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Variation in virulence of bovine rotaviruses

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SUMMARY

Forty-six gnotobiotic calves aged less than 16 days or 42–116 days were infected with three strains of bovine rotavirus designated C3-160, CP-1 and PP-1. Each virus was passaged and cloned in cell culture (cloned viruses) but CP-1 and PP-1 were also used before culture (faecal viruses).

Infection of calves aged less than 16 days with faecal or cloned CP-1 caused disease whereas cloned C3-160 and faecal or cloned PP-1 caused subclinical infections. The clinical signs of disease were change in faecal colour to pale yellow or cream, increase of 2- to 7-fold in the volume of faecal output and, usually, anorexia. With the virulent CP-1 virus and the avirulent C3-160, similar amounts of virus were excreted in the faeces for 4–6 days.

Infection of calves aged 56–116 days with faecal CP-1 produced disease of similar severity to that seen in calves aged 7–10 days infected with the same virus. No differences in clinical signs, virus excretion or levels of convalescent antibody were seen between the two groups. With cloned CP-1, 5 of 8 older calves developed disease but 3 showed only mild signs of infection.

It was concluded that two strains of rotavirus caused sub-clinical infections in young calves while a third was virulent in calves up to at least 116 days of age.

INTRODUCTION

Rotaviruses are accepted as enteric pathogens in many animal species. In the UK and North America they have been associated with disease in calves aged under 4 weeks, frequently in the second week of life (Woode & Bridger, 1975; Acres & Babiuk, 1978; D. J. Reynolds and J. H. Morgan, personal communication) but the occurrence of antibody in nearly 100% of cattle of all ages indicates that sub-clinical infections are common (de Leeuw *et al.* 1980; Schusser, Hinaidy & Bürki, 1982; McNulty & Logan, 1983). There are at least three possible explanations for these observations. Firstly, sub-clinical infections, which resulted in the development of serum antibody, were produced by feeding rotavirus antibody at the time of challenge with virulent virus (Snodgrass & Wells 1976; Bridger & Brown, 1981); this probably occurs frequently under natural conditions. Secondly, differences in virulence between isolates of rotavirus have been suggested in studies using intestinal loops (Carpio, Bellamyu & Babiuk, 1981) and infection with avirulent strains could account for sub-clinical infections. A third explanation could be an

age-related resistance to disease; virus may multiply and produce an immune response in calves of all ages but produce disease only in the young. Evidence to support this hypothesis was reported by Tzipori *et al.* (1981) with four rotaviruses isolated in Australia which caused disease only in calves aged less than 7 days but these results contrast with those obtained with a rotavirus studied in the United Kingdom which was said to cause disease in gnotobiotic and colostrum-deprived calves aged up to 56 days (Bridger & Woode, 1975).

The present paper examines the second and third explanations for sub-clinical infections. The virulence of three strains of bovine rotavirus in gnotobiotic calves infected with either uncultured (faecal) or cloned viruses is described. Firstly, infection of calves aged 16 days and under was studied with cloned rotaviruses CP-1, C3-160 and PP-1 plus faecal CP-1 and PP-1. Secondly, infection of calves aged 42–116 days with the faecal and cloned CP-1 strain is described.

MATERIALS AND METHODS

Viruses.

Bovine rotaviruses CP-1 and PP-1 were obtained from an outbreak of diarrhoea in colostrum-deprived calves at this Institute in 1973 (Woode *et al.* 1974). The faecal CP-1 inocula were prepared from faeces of a gnotobiotic calf inoculated with virus from the outbreak which had been passaged serially seven times in gnotobiotic calves; the faeces were filtered through 0·22 or 0·45 μm membranes (Millipore). The faecal PP-1 inocula were prepared as filtrates of diarrhoeic faeces from a gnotobiotic pig inoculated with the same outbreak material which had been passaged serially five times in gnotobiotic pigs. By electron microscopy, viruses other than rotaviruses were not observed in the faeces of these animals. Both faecal CP-1 and PP-1 were passaged and cloned in cell cultures as described previously (Bridger & Brown, 1984). Cloned CP-1 had been passaged serially a total of nine times in cell cultures and cloned PP-1 a total of eight times.

Rotavirus C3-160 was obtained from the Institute's herd in 1980 from a normal calf in the second week of life when there was no diarrhoea in the herd. It was adapted for growth in MA104 cells (continuous Rhesus monkey kidney cells, Microbiological Associates, USA) and cloned by three rounds of plaque purification (D. H. Pocock, personal communication). It had been passaged serially a total of 10 times in cell culture.

