A definition of bovine rotavirus virulence

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Rotaviruses are accepted as enteric pathogens of calves but many natural infections are subclinical. In the present paper, the outcome of inoculation of gnotobiotic calves of three ages (the second day of life, the second week of life and calves aged 6 weeks and over) with doses of $10^{5.0}$ to $10^{6.5}$ TCID$_{50}$ was compared for three bovine rotavirus isolates (C3-160, 17/4 and 39/58). The clinical outcome of infection was dependent on both calf age and rotavirus isolate. Age-dependent resistance to infection was not found. By contrast, age-dependent resistance to disease was found with rotavirus isolates C3-160 and 17/4 but not with 39/58. All three isolates caused disease in calves inoculated on the second day of life but only one, 39/58, caused disease in the two older groups. Peak levels and duration of virus excretion were similar in clinically normal ($10^{6.7} \pm 1.08$ TCID$_{50}$ per g of faeces for 4.6 $\pm$ 1.2 days) and diseased ($10^{7.45} \pm 0.94$ TCID$_{50}$ per g of faeces for 5.3 $\pm$ 0.98 days) calves of all ages, but the onset of virus excretion occurred sooner in clinically affected calves ($1.6 \pm 0.63$ days in clinically affected compared with $3.7 \pm 1.5$ days in clinically normal calves, $P < 0.01$). The present study confirmed the findings of an earlier study (Bridger & Pocock, 1986) which showed that bovine rotaviruses differ in virulence for calves in the second week of life and that older calves are susceptible to rotavirus infection and disease. In addition, the present study demonstrated for the first time, that differences in rotavirus virulence are not apparent with calves inoculated on the second day of life, an age which has been used commonly to assess rotavirus virulence. It is suggested that rotaviruses that cause disease in calves only on the second day of life should be described as of low virulence whereas those that cause disease in all ages should be described as virulent.

Rotaviruses are accepted as enteric pathogens of children and young farm animals, including calves. However, natural subclinical infections are common in cattle in the second week of life (de Leeuw et al., 1980; Schusser et al., 1982; McNulty & Logan, 1983), and this observation raises doubts about rotavirus pathogenicity. Clinical and subclinical infections have been reproduced experimentally after rotavirus inoculation in calves of different ages. In the first studies of rotavirus pathogenicity, day old calves inoculated with American bovine rotaviruses became diarrhoeic (Mebus et al., 1969). However, disease was not produced after infection of calves older than 7 days with four Australian rotaviruses (Tzipori et al., 1981), and these authors concluded that, on their own, rotaviruses were not pathogenic to calves of this age. In contrast, studies in Europe described natural and experimental rotavirus disease in calves up to 8 weeks old (Bridger & Woode, 1975; Woode et al., 1978; Sibalin et al., 1980; Schusser et al., 1982). These conflicting results were partially resolved when the virulence of two bovine rotaviruses was compared in calves in the second week of life (Bridger & Pocock, 1986). One multiplied and caused disease whereas the second multiplied subclinically demonstrating that virulence variation occurred in bovine rotaviruses. The influence of calf age on the outcome of rotavirus infection however, remained unresolved.

The present paper examines the influence of calf age on the outcome of rotavirus infection using three bovine rotavirus isolates (C3-160, 17/4 and 39/58) obtained from different sources in the United Kingdom. Gnotobiotic calves of three ages were used: calves on the second day of life, in the second week of life and calves aged 6 weeks and over. The three ages were chosen for specific reasons: the second day of life because several previous investigators had used this age to study rotavirus pathogenesis (Mebus et al., 1969; Logan et al., 1979; Castrucci et al., 1983); the second week of life because natural rotavirus excretion, with or without disease, commonly occurs in calves of this age (Acres et al., 1977; Reynolds et al., 1986), and 6 weeks and over to assess the development of age-related resistance and the

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need for active immunity in disease prevention. In the present paper, the terms ‘virulence’ and ‘pathogenicity’ are used as defined by Mims & White (1984) and Tyler & Fields (1990).

Virus inocula were prepared by filtration of faeces through 0-45 μm filters and checked for bacterial sterility. A sample of each inoculum was taken for infectivity assay on the day of inoculation before transfer in sealed glass ampoules into gnotobiotic isolators. Small aliquots of the inocula were saved, transferred from the isolator after calf inoculation and assayed for infectious virus to confirm the inoculum dose. Calves were inoculated with between $10^{4.6}$ and $10^{6.5} \text{TCID}_{50}$. The rotavirus isolates C3-160 and 17/4 have been described previously (Bridger & Pocock, 1986; Bridger & Oldham, 1987). Rotavirus 39/59 was obtained from the faeces of a diarrhoeic calf aged 10 weeks in an outbreak of rotavirus-associated diarrhoea on a farm at Avebury, Wiltshire, U.K. in 1983. It had not been passaged in cell culture to avoid the possible selection of less virulent variants from the original material. The absence of other enteric viral pathogens was established by inoculation of two gnotobiotic calves with one of two cloned rotaviruses, C5-175 or C6-167 (Pocock, 1990), followed by challenge 20 to 28 days later with the rotavirus inoculum 39/58. No disease occurred, suggesting the absence of enteropathogenic viruses other than rotaviruses in the 39/58 inoculum.

