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Rotaviral and Coronaviral Diarrhea

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VIRUSES AS ETIOLOGIC AGENTS OF NEONATAL CALF DIARRHEA

Historical Perspective

The concept of viruses as primary etiologic agents of neonatal calf diarrhea was not finally accepted until the report of Mebus and coworkers at the University of Nebraska in 1969[52] of the reproduction of diarrhea in colostrum-deprived gnotobiotic calves with bacteria-free fecal filtrates from natural cases of calf diarrhea. Several publications followed, including the characterization and cell-culture propagation of the "neonatal calf diarrhea" virus, or NCDV.[45,87] Prior to the work at the University of Nebraska, there were several reports of viruses causing diarrhea in young calves. In 1943, Baker described the isolation of a filterable virus causing enteritis and pneumonia in calves.[6] He was able to infect mice with lung homogenates from affected calves and reinfect calves with mice lung homogenates with the production of diarrhea and pneumonia. In 1943, Light and Hodes isolated a filterable agent from fecal samples of children with acute gastroenteritis that was capable of producing a severe diarrhea in experimentally inoculated calves.[29] It is of historical interest that a lyophilized aliquot of a calf fecal filtrate studied by Lights and Hodes contained numerous rotavirus particles when examined 32 years later by electron microscopy.[29]

In 1973, Australian and British[23,41,93] scientists discovered viruses in fecal extracts of children with acute gastroenteritis that were morphologically identical to the NCDV agent. Subsequent to these reports, several inves-

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tigators around the world reported on the presence of similar viruses in the feces of diarrheic children and from a variety of domestic, laboratory, and wild animals.[23,41,93] The initial agent isolated by Mebus was classified as a reovirus-like agent based on its morphologic and biochemical characteristics, and on its lack of serologic relationship to reoviruses.[87] In 1974, Flewett[22] suggested the name "rotavirus" for these reovirus-like agents, coining the name from the Latin word "rota," meaning wheel, in view of the characteristic morphology of this virus when examined under negative staining by transmission electron microscopy.

In 1971, Stair, Mebus, and coworkers isolated a coronavirus from cases of neonatal calf diarrhea in Nebraska during an experimental trial of a vaccine against calf rotavirus.[49] Several subsequent publications described the characteristics of this coronavirus and its pathogenicity in infected neonatal gnotobiotic calves.[47,49,50]

Several other viral agents have been found to be implicated in the etiology of neonatal calf diarrhea in recent years. They include astroviruses,[89] caliciviruses,[89] parvoviruses,[76] Breda virus,[92] and a virus that causes villous epithelial syncytia.[48] A number of other unclassified virions have also been observed by electron microscopy in diarrheic fecal samples.[19,21]

Characteristics of the Calf Rotavirus

Rotaviruses are classified as a genus of the Reoviridae family of RNA-containing viruses having the unique characteristic of containing double-stranded (ds) RNA.[23,41,93] Morphologically, all rotaviruses are identical, having a distinct appearance when examined by electron microscopy under negative staining (Fig. 1). Particles are round, 65 to 75 nm in diameter, and have a hexagonal core from which the capsomers radiate, giving them a "wheel-like" appearance. Rotaviruses contain a double layer of capsomers. Complete rotavirus particles have a buoyant density of 1.36 gm per cm^3 and present a smooth outline when examined by negative-staining electron microscopy; incomplete particles, however, which lack the outer layer of capsomers, have a density of 1.38 gm per cm^3 and a rough outline. Calf rotavirus is resistant to pH 3.0 and ether and is relatively stable to heat.[29,41,93] Some calf-rotavirus isolates have the ability to hemagglutinate human erythrocytes.[73] Calf rotavirus has survived up to 9 months in fecal material stored at room temperature,[88] or 1 hour at 6°C.[41] Rotaviruses can survive several days in distilled water as well as in wastewater[39]—characteristics that have important implications in the epidemiology of rotavirus infection.

Rotaviruses contain 11 segments of dsRNA of molecular weights ranging between 0.2 to 2.3 $\times$ 10^6 daltons.[41,93] These segments of RNA can be separated electrophoretically into four main groups, each segment having its own characteristic mobility. This technique of electropherotyping has been used in recent years to study the epidemiology of rotaviruses in large populations and to help in the distinction of rotaviruses from different animal species.[33] It has been estimated that rotaviruses have 8 to 10 polypeptides that have a molecular weight ranging from 15 to 130 $\times$ 10^3 daltons.[41,93] The polypeptides contained in the internal layer of capsomers have the group-antigenic determinants, whereas those present in the outer surface of the virion are thought to have the species-specific antigens.[8]

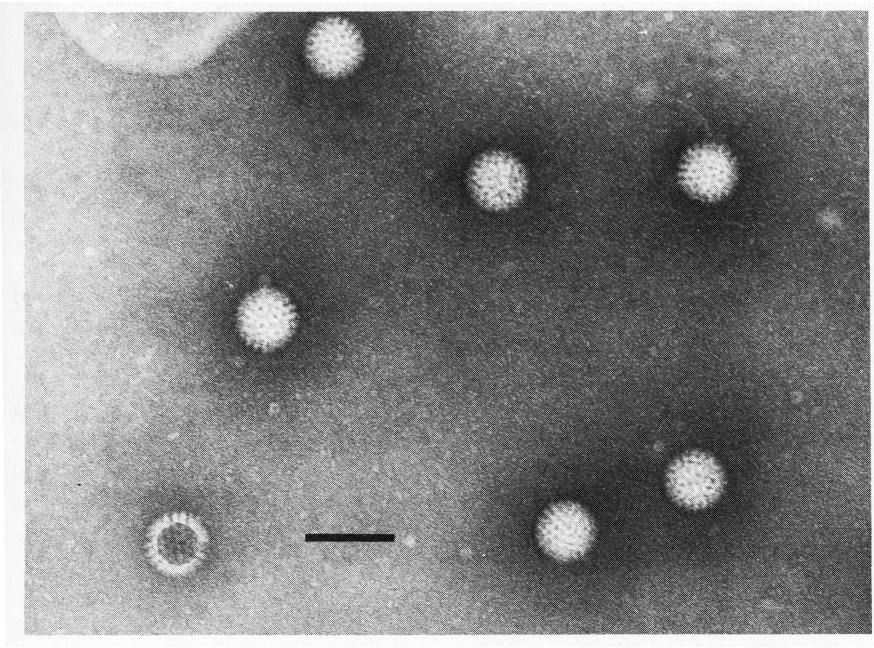

Figure 1. Calf rotavirus particles as observed by electron microscopy under "negative staining." Bar = 100 nm.

