Propagation of porcine group C rotavirus in an intestinal cell line

by

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INTRODUCTION

Rotaviruses are an important cause of porcine viral enteritis, with a worldwide distribution. While pigs of any age may be infected, clinical disease has been shown to be most severe in nursing and immediate postweaning pigs (Woode, 1986). Within these age groups, morbidity may be 50 to 80% with a mortality of 7 to 20% (Straw, 1986). Recovered piglets often take longer to reach market, resulting in further economic loss to the producer (Woode, 1986).

Serologic studies have shown that rotaviruses are divided into a number of antigenically distinct serogroups, with no serologic cross-reactivity between these groups. Currently, groups A through F, and tentatively G, are known (Bridger, 1987). Only groups A, B, and C have been found in pigs in the United States; in addition, group E has been detected in pigs in Europe.

Group A rotavirus is the most prevalent type detected in pigs worldwide. Many group A isolates can be cultivated in vitro, allowing the group A rotaviruses to be the most characterized serogroup of the genus and permitting the production of vaccines. Groups B and C, although less commonly a cause of gastroenteritis in pigs than group A, are also important porcine enteric pathogens and are much less

well characterized. At the time this experiment was planned and begun, group B rotaviruses had not yet been propagated *in vitro* and only one group C isolate had been grown in cell culture (Terrett and Saif, 1987).

The intent of this project was to discover an *in vitro* culture system that would allow the propagation of some of these fastidious rotaviruses. Once such a system was standardized, these viruses could be propagated more easily and economically than current *in vivo* methods, which would allow faster characterization, development of better diagnostics, and the possibilities of vaccine production.

LITERATURE REVIEW

Rotavirus

Rotaviruses, members of the family Reoviridae, are found throughout the world and are one of the major causes of gastroenteritis in man, other mammals, and many avian species (Estes et al., 1984). In man as well as other mammals, gastroenteritis due to rotaviruses is most severe in nursing and post-weaning individuals; infection in juvenile or in adult animals is usually milder or even subclinical. Death rates for domestic animals are highly variable; death is often due to dehydration and secondary bacterial infections (Pearson and McNulty, 1977). Recovered animals often grow more slowly than uninfected animals of the same age, resulting in economic loss to the producer.

Generally, it has been assumed that rotaviruses were host-specific and that infections across species lines were rare or clinically mild. For example, Ryder et al. (1986) showed that farm workers in Panama with frequent contact with cattle would transmit rotaviruses within family units but transmission was not to or from cattle. However, exceptions to this assumption have recently been noted: a group B rotavirus has been isolated that caused gastroenteritis in human adults and children and also in infant rats (Eiden et al., 1985; and Vonderfecht et al., 1985); and Nakagomi and Nakagomi (1989) reported transmission of a feline group A

rotavirus to humans.

Structure and physical properties

The characteristic structure of the genus Rotavirus is a non-enveloped, icosahedral virion of approximately 75 nm with a density of 1.36 gm/cm^3 in CsCl. The name rota derives from Latin for "wheel-like", since the appearance of the double-shelled capsid by electron microscopy is smooth and round with short spoke-like structures. The outer shell is composed of 132 capsomeres and is penetrated by 132 channels which communicate from the inner core to the outside (Estes, 1990). Since rotaviruses lack an envelope, they are resistant to fluorocarbon, ether, chloroform, and deoxycholate. The virus can be inactivated by disinfectants such as ethanol, phenols, formalin, chlorine, and beta-propiolactone (Estes, 1990). Infectivity is retained over a pH range of 3.5 to 10.0 (Kapikian and Chanock, 1990).

The outer capsid can be removed using calcium chelating agents such as EDTA and EGTA, resulting in a non-infectious single-shelled virion. Compared to double-shelled virions, single-shelled capsids appear rough by electron microscopy. They are approximately 55 nm in diameter and have a density of 1.38 gm/cm^3 in CsCl. The loss of the outer capsid activates the viral RNA-dependent RNA polymerase.

The inner capsid can be removed using chaotropic compounds (sodium thiocyanate, calcium chloride) leaving core

particles of 37 nm with a density of 1.44 gm/cm^3 in CsCl (Estes, 1990). With the removal of the inner shell, polymerase activity is lost even though the polymerase resides in the inner core.

Genetics

The double-stranded RNA genome is composed of 11 gene segments that are numbered 1 through 11 on the basis of their mobility using polyacrylamide gel electrophoresis (PAGE). Segment 1 is the largest, slowest-moving segment and is seen at the top of the gel; segment 11 is the smallest and is found at the bottom. A number of group A gene segments have now been sequenced, showing that this group has a number of common features, as reviewed by Estes (1990). Conserved 5' and 3' terminal sequences are contained within the non-coding sequences that flank the open reading frame. There is no 3' polyadenylation signal. All genes are thought to be monocistronic except possibly gene segment 9. Gene coding assignments have been determined using in vitro translation methods for a number of gene segments. Those for the simian group A rotavirus SA11 are most complete (Table 1). Like other segmented RNA viruses, these gene segments are capable of reassorting with homologous gene segments of other rotaviruses during mixed infections (Hoshino et al., 1987).

Viral proteins

As with the gene coding assignments, the majority of the

Table 1. Polypeptides encoded by gene segments of SA11[a]

Genome Segment	Primary Product	Modification	Modification Product
1	VP1[b] (125 kDa)	None	None
2	VP2 (94 kDa)	None	None
3	VP3 (88 kDa)	None	None
4	VP4 (88 kDa)	Cleaved	VP5 (60 kDa) VP8 (28 kDa)
5	NS53[c] (58 kDa)	None	None
6	VP6 (44 kDa)	None	None
7	NS34 (34 kDa)	None	None
8	NS35 (35 kDa)	None	None
9	VP7 (34 kDa)	Glycosylated Trimmed	VP7 (38 kDa)
10	NS20 (20 kDa)	Glycosylated Trimmed	NS29
11	NS26 (26 kDa)	Phosphorylated	NS28

[a]Estes, 1990.

[b]VP = viral protein.

[c]NS = non-structural protein.

knowledge about viral proteins comes from work with group A rotaviruses. Information presented in this section is derived from group A models; group B and C information will be presented separately.

Viral proteins 1, 2, and 3, found in the core particles, are coded for by gene segments 1 through 3 respectively. VP1 is present in small amounts in the core particle, suggesting that its function is enzymatic rather than structural, and its amino acid sequence suggests similarities to RNA polymerases of a number of RNA viruses (Estes and Cohen, 1989). VP1 is usually shielded from immune recognition in intact viral particles, generally failing to react with immune or hyperimmune sera; purified VP1, however, is highly immunogenic (Cohen et al., 1989).

VP2 is partly exposed on single-shelled particles and is also highly immunogenic (Estes and Cohen, 1989). It is the most abundant protein of the core particle and the third most abundant protein in the intact capsid (Estes and Cohen, 1989). Boyle and Holmes (1986) have shown that VP2 possesses nucleic acid binding activity to dsRNA, ssRNA, and dsDNA, apparently acting as a leucine zipper.

Like VP1, VP3 is present in small amounts and is thought to play a role in RNA replication.

VP4 is a multifunctional non-glycosylated protein of the outer capsid coded for by gene segment 4. Prior to the

discovery by Liu et al. (1988) of VP3 as the protein product of gene segment 3, VP4 was referred to as VP3. In many rotavirus strains, VP4 is a hemagglutinin (Kalica and Greenberg, 1983); human type O red blood cells are most commonly used for hemagglutination and hemagglutination inhibition. VP4 is cleaved by trypsin into VP5 and VP8, resulting in increased viral infectivity due to enhanced cell penetration but not enhanced binding (Clark et al., 1981; Estes et al., 1981; and Graham and Estes, 1980). Infectivity is also enhanced by elastase and by an as yet unknown acid-stable enzyme in pancreatin (Graham and Estes, 1980). Most importantly, VP4 is one of the two proteins which react with neutralizing antibodies against the virus. This neutralization can be detected with either in vitro or in vivo models (Offit et al., 1986b). VP4 is also suspected to be a virulence factor (Flores et al., 1986; and Offit et al., 1986a) and a fusion protein (Mackow et al., 1988).

VP6, coded for by gene segment 6, contains the common group A antigen and the subgroup antigens. While it is highly immunogenic and antigenic, antibodies to it have little or no neutralizing activity. Present as a trimer, VP6 forms the inner capsid shell and is the major structural protein of the entire capsid (Estes and Cohen, 1989). It is required for transcription, but appears to act as a structural scaffold rather than an enzyme (Bican, 1982). Serologic diagnostic

assays for group A rotaviruses are directed toward this protein.

VP7 is coded for by gene segment 7, 8, or 9, depending on the viral strain. It is glycosylated and highly immunogenic. Like VP4, it is found in the outer capsid and induces neutralizing antibodies. It is the second most abundant capsid protein and has been shown to be the cell attachment protein (Fukuhara et al., 1988). Classification of serotypes of rotaviruses within serogroup A is done by neutralization tests using hyperimmune antisera. Neutralizing antibodies are primarily directed against the VP7 proteins. Currently, thirteen serotypes are recognized (Estes, 1990). While polyclonal and monoclonal antibodies to VP7 have been shown to inhibit hemagglutination, this is thought to be due to steric hinderance of VP4, since VP7 is not itself a hemagglutinin. Although there is this close interaction of VP4 and VP7, different strains can reassort either <u>in vitro</u> or <u>in vivo</u> during mixed rotavirus infections, giving progeny with a VP7 of one parent and a VP4 of the other. Since there are now at least nine known VP4 serotypes (Estes and Cohen, 1989), suggestions have been made that in the future a dual serotyping system similar to that used for influenza viruses may be necessary.

The products of other rotaviral gene segments are non-structural proteins, found in infected cells but not in the

mature virions (Estes and Cohen, 1989). Their functions remain uncertain.

Rotavirus classification

Rotaviruses can be divided into a number of antigenically distinct serogroups; groups A through F, and tentatively G, have so far been detected (Bridger, 1987). There is no serologic cross reactivity between these groups. Each group also gives a distinct migration pattern of its gene segments by polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970) with the silver stain technique of Herring et al. (1982), although the patterns for groups B and E are somewhat similar. These segments migrate to form four different regions within the gel, resulting in the distinctive group patterns. Group A gives a 4-2-3-2 pattern (from the top); group B is 4-2-2-3; and group C is 4-3-2-2 (Figure 1).

Because rotaviruses are the only known enteric mammalian virus with 11 gene segments, detection and diagnosis by PAGE are commonly done (Estes and Cohen, 1989). Epidemiological studies are also frequently done since isolates often show individual differences of PAGE signature within the overall group pattern. However, PAGE pattern alone should not be used to assign a new virus isolate to its group; other confirmation such as serologic testing (Estes and Cohen, 1989) or nucleic-acid hybridization (Eiden et al., 1986c) should also be done. This paper will discuss groups A through C in mammals; groups

Figure 1. PAGE of the major rotavirus serogroups prevalent in U.S. swine herds. Left lane: group A (isolate 1645); middle lane: group B (isolate B1146); right lane: group C (isolate C850). Note that some gene segments migrate very close together.

D and F are avian pathogens and group E is rare.

Serotype classification

Group A rotaviruses are further classified into serotypes on the basis of virus neutralization tests such as fluorescent focus neutralization (Woode et al., 1983) and plaque-reduction assays (Estes and Graham, 1980; and Hoshino et al., 1984), using hyperimmune sera. These antibodies are primarily directed against VP7. Antibodies against VP4 also have neutralizing activity and play a role in serotype determination (Hoshino et al., 1987). Serotypes are defined as having reciprocal 20-fold or greater serum antibody titer differences (Hoshino et al., 1984).

Other methods of serotyping have been reported but are not yet routinely used. Monoclonal antibodies used for serotyping by ELISA may not detect all members of a given serotype (Coulson et al., 1987; and Taniguchi et al., 1987). Nucleic acid hybridization allows assignment of serotypes (Dimitrov et al., 1985; Flores et al., 1989; and Lin et al., 1987), but radioactive probes restrict the usage of this method. Serotyping can also be determined by nucleotide sequencing (Glass et al., 1985; Green et al., 1987; and Green et al., 1988), although this technique is not yet practical for wide scale usage.

