

Pathology of Experimental CV777 Coronavirus Enteritis in Piglets.

I. Histological and Histochemical Study

W. COUSSEMENT, R. DUCATELLE, P. DEBOUCK and J. HOORENS

Departments of Veterinary Pathology and Virology, State University of Ghent, Ghent, Belgium

Abstract. Sixteen cesarean-derived colostrum-deprived piglets were infected oronasally with CV777 coronavirus on the second or third day of life. Two uninfected piglets were controls. They were killed at 96 and 120 hours after birth. After an incubation period of 22 to 36 hours, all principals showed severe diarrhea. The principals were killed between 12 and 120 hours after infection.

Exfoliation of enterocytes was seen first in the piglet killed 24 hours after infection (two hours after the diarrhea began). From that time on, shortening and fusion of villi was present in all small intestinal parts. Affected cells showed vacuolation. The histochemical study showed that infected piglets had decreased activity of all four enzymes studied. The light microscope showed no lesions in the absorptive colonic epithelium.

The significance of the lesions in relation to intestinal dysfunction is discussed, and lesions are compared to those of transmissible gastroenteritis and porcine rotavirus infection.

Viral diarrhea in pigs is known to be caused by transmissible gastroenteritis and rotavirus. Anatomical studies on transmissible gastroenteritis [1, 12, 16, 21, 24, 26] and rotavirus enteritis [2, 19, 25] are reported frequently. These two viruses affect the mucosal epithelium of the villi of the small intestine. No lesions have been seen in the crypt epithelium. Exfoliation of enterocytes, and shortening and fusion of villi, have been seen. In 1978 it was reported [20] that another virus, a coronavirus-like agent, may be involved in porcine diarrhea. This virus has been differentiated antigenically from transmissible gastroenteritis virus and hemagglutinating encephalomyelitis virus [20]. Intestinal lesions caused by this virus in hysterectomy-derived colostrum-deprived pigs and in conventional pigs included marked shortening of villi [5].

We conducted a sequential light microscopic and histochemical study of intestinal lesions following experimental infection with this virus.

Materials and Methods

We used 18 cesarean-derived colostrum-deprived piglets from four litters, delivered by hysterectomy at day 115 of gestation and kept in individual isolation units as described [5]. On the second or third day of life, 16 piglets were infected oronasally with 10^4 pig infective doses of a CV777 virus stock in 2 ml inoculum, as described [5].

Two uninfected piglets were controls. Clinically, the piglets were observed for vomiting, diarrhea and vitality. They were killed by electrocution and exsanguination at 12, 18, 24, 30, 32, 36, 38, 41, 48, 60, 72, 96, and 120 hours after infection. The control piglets were killed at 96 and 120 hours after birth.

Immediately after death, tissue specimens for histological study were collected from the tonsil, duodenum, jejunum (five pieces taken at 50-cm intervals), ileum, the middle of the colon, and the rectum. Specimens also were taken from the mesenteric lymph nodes, lung, liver, spleen, and kidney. Tissues were fixed in 10% phosphate-buffered formalin, dehydrated, embedded in paraplast, cut at 4 μm , and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), van Gieson, and Giemsa stains. Scharlach stains were used for the first part of the jejunum of piglet A 688; middle of the jejunum of piglets A 690, A 693, and A 696; terminal part of the jejunum of piglets A 688 and A 696; and colon of piglets A 688, A 689, A 690, A 691, A 692, A 694, A 695, B 711, and D 748. Semithin sections were made as described [8].

The length of the villi (from the tip of the villus to villus/crypt junction) and depth of the crypts (junction of villus/crypt to base of the crypt) were measured with an ocular micrometer on a minimum of seven different places per small intestinal segment.

Histochemically, we studied the localization and concentration of four enzymes: alkaline and acid phosphatase, succinic dehydrogenase, and monoamine oxydase. Intestinal tissue from control pig A 690 and from piglets with diarrhea for 2, 21, and 26 hours was embedded in Tissue-Tec medium (Lab-Tec Products, Miles Laboratories Inc., Naperville, Ill.) and enclosed in gelatinous capsules. These capsules then were frozen in ethanol at -70°C . Cryostat sections 10 μm thick were cut from the duodenum, the first, middle, and terminal parts of the jejunum, the ileum, colon, and rectum. The sections were processed simultaneously through chemical reaction baths. Described methods were used for alkaline phosphatase and succinic dehydrogenase [18]; for the localization of monoamine oxydase [10]; and for acid phosphatase [11].

Results

The control pigs had no diarrhea.