In the last few years, viruses morphogically undistinguishable from rotaviruses have been isolated from several animal species from cases of neonatal diarrheas.[72] These rotaviruses are atypical in that they lack the common antigen; thus, they are serologically different. These atypical rotaviruses, also known as pararotaviruses or rotavirus-like viruses, are pathogenic for susceptible animals.[72] The incidence of diarrheas in calves due to atypical rotaviruses has been estimated in less than 1 per cent of all rotaviral diarrheas.[72] This group of atypical rotaviruses (designated as group B rotaviruses) may turn out to be a separate genus of the Reoviridae family as more information on their characteristics becomes available.

Rotaviruses replicate entirely in the cytoplasm of susceptible cells.[23,41,93] For several years, the only rotavirus isolate that could be propagated in cell cultures was the original NCDV strain.[45] Cultivation of additional rotavirus isolates from calves, as well as from a variety of animals and humans, was made possible by the treatment of the virus with proteolytic enzymes.[80] Today, there are several cultivable isolates of calf rotavirus,[5] with recent evidence suggesting that there may be more than one antigenic serotype of calf rotavirus;[32,59,91] this is similar to the situation found for rotaviruses in other animal species, in which there is clear evidence of distinct serotypic variations.[33] Recent serologic comparison of rotaviruses from a variety of animal species indicates that the NCDV strain and its related UK strain of calf rotavirus are a distinct serotypic group.[30] Other human and animal ro-

taviruses share some antigenic relationships, which suggests the possibility of cross-species infectivities.[30]

Experimentally, some isolates of human rotaviruses can be pathogenic for newborn calves with the production of diarrhea.[53] On the other hand, although the calf rotavirus has been demonstrated not to be pathogenic for humans, nor for a variety of laboratory animals, some strains are pathogenic for piglets[27] and can infect young dogs, resulting in virus shedding but not diarrhea.[70] Recent evidence suggests that the NCDV strain of calf rotavirus appears to offer some possibilities as a potential human vaccine strain for the prevention of human rotaviral diarrhea.[85]

Calf rotaviruses have been isolated from numerous countries around the world, and their distribution appears to be ubiquitous.[23] In temperate countries, the incidence of rotaviral diarrhea in calves is higher during the winter months.[23,41,93] The prevalence of calf rotavirus in the normal bovine population is high. Serologic surveys indicate that rotavirus infection in cattle could be as high as 90 to 100 per cent,[69] with morbidity rates for clinical rotaviral diarrhea in the order of 20 to 40 per cent,[31] and with occasional outbreaks approaching 100 per cent.[55] Mortality in uncomplicated rotavirus diarrhea in neonatal calves is less than 10 per cent, but it increases in cases of secondary infection with enteropathogenic *Escherichia coli* or with other enteric viruses.[31,55] Most cases of calf rotaviral diarrhea are found in calves under 10 days of age. In some instances, however, rotaviral diarrhea has been diagnosed in calves 3 to 4 months of age, as well as in some adult animals.[88,90] A recent report on a longitudinal survey of rotavirus infection in a closed dairy herd indicates that rotaviruses were present in 79 per cent of the fecal samples collected during two consecutive calving seasons. Of these rotavirus-infected animals, only 58 per cent developed diarrhea, leaving a 42 per cent incidence of subclinical rotavirus infection.[43] These data are similar to that found in children with rotavirus infections.[33]

Characteristics of the Calf Coronavirus

All known coronaviruses are currently classified as members of a common genus in the Coronaviridae family of RNA viruses. Although all rotaviruses are associated with enteric infections, coronaviruses are important causative agents of several diseases including hepatitis, pneumonitis, nasopharyngitis, peritonitis, encephalitis, and gastroenteritis in a wide variety of animal species.[86] Coronaviruses have a common morphologic characteristic. Particles are quite pleomorphic, but mostly round to oval, all having typical peplomers projecting from the virion. When observed by negative-staining under electron microscopy, calf coronavirus particles have a mean diameter of 100 to 120 nm with uniformly spaced petal-shaped projections 10- to 20-nm long (Fig. 2).[71] Calf coronavirus has a buoyant density of 1.18 to 1.21 gm per cm^3 in sucrose.[15,71] The calf coronavirus contains essential lipids; thus, it is inactivated by ether, chloroform, and deoxycholate but is stable at pH 3.0,[71] as are all enteric coronaviruses. However, all other non-enteric coronaviruses are acid labile.[86] The calf coronavirus is unstable at temperatures above 45°C, and it has the ability to hemagglutinate (HA) erythrocytes of hamster, rat, and mouse.[71] Cells in which the calf coronavirus is replicating have the ability to hemadsorb the same erythrocytes.[71] The

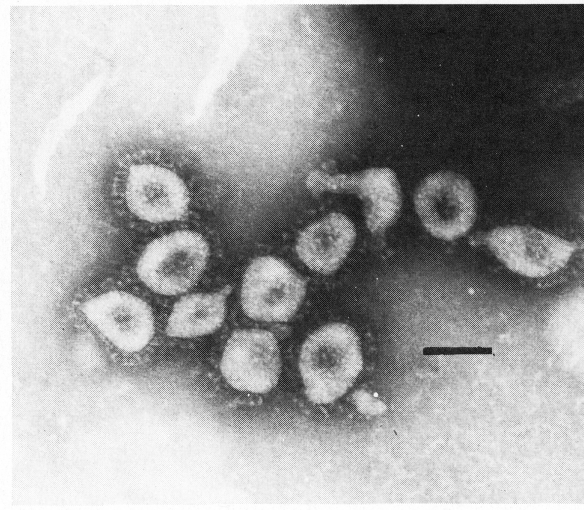

Figure 2. Calf coronavirus particles as observed by electron microscopy under "negative staining." Bar = 100 nm. (From Sharpee, R.L., Mebus, C.A., and Bass, E.P.: Characterization of a calf diarrheal coronavirus. Am. J. Vet. Res., 37(9):1031–1041, 1976; with permission.)