Group A rotaviruses

Because group A rotaviruses are the most prevalent and

the best characterized group, they will be used as a model for the description of this genus. Worldwide in occurrence, they have been found in man and a large number of domestic animals, including pigs, cows, horses, monkeys, lambs, dogs, cats, poultry, and laboratory rabbits and mice (Kapikian and Chanock, 1990). Serologic surveys have shown the ubiquitous nature of group A rotaviruses. Woode (1978) showed that ninety to one hundred percent of humans, cattle, and pigs have antibodies against this serogroup. Bridger and Brown (1985) showed that virtually all swine herds were seropositive. Mebus (1980) reported that rotavirus was second only to coronavirus as a cause of viral diarrhea in calves.

Generally, disease is more severe in the young; disease in adults is often subclinical. In pigs, Woode (1986) reported that the severity of the disease varied with the age and immune status of the pig. Young piglets in the first week of life are usually protected by colostral antibodies. However, in those that are colostrum-deprived or are nursing sows that are lacking antibodies against rotavirus, mortality can be as high as 100%. Disease is most commonly seen in suckling piglets 10 to 21 days old, as colostral immunity wanes, and in those immediately postweaning, which are no longer receiving lactogenic antibodies. In the suckling pigs, it is usually a mild diarrheal disease with quick recovery and low mortality. In the postweaning pigs of 3 to 8 weeks of age, clinical

symptoms may be more severe; mortality is usually 3 to 10% but can reach 50%. Disease in adults is usually subclinical, but infection is common enough that most sows have antibodies to rotavirus.

In cattle, like in pigs, the young are most susceptible (Woode, 1978). Rotavirus has been associated with enteritis and mortality in calves from birth to nine weeks of age. Calves under seven days of age are the most susceptible (Tzipori et al., 1981). Unlike in pigs, severe disease may also be seen in adult cattle, but mortality is rare (Woode and Crouch, 1978).

In children, group A rotaviruses are the single most important cause of severe diarrheal diseases worldwide (reviewed by Kapikian and Chanock, 1990). Although diarrheal diseases are not a major cause of mortality for children in the United States, the World Health Organization (1973) reported that diarrheal diseases result in a large proportion of the total deaths of children in developing countries.

Group B rotaviruses

Formerly called rotavirus-like viruses, group B rotaviruses have been associated primarily with diarrheas of newborn domestic animals (Bridger and Brown, 1985; Brown et al., 1987; and Nagesha et al., 1988), although they have been recovered from man, pig, cattle, sheep, and rat (Chen et al., 1985; Saif and Theil, 1985; and Vonderfecht et al., 1985).

While considered much less common as a cause of diarrheas of domestic animals than group A rotavirus, group B rotavirus infections may be under-reported since sample collection must be quite early in the infection to allow demonstration of the virus (Huber et al., 1989; and Vonderfecht et al., 1988). Epidemics of severe human cholera-like diarrheal disease in China, affecting hundreds of thousands, have been caused by a group B rotavirus termed adult diarrhea rotavirus (ADRV) (Chen et al., 1985; Hung et al., 1984; Hung et al., 1985; Su et al., 1986; and Wang et al., 1985). As the name implied, this unusual rotavirus was most frequent and severe in adults rather than in infants. However, Dai et al. (1987) reported that neonates may be infected but show milder clinical symptoms, while Eiden et al. (1986a) reported that an antigenically similar group B rotavirus caused a severe outbreak of gastroenteritis in a nursery.

Eiden et al. (1985) and Vonderfecht et al. (1985) found that the group B infectious diarrhea of infant rats (IDIR) virus could also cause disease in children, helping to refute the theory that all rotaviruses are host-specific. This finding, coupled with the reports of ADRV epidemics, has focused more attention onto group B rotaviruses. Antibodies to group B rotaviruses have been found worldwide (Mattion et al., 1989; and Nagesha et al., 1988). Antibody prevalence for group B rotaviruses has ranged from 23 to 97% in pigs (Bridger

and Brown, 1985; Brown et al., 1987; and Theil and Saif, 1985); in man, the prevalence ranged from 4.5 to 9.5% in the United States up to 45% in provinces of China (Hung et al., 1987; Hung, 1988; and Nakata et al., 1987). Saif and Theil (1985), using reciprocal cross-protection experiments, suggested that there may be at least two distinct serotypes within group B.

The protein characteristics of group B rotaviruses are less well known than those of group A. Fang et al. (1989), using SDS-PAGE and Western blotting techniques with ADRV purified from Chinese clinical fecal samples, have produced the bulk of the information known about group B viral proteins. They have found

1. a viral core protein, tentatively called an analog to VP1.
2. an antigenic core protein that may be a VP2 analog.
3. a moderately antigenic protein of the outer capsid that was designated VP5.
4. an inner capsid protein that was the most immunologically reactive protein when using antisera to ADRV; it also reacted with antisera to group B rotaviruses of other species. This protein was a good candidate for the common group antigen, similar to VP6 of group A.

5. a glycosylated outer capsid protein that was a probable analog for VP7.
6. a protein that reacted strongly with convalescent sera and was tentatively called VP8.

The Western immunoblotting procedures confirmed that there was no reaction of these group B rotavirus proteins with group A antisera, as has also been shown by immunoelectron microscopy, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) (Nakata et al., 1986; Pedley et al., 1983; and Saif and Theil, 1985). Studies to determine the gene segments coding for these proteins were not done.

Group C rotaviruses

Group C rotaviruses, formerly called pararotaviruses, have only been recovered from swine, guinea pigs, and humans (Rodger et al., 1982; Saif et al., 1980; and Ushijima et al. 1989). While worldwide in occurrence (Bridger and Brown, 1985; Brown et al., 1988; Matsumota et al., 1989; Mattion et al., 1989; Nagesha et al., 1988; and Penaranda et al., 1989) and shown to be an important cause of gastroenteritis in man (Bridger et al, 1986; Brown et al., 1988; Espejo et al., 1984; Penaranda et al., 1989; and Ushijima et al., 1989), the prevalence and importance of group C rotavirus relative to that of group A in pigs is still unclear. Studies by Fu et al. (1989) in New Zealand, Nagesha et al. (1988) in Australia, and Bridger (1985) in Great Britain suggested that group C

rotaviruses in pigs may be almost as common as group A. Fu (1987) showed that inapparent infection of pigs with group C rotavirus was similar to that of group A.

Bremont et al. (1988) reported five major proteins of a porcine group C rotavirus. Two of these proteins were shown to be from the outer capsid; the smaller of these two proteins was glycosylated and a likely candidate for the VP7 analog. One porcine group C isolate, known as the Cowden strain, has recently been adapted to cell culture (Terrett and Saif, 1987). Working with this Cowden strain, Jiang et al. (1990) reported ten viral proteins for this virus, seven structural and three non-structural. On the basis of molecular mass and location within the virion, proteins corresponding to group A VP1, VP2, VP6, and VP7 were identified. The VP6 analog formed an oligomer of four units, unlike the trimer of group A. The VP7 analog was glycosylated and was of a similar molecular mass as the protein that Bremont et al. (1988) reported to be a likely VP7. No protein was identified as a VP4 counterpart, although two other outer capsid proteins, one of them glycosylated, were reported but were not classified. It is possible that these two proteins correspond to VP5 and VP8; however, there is not a good correlation between the sum of the molecular masses of these two proteins with the mass of the single remaining outer capsid protein seen by Bremont et al. (1988). If these two proteins do represent cleavage

products of a VP4 analog, the group C protein corresponding to VP4 is glycosylated, while the group A VP4 is not.

Pathology

Rotaviruses, like most other gastroenteritis viruses, infect the cells of the small intestine. Despite the species infected or the serogroup of rotavirus, preferred sites are the jejunum and the upper to middle ileum, although virus can be found by fluorescent antibody (FA) staining from the pylorus into the cecum (Bohl et al., 1982; Chasey and Banks, 1986; McAdaragh et al., 1980; Pearson and McNulty, 1977; and Shaw et al., 1989). Transmission is primarily by the fecal-oral route. Uptake of the virions into the cell is mediated by the binding of VP7 to a receptor, but penetration into the cell is associated with the proteolytic cleavage of VP4 (Estes and Cohen, 1989). Sialic acid appears to be the receptor or an essential part of it, since Bastardo and Holmes (1980) showed neuraminidase treatment of target cells reduced virus binding, and Yolken et al. (1987) showed sialic acid-containing compounds inhibit binding. The exact mechanism of entry into the cell is unclear. Ludert et al. (1987) and Estes (1990) stated that uptake was by receptor-mediated endocytosis; Kaljot et al. (1988) stated that the virus entered the cell by direct penetration.

Rotaviruses selectively infect mature columnar intestinal epithelial cells, which are located on the distal villi, and

replicate in the cytoplasm. The virus particles bud into the endoplasmic reticulum (Chasey, 1977), gaining a temporary envelope which is lost before the release of mature virions with cell lysis. The degenerating infected enterocytes appear to slough from the villi before the release of the virions (Saif et al., 1978). Loss of these infected enterocytes then leads to shortening, thickening, and fusing of the villi. Lost enterocytes are replaced by immature epithelial cells from the crypts, resulting in crypt hyperplasia. These immature squamous to cuboidal epithelial cells do not have the absorptive ability of the mature enterocytes. Infiltration of the underlying lamina propria with inflammatory cells is sometimes seen. Diarrhea is due to loss of absorptive ability, impairment of electrolyte and glucose transport, and the osmotic action of the unabsorbed nutrients (Shaw et al., 1989).

Stevenson (1990) pointed out that all previous descriptions of intestinal pathology in pigs are from young (less than two weeks old) piglets on liquid diets. When he exposed three-week-old weaned piglets to a group A rotavirus, they remained clinically normal and showed much less severe villous atrophy when compared to neonatal gnotobiotic piglets.

Group B rotaviruses typically produce syncytia from the intestinal epithelial cells of various species (Chasey and Banks, 1986; Chasey et al., 1989; Mebus et al., 1978; and

Vonderfecht et al., 1984) as well as in cell culture monolayers (Theil and Saif, 1985). Askaa and Bloch (1984) showed that, while syncytia formation from the enterocytes may be extensive, goblet cells were unaffected. In rats, Huber et al. (1989) showed that syncytia were only present on the first day of disease. Syncytia alone, however, can not be used to diagnose group B rotavirus infection, since Gelberg et al. (1990) reported that group A rotavirus may rarely form syncytia.

Immunity

As was shown by Estes and Graham (1980) and by Gaul et al. (1982), neutralizing antibodies against rotaviruses are serotype specific. While experimentally hyperimmunized animals produce antibodies primarily against VP7, naturally infected animals produce antibodies against both VP4 and VP7. Protection is primarily due to antibodies in the lumen of the intestine, either acquired passively with milk or colostrum or actively produced due to rotavirus exposure. Offit and Clark (1985) showed that either intestinal IgA or IgG was protective, although IgA was more potent *in vivo*. Since most adult pigs have antibody to rotavirus, colostral and lactogenic protection will usually prevent the most severe clinical disease seen in the first week of life (Askaa et al., 1983). Offit and Clark (1985), Shaw et al. (1989) and Woode (1986) stated that circulating antibodies alone give no

protection.

The role of cell-mediated immunity is less clear. Children (Losonsky et al., 1985) and mice (Riepenhoff-Talty et al., 1987) with severe combined immunodeficiency disease (SCID) became chronically infected with rotavirus. However, Eiden et al. (1986b) showed that athymic mice recovered from rotavirus infection at the same rate as normal controls, despite the failure of the athymic mice to produce antibodies against rotavirus.

Clinical disease in pigs

Field and experimental studies have shown that pigs were most susceptible to rotaviral disease from birth to six weeks of age (Bohl et al., 1978; McNulty et al., 1976; Shaw et al., 1989; and Woode, 1986). In experiments with pigs less than five days old, Crouch and Woode (1978) and Janke et al. (1988) showed that severe watery diarrhea often began by 24 hours after inoculation, leading to high mortality. These infected piglets often became inappetant, reluctant to move, and dehydrated, commonly dying within 72 to 96 hours postinfection (Bohl et al., 1978). Vomiting was occasionally seen early in the course of the disease.