Gross lesions

All experimental piglets except those killed in the incubation period (22 to 36 hours) of the disease had diarrhea. They had a dirty yellow perineum and hind legs. In the early stage of the disease, the stools were profuse and watery. Piglets with diarrhea for 84 and 96 hours were severely dehydrated; their feces became more mucous. At necropsy, piglets with diarrhea had distended intestines, with watery to yellow contents. The intestinal wall looked thinner. Congestion of the mesenteric vessels was common, and mesenteric lymph nodes were edematous.

Histology

Control (normal) piglets: The villi of the small intestine were long and narrow (fig. 1). In both piglets, the length of villi was about 640 μm in the duodenum, reached

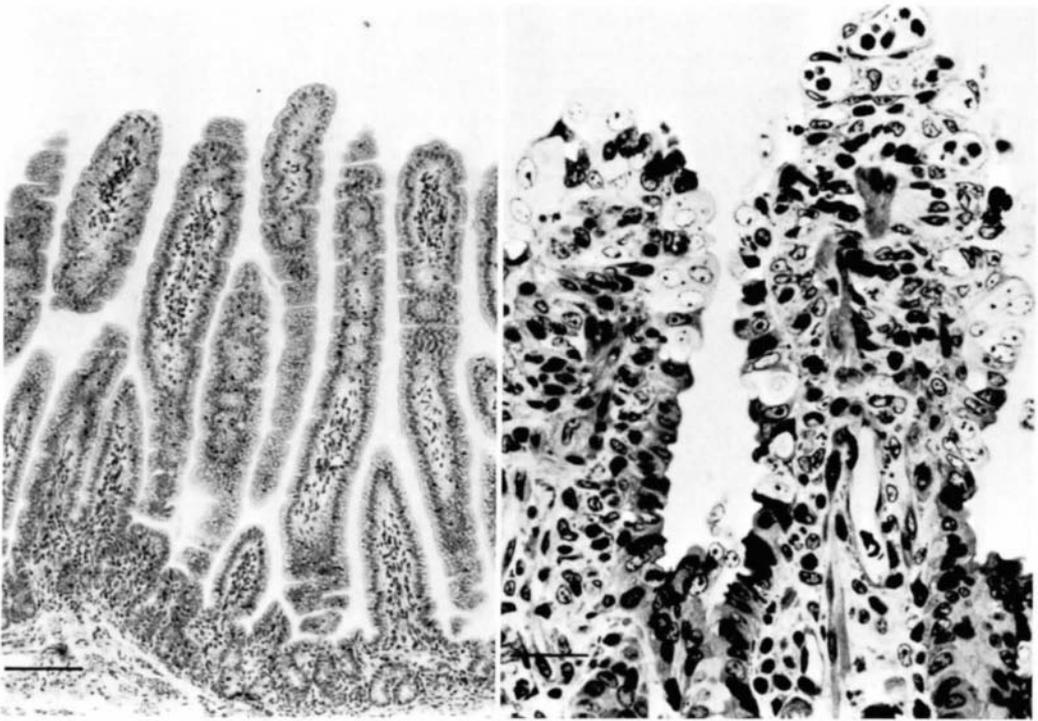


Fig. 1: Normal mucosa of jejunum. Villi long and covered with high columnar cells. HE. Bar = 100 μm .

Fig. 2: Semi-thin section (1 μm) of middle of jejunum of piglet 689 (two hours diarrhea). Massive cell exfoliation on upper half of right villus tip. On left villus, few enterocytes detached. Toluidine blue. Bar = 25 μm .

a maximum in the middle of the jejunum (780 to 930 μm), and varied from 700 to 780 μm in the ileum (table I). The depth of the crypts was 110 to 120 μm in all parts of the small intestine. The enterocytes were high columnar. Nuclei were located in the basal region on the cells. PAS-negative vacuoles were seen in the cytoplasm of cells on the tips of villi. Scharlach stain showed small fat droplets throughout the cytoplasm. The brush border was well developed. On the tips of villi, an occasional effete enterocyte was seen. The lamina propria contained a few eosinophils but no other inflammatory cells. The mucosa of the pylorus, cecum, colon, and rectum was normal. No alterations were found in the spleen, liver, lung, kidney and mesenteric lymph nodes.

Infected piglets: Piglets A 688, B 711, C 736, and C 734 were killed during the incubation period. The length of intestinal villi was 420 to 560 μm in the duodenum, 740 to 880 μm in the middle of the jejunum, and 420 to 620 μm in the ileum (table I). The depth of the crypts was below 100 μm in piglet A 688 and varied from 100 to 130 μm in all other piglets.

