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## An Immunohistochemical Investigation of Porcine Epidemic Diarrhoea

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### Summary

A sudden outbreak of epidemic diarrhoea of piglets occurred in Japan, the principal features being watery diarrhoea, dehydration and high mortality in newborn animals. The microscopical lesions were villous atrophy in the small intestine, the villous enterocytes being vacuolated and cuboidal in shape. The villus-crypt ratio was severely reduced, varying from 1:1 to 3:1. Transmission electron microscopy showed numerous coronaviruses within the cytoplasm of enterocytes and among microvilli. Specific antigens of porcine epidemic diarrhoea (PED) virus were detected in the cytoplasm of enterocytes by the streptavidin-biotin (SAB) technique. Infected cells, which were most abundant in the villous epithelia of the jejunum and ileum, were present in small numbers in the large intestine, the crypt epithelia, the lamina propria and Peyer's patches. The study suggests that the SAB technique is useful for the diagnosis of PED.

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### Introduction

Porcine epidemic diarrhoea (PED) virus is distinguishable antigenically from transmissible gastroenteritis virus (TGEV) and haemagglutinating encephalomyelitis virus (HEV) (Horvath and Mocsari, 1981; Pensaert *et al.*, 1981).

PED has been reported in England (Wood, 1977; Chasey and Cartwright, 1978), Belgium (Pensaert and Debouck, 1978), Canada (Turgeon *et al.*, 1980), Hungary (Horvath and Mocsari, 1981) and Germany (Pospischil *et al.*, 1981). In Japan, PED or PED-like disease occurred from late 1982 to early 1983 (Takahashi *et al.*, 1983; Kuwahara *et al.*, 1988) but was not reported again until outbreaks occurred on a number of farms between September 1993 and June 1994. Newborn piglets developed signs of diarrhoea, dehydration and vomiting, death occurring within a few days. Mortality ranged from 30% to 80% and *c.* 14 000 deaths occurred. Adult pigs showed only transient inappetence and the milk secretion of sows was sometimes depressed.

PED virus antigens were demonstrated by the direct immunofluorescence technique in the intestine of experimentally infected pigs (Debouck and Pensaert, 1980; Debouck *et al.*, 1981; Pospischil *et al.*, 1981). This paper

describes the demonstration of PED virus antigens in the intestine of naturally infected piglets by the hitherto untried streptavidin-biotin technique.

### Materials and Methods

#### *Animals*

Twenty-one piglets aged 2 to 7 days were collected from five large Japanese swine breeding farms, with 100–2600 sows, during outbreaks of diarrhoeal disease (Table 1). The piglet mortality varied from 30% to 60% and *c.* 10 000 newborn piglets died. On these farms, vaccines against transmissible gastroenteritis (TGE), Japanese porcine encephalitis, porcine parvovirus infection, swine fever, Aujeszky's disease and swine erysipelas had been in regular use. Piglets were treated for diarrhoea with colistin or oxolinic acid, but without success.

TGE was excluded by failure to isolate the virus from the small and large intestine of all 21 piglets on a porcine kidney cell line (CPK cell) and in Vero cell cultures, and by failure to demonstrate elevated neutralizing antibody titres against TGEV in the serum of nine convalescent piglets (19-day-old) on farms 1 and 2 (Table 1). Rotavirus infection was also excluded by the absence of specific antigen in the intestinal content of all 21 piglets collected, as shown by the Rotaclone Kit (Cambridge Biotech, MA, USA). Bacteriological examination of samples from 13 diarrhoeic piglets (intestinal contents, liver, lung and kidney) failed to reveal pathogenic bacteria.

Control material for the PED virus antigen tests (see below) was obtained from a 25-day-old specific pathogen-free (SPF) piglet, a 25-day-old SPF piglet infected orally with the virulent TGEV strain Shizuoka-20, and four newborn piglets in which TGE had been diagnosed by direct immunofluorescence.

#### *Pathological Examination*

Specimens were taken after slaughter from the stomach, duodenum, jejunum, ileum, caecum, colon, liver, spleen, lung, heart, kidney, adrenal, tonsil, mesenteric lymph node, pancreas, cerebrum, cerebellum, mesencephalon, medulla oblongata, hypophysis, spinal cord, trigeminal ganglion, and coeliac ganglion. The tissues were fixed in 10% phosphate-buffered formalin, embedded in paraffin wax, sectioned, stained with haematoxylin and eosin (HE), and examined by light microscopy.

