550; segment 6 = 1,340; segment 7 = 1,050; segment 8 = 1,050; segment 9 = 1,050; segment 10 = 760; and segment 11 = 680 (Holmes 1983).

The 11 dsRNA genome segments demonstrate a distinctive pattern in polyacrylamide gel electrophoresis (PAGE) which is unique to rotaviruses and can be used to differentiate rotaviruses from reovirus and orbiviruses (Todd and McNulty, 1976). Electropherotyping has been used to detect rotavirus in clinical samples, as a tool for molecular epidemiological studies, and also to distinguish members within a rotaviral group (Kalica et al., 1978a). This technique has proved very useful in the study of rotaviruses however it does have limitations. The analysis of different human and animal rotaviruses have revealed considerable inter- and intra-species variability in the migration of many of the dsRNA segments (Kalica et al., 1978b; Rodger and Holmes 1979; Sabara et al., 1982; Paul et al., 1988). Due to considerable amounts of variation seen in migration patterns Clark and McCrae (1983) analyzed genome segments using base specific nucleases and sequencing gels which determined that variations in migration did not necessarily reflect a sequence change in the RNA. Another variation seen in migration patterns has been the absolute order of the genome segments not always corresponding to the same genes among rotaviruses within the tight 7, 8, 9 triplet (Class III). It has been shown that either gene 8 or 9, depending on the species, codes for the outer capsid protein VP7 (Offit and Blavat 1986; Dyall-Smith et al., 1983). For example, in the UK strain of bovine rotaviruses gene segment 8 codes for VP7, while in the

NCDV strain of bovine rotavirus gene segment 9 codes for the neutralization protein (Dyall-Smith and Holmes, 1981; McCrae and McCorquodale, 1982). Short and "supershort" migration patterns have been demonstrated and attributed to the inversion of gene segments ten and eleven (Dyall-Smith and Holmes, 1981; Paul et al., 1988). The high degree of variation among rotaviruses in the size of gene segments suggests that genome diversity exists among bovine rotaviruses. Careful evaluation of an isolate using several criteria is necessary before an isolate can be fully characterized.

Virus Composition and Virus Proteins

The RNA segments and the RNA dependent RNA polymerase of the simian SA11 rotavirus are contained within a core particle which is composed of three major proteins, VP1, VP2 and the recently identified product of gene segment 3, VP3 (Estes et al., 1983; Liu et al., 1988). Rotavirus polymerase activity has been found to be optimal between 45 and 50 C at pH 8.0 in the presence of 8-10 mM magnesium ions (Cohen, 1977). Polymerase activity is known to be associated with single shelled particles and is activated by chelating agents such as EDTA (Estes et al., 1979). The proteins involved in polymerase activity are not known, however, it was recently suggested that the newly discovered product of gene 3, VP3, might function as part of the virion-associated RNA polymerase (Liu et al., 1988; Estes et al., 1983). The inner capsid is composed exclusively of VP6 and encloses the core particle (Estes et al., 1983). The outer capsid contains two structural proteins VP7 and VP4 in a molar ratio of 13 to 1 respectively (Prasad et al., 1988). VP7 has

been identified as the cell attachment protein (Sabara et al., 1985; Fukuhara et al., 1988) and cell attachment occurs independent of trypsin treatment. The pathway of internalization of human rotavirus (probably animal viruses also), however, is determined by trypsin treatment. Kaljot et al. (1988) suggested that proteolytic cleavage of VP4 determines which of two entry pathways rotavirus follows. Trypsin treatment of the virus is associated with the direct plasma membrane penetration pathway in which only inner components of the virus are internalized leading to a productive infection (Kaljot et al., 1988; Fukuhara et al., 1988). Non-trypsin treated virions will attach to cells, enter by endocytosis and are subsequently destroyed in the lysosomes (Kaljot et al., 1988). Kaljot et al. (1988) suggests that infectious rotavirus employ a novel entry strategy: protease-dependent direct cell membrane penetration which has also been suggested by EM studies. He further suggests this virus-cell membrane interaction leads to virus capsid rearrangement, viral transcriptase activation, cell membrane pore formation, and the passage of viral RNA into the cytoplasm by a mechanism analogous to bacteriophage infection. The remaining RNA segments are believed to code for nonstructural proteins (Estes et al., 1983; Liu et al., 1988; Chan et al., 1986).

Many investigators have studied the number and molecular weight (MW) of structural proteins of bovine rotaviruses and have reported as many as 8-10 structural proteins (Matsuno and Mukoyama, 1979; McCrae and McCorquodale, 1982), but it is now believed the number is closer to 5-6 (Estes et al., 1983; Liu et al., 1988). These discrepancies may reflect

different methodologies used for virus growth and purification, polypeptide analysis, failure to use molecular weight standards, differing PAGE systems and different rotavirus strains (Estes et al., 1983). Although rotaviruses contain several structural proteins, three proteins are most significant and will be discussed in more detail. VP6 is the product of the sixth gene segment, approximately 42 kd in size and associated with the inner capsid as determined by agglutination of single shelled virus particles with heterologous antisera (Woode et al., 1976; Bridger, 1978). VP6 contains an antigenic region which codes for the common group antigen of group A rotavirus (Kalica et al., 1981). It was thought all rotaviruses shared a common antigen and several tests to detect rotavirus were developed using this common antigen including complement fixation (CF), immunofluorescence (IF), gel diffusion and immune electron microscopy (IEM) (Woode et al., 1976; Kapikian et al., 1981; Estes et al., 1983). As more isolates were adapted to cell culture, at least two antigenically distinct type of rotaviruses were identified (Thouless et al., 1977; Yolken et al., 1978b) by CF, IEM and enzyme linked immunosorbant assay (ELISA). Kalica et al. (1981) has shown that VP6 was also the polypeptide associated with this antigenic specificity (Yolken et al., 1978). Greenberg et al. (1983a), using monoclonal antibodies, demonstrated the existence of at least two distinct domains on VP6 with one region representing the common antigen and a second domain with at least two specificities. The second more diverse domain was termed the subgroup antigen. Kalica et al. (1981) demonstrated the antigenic determinants involved in non-neutralizing

tests were associated with VP6 and were separate from determinants involved in neutralization. Kapikian et al., (1981) suggested the term subgroup refer to antigenic determinants involved in non-neutralization tests (CF, IF, Immune adherence hemadsorption assay; IAHA) and the term serotype be reserved for neutralization antigens.

VP7 is the major outer-shell glycoprotein which contains neutralization epitopes and determines the virus serotype (Greenberg et al., 1983b; Kalica et al., 1981). VP7 is important in the development of neutralizing antibodies which can neutralize the virus in vivo and in vitro. VP7 is coded for by either gene 8 or 9 depending on the strain of virus (Both et al., 1983; Mason et al., 1980). The sequence analysis data has shown that VP7 gene is 1062 nucleotides in length. The sequence of the first 10 nucleotides at the 5' end of VP7 genes of all group A rotaviruses is conserved in rotaviruses sequenced so far (Both et al., 1983; Gorziglia et al., 1986). Studies have demonstrated that VP7 is composed of two molecular species identified as VP7-1 which is 37 kd in size and VP7-2 35 kd in size (Sato et al., 1986; Suzuki et al., 1984; Chan et al., 1986; Fukuhara et al., 1988). The origin of the two proteins is unknown at this time but both proteins can be reduced to 29 kd in the presence of tunicamycin which suggests the molecular weight difference is due to different glycosylation pathways (Sato et al., 1986; Suzuki et al., 1984). Chan et al. (1986) however suggests the two proteins originate from two in-phase codons at nucleotides 49 to 51 and 136 to 138 and differ in amino terminal sequences. The two VP7 glycoproteins are functionally distinct since cell binding activity is

associated with the 35 kd species only which suggests the importance of the carbohydrate moiety structure in cell binding (Fukuhara et al., 1988). Both molecular species may perform an essential function such as assembly of the outer capsid of mature viral particles since both proteins are found in the lysate of infected MA104 cells as well as mature viral particles (Sato et al., 1986; Suzuki et al., 1984; Chan et al., 1986).

Identification of the antigenic domains on VP7 is important since VP7 induces protective antibodies and determines serotype specificity. Monoclonal antibody resistant (Mar) variants have been used successfully in the analysis of neutralization epitopes of influenza virus (Lubeck and Gerhard, 1981), poliovirus (Minor et al., 1983) and other viruses (Wiktor and Koprowski, 1980; Yewdell and Gerhard, 1981). Monoclonal antibody resistant (Mar) variants have been used to identify two distinct domains on VP7 (Sonza et al., 1984; Dyall-Smith et al., 1986; Shaw et al., 1986). Further studies indicated that two areas of VP7 mediate serotype specific neutralization (amino acids 94 to 99 and 211). The areas are physically approximated in the intact virion and the neutralization domain is comprised of these two discontinuous epitopes (Mackow et al., 1988b; Dyall-Smith et al., 1986). The possibility that the neutralization structure of VP7 was dependent on tertiary and quaternary structure was evidenced by: (i) antibodies raised against denatured proteins failed to recognize the virus, (ii) linear synthetic polypeptides do not elicit neutralizing antibodies, (iii) most neutralizing VP7 monoclonal antibodies (Mab) will not immunoprecipitate protein present in infected

cell lysates (Bastardo et al., 1981; Gunn et al., 1985; Taniguchi et al., 1985; Coulson et al., 1985; Heath et al., 1986). All the evidence suggests that distinct regions of the "linear" protein are brought together and only assume the ultimate conformation when incorporated into the mature virion.

The nucleotide sequence of the VP7 gene of more than 35 rotavirus strains has been determined, and the deduced amino acid sequence comparison has revealed six discrete divergent regions (A-F) among different serotypes (Glass et al., 1985; Green et al., 1987). The six regions correspond to amino acids: 39-50 (region A); 87-101 (region B); 120-130 (region C); 143-152 (region D); 208-221 (region E); and 233-242 (region F). Dyall-Smith et al., (1986) identified three regions (B, D and E) as the antigenic sites involved in serotype-specific neutralization which corresponds to the two regions previously identified as the conformation neutralization epitope. Identification of specific antigenic epitopes is essential in developing vaccine strategies.

VP4 is the second outer capsid protein which was previously referred to as VP3 (Liu et al., 1988). VP4 is an 82 to 88 kd protein encoded by gene segment 4. Gene 4 is 2,359 - 2,364 base pair in length depending on the strain sequenced (Gorziglia et al., 1988; Kantharidis et al., 1988; Potter et al., 1987; Taniguchi et al., 1988). Gene 4 contains one long open reading frame of 2,325 bases which codes for a protein 775-776 amino acids in length. The 5′ and 3′ terminal non-coding regions of 9 and 22-25 nucleotides respectively are highly conserved (Kantharidis et al., 1988; Taniguchi et al., 1988; Gorziglia et al., 1988) but the 3′

noncoding region of C486 bovine isolate contains two additional bases (Potter et al., 1987). In human rotaviruses, twenty-three proline residues are conserved which may have a major influence on the conformation since proline is known to distort three-dimensional structure (Gorziglia et al., 1988). Conserved trypsin cleavage sites are located at amino acids 241 and amino acids 247 with amino acid 247 being the preferred site identified by Lopez et al., (1985). The preferred trypsin cleavage site in the UK virus is located one amino acid toward the N terminus (Kantharidis et al., 1988). A second potential trypsin cleavage site has been identified at amino acid 245 in symptomatic viruses (Gorziglia et al., 1988; Mackow et al., 1988).