The enterocytes were high columnar and no exfoliation was seen. The brush

Table I. Measurements of length of villi in the small intestine after experimental infection with CV777 coronavirus

Pig number	Duration of diarrhea at time of death (hours)	Length of villi in μm^1		
		Duodenum	Middle of jejunum	Ileum
A 690 ⁽²⁾	none	640 \pm 28	930 \pm 50	780 \pm 17
A 1 ⁽²⁾	none	620 \pm 40	780 \pm 52	700 \pm 55
A 688	none	530 \pm 25	880 \pm 18	570 \pm 75
C 736	none	540 \pm 42	740 \pm 40	610 \pm 70
B 711	none	560 \pm 34	770 \pm 34	620 \pm 3
C 734	none	420 \pm 54	840 \pm 14	420 \pm 17
D 744	1	600 \pm 48	870 \pm 23	560 \pm 34
A 689	2	190 \pm 23	270 \pm 30	400 \pm 23
B 710	6	400 \pm 37	270 \pm 28	250 \pm 9
A 691	6	540 \pm 54	420 \pm 37	470 \pm 45
C 737	6	450 \pm 57	240 \pm 23	240 \pm 68
D 748	12	640 \pm 47	150 \pm 20	170 \pm 20
A 696	21	420 \pm 37	260 \pm 6	240 \pm 31
A 693	36	600 \pm 65	290 \pm 17	260 \pm 14
A 695	45	330 \pm 34	390 \pm 37	330 \pm 17
A 694	60	360 \pm 23	350 \pm 28	320 \pm 37
A 692	84	520 \pm 11	360 \pm 23	310 \pm 62
D 745	96	350 \pm 28	270 \pm 14	230 \pm 17

¹ Expressed as average measurements \pm standard error.

² Control pigs.

border was normal. Vacuolated enterocytes were seen on the tips of villi or spread over the entire villus. Small and large vacuoles of fat were seen in these cells in the first and terminal parts of the jejunum of piglet A 688. The lamina propria contained eosinophils and a few neutrophils. The crypts of Lieberkühn were normal. The absorptive epithelium of the colon of piglet A 688 contained many fat globules, in contrast to piglet B 711, which had only a few fat droplets.

Six piglets (D 744, A 689, B 710, A 691, C 737, and D 748) had diarrhea for up to 12 hours, and were killed during this time. The intestines of piglet D 744, which had diarrhea for only one hour, were normal. The length of the villi and depth of the crypts were about the same as in control piglet A 690. Piglet A 689, with diarrhea for two hours, showed exfoliation of enterocytes. The villus on the left in fig. 2 shows only slight exfoliation of enterocytes on the tip. On the villus on the right, all enterocytes of the upper half were detached, with distention of the lymphatics on the tip. Several naked tips were seen (fig. 3). The nuclei of detaching enterocytes were pale and swollen, and their brush borders had disappeared. Rarely, a totally naked villus, with the propria exposed to the lumen of the intestines, was present. The lamina propria was thick and sometimes strongly infiltrated with polymorphonuclear cells and a few lymphocytes (fig. 4). The remaining enterocytes were markedly vacuolated. The villi of this piglet were short (table I). The crypts of Lieberkühn