Clinical rotaviral disease has most commonly been reported in 2 to 3 week old suckling pigs, as colostral antibody wanes (Askaa et al., 1983; and Woode, 1986). McAdaragh et al. (1980) and Theil et al. (1978) showed that

these older pigs developed profuse diarrhea within 30 hours post-infection and usually recovered in 4 to 6 days. Pearson and McNulty (1977) stated that the mortality rate was low unless there was a concurrent infection with E. coli or other pathogens. Askaa et al. (1983) showed that litters of gilts often were infected at a younger age and showed a higher proportion of pigs with clinical disease than did litters of sows, since gilts tended to have lower colostral antibody levels. Postweaning diarrhea in 3 to 4 week old pigs has also been reported to be caused by rotaviruses (Lecce and King, 1978; Woode et al., 1976). Disease in adults was usually subclinical.

Detection, quantitation, and diagnosis of rotaviruses

Rotaviruses can be detected through a number of techniques that fall into three general categories. Often more than one of the following methods is used in combination, such as with immunoelectron microscopy. Timing of sample collection, however, is critical. Benfield et al. (1984) showed that group A antigen can be reliably detected in intestinal tissues for only 24 to 72 hours after onset of diarrhea. Huber et al. (1989) and Vonderfecht et al. (1988) stated that the rat group B rotaviruses were unlikely to be detected after the first day of infection. Detection of the virus may not always relate to clinical disease, however, since rotaviruses are ubiquitous and infections are often

subclinical.

Visualization of virus or virus-specific molecules

Direct visualization of rotaviruses by electron microscopy (EM) was the first major method of detection and is still in use. An advantage of EM is that rotaviruses have a distinct morphologic appearance, allowing high specificity, and that all serogroups can be detected. Brandt et al. (1981) reported that detection rates ran as high as 90% in virus positive specimens. Benfield et al. (1984) showed that EM was more sensitive than virus isolation, fluorescent antibody, or ELISA for detection of group A rotavirus from porcine fecal samples; for bovine samples, ELISA and EM were equally sensitive. The stated disadvantages of EM were that it was not ideal for quantifying virus and that the technique was time-consuming and expensive. Flewett (1976) stated that EM will reveal rotavirus only when virus concentrations exceed 10^6 particles/ml. Nakata et al. (1987b) reported that group B rotaviruses were much more easily degraded by standard negative staining techniques than were groups A and C; they recommended phosphotungstic acid at pH 4.5 or uranyl acetate for negative staining and phosphotungstic acid at pH 4.5 for immunoelectron microscopy.

Immunoelectron microscopy (IEM) is used less frequently than EM. Its main advantages over EM are the ability to confirm serogroup and, when used with convalescent sera, to

confirm prior infection. Gilchrist et al. (1987) stated that IEM was generally more sensitive than direct EM for detection of viruses.

Detection of rotaviral RNA by PAGE can be done with extracts from fecal specimens or from cell cultures. Advantages of this technique are economy, speed, and simplicity. The silver stain developed by Herring et al. (1982) can detect as little as 300 to 400 pg of RNA in a single band. Like EM, PAGE is able to detect all rotavirus serogroups and has the added advantage of identifying to which serogroup the isolate probably belongs.

Flores et al. (1983) developed a dot blot hybridization assay for the detection of rotaviral RNA. They reported that the method was highly specific and was 10 to 100 times more sensitive than ELISA, able to detect as little as 8 pg. of RNA. Usage of this method is restricted, however, because of the radioactive labeling.

Serologic methods

Serology can be used to detect either the presence of rotaviruses or of antibodies to them. However, unless polyvalent sera are used, all serologic techniques are limited to the detection of a single serogroup or sometimes a single serotype within a serogroup. Also, since rotaviral infections may be subclinical, detection of antibodies to rotavirus may not be indicative of clinical disease.

Fluorescent antibody (FA) methods, both direct and indirect, can be used to detect rotaviral antigens in frozen intestinal sections, as well as in fixed cell culture monolayers. While the technique is simple, accurate, and rapid, timing is important due to the fairly rapid shedding of infected villous epithelial cells. Benfield et al. (1984) stated that FA and EM were the preferred tests for porcine group A rotavirus.

Enzyme-linked immunosorbent assays (ELISA) for rotavirus detection are widely used, since they are simple, sensitive, specific, and inexpensive. Studies have shown that ELISA was as sensitive as EM for group A rotaviruses of children (Brandt et al., 1981; Cheung et al., 1982; Grauballe et al., 1980; Hammond et al., 1982; and Rubenstein et al., 1982) and of calves (Benfield et al., 1984; Cornell et al., 1982; Ellens and DeLeeuw, 1977; Grauballe et al., 1980; and McGuire et al., 1982) but not as sensitive for porcine group A rotaviruses (Benfield et al., 1984). ELISA tests for IgG, IgM, and IgA against group A rotaviruses have also been developed (Delem and Vesikari, 1987; and Losonsky et al., 1986). Commercial group A rotavirus kits currently reported available by Christensen (1989), Gilchrist et al. (1987), Goyal et al. (1987), and Prey et al. (1988) were Pathfinder (Kallestadt, Austin, TX), Rotavirus Bio-EnzaBead (Litton Bionetics, Charleston, SC), Rotavirus EIA (International Diagnostic

Laboratories, St. Louis, MO), and Rotazyme (Abbott Laboratories, N. Chicago, IL).

ELISA tests for group B rotavirus detection have also been developed by Nakata et al. (1986) and by Vonderfecht et al. (1985). ELISA tests for the detection of antibodies against group B rotavirus have been developed by Nakata et al. (1987) and by Qiu et al. (1986).

Latex agglutination is popular due to its simplicity, excellent specificity, and lack of required instrumentation (Doern et al., 1986). Gilchrist et al. (1987) found that latex agglutination kits were less sensitive than ELISA kits, but were of equal specificities. Commercial latex agglutination kits reported available by Christensen (1989), Gilchrist et al. (1987), Goyal et al. (1987), and Prey et al. (1988) were Meritec-Rotavirus (Meridian, Cincinnati, OH), Rotalex (Medical Technology Corporation, Somerset, NJ), Slidex Rota-kit (bioMerieux, Marcy-l'Etoile, France), and Virogen-Rotatest (Wampole Labs, Cranbury, NJ).

Many, although not all, rotaviruses hemagglutinate. Testing for presence of these rotaviruses by hemagglutination (HA) or for antibodies against rotavirus by hemagglutination inhibition (HI) is a relatively simple laboratory procedure. A modification developed by Sanekata et al. (1982), reverse passive hemagglutination assay (RPHA), was stated to be 10 times more sensitive than complement fixation. In this test,

sheep red blood cells were coated with antibodies to rotavirus, and the presence of rotavirus caused hemagglutination. Inhibition of this reaction could be used to test for the presence of antibodies.

Other less commonly used methods are complement fixation (Su et al., 1986), radioimmunoassay or RIA (Hughes et al., 1984), immunoelectrophoresis (Hammond et al., 1984), immunodiffusion, and immunoelectro-osmophoresis (Grauballe et al., 1977).

Culture techniques

Culture techniques can be used to complement those methods discussed above. For example, samples suspected of containing very low levels of rotaviruses can be first amplified by either in vitro or in vivo passages to the point where virus concentration is more appropriate for its detection by other means. Generally, group A rotaviruses are amplified in vitro and other serogroups are propagated in vivo. Plaque assays, first developed by Matsuno et al. (1977), are commonly used for viral quantitation. Virus neutralization assays, used to quantitate, to serotype, and to measure neutralizing antibodies, are most commonly done as a plaque-reduction method (Estes and Graham, 1980), but have also been done by measuring infectivity to either susceptible animals (Gaul et al., 1982; and Offit et al., 1986b) or to cell monolayers.

Propagation of rotaviruses

While rotaviruses can be propagated in the species of origin, this is time consuming and expensive compared to tissue culture techniques. With the human rotaviruses, host propagation also raises ethical questions. Propagation of human rotaviruses in other mammalian species has not been highly successful. Mebus et al. (1976) were able to propagate a group A human rotavirus in gnotobiotic calves; Fang et al. (1989) failed to propagate human group B ADRV in pigs; and Bridger et al. (1986) failed to propagate human group C rotavirus in gnotobiotic pigs.

Initial success with in vitro propagation of rotaviruses of animal origin was through the use of primary cell cultures, usually taken from the same species as the virus source. Malherbe and Harwin (1957) isolated the monkey rotavirus SA11 on primary vervet monkey kidney cells in roller tubes. Mebus et al. (1971) initially grew neonatal calf diarrhea virus (now called Nebraska calf diarrhea virus or NCDV) in primary cell cultures of bovine lung, thyroid, choroid plexus, and kidney using roller tubes. Although NCDV grew in all of these cell types, infectivity gradually decreased and back passage through a calf was necessary to rescue the virus. Fernelius et al. (1972) found that NCDV could also be propagated in the continual cell lines PK-15 (porcine kidney), Mouse L, VERO (African green monkey kidney), bovine embryonic skin (BES),

and embryonic bovine trachea (EBT).

Rotaviruses of human origin were first grown in human embryonic intestinal organ cultures in petri dishes, although Wyatt et al. (1974) reported that conditions for virus growth were not optimal. Later, Banatvala et al. (1975) found that the human origin rotaviruses could be propagated in the porcine kidney line IB-RS-2. Matsuno et al. (1977) developed a rotavirus plaque assay using the rhesus monkey kidney cell line MA104, which has now become the most commonly used cell line for rotavirus propagation. They also found that the best plaque formation occurred with the addition of trypsin (2 μg/ml) and DEAE-Dextran (100 μg/ml).

In the same year, Theil et al. (1977) reported that porcine rotaviruses could be propagated in primary porcine kidney (PPK) cells only if the viruses were incubated with pancreatin (25 μg/ml) or trypsin (1 μg/ml). While pancreatin is a mixture of trypsin, amylase, lipase, and perhaps other proteolytic enzymes, trypsin alone was found to be as effective as pancreatin. From these discoveries, the standard method of rotavirus propagation was developed: using MA104 cells in roller tubes with trypsin as the proteolytic enzyme. Once adapted in cell cultures, many rotavirus isolates could be switched from roller tubes to stationary flasks.

With the detection of the non-group A rotaviruses came the realization that this method of propagation was only

successful with the group A rotaviruses and that even some fastidious group A rotaviruses could not be grown in vitro. For instance, Ward et al. (1984) reported that only approximately 75% of human group A rotaviruses could be propagated from fecal samples.

Theil and Saif (1985) have found that group B rotaviruses could be centrifuged onto MA104 cells, allowing quantitation of virus and detection of antibodies to them. Using immunofluorescence, viral antigens could be shown to be present in the syncytia that formed. However, complete viral replication did not occur since the virus could not be passaged. Recently, after the bulk of this experiment had been completed, there appeared a brief report by Chambers et al. (1990) of successful propagation of a porcine group B rotavirus in a monkey kidney cell line using a chelating agent treatment.

Terrett and Saif (1987) were the first to propagate a group C rotavirus in vitro, using PPK cells in roller tubes with pancreatin. Pancreatin was used because, in previous experiments, it gave much higher cell culture immunofluorescence (CCIF) titers for those group C rotaviruses that were centrifuged onto the cell monolayers (Terrett et al., 1987). This virus, now known as the Cowden prototype strain, became tissue culture adapted and could be transferred into MA104 cells in roller tubes after nine passages in the

PPK cells (Saif et al., 1988). This virus would not grow in either PPK or MA104 cells in stationary flasks.

Avian (group D) rotavirus isolates have been propagated in primary chicken embryo liver cells (McNulty et al., 1981; and Todd and McNulty, 1986).

Other fastidious porcine enteric viruses may provide clues for previously non-cultivable porcine rotavirus propagation. Flynn and Saif (1988) were able to propagate a porcine enteric calicivirus-like virus in roller tubes using PPK cell cultures plus intestinal content extract; pancreatin and trypsin proved ineffective. After twenty passages in PPK cells, this virus was capable of being transferred into two continuous porcine kidney cell lines, LLC-PK and PK-GL. Intestinal content extract of porcine origin was still critical; bovine or avian intestinal content extract, alkaline phosphatase, enterokinase, elastase, protease, lipase, or no supplementation were ineffective (Parwani et al., 1990).

MATERIALS AND METHODS

Materials

Cell lines

MA104, derived from rhesus monkey kidney cells, was used for rotavirus propagation and was obtained from the National Veterinary Services Laboratory, Ames, IA. These cells were grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, NY) with 10% serum supplement (Hazelton Research Products, Inc., Lenexa, KS).