In vitro exposure of virions to proteolytic enzymes cleaves VP4 into VP5 (58-60 kd) and VP8 (27-28 kd) (Estes et al., 1981; Espejo et al., 1981; Offit et al., 1983; Kalica et al., 1983) resulting in a two to nine-fold increase in infectivity in cell culture (Estes et al., 1981). Conserved regions flanking the trypsin cleavage sites corresponding to amino acids 224-236 and 257-271 have been observed in RRV, SA11 and RV5 possibly holding sites in proper conformation for cleavage (Mackow et al., 1988). Gorziglia et al. (1988) observed the region between the cleavage sites was highly conserved and suggested it as a possible molecular candidate for virulence since the extra cleavage site at amino acid 245 was present in all sequenced symptomatic viruses.

Several functions have been assigned to VP4 including: hemagglutination (Greenberg et al., 1983b), increased infectivity in cell culture (Kalica et al., 1983; Estes et al., 1981), restriction of

virulence of certain strains in mice and humans, (Offit et al., 1986c; Flores et al., 1986b) and induction of protective neutralizing antibodies (Hoshino et al., 1988; Offit et al., 1986). Antibodies directed to VP4 inhibit viral hemagglutination (Greenberg et al., 1983a) and neutralize the virus in vitro and in vivo (Greenberg et al, 1983a; Offit and Blavat, 1986; Offit et al., 1986b). As the number of functions assigned to VP4 increased it has become clear that VP4 plays a significant role in pathogenesis and recovery from rotavirus infections.

Early studies using VP4 specific Mab demonstrated virus neutralization which was inconsistent with previous findings that associated neutralization with VP7 (Taniguchi et al., 1985; Greenberg et al., 1983c). A study by Hoshino et al. (1985) demonstrated that VP4 was an important immunogen and could elicit neutralizing antibodies. Hoshino used a naturally occurring interserotypic virus M37 and reassortants made from the UK strain of rotavirus to demonstrate that serotype specificity of VP4 could be determined and differentiated from VP7. Hoshino suggested a dual classification system for rotavirus based on neutralization specificities of both VP4 and VP7 was needed. Reassortant viruses containing VP4 and VP7 from different serotypes have been used to demonstrate that both passive and active immunity can offer protection against challenge from both parental serotypes in mice and pigs (Offit et al., 1986a; Hoshino et al., 1988). These findings support the development of a reassortant rotavirus vaccine which could protect against more than one serotype.

As with VP7, Mab were used to identify neutralization domains on VP4. A study by Shaw et al. (1986) identified two domains on VP4 which mediated viral neutralization and hemagglutination. Shaw's data suggested both homotypic and heterotypic neutralizing epitopes existed on VP4 suggesting that antibodies could be induced which could neutralize more than one serotype of rotavirus. Evidence to suggest the cross-reactive neutralizing antigenic site(s) may exist among heterotypic rotaviruses include: (i) neutralization of heterotypic rotaviruses using hyperimmune antisera or convalescent-phase sera, although titers are low (Bishop et al., 1983; Estes et al., 1983; Wyatt et al., 1983); (ii) heterotypic cross protection induced by vaccination with human and animal rotavirus strains (Kapikian et al., 1986; Vesikari et al., 1984; Zissis et al., 1983); (iii) cross-reactive neutralizing Mab which react with rotaviruses of different serotypes which have been produced (Offit et al., 1986b; Taniguchi et al., 1985; Taniguchi et al., 1987).

Taniguchi et al. (1987) used neutralizing Mab's against 5 serotypes of viruses and the results suggested the existence of three operationally distinct cross-reactive neutralization epitopes on VP4. Burns et al. (1988) also identified a cross reactive neutralization site on SA11 which corresponded to a site identified by Taniguchi et al. (1987).

Taniguchi et al. (1987) using the same neutralizing Mabs also demonstrated intraserotypic variations within some human rotavirus strains and a marked difference between the reactivity of serotype 2 when compared with serotype 1, 3 and 4. This difference between serotype 2

and 1, 3 and 4 had been previously noted by RNA-RNA Hybridization (Flores et al., 1986).

The difference between serotype 2 and 1, 3 and 4 was further compared by sequence analysis of human rotaviruses which indicated serotype 2 had several unique regions which were divergent from the other serotypes (Gorziglia et al., 1988).

Taniguchi et al. (1988) developed Mar mutants and analyzed the sequences to determine the serotype-specific and heterotypic neutralization epitopes on VP3. Three distinct neutralization epitopes which corresponded to amino acid position 305 (epitope 1), and 392 and 439 (epitope 2) and 433 (epitope 3) were clustered on VP5, the larger cleavage product of VP4. Taniguchi also suggested that epitope II was conformational epitope while epitope I was a sequential determinant. Epitope II has been shown to be cross reactive to serotype 1, 3 and 4 strains while specific to serotype 2 and evidence suggests epitope I may also be site specific to serotype 2 and cross reactive to 1, 3 and 4. Identification of cross-reactive neutralization antigenic determinants will prove useful in developing vaccines against rotavirus infection.

Streckert et al. (1988) tested a synthetic peptide vaccine corresponding to the C terminal of VP8 and demonstrated production of antibodies which had strain specific neutralization activity. The region selected for the synthetic peptide did not correspond to regions identified by Taniguchi et al. (1988) indicating additional neutralization epitopes may exist on VP8.

A recent study expressed RRV gene product VP4 using a baculovirus recombinant which retained functional and antigenic conformation (Mackow et al., 1989). The ability to express large amounts of VP4 will aid in the study of functions associated with VP4 and in measuring the immune response to a single rotavirus polypeptide determining its usefulness as a synthetic rotavirus vaccine.

Gene Coding Assignments

Gene coding assignments for rotavirus genes have been determined in various ways, including: a) in vitro translation of denatured dsRNA b) phenotype and genotype correlation using reassortant viruses and c) Mab reactions to gene products. The simian rotavirus SA11 has been extensively studied by the above methods. Table 1 summarizes the coding assignments of the various gene products (Kapikian and Chanock, 1985).

Classification of Rotaviruses

Group and Subgroup Classification

Early studies established that rotavirus from birds and a range of mammals possess a common group antigen associated with the inner capsid of the virion and later assigned to VP6 (Woode et al., 1976; Flewett and Woode, 1978; Kalica et al., 1981). The common group antigen can be detected by numerous serological tests including immunoelectron microscopy (IEM), complement fixation (CF), immunofluorescence (IF), enzyme-linked immunosorbent assays (ELISA) and immune adherence hemagglutination assay (IAHA) (Woode et al., 1976; Flewett and Woode,

Table 1. Summary of polypeptides encoded by gene segments of SA-11[a]

Genome Segment	Primary Product	Modification	Modification Product(s)
1	125 kDa (VP1)[b]	None	None
2	94 kDa (VP2)	Cleaved	
3	88 kDa (VP3)		
4	88 kDa (VP4)	Cleaved	60K VPS* 28K VPS*
5	53 kDa (NS)[c]	None	None
6	41 kDa (VP6)	None	None
7	34 kDa (NS)	None	None
8	35 kDa (NS)	None	None
9	37 kDa (VP7)	Glycosylated & Trimmed	38 kDa VP7
10	20 kDa (NS)	Glycosylated & Trimmed	29 kDa NS
11	26 kDa (VP9)	Yes - Type Unknown	27 kDa VP9

[a]Kapikian and Chanock, 1985.
[b]VP = viral polypeptide.
[c]NS = nonstructural polypeptide.

1978; Estes et al., 1983; Holmes, 1983). The distinctive migration pattern of the 11 dsRNA segment of the rotavirus has also been used to classify viruses according to groups. Group A rotaviruses have an RNA electrophoretic pattern in PAGE described as four regions containing 4, 2, 3 and 2 segments respectively (Kalica et al., 1978a). Viruses that are morphologically identical but lack the common group antigen have also been isolated. Serologic studies (indirect immunofluorescence) indicate these atypical rotaviruses do not cross react with the common group A antigen and can be further divided into four separate groups based on the group specific antigen (Bridger et al., 1982; Saif et al., 1980; NcNulty et al., 1981a; Pedley et al., 1983; Pedley et al., 1986). These atypical viruses contain 11 dsRNA segments but demonstrate RNA electrophoretic patterns which are distinct from Group A rotaviruses (Snodgrass et al., 1984a; Pedley et al., 1983; Pedley et al., 1986). Group B rotaviruses have an electrophoretic pattern in PAGE divided into four regions containing 4, 2, 2 and 3 segments respectively. The group C rotavirus electrophoretic pattern has 4 regions containing 4, 3, 2 and 2 segments. The electrophoretic pattern of group D rotaviruses has 4 regions containing 5, 2, 2 and 2 segments and group E rotavirus electrophoretic pattern has 4 regions containing 4, 2, 2 and 3 segments (Pedley et al., 1983; Pedley et al., 1986). The use of electrophoretic patterns in PAGE to group rotaviruses is rapid, but confirmation by serological tests is necessary.

Group A rotaviruses have been isolated from most mammals including humans, calves, piglets, foals and the avian species (Mebus et al., 1969;

Bishop et al., 1973; Leece et al., 1976; Flewett et al., 1975; McNulty et al., 1976). Group B rotaviruses have been isolated from pigs in the United States and England (Bridger, 1980; Theil et al., 1985) and humans in China (Hung et al., 1984). Group C rotavirus previously referred to as pararotavirus have been isolated from pigs in the United States (Bohl et al., 1982). Group D rotaviruses have been isolated from chickens in England, and turkeys and pheasants in the United States (Pedley et al., 1986). Group E rotaviruses have been isolated from pigs in England (Chasey et al., 1985).

Group A rotaviruses can be further classified into two subgroups based on an additional antigenic specificity encoded in gene 6. Subgrouping of rotaviruses can be determined by ELISA and IAHA. The majority of animal rotaviruses possess the subgroup I specificity while the majority of human rotaviruses possess the subgroup II antigen (Hoshino et al., 1984) although several exceptions do exist.