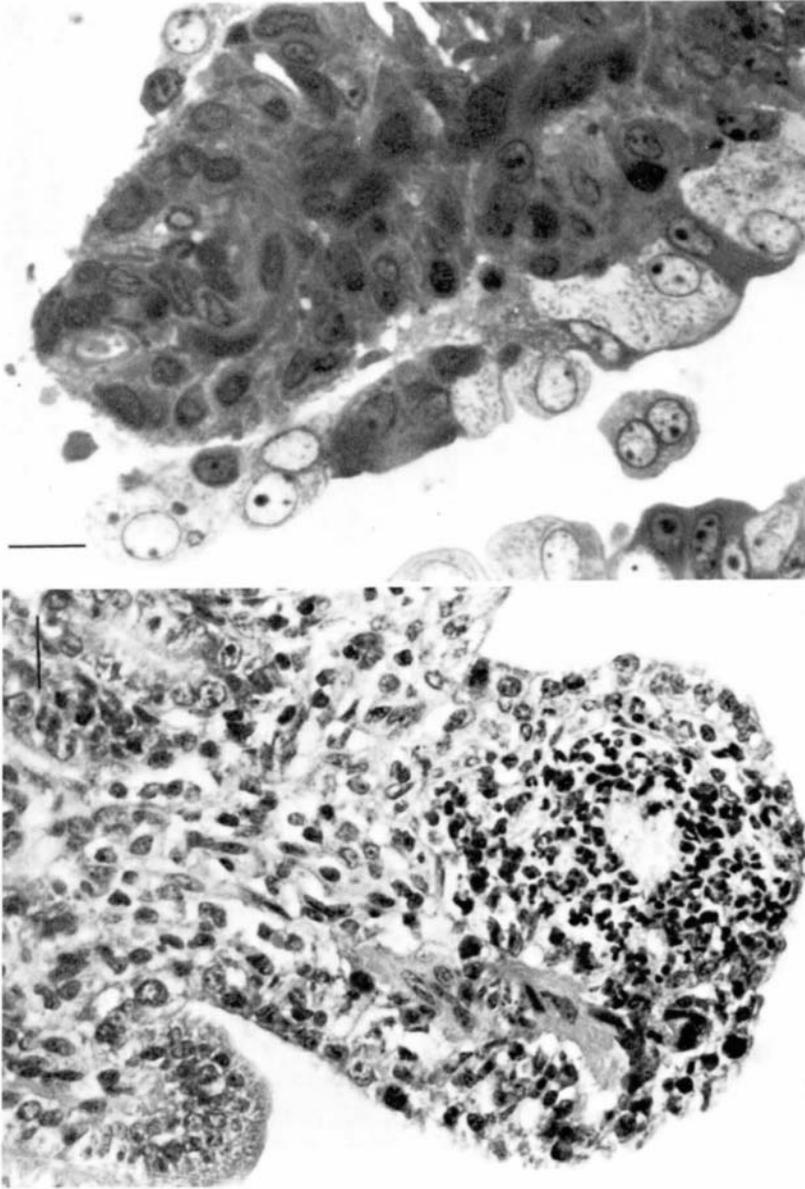


Fig. 3: Semi-thin section of middle of jejunum of piglet 689 (two hours diarrhea). Several enterocytes exfoliated. Toluidine blue. Bar = 10 μ m.

Fig. 4: Blunted villus covered with cuboidal enterocytes. Lamina propria filled with neutrophils. Piglet 689. HE. Bar = 25 μ m.

were not affected. The shortest villi were measured in piglet D 748 (table I), whose intestines showed slight or no exfoliation. Shortened villi were covered by flat enterocytes. The crypts contained more mitotic figures than those of control piglets. Fusion of villi was common. Length of villi in piglets B 710, A 691, and C 737 varied

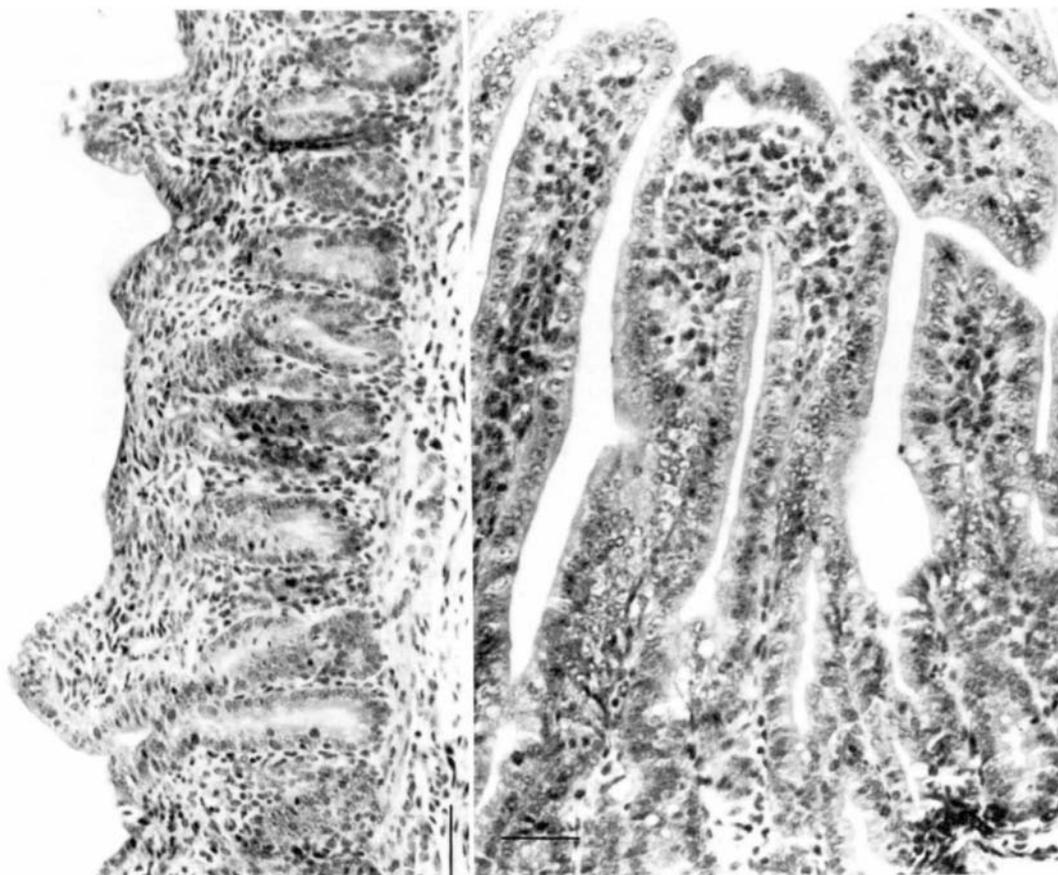


Fig. 5: Section of middle of jejunum of piglet 710 (six hours diarrhea). Flat, nearly avillous mucosa covered with cuboidal cells. HE. Bar = 62.5 μm .