Gnotobiotic calves were produced, reared and fed on a milk-based diet twice daily as described previously (Dennis et al., 1976; Hoare et al., 1976). Calves inoculated on the second day of life had been delivered by hysterotomy 26 h earlier. Groups of two to four calves were inoculated at midday at the specified ages with 2.0 ml volumes of the inocula. Changes in faecal colour, visible consistency, dry matter content and output plus anorexia, dehydration and demeanour were noted once or twice daily. Daily faecal output was measured in some male calves with the aid of a harness. Daily output was not measured with calves inoculated on the second day of life because they reacted adversely to the harness. Dry matter content was determined by evaporation to constant weight at 60 °C. Rectal temperatures were taken daily at 09.00. Anorexia 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 to 10 min. Calves were considered to be depressed when they were slow in their responses, were reluctant to move or unsteady on their feet. Dehydration was assessed by the skin-fold test. Faecal samples, stored at 4 °C, were assayed for infectivity as described previously but using MA104 cells (Bridger & Brown, 1981). Each sample was diluted in duplicate and a total of eight microtitre wells were inoculated for each dilution step. Endpoints were expressed as TCID$_{50}$ per g of faeces or ml of culture fluid.

All three calves inoculated on the second day of life with $10^{5.7} \text{TCID}_{50}$ of rotavirus isolate C3-160 developed clinical signs on the day after inoculation (two calves) or on the second day after inoculation (one calf). Clinical signs, including diarrhoea, fever and depression, persisted for 2 to 4 days (Table 1). The first clinical sign was an abrupt change in the faeces from dark solid meconium to large volumes of watery yellow-green faeces with dry matter contents falling from around 30% to between 6% and 9%. None of the calves required treatment with oral electrolytes and none died. Virus excretion commenced with the onset of clinical signs and lasted for 5 to 7 days (Fig. 1a). High levels of virus were detected by infectivity assay; the peak levels ranged from $10^{4.4}$ to $10^{8.9}$ (mean $10^{8.7}$) TCID$_{50}$ per g of faeces.

Infection of three calves in the second week of life with a similar dose confirmed the earlier findings of Bridger & Pocock (1986). Clinical signs did not develop but rotavirus excretion began on day 3 or 4 after inoculation and lasted for 4 to 6 days (Table 1; Fig. 1b). Peak levels ranged from $10^{7.2}$ to $10^{8.5}$ (mean $10^{7.6}$) TCID$_{50}$ per g of faeces. Two of two calves aged 6 weeks and over (72 and 87 days) inoculated with $10^{5.7} \text{TCID}_{50}$ excreted rotavirus from day 5 or 6 after inoculation for 3 to 5 days (Table 1; Fig. 1c). The peak levels of rotavirus excreted were $10^{6.5}$ and $10^{8.2} \text{TCID}_{50}$ per g of faeces but neither showed any clinical signs.

All three calves inoculated on the second day of life with $10^{6.6} \text{TCID}_{50}$ of rotavirus isolate 17/4 developed clinical signs on the day after inoculation that were similar in severity and duration to those seen with calves inoculated with isolate C3-160 on the second day of life (Table 1). Virus excretion commenced with the onset of clinical signs and its duration was similar to that for isolate C3-160 but the mean peak levels of virus were lower; $10^{6.9} \text{TCID}_{50}$ per g of faeces. The three calves inoculated with isolate 17/4 in the second week of life failed to develop disease but excreted $10^{4.4}$ to $10^{6.8}$ (mean $10^{5.9}$) TCID$_{50}$ per g of faeces from day 2 after inoculation for 3 to 6 days (Fig. 1b). Calves aged 6 weeks and older were not inoculated with this virus.

