THE ISOLATION OF REOVIRUS-LIKE AGENTS (ROTA-VIRUSES) FROM ACUTE GASTROENTERITIS OF PIGLETS

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PLATE XVI

Reovirus-like agents, now commonly known as rotaviruses (Flewett et al., 1974) or duoviruses (Davidson et al., 1975), were first seen in preparations from diarrhoeic faeces of calves in the United States (Mebus et al., 1969) and subsequently in Australia (Turner, Caple and Craven, 1973) and in the United Kingdom (Woode et al., 1974). Similar viruses have been demonstrated in the faeces of cases of acute gastroenteritis of children (Bishop et al., 1973; Flewett, Bryden and Davies, 1973), monkeys (Els and Lecatsas, 1972), mice (Much and Zajac, 1972; Kapikian et al., 1974; Flewett, Bryden and Davies, personal communication) and foals (Flewett, Bryden and Davies, 1975). Infection was established in piglets with the viruses isolated from calves and children, and the calf virus was shown to be pathogenic to piglets (Bridger et al., 1975; Woode and Bridger, 1975; Hall et al., 1976). After serological evidence had been obtained that pigs from one herd were infected with a virus antigenically similar to the calf rotavirus (Woode and Bridger, 1975), attempts were made to isolate naturally-occurring pig rotaviruses. This paper describes the isolation of pig rotaviruses from outbreaks of diarrhoea in both weaned and unweaned piglets and the antigenic relation between human, calf and pig rotaviruses passed in pigs.

MATERIALS AND METHODS

Source of virus. Two herds with a history of piglet diarrhoea were studied. One was a minimal-disease herd (MD), formed by the purchase of breeding stock bred from caesarean-derived animals, and the other was a conventional pig herd. One isolate, no. SW1/2, was obtained from diarrhoeic material of 4-week-old, weaned pigs in the MD herd. Diarrhoea began in the majority of a group of 45 pigs 4-5 days after weaning. Four of the 45 pigs died.

The other isolate, no. SW20/21, was obtained by pooling diarrhoeic material from two pigs of a group of 40 from the conventional herd. Three of the 40 pigs died, and at necropsy Escherichia coli, strain Abbotstown, was isolated from the jejunum of one of them in pure and profuse culture; the other two pigs did not yield haemolytic E. coli. Virus studies were made in outbreaks of diarrhoea on 23 farms.

Animal inoculation. Preparations no. SW1/2 and no. SW20/21 were shown to contain rotavirus particles by electron microscopy (EM). They were passed through 0.45 μm...
membrane filters after dilution 1 in 3 (v/v) in phosphate-buffered saline, pH 7.2. Gnotobiotic piglets aged 2–28 days (Tavernor et al., 1971) were given intranasal inoculations of 0.5–1.0 ml of each filtrate. Similar preparations of a calf virus described by Woode et al. (1974), a virus isolated from children (Flewett et al., 1973), and three other pig isolates from different farms were inoculated intranasally into gnotobiotic piglets. For assay of the infectivity of isolate SW20/21 ten-fold dilutions were made in phosphate-buffered saline and 1 ml of each dilution was inoculated intranasally into each of three pigs, and diluent alone into three control pigs. Faecal samples were taken at the commencement of diarrhoea and serum samples 4 weeks later.

Antiserum production. Serum samples were taken from the experimentally infected piglets before inoculation and 2–4 weeks after infection. Paired serum samples were taken from naturally affected pigs during the acute phase of the disease and 3 weeks later.

Pathology. Histopathological studies have been made of six gnotobiotic piglets infected with calf rotavirus (Hall et al., 1976) and nine gnotobiotic piglets infected with pig rotavirus. Tissues for histological examination were removed under pentobarbitone sodium anaesthesia. Short lengths of upper, middle and lower small intestine were tied off and filled with 12% neutral buffered formalin; fixation was completed by immersion in fresh fixative. Blocks of fixed tissue were dehydrated and embedded in paraffin wax; sections were cut at 5 μm and stained with haematoxylin and eosin.

Tissue culture. Primary calf-kidney (CK) and pig-kidney (PK) cell cultures were prepared in tubes with flying coverslips. Growth medium was Earle's salt solution with galactose 0.1% (w/v) in place of glucose, lactalbumin hydrolysate (Nutritional Biochemicals) 0.5% (w/v) and foetal calf serum 10% (v/v). For maintenance of infected cultures 2% of foetal calf serum was added to the medium.