Fig. 6: Middle of jejunum of piglet 694 (60 hours diarrhea). Villi longer, fused only at tips. HE. Bar = 40 μm .

from 400 to 540 μm in the duodenum and from 270 to 420 μm in the middle of the jejunum and ileum (see table I). Crypts measured from 120 to 130 μm in all parts of the intestines. On the villi, a few exfoliating enterocytes were seen. Shrunken, fused villi were covered by flat to low cuboidal enterocytes (fig. 5). In the crypts a higher rate of mitosis was seen. The colon had vacuoles in the cytoplasm of the absorptive cells. In piglets A 689, A 691, and D 748 these vacuoles were strongly fat-positive.

Piglets A 696, A 693, A 695, A 694, A 692, and D 745 had diarrhea for 21 to 96 hours. Villi of piglet A 696 (21 hours diarrhea) measured 420 μm in the duodenum and about 250 μm in the middle of the jejunum and ileum. The intestinal villi of all other piglets of this group were longer, except in piglet D 745, where the villi were shorter again, and fused. The enterocytes were cuboidal to low columnar. Many mitoses were seen in the crypts. The lamina propria contained more plasma cells and lymphocytes than neutrophils. Piglets A 695, A 694, and A 692 had longer villi

covered by columnar epithelial cells. The villi were fused only at the tips (fig. 6). No remarkable lesions were seen in the colon, although piglet A 695 had many fat vacuoles. In piglets A 692 and A 694, only a few fat vacuoles were seen.

Histochemistry

In control piglet A 690, the staining intensities of all four enzymes were pronounced in the enterocytes of the small intestine and colon. In the rectum, the intensity was lower and in the crypts only slight. The reactions of succinic dehydrogenase and monoamine oxydase were spread over the entire cytoplasm of the enterocytes, with a greater intensity in the basal region of the cells. Alkaline phosphatase was found only in the apical border on the enterocytes. Acid phosphatase was seen in the apical cytoplasm.

In the infected piglets, the cellular localization of all enzymes remained the same. In piglet A 696 (21 hours diarrhea), staining intensities for all four enzymes were slight from the duodenum to the ileum (fig. 7, 8), while in the colon and the rectum, reactions were similar to those in the control piglet. Higher intensities were seen in piglet A 689 (two hours diarrhea) than in piglet A 696. In the small intestine, the tips of the villi sometimes had no reaction. In the colon, staining intensities of succinic dehydrogenase and monoamine oxydase were lower than in the control piglet. In piglet D 745 (96 hours diarrhea), the intensities in the small intestine were between those of the control piglet and piglet A 689.

Discussion

Vacuolation was found in the epithelial cells covering the villi of the small intestine of control and infected pigs, but was more pronounced in infected pigs. These vacuoles were fat-positive with the Scharlach stain. Vacuolation was seen in rotavirus enteritis as well [19]; but in contrast to our findings, the vacuolation was more pronounced in the control piglets and no fat was found with Sudan IV stain. Our results are more similar to those described in transmissible gastroenteritis [12], where vacuolated enterocytes were established especially in intestines of pigs killed two to five days after infection. These vacuoles contained fat [24]; in normal pigs, vacuolation was rare [12]. It is assumed that this fatty degeneration may be due to an inability of the cell to transport fat to the lymphatics [24]. Active fat transport requires energy, which comes from the mitochondria. Decreased activity of succinic dehydrogenase reflects an alteration in the biochemical function of the mitochondria or a decrease in their numbers [24]. This results in a decreased energy-producing capacity of the enterocyte [24], and therefore in decreased intracellular fat transport. The same findings were reported in coronavirus enteritis of turkeys [22] and in canine coronavirus enteritis [13, 23].