Calves and inoculation.

Forty-six gnobiotic calves were produced and reared on a milk based diet (Dennis, Davies & Hoare, 1976; Hoare, Davies & Dennis, 1976) They were infected as follows: 3, aged 7–10 days, and 18, aged 56–116 days, with faecal CP-1; 5, aged 8–12 days and 8, aged 42–85 days, with cloned CP-1; 6, aged 1–4 days, with faecal PP-1; 3, aged 4–16 days, with cloned PP-1; 3, aged 6–15 days with cloned C3-160. With the cloned viruses, these infections included three to five serial calf passes after culture. Calves were either bacteriologically sterile during the period of observation or were accidently contaminated with species of *Staphylococcus*, *Bacillus*, *Edwardsiella*, *Micrococcus*, *Citrobacter*, *Streptococcus* or *Enterobacter*. Accidental contamination of calves with these species did not result in changes in

faecal colour, faecal output or in anorexia. Serum samples were obtained at the time of inoculation and 3 weeks later. Before inoculation, the serum of each calf was free of rotavirus antibody. Calves were inoculated orally with 2 ml of either a faecal filtrate or cell culture fluid.

The daily faecal output of male calves and dry matter content of the faeces was determined as described previously (Bridger, Hall & Brown, 1984). The mean daily faecal output before infection was 320 g (S.D. ± 80 g). Samples of faeces, passed naturally, were collected from female calves and the colour and visible consistency of all faecal samples noted. Anorexia was assessed by a comparison of feeding habits before and after inoculation and was recorded as present when, for one or more feeds, calves did not consume the milk offered within 1 h; normally calves drank within 5–10 min.

Virus detection

Infectivity assays. Assays of faecal samples and cell culture fluids were conducted in duplicate in trypsin-free medium as described previously (Bridger & Brown, 1981) except that MA104 cells were used. Titres were expressed as $\log_{10}$ TCID50 per g of faeces or ml of culture fluid.

Electron microscopy. Three grams of faeces were diluted 1:4 with phosphate-buffered saline, centrifuged twice at 8000 ***g*** for 30 min and the supernatant fluid layered over 5 ml of 40% (w/w) sucrose solution followed by centrifugation at 83000 ***g***. The resultant pellet was resuspended in phosphate buffered saline and examined by electron microscopy after staining with 2% potassium phosphotungstate (pH 6·0).

Antibody detection

Immunofluorescent activity was determined indirectly as described previously (Bridger, 1978) except that MA104 cells and rabbit anti-bovine immunoglobulin conjugated with fluorescein isothiocyanate (Nordic Laboratories Ltd) were used. Neutralizing activity was determined by a fluorescent focus reduction test with homologous virus (Bridger & Brown, 1984).

RESULTS

Comparison of the virulence of three rotavirus strains in calves aged 1–16 days

Oral inoculation of five calves aged 8–12 days and three calves aged 6–15 days with cloned viruses CP-1 and C3-160 respectively produced different clinical effects (Table 1). Similar levels of infectious virus and serum neutralizing antibody were found with both viruses but infection with C3-160 was sub-clinical in all three serial calf passes. In contrast, clinical signs developed in all three calf passes with CP-1. The presence of rotavirus in the faeces was confirmed for both viruses by electron microscopy, beginning 2–4 days after infection. The clinical effects of CP-1 before passage in cell culture (faecal CP-1) were similar to those after culture and cloning (compare Tables 1 and 2). Samples of C3-160 were not available before culture.

Sub-clinical infections also occurred in nine calves aged 1–16 days inoculated with $10^{2\cdot0}$–$10^{7\cdot5}$ TCID50 of PP-1, a virus from bovine faeces passaged in piglets. During three (cloned virus) or five (faecal virus) serial passages rotavirus was

Table 1. *Inoculation of young gnotobiotic calves with cloned bovine rotaviruses CP-1 and C3-160*

Virus	Age of calves (days)	Dose (log_{10} TCID50)	No. calves tested	No. calves positive (days duration)				Mean (range) of		
					Faecal changes					
				Anorexia	Colour	Daily output†	Virus excretion	Peak faecal output /day (g)	Peak virus excretion log_{10} TCID50 /g faeces)	Antibody titre in convalescent serum§
CP-1*	8–12	2·0–7·0	5	3 (1–3)	5 (6–7)	3‡ (3–6)	5 (4–6)	1570‡ (750–2250)	5·6 (4·5–6·6)	6800
C3-160*	6–15	4·0–6·0	3	0	0	0	3 (4–5)	374 (331–440)	6·4 (6·0–6·8)	1560 (1100–2180)

* CP-1 was passaged nine times in cell culture followed by three serial passes in calves. C3-160 was passaged 10 times in cell culture followed by three serial passes in calves.