IPEC-J2 and IPEC-1 lines contain mixed populations of porcine intestinal epithelial cells that were derived from the middle jejunum and a combination of jejunum and ileum, respectively. Berschneider (1989) reported that physiologically these cells behaved like intestinal organ explants. Both cell lines were grown in Dulbecco's modified Eagle medium:nutrient mixture F-12 (Ham) 1:1 (DMEM/F12) (GIBCO Laboratories, Grand Island, NY) with 5% fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO). In addition, epidermal growth factor (5μg/l) (EGF, culture grade, Collaborative Research, Inc., Bedford, MA), insulin (5 mg/l), human transferrin (5 mg/l), and sodium selenite (5 μg/l) (ITS Premix, Collaborative Research, Inc., Bedford, MA) were added.

Cell line HEp-2, derived from a human epidermoid carcinoma of the larynx, was obtained from the American Type Culture Collection (ATCC), Rockville, MD. These cells were

grown in Eagle's minimum essential medium (MEM) with Earle's balanced salt solution (BSS) (GIBCO Laboratories, Grand Island, NY) with the addition of 10% FBS.

Proteolytic enzymes

Treatment of all rotaviruses with proteolytic enzymes has been shown to be important for in vitro propagation of groups A and C rotaviruses (Terrett and Saif, 1987). Rotaviruses were preincubated with a high concentration of proteolytic enzyme for 30 minutes, allowed to absorb onto the cells, and then refed with fresh media with a lower level of enzyme. Serum-free media was used to avoid the inhibitory effect that serum has on proteolytic enzymes.

Type IX trypsin (Sigma Chemical Co., St. Louis, MO) was used at 10 μg/ml for the 30-minute pre-incubation and at 0.5 μg/ml in the maintenance medium.

Pancreatin, a mixture of trypsin, amylase, lipase, and other pancreatic enzymes, proved the optimal proteolytic source for the initial isolation of the Cowden strain of group C rotavirus (Territt and Saif, 1987). Pancreatin 4X NF (10X) liquid (Gibco Laboratories, Grand Island, NY) was used at the levels of 150 μg/ml for pre-incubation and 75 μg/ml in the maintenance media. These concentrations did not produce cytotoxic effects on the cells.

The effect of intestinal content extract on virus propagation was also determined. The extract was prepared by

a procedure similar to that discussed by Flynn and Saif (1988). Specifically, pooled intestinal contents of two gnotobiotic pigs were diluted 1:10 with phosphate-buffered saline (PBS), clarified by centrifugation at 650xg for 30 minutes, filtered through a 0.45 μm filter, and frozen in aliquots until use. Levels used, which were found to be sublethal to the cell monolayers, were 75 μl/ml of media for pre-incubation and 37.5 μl/ml for maintenance.

Control passages were also prepared in which no proteolytic enzymes were used. All seven virus isolates were passaged in this manner to show the effect of proteolytic enzymes.

Virus isolates

One group A rotavirus, designated A1645, was used as a positive control. This virus was isolated from diarrheic pig feces and propagated in MA104 cell culture, using trypsin as the proteolytic enzyme.

Of the group B rotaviruses, B-Ohio, the group B prototype, was obtained from Dr. Kenneth Thiel (OARDC, Ohio State University). Field isolates used were designated B734 and B1146.

C-Cowden, the group C prototype, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). This isolate was propagated by ATCC in pig intestines and had never been passed in cell culture. Group C field isolates were

designated C319 and C850.

Methods

Viral amplification

The original samples of porcine fecal material obtained from the ISU Veterinary Diagnostic Laboratory were screened by PAGE to detect rotavirus-positive specimens. Since the volume of most of the positive samples was often quite small, promising isolates were propagated to produce a viral stock. Newborn gnotobiotic piglets were acquired from the National Animal Disease Center (NADC, Ames, IA), and newborn caesarian-derived, colostrum-deprived (CD/CD) piglets were acquired from the ISU Laboratory Animal Resources herd. These piglets were transferred into sterilized isolators (Class Biologically Clean, Ltd., Madison, WI), and gnotobiotic procedures were followed to prevent the introduction of extraneous organisms. The piglets were inoculated with rotavirus at three days of age. To avoid mixed infections, only one rotavirus isolate was used per incubator.

Inoculum was prepared by diluting a portion of the original sample 1:5 to 1:10, depending on the relative viral concentration estimated by PAGE, in serum-free medium. This inoculum was then dispersed by sonication for two 30-second bursts, clarified by low-speed centrifugation (650xg) for 15 minutes, and filtered through a 0.45 μm filter. The piglets were monitored for onset of gastroenteritis, and were

euthanized when the diarrhea was at its peak, usually 8 to 12 hours post-infection. Diarrhea, intestinal contents, and intestines were collected and frozen. Presence of rotavirus was confirmed by PAGE.

Sample preparation

Inoculum for cell culture attempts was prepared from rotavirus-positive fecal samples diluted 1:25 in serum-free media. This suspension was dispersed by two 30-second sonicator bursts, clarified by low-speed centrifugation (650xg for 15 minutes), and filtered through 0.45 μm filters. The inoculum was incubated for 30 minutes with pre-incubation levels of the appropriate proteolytic enzyme prior to placement on cells.

Roller tube culture

Cell lines were grown to confluency in roller tubes held in roller tube drums (Bellco, Vineland, NJ) revolving at the rate of one revolution in four minutes. Media containing 20 mM HEPES (N-[2-Hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid], Sigma Chemical Co., St. Louis, MO) buffer was used in all roller tubes to maintain the proper pH. All media used for viral cultures was supplemented with 10 ml/l antibiotic-antimycotic solution (100x liquid; 10,000 U penicillin, 10,000 μg streptomycin, and 25 μg amphotericin B/ml; Gibco Laboratories, Grand Island, NY).

Before inoculation, growth medium was removed, and the

tubes were rinsed twice with serum-free media. Each tube was inoculated with 0.2 ml of pre-incubated suspension of the 1:25 fecal material or of the previous passage. The tubes were replaced in the roller drum for one hour to allow any virus present to absorb onto the cells. After this incubation, the inoculum was removed and the cells were refed with serum-free medium containing the appropriate proteolytic enzyme at maintenance levels. The tubes were observed daily for cytopathic effect (CPE) and were harvested by freezing and thawing three times when either CPE became extensive or at the end of three days. Presence of rotavirus was determined for each sample by PAGE and/or by a fluorescent antibody assay (FA). Each isolate was passaged ten times in every combination of enzyme and cell line.

Stationary culture

Cell lines were grown to confluency in 6- or 12-well plates. Growth media was removed from the wells and replaced by inoculum, which had been treated with pre-incubation levels of proteolytic enzymes. Inoculum was either the 1:25 fecal dilution or the previous passage diluted 1:1 with serum-free medium. The 6-well plates received 2 ml/well, while the 12-well plates received 1 ml/well. The plates were then centrifuged in plate carriers for one hour at 500xg to force virus onto the cells. After centrifugation, the inoculum was aspirated and the cells were refed with serum-free medium

containing maintenance levels of proteolytic enzymes. As with the roller tubes, the plates were observed and harvested, using a cell scraper, when the CPE became extensive or after three days. Presence of rotavirus was determined by PAGE and/or FA. As with the roller tube cultures, each isolate was taken through ten passages.

Antisera production

Antisera previously prepared in rabbits from a prior experiment was used for detecting group A rotaviruses (Paul et al., 1988). Equal volumes of antisera to the Gottfried prototype (serotype 4) and to the OSU prototype (serotype 5) were diluted 1:100 in PBS for use in the fluorescent antibody assay.

The group B rotavirus prototype, B-Ohio, was obtained from Dr. Kenneth Theil, OARDC, Ohio State University. One two-week-old gnotobiotic pig was infected orally with this inoculum. When diarrhea was at its peak the next day, the pig was euthanized and intestinal contents and intestines were collected. Diarrhea collected at the time of euthanasia was positive for group B rotavirus by PAGE. The intestines that were collected were homogenized in a blender with four parts of serum-free media, clarified by low-speed centrifugation, and frozen in aliquots. A second gnotobiotic pig was infected orally with 2 ml of this intestinal homogenate. Two weeks later, this pig was injected subcutaneously in multiple sites

with 5 ml of this homogenate which had been emulsified with 5 ml of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO). Two weeks after this, the pig was inoculated intramuscularly with 1 ml of Freund's complete adjuvant emulsified with 1 ml of homogenate concentrate, which was made by pelleting 5 ml of homogenate and resuspending in 1 ml PBS. The pig died 16 days after the second immunization. A small amount of serum was harvested and frozen in aliquots.

Four gnotobiotic pigs were infected orally with the group C rotavirus prototype, C-Cowden, which was obtained from ATCC. Three pigs were euthanized at the peak of the gastroenteritis symptoms at one day postinfection. The pooled intestines were homogenized with four parts of serum-free medium, clarified by low-speed centrifugation, and frozen in aliquots. PAGE of the intestinal contents of these pigs and of a 5:1 concentrate of the intestinal homogenate was positive for group C rotavirus. An uninfected gnotobiotic pig was placed with the remaining pig in the isolator to be naturally infected with the Cowden isolate. Two weeks later, each pig received multiple subcutaneous injections comprised of 2 1/2 ml intestinal homogenate emulsified with 2 1/2 ml Freund's complete adjuvant. Two weeks later, each pig received a subcutaneous injection comprised of 1 ml of Freund's complete adjuvant emulsified with 1 ml intestinal homogenate concentrate, which was made by pelleting 50 ml of homogenate and resuspending in

2 ml PBS. Two weeks after this injection, the pigs were euthanized and serum was collected.

Before fluorescent antibody (FA) tests were performed, these antisera were tested for specificity. All antisera reacted only with viruses of the homologous serogroup.

Fluorescent antibody test

All FA tests for the roller tube passages were performed on IPEC-1 cells, as these cells gave noticeably less background fluorescence with the antisera than did MA104 cells. Roller tube culture samples were centrifuged onto monolayers as described by Theil and Saif (1985); specifically, growth medium was aspirated from the plates, the monolayers were rinsed with serum-free medium, inoculum was added, and the plates were centrifuged for 1 hour at 500xg using plate carriers. After centrifugation, the inoculum was aspirated and replaced with serum-free medium. The plates were incubated for 16 to 18 hours and then fixed as described below. Proteolytic enzymes were not used to preserve the cellular morphology. Both the stationary cultures and the centrifuged culture plates for the roller tube samples were prepared for FA by aspirating medium, fixing with cold methanol for 10 minutes, aspirating, and adding PBS for 5 minutes. The PBS was aspirated and the plate was either used immediately for FA or sealed against moisture loss and refrigerated for later use.

Antiserum dilutions were added to the appropriate wells and incubated for 30 minutes at 37^0 C. Both the group B and the group C antisera were diluted 1:40 with PBS; as mentioned above, the group A antiserum was diluted 1:100. After incubation, the antiserum was aspirated, and the wells were rinsed with PBS. The PBS was aspirated, fluorescein conjugate was added, and the plates were again incubated for 30 minutes at 37^0 C. For the group A antiserum, sheep-origin anti-rabbit IgG conjugate (lot 31589, Cappel, Organon Teknika Corp., West Chester, PA) was used at a 1:50 dilution in PBS; for groups B and C, rabbit-origin anti-swine IgG conjugate (lot 30044, Cappel) was used at a 1:50 dilution. After incubation, the conjugate was aspirated, and the wells were rinsed with PBS. Five percent glycerol in PBS was added to the wells, and the cells were examined for immunofluorescence under an ultraviolet microscope (Leitz, Wetzlar, Germany).

RESULTS

Rotavirus Propagation

Cell lines

The HEp-2 cells were extremely sensitive to proteolytic enzymes and to centrifugation, hence this cell line was not used further. In preliminary experiments, the IPEC-1 and IPEC-J2 cell lines equally supported replication of group A rotavirus. The IPEC-J2 line did, however, seem slightly more sensitive to centrifugation than did the IPEC-1 line. Also, since the IPEC-1 line contained cells derived from both the jejunum and the ileum, potentially giving a more complete array of small intestinal cell types, the use of IPEC-J2 was discontinued. Further work was concentrated on IPEC-1.