The first histological lesions were found in the piglet killed 24 hours after infection, which had diarrhea for two hours. These clinical signs of diarrhea were present before cell exfoliation had become significant. Exfoliation of enterocytes was seen in several piglets. Totally naked villi were rare, in contrast with transmissible gastroen-

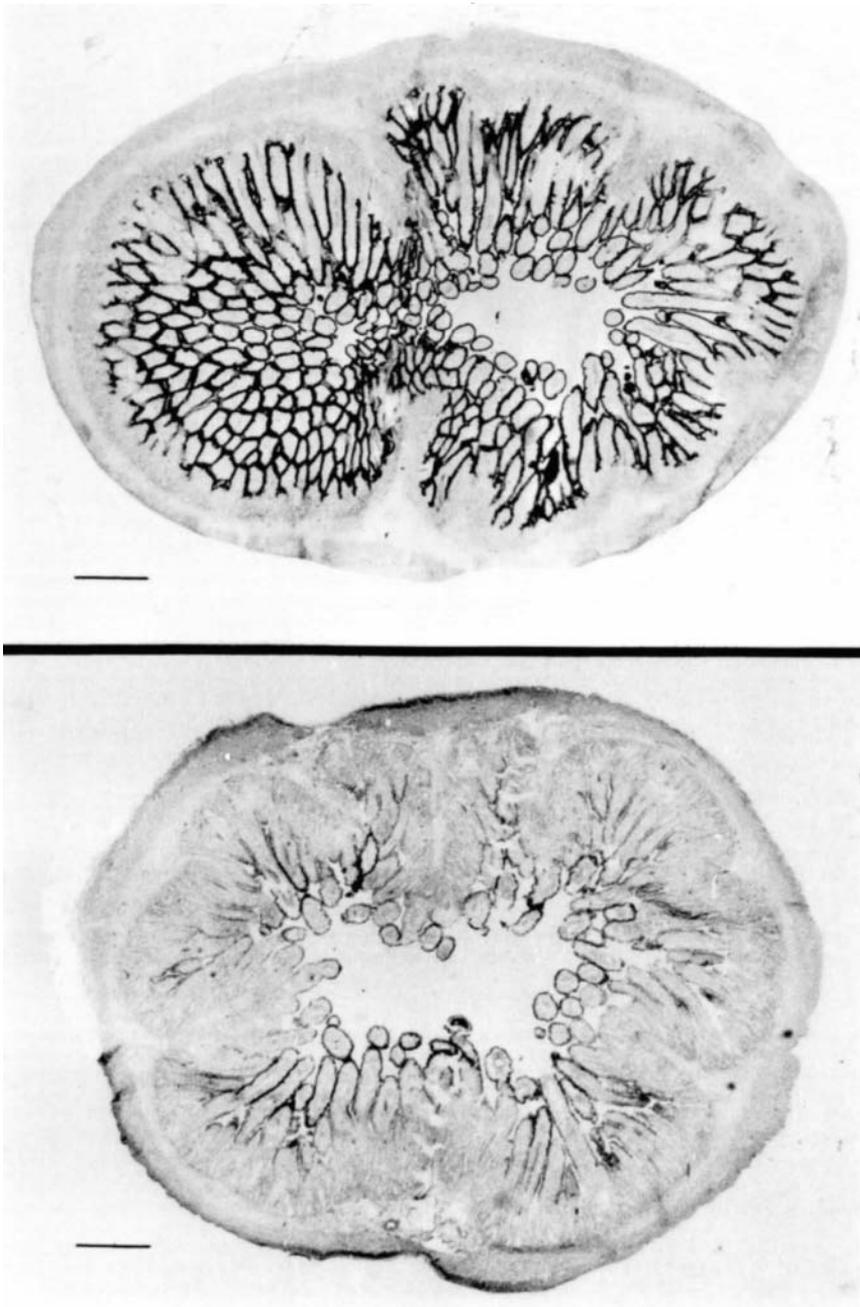


Fig. 7: Alkaline phosphatase stain of jejunum of control piglet. Pronounced staining intensity of villous epithelium. Crypts nearly negative. Bar = 417 μm .

Fig. 8: Alkaline phosphatase stain of jejunum of piglet 696 (21 hours diarrhea). Lower staining intensity compared with control jejunum. Bar = 417 μm .

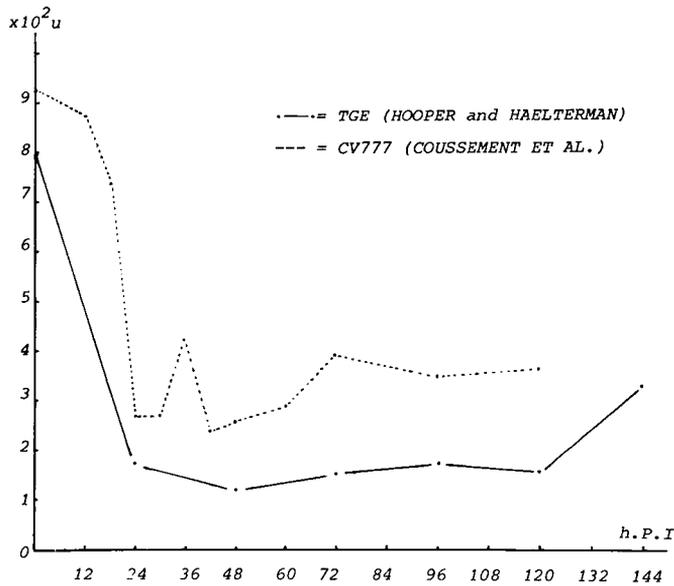


Fig. 9: Comparison of lengths of villi in middle of jejunum between transmissible gastroenteritis and CV777.

teritis, in which exfoliation is severe and nearly all enterocytes are detached [1, 16, 21, 26]. Thus in transmissible gastroenteritis, cell destruction and exfoliation are more pronounced or occur in a shorter period of time. Shortening of villi is caused by epithelial cell exfoliation. The decrease in length of villi is more drastic in transmissible gastroenteritis than in CV777 enteritis (fig. 9).