HA activity of calf coronavirus is destroyed by ether and chloroform, but not by deoxycholate or neuroaminidase, and is retained in heat-inactivated viral preparations.[71] The calf coronavirus replicates entirely in the cytoplasm, with budding into cytoplasmic vesicles, like most coronavirus, and produces syncytial cells in infected cell cultures.[49,71] The calf coronavirus contains a single plus-stranded polyadenylated infectious RNA of a moelcular weight in the order of 5.0 to 8.0 $\times$ 10^6 daltons.[26] The calf coronavirus contains five polypeptides of molecular weight in the range of 26 to 140 $\times$ 10^3 daltons.[34] Calf coronavirus can be propagated in a variety of cells,[13,49,71,83] and its replication is enhanced by trypsin.[83] All bovine coronaviruses appear to be of the same serotype. The calf coronavirus is serologically related to other coronaviruses, including mouse hepatitis virus type 3 and human coronavirus strain OC 43, and to the hemagglutinating encephalomyelitis virus of swine. However, it is not serologically related to the human coronavirus strain 229E, canine coronavirus, transmissible gastroenteritis virus, or feline infectious peritonitis virus.[24,61,67] Antibodies to calf coronavirus have been found in a variety of animal species, including humans.[68] Some isolates of calf coronaviruses can be experimentally pathogenic for mice producing encephalitis.[35]

Coronaviruses associated with neonatal calf diarrhea have worldwide distribution.[10] The prevalence of antibodies to the calf coronavirus is high in the cattle population, closer to 100 per cent.[65] Morbidity of coronavirus enteritis has been estimated at 15 to 25 per cent,[36] with mortality rates of 5 to 10 per cent in uncomplicated coronavirus infections. Mortality rates are significantly higher in cases of secondary viral or bacterial infections, especially those of enteropathogenic *E. coli*.[31] Most cases of coronavirus diarrhea are observed in calves 5 to 20 days of age,[11,36,49] but epizootics of coronaviral diarrhea have been reported in adult cattle.[79] There is evidence that strains of calf coronavirus can infect the bovine respiratory tract.[63]

CLINICAL PRESENTATION AND PATHOGENESIS

Calf Rotavirus Infection

Clinical Signs. The course of natural rotavirus infections in the field is chararacterized by a sudden onset of diarrhea that spreads very rapidly among neonatal calves present on the farm. The incubation period has been estimated to be 12 to 36 hours under experimental conditions. The incubation period in field cases may be somewhat longer (24 to 48 hours) depending on the dose of the virus challenge. Typically, the signs of rotavirus infection include reluctance to stand and nurse, mild depression, salivation, and watery, yellow diarrhea. Diarrhea may last for 1 to 2 days in uncomplicated cases, and much longer (3 to 5 days) in cases with secondary bacterial infections. Under germ-free conditions, the rotaviral diarrhea is self-limiting and of short (6 to 10 hours) duration, with full recovery by 24 hours after infection. Recovery from natural outbreaks of rotaviral diarrhea depends upon the degree of secondary intestinal infection and dehydration. Mortality can be high if there is a concomitant infection with enterotoxigenic *E. coli* strains. There are no gross lesions in calves with rotaviral diarrhea, except for the presence of liquid intestinal contents throughout the intestinal tract.

Epidemiologic studies of acute diarrhea of calves have demonstrated that bovine rotavirus usually affects calves between 1 day and 3 weeks of age. A recent field study reported that the average age when rotaviruses were first detected in feces of affected calves was 6.1 days.[43] Research has shown, however, that older animals can be infected. Yearlings and adult cattle have been shown to be infected.[20,88,90]

There are many factors that influence the clinical course of calf rotavirus infection in field cases. Rotavirus infection can have a broad spectrum of clinical effects, ranging from severe illness with high mortality to infection with no clinical abnormality at all. Serotype of virus and the effect of colostral antibody are important factors. Recent reports from the United Kingdom and Japan[57,59] have demonstrated that there are distinct serotypes of rotavirus based on neutralization reactions. Virulence between different bovine rotavirus isolates varies.[12] Morphologic examination of microscopic changes in intestinal segments infected with different rotavirus isolates in one study showed that there was a difference between the severity of the lesion caused by isolates with different polypeptide electrophoretic patterns.

Another factor important in the course of rotavirus infection is the presence of passively transferred antibody.[42] Calves fed colostrum containing sufficiently high levels of rotavirus antibody are protected from infection, yet the effect is thought to be due to local action of colostral and milk antibodies within the gut lumen. Serum antibody does not protect from infection. Because the amount of antibody in the dam's milk decreases to negligible amounts within a few days, calves become susceptible to infection at a very early age. Partial protection probably occurs, and subclinical infections are thought to be common.

Pathogenesis of Calf Rotavirus Infection. The calf is most commonly infected by rotavirus through ingestion of fecal contaminated material. As indicated before, rotaviruses are quite resistant to inactivation; therefore, it

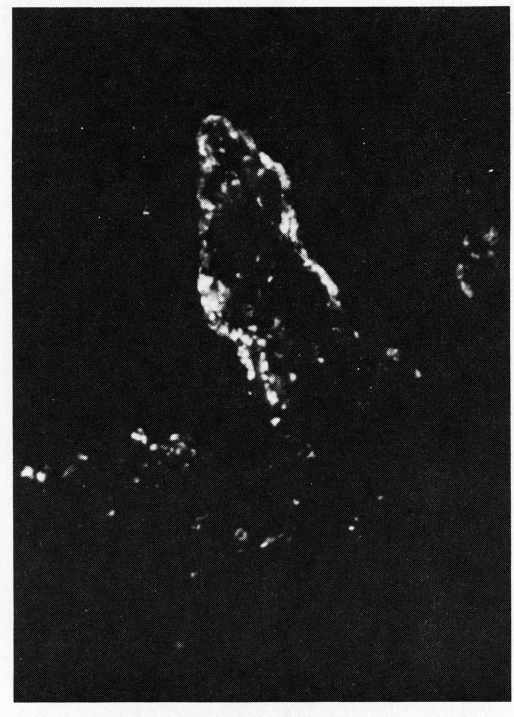

Figure 3. Section of small intestine infected with bovine rotavirus and reacted with fluorescein-labeled bovine rotavirus antibody. Note the presence of viral antigen within epithelial cells lining the upper two thirds of the villi.

is not surprising that environmental contamination is recognized as an important factor in the epizootiology of rotavirus infections.[62]

Recent work indicates that once the virus enters the gastrointestinal system, it may undergo enzymatic activation. It seems that proteolytic enzymes found in the gastrointestinal tract of calves probably play an important role in the pathogenesis of rotavirus infections, but the mechanisms through which they exert their effects have yet to be elucidated. The infectivity of rotaviruses has been greatly enhanced in in-vitro experiments by reaction with certain proteolytic enzymes.[2,4] The addition of trypsin to cell-culture systems has been reported to enhance bovine rotavirus production by 1000 times.[2] Activation of rotavirus in vitro by intestinal trypsin has been postulated to occur in rotavirus infections in children[40] and in domestic animals.[80] Proteolytic enzymes fed to piglets infected with human rotavirus have been shown to exacerbate diarrhea.[75]

The primary target cells for rotavirus infection are the epithelial cells lining the small intestine (enterocytes). Epithelial cells covering the villous surface of the upper small intestine are the first to become infected (Fig. 3). This is followed by progression of infection to epithelial cells on the villi in the mid and lower small intestine.