Serotype Classification of Rotaviruses

Serotypes of rotaviruses can be discriminated by virus neutralization tests including plaque-reduction assays (PRN) (Hoshino et al., 1984) and fluorescent focus neutralization (FFN) (Woode et al., 1983). The serotype-specific antigen involved in virus neutralization is mainly defined by VP7 which is encoded by RNA segment 8 or 9 (Kalica et al., 1981; Greenberg et al., 1983b). Although antibodies directed to VP4 can neutralize the virus and result in ambiguous data, their role in determining serotype has not been determined (Hoshino et al., 1987). At least seven serotypes of group A rotaviruses have been reported in

accordance with the unified scheme of serotyping rotaviruses from animals and man (Hoshino et al., 1984). A summary of current serotype classification of a limited number of rotavirus is presented in Table 2.

Table 2. Rotavirus serotypes determined by neutralization[a]

Serotype	Animal rotavirus prototype strain	Human rotavirus prototype strain
1	none	Wa, K8, KU, D, DB
2	none	DS-1, S2, KUN, 390
3	S-SA11, S-RRV, C-Cu-1 F-(Taka), E-H-2	P, M, Walk 57/14, Mo
4	P-Sb-2, P-Gottfried, P-SB-1A	St. Thomas No. 3 and No. 4
5	P-OSU, E-H-1, P-SB-1A	None
6	B-NCDV, B-UK	None
7	C-ch.2 T-Ty.1	None
8	none	69M
9	none	WI61

[a]Kapikian and Chanock, 1985.

B = bovine
S = simian
C = canine
F = feline
E = equine
P = porcine
C = chicken
T = turkey

At least two additional serotypes have been proposed with 69M (serotype 8) (Matsuno et al., 1985) and WI61 (serotype 9) (Clark et al., 1987) serving as the prototype viruses. While traditional methods of serotyping have used PRN or FFN assays, several groups have investigated

the use of VP7 serotype-specific Mab for use in serotyping ELISA (Shaw et al., 1985; Taniguchi et al., 1987; Coulson et al., 1987). ELISA is a sensitive and efficient method to serotype rotaviruses, however, problems seen with ELISA serotyping have included the inability to serotype all isolates and problems selecting a Mab which detects all isolates of the same serotype (Coulson et al., 1987; Taniguchi et al., 1987). Nucleic acid hybridization has also been utilized in the differentiation of rotavirus serotypes (Dimitrov et al., 1985; Lin et al., 1987; Johnson et al., 1989; Flores et al., 1989). Dot blot hybridization studies have distinguished serotypes 1, 2, 3, 4 and 5 and have also serotyped rotavirus isolate Mabs have not recognized (Flores et al., 1989; Johnson et al., 1989). Modification of the dot blot test to include use of non-radioactive probes may allow practical application of dot blot hybridization to serotyping.

Comparison of the nucleotide sequence of rotavirus VP7 (gene 8 or 9) has also been used to determine serotype. Glass et al. (1985) identified six serotype-specific regions designated A-F which were conserved within serotypes while differing among serotypes. Green et al. (1987) observed an overall 15-29% divergence in the VP7 proteins of the four human serotypes and a 91-99% homology within a serotype in the six serotype specific regions. Green et al. (1989) examined 35 rotavirus strains using sequence analysis and reported the predicted serotype correlated with the serotype determined by PRN or Mab based ELISA. Although routine serotyping by sequence analysis may not be practical, it has shown to be accurate and offers an additional method to determine serotype.

Detection of Rotavirus

Many assays have been developed to detect rotavirus. Visualization of rotaviruses with electron microscopy was the first method used to detect rotavirus and still remains useful, however, additional methods which are easier and require less time and equipment have been developed. Electron microscopy (EM) has the advantage of being highly specific and detection rates run as high as 90% in virus-positive specimens (Brandt et al., 1981).

Since cell culture replication of animal rotaviruses is sometimes difficult, a fluorescent antibody (FA) test using frozen sections of intestine was developed. The FA technique is simple, rapid and accurate, but due to rapid shedding of rotavirus infected epithelial cells from the tips of the villi into the intestinal lumen, detection of rotavirus antigen 24 to 72 after the onset of diarrhea can be difficult (Benfield et al., 1984; Rhodes et al., 1979). In an effort to overcome disadvantages of the EM and FA techniques, several immunoassays including: complement fixation (CF), immunoelectroosmophoresis, IAHA, immunodiffusion (ID) and ELISA have been developed for the detection of rotaviruses (Estes et al., 1983). The ELISA is the most sensitive and widely used method used to detect rotavirus in clinical samples of human, pig, and cattle feces. Rotazyme (Abbott Laboratories, North Chicago, IL), a commercially available ELISA, uses antibody directed to the common group A antigen to detect rotavirus antigen. Disadvantages of ELISA include false positives and lack of detection of other rotavirus groups.

Cultivation of rotavirus in cell culture has been used to detect and amplify rotavirus. The addition of trypsin in the media and the use of roller tubes has increased the number of rotaviruses isolated (Sato et al., 1981; Urasawa et al., 1981). One disadvantage to direct cell culture is many rotavirus isolates including group B and C cannot be grown in cell culture and will go undetected. Direct detection of rotaviral nucleic acid in silver stained polyacrylamide gels (PAGE) has also been developed. This method has several advantages including simplicity, economy, speed and tentative identity of electropherotype (Herring et al., 1982). Herring reported that PAGE, EM and ELISA were equal in sensitivity and the detection limit of the silver stain for a single band was 300 to 400 pg of RNA. A distinct advantage to PAGE is the ability to detect additional groups of rotaviruses and the unambiguous nature of positive results (Herring et al., 1982).

A recently developed dot hybridization assay is based on the in situ hybridization of labeled rotavirus ssRNA transcripts to heat denatured rotavirus RNA immobilized on nitrocellulose or nylon membranes (Flores et al., 1983; Pedley and McCrae, 1984; Lin et al., 1987; Flores et al., 1989). The test is highly specific and 10 to 100 fold more sensitive than ELISA, allowing the detection of as little as 8pg purified RNA in homologous reactions (Kapikian and Chanock, 1985). A major disadvantage to dot hybridization is the use of radiolabeled probes which are time consuming and pose a potential hazard.

Many techniques for measuring serological response to rotavirus infection including IEM, immunofluorescence (IF), virus neutralization

and CF (Kapikian and Chanock, 1985) have been developed. Serological diagnosis can be made when acute and convalescent sera are available using the afore mentioned techniques.

Detection of rotavirus and/or demonstration of a serologic response to rotavirus does not necessarily indicate rotavirus as the cause of illness since infants and adults commonly undergo subclinical infection.

Bovine Rotavirus

Rotavirus Infections in Cattle

Neonatal calf diarrhea, or calf scours, in calves less than 30 days of age is considered the most important disease in both dairy and beef herds in the United States, the United Kingdom and New Zealand (Woode et al., 1975; Woode and Bridger, 1975; Acres and Babiuk, 1978; Moon et al., 1978; Woode, 1978). Neonatal calf diarrhea cannot be attributed to a single disease but rather a group of organisms that share many clinical characteristics. Organisms involved in neonatal calf diarrhea include: Enterotoxigenic Escherichia coli (ETEC), Campylobactor spp., Salmonella spp., rotavirus, coronavirus, astrovirus, calcivirus, parvovirus and cryptosporidium (Tzipori, 1981; Woode and Bridger, 1975; Moon et al., 1978).

Since the isolation of the first bovine rotavirus (NCDV) was reported in the United States, its role in neonatal calf diarrhea has been extensively explored (Mebus et al., 1969). Rotaviruses have been isolated from calves in the United Kingdom (Woode et al., 1974), Ireland

(McNulty et al., 1976), Australia (Turner et al., 1973), Canada (Babiuk et al., 1977), Germany (Brussow et al., 1987), Argentina (Bellizoni et al., 1987) and Japan (Murakami et al., 1983). Rotaviruses are widely distributed as evidenced by serological studies which indicate that ninety to one hundred percent of cattle, humans and pigs possess serum antibodies to rotavirus (Woode, 1978). A survey conducted to determine the cause of viral diarrhea in calves revealed that rotavirus was second only to coronavirus (Mebus, 1980). A survey in the United Kingdom and Northern Ireland indicated rotavirus was isolated from eighty percent of diarrhea outbreaks from both dairy and beef herds. Rotavirus has been demonstrated in 69 herds in Nebraska, and from herds from South Dakota, Illinois, California, Wisconsin, Arizona, Colorado, Kansas, New Mexico and Ohio (Mebus et al., 1971). Economic losses to the cattle industry ranging from three to eight million dollars per year resulted from lower feed efficiency and retarded weight gain from rotavirus infected calves (House, 1978; Woode and Crouch, 1978).

Rotavirus Strains Associated with Cattle

Bovine rotavirus was first isolated in the United States and referred to as reovirus-like virus or Nebraska (Neonatal) calf diarrhea virus (NCDV) (Mebus et al., 1969). In 1974, a morphologically similar virus was isolated in England which was later shown to be antigenically related (Woode et al., 1974; Ojeh et al., 1984; Wyatt et al., 1980). Several additional bovine rotaviruses have been isolated and some representative isolates are listed in Table 3. Group A rotaviruses are the most prevalent group of rotaviruses isolated from diarrheic calves

Table 3. Bovine rotavirus isolates[a]

Virus	Bovine Serotype	Reference
NCDV	1	Mebus et al., 1969
UK (England)	1	Woode et al., 1974
B641	1	Woode et al., 1983
B223	2	Woode et al., 1983
B2352 (Canada)	3 (?)	Spence et al., 1978
B14		Gaul et al., 1982
C486 (Canada)	1	Babiuk et al., 1977
KK3 (Japan)	2	Murakami et al., 1983
KN4 (Japan)	3	Murakami et al., 1983
639 (UK)	1	Snodgrass et al., 1984b
678 (UK)	2	Snodgrass et al., 1984b
V1005 (Germany)	2	Brussow et al., 1987
ESV (Group B)	---	Vonderfecht et al., 1986
D522 (Group B) (UK)		Snodgrass et al., 1984a
D531 (Group B) (UK)		Snodgrass et al., 1984a
ID		Theil and McCloskey, 1988
VMRI	1	Paul et al., 1988

[a]Unless noted otherwise, viruses were isolated in the United States.

(Theil and McCloskey, 1988; Bellinzoni et al., 1987; Snodgrass et al., 1984b). This may be due to methods used to detect rotavirus which usually consists of an ELISA using an antibody to the group A antigen and would not detect additional groups. Recently several groups have reported on the isolation of atypical rotaviruses in cattle (Snodgrass et al., 1984a, Vonderfecht et al., 1986; Chasey and Davies, 1984). Prevalence of other rotavirus groups in cattle has not been determined since rapid detection methods such as ELISA do not exist and atypical rotaviruses can not be isolated in cell culture.