† Above 500 g: in uninoculated controls daily faecal output averaged 320 g (standard deviation ±80 g).

‡ Three calves tested.

§ By neutralization of homologous virus. A single calf was tested for CP-1.

Table 2. *Inoculation of gnotobiotic calves of different ages with faecal virus, CP-1*

Age of calves (days)	Dose of virus (log_{10} TCID50)	No. calves tested	No. calves positive (days duration)				Mean (range) of		
				Faecal changes					
			Anorexia	Colour	Daily output*	Virus excretion	Peak faecal output /day (g)	Peak virus excretion log_{10} TCID50 /g faeces)	Antibody titre in convalescent serum§
7–10	5·0–5·3	3	3 (1–4)	3 (6–9)	3 (3–7)	3 (6)	1370 (900–2000)	6·2 5·7–6·7)	320 (160–640)
56–116	2·7–5·6	18	17 (1–7)	18 (5–9)	9† (5–7)	9† (5–6)	2000† (1250–2800)	5·9‡ (4·8–6·3)	640 (640)

* Above 500 g: in uninoculated controls, daily faecal output averaged 320 g (standard deviation ±80 g).

† Nine calves tested.

‡ Five calves tested.

§ Measured by indirect immunofluorescence; three calves tested per group.

detected in faeces of each calf for 1–4 days with peak titres per calf ranging from $10^{1.7}$ to $10^{5.7}$ TCID50 /g followed by seroconversion. There was no disease although two calves, at the second and third passages of the faecal virus, showed a slight colour change of the faeces which coincided with virus excretion.

With pathogenic infections produced by faecal and cloned CP-1 virus in young calves, clinical signs commenced within 2–3 days after inoculation. The first signs were anorexia and/or a colour change of the faeces from dark green or brown to pale yellow or cream, occasionally light green or grey. The amount of faeces produced was a good indication of the severity of disease; increased volumes above 500 g/day were produced for 3–7 days with maximum daily outputs of 2- to 7-fold above normal. In the majority of calves, the faeces were less fluid after infection than before, although some calves showed no visible change. Occasional individual samples were watery but these occurred sporadically. The dry matter content of the faeces was a poor indication of disease, ranging from 11·4–28·1 % (mean 17·2 %) before infection to 7·3–20·6 % (mean 13·3 %) after infection (data from 12 calves). Anorexia occurred in 6 of 8 calves and 2 inoculated with faecal CP-1 became severely ill; they were unable to rise without assistance and bellowed repeatedly. Milk was withheld and the calves recovered.

Influence of age on susceptibility to rotavirus CP-1

Faecal CP-1. Faecal CP-1 produced clinical disease of equal severity in calves aged 56–116 days as in calves aged 7–10 days (Table 2). In older calves the faeces became pale yellow in colour, the volume increased about 4- to 9-fold and all but one calf developed anorexia. Virus was excreted concurrently in the faeces at similar levels to younger calves and, by indirect immunofluorescence, the levels of rotavirus antibody in convalescent sera were similar to those of younger calves. Four of the 18 older calves were more severely affected. They were unable to rise without assistance and bellowed repeatedly. As with younger calves, they recovered after milk was withheld.

Cloned CP-1. Eight calves aged 42–85 days, were inoculated with the first to third calf passage after culture. Five, aged 42–85 days and inoculated with $10^{2.7}$ TCID50 (1 calf) or 10^{6}–$10^{7.6}$ TCID50 (4 calves) of the first and second calf passages developed disease indistinguishable from that after infection with faecal and cloned CP-1 in young calves. They excreted virus for 3–6 days with maximum levels of $10^{4.3}$–$10^{7.5}$ TCID50/g of faeces. One calf, aged 46 days and inoculated with $10^{2.7}$ TCID50 was killed *in extremis* 13 days after inoculation when it had been lethargic for 6 days and anorexic from day 2 after inoculation. *Streptoccus* sp. and *Staphylococcus* sp. were isolated from the faeces. In the remaining three calves, aged 45–70 days and inoculated with low doses of virus ($10^{2.0}$–$10^{4.0}$ TCID50) at the second and third calf passages after culture, only mild clinical signs developed. The colour of the faeces became slightly lighter and the maximum daily faecal outputs ranged from 583 to 790 g; anorexia was not observed. Rotavirus was detected for 2–4 more days with maximum levels of $10^{3.7}$–$10^{4.5}$ TCID50/g. *Staphylococcus* sp., *Bacillus* sp. and *Edwardsiella* sp. were isolated from the faeces. The mean titre of neutralizing antibody in the convalescent sera (8150) was similar to that in the clinically affected animals (8640).