A pathogenesis study of a CV777 infection in piglets has shown that viral antigen can be detected as early as 18 hours after infection, which is before the onset of diarrhea [6]. It was assumed that diarrhea is induced initially by malabsorption due to functional disorder of absorptive epithelial cells. Infiltration of polymorphonuclear cells and lymphocytes into the lamina propria is due to the exposure of the lamina propria to intestinal contents [21]. Atrophy of villi is the result of exfoliation of enterocytes. The gaps in a villus are closed by contraction of villi and flattening of adjacent cells [21]. Fusion of villi like that in transmissible gastroenteritis [21, 24] and coronavirus enteritis in the calf [7, 14] was seen in our piglets. It has been suggested that swollen and shortened villi are less mobile and tend to adhere to each other [7]. The separation of fused villi, beginning at the base, is caused by epithelial cell migration from the crypts [21]. Indeed, the crypt epithelium was not affected in piglets with CV777, thus regeneration capacity was preserved. Marked elongation and hyperplasia of the crypts as an answer to villous atrophy, and as described in transmissible gastroenteritis [12] and in porcine rotaviral enteritis [25], however, was not a constant feature in the piglets infected experimentally with CV777 corona-like virus.

As a result of production of new epithelial cells, the intestine of the piglets with

diarrhea for 45 to 85 hours had longer villi than those of piglets with diarrhea for 20 to 30 hours. The enterocytes also had become more columnar. Although CV777 is known to infect the colon [6], this apparently does not result in severe histologic lesions. As a matter of fact, few fat droplets could be found in the absorptive cells of the colonic surface epithelium. This may point to a slight temporary decrease of the cell function. Histochemically, in infected piglets all four enzymes had a slighter staining intensity from the duodenum to the ileum compared with the control piglet. This is in accordance with histochemical studies in transmissible gastroenteritis [24] and in canine coronavirus enteritis [13]. Alkaline phosphatase is located in the plasma membrane of the microvilli [9, 17] and is considered as a measurement of the digestive-absorptive surface [4, 15, 17]. Decreased alkaline phosphatase staining intensity indicates that microvilli are shortened. Electron microscope findings are in agreement with this thesis [8]. The staining intensities of acid phosphatase, a chiefly lysosomal enzyme [3], also is reduced, indicating either a decreased number of lysosomes or a loss of enzymatic activity within the lysosomes. The ultrastructural findings seem to favor the latter hypothesis [8].

Acknowledgements

This study was supported by the Institute for Encouragement of Research Industry and Agriculture (IWONL-Brussels). Appreciation is expressed to Mr. Gheyle, C. Puttevils, J.P. Logghe, and P. De Groot for their excellent technical assistance. We are indebted to the department of Virology (Director: Prof. Dr. M.B. Pensaert) for preparing the infectious material.

References

- 1 BAY, W.W.; DOYLE, L.P.; HUTCHINGS, L.M.: The pathology and symptomatology of transmissible gastroenteritis. *Am J Vet Res* **12**:215–218, 1951
- 2 BOHL, E.H.; KOHLER, E.M.; SAIF, L.J.; CROSS, R.F.; AGNES, A.G.; THEIL, K.W.: Rotavirus as a cause of diarrhea in pigs. *J Am Vet Med Assoc* **172**:458–463, 1978
- 3 BROWN, H.H.; MOON, H.W.: Localization and activities of lysosomal enzymes in jejunal and ileal epithelial cells of the young pig. *Am J Vet Res* **40**:1573–1577, 1979
- 4 CRANE, R.K.: Enzymes and malabsorption: A concept of brush border membrane disease. *Gastroenterology* **50**:254–262, 1966
- 5 DEBOUCK, P.; PENZAERT, M.: Experimental infections of pigs with a new porcine enteric coronavirus CV777. *Am J Vet Res* **41**:219–223, 1980
- 6 DEBOUCK, P.; PENZAERT, M.; COUSSEMENT, W.: The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV777. *Vet Microbiol* **5**:1981 (in press)
- 7 DOUGHRI, A.M.; STORZ, J.: Light and ultrastructural pathological changes in intestinal coronavirus infection in newborn calves. *Zentralbl Veterinärmed [B]* **24**:367–385, 1977
- 8 DUCATELLE, R.; COUSSEMENT, W.; DEBOUCK, P.; HOORENS, J.: Pathology of experimental CV777 coronavirus enteritis in piglets. II. Electron microscopic study. *Vet Pathol* **19**:57–60, 1982
- 9 EICHOLZ, A.; CRANE, R.K.: Studies on the organization of the brush border in intestinal epithelial cells. I. Tris disruption of isolated hamster brush border and density gradient separation of fractions. *J Cell Biol* **26**:687–691, 1965