Prior to infection, the cells covering the villi form a single layer of tall columnar cells (Figs. 4 and 5). Early in the infection, large quantities of viral antigen (see Fig. 3) are present in the cytoplasm of these cells coinciding with the onset of diarrhea. Viral infection alters cellular function, and although the integrity of the epithelial cell layer is initially maintained, within

Figure 4. Scanning electron micrograph of mid-ileum from a normal 48-hour-old calf. Note the length of the villi and the smooth appearance of the epithelial cells covering the villi. Bar = 100 μm. (From Torres-Medina, A.: Effect of combined rotavirus and *Escherichia coli* in neonatal gnotobiotic calves. Am. J. Vet. Res., 45(4):643–651, 1984; with permission.)

a very short period infected cells are desquamated into the intestinal lumen. Exfoliated cells containing viral antigen are found in the feces. Identification of infected cells present in the feces by immunofluorescent microscopy was the original method used to diagnose calf rotavirus infection.

Following the exfoliation of infected cells, the villi become atrophied (Fig. 6), covered by immature cells with a squamous to cuboidal morphology (Fig. 7) that migrate up from the unaffected crypts. The replacement of infected cells by immature cells begins within 4 hours after the onset of diarrhea.[44] The immature cells are refractory to virus infection and are functionally unprepared for the digestive and absorptive roles of the normal villous epithelium; this results in continued diarrhea. Crypt epithelial cells are not involved. Although desquamation of the epithelium of the abomasum and large intestine has been reported,[16] for all practical purposes, rotavirus infection is limited to the small intestine.

Examination of intestinal tissues collected from infected calves reveal rotavirus replication within the cistern of the endoplasmic reticulum of mature villous epithelial cells and macrophages of the lamina propria of the small intestine. Free virus particles are present in the luminal contents approximately 30 hours after infection. The number of virions increases very rapidly, and quantities up to 10×10^9 per gm of feces are not unusual. The

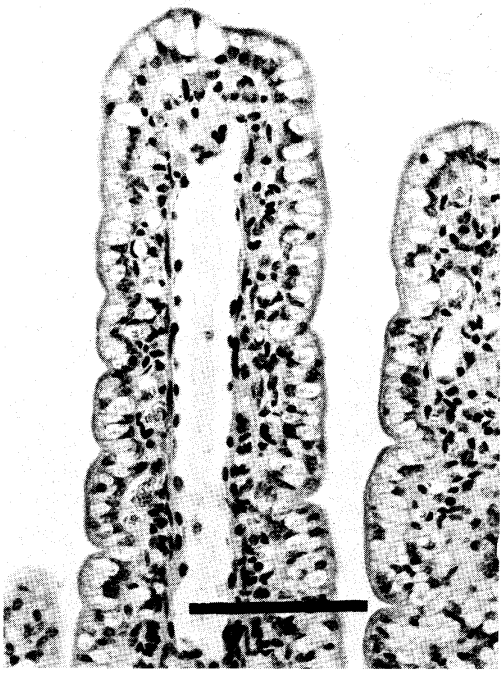

Figure 5. Light microscopic photomicrograph of mid-ileum from a normal 48-hour-old calf. The villus is covered by a uniform layer of mature columnar intestinal epithelial cells. Bar = 100 μm. (From Torres-Medina, A.: Effect of combined rotavirus and *Escherichia coli* in neonatal gnotobiotic calves. Am. J. Vet. Res., *45*(4):643–651, 1984; with permission.)

number of rotaviruses in the feces decreases rapidly after the onset of diarrhea and continues at lower levels over the next 5 to 6 days.

The pathophysiology of rotavirus diarrhea and the serious alterations in fluid, electrolyte, and vascular homeostasis that develop secondarily are complex. The cells that the virus affects have important functional roles in primary intestinal defense as well as secretion and absorption. Their relative importance is underscored by the devastating series of events that develop during rotaviral infection. In addition to physically covering the surface of the intestine, intestinal epithelial cells secrete compounds important in local defense against bacterial invasion. Two such important products are lysozymes and lactoferrin. Secretory products are also important in digestive and absorptive functions. The microvilli of the tall columnar cells are covered with a surface glycocalyx layer that contains important digestive enzymes. When these cells are infected with rotavirus, cellular metabolism is altered, which results in deranged function.

In the small intestine of a normal animal, cells lining the crypts have a secretory function, which is in contrast to the more mature and more differentiated epithelial cells on the villi, which perform an absorption function. When absorptive enterocytes are infected with rotavirus, their absorption decreases markedly, whereas cells lining the crypts are spared and continue to secrete fluids. This results in an imbalance with net accumulation of fluid in the lumen of the intestine, which contributes to the diarrhea.

Even after the infected cells are lost, intestinal function is still greatly

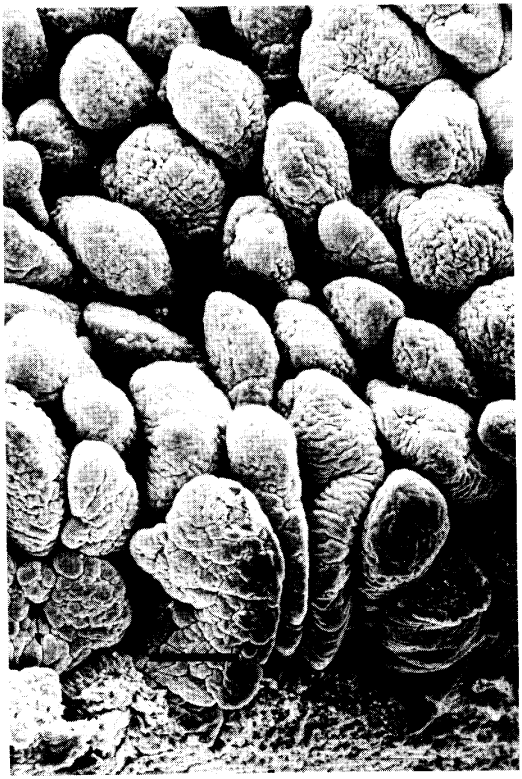

Figure 6. Scanning electron micrograph of mid-ileum from a 48-hour-old calf infected 24 hours before with bovine rotavirus. Contrast this photomicrograph with Figure 4, a photomicrograph of tissue from a normal calf. Villi from the calf infected with rotavirus are markedly atrophied. Note the significant loss in relative surface area. Bar = 100 μm. (From Torres-Medina, A.: Effect of combined rotavirus and *Escherichia coli* in neonatal gnotobiotic calves. Am. J. Vet. Res., *45*(4):643–651, 1984; with permission.)