Since group A rotavirus is the most frequently detected bovine rotavirus, it is the most studied. Serologic analysis of bovine isolates has determined that at least two and possibly three serotypes of rotavirus exist in cattle (Woode et al., 1983; Murakami et al., 1983; Snodgrass et al., 1984b; Brussow et al., 1987). The relationship between isolates from the U.S. and other countries has not been fully determined due to import restrictions, however, the most common isolate within each country was serotypically related to NCDV (Snodgrass et al., 1984b; Brussow et al., 1987; Murakami et al., 1983). Serologic analysis of bovine rotavirus field isolates has indicated most are bovine rotavirus serotype 1 based on the 20-fold difference in neutralization titers (Woode et al., 1983; Snodgrass et al., 1984b). Even though most isolates are serotype 1, some degree of antigenic diversity exists between the field isolates and the established bovine serotype 1 rotavirus (Gaul et al., 1982; Woode et al., 1983; Paul et al., 1988). Antigenic differences also exist between the two prototype bovine serotype 1 rotaviruses NCDV

and UK. In a study using reassortant viruses containing UK gene 4, it was demonstrated that no cross reaction occurred between NCDV and UK (Hoshino et al., 1985), and has since been determined that gene 4 of UK and NCDV is distinct (Kantharidis et al., 1988). Reassortant viruses containing either NCDV gene 4 or gene 9 were used to vaccinate mice which were then challenged with UK bovine rotavirus. Mice vaccinated with reassortant virus containing NCDV gene 4 were not protected against UK challenge while mice vaccinated with the reassortant virus containing NCDV gene 9 were protected. Cross protection studies conducted in calves and mice using NCDV and UK virus, demonstrate cross-protection (Woode et al., 1978; Offit et al., 1986a). Further antigenic diversity between serotype 1 bovine rotavirus has been noted between NCDV and B641 (Woode et al., 1983; Woode et al., 1987). Calves vaccinated with NCDV were not protected against B641 challenge even though convalescent antiserum titers to both viruses are similar. Woode could not explain this phenomenon and suggested B641 was a minor serotype variant of NCDV. Results from these studies indicate that in vitro neutralization does not necessarily correlate with in vivo cross protection studies and suggest in vivo testing is a more sensitive method to determine antigenic relatedness.

In addition to group A rotaviruses and atypical rotaviruses, group A rotaviruses with novel RNA electrophoretic patterns have also been isolated from calves (Theil and McCloskey, 1989; Allen et al., 1989). The significance of these novel patterns has not yet been determined but it does indicate that substantial genomic diversity exists within bovine

group A rotaviruses. In addition to these group A rotaviruses possessing novel RNA patterns, two reports have indicated the existence of group A bovine rotaviruses with a short (Theil and McCloskey, 1988) and a "super-short" (Paul et al., 1988) electrophoretic patterns. The isolates have been shown to be serotype 1 bovine rotaviruses. In these viruses, gene segments 10 and 11 have slower electrophoretic mobilities. The significance of the altered electrophoretic pattern has not been fully determined.

Clinical Features of Rotavirus Infection

Rotaviruses have been shown to produce diarrhea in gnotobiotic calves (GC), colostrum-deprived caesarean-derived (CDCD) calves and conventionally reared calves (Mebus et al., 1971; Tzipori et al., 1981; Woode, 1978; Woode and Bridger, 1975). Infection occurs suddenly and explosively spreads within a herd of susceptible animals (Woode, 1978; Woode and Crouch, 1978). The incubation period can range from 18 to 96 hours which is followed by a short period of depression followed by diarrhea. In infected calves, feces can range from watery brown, gray, light yellow or light green which may contain fresh blood and mucus (Tzipori et al., 1981; Torres-Medina, 1984; Woode and Crouch, 1978). Usually, the disease lasts from one to eight days but sometimes longer in young calves (Tzipori et al., 1981). Prolonged diarrhea results in weight loss (body weight loss of 10 to 25%) and dehydration. The individual may die 4 to 7 days after commencement of diarrhea if the dehydration is severe (Woode, 1978; Woode and Crouch, 1978). The susceptibility of GN, CDCD and conventional calves to rotaviruses has

been shown to be age dependent with calves under 7 days most susceptible (Tzipori et al., 1981) although rotavirus can occur in all ages including adult cattle. Rotavirus has been shown to be associated with enteritis and death in calves from 0 to 9 weeks of age. Clinical signs in adult animals can be severe and result in temporary physiologic disturbances, such as, a marked decline in milk production of cows but rarely does mortality occur (Woode and Bridger, 1975; Woode and Crouch, 1978). Age dependent susceptibility may be related to different rotavirus strains since it has been demonstrated that the UK isolate can cause clinically severe disease in GN calves up to 35 days of age while Tzipori et al. (1981) could only induce diarrhea in calves up to 7 days of age with four Australian isolates (Woode et al., 1978).

During the acute phase of the disease, it has been shown that calves can excrete approximately 10^6 - 10^8 infectious doses of rotavirus per gram of feces for five to nine days (Woode et al., 1976; Woode and Crouch, 1978). Natural transmission of rotavirus has been associated with direct or indirect contact (human handlers, contaminated utensils) when calves are in intensive housing systems (dairy herds) (Woode, 1978). However, explosive outbreaks of rotavirus also occur in grazing beef herds. Woode (1978) suggested adults are the major source of infection since outbreaks of disease in calves are frequently associated with mild diarrhea in one or more cows. Subclinical infections within a herd may exist and may be as frequent as clinical infections. Subclinical infections may result from infection with a pathogenic strain in the presence of passively derived immunity or infection with a strain which

is not pathogenic to the infected animal (Woode, 1978; Woode and Crouch, 1978). Long survival rate of the virus in the environment may also play a role in herds with recurrent infections (Woode et al., 1975).

Morbidity in previously unexposed herds can run as high as 80 to 90%, however, mortality is generally low (Acres and Babiuk, 1978; Woode, 1978) unless complicated with bacterial infections. In nearly all outbreaks of rotavirus-induced diarrhea, one or more additional agents including ETEC, Salmonella spp., coronavirus and coccidia can be isolated although the interaction between these agents has not been fully determined (Woode and Bridger, 1975; Acres and Babiuk, 1978). ETEC is the most common cause of diarrhea in calves under three days of age. The ETEC incubation period is short, 12 to 18 hours, calves become depressed and anorexic. Dehydration and death may result from profuse watery diarrhea. Two important virulence factors which distinguish ETEC from non-pathogenic Escherichia coli strains are adhesion to gut mucosal cells and production of a stable toxin (ST) (Tzipori et al., 1981). Several studies have examined the pathogenicity of combined rotavirus and ETEC infection. Early studies indicated that a synergistic effect between ETEC and rotavirus caused a sublethal dose of ETEC to become lethal (Gouet et al., 1978). Subsequent studies have indicated an additive effect rather than synergistic. Tzipori et al. (1981) infected GN calves with rotavirus and ETEC at different intervals and found they were able to induce diarrhea in calves independent of age. Diarrhea appeared approximately 3 days after inoculation of rotavirus, independent of which organism had been given first. Diarrhea coincided with the excretion of

rotavirus in the feces rather than ETEC. A study by Snodgrass et al. (1982) using naturally derived calves and Torres-Medina (1984) using GN calves also supported a synergistic rather than additive effect.

Pathological Findings

By the time rotaviral diarrhea appears, a considerable chain of events has already occurred in the mucosa of the small intestine. Diarrhea itself is largely a manifestation of the immaturity of the crypt enterocytes, which have migrated to replace infected cells on the villi. Several investigators have examined the effect of rotaviruses on the intestinal mucosa (Mebus et al., 1971; Middleton, 1978; Snodgrass et al., 1982; Torres-Medina, 1984; Pearson et al., 1978; Woode et al., 1974, Woode and Crouch, 1978). Histological examination has shown rotavirus infection is limited to the small intestine with most infection occurring in the ileum (Torres-Medina, 1984; Snodgrass et al., 1982; Middleton, 1978; Pearson et al., 1978) although others have reported infection in the anterior jejunum and duodenum. Infection is limited to the epithelial cells of the absorptive portion of the villus and not crypt cells (Woode and Crouch, 1978). Principal lesions seen are villous atrophy indicated by club shaped stunted villi and increased numbers of round cells in the lamina propria (Torres-Medina, 1984; Middleton, 1978). The lamina propria is infiltrated with mononuclear cells and polymorphonuclear leukocytes. Crypts are lengthened and many contained mitotic figures (Snodgrass et al., 1982). Following oral inoculation, microscopic examination for rotavirus indicates columnar epithelial cells over the distal two thirds of the villi of the upper small intestine

become infected. Mebus et al. (1971) described a wave of infection progressing posteriorly, however, not all studies are in agreement with this observation. As the infection proceeds, infected epithelial cells move toward the tips of the villi and are lost into the lumen. Migration of the new epithelial cells from the base of the villi is not rapid enough to keep the tips of all villi covered (Mebus et al., 1971). The lost cells are replaced with squamous or cuboidal cells which lack a brush border (Middleton, 1978; Crouch, 1985). Enzyme profiles of the immature cells at the tips of villi shows a profile similar to that of crypt cells which are rich in thymidine kinase rather than sucrose, supporting the idea that rotavirus infection accelerates the migration of secretory crypt cells to villi which are normally lined by mature adsorptive cells. The loss of the adsorptive cells of the small intestine and replacement with immature cells is assumed to be responsible for the observed malabsorption syndrome (Woode and Crouch, 1978; Crouch, 1985). Malabsorption is further complicated by the decreased ability of the intestine to utilize dietary lactose which results in the accumulation in the large intestine. The lactose prevents normal absorption of water by exerting an osmotic effect (Crouch, 1985; Woode and Crouch, 1978). Others have suggested rotavirus induced diarrhea results from a disordered sodium transport system with a net extracellular fluid-to-lumen flux of sodium ions (Middleton, 1978). If pathogenic E. coli are present, the altered epithelium and intestinal function permits an overgrowth of the bacteria which may explain some of the synergistic effect of the two organisms.

Diagnosis and Treatment

The specific cause of neonatal calf diarrhea is often difficult to determine because: (i) several enteropathogens cause similar clinical syndromes, (ii) most outbreaks result from a combination of enteropathogens, (iii) the illness has progressed to a stage where the probability of isolating the etiologic agent(s) has decreased, (iv) sensitive, inexpensive and easy to use diagnostic techniques are not yet available for all enteropathogens, (v) enteropathogens can be isolated from healthy calves (Radostits and Acres, 1980; Acres et al., 1975; House, 1978; Moon et al., 1978). A definitive diagnosis of rotavirus can be achieved by: (i) demonstrating virions in feces by EM, ELISA, direct isolation of the virus in cell culture or demonstration of viral nucleic acid by PAGE (Tzipori, 1981; Mebus et al., 1969; Herring et al., 1982); (ii) demonstration of typical lesions in the small intestine using histochemical stains or immunofluorescence (Tzipori, 1981; Woode and Bridger, 1975; Morin et al., 1976).