Stationary culture

Group A rotavirus

The group A rotavirus, isolate 1645, was initially detected in both cell types with all treatments. Virus concentration decreased with each passage in wells receiving no proteolytic enzyme. In the MA104 cells, there was no detectible rotavirus present at passage 10 as determined by PAGE, but the FA technique still showed a few weakly positive cells. In the IPEC-1 cell line, group A rotavirus could not be detected by passage 7 using either PAGE or FA.

In the MA104 cells with the proteolytic enzyme treatments, the group A rotavirus appeared to replicate

equally well using any of the three treatments. While titers for these group A passages were not determined, virus concentration appeared to be increasing with each passage based on the rapidity of onset and completeness of CPE, the amount of RNA seen by PAGE, and the number of positive cells seen by FA.

In IPEC-1 cells, trypsin and pancreatin treatments appeared as effective in promoting viral replication as they were in MA104 cells. With the intestinal content extract treatment of the virions, however, detectible virus decreased, so that by passage 7, no virus was detected by either PAGE or FA.

Group B and C rotaviruses

Replication of group B or C isolates in stationary culture was not detected in either cell type, although rotavirus antigen could be detected by FA in the first passage. Interestingly, even though group B rotavirus has been reported to form syncytia when centrifuged onto MA104 cells (Theil and Saif, 1985), no increased syncytia formation, beyond the few small syncytial cells that were normally present in the uninfected control wells, was seen in the IPEC-1 cell line. In this experiment, syncytia were seen in the first passage of the group B rotaviruses centrifuged onto the MA104 cells, but the numbers and sizes of syncytia seen were smaller than that reported by Theil and Saif (1985), perhaps

indicating a lower titer. No morphologic changes in either cell line were seen with the group C rotaviruses.

Roller tube culture

Group A rotavirus

In MA104 cells, isolate 1645 replicated in roller cultures much like it did in MA104 stationary culture. It replicated well with any of the three proteolytic treatments. Without any treatment, the number of fluorescent cells decreased at each passage so that there were just a few positive cells by FA at passage 10.

In IPEC-1 cells, this group A rotavirus replicated using either trypsin or pancreatin for the proteolytic treatment, but the titers estimated using PAGE or FA did not appear to be quite as high as what was seen using MA104 cells. There was no evidence of viral replication at any passage when using either intestinal content extract or no proteolytic treatment.

Group B rotavirus

There was no evidence of viral replication with any combination of cell line, treatment, and testing procedure. Syncytia were not seen even for the first passage in either MA104 or IPEC-1 cell lines.

Group C rotavirus

No evidence of group C rotavirus replication was seen in MA104 cells. In IPEC-1 cells, however, pancreatin-treated isolate C850 showed weak, but characteristic, bands on PAGE at

passage 3. By passages 4 and 5, the virus concentration increased so that intense RNA bands were visible by PAGE. The PAGE pattern of this cell culture propagated virus was the same as that of the parent virus propagated in gnotobiotic pigs. Fluorescent antibody assays, using the antisera produced in gnotobiotic pigs against the group C prototype Cowden strain, showed numerous positive cells with the staining confined to the cytoplasm, a pattern that is typical of rotavirus and most other RNA viruses (Figure 2). The cytopathic effect (CPE) observed with passages 4 and 5 was primarily an increase in the rounding of individual cells when compared to the controls, although some cell rounding and sloughing also occurred in the control tubes due to the presence of pancreatin. The formation of syncytia was not observed.

The intensity of the RNA bands became weaker at passage 6, and viral RNA could no longer be detected at passage 7 and beyond, even using 10x concentrations for the extracts. By FA, passage 6 was weakly positive, and succeeding passages were only very faintly positive or were negative. CPE, however, became much more extensive during these later passages. Electron microscopy performed on passage 7 (done by Jane Fagerland, Dept. of Veterinary Pathology, ISU) showed no rotavirus particles but did show numerous picornavirus-sized virions that were tentatively identified as enteroviruses.

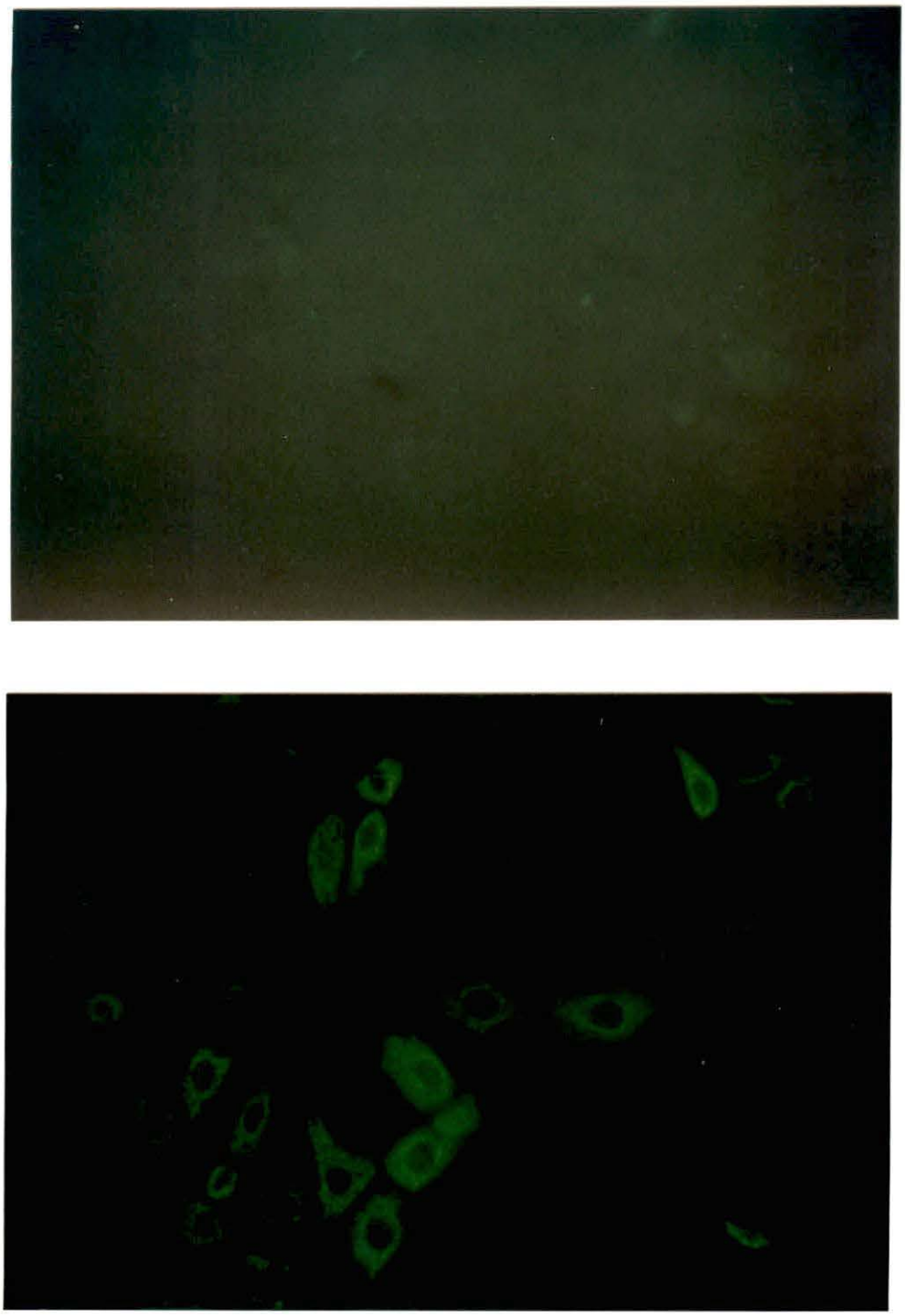

Figure 2. IFA of the IPEC-1 cell control (top) and of the fourth passage of C850 in IPEC-1 cells (bottom) prepared by centrifuging the virus onto a monolayer. Both plates were stained with antisera to Cowden, the group C prototype. Cytoplasmic staining is characteristic of rotavirus.

Numerous attempts to eliminate this contaminant were made by pre-incubating various passages (passages 3 through 11) with anti-enterovirus sera. The first antisera used were of SPF porcine and of gnotobiotic porcine origin, both directed against serotype 8, the most common serotype found in swine. The CPE decreased but not completely; PAGE and FA were still negative for rotavirus. Later, polyvalent swine-origin antisera against porcine enterovirus serotypes 1 through 8 was acquired from the National Veterinary Services Laboratory, Ames, Iowa. This caused a marked, but not total, decrease in CPE, but PAGE and FA for rotavirus continued to be negative.

Pancreatin-treated IPEC-1 passages 5 and 6 of C850 were inoculated into MA104 roller tubes, both with and without ITS and EGF in the media, and carried out to passage 10. There was a marked decrease in CPE when compared to corresponding IPEC-1 passages, but no rotavirus was detected. In an attempt to make the MA104 cells more porous to viral entry, this experiment was repeated using cells that had been pretreated for 30 minutes with media containing 25 μg/ml of DEAE-Dextran (diethyl-aminoethyl-Dextran, Sigma Chemical Co., St. Louis, MO). Again, no rotavirus was detected by either PAGE or FA.

Of the other pancreatin-treated group C rotaviruses, C319 was not detected by either method at any passage. Cowden was never detected by PAGE; however, a very few FA-positive cells were seen at passage 6 of Cowden in the IPEC-1 cells. Passage

7 was ambiguous at best, and passage 8 was clearly negative. EM of passage 7 showed some enterovirus particles but no rotavirus.

Group C rotaviruses treated with the other proteolytic enzymes gave no evidence of replication when tested by PAGE or by FA. Also, the CPE that appeared to be due to the enterovirus contaminant was not seen with other proteolytic treatments.

DISCUSSION

Rotavirus Propagation

Group A rotavirus

The culture-adapted group A rotavirus, isolate 1645, which served as a positive control, also allowed some comparisons between the two cell lines. This isolate was easily propagated in both cell lines using either stationary or roller tube systems when pancreatin or trypsin were used as the proteolytic enzyme treatments.

In the passages with no proteolytic enzyme treatment, the amount of detectible virus decreased in each successive passage in stationary culture. This was expected given the requirement of group A rotaviruses for proteolytic cleavage of VP4 to allow maximum infectivity. Graham and Estes (1980), using MA104 and other kidney-origin continuous cell lines, showed that trypsin enhanced viral titer in cell cultures 10- to 1000-fold. The decrease of titer was more rapid in the stationary IPEC-1 cells than in the MA104 cells. In the IPEC-1 roller tube cultures, virus was not detected at any passage.

When intestinal content extract was used, the group A rotavirus was propagated in MA104 cells much as if trypsin or pancreatin were used. In IPEC-1 cells, virus was no longer detected by passage 7 in stationary culture, and virus was not detected in any passage in the roller tube cultures.

The information above allows some comparisons to be

drawn:

1. Under less than optimal enzymatic conditions for rotaviral propagation, the more rapid loss of viral titer in the stationary IPEC-1 cells implied that MA104 cells are more favorable to the growth of this cell-adapted group A rotavirus than are the IPEC-1 cells. However, since isolate 1645 was originally propagated and cell-adapted in MA104 cells, this cell preference may only apply to those group A rotaviruses that have been previously propagated in cell culture.
2. This difference of cell tropism was even more marked in the roller tube cultures, implying that proteolytic enzymes are essential for viral replication of group A rotaviruses in IPEC-1 cells. Since the roller tube technique relied upon normal virus-host cell interactions for viral uptake instead of using centrifugation of the virus onto the cells, the varied results between roller tube and stationary cell culture systems were not surprising.
3. Testing of these terminal no-treatment passages for viral presence showed that the FA technique was more sensitive than PAGE for detecting low numbers of virus.

4. In IPEC-1 cells, intestinal content extract proved to be much less effective than either trypsin or pancreatin. In fact, it was no better than using no proteolytic enzyme treatment. However, the intestinal content extract did prove to be an effective treatment with the MA104 cells.

Group B rotavirus

Using the methods of this study, the IPEC-1 cells proved no more able to support the growth of group B rotaviruses than MA104 cells. Theil and Saif (1985) first reported that group B rotavirus caused the formation of syncytia when centrifuged onto MA104 cells, even though no replication occurred. This was also observed in this study with the MA104 cells. However, no syncytia were seen in the MA104 roller tubes, apparently indicating that no internalization of virus occurred.