affected during the period of repair. During this stage of the infection, there is an accelerated production of immature, undifferentiated cells in the crypts. Although these immature epithelial cells move up over the denuded villi, they are not capable of performing normal absorptive and digestive functions. The immature cuboidal cells contain low levels of disaccharidases that are insufficient for normal glucose and galactose absorption. Clinically, D-xylose malabsorption can be demonstrated[88] with reductions of 60 to 90 per cent below normal. The results of inadequate digestion and absorption include accumulation of lactose and digestible carbohydrates that undergo fermentation by bacteria, exacerbating the diarrhea because of increased osmotic pressure. Although poorly studied, intestinal hypermotility may occur, causing a decrease in transit time for ingesta and compounding problems of digestion and absorption. These changes lead to net alterations in fluid and electrolyte homeostasis that result in dehydration, hemoconcentration, acidosis (with secondary hyperkalemia), and, in severe cases, shock.

There has been considerable interest and recent research in the area of multiple infections. The interaction of rotavirus infection and coinfection with other enteric viruses (coronavirus, parvovirus, and so on), protozoa (cryptosporidia, coccidia, and so on) and bacteria, especially *E. coli*, is of practical importance because field studies have shown that more than one of these agents is commonly encountered.

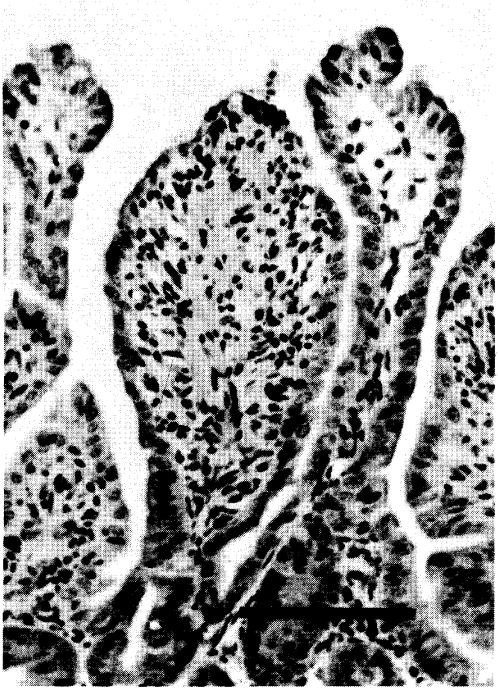

Figure 7. Light microscopic photomicrograph of mid-ileum from a 48-hour-old calf infected 24 hours before with bovine rotavirus (see Fig. 6). Mature epithelial cells that were infected with rotavirus have been lost and replaced by squamous and cuboidal immature epithelial cells. Note the increased cellularity of the lamina propria as compared with Figure 5. Bar = 100 μm. (From Torres-Medina, A.: Effect of combined rotavirus and *Escherichia coli* in neonatal and gnotobiotic calves. Am. J. Vet. Res., *45*(4):643–651, 1984; with permission.)

The relative frequency of multiple infections in field situations has been reported in several studies.[1,28,54,55] During a study of 59 beef cattle herds in Canada, rotavirus infection alone was found in 33.3 per cent of 39 herds. Enterotoxigenic *E. coli* was found in 38.1 per cent of 55 herds, and 14.3 per cent of 35 herds had infections with both agents.[1] In another study,[54] 10 of 13 calves with rotavirus infection were also infected with other agents. Because infections are frequently mixed, several different animals should be included in any clinical diagnostic work-up of an epizootic of calf diarrhea.

The importance of multiple infections with enteropathogens has been investigated experimentally by a number of investigators.[16,25,51,55,60,66,81,82,84] Direct comparisons between these studies is difficult because of differences in the ages of animals used, virus and bacterial isolates, dosages, and feeding regimens. In general, these studies have shown that simultaneous inoculations of calf rotavirus and enterotoxigenic *E. coli* cause a more severe diarrhea than either agent causes alone, but the effect is additive and not synergistic.

Calf Coronavirus Infection

Clinical Signs. The clinical manifestations of natural outbreaks of calf coronavirus diarrhea are very similar to those observed for rotavirus infections. However, clinical manifestations are more severe in cases of coronavirus diarrhea. Initially, there is a sudden onset of diarrhea in susceptible calves after an incubation period of 36 to 60 hours. Typical signs include moderate depression, reluctance to nurse, and the passage of feces containing mucus and milk curds. After 2 to 4 days of diarrhea, calves can become

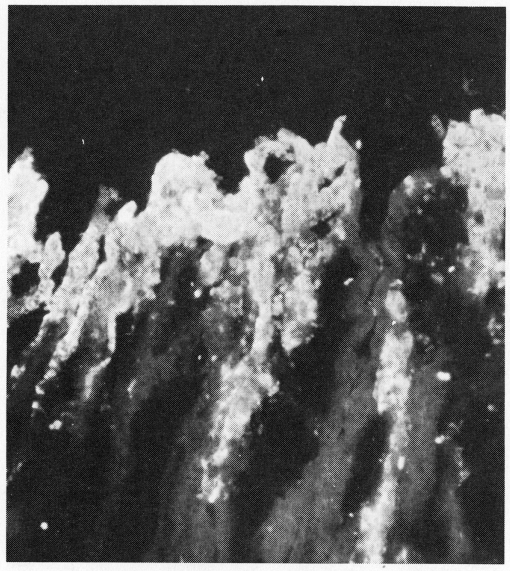

Figure 8. Section of colon infected with bovine coronavirus and stained with fluorescein-labeled antibovine coronavirus antibody. Coronavirus antigen is present in both surface epithelial cells and in epithelial cells lining the crypts.

severly depressed, weak, and gaunt; death follows in severe cases. As in rotavirus infections, postmortem examination reveals no gross lesions, and the intestines have moderate distention with liquid contents. Under germ-free conditions, the coronavirus diarrhea is significantly more severe than rotavirus diarrhea and can lead to death, even in the absence of secondary enteric infections.

Epidemiologic studies have indicated that calves can be affected with coronavirus diarrhea up to 3 weeks of age; they are generally affected at 7 to 10 days of age, which is a little older than the mean susceptible age for rotavirus diarrhea.

Pathogenesis of Calf Coronavirus Infection. Viral infections with the bovine rotavirus and bovine coronavirus differ in that the coronavirus replicates and causes severe damage in both the small intestine *and* in the colon. In the initial stages of infection, coronavirus replicates in the epithelial cells lining the villi in the cranial part of the small intestine, and infection progresses caudally. The epithelial cells in the crypts are spared.