#### *Transmission Electron Microscopy*

The samples obtained from the jejunum were used. They were fixed in 2.5% glutaraldehyde-phosphate buffer (pH 7.4) at 4°C for 2 h, washed in 0.1 M phosphate buffer and post-fixed in 1% osmium tetroxide-phosphate buffer at 4°C for 1 h. After having been dehydrated, they were embedded in epoxy resin. Ultrathin sections were then cut, stained with uranyl acetate and lead citrate, and examined with a Jeol transmission electron microscope (JEM-1010).

#### *Immunohistochemical Examination*

The distribution of PEDV antigens was investigated by the streptavidin-biotin (SAB) technique, with light microscopy. The SAB kit (Scytek Laboratories, Logan, Utah, USA) was used to examine formalin-fixed, paraffin wax-embedded sections of the gastrointestinal tract and lung. After de-waxing, endogenous peroxidase activity was blocked by treatment with 0.3% H<sub>2</sub>O<sub>2</sub>, and the sections were then washed in 0.05 M Tris buffer (pH 7.6). Reagents in the SAB kit were prepared according to the manufacturer's instructions. Sections were counterstained with methyl green. Rabbit antiserum against PED virus (strain 83P-5) was used as primary antibody. The specificity of the antiserum has been reported previously by Kusanagi *et al.* (1992),

**Table 1**  
**Outbreaks on five farms and pathological findings in 21 piglets**

Farm no.	Duration of outbreak (months)	Mortality rate (%)	Number of dead piglets	Number of piglets examined	Age of piglets (days)	Number of piglets with			
						watery diarrhoea	curdled milk in stomach	villous atrophy	vacuolated enterocytes
1	5	30	5670	4	2-5	2	2	3	2
2	10	50	2900*	4	4	4	3	4	4
3	3	60	700*	4	3-7	3	1	4	4
4	2	55	660*	4	4-6	3	4	4	4
5	2	39	545	5	4-7	4	4	5	5

\*This value is estimated approximately from the number of breeding sows, the duration of the outbreak and the mortality rate.

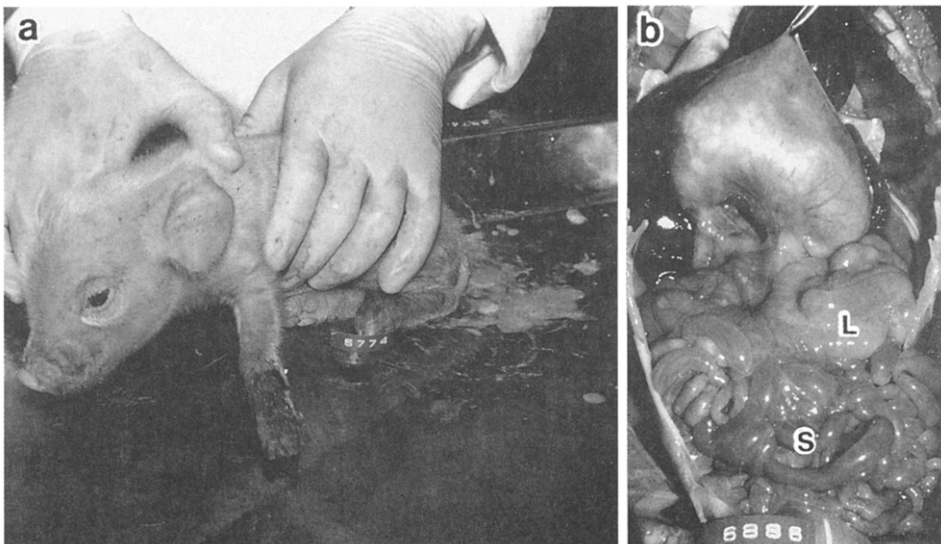


Fig. 1. A 4-day-old piglet shows diarrhoea. (a) The diarrhoea is yellowish and watery. (b) The intestine is distended with fluid contents, and the wall is very thin and transparent. The stomach is also distended with curdled milk. S=small intestine. L=large intestine.

who found that PED virus (83P-5), TGEV and HEV were neutralized only by homologous antiserum and that no antigenic relationship between TGEV and PED virus (83P-5) could be demonstrated by the indirect immunofluorescence test.