Virus isolation in tissue culture. Bacteria-free filtrates of faecal material from field cases of disease and from experimentally infected pigs were inoculated into coverslip cultures at final dilutions of 1 in 30 of faecal material. These cultures were fixed in acetone 24–72 h after inoculation and examined for evidence of infection by immunofluorescence with gnotobiotic-calf antiserum to the calf virus and gnotobiotic-pig antiserum to the calf, pig and human viruses (Woode et al., 1974).

Virus detection by electron microscopy. Piglet faeces or gut contents were prepared by differential centrifugation, sometimes followed by centrifugation through sucrose, before examination with the electron microscope (Flewett et al., 1974). For the determination of virus-particle size a Phillips EM 300 electron microscope was calibrated with beef-liver catalase crystals (Boehringer Ltd, Mannheim, Germany), and calculations of the true microscope magnification were made by taking the period of the crystals as 8.44 nm (Cox and Horne, 1968).

Serology. Sera were tested by an indirect immunofluorescent (FA) technique and a neutralisation test (NT) with rotavirus adapted to calf-cell culture (Bridger and Woode, 1975).

RESULTS

Clinical signs of disease. Isolates SW1/2 and SW20/21 caused diarrhoea in gnotobiotic piglets within 20–48 h of infection and in all subsequent passages in gnotobiotic piglets of the isolates, of which there were three of SW1/2 and four of SW/21. Similarly, calf rotavirus caused diarrhoea in all six passages of the virus in gnotobiotic piglets.

Naturally infected pigs, and gnotobiotic pigs experimentally infected with pig-rotavirus isolates SW1/2 and SW20/21 and calf rotavirus, showed similar clinical signs. Within 18–24 h of infection there was depression, anorexia and reluctance to move. In piglets experimentally infected with pig virus, vomiting was observed after 18–24 h, followed a few hours later by profuse diarrhoea. Conventionally reared farm piglets on a solid food diet showed diarrhoea varying
in colour from yellow to dark grey, whereas in experimental pigs on an all-milk diet the diarrhoea was invariably yellow, frequently with floccules floating in a whey-like fluid. In both naturally infected and experimentally infected pigs there was a rapid loss of condition with the backbone and ribs becoming prominent. During the period 24–72 h after infection, pigs showed a reduced food intake but thereafter the appetite recovered. Clinical signs regressed 4–6 days after infection, although loose, yellow faeces persisted for 7–14 days. These clinical signs were observed in all pigs infected with all passages of isolates SW1/2 and SW20/21 and calf rotavirus. The rate of recovery was enhanced when water replaced milk in the diet for 24 h.

**Electronmicroscopic examination.** Pig rotaviruses obtained from naturally and experimentally infected piglets were indistinguishable in size and appearance from the calf and human rotavirus described by Flewett et al. (1974). Particles with and without the outer capsid layer were found; the former were c. 69 nm and the latter c. 58 nm in diameter. Both types of particle have been observed in preparations of calf and mouse rotaviruses (Bridger and Woode, 1975; Woode et al., unpublished). Rotavirus particles were observed in the faeces of all gnotobiotic pigs given experimental inoculations of SW1/2 and SW20/21 material, and in the original SW1/2 diarrhoea material. However, virus was not observed in the original SW20/21 preparation. A small virus with a mean diameter of 28 nm was also observed in the SW1/2 preparations and in all passages of this isolate. As no other virus particles were observed in SW 20/21 passages, this isolate has been established as the standard pig rotavirus for experimental purposes (the figure).

Virus particles similar to rotaviruses were observed in faecal filtrates from 23 of 65 piglets with diarrhoea—whether weaned or unweaned—on nine of the 23 farms studied. Three of these filtrates were inoculated intranasally into gnotobiotic piglets and caused diarrhoea and clinical illness.

**Culture of the virus.** Coverslip cultures of CK and PK cells that had been inoculated with faecal filtrates of calf and pig rotaviruses obtained from experimentally infected piglets showed individual immunofluorescent cells when examined by the indirect test with calf-rotavirus monospecific antiserum, and with convalescent gnotobiotic-pig antiserum to the calf, pig and human rotavirus isolates. Granular intracytoplasmic fluorescence was seen, and was similar in appearance for all viruses studied, which included calf rotavirus isolated from calves, calf rotavirus passed in gnotobiotic pigs, pig rotavirus passed in gnotobiotic pigs and the cell-culture-adapted calf virus.