Diarrhea begins at approximately the time that most of the villous epithelial cells contain coronavirus antigen but before any morphologic changes that are detectable at the light microscopic level occur (Fig. 8). Functional alterations of the epithelial cells due to virus infection are thought to be responsible for abnormal absorption and secretion at this stage of the infection.

Coronavirus infection is cytocidal, and within a short period of time, infected epithelial cells are sloughed into the intestinal lumen. Forty to ninety hours after diarrhea begins, the loss of epithelial cells from the villi is extensive. Villi appear shortened (Fig. 9), and occasional adjacent villi fuse (Fig. 10). The tall columnar cells are replaced by cuboidal epithelium.

The tall columnar cells lining the colon also become infected. The co-

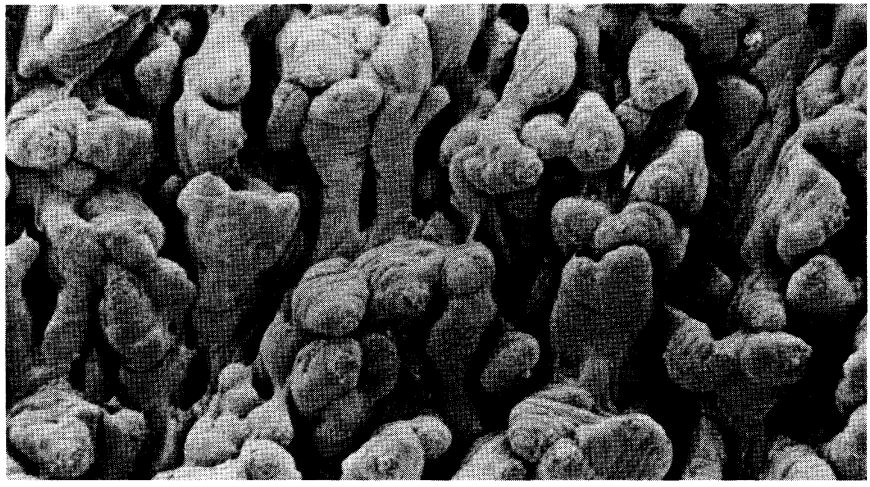

Figure 9. Scanning electron micrograph of the small intestine of a calf inoculated 30 hours after birth with bovine coronavirus and killed 49 hours later. Note the marked atrophy and fusion of villi compared with Figure 4. (From Mebus, C.A., Newman, L.E., and Stair, E.L.: Scanning electron, light, and immunofluorescent microscopy of intestine of gnotobiotic calf with calf diarrheal coronavirus. Am. J. Vet. Res., 36(12):1719–1725, 1975; with permission.)

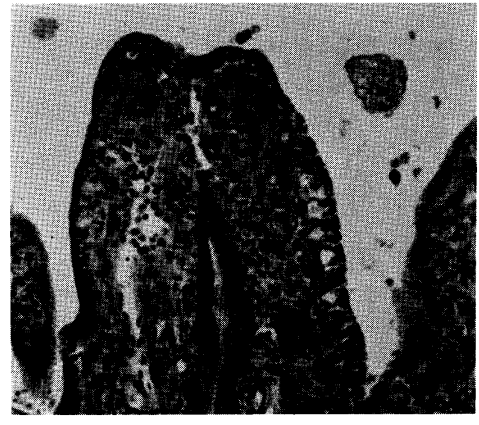

Figure 10. Light microscopic photomicrograph of the small intestine of a calf that was inoculated 30 hours after birth with bovine coronavirus and killed 49 hours later. Note the markedly shortened villi and the presence of immature epithelial cells covering the villi. Damaged villi frequently fuse to each other. (From Mebus, C.A., Newman, L.E., and Stair, E.L.: Scanning electron, light, and immunofluorescent microscopy of intestine of gnotobiotic calf with calf diarrheal coronavirus. Am. J. Vet. Res., 36(12):1719–1725, 1975; with permission.)

Figure 11. Scanning electron micrograph of the colonic mucosa from a normal 79-hour-old calf. Flattened ridges are separated by furrows. Openings of the crypts can also be seen. (From Mebus, C.A., Newman, L.E., Stair, E.L.: Scanning electron, light, and immunofluorescent microscopy of intestine of gnotobiotic calf with calf diarrheal coronavirus. Am. J. Vet. Res., 36(12):1719–1725, 1975; with permission.)

lonic ridges of the large intestine (Fig. 11) become atrophied owing to the destruction of the epithelial cells lining the crypts and the surface of the ridges (Fig. 12). Crypts are frequently distended and contain cellular debris. There may also be congestion of the capillary bed and mild plasma cell and lymphocytic infiltration of the lamina propria.[47]

Although the calf coronavirus replicates primarily in the mature epithelial cells of the intestinal mucosa, other cells, including undifferentiated epithelial cells, goblet cells, fibroblasts, and endothelial cells in the lamina propria, are also infected.[77] Enteric bovine coronavirus has recently been reported to replicate in organ cultures of the fetal bovine trachea[9] and within the nasal epithelial cells.[63] The relative importance of these findings in the epizootiology of bovine coronavirus infection is not yet known.

As the tissues undergo repair, functionally immature cells begin to cover the villi of the small intestine and the mucosa of the colon. As was noted in the case of rotavirus infection, these young cells have not developed enough to be able to perform the normal absorptive or secretory functions.

Severe water and electrolyte alterations occur as a result of coronaviral enteric infections. As has been noted previously, alterations in permeability,

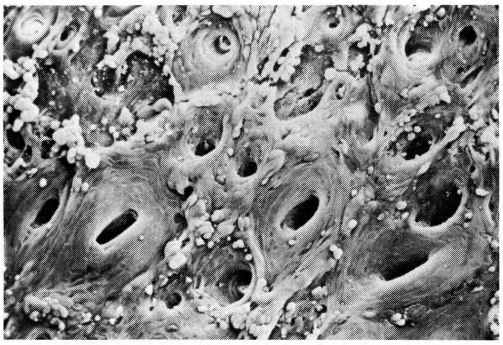

Figure 12. Scanning electron micrograph of the colonic mucosa from a calf that was inoculated 30 hours after birth with bovine coronavirus and killed 49 hours later. The ridges and furrows are nearly entirely destroyed. Openings of the crypts have become much more obvious. Irregular clusters of rounded epithelial cells are all that remain of the surface epithelium. (From Mebus, C.A., Newman, L.E., and Stair, E.L.: Scanning electron, light, and immunofluorescent microscopy of intestine of gnotobiotic calf with calf diarrheal coronavirus. Am. J. Vet. Res., 36(12):1719–1725, 1975; with permission.)

secretion, and absorption by the epithelial cells lining the intestine can occur before any morphologic changes are detected, and severe destruction of infected epithelial cells and replacement by immature cells are responsible for continued functional deficiency of the intestinal system.