All ten calves inoculated with doses of 39/58 ranging from $10^{4.4}$ to $10^{6.0} \text{TCID}_{50}$ either on the second day of life, in the second week of life or at over 6 weeks of age, developed diarrhoea. Clinical signs commenced on days 2 or 3 after inoculation in all three age groups, with faecal colour changing from dark brown/green to light yellow (Table 1). Clinical signs lasted for 5 to 9 days. Faecal outputs were measured in calves inoculated in the second week of life and were above mean pre-inoculation levels of 320 ± 80 g per day for 5 to 7 days, peak outputs ranging from 1600 to 2890 g per day. Faecal dry matter
Table 1. Infection of calves with C3-160, 17/4 and 39/58 on the second day of life, in the second week of life and at 6 weeks and over

<table>
<thead>
<tr>
<th>Virus</th>
<th>Calf age (days)</th>
<th>No. of calves with diarrhoea/ no. of calves tested</th>
<th>Mean no. of days with clinical signs (range)</th>
<th>Clinical parameters</th>
<th>Virus excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. calves positive (days duration)</td>
<td>Mean peak level (log_{10} TCID_{50}/g) (range)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3-160</td>
<td>1</td>
<td>3/3</td>
<td>3 (2-4)*</td>
<td>3 (2-4)*</td>
<td>3 (1)</td>
</tr>
<tr>
<td></td>
<td>7-8</td>
<td>0/3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>72, 87</td>
<td>0/2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17/4</td>
<td>1</td>
<td>3/3</td>
<td>4.5 (3.5-5)</td>
<td>3 (3.5-5)</td>
<td>3 (1)</td>
</tr>
<tr>
<td></td>
<td>7-8</td>
<td>0/3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>39/58</td>
<td>1</td>
<td>3/3</td>
<td>6 (5-7)</td>
<td>3 (5-7)</td>
<td>2 (0-1)</td>
</tr>
<tr>
<td></td>
<td>8-10</td>
<td>3/3</td>
<td>6 (5-9)</td>
<td>3 (5-7)</td>
<td>2 (0-2)</td>
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<tr>
<td></td>
<td>74-97</td>
<td>4/4</td>
<td>5 (5-6)</td>
<td>4 (5)</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Only two calves monitored.
† Only one calf monitored.
‡ NT, Not tested.

content ranged from 17% to 41% before inoculation and fell to 10% or below in three of six calves tested. Three of the 10 calves became anorexic, and rectal temperatures were above 39.2 °C for one or more days in half of the calves tested. All three calves inoculated on the second day of life were depressed for 1 to 2 days. None of the calves required treatment with oral electrolytes and none died. The pattern of virus excretion was similar, irrespective of age (Table 1; Fig. 1). Virus excretion commenced on days 2 or 3 after inoculation and the duration and levels of rotavirus excretion were similar in all age groups.

The clinical infections produced by the three rotavirus isolates were not associated with higher concentrations of rotavirus in faeces. The peak levels found in the subclinical infections with isolates C3-160 and 17/4 in the second week of life and at 6 weeks and over (mean $10^{6.7\pm1.08}$ TCID_{50} per gram of faeces) were similar to those found in clinical infections with isolates C3-160 and 17/4 on the second day of life and with isolate 39/58 at all three ages (mean $10^{7.45\pm0.99}$ TCID_{50} per g of faeces). High concentrations of rotavirus (in excess of $10^{6}$ TCID_{50} per g of faeces) were found in all three diarrhoeic calves inoculated on the second day of life with isolate C3-160 but titres of over $10^{8}$ TCID_{50} were observed occasionally with this rotavirus in disease-free older calves in both the present and a previous study (Bridger & Oldham, 1987). Levels of rotavirus antigen, detected by enzyme immunoassay, were also found to be similar in the faeces of diarrhoeic and clinically normal calves in a study in Canada (Archambault et al., 1990) but, in mice, Eydeltoth et al. (1984) noted that disease correlated with higher intestinal levels of rotavirus as measured by enzyme immunoassay. The clinical infections were not associated with longer periods of virus excretion; diarrhoeic calves excreted rotavirus for 3 to 7 (mean 5.3 ± 0.98) days whereas non-diarrhoeic calves did so for 3 to 6 (mean 4.6 ± 1.2) days. Similarly, in a previous study with a virulent and avirulent porcine rotavirus (Bridger et al., 1992), neither the duration of virus excretion nor the peak levels of rotavirus infectivity differed significantly.

Clinical infections were associated with earlier virus excretion. Diarrhoeic calves, infected with isolates C3-160 or 17/4 on the second day of life or isolate 39/58 at all ages, first excreted virus within 1 to 3 (mean 1.6 ± 0.6) days whereas clinically normal calves, infected with isolates C3-160 or 17/4 in the second week of life or beyond, did so within 2 to 6 (mean 3.7 ± 1.5) days ($P < 0.01$). In pigs, the virulent porcine rotavirus 4F was excreted more quickly after inoculation than the avirulent porcine rotavirus 4S (Bridger et al., 1992). Similarly, a porcine rotavirus that had been attenuated in cell culture grew at a slower rate in pigs than the virulent parental virus (Tzipori et al., 1989). In a study of pathology aimed at investigating the reasons for differences in bovine rotavirus virulence, the area of rotavirus-infected villous epithelium was found to be eight times greater in 10-day-old diarrhoeic calves inoculated with the virulent rotavirus isolate CP-1 compared to 10-day-old clinically normal calves infected with the low virulence rotavirus isolate C3-160 (Hall et al., 1993) supporting the suggestion that virulent rotaviruses replicate more quickly. Differences in rotavirus replication rates in the gut and age-dependent differences in the rate of enterocyte loss and replacement may explain the differences in clinical outcome depending on calf age seen in the present study. Enterocytes lining the intestinal villi are constantly shed and replaced in uninfected animals. Rotavirus diarrhoea is thought to
occur when villi shorten because infected enterocytes are lost faster than they can be replaced from the crypts (Hall et al., 1993). In pigs, the rate of natural enterocyte replacement was faster in 3-week-old pigs than in day-old pigs (Moon, 1971) and this was proposed as an explanation for the age-dependent resistance to transmissible gastroenteritis virus (TGEV) in pigs (Moon et al., 1975). It is conceivable that the virulent bovine rotavirus isolate 39/58, caused enterocytes to be lost faster than they could be replaced in all three ages of gnotobiotic calves.