## Results

### *Clinical Observations*

Sixteen out of the 21 newborn piglets examined showed whitish or yellowish watery diarrhoea, dehydration (Table 1; Fig. 1a) and sometimes vomiting. One piglet from farm 4 died in transit.

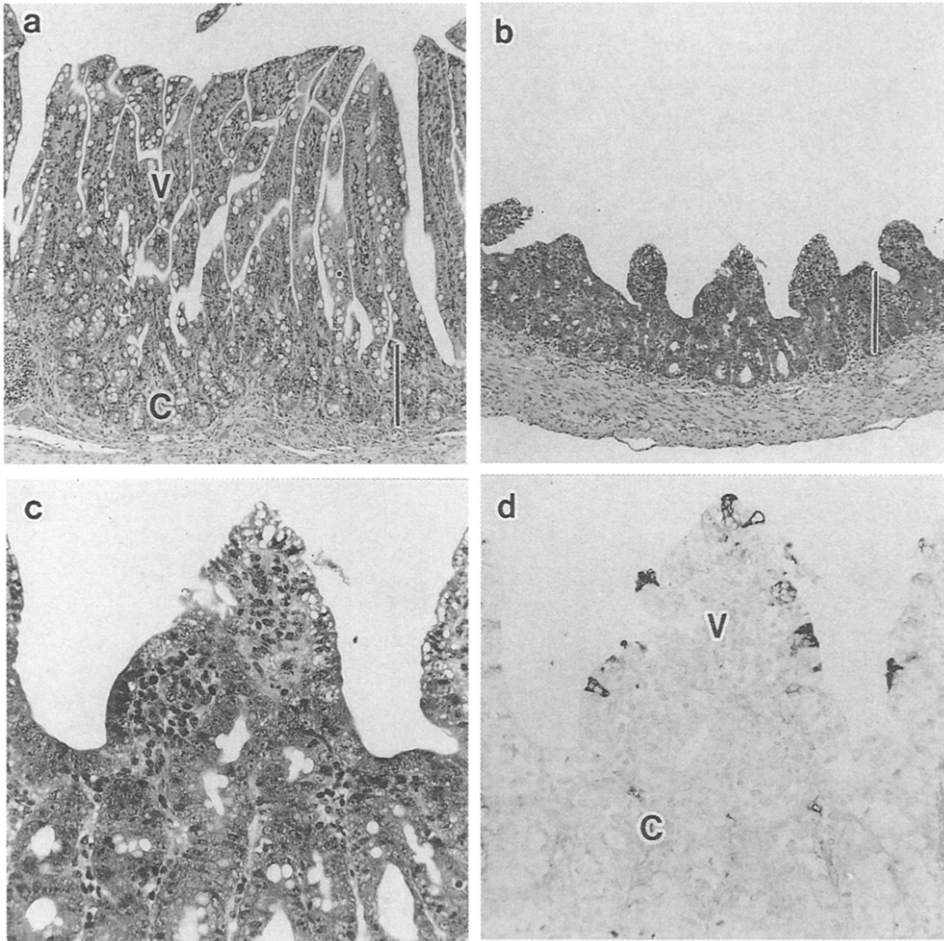


Fig. 2. Transverse sections of the jejunum. (a) A piglet from farm 1, showing no diarrhoea. This piglet was PED-antigen negative. The villi (V) are still of normal length (*c.* 700  $\mu$ m). C=crypt. HE. Bar=200  $\mu$ m. (b) A piglet with severe diarrhoea. The villus-crypt ratio is about 1:1. HE. Bar=200  $\mu$ m. (c) Higher magnification of Fig. 2b. Villi are covered with cuboidal and vacuolated enterocytes. There is a slight lymphocytic infiltrate in the lamina propria. Crypt enterocytes are intact. HE.  $\times$  182. (d) Serial transverse section adjacent to that in Fig. 2c. PEDV antigen is seen in low concentration in the villous enterocytes. V=villus. C=crypt. SAB technique.  $\times$  182.