Diarrhea in the calf with rotavirus and coronavirus infection results in extensive losses of water, sodium, chloride, and bicarbonate. Fecal bicarbonate loss combined with lactic acidosis and decreased renal function produce a general acidosis with secondary tissue buffering. This results in movement of potassium ions out of cells because of hydrogen-ion replacement. These changes produce an elevation in plasma potassium levels, but a loss in total body potassium.

Altered potassium levels, combined with hypoglycemia that is associated with severe diarrhea, contribute to the clinical lethargy. Potassium cardiotoxicosis is felt to be an important factor leading to death.[37]

Another important feature in the pathophysiology of viral enteritis is the development of hypovolemic shock. Following water loss through alterations in intestinal fluid absorption and secretion, changes occur in total circulatory fluid volume. To maintain sufficient blood pressure for perfusion of vital organs, peripheral vasoconstriction occurs. Decreased blood flow to the extremities causes them to feel cool. Death can occur because of the hypovolemic shock. The reader is referred to other publications for more detailed information about electrolyte and fluid imbalances that occur during neonatal calf diarrhea.[3,37,38]

DIAGNOSIS OF VIRAL NEONATAL CALF DIARRHEA

Because of the clinical similarities in the diarrheas caused by a variety of pathogens and conditions, the etiologic diagnosis of neonatal calf diarrheas requires the help and support of a veterinary diagnostic laboratory. The accurate diagnosis of calfhood diarrheas is complicated by the sudden onset of illness, the potential interaction of several enteric pathogens, the presence of the natural intestinal microbial flora, and the high prevalence of serum antibodies to most viral enteric pathogens in the cattle population.

The incubation period for infectious neonatal calf diarrheas is rather short, with most infected enterocytes being shed in the diarrheic feces during the first 24 hours of diarrhea, which severely limits the time of collection of diagnostic fecal or tissue samples. The fact that most cases of neonatal calf diarrhea are the result of mixed infections (two or more viral, bacterial, or protozoal agents acting simultaneously in the intestinal tract, along with the normal microbial flora of the gut) complicates the identification of etiologic agents and the interpretation of laboratory results. For example, there are many new viral agents that are only detected by electron microscopy for which very little is known about their pathogenicity.

In addition, limited information is available on the interaction of several viruses or the interaction of viruses and bacteria in the development of diarrhea. The high prevalence of serum antibodies in the cattle population against enteric viruses and the fact that serum antibody levels are not cor-

related with the degree of protection against intestinal infection preclude the use of serology for diagnosis of enteric infections of calves.

Sample Collection and Submission

The timing of collection of samples for laboratory diagnosis is the single most important factor in the successful diagnosis of neonatal calf diarrhea. Fecal samples or intestinal segments should be collected less than 24 hours after the onset of diarrhea. In the majority of the cases, the sample of choice is the diarrheic feces. The relatively low mortality of uncomplicated cases and the rapid progression of intestinal alterations and intestinal postmortem degeneration greatly limit the use of intestinal samples for histologic diagnosis to cases of selected euthanasia and necropsy for diagnostic purposes.

Feces must be collected from the live calf after manual stimulation or from the rectum or colon of dead calves, never from the floor. Fecal samples (10 to 20 gm) should be placed in a wide-mouth container with a tight-closing lid. Intestinal segments submitted for immunofluorescence should be collected from the mid ileum (2 to 3 inches), and it is preferred that they be tied at the two ends to retain their intestinal contents.

Fecal and intestinal samples should be mailed to the laboratory under refrigerated conditions, but they should not be frozen unless dry ice is used for freezing and transport of the samples. Freezing the samples at home-freezer temperatures (-15 to -20°C) and mailing the samples under wet-ice refrigeration cause disruptions in the morphology of coronaviruses and in the integrity of the mucosa of intestinal samples, which cause problems of interpretation in the laboratory. In the case of liquid fecal samples submitted for electron microscopy only (not for bacteriologic examination), the addition of one or two drops of a 10 per cent formalin solution may prevent the autolytic processes associated with the microbial flora present in the sample. The submission of serum samples for the detection of antibodies to the enteric viruses is not advisable, except for prevalence survey studies to determine the degree of exposure of a given cattle population to these agents. With this in mind, the majority of the diagnostic tests available for the etiologic diagnosis of viral calf enteritis are those tests done in fecal samples or intestinal segments for the presence of viral antigens.

Antigen Detection Procedures

Electron Microscopy. Transmission electron microscopy has been a method utilized for the discovery of enteric viruses and continues to be a diagnostic procedure of choice for several reasons.[21] Electron microscopy can detect a number of different virus particles in the same fecal preparation, thus providing a broad, but nonspecific, diagnostic tool for a variety of serologically different viruses, most of which cannot be propagated in cell cultures.[19] Electron microscopy procedures are also rapid and comparatively inexpensive for diagnostic laboratories having access to an electron microscope. Some of the disadvantages of the electron microscopy procedure are its rather low sensitivity (estimated at 1×10^6 virions per ml of feces) and the need to have personnel trained in the operation of the electron microscope and in the interpretation of the images.[21]

A rapid electron microscopy procedure for detection of fecal viruses

under "negative staining" that has been used successfully for many years by one of us (A.T-M.), is as follows: A 15 to 20 per cent suspension of feces is prepared in buffered saline and agitated vigorously in a vortex mixer. This suspension is then centrifuged at low speed (less than 400 × g) for 20 to 30 minutes, and the supernatant is saved. This is an important step, because many enteric viruses are found to be present in large aggregates that could be pelleted and discarded at higher centrifugal forces.[58] The low-speed supernatant is then transferred to ultracentrifuge tubes, and centrifuged at 100,000 × g for 60 minutes. The supernatant is decanted, the resulting pellet is saved and reconstituted in 0.5 ml of distilled water. This concentrated specimen is now ready to be sprayed onto collodion- and carbon-coated 200-mesh copper grids. For this, glass nebulizers are utilized.* The mixture to be sprayed consists of 20 drops of distilled water, 4 drops of a 2.0 to 4.0 per cent phosphotungstic acid of neutral pH, 1 drop of a 0.1 per cent aqueous solution of bovine serum albumin, and 1 to 2 drops of the fecal pellet. This mixture can be prepared in the nebulizer or in a separate glass test tube, and then transferred to the nebulizer. The copper grids are sprayed 10 times, from a distance of about 6 inches. Spraying grids must be done in a biosafety laminar flow hood, for potentially pathogenic aerosols are formed. The sprayed grids are then air dried and examined under a transmission electron microscope at a screen magnification of 45,000. Observation should concentrate on the edges of the small droplets formed on the grids, becaue virions tend to concentrate in these areas owing to surface tension. At least five grid squares containing at least one droplet should be examined. A total of 15 mintues of observation per specimen is generally sufficient to warrant designating a specimen negative if virions are not observed.