- 10 GLENNER, G.G.: Histochemical demonstration of monoamine oxydase activity by tetrazolium salts. *J Histochem Cytochem* **5**:591, 1957
- 11 GOMORI, G.: An improved histochemical technique for acid phosphatase. *Stain Technol* **25**:81, 1950
- 12 HOOPER, B.E.; HAELTERMAN, E.O.: Lesions of the gastrointestinal tract of pigs infected with transmissible gastroenteritis. *Can J Comp Med* **33**:29–36, 1969
- 13 KEENAN, K.P.; JERVIS, H.R.; MARCHWICKI, R.H.; BINN, L.N.: Intestinal infection of neonatal dogs with canine coronavirus 1–71: Studies by virologic, histologic, histochemical and immunofluorescent techniques. *Am J Vet Res* **37**:247–256, 1976
- 14 MEBUS, C.A.; NEWMAN, L.E.; STAIR, E.L.: Scanning electron, light and immunofluorescent microscopy of intestine of gnotobiotic calf infected with calf diarrheal coronavirus. *Am J Vet Res* **36**:1719–1725, 1975
- 15 MILLER, D.; CRANE, R.K.: The digestive function of the epithelium of the small intestine. I. An intracellular locus of disaccharidase and sugar phosphatase hydrolysis. *Biochem Biophys Acta* **52**:281–293, 1961
- 16 MORIN, M.; MOREHOUSE, L.G.; SOLORZANA, R.F.; OLSON, L.D.: Transmissible gastroenteritis in feeder swine: clinical, immunofluorescence and histopathological observations. *Can J Comp Med* **37**:239–248, 1973
- 17 OVERTON, J.R.; EICHOLZ, A.; CRANE, R.K.: Studies on the organization of the brush border in intestinal cells. II. Fine structure of fractions of tris-disrupted hamster brush border. *J Cell Biol* **26**:693–706, 1965
- 18 PEARSE, A.G.E.: *Histochemistry, Theoretical and Applied*. Churchill, London, 1961
- 19 PEARSON, G.R.; MCNULTY, M.S.: Pathological changes in the small intestine of neonatal pigs infected with a pig reovirus-like agent (rotavirus). *J Comp Pathol* **87**:363–375, 1977
- 20 PENSART, M.; DEBOUCK, P.: A new coronavirus-like particle associated with diarrhea in swine. *Arch Virol* **58**:243–247, 1978
- 21 PENSART, M.; HAELTERMAN, E.O.; BURNSTEIN, T.: Transmissible gastroenteritis of swine: virus—intestinal cell interactions. *Arch Gesamte Virusforsch* **31**:321–334, 1970
- 22 POMEROY, K.A.; PATEL, B.L.; LARSEN, C.T.; POMEROY, B.S.: Combined immunofluorescence and transmission electron microscopic studies of sequential intestinal samples from turkey embryos and poults infected with turkey enteritis coronavirus. *Am J Vet Res* **39**:1348–1354, 1978
- 23 TAKEUCHI, A.; BINN, L.N.; JERVIS, H.R.; KEENAN, K.P.; HILDEBRAND, P.K.; VALAS, T.R.B.; BLAND, F.F.: Electron microscope study of experimental enteric infection in neonatal dogs with a canine coronavirus. *Lab Invest* **34**:539–549, 1976
- 24 THAKE, D.C.: Jejunal epithelium in transmissible gastroenteritis of swine. An electron microscopic and histological study. *Am J Pathol* **53**:149–168, 1968
- 25 THEIL, K.W.; BOHL, E.M.; CROSS, R.F.; KOHLER, E.M.; AGNES, A.G.: Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. *Am J Vet Res* **39**:213–220, 1978
- 26 TRAPP, A.L.; SANGER, V.L.; STAINAKER, E.: Lesions of the small intestinal mucosa in transmissible gastroenteritis-infected germfree pigs. *Am J Vet Res* **27**:1695–1702, 1966

Request reprints from Dr. W. Coussement, Department of Veterinary Pathology, State University of Ghent, Casinoplein 24, 9000-Ghent, (Belgium).