### Pathology

Macroscopically, the intestine of diarrhoeic piglets was distended with yellowish watery contents and sometimes contained flecks of curdled undigested milk. The intestinal wall was thin and transparent (Fig. 1b). The stomach was also distended and in 14 piglets the wall was thin. The mesenteric lymph nodes were occasionally enlarged.

Microscopically, the villi were atrophied in the small intestine of 20 piglets (Table 1). The villus-crypt ratio was reduced, varying from *c.* 1:1 to 3:1 (Fig. 2a, b). Atrophied villi often consisted of strikingly vacuolated enterocytes. Numerous enterocytes on the villi were cuboidal to low columnar and the

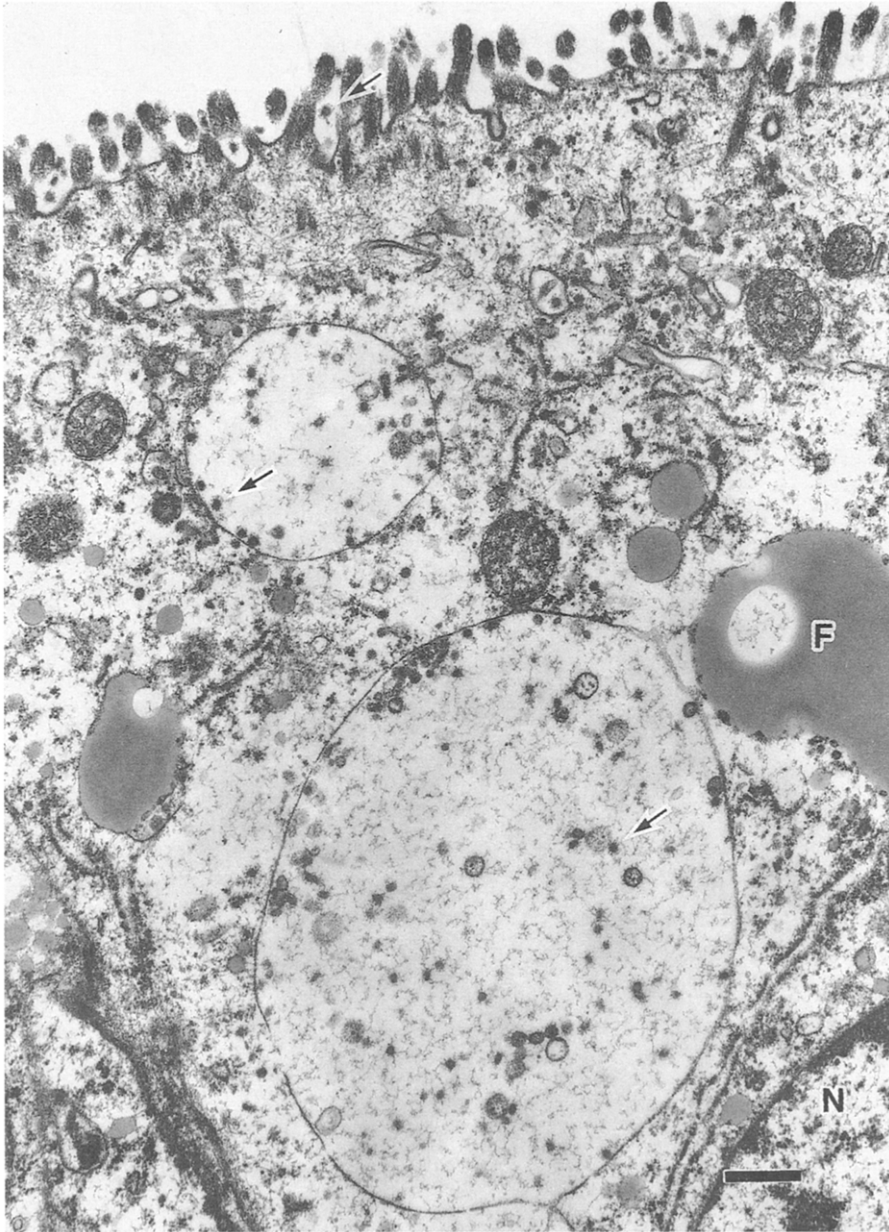


Fig. 3. Transmission electron micrograph of the enterocytes of the jejunum. Mature viral particles (arrows) covered with projections are seen in the membrane-bounded vacuoles and among microvilli. F= fat droplet. N=nucleus. Bar=500 nm.