In contrast, no increase in syncytia was seen in the IPEC-1 cells in either stationary or roller tube systems, even though group B rotaviral antigens could be detected by FA in the first passage of the stationary culture. Since the positive FA would appear to indicate cellular entry, the IPEC-1 cells, when used under the parameters of this experiment, seem to be unfavorable to syncytial formation by group B rotaviruses.

Group C rotavirus

Despite the inability to eliminate the enterovirus contaminant to allow the production of a relatively high concentration of group C rotavirus in pure culture for characterization, the use of pancreatin and IPEC-1 cells in roller tubes has been shown to be another method of propagating this fastidious serogroup. The results indicate that this group C isolate was indeed propagated in cell culture, rather than the original inoculum was detected for a few passages without replication, since the intensity of the RNA bands increased from passages 3 to 5. The identical PAGE pattern of this in vitro propagated virus with that of the fecal-origin field isolate provided evidence that the cultivated virus was group C and that there was no recombination with a more easily cultivable group A rotavirus. The positive FA with anti-Cowden serum also showed that this was not likely a recombinant, since the anti-Cowden sera was shown to be specific for group C rotaviruses.

If there is some variation in cultural requirements of this serogroup, as there appears to be within the group A rotaviruses, a number of methods may be necessary to allow the propagation of representative group C rotavirus field isolates, and the IPEC-1 cells may prove useful in doing this. Ideally, the field isolates could be initially propagated in the IPEC-1 cells until they are adapted to cell culture and

then transferred to MA104 cells, as was done with the initial cultivation of the Cowden strain in swine embryonic kidney and MA104 cell lines by Terrett and Saif (1987). This would allow for a more standardized characterization of isolates, and it would also be a more efficient use of laboratory time and resources. MA104 is preferable as the final cell line for propagating these viruses because the IPEC-1 cell line is more demanding of media supplements and is restrictive in its growth characteristics: the maximum split ratio is 1:2.5, and at least 7 days are required to reach confluency.

Attempts in this study to transfer the group C rotavirus from IPEC-1 cells to MA104 cells probably failed for two reasons: the enterovirus contaminant overgrew and out-competed the group C isolate, and not enough replication cycles of the group C isolate occurred before this enterovirus overgrowth to allow total adaptation of the group C rotavirus isolate to cell culture. Saif et al. (1988) needed to pass the Cowden isolate nine times in PPK cells before they were able to make this transfer to MA104 cells. The marked loss of enterovirus CPE seen with the transfer from IPEC-1 to MA104 cells seemed to imply that the enterovirus greatly preferred the IPEC-1 cell line to the MA104 line; perhaps, if enough passages to allow adaptation of the group C rotavirus had occurred, this transfer may have been useful in eliminating the enterovirus. The origin of the enterovirus contaminant

was most likely from the C850 field sample, with subsequent multiplication of the virus during passage. Other pancreatin-treated IPEC-1 roller tubes that were used for the group B and the C319 isolates did not show evidence of the enterovirus.

CONCLUSION

The IPEC-1 cell line used in roller tubes, plus pancreatin as the proteolytic enzyme, shows promise as another method to propagate the fastidious group C rotaviruses. A majority of group B and C rotavirus isolates, and still many group A isolates, have yet to be propagated *in vitro* for characterization and serotyping. Because of this, it is possible that important members of these serogroups have not yet been reported. It may be necessary to use a combination of various cell lines, including both IPEC-1 and MA104, with appropriate enzyme or chelating agent treatment to adequately propagate representative samples of the various serogroups for better characterization and possibly vaccine production.

LITERATURE CITED

Askaa, J., and B. Bloch. 1984. Infection in piglets with a porcine rotavirus-like virus. Experimental inoculation and ultrastructural examination. Arch. Virol. 80:291-303.

Askaa, J., B. Bloch, G. Bertelsen, and K. O. Rasmussen. 1983. Rotavirus associated diarrhoea in nursing piglets and detection of antibody against rotavirus in colostrum, milk, and serum. Nord. Vet. Med. 35:441-447.

Banatvala, J. E., B. Totterdell, I. L. Chrystie, and G. N. Woode. 1975. In vitro detection of human rotavirus. Lancet ii:821.

Bastardo, J. W., and I. H. Holmes. 1980. Attachment of SA-11 rotavirus to erythrocyte receptors. Infect. Immun. 29:1134-1140.

Benfield, D. A., I. J. Stotz, E. A. Nelson, and K. S. Groon. 1984. Comparison of a commercial enzyme-linked immunosorbent assay with electron microscopy, fluorescent antibody, and virus isolation for the detection of bovine and porcine rotavirus. Am. J. Vet. Res. 45:1998-2002.

Berschneider, H. M. 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology 96:A41. (Abstr.)

Bican, P., J. Cohen, A. Charpiliene, and R. Scherrer. 1982. Purification and characterization of bovine rotavirus cores. J. Virol. 43:1113-1117.

Bohl, E. H., E. M. Kohler, L. J. Saif, R. F. Cross, A. G. Agnes, and K. W. Theil. 1978. Rotavirus as a cause of diarrhea in pigs. J. Am. Vet. Med. Assoc. 172:458-463.

Bohl, E. H., L. J. Saif, K. W. Theil, A. G. Agnes, and R. F. Cross. 1982. Porcine pararotavirus: detection, differentiation from rotavirus, and pathogenesis in gnotobiotic pigs. J. Clin. Microbiol. 15:312-319.

Both, G. W., L. J. Siegman, A. R. Bellamy, N. Ikegami, A. J. Shatkin, and Y. Furuichi. 1984. Comparative sequence analysis of rotavirus genomic segment 6--the gene specifying viral subgroups 1 and 2. J. Virol. 51:97-101.

Boyle, J. F., and K. V. Holmes. 1986. RNA-binding proteins

of bovine rotavirus. J. Virol. 58:561-568.

Brandt, C. D., H. W. Kim, W. J. Rodriguez, L. Thomas, R. H. Yolken, J. O. Arrobio, A. Z. Kapikian, R. H. Parrott, and R. M. Chanock. 1981. Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis virus in children. J. Clin. Microbiol. 13:976-981.

Bremont, M., J. Cohen, and M. A. McCrae. 1988. Analysis of the structural polypeptides of a porcine group C rotavirus. J. Virol. 62:2183-2185.

Bridger, J. C. Novel rotaviruses in animals and man. 1987. CIBA Found. Symp. 128:6-23.

Bridger, J. C., and J. F. Brown. 1985. Prevalence of antibody to typical and atypical rotavirus in pigs. Vet. Rec. 116:50.

Bridger, J. C., S. Pedley, and M. A. McCrae. 1986. Group C rotaviruses in humans. J. Clin. Microbiol. 23:760-763.

Brown, D. W. G., G. M. Beards, G.-M. Chen, and T. H. Flewett. 1987. Prevalence of antibody to group B (atypical) rotavirus in humans and animals. J. Clin. Microbiol. 25:316-319.

Brown, D. W. G., M. M. Mathan, M. Mathew, R. Martin, G. M. Beards, and V. I. Mathan. 1988. Rotavirus epidemiology in Vellore, South India: group, subgroup, serotype, and electropherotype. J. Clin. Microbiol. 26:2410-2414.

Chambers, D. M., M. W. Welter, and C. J. Welter. 1990. Propagation of porcine group B rotavirus in a continuous cell line. CRWAD 71:16. (Abstr.)

Chasey, D. 1977. Different particle types in tissue culture and intestinal epithelium infected with rotavirus. J. Gen. Virol. 37:443-451.

Chasey, D., and J. Banks. 1986. Replication of atypical ovine rotavirus in small intestine and cell culture. J. Gen. Virol. 67:567-576.

Chasey, D., R. J. Higgins, M. Jeffrey, and J. Banks. 1989. Atypical rotavirus and villous epithelial cell syncytia in piglets. J. Comp. Path. 100:217-222.

Chen, G.-M., T. Hung, J. C. Bridger, and M. A. McCrae. 1985. Chinese adult rotavirus is a group B rotavirus. Lancet ii:1123-1124.

Cheung, E. Y., S. I. Hnatko, H. Gunning, and J. Wilson. 1982. Comparison of rotazyme and direct electron microscopy for detection of rotavirus in human stools. J. Clin. Microbiol. 16:562-563.

Chriatensen, M. L. 1989. Human viral gastroenteritis. Clin. Microbiol. Rev. 2:51-89.

Clark, S. M., J. R. Roth, M. L. Clark, B. B. Barnett, and R. S. Spendlove. 1981. Trypsin enhancement of rotavirus infectivity: mechanism of enhancement. J. Virol. 39:816-822.

Cohen, J., A. Charpilienne, S. Chilmonczyk, and M. K. Estes. 1989. Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. Virology 171:131-140.

Cornell, W. D., H. K. Whitaker, J. Craft, and C. Alderson. 1982. A comparison of negative contrast electron microscopy and the enzyme-linked immunosorbent assay in the detection of rotavirus infection in pigs and calves. Proc. Annu. Meet. Am. Assoc. Vet. Lab. Diagn. 25:209-220.

Coulson, B. S., L. E. Unicomb, G. A. Pitson, and R. F. Bishop. 1987. Simple and specific enzyme immunoassay using monoclonal antibodies for serotyping human rotaviruses. J. Clin. Microbiol. 25:509-515.

Crouch, C. F., and G. N. Woode. 1978. Serial studies of virus multiplication and intestinal damage in gnotobiotic piglets infected with rotavirus. J. Med. Microbiol. 11:325-334.

Dai, G., M. Sun, S. Liu, X. Ding, Y. Chen, L. Wang, D. Du, G. Zhao, Y. Su, J. Li, W. Xu, T. Li, and X. Chen. 1987. First report of an epidemic of diarrhoea in human neonates involving the new rotavirus and biological characteristics of the epidemic virus strain (KMB/R85). J. Med. Virol. 22:365-373.

Delem, A., and T. Vesikari. 1987. Detection of serum antibody responses to RIT 4237 rotavirus vaccine by ELISA and neutralization assays. J. Med. Virol. 21:231-238.

Dimitrov, D. H., D. Y. Graham, and M. K. Estes. 1985.

Detection of rotavirus by nucleic acid hybridization with cloned DNA of simian rotavirus SA11 genes. J. Infect. Dis. 152:293-300.

Doern, G. V., J. E. Herrmann, P. Henderson, D. Stobbs-Walro, D. M. Perron, and N. R. Blacklow. 1986. Detection of rotavirus with a new polyclonal antibody enzyme immunoassay (Rotazyme II) and a commercial latex agglutination test (Rotalex): comparison with a monoclonal antibody enzyme immunoassay. J. Clin Microbiol. 23:226-229.

Eiden, J., J. Kinney, S. Vonderfecht, K. Theil, and R. Yolken. 1986. Gastroenteritis due to a newly recognized rotavirus-like agent (RVLA), identification of the virus as a group B rotavirus and association with nosocomial gastroenteritis in a nursery. Clinical Res. 34:850A. (Abstr.)

Eiden, J., H. M. Lederman, S. Vonderfecht, and R. Yolken. 1986. T-cell-deficient mice display normal recovery from experimental rotavirus infection. J. Virol. 57:706-708.

Eiden, J., S. Vonderfecht, K. Theil, A. Torres-Medina, and R. H. Yolken. 1986. Genetic and antigenic relatedness of human and animal strains of antigenically distinct rotaviruses. J. Infect. Dis. 154:972-982.

Eiden, J., S. Vonderfecht, and R. H. Yolken. 1985. Evidence that a novel rotavirus-like agent of rats can cause gastroenteritis in man. Lancet ii:8-11.

Ellens, D. J., and P. W. DeLeeuw. 1977. Enzyme-linked immunosorbent assay for diagnosis of rotavirus infections in calves. J. Clin. Microbiol. 6:530-532.

Espejo, R. T., F. Puerto, C. Soler, and N. Gonzalez. 1984. Characterization of a human pararotavirus. Infect. Immun. 44:112-116.

Estes, M. K. 1990. Rotaviruses and their replication. Pages 1329-1352 in B. N. Fields and D. M. Knipe, eds. Virology. Second edition. Raven Press, Ltd., N. Y.

Estes, M. K., and J. Cohen. 1989. Rotavirus gene structure and function. Microbiol. Rev. 53:410-449.

Estes, M. K., and D. Y. Graham. 1980. Identification of rotaviruses of different origins by the plaque reduction test. Am. J. Vet. Res. 41:151-152.