The treatment of acute neonatal diarrhea includes: (i) isolating sick calves from the rest of the herd, (ii) changing the diet, (iii) replacing fluids and electrolytes, (iv) administering antimicrobial drugs and immunoglobulin, and (v) possible administration of antiparasympathomimetics and intestinal protectants (Radostits and Acres, 1980). In rotavirus-induced diarrhea, treatment of diarrheic calves include isolation of affected calves to prevent the spread of disease, changing from a milk diet to ionic solutions, water replacement, and

treatment with bicarbonate (Woode et al., 1975; Radostits and Acres, 1980).

Role of Passive Immunity in Rotaviral Infections

Since rotavirus infection usually strike within the first few weeks of life, passively acquired antibodies are the major source of protection against rotavirus-induced diarrhea. Calves receive antibodies exclusively through colostrum since antibodies are not transferred across the placenta in the bovine (Crouch, 1985). In bovine colostrum IgG1 accounts for 75 to 85% of total Ig's with IgG2, IgM and IgA each accounting for 3 to 7% of the total. IgG1, the primary Ig present in bovine mammary secretions, is selectively transported unchanged from blood into lacteal secretions by glandular epithelial cells. Transfer of serum IgG1 is higher during colostrum formation and decreases during lactation. It has been demonstrated that the prevalence of antibodies associated with a given Ig isotope in mammary secretions closely resembles the relative concentration of that isotype in the serum sample. In serum, rotavirus antibodies are associated equally with IgG1 and IgG2, however, due to selective transport, IgG1 rotavirus antibodies predominate in colostrum and milk (Radostits and Acres, 1980; Saif et al., 1984). Colostrum supplies the calf with antibodies which are eventually absorbed from the lumen of the intestine and extruded into the lymph although colostrum antibodies also function locally in the lumen to prevent enteric disease. Maximum absorption of colostrum into the lymph occurs during the first 2 to 6 hours after birth which emphasizes the importance of early feeding of colostrum (Radostits and Acres, 1980).

Ingestion of colostrum containing rotavirus antibodies during the first 12 to 24 hours after birth results in local immunity at the epithelial surface of the intestine. Humoral antibodies also appear as a result of colostral intake but have little effect on the virus (Waltner-Toews et al., 1985; Crouch, 1985; Woode, 1978). Several investigators have determined that colostral antibody in the gut lumen at the time of virus challenge is necessary for protection since circulating antibody affords no protection (Mebus et al., 1972; Woode et al., 1975; Snodgrass and Wells, 1978).

Early observations suggested ingestion of antibodies to rotavirus by neonates could prevent infection and diarrhea (Bridger and Woode 1976; Leece et al., 1976; Snodgrass and Wells, 1978; Fahey et al., 1981). Other researchers have shown anti-rotavirus antibodies delay the onset but do not prevent the disease (Snodgrass et al., 1980). A possible explanation is although antibody titers to rotaviruses are generally high in colostrum, titers decline within five days after calving leaving calves susceptible to rotavirus infection (Woode et al., 1975; Woode and Bridger, 1975; Acres and Babiuk, 1978).

Control of Rotavirus Infection

Vaccination appears to be the method most likely to control rotavirus-induced enteritis in cattle, however, current vaccines do not offer complete protection. Two vaccination protocols have been explored in attempts to decrease rotavirus-induced diarrhea. The first vaccine introduced in 1973 was a modified live bovine rotavirus-coronavirus vaccine (Scourvax-Reo, Norden Laboratories, Lincoln NE), given orally to

the calf as soon as possible after birth (Anon. 1985). A second modified live rotavirus-coronavirus vaccine was introduced in 1979 (CalfGuard) which was approved for IM inoculation of pregnant cows. In 1987, Norden introduced ScourGuard 3(K) which contained inactivated strains of bovine rotavirus and coronavirus and was adjuvanted. Field trial results using the modified live rotavirus vaccine given orally to calves indicated lower incidence of diarrhea in vaccinated calves (Mebus et al., 1972), however, subsequent studies have reported no difference between vaccinates and controls (Thurber et al., 1977; Acres and Radostits, 1976; DeLeeuw et al., 1980). Studies have also indicated poor performance with the modified live vaccine given to pregnant cows (Waltner-Toews et al., 1985). Examination of serum, colostrum and milk indicated no increase in rotavirus antibodies in vaccinated animals (Saif et al., 1984; Myers and Snodgrass, 1982). In a study by Saif et al. (1984), use of an adjuvanted rotavirus vaccine given intramuscularly and intramammarily increased titers in sera, colostrum and milk significantly. Norden recently introduced an adjuvanted rotavirus vaccine (1989) which may increase protection.

The currently available vaccine from Norden contains the bovine serotype 1 isolate NCDV. Since it has been demonstrated that at least one additional serotype exists in the U.S. and cross protection between all bovine serotype 1 isolates is not complete (Woode et al., 1983; Woode et al., 1987) the selection of additional isolates for use as vaccine candidates should be investigated.

ANTIGENIC RELATEDNESS AND GENE HOMOLOGY AMONG NCDV, B641, VMRI, AND B223 STRAINS OF BOVINE ROTAVIRUS

Summary

The antigenic relatedness of four bovine rotavirus isolates was examined at the antigenic and molecular level using virus neutralization, radioimmunoprecipitation and RNA-RNA hybridization. Neutralization tests demonstrated that NCDV, VMRI and B641 were serotypically related with some antigenic heterogenicity indicated by homologous-heterologous titer ratios. Two way neutralization tests using reassortant viruses containing either gene 4 or gene 9 which code for separate neutralization specificities indicated antigenic differences between the viruses corresponded to VP4. Analysis of radiolabeled viral proteins indicated that both molecular weight and antigenic differences existed between VP4 of the three serotype 1 bovine rotaviruses. RNA-RNA hybridization using stringency conditions allowing a base pair mismatch of 21% identified three VP4 gene types among bovine rotavirus used in the study. These findings are significant in developing strategies for rotavirus-induced gastroenteritis in cattle as VP4 has been shown to be an important immunogen.

Introduction

Rotaviruses have been isolated from humans and most animals (reviewed by Estes et al., 1983) and have been established as an important cause of enteric disease in cattle (Mebus et al., 1969; Woode, 1978; Woode and Bridger, 1975: Acres and Babiuk, 1978). In the United States, at least two serotypes of bovine rotaviruses have been isolated and described with NCDV serving as the prototype bovine rotavirus serotype 1 (Mebus et al., 1969) and B223 as the prototype bovine

rotavirus serotype 2 (Woode et al., 1983). In addition to these two viruses, several other bovine rotaviruses, including C486 (Babiuk et al., 1977), B14 (Gaul et al., 1982), B641 (Woode et al., 1983), ID (Theil and McCloskey, 1988) and VMRI (Paul et al., 1988), have been isolated and the majority classified as either bovine rotavirus serotype 1 or 2 (Gaul et al., 1982; Woode et al., 1983; Theil and McCloskey, 1988; Paul et al., 1988). Reports from England (Ojeh et al., 1984), Germany (Brussow et al., 1987), and Japan (Murakami et al., 1983) suggest the existence of at least two serotypes within each country although the relationship of these viruses to the U.S. isolates has not been determined. A majority of the isolates have been characterized as either bovine rotavirus serotype 1 or 2 based on less than a 20-fold difference in virus neutralization titers, but differences have been observed in both in vitro neutralization assays and in vivo cross protection studies (Woode et al., 1983, Woode et al., 1987; Ojeh et al., 1984; Hoshino et al., 1985; Paul et al., 1988) suggesting antigenic diversity exists among isolates belonging to the same serotype. NCDV and the UK bovine rotavirus isolates are both classified as serotype 1 (Ojeh et al., 1984; Wyatt et al., 1980), but it has been demonstrated that both gene 4 (Kantharidis et al., 1988) and gene 4 product VP4 (formerly designated VP3, Liu et al., 1988), are different in each virus (Hoshino et al,. 1985). VP4 is an outer capsid protein responsible for hemagglutination (Greenberg et al., 1983; Kalica et al., 1983), growth restriction in cell culture and has been recently shown to play a role in generating neutralizing antibodies (Offit and Blavat, 1986). VP7, encoded by gene

segment 8 or 9 depending on the virus strain, is the second outer capsid protein involved in neutralization, and is the major protein responsible for serotype determination (Kalica et al., 1981; Offit et al., 1986). Recently, isolation and partial characterization of a VMRI strain of bovine rotavirus was reported and characterized as a bovine rotavirus serotype 1, but differences between neutralization titers with homologous and heterologous bovine serotype 1 rotavirus isolates suggested some antigenic diversity (Paul et al., 1988). In this study, we examined whether the antigenic diversity noted earlier between NCDV, B641, and VMRI isolates (Paul et al., 1988) and lack of cross protection observed between NCDV and B641 (Woode et al., 1983) were due to diversity in VP4 or VP7.

Materials and Methods

Viruses

The rotaviruses used in this study were bovine isolates NCDV (Mebus et al., 1969), kindly supplied by Harry B. Greenberg, Veterans Administration Medical Center, Palo Alto, CA; B641 and B223 (Woode et al., 1983) kindly supplied by Gerald N. Woode, Texas A & M University, College Station, TX; and VMRI, isolated from a diarrheic calf from Missouri (Paul et al., 1988). Reassortant viruses N4 and N9 (Offit and Blavat, 1986) were kindly supplied by Paul Offit, Children's Hospital of Philadelphia, Philadelphia, PA. An established rhesus monkey kidney cell line, MA104, was used to grow all viruses. Cells were cultivated in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Grand Island, NY)

containing 10% serum supplement (Serum Plus, Hazelton Biologics, Lenexa, KS). The viruses were pretreated with 10 ug of trypsin (type IX, Sigma Chemical Co., St. Louis, MO) per ml for 30 minutes at 37 C. Virus was adsorbed onto cell monolayers for 90 minutes and the monolayers refed with DMEM.

Virus Purification

Cells infected with rotavirus were frozen and thawed twice, extracted with one-fourth volume of trichlorotrifluoroethane (Genetron, E. I. duPont de Nemours and Co., Wilmington, DE) and separated into two phases by centrifugation. The aqueous phase was collected and pelleted through a cushion of 30% sucrose in 50 mM Tris HCl (pH 8.0) at 72,000 X g for 4 hours. The pellets were resuspended in 50 mM Tris HCl (pH 8.0) and either frozen or further purified by isopycnic gradient centrifugation in a cesium chloride gradient. Gradients were prepared with cesium chloride solutions with densities of 1.2 g/ml and 1.4 g/ml containing 5% glycerol using a linear gradient maker (Hoefer Scientific Instruments, San Francisco, CA). Gradients were centrifuged at 112,000 X g for 2 hours (McCrae, 1985). Visible viral bands containing double shelled capsids, single shelled capsids and empty capsids were collected and diluted in 50 mM Tris HCl (pH 8.0) and pelleted at 112,000 X g for 1 hour. The pellet was resuspended in 50 mM Tris HCl (pH 8.0) and stored at -70 C.