brush border was indistinct (Fig. 2c). Some cells on the crests of the villi appeared distorted and the nuclei were pyknotic. The mucosal lamina propria was infiltrated slightly with lymphocytes, eosinophils and neutrophils, and was occasionally congested and oedematous. However, inflammatory exudate was

**Table 2**  
**Distribution of PEDV antigen as shown by the SAB technique**  
**in the gastrointestinal tract and lung**

Farm no.	Number of piglets	Results (number of antigen-positive piglets/antigen concentration) in				
		duodenum	jejunum	ileum	caecum	colon
1	4	2/+~+++	3/+++~++++	2/+++~++++	0/	2/+~+++
2	4	1/+	4/+++~++++	4/+++	3/+	3/+
3	4	1/+++	4/+~+++	3/+~+++	0/	0/
4	4	3/+++	4/+++~++++	4/+++~++++	3/+~+++	2/+~+++
5	5	ND	3/+++	3/+++~++++	1/+	0/

+, Low; ++, moderate; +++, high.

ND, not done.

Stomach and lung gave negative results in all pigs.

not a striking feature. The lesions were most common and severe in the jejunum and ileum, and were sometimes observed in the duodenum. The caecum and colon showed no obvious changes except for vacuolation of the superficial enterocytes. No characteristic lesions were observed in other organs except slight pneumonia and slight renal nephrosis in some piglets.

#### *Transmission Electron Microscopy*

Microvilli of infected superficial enterocytes were disoriented. Many viral particles were seen in vacuoles or cytoplasmic organelles situated mainly in the supranuclear cytoplasm. Some viruses were seen between the microvilli. The viral particles were pleomorphic, measuring between 70 nm and 140 nm. They were mainly spherical or oval. Mature viruses (100–140 nm diameter) with a number of projections (*c.* 20 nm) were observed within the membrane-bounded vacuoles in the enterocytes (Fig. 3).

#### *Immunohistochemistry*

PEDV antigens were detected in high concentration in the enterocytes in 18 of 21 piglets by the SAB technique. The antigen-containing enterocytes were profuse in the small intestinal mucosa but sparse in the large intestine (Table 2; Fig. 4). The infected cells in the duodenum were fewer than in the jejunum and ileum but greater than in the caecum and colon. In infected piglets, antigen-containing cells invariably occurred in the jejunum. These infected enterocytes tended to be less numerous in severe (Fig. 2d) than in moderate (Fig. 4b) villous atrophy. Infected cells were not seen in two piglets that showed severely atrophied villi. Antigen was confined to the cytoplasm, and could not be detected in the nuclei. Most infected cells were not arranged continuously in the epithelial layer (Figs 2d and 4a–d) and the border between infected and uninfected cells was distinct. Infected cells were observed occasionally in the crypts (Fig. 4a) and in the lamina propria and Peyer's patches in the ileum (Fig. 4c).