Estes, M. K., D. Y. Graham, and D. H. Dimitrov. 1984. The molecular epidemiology of rotavirus gastroenteritis. Pages 1-22 in J. L. Melnick, ed. Progress in medical virology. S. Karger, Basel.

Estes, M. K., D. Y. Graham, and B. B. Mason. 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. 39:879-888.

Fang, Z.-Y., R. I. Glass, M. Penaranda, H. Dong, S. S. Monroe, L. Wen, M. K. Estes, J. Eiden, R. H. Yolken, L. Saif, V. Gouvea, and T. Hung. 1989. Purification and characterization of adult diarrhea rotavirus: identification of viral structural proteins. J. Virol. 63:2191-2197.

Fernelius, A. L., A. E. Ritchie, L. G. Classik, J. O. Norman, and C. A. Mebus. 1972. Cell culture adaption and propagation of a reovirus-like agent of calf diarrhea from a field outbreak in Nebraska. Arch. Gesamte Virusforsch. 37:114-130.

Flewett, T. H. 1976. Diagnosis of enteritis virus. Proc. R. Soc. Med. 69:693-696.

Flores, J., E. Boeggeman, R. H. Purcell, M. Sereno, I. Perez, L. White, R. G. Wyatt, R. M. Chanock, and A. Z. Kapikian. 1983. A dot hybridization assay for detection of rotavirus. Lancet i:555-559.

Flores, J., K. Y. Green, D. Garcia, J. Sears, I. Perez-Schael, L. F. Avendano, W. B. Rodriguez, K. Taniguchi, S. Urasawa, and A. Z. Kapikian. 1989. Dot hybridization assay for distinction of rotavirus serotypes. J. Clin. Microbiol. 27:29-34.

Flores, J., K. Midthun, Y. Hoshino, K. Green, M. Gorziglia, A. Z. Kapikian, and R. M. Chanock. 1986. Conservation of the fourth gene among rotaviruses recovered from asymptomatic newborn infants and its possible role in attenuation. J. Virol. 60:972-979.

Flynn, W. T., and L. J. Saif. 1988. Serial propagation of porcine enteric calicivirus-like virus in primary porcine kidney cell cultures. J. Clin. Microbiol. 26:206-212.

Fu, Z. F. 1987. Detection of an atypical (possibly group C) rotavirus in New Zealand pigs. N. Z. Vet. J. 35:115-116.

Fu, Z. F., D. K. Blackmore, D. J. Hampson, and C. R. Wilks.

1989. Epidemiology of typical and atypical rotavirus infections in New Zealand pigs. N. Z. Vet. J. 37:102-106.

Fukuhara, N., O. Yoshie, S. Kitaoka, and T. Konno. 1988. Role of VP3 in human rotavirus internalization after target cell attachment via VP7. J. Virol. 62:2209-2218.

Gaul, S. K., T. F. Simpson, G. N. Woode, and R. W. Fulton. 1982. Antigenic relationships among some animal rotaviruses: virus neutralization in vitro and cross-protection in piglets. J. Clin. Microbiol. 16:495-503.

Gelberg, H. B., W. F. Hall, G. N. Woode, E. J. Basgall, and G. Scherba. 1990. Multinucleate enterocytes associated with experimental group A porcine rotavirus infection. Vet. Pathol. 27:453-454.

Gilchrist, M. J. R., T. S. Bretl, K. Moultney, D. R. Knowlton, and R. L. Ward. 1987. Comparison of seven kits for detection of rotavirus in fecal specimens with a sensitive, specific enzyme immunoassay. Diagn. Microbiol. Infect. Dis. 8:221-228.

Glass, R. I., J. Keith, O. Nakagomi, T. Nakagomi, J. Askaa, A. Z. Kapikian, R. M. Chanock, and J. Flores. 1985. Nucleotide sequence of the structural glycoprotein VP7 gene of Nebraska calf diarrhea virus rotavirus: comparison with homologous genes from four strains of human and animal rotaviruses. Virology 141:292-298.

Goyal, S. M., R. A. Rademacher, and K. A. Pomeroy. 1987. Comparison of electron microscopy with three commercial tests for the detection of rotavirus in animal feces. Diagn. Microbiol. Infect. Dis. 6:249-254.

Graham, D. Y., and M. K. Estes. 1980. Proteolytic enhancement of rotavirus infectivity: biologic mechanisms. Virology 101:432-439.

Grauballe, P. C., J. Genner, A. Meyling, and A. Hornsleth. 1977. Rapid diagnosis of rotavirus infections: comparison of electron microscopy and immunoelectro-osmophoresis for the detection of rotavirus in human infantile gastroenteritis. J. Gen. Virol. 35:203-218.

Grauballe, P. C., B. F. Vestergaard, A. Meyling, and J. Genner. 1980. Optimized enzyme-linked immunosorbent assay for detection of human and bovine rotavirus in stools: comparison with electron microscopy,

immunoelectro-osmophoresis and fluorescent antibody techniques. J. Med.Virol. 7:29-40.

Green, K. Y., K. Midthun, M. Gorziglia, Y. Hoshino, A. Z. Kapikian, R. M. Chanock, and J. Flores. 1987. Comparison of the amino acid sequences of the major neutralization protein of four human rotavirus serotypes. Virology 161:153-159.

Green, K. Y., J. F. Sears, K. Taniguchi, K. Midthun, Y. Hoshino, M. Gorziglia, K. Nishikawa, S. Urasawa, A. Z. Kapikian, R. M. Chanock, and J. Flores. 1988. Prediction of human rotavirus serotype by nucleotide sequence analysis of the VP7 protein gene. J. Virol. 62:1819-1823.

Hammond, G. W., G. S. Ahluwalia, F. G. Barker, G. Horsman, and P. R. Hazelton. 1982. Comparison of direct and indirect enzyme immunoassays with direct ultracentrifugation before electron microscopy for detection of rotaviruses. J. Clin. Microbiol. 16:53-59.

Hammond, G. W., G. S. Ahluwalia, B. Klisko, and P. Hazelton. 1984. Human rotavirus detection by counter-immunoelectrophoresis versus enzyme immunoassay and electron microscopy after direct ultracentrifugation. J. Clin. Microbiol. 19:439-441.

Herring, A. J., N. F. Inglis, C. K. Ojeh, D. R. Snodgrass, and J. D. Menzies. 1982. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J. Clin. Microbiol. 16:473-477.

Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, R. M. Chanock, and A. Z. Kapikian. 1987. Analysis by plaque reduction neutralization assay of intertypic rotaviruses suggests that gene reassortment occurs in vivo. J. Clin. Microbiol. 25:290-294.

Hoshino, Y., R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian. 1984. Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque-reduction neutralization. J. Infect. Dis. 149:694-702.

Huber, A. C., R. H. Yolken, L. C. Mader, J. D. Strandberg, and S. L. Vonderfecht. 1989. Pathology of infectious diarrhea of infant rats (IDIR) induced by an antigenically distinct rotavirus. Vet. Pathol. 26:376-

385.

Hughes, J. H., A. V. Tuomari, D. R. Mann, and V. V. Hamparian. 1984. Latex immunoassay for rapid detection of rotavirus. J. Clin. Microbiol. 20:441-447.

Hung, T. 1988. Rotavirus and adult diarrhea. Adv. Virus Res. 35:193-218.

Hung, T., R. Fan, C. Wang, G. Chen, D. Chou, J. Chang, M. A. McCrae, W. Wang, W. Se, R. Dan, and M. H. Ng. 1985. Seroepidemiology of adult rotavirus. Lancet ii:325-326.

Hung, T., C. Wang, Z. Fang, Z. Chou, X. Chang, X. Liong, G. Chen, H. Yao, T. Chao, W. Ye, S. Den, and W. Chang. 1984. Waterborne outbreak of rotavirus diarrhea in adults in China caused by a novel rotavirus. Lancet i:1139-1142.

Janke, B. H., L. G. Morehouse, and R. F. Solorzano. 1988. Single and mixed infections of neonatal pigs with rotaviruses and enteroviruses: clinical signs and microscopic lesions. Can. J. Vet. Res. 52:360-363.

Jiang, B. M., L. J. Saif, S. Y. Kang, and J. H. Kim. 1990. Biochemical characterization of the structural and nonstructural polypeptides of a porcine group C rotavirus. J. Virol. 64:3171-3178.

Kalica, A. R., J. Flores, and H. B. Greenberg. 1983. Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. Virology 125:194-205.

Kaljot, K. T., R. D. Shaw, D. H. Rubin, and H. B. Greenberg. 1988. Infectious rotavirus enters cells by direct cell membrane penetration, not endocytosis. J. Virol. 62:1136-1144.

Kapikian, A. Z., and R. M. Chanock. 1990. Rotaviruses. Pages 1353-1404 in B. N. Fields and D. M. Knipe, eds. Virology. Second edition. Raven Press, Ltd., N. Y.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lecce, J. G., and M. W. King. 1978. Role of rotavirus (reo-like) in weanling diarrhea of pigs. J. Clin. Microbiol. 8:454-458.

Lin, M., M. Imai, N. Ikegami, A. R. Bellamy, D. Summers, D. L. Nuss, R. Deibel, and Y. Furuichi. 1987. cDNA probes of individual genes of human rotavirus distinguish viral subgroups and serotypes. J. Virol. Methods 15:285-289.

Liu, M., P. A. Offit, and M. K. Estes. 1988. Identification of the simian rotavirus SA11 genome segment 3 product. Virology 163:26-32.

Losonsky, G. A., J. P. Johnson, J. A. Winkelstein, and R. H. Yolken. 1985. Oral administration of human serum immunoglobulin in immunodeficient patients with viral gastroenteritis. J. Clin. Invest. 76:2362-2367.

Losonsky, G. A., M. B. Runnels, Y. Kim, G. Krall, A. Z. Kapikian, and M. M. Levine. 1988. Systemic and mucosal immune responses to rhesus rotavirus vaccine MMU 18006. Pediatr. Infect. Dis. J. 7:388-393.

Ludert, J. E., F. Michelangi, F. Gill, F. Liprandi, and J. Esparza. 1987. Penetration and uncoating of rotaviruses in cultured cells. Intervirology 27:95-101.

Mackow, E. R., R. D. Shaw, S. M. Matsui, P. T. Vo, M. N. Dang, and H. B. Greenberg. 1988. Characterization of the rhesus rotavirus VP3 gene: location of amino acids involved in homologous and heterologous rotavirus neutralization and identification of a putative fusion region. Proc. Natl. Acad. Sci. USA 85:645-649.

Malherbe, H., and R. Harwin. 1957. Seven viruses isolated fron the vervet monkey. Brit. J. Exp. Path. 38:539-541.

Matsumoto, K., M. Hatano, K. Kobayashi, A. Hasegawa, S. Yamazaki, S. Nakata, S. Chiba, and Y. Kimura. 1989. An outbreak of gastroenteritis associated with acute rotaviral infection in schoolchildren. J. Infect. Dis. 160:611-615.

Matsuno, S., S. Inouye, and R. Kono. 1977. Plaque assay of neonatal calf diarrhea virus and the neutralizing antibody in human sera. J. Clin. Microbiol. 5:1-4.

Mattion, N. M., R. C. Bellinzoni, J. O. Blackwell, J. L. La Torre, and E. A. Scodeller. 1989. Antigenic characterization of swine rotaviruses in Argentina. J. Clin. Microbiol. 27:795-798.

McAdaragh, J. P., M. E. Bergeland, R. C. Meyer, M. W. Johnshoy, I. J. Stotz, D. A. Benfield, and R. Hammer.

1980. Pathogenesis of rotaviral enteritis in gnotobiotic pigs: a microscopic study. Am. J. Vet. Res. 41:1572-1581.

McGuire, S. J., and A. E. Castro. 1982. Rapid diagnosis of rotavirus by an enzyme-linked immunosorbent assay (ELISA) in an outbreak of neonatal diarrhea in dairy calves. Bovine Pract. 17:66-71.

McNulty, M. S., G. M. Allan, D. Todd, J. B. McFerran, and R. M. McCracken. 1981. Isolation from chickens of a rotavirus lacking the rotavirus group antigen. J. Gen. Virol. 55:405-413.

McNulty, M. S., G. R. Pearson. J. B. McFerran, D. S. Collins, and G. M. Allan. 1976. A reovirus-like agent (rotavirus) associated with diarrhea in neonatal pigs. Vet. Microbiol. 1:55-63.