CK and PK cells appeared to be equally susceptible to infection with calf and pig rotaviruses. Although some cytopathic effect was observed with the first passage of field virus, this effect was not seen with subsequent passages; a similar effect was observed with the field isolates of the calf rotavirus (Bridger and Woode, 1975). Unlike the calf virus, the pig rotavirus could not be adapted to replicate in cell culture; infectivity was lost after two or three passages.

Few faecal preparations from pigs naturally infected with rotavirus were infectious in cell culture, although virus was demonstrated by electron microscopy. The morphological appearance and the infectivity of faecal preparations
in cell cultures was improved by sampling from the pig directly into bottles with screw caps. Exposure of calf and pig faeces to the air appeared to reduce the ability to demonstrate well-formed particles by EM, and the preparations usually were not infectious in cell culture.

_Serological studies._ The two tests employed revealed differences in the response to infection of pigs with rotaviruses. Sera from adult sows in the herd of pigs from which isolates SW1/2 and SW20/21 were made neutralised the calf cell-culture-adapted virus at titres of 40 or greater. These sera also possessed antibody by the FA test at titres of 40–160. However, convalescent sera from 13 gnotobiotic piglets experimentally infected with the two pig-rotavirus isolates had neutralising-antibody titres of <10; although they had FA titres of 160–640.

Piglets convalescent from calf-rotavirus infection showed a variable antibody response when tested against the same isolate of calf rotavirus. Only four of six piglets produced neutralising antibodies but all six produced FA antibodies at titres of 80–320. When pigs were bled at 8 and 12 weeks the pattern of antibody response had not altered. Similar variations were observed in the activity of sera of sows that were randomly sampled from several farms. Thirty-six of 56 sera had neutralising activity with titres of 40–320, and 54 of 56 had FA activity with titres of 40–160.

Paired sera from 12 piglets concerned in the outbreak from which the isolate SW20/21 was obtained were tested for rotavirus antibody by NT and FA. Six had NT titres of 10–80 and 11 had FA titres of 40–80 during the acute phase of the disease. Two of the 12 piglets showed a four-fold to eight-fold rise in NT titres and seven a four-fold to eight-fold rise in FA titre.

_Infectivity assay in piglets._ The titre of piglet infectivity of isolate SW20/21 was 10^7 on two separate occasions. Virus was reisolated from all the piglets given dilutions of virus from 10^{-1}–10^{-7}. Convalescent sera had FA titres of 320–640, but no neutralising antibody was detected. The piglets inoculated with 10^{-8} dilution of virus and the control piglets remained clinically normal, and did not excrete virus nor develop specific antibody. The incubation period for the onset of diarrhoea and clinical illness varied from 24 h for the 10^{-1} dilution to 72–96 h for the 10^{-7} dilution. No difference was observed in the severity of disease according to the dose of virus inoculated. However, 33% died at dilutions of 10^{-3}, 10^{-6} and 10^{-7}.

_Pathological changes._ In the piglets infected with pig rotavirus, lesions were detected in the small intestine. There was desquamation of the epithelial cells lining the distal three-quarters of the villi, and degenerate cells were observed in the exposed lamina propria. Eventually the villi were severely stunted. The remaining cells on the surface of the villi were cuboidal or flattened, had no brush-border and contained cytoplasmic vacuoles. The middle small intestine was most severely affected.

These lesions produced by pig rotavirus in the small intestine of gnotobiotic pigs were identical in nature and severity with those produced by the calf rotavirus (Hall et al., 1976).
DISCUSSION

The possibility that pathogenic viruses are responsible for many outbreaks of diarrhoea in pigs has long been suspected. Smith and Jones (1963) studied the bacterial flora of healthy and diseased pigs and found that bacteria did not appear to play any part in 12 of 25 outbreaks of diarrhoea in unweaned and in three of 24 outbreaks in weaned pigs. Before the discovery of rotaviruses, clear evidence for the existence of viruses that caused diarrhoea had been obtained mainly in studies of transmissible gastroenteritis of pigs and the vomiting and wasting disease, in both of which coronaviruses had been incriminated (Tajima, 1970; Phillip, Cartwright and Scott, 1971). Little evidence had been produced that other viruses caused diarrhoea, although enteroviruses and adenoviruses are frequently isolated from normal and diseased pigs.