Thus, the clinical outcome of experimental rotavirus infection in rotavirus-naive gnotobiotic calves varied and was dependent on calf age and rotavirus isolate within the dose range of $10^{6.0}$ to $10^{9.4}$ TCID$_{50}$. The clinical outcome was influenced by calf age with two rotavirus isolates (C3-160 and 17/4), but not with a third (39/58). All three isolates caused disease in calves inoculated on the second day of life but, as shown in the present and previous studies, isolates C3-160 (Bridger & Pocock, 1986; Bridger & Oldham, 1987) and 17/4 (Bridger & Oldham, 1987) failed to do so in older calves whereas rotavirus isolates 39/58, CP-1 (Bridger & Pocock, 1986) and J160 (Bridger & Pocock, 1986) did. The differences in rotavirus virulence which were observed previously in calves in the second week of life (Bridger & Pocock, 1986) were not apparent with calves inoculated on the second day of life, an age which has been used frequently to assess rotavirus pathogenicity. The results of the present study indicate that, at this age, rotavirus virulence differences are not apparent. It is suggested that rotaviruses that cause disease only in newborn calves should be described as of low virulence and those that cause disease irrespective of calf age should be described as virulent. The four Australian bovine rotaviruses described by Tzipori et al. (1981) appear similar to isolates C3-160 and 17/4 in the present study but contrast markedly with isolates CP-1, J160 and 39/58 as described in the present or the previous study (Bridger & Pocock, 1986).

There were some minor differences in clinical outcome between calves inoculated on the second day of life with the low virulence rotavirus isolates C3-160, 17/4 and the virulent 39/58 isolate. Disease was present for 5 to 7 days with isolate 39/58 but only for 2 to 5 days with isolates C3-160 and 17/4. Five of six calves inoculated with isolates C3-160 or 17/4 developed watery diarrhoea, with dry matter contents of 10% or below, on the day after inoculation. The sixth calf developed watery diarrhoea on the second day after inoculation. However, all three calves inoculated with the virulent rotavirus isolate 39/58 developed clinical signs on the second day after inoculation and in only one was the dry matter content of the faeces below 10%.

No evidence was found in the present study for age-dependent resistance to rotavirus infection. All ages became infected and the duration of excretion and levels of virus excreted were similar in all ages. Gelberg (1992) found no evidence for age-related resistance to infection with rotavirus OSU in pigs, in contrast to earlier studies in pigs by Kirstein et al. (1985). Resistance to rotavirus infection increasing with age was reported in mice infected with murine rotaviruses (Wolf et al., 1981; Riepenhoff-Talty et al., 1982; Sheridan et al., 1983; Eydelloth et al., 1984), but mice beyond the neonatal period were susceptible to rotavirus infection (Sheridan et al., 1983; Eydelloth et al., 1984; Eiden et al., 1986; Ward et al., 1990). Older turkeys and chickens were found to be more susceptible to homologous rotavirus
infection than younger birds (Yason & Schat, 1987). None of the above studies compared different rotavirus isolates from the homologous species as the present study does. Age-related differences in rotavirus infection and disease susceptibility have been explained by maturation of enterocytes, with decreased macromolecular uptake in older animals (Woff et al., 1981) and differences in the numbers of rotavirus receptors on enterocytes with age (Riepenhoff-Talty et al., 1982; Kirsten et al., 1985) in mice and pigs.

The observed variations in rotavirus virulence are relevant to the diagnosis of and resistance to rotavirus disease. The existence of rotaviruses that multiply without causing clinical signs can confuse diagnosis of infection, the development of in vitro methods for the identification of virulent rotaviruses would be useful. Passive immunity from the mother is effective at preventing rotavirus disease in calves but passive immunity cannot be maintained indefinitely. The finding that virulent rotaviruses cause disease in calves beyond the first few days of life indicates that active immunity is required for complete protection from rotavirus disease.

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References


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