One can enhance electron microscopic detection of enteric viruses by using specific antibodies mixed with the clarified fecal preparation before ultracentrifugation; this procedure is known as immunoelectron microscopy. This procedure may be useful in viewing small virions but is not practical for routine diagnosis, because virions with different antigenic composition will not be clumped by the antibody preparation.

Enzyme and Radiolabel Immunoassays. The ability to purify large amounts of rotavirus from diarrheic fecal samples and the presence of a common antigen for rotaviruses from different animal species led to the development of rapid and sensitive methods for the detection of rotavirus antigens in fecal samples. Both enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) have been used for rotavirus-antigen detection with excellent results.[4,17] Initially, ELISA and RIA procedures were mostly used in research, but in recent years, commercially available ELISA tests have opened the opportunity of rotavirus diagnosis to most clinical or diagnostic laboratories. The use of RIA tests continues to be mostly of research use owing to its instrumentation and restrictions in the use of radioactive materials. Most of the commercially available ELISA tests have been developed for humans, but they work equally well for animal rotaviruses that share the common antigen. ELISA tests are more sensitive than electron microscopy procedures and are rapid and accurate.[7,18] Their dis-

*Ted Pella Company, Tustin, California.

advantage is that they are very specific for rotaviruses and will not detect the newly recognized atypical rotaviruses nor other enteric viruses that may be present in diarrheic fecal samples. If there is interest in detecting only rotavirues, ELISA tests are more than adequate, but if a comprehensive etiologic diagnosis of neonatal calf diarrhea is to be established, then electron microscopy procedures are recommended because of their broad specificity, even though they may be slightly less sensitive. The same applies to RIA tests, In the case of calf coronavirus, both ELISA and RIA tests have been developed,[65] but they are still limited to use in research environments. No such tests are commercially available at the present time.

Immunostaining. Rotavirus and coronavirus antigens can be detected in intestinal samples from infected calves by immunofluorescent (fluorescent antibody) staining of frozen sections or of fecal smears. Fluorescent antibody procedures were very useful in the early work with both calf rotavirus and calf coronavirus. In the case of rotavirus, smears of fecal samples, as well as frozen sections from segments of mid to caudal small intestine, are good samples for fluorescent antibody staining. For intestinal samples, this is a limiting factor, unless euthanasia and postmortem examination are performed in the acutely ill calf. The reason for this requirement is that the number of rotavirus-infected enterocytes attached to the intestinal villi and present in the intestinal lumen decreases very rapidly after the onset of diarrhea.[46] The number of enterocytes containing rotavirus antigen that are present in the feces or in the intestinal mucosa is very low 18 to 24 hours after the onset of diarrhea. In the case of calf coronavirus, fluorescent antibody staining of mid to caudal frozen sections of small intestine has the same limitations as for cases of rotavirus infections. However, examination of fecal smears for detection of cells containing coronavirus antigen by fluorescent antibody procedures is a futile enterprise due to the cytolytic action of coronavirus for infected enterocytes.[47] Fluorescent antibody staining may be applied in a case of coronavirus on frozen segments of colon, because of the fact that coronavirus infects the colonic mucosa of calves and coronavirus antigen persists in the crypt cells for 3 to 4 days after the onset of diarrhea.[47] Still there is a limitation of obtaining colon samples from either dead or euthanatized calves. Reagents for fluorescent antibody staining of intestinal samples for calf rotavirus and coronavirus are available in most state diagnostic laboratories as a service provided by the National Veterinary Service Laboratory of the U.S. Department of Agriculture. Other laboratories in the United States and especially abroad have limitations in procurement of fluorescein-conjugated antibodies for these tests.

Other Procedures. Several other procedures have been used for the detection of rotavirus or coronavirus antigen in fecal samples from diarrheic calves. They are based in immunologic reactions with specific antibodies, and include techniques such as latex agglutination tests (LA),[56] counterimmunoelectro-osmophoresis (CIE),[14] and immunodiffusion (ID) procedures.[64] Recently, LA tests for rotavirus have become commercially available.[56] Of all these tests, probably the most useful have been the CIE procedures, especially in laboratories limited in resources. CIE is fast and relatively sensitive, although less so than ELISA or electron microscopy procedures. Their limitation is their specificity and, thus, their inability to detect rotavirus

or coronaviruses of different antigenic composition or other unrelated viruses associated with neonatal calf diarrheas.

SUMMARY

A number of different viruses can be primary pathogens in the neonatal calf diarrhea complex. By far the most common viruses causing calfhood diarrhea found throughout the world are rotaviruses and coronaviruses. Primary infection of newborn calves with either one of these viruses can cause severe intestinal alterations and diarrhea.

Rotaviruses can produce high-morbidity outbreaks of diarrhea in calves under 10 days of age. Mortality is variable mainly owing to secondary bacterial infections and electrolyte imbalances. Rotavirus infection of the small intestinal mucosa leads to loss of enterocytes of the upper third of the intestinal villi with subsequent villous atrophy and malabsorption. There is growing evidence that different rotavirus serotypes of different pathogenicity exist.

Coronavirus infections can produce high-morbidity outbreaks of diarrhea in calves under 20 days of age, with variable mortality due to secondary complications. Coronaviruses affect not only the small intestinal mucosa, producing significant villous atrophy, but also the colon, causing a very severe intestinal damage that can lead to death due to subsequent electrolyte disturbances. All coronaviruses associated with neonatal calf diarrhea appear to be of the same serotype.

The etiologic diagnosis of viral diarrheas of calves requires the support of the laboratory. One of the most useful diagnostic methods is the examination of fecal extracts for the presence of virus particles by electron microscopy. Other antigen-detection procedures like enzyme immunoassays have been found to be useful in the diagnosis of rotaviral diarrheas. The sample of choice for these diagnostic tests is a fresh fecal sample collected directly from the calf as close as possible to the onset of diarrhea. Samples from more than one calf during the outbreak enhance the laboratory ability to establish a proper viral diagnosis.

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