Cloned CP-1 followed by faecal CP-1. The possiblity that faecal CP-1 contained a filterable agent other than rotavirus was examined by challenge of two gnotobiotic calves infected at 45 and 51 days of age with cloned CP-1. When challenged at 66 and 72 days of age with $10^{5\cdot0}$ TCID50 of faecal CP-1 no clinical signs or excretion of rotavirus occurred whereas two control calves aged 71 and 73 days developed diarrhoea. These results confirmed that rotavirus in the faecal CP-1 inocula was responsible for the disease in the older calves.

DISCUSSION

The results presented here show that two strains of rotavirus (C3-160 and PP-1) multiplied avirulently while another (CP-1) was virulent in calves of all ages. These observations support the hypothesis that infection with avirulent rotaviruses could account for natural sub-clinical infections but do not support the hypothesis that rotaviruses are pathogenic only in the young calf. In natural infections, rotaviruses have been associated with disease in calves aged up to 49 days (Sibalin, Szekely & Bürki, 1980; Van Opdenbosch & Wellemans, 1982) and in man, rotavirus-associated outbreaks of diarrhoea have been observed in adults (Linhares *et al.* 1981; Echeverria *et al.* 1983). In contrast, age-dependent resistance to disease have been reported in cattle and mice (Tzipori *et al.* 1981; Wolf *et al.* 1981; Riepenhoff-Talty *et al.* 1982; Sheridan *et al.* 1983). These data, together with the observations presented here, lead us to conclude that some strains cause disease in all ages, some infect all ages but cause disease only in the young, while other strains infect the young without disease.

The use of viruses which had been cloned in cell culture reduced the chance of the presence of adventitious viruses and mixed rotavirus populations. However, after cloning, CP-1 was pathogenic less consistently in older calves, 3 of 8 infected calves developed only mild clinical signs. This may have been due to attenuation by passage in cell culture or due to selection of a less virulent virus. The latter explanation is supported by the finding that, in neutralization tests with standard sera, the cloned virus was neutralized whereas the faecal virus was not (J. C. Bridger, personal observation). Whether the avirulent virus C3-160 was pathogenic before culture could not be tested but as CP-1 was not rendered avirulent in young calves by a similar number of passages in cell cultures, nor were two other rotaviruses (Bridger & Brown, 1984), this seems unlikely. In addition, Carpio, Bellamyu & Babiuk (1981) reported that rotaviruses were not readily attenuated by passage in culture.

The reason for the failure of cloned CP-1 to cause disease in 3 of 8 older calves while causing disease in 5 is unclear but these calves were inoculated with low doses, $10^{2\cdot0}$–$10^{4\cdot0}$ TCID50 of virus. Dose alone does not appear to determine the clinical outcome, however, as one older calf inoculated with $10^{2\cdot7}$ TCID50 and contaminated with *Streptococcus* sp. and *Staphylococcus* sp succumbed to infection. Other factors such as animal variation and nature of the bacterial flora (Gouet *et al.* 1978; Runnels *et al.* 1980) may contribute to the clinical outcome of infection, but on their own, these bacteria did not cause the clinical signs observed following infection with virulent rotavirus.

The avirulent strain, PP-1 was identified and isolated by passage in gnotobiotic

piglets. It originated from an outbreak of diarrhoea in cattle, was pathogenic for pigs at the first passage (Hall *et al.* 1976) and was found to be related antigenically to both bovine and porcine rotaviruses (Bridger & Brown, 1984). On subsequent serial passage of the faecal PP-1 from gnotobiotic pigs in calves, it was avirulent but its pathogenicity for calves may have been modified by passage in pigs. If so, then passage in a heterologous host may be a mechanism by which rotaviruses became avirulent naturally.

Thus, taking all the available evidence together, it is likely that the outcome of rotavirus infection depends on the immune status at the time of infection, the virulence of the infecting virus strain and the age of the infected animal. The reasons for differences in virulence between strains are unclear from the present study but may be elucidated by studies of the molecular basis of virulence and the pathology of virulent and avirulent virus infections.

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