Mebus, C. A. 1980. Reovirus and coronavirus infections. Pages 127-136 in H. E. Amstutz, ed. Bovine medicine and surgery. Vol. I. American Veterinary Publications, Santa Barbara, CA.

Mebus, C. A., M. Kono, N. R. Underdahl, and M. J. Twiehaus. 1971. Cell culture propagation of neonatal calf diarrhea (scours) virus. Can. Vet. Jour. 12:69-72.

Mebus, C. A., M. B. Rhodes, and N. R. Underdahl. 1978. Neonatal calf diarrhea caused by a virus that induces villous epithelial cell syncytia. Am. J. Vet. Res. 39:1223-1228.

Mebus, C. A., R. G. Wyatt, R. L. Sharpee, M. M. Sereno, A. R. Kalica, A. Z. Kapikian, and M. J. Twiehaus. 1976. Diarrhea in gnotobiotic calves caused by the reovirus-like agent of human infantile gastroenteritis. Infect. Immun. 14:471-474.

Nagesha, H. S., C. P. Hum, J. C. Bridger, and I. H. Holmes. 1988. Atypical rotaviruses in Australian pigs. Arch. Virol. 102:91-98.

Nakagomi, T., and O. Nakagomi. 1989. RNA-RNA hybridization identifies a human rotavirus that is genetically related to feline rotavirus. J. Virol. 63:1431-1434.

Nakata, S., M. K. Estes, D. Y. Graham, R. Loosle, T. Hung, S. Wang, L. J. Saif, and J. L. Melnick. 1986. Antigenic characterization and ELISA detection of adult diarrhea

rotavirus. J. Infect. Dis. 154:448-455.

Nakata, S., M. K. Estes, D. Y. Graham, S. Wang, G. W. Gary, and J. L. Melnick. 1987. Detection of antibody to group B adult diarrhea rotaviruses in humans. J. Clin. Microbiol. 25:812-818.

Nakata, S., B. L. Petrie, E. P. Calomeni, and M. K. Estes. 1987. Electron microscopy procedure influences detection of rotaviruses. J. Clin. Microbiol. 25:1902-1906.

Offit, P. A., G. Blavat, H. B. Greenberg, and H. F. Clark. 1986. Molecular basis of rotavirus virulence: role of gene segment 4. J. Virol. 57:46-49.

Offit, P. A., and H. F. Clark. 1985. Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. J. Virol. 54:58-64.

Offit, P. A., R. D. Shaw, and H. B. Greenberg. 1986. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins VP3 and VP7. J. Virol. 58:700-703.

Okada, Y., M. A. Richardson, N. Ikegami, A. Nomoto, and Y. Furuichi. 1984. Nucleotide sequence of human rotavirus genome segment 10, an RNA encoding a glycosylated virus protein. J. Virol. 51:856-859.

Parwani, A. V., W. T. Flynn, K. L. Gadfield, and L. J. Saif. 1990. Serial propagation of porcine enteric calicivirus in continuous cell lines and effects of medium supplementation with various additives. CRWAD 71:41. (Abstr.)

Paul, P. S., Y. S. Lyoo, J. J. Andrews, and H. T. Hill. 1988. Isolation of two new serotypes of porcine rotavirus from pigs with diarrhea. Arch. Virol. 100:139-143.

Pearson, G. R., and M. S. McNulty. 1977. Pathological changes in the small intestine of neonatal pigs infected with a pig reovirus-like agent (rotavirus). J. Comp. Path. 87:363-375.

Pedley, S., J. C. Bridger, J. F. Brown, and M. A. McCrae. 1983. Molecular characterization of rotaviruses with distinct group antigens. J. Gen. Virol. 64:2093-2101.

Penaranda, M. E., W. D. Cubitt, P. Sinarachatanant, D. N.

Taylor, S. Likanonsakul, L. Saif, and R. I. Glass. 1989. Group C rotavirus infections in patients with diarrhea in Thailand, Nepal, and England. J. Infect. Dis. 160:392-397.

Prey, M. U., C. A. Lorelle, T. A. Taff, L. Sonsouchie, M. S. Webb, T. D. Gardner, and T. I. Aquino. 1988. Evaluation of three commercially available rotavirus detection methods for neonatal specimens. Am. J. Clin. Path. 89:675-678.

Qiu, F. X., Y. Tian, J. C. Liu, X. S. Zhang, and Y. P. Hao. 1986. Antibody against adult diarrhea rotavirus among healthy adult population in China. J. Virol. Methods 14:127-132.

Riepenhoff-Talty, M., T. Dharakul, E. Kowalski, S. Michalak, and P. L. Ogra. 1987. Persistent rotavirus infection in mice with severe combined immunodeficiency. J. Virol. 61:3345-3348.

Rodger, S. M., R. F. Bishop, and I. H. Holmes. 1982. Detection of a rotavirus-like agent associated with diarrhea in an infant. J. Clin. Microbiol. 16:724-726.

Rubenstein, A. S., and M. F. Miller. 1982. Comparison of an enzyme immunoassay with electron microscopic procedures for detecting rotavirus. J. Clin. Microbiol. 15:938-944.

Ryder, R. W., R. H. Yolken, W. C. Reeves, and R. B. Sack. 1986. Enzootic bovine rotavirus is not a source of infection in Panamanian cattle ranchers and their families. J. Infect. Dis. 153:1139-1144.

Saif, L. J., E. H. Bohl, K. W. Theil, R. F. Cross, and J. A. House. 1980. Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. J. Clin. Microbiol. 12:105-111.

Saif, L. J., L. A. Terrett, K. L. Miller, and R. F. Cross. 1988. Serial propagation of porcine group C rotavirus (pararotavirus) in a continuous cell line and characterization of the passaged virus. J. Clin. Microbiol. 26:1277-1282.

Saif, L. J., and K. W. Theil. 1985. Antigenically distinct rotaviruses of human and animal origin. Pages 208-214 in S. Tzipori et al., eds. Infectious diarrhoea in the young. Elsevier Science Publishing, Inc., Amsterdam.

Saif, L. J., K. W. Theil, and E. H. Bohl. 1978. Morphogenesis of porcine rotavirus in porcine kidney cell cultures and intestinal epithelial cells. J. Gen. Virol. 39:205-217.

Sanekata, T., Y. Yoshida, K. Oda, and H. Okada. 1982. Detection of rotavirus antibody by inhibition of reverse passive hemagglutination. J. Clin. Microbiol. 15:148-155.

Shaw, D. P., L. G. Morehouse, and R. F. Solorzano. 1989. Experimental rotavirus infection in three-week-old pigs. Am. J. Vet. Res. 50:1961-1965.

Stevenson, G. W. 1990. Pathogenesis of a new porcine serotype of group A rotavirus in neonatal gnotobiotic and weaned conventional pigs. Ph.D. Thesis. Iowa State University (Call number ISU 1990 St48). 123 pp.

Straw, B. E. Differential diagnosis of swine diseases. 1986. Pages 214-242 in A. D. Leman et al., eds. Diseases of swine. Sixth edition. Iowa State University Press, Ames, Iowa.

Su, C., Y. Wu, H. Shen, D. Wang, Y. Chen, D. Wu, L. He, and Z. Yang. 1986. An outbreak of epidemic diarrhoea in adults caused by a new rotavirus in Anhui province of China in the summer of 1983. J. Med. Virol. 19:167-173.

Taniguchi, K., T. Urasawa, Y. Morita, H. B. Greenberg, and S. Urasawa. 1987. Direct serotyping of human rotavirus in stools by an enzyme-linked immunosorbent assay using serotypes 1-,2-,3-, and 4-specific monoclonal antibodies to VP7. J. Infect. Dis. 155:1159-1166.

Terrett, L. A., and L. J. Saif. 1987. Serial propagation of porcine rotavirus (pararotavirus) in primary porcine kidney cell cultures. J. Clin. Microbiol. 25:1316-1319.

Terrett, L. A., L. J. Saif, K. W. Theil, and E. M. Kohler. 1987. Physicochemical characterization of porcine pararotavirus and detection of virus and viral antibodies using cell culture immunofluorescence. J. Clin. Microbiol. 25:268-272.

Theil, K. W., E. H. Bohl, and A. G. Agnes. 1977. Cell culture propagation of porcine rotavirus (reovirus-like agent). Am. J. Vet. Res. 38:1765-1768.

Theil, K. W., E. H. Bohl, R. F. Cross, E. M. Kohler, and A. G.

Agnes. 1978. Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. Am. J. Vet. Res. 39:213-220.

Theil, K. W., and L. J. Saif. 1985. In vitro detection of procine rotavirus-like virus (group B rotavirus) and its antibody. J. Clin. Microbiol. 21:844-846.

Todd, D., and M. S. McNulty. 1986. Electrophoretic variation of avian rotavirus RNA in polyacrylamide gels. Avian Path. 15:149-159.

Tzipori, S. R., T. J. Makin, M. L. Smith, and F. L. Knautil. 1981. Clinical manifestations of diarrhea in calves infected with rotavirus and enterotoxigenic Escherichia coli. J. Clin. Microbiol. 13:1011-1016.

Ushijima, H., H. Honma, A. Mukoyama, T. Shinozaki, Y. Fujita, M.Kobayshi, M. Ohseto, S. Morikawa, and T. Kitamura. 1989. Detection of group C rotaviruses in Tokyo. J. Med. Virol. 27:299-303.

Vonderfecht, S. L., J. J. Eiden, R. L. Miskuff, and R. H. Yolken. 1988. Kinetics of intestinal replication of group B rotavirus and relevance to diagnostic methods. J. Clin. Microbiol. 26:216-221.

Vonderfecht, S. L., A. C. Huber, J. Eiden, L. C. Mader, and R. H. Yolken. 1984. Infectious diarrhea of infant rats produced by a rotavirus-like agent. J. Virol. 52:94-98.

Vonderfecht, S. L., R. L. Miskuff, J. J. Eiden, and R. H. Yolken. 1985. Enzyme immunoassay inhibition assay for the detection of rat rotavirus-like agent in intestinal and fecal specimens obtained from diarrheic rats and humans. J. Clin. Microbiol. 22:726-730.

Wang, S., R. Cai, J. Chen, R. Li, and R. Jiang. 1985. Etiologic studies of the 1983 and 1984 outbreaks of epidemic diarrhea in Guangxi. Intervirology 24:140-146.

Ward, R. L., D. R. Knowlton, and M. J. Pierce. 1984. Efficiency of human rotavirus propagation in cell culture. J. Clin. Microbiol. 19:748-753.

Woode, G. N. 1978. Epizootiology of bovine rotavirus infection. Vet Rec. 103:44-46.

Woode, G. N. Porcine rotavirus infection. 1986. Pages 368-382 in A. D. Leman et al, eds. Diseases of swine. Sixth

edition. Iowa State University Press, Ames, Iowa.

Woode, G. N., J. Bridger, G. A. Hall, J. M. Jones, and G. Jackson. 1976. The isolation of reovirus-like agents (rotavirus) from acute gastroenteritis of piglets. J. Med. Microbiol. 9:203-209.

Woode, G. N., and C. F. Crouch. 1978. Naturally occurring and experimentally induced rotaviral infections of domestic and laboratory animals. J. Am. Vet. Med. Assoc. 173:522-526.

Woode, G. N., N. E. Kelso, T. F. Simpson, S. K. Gaul, L. E. Evans, and L. Babiuk. 1983. Antigenic relationships among some bovine rotaviruses: serum neutralization and cross-protection in gnotobiotic calves. J. Clin. Microbiol. 25:1052-1058.

World Health Organization. 1973. Mortality due to diarrheal diseases in the world. WHO Weekly Epidemiol. Rec. 48:409-416.

Wyatt, P. G., A. Z. Kapikian, T. S. Thornhill, M. M. Sereno, H. W. Kim, and R. M. Channock. 1974. In vitro cultivation in human fetal intestinal organ culture of a reovirus-like agent associated with nonbacterial gastroenteritis in infants and children. J. Infect. Dis. 130:523-533.

Yolken, R. H., R. Willoughby, S.-B. Wee, R. Miskuff, and S. Vonderfecht. 1987. Sialic acid glycoproteins inhibit in vitro and in vivo replication of rotaviruses. J. Clin. Invest. 79:148-154.

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