RNA Extraction and Electrophoresis

Genomic dsRNA was obtained from infected cell culture or purified virus by phenol chloroform extraction as described previously (Paul et al., 1988). Electrophoresis of RNA segments was performed in 0.75 mm-

thick polyacrylamide slab gels with 10% resolving and 3.5% stacking gels (Laemmli, 1970). Electrophoresis was done at a constant current of 10 mA for 15 hrs at 4 C. Gels were stained with silver (Herring et al., 1982) or ethidium bromide.

Antisera

Antisera were prepared as previously described (Paul et al., 1988). Either partially purified virions or cesium chloride purified double capsid virions mixed with equal volumes of Freunds complete adjuvant (first injection) or Freunds incomplete adjuvant (second injection) were used to immunize guinea pigs. Injections were given intramuscularly at four week intervals and then guinea pigs were bled and sera titer tested using virus neutralization tests. Additional immunizations with virus alone were given until high titers (>20,000), were induced, then the animals were bled and sera was collected.

Virus Neutralization

Neutralization assays were performed by fluorescent focus neutralization test (FFN) as previously described (Woode et al., 1987). Approximately 200 fluorescent-focus forming units (ffu) of the virus were mixed with equal volumes of diluted antisera and incubated for 1 hour at 37 C. The virus-serum mixture was inoculated into each of 4 wells (0.2 ml/well) of an MA104 cell monolayer in a 96 well plate. Ninety minutes post infection, the inoculum was replaced with DMEM. At 20 to 24 hours post infection, cells were fixed with methanol and stained for rotavirus antigen by an indirect immunofluorescence test. The neutralization titer

was the reciprocal of the highest dilution of serum that neutralized 50% or more of the virus.

Radiolabeling of Viral Proteins

MA104 cells were infected with rotavirus as described earlier. Seven and one half hours post infection, cell monolayers were rinsed and refed with methionine-free (MF) MEM containing 2.5 ug/ml actinomycin D (Sigma Chemical Co., St. Louis, MO). Thirty minutes later, the monolayers were refed with MF-MEM containing 100 uci/ml ^{35}S methionine (Amersham, Arlington Heights, IL) and actinomycin D. Four hours after the addition of the label, the cells were harvested and pelleted. The pellet was resuspended in lysis buffer, (500mM NaCl, 50mM Tris Base, 5mM EDTA, and 1% Triton X-100) lysed at 4 C, and cell debris removed by pelleting in a microfuge. Cell lysates were stored at -70 C. Molecular weights were calibrated using a mixture of ^{14}C-methylated polypeptides (Amersham).

Radioimmunoprecipitation

Hyperimmune guinea pig sera was mixed with MA104 cell lysates for 2 hours and adsorbed onto Protein A sepharose beads for 2 hours (Pharmacia LKB, Uppsala, Sweden). The beads were pelleted and resuspended in the presence of the radiolabeled viral antigen and mixed overnight at 4 C. The beads were washed several times, resuspended in sample buffer (0.125 Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.5% bromphenol blue) boiled for 5 minutes, and analyzed by SDS-PAGE (Laemmli, 1970). The gels were fluorographed, (ENHANCE, NEN Research Products,

Boston, MA) dried and exposed to X-OMAT film (Eastman Kodak, Rochester, NY) for 24 hours.

In vitro Transcription

Rotavirus virions were partially purified as described earlier except pellets were resuspended in 50 mM Tris HCl pH 8.0 and 5 mM EDTA. The ssRNA transcripts were prepared by a method similar to that described by Flores et al. (1982). To generate probes, a 400 ul transcription reaction mixture containing approximately 10 ug of virus, 8 mM ATP, 0.5 mMGTP, 2.5 mM each CTP and UTP; 0.5 mM S-adenosylmethionine; 0.1% bentonite; 15 mM Magnesium acetate; 70 mM Tris base; 260 mM sodium acetate and ^{32}P GTP (125-250 uCi per reaction) was incubated for 6 hours at 42 C. The dsRNA was removed by pelleting the bentonite and the supernate containing the ssRNA was extracted with phenol-chloroform and precipitated overnight with 2 M LiCl at 4 C.

Northern Blot Hybridization

Genomic dsRNA was extracted from partially purified NCDV, VMRI, B641 and B223 bovine rotaviruses and separated by PAGE (400 ng/lane) as described earlier. The gels were stained with ethidium bromide and photographed. The dsRNA was electrophorectically transferred to nylon membrane (Zeta Probe, Bio-Rad Laboratories, Richmond, CA) according to manufacturer's instructions with the following modifications. The dsRNA was partially denatured by immersion in 0.1 M NaOH for 5 minutes, neutralized and transferred to the nylon membrane at 80V overnight. The membranes were air dried, baked for 2 hrs at 80 C and stored at room temperature.

Two stringencies were used in the analysis of hybridization between corresponding genes of the four bovine strains. Lower stringency hybridization was similar to conditions described by Dimitrov et al. (1985). Prehybridization was performed at 65 C for two hours in a solution containing 6X SSC (1X SSC = 0.15 M NaCl, 0.15 M sodium citrate), 4X Denhardt's solution, (1X Denhardt's solution = 0.02% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll), 100 mM Tris (pH 8.0), 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 200 ug/ml sonicated, denatured salmon testes DNA (Sigma Chemical Co.) and 10% deionized formamide. Hybridization was performed at 65 C for 18-24 hours in a solution (70-140 ul/cm^2 of membrane) of 4X SSC, 2X Denhardt's solution, Tris, EDTA, SDS, DNA, Formamide and ^{32}P-labeled (1.25 -2.5 X 10^8 cpm/ml) probe which had been denatured by boiling for five minutes. After hybridization, the filters were washed 3 times in 3X SSC - 0.2% SDS, twice in 1.5X SSC - 0.2% SDS and twice in 1X SSC at 65 C. Higher stringency conditions were adapted from Flores et al. (1986). Membranes were prehybridized for two hours at 52 C in a solution containing 2.5X SSC, 4X Denhardt's solution, 100 mM Tris pH (8.0), 2 mM EDTA, 7% SDS, 200 ug/ml salmon testes DNA and 50% deionized formamide. Hybridization was performed at 52 C for 18-24 hours in a solution (70 - 140 ul/cm^2 of membrane) of 2.5X SSC, 2X Denhardt's solution, Tris, EDTA, SDS, salmon testes DNA, 50% formamide and ^{32}P-labeled (1.25 - 2.5 X 10^8 cpm/ml) probe which had been denatured by boiling for five minutes. Following hybridization, membranes were washed four times at room temperature in 1X SSC - 0.1% SDS, twice in 2X SSC - 0.1% SDS, and twice in 1X SSC at 52 C.

The filters were kept moist and exposed to X-OMAT film (Eastman Kodak Co., Rochester, NY).

Under conditions of lower stringency (65 C, 4X SSC), the effective hybridization temperature was T_M (RNA) - 34 C while using conditions of higher stringency (52 C, 2.5X SSC, 50% formamide) the effective hybridization temperature was T_M (RNA) - 29 C.

Results

Antigenic Relatedness

Neutralization titers of hyperimmune antisera are given in Table 1. Cross-neutralization tests with hyperimmune antisera to the three viruses demonstrated that NCDV, VMRI and B641 belong to the same serotype; however, 2- to 13-fold differences between homologous and heterologous titers suggest some antigenic differences exist between the viruses. Since two proteins VP7 and VP4 are involved in neutralization, two way cross neutralization with genetic reassortant viruses containing either NCDV VP7 (N9) or VP4 (N4) were performed to determine whether VP7 or VP4 differed among these viruses. Homologous:heterlogous titers ranged from .5 to 1 for N9 sera when titrated against the three bovine serotype 1 viruses (Table 2). If 20-fold differences are required to define serotypes, these viruses would then belong to the same serotype. Comparison of the three viruses by reciprocal cross neutralization with N4 sera indicated NCDV differed from VMRI and B641 with homologous:heterologous titer ratios ranging from 4 to >1000 demonstrating VP4 of VMRI and B641 is antigenically distinct from NCDV VP4.

Table 1. Antigenic relatedness among bovine rotavirus strains using virus neutralization

	Neutralization titer with bovine rotavirus strains[a]								
	Serum								
	NCDV		VMRI	B641		N4		N9	
Virus	1	2	1	1	2	1	2	1	2
NCDV	32,000	22,600	15,885	8,038	565	5,024	5,024	22,600	45,201
VMRI	16,000	16,000	68,086	22,600	5,650	1,280	5,640	22,000	22,600
B641	12,708	12,708	31,770	11,721	45,201	<40	<40	22,600	45,201
N4	508	502	5,024	100	<100	45,201	22,600	<100	<100
N9	11,300	6,400	8,000	22,600	45,201	<40	<100	12,708	22,600

[a]Reciprocal of dilution giving 50% reduction in fluorescing foci.

Table 2. Ratios of homologous:heterologous neutralization titers[a]

	Serum								
	NCDV		VMRI	B641		N4		N9	
Virus	1	2	1	1	2	1	2	1	2
NCDV	1	1	4.3	1.45	80	9	4.5	.5	.5
VMRI	2	1.4	1	.5	8	35	4	.6	1
B641	2.5	1.8	2.14	1	1	>1,000	>565	.5	.5
N4	63	45	13	>100	>450	1	1	>450	>226
N9	2.8	.4	8.5	.5	1	>1,000	565	1	1

[a]Titer of serum with homologous virus/titer of serum with heterologous virus.

Comparison of Viral Proteins

Viral proteins from each virus were radiolabeled in the presence of ^{35}S-methionine and analyzed by SDS-PAGE. Eight proteins were detected with calculated molecular weights of 98,000 (VP1), 87,000 (VP2), 82,000 (VP4), 78,000 (VP4*), 57,000 (VP5), 43,000 (VP6), 35,000 (VP7a), 33,000 (VP7b) and 24,000 (VP8) (Fig. 1). The molecular weight of the rotavirus polypeptides were determined by running labeled molecular weight markers of the same gel. In the calculation of molecular weight, a linear relation between log (molecular weight) and relative mobility is assumed. The relative mobility is defined as the ratio of the distance moved by the protein to the distance moved by the bromophenol blue marker. The coefficient r for the linear regression of the molecular weight markers was between 0.9936 and 0.9897 for different electrophoresis of the proteins.

The VP4 of VMRI and B641 migrated faster than the VP4 of NCDV and therefore the term VP4* was assigned to indicate the faster migrating BP4. Two proteins were labeled in the VP7 region and were assigned VP7a and VP7b.