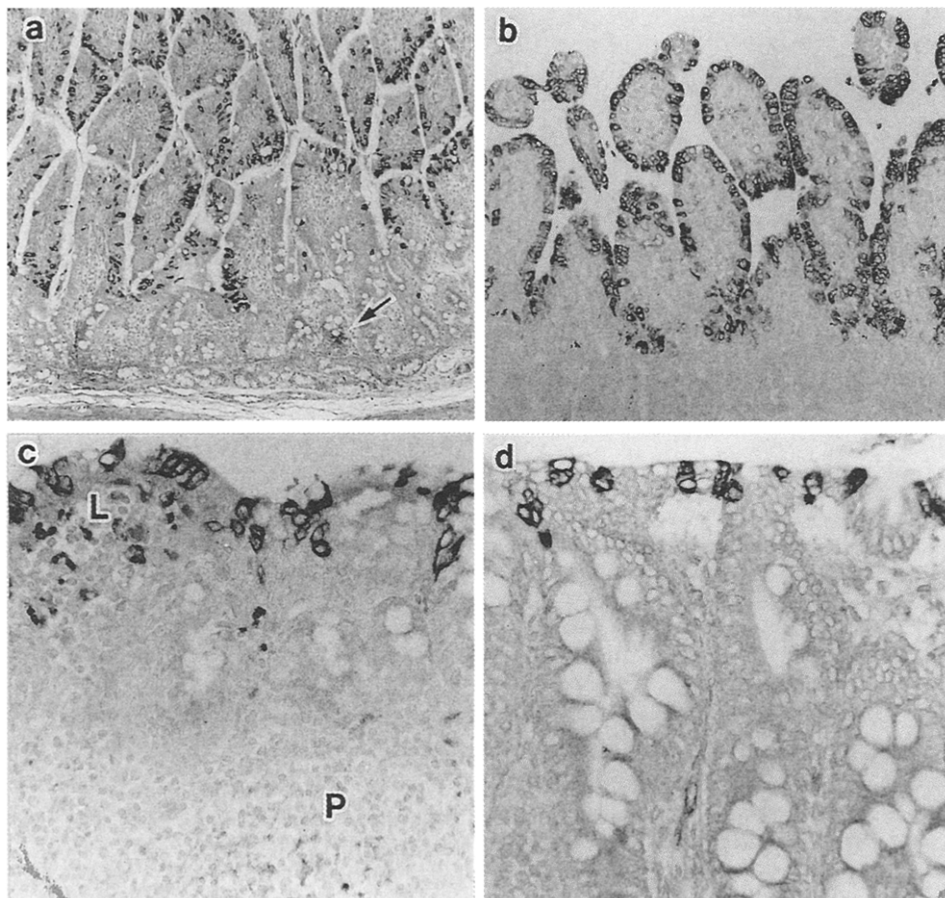


Fig. 4. SAB technique with PEDV antiserum. (a) The duodenum. Infected cells, though diffusely scattered in most piglets, were sometimes distributed as seen in this photograph. The antigens (arrow) are also seen in the crypt epithelia.  $\times 54$ . (b) The jejunum. Villi are densely covered with infected cells.  $\times 93$ . (c) The ileum. The PEDV antigens are confirmed not only in the superficial enterocytes, but also in the lamina propria (L). P=Peyer's patch.  $\times 273$ . (d) The colon. PEDV antigen is seen in a few superficial enterocytes.  $\times 285$ .

PEDV antigen could not be detected in Brunner's glands of the duodenum, stomach or lung, or in the intestine of control animals (a healthy SPF piglet, a piglet experimentally infected with TGEV, and four piglets in which natural TGE had been diagnosed).

### Discussion

In an outbreak of epidemic diarrhoea in piglets in Japan, TGEV, rotavirus and bacteria were excluded as possible aetiological agents by appropriate tests. However, the SAB technique, with PEDV antiserum, gave positive results in the small intestine, caecum and colon. This closely resembled the findings reported by Debouck *et al.* (1981) in pigs experimentally infected with PEDV



and examined by the immunofluorescence technique. The site of viral replication of PEDV was clearly different from that of TGEV, which is the small intestine (Hooper and Haelterman, 1966). In addition, PEDV was absent from the lung, thus differing from porcine respiratory coronavirus (Cox *et al.*, 1990). From the results, the diarrhoeal disease was diagnosed as PED.

This infection seems to lead to diffuse and severe villous atrophy in the small intestine. Debouck *et al.* (1981) reported that the mean villus-crypt ratio varied from 2·3:1 to 1·5:1 in experimental PED. In the present study, however, the villus-crypt ratio in the most severely affected piglets was 1:1 and the lesions were as severe as those of TGE. Furthermore, villous atrophy was observed in the duodenum, in contrast to the findings of Ducatelle *et al.* (1981) in experimental pigs. The differences between the results obtained in experimental infection and those in the present study may have been due to differences in the virulence of the strains or in the infecting dose.

The number of enterocytes infected with PEDV tended to decrease with increasing severity of villous atrophy. Possibly in piglets with severe villous atrophy, the PEDV may already have left the enterocytes, or the infected cells may already have been eroded. Thus the distribution of PEDV antigens may vary according to the stage of the disease.

In the SAB technique, fixed material may be used, no cryostat is needed, and the sections show adequate detail and are relatively permanent. The method would therefore seem useful as a diagnostic method for PED and for studying the distribution of PEDV antigens.

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