The results of our studies on the pig provide another instance of the causal association of rotaviruses with diarrhoea in man and animals. The rotaviruses of children, calves, pigs, mice and monkeys are indistinguishable morphologically and all share a common antigen. One member of this group of viruses can be used in serological tests for rotavirus infection in other species. There is serological evidence of rotavirus infection in rabbits, sheep, guinea-pigs and goats (Bridger et al., 1975; Woode et al., unpublished). Rotaviruses are commonly associated with diarrhoea in children (Flewett et al., 1974) and in calves (Woode and Bridger, 1975). Unfortunately the diagnostic tests for diarrhoea due to rotavirus are less satisfactory in piglets than in calves and children, because fewer particles are observed by EM, and occasionally the presence of a pathogenic virus is demonstrable only by inoculation into piglets. However, pig rotavirus was isolated at the first attempt in nine of 23 outbreaks of disease. Twelve herds have been studied serologically and all were shown to be infected with rotavirus. Thus rotavirus infection appears to be as common in pigs as in children and calves.

The fact that the pig is readily infected by rotaviruses isolated from children and calves may be of great epidemiological importance. Although we have been unsuccessful in transmitting human and pig rotaviruses to gnotobiotic calves, viruses of this group may well be transmissible between mammalian species. Thus, control of diarrhoea in the pig, which in the past has been based on hygienic precautions or the production by hysterotomy of pathogen-free pigs, must now include measures to prevent infection from man and other animals.

The lack of neutralising activity in 60% of sow sera, all of which possess antibodies detectable by immunofluorescence, is also observed in some human sera. Five of 23 human sera had neutralising activity to the calf virus, but 15 of 23 possessed immunofluorescent antibody (Bridger et al., 1975). In contrast, all bovine antisera to the calf virus, obtained after experimental infection or by random selection, possessed both neutralising and immunofluorescent activity (Bridger and Woode, 1975). The variation in activity of human and sow antisera may depend on differences between strains of rotavirus.
or variation in the serological response to infection. The response of pigs to experimental infection with particular isolates of rotavirus was similar in the majority of animals, although a minority showed an atypical response. All of six piglets convalescent from calf-rotavirus infection and all of 11 piglets convalescent from human rotavirus infection produced immunofluorescent antibody to the calf rotavirus, whereas only four of the six and two of the 11 produced neutralising antibody (Bridger et al., 1975).

Studies are continuing of the frequency and importance of rotaviruses in outbreaks of diarrhoea in pigs. However, the diagnosis of rotavirus infection is complicated by the difficulty of isolating the virus, the occurrence of subclinical infection in pigs and calves with both virulent and avirulent strains, and the excretion of virus from some animals for at least 3 weeks (Bridger et al., 1975; Woode, unpublished). The incidence of subclinical infection probably exceeds that of clinical infection, because it is estimated that 10% of animals develop clinical diarrhoea but 80–100% are serologically positive at 6–9 months (Woode, unpublished). Thus, in the absence of a simple laboratory test for virulence, the presence of a rotavirus in faeces is not conclusive evidence of its causal role in the disease.

**SUMMARY**

Isolations of reovirus-like agents (rotaviruses) were made from nine of 23 outbreaks of piglet diarrhoea on different farms and from both weaned and unweaned piglets. The viruses were shown to be morphologically and antigenically similar to the rotaviruses of children and calves. Gnotobiotic piglets given intranasal inoculations of five different isolates developed acute gastroenteritis, and the virus was re-isolated from the faeces or intestinal contents. The piglet virus was not adapted to replicate in cell culture. We conclude that the pig rotavirus is commonly associated with outbreaks of gastroenteritis and is probably an important aetiological factor in this disease.

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**REFERENCES**


FIGURE.—Rotavirus particles isolated from diarrhoeic faeces of naturally infected piglets. a. Particles without the outer capsid layer. b. Particle with the outer capsid layer. × 190 000.
ROTAVIRUSES FROM PIGLETS