Radioimmunoprecipitation (RIP) using hyperimmune antisera was used to analyze the antigenic composition of the radiolabeled viral proteins (Fig. 2). Antisera to NCDV immunoprecipitated a protein in the VP4 region of VMRI and B641 while reacting with possibly two proteins of NCDV. Immunoprecipitation with VMRI antisera indicated a molecular weight difference of 4 Kd in the VP4 region of VMRI and B641 compared to NCDV. The data suggest homologous antisera and virus combinations

recognize two proteins in the VP4 region while heterologous combinations immunoprecipitate a single protein. Antisera to B641 precipitated the faster migrating protein of B641 and VMRI in the VP4 area while recognizing the slower migrating protein of NCDV. Immunoprecipiation data suggested some antigenic differences existed between VP4 of NCDV, VMRI and B641 and indicated that VP4* of VMRI and B641 are more closely related to each other than to VP4 of NCDV. The extent of antigenic heterogenicity could not be determined using RIP.

Gene Segment Homology Among Bovine Rotaviruses

Genetic relatedness among the four bovine rotaviruses was examined by Northern blot RNA-RNA hybridization. The radiolabeled ssRNA probes of NCDV and VMRI were hybridized to homologous viral RNA, RNA from additional bovine rotavirus serotype 1 isolate B641, and RNA from the bovine rotavirus serotype 2 isolate (B223). Using conditions of lower stringency which allowed a base pair mismatch of 24% (65 C, no formamide), NCDV and VMRI probes hybridized to all segments of both viruses with a slightly weaker hybridization to heterologous gene 4. Using the lower stringency conditions, differences between gene 4 of NCDV and VMRI were not clearly evident (data not shown). Increasing the conditions of stringency (52 C, 2.5X SSC, 50% formamide) decreased the allowable base pair mismatch to 21%. Using these conditions, NCDV probe hybridized to all segments of homologous NCDV RNA and all heterologous gene segments except gene segment 4 of VMRI and B641 (Fig. 3). NCDV probe hybridized to heterologous B223 gene segments with the exception of gene 4, gene 5 and gene 9. VMRI probe hybridized to all segments of

Fig. 1. Polyacrylamide gel electrophoresis of ^{14}C-labeled molecular weight standards (lanes 1 and 6) and ^{35}S-methionine labeled polypeptides of bovine rotavirus strains NCDV (lane 2), VMRI (lane 3), B641 (lane 4) and B223 (lane 5)

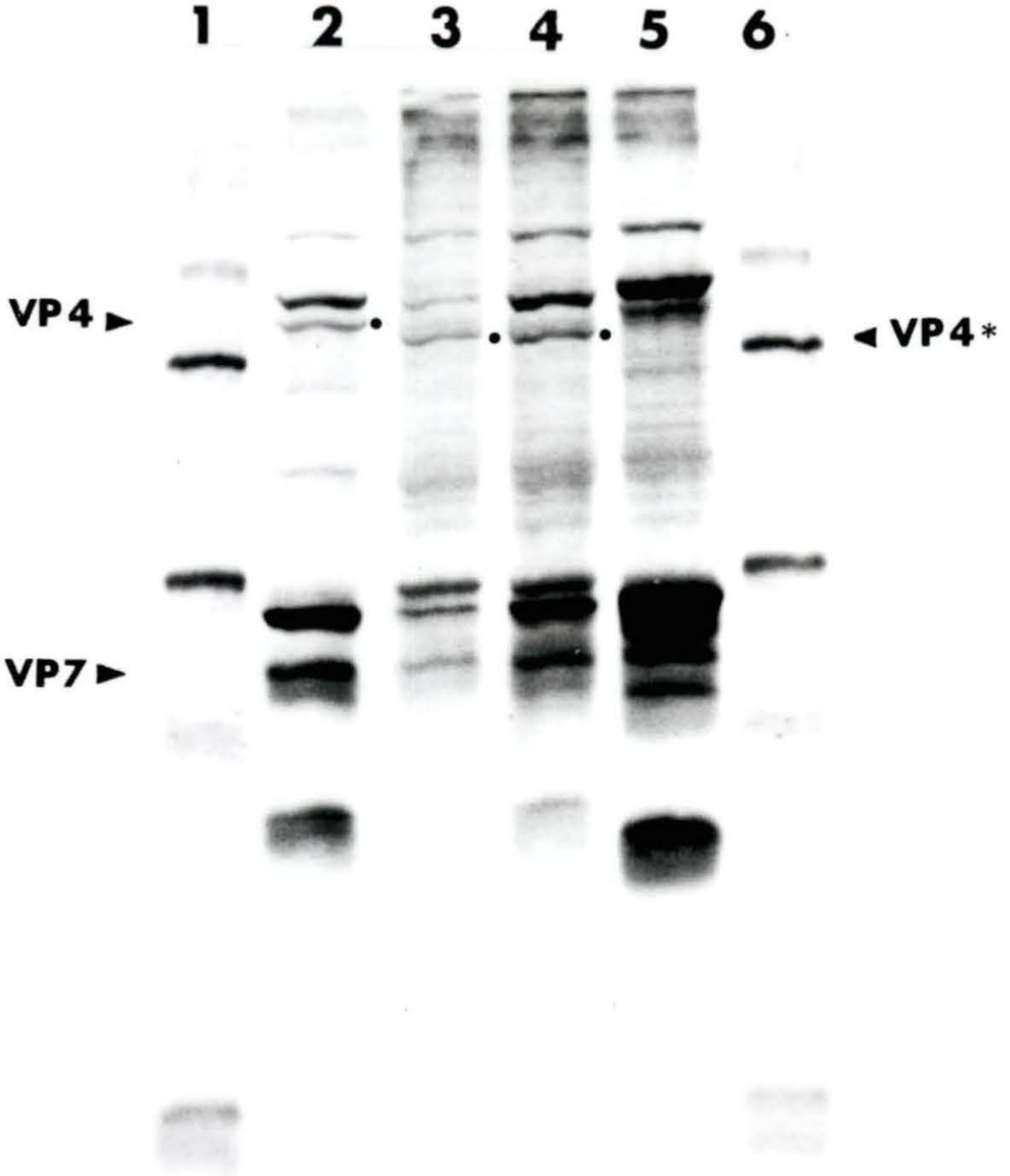

1
2
3
4
5
6
VP4
VP7
VP4*

Fig. 2. Immunoprecipitation of ^{35}S-methionine labeled bovine rotavirus infected MA-104 cell lysate with NCDV hyperimmune antisera (Panel A), VMRI hyperimmune antisera (Panel B) and B641 hyperimmune antisera (Panel C). Control uninfected MA-104 cell lysate (lane 1), NCDV infected cell lysate (lane 2), VMRI infected cell lysate (lane 3), B641 infected cell lysate (lane 4) and B223 infected cell lysate (lane 5)

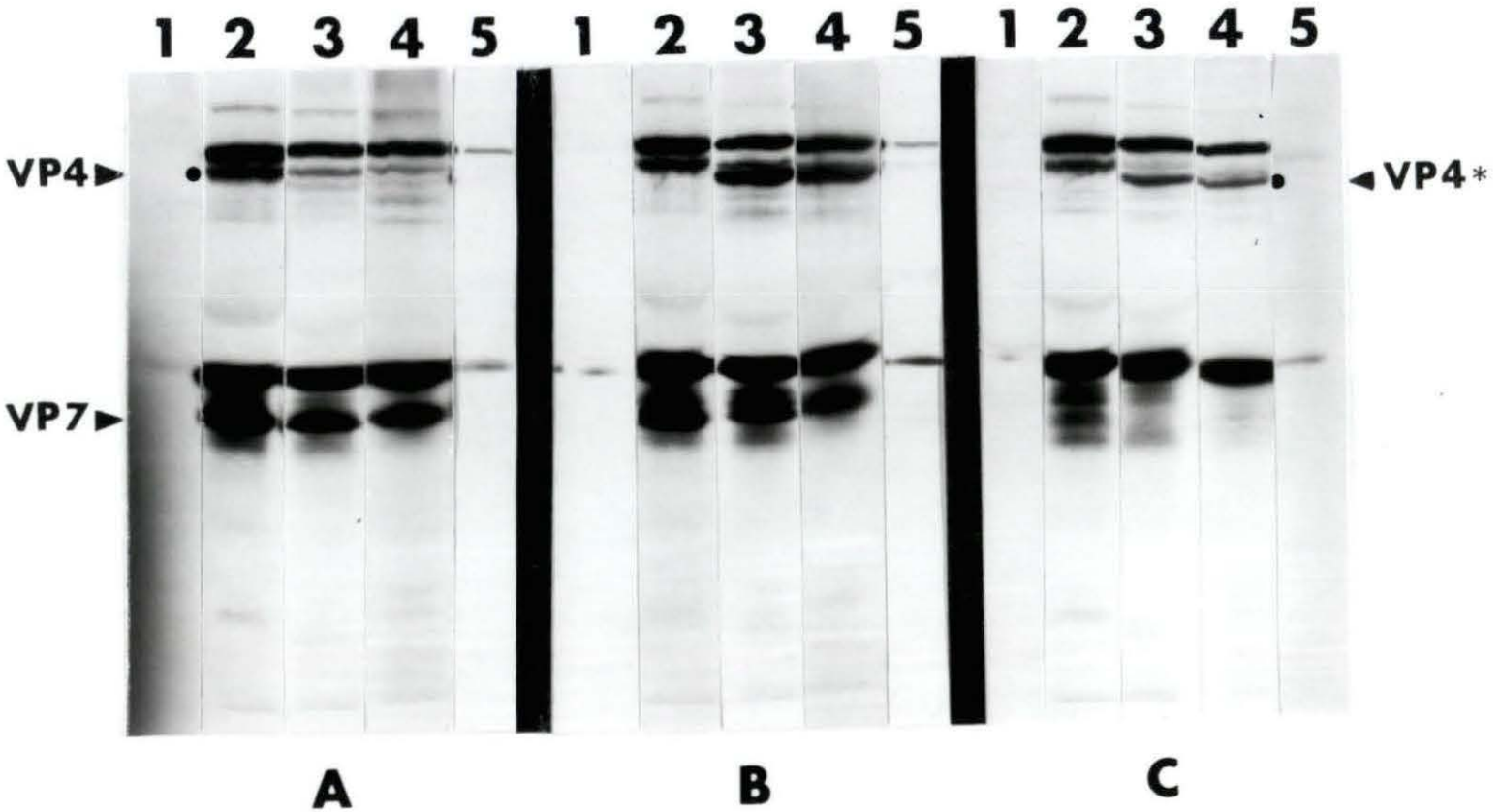
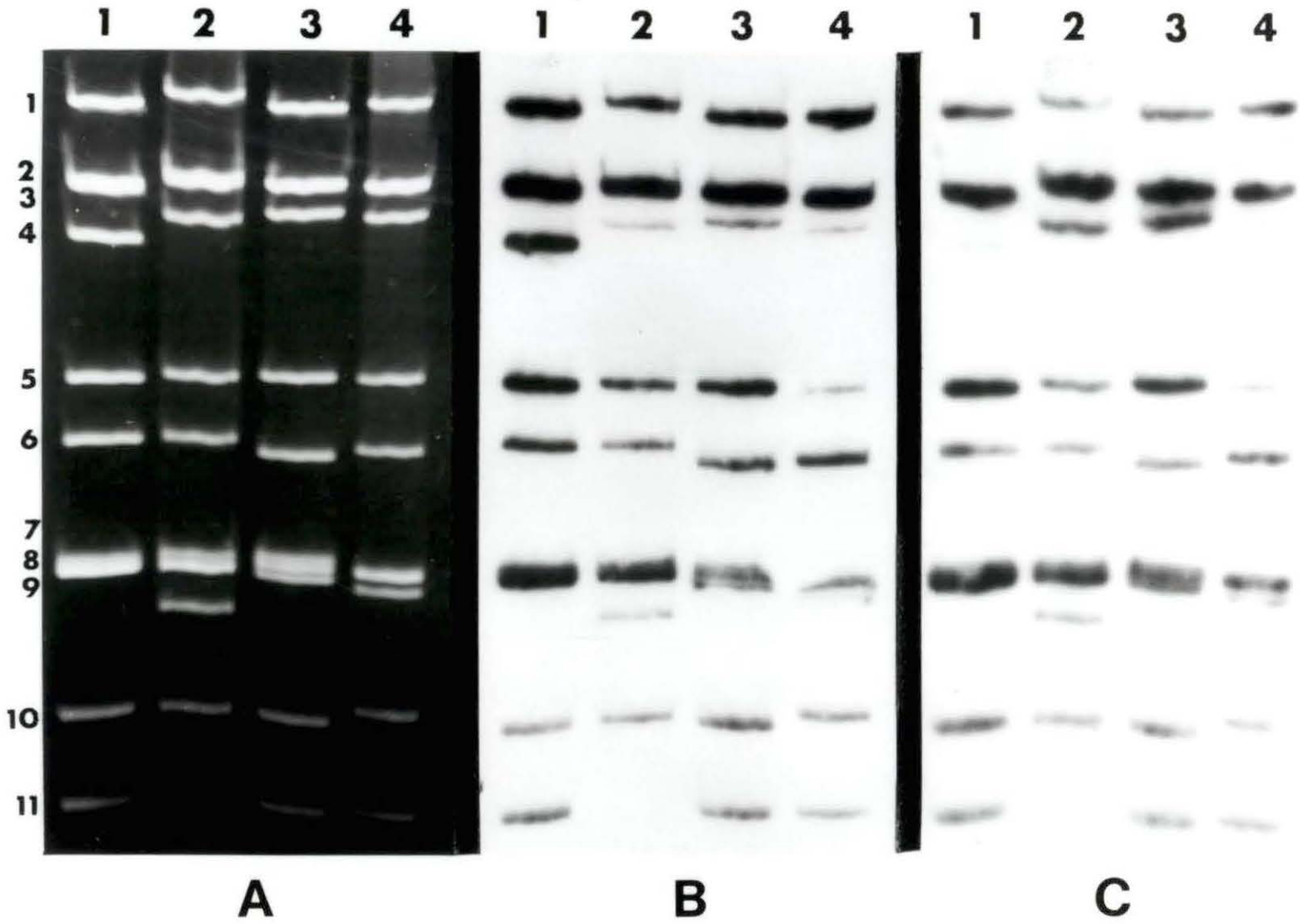

1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
VP4►
VP7►
◄VP4*
A
B
C

Fig. 3. Northern blot hybridization of dsRNA preparations at 52 C, 50% formamide. Panel A: UV light photography of gels after ethidium bromide staining. Panel B: Autoradiogram after hybridization to NCDV probe. Panel C: Autoradiogram after hybridization to VMRI probe. NCDV (lane 1), VMRI (lane 2), B641 (lane 3) and B223 (lane 4)


1 2 3 4
1 2 3 4
1 2 3 4
1
2
3
4
5
6
7
8
9
10
11
A
B
C

VMRI, B641, and NCDV except gene 4 segment of NCDV under conditions of high stringency. As observed with the NCDV probe, the VMRI probe hybridized to 8 segments of B223 with no apparent hybridization to gene segments 4, 5, and 9.

Discussion

We had previously shown that antigenic differences existed between serotype 1 bovine rotaviruses (Paul et al., 1988). The present study used reassortant viruses, radioimmunoprecipitation and RNA-RNA hybridization to further examine antigenic differences between bovine rotaviruses. Data from the study suggest NCDV, VMRI and B641 (bovine serotype 1 rotaviruses) share a common VP7 type but have distinct VP4 types. Analysis of neutralization and radioimmunoprecipitation data from serotype 1 bovine rotaviruses indicated B641 and VMRI are antigenically more related to each other than to the prototype strain NCDV bovine serotype 1 rotavirus. Neutralization results using NCDV VP4 specific antisera suggested VP4 was responsible for the neutralization differences between the three viruses. Other investigators have demonstrated neutralization differences occur between viruses containing identical gene 9 segments and different gene 4 segments (Hoshino et al., 1985; Hoshino et al., 1987). In view of the recent evidence that VP4 plays a role in determining serotype specificity, ability to generate neutralizing antibodies and passive protection of mice against rotavirus challenge (Hoshino et al., 1985; Offit and Blavat 1986; Hoshino et al., 1988; Offit et al., 1986) evidence for VP4 antigenic diversity within a

serotype could influence vaccine development. From this study it appears B641 and VMRI VP4* and NCDV VP4 are antigenically distinct which offers a possible explanation for the results of a cross protection study by Woode et al. (1983). In the later study, calves were orally vaccinated with NCDV then challenged with the serotypically related bovine rotavirus B641. Unexpectedly, calves developed diarrhea and excreted virus. Lack of antibodies directed to the common VP7 could be one explanation for the lack of cross protection since it has been demonstrated that 80% of the serum neutralizing antibodies detected following rotavirus infection of adult volunteers are directed toward the VP4 protein (Ward et al., 1988). Convelascent antisera from the calves indicated similar titers to both NCDV and B641, however, it was not determined to what protein the antibodies were directed. VP4 plays an important role in inducing neutralizing response after oral infection as opposed to systemic immunization (Shaw et al., 1987). In comparison, hyperimmune antisera prepared by systemic immunization contains antibodies to both VP4 and VP7.

In contrast, challenge studies in calves and mice using bovine rotavirus isolates NCDV and UK, which share a common VP7 but have a distinct VP4, resulted in cross protection (Offit et al., 1986; Woode et al., 1978). In addition reassortant viruses containing VP4 and VP7 from serologically distinct viruses have been used to vaccinate and protect animals from challenge to both serotypes (Hoshino et al., 1988; Offit et al., 1986). The inability of NCDV to fully protect against challenge from a serotypically related virus may suggest B641 may represent a

unique isolate and perhaps offer an explanation for outbreaks of rotavirus in vaccinated herds.

RNA-RNA hybridization was used to examine the extent of gene segment homology among four bovine rotaviruses. In the present study the percent base pair mismatch allowed under the different hybridization stringencies was calculated from the equation $T_m(RNA) = 79.8 + 18.5(\log M) + 58.4(H_G)^2 - 0.35(\%\ \text{formamide})$ where M is the Na^+ molarity and H_G as the guanine-plus-cytosine content expressed as a fraction; a value of 0.43 was used in our calculations (Flores et al., 1982). At low stringency conditions, (65 C, 4X SSC, 0 formamide) the allowable base pair mismatch is 26% and extensive cross hybridization among strains was evident. Increasing the stringency (52 C, 2.5X SSC, 50% formamide) decreased the allowable base pair mismatch to 21% eliminated cross hybridization at gene 4 although probes remained specific for gene 9 across the three serotype 1 rotaviruses. Using conditions of high stringency three distinct gene segment 4 types were differentiated with two types associated with serotype 1, and a second type associated with serotype 2. At least three different VP4 genes have been reported among the human rotaviruses and sequence analysis has indicated one VP4 type is associated with human serotype 1,3 and 4 while the second type is associated with human serotype 2 (Gorziglia et al., 1988).

Under both stringency conditions no homology was seen between serotype 1 NCDV and VMRI gene 9 and B223 serotype 2 gene 9 suggesting gene 9 codes for VP7 in B223. This observation was facilitated by the separation of gene 9 from 7 and 8 in B223. In addition to gene 4 and 9,

gene 5 also failed to hybridize to either serotype 1 probe suggesting diversity may exist in other gene segments.

The MW estimates of the rotavirus proteins are intended to illustrate relative differences and not absolute values. The estimated MW was the mean of three gels. Our results are in close agreement with a previous report which had used similar conditions with variations $^{+}$ 5000 (Novo and Esparza 1981). Results from both protein gels and RIP indicated NCDV VP4 and VP4 of VMRI and B641 differed in MW by 4kDa and therefore was designated VP4*. RIP data suggested the proteins were antigenically different which further supported the NT data. Recent sequencing data has indicated gene segment 4 is 2359-2362 bases in length and codes for a protein 775-776 amino acids in length (Kantharidis et al., 1988; Gorziglia et al., 1988). In our study gene 4 of NCDV migrated farther than gene 4 of VMRI and B641 but VP4* migrated farther than NCDV VP4. Sequencing of the different bovine gene 4 segments will play a role in classifying VP4 and determine differences which may lead to identification of serotypic sequences which will be important in classifying VP4.

The presence of several distinct VP4 types in different bovine rotavirus strains of the same serotype may greatly influence future vaccine strategies. The finding that VP4 codes for neutralization specificities that may influence immunity and neutralization titers suggest a better understanding of VP4 is necessary and could lead to a dual classification system for rotaviruses based on VP4 and VP7 as suggested by Hoshino et al. (1985). The prevalence of different VP4

types needs to be determined as will be essential in developing improved vaccines.

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SUMMARY AND CONCLUSIONS

Neonatal calf scours remains a significant problem even in vaccinated herds. One explanation for the lack of protection afforded by the vaccine may be antigenic diversity in field strains of bovine rotavirus. In the preceding study, antigenic diversity among four bovine rotavirus isolates was examined by virus neutralization with hyperimmune antisera, radioimmunoprecipitation using radiolabeled viral proteins and hyperimmune antisera and gene segment homology using Northern blot hybridization.

Results from the experiments indicated antigenic, as well as molecular, diversity exists between bovine rotavirus strains. Virus neutralization test results indicated slight antigenic variation occurs in isolates of the same serotype. Use of hyperimmune sera directed to each of the outer capsid proteins involved in neutralization indicated VP4 was responsible for the antigenic variation. Examination of the radiolabeled proteins indicated VP4 differed in size among serotype 1 isolates and reactions with hyperimmune antisera further correlated antigenic differences with VP4. Northern blot hybridization determined at least three distinct gene 4 segments existed among the four bovine isolates. These finding may have important implications in future vaccine development and development of a dual system of classification involving both rotavirus outer capsid proteins VP4 and VP7.

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