Characterization of a Calici-Like Virus (Newbury Agent) Found in Association with Astrovirus in Bovine Diarrhea

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Received 10 June 1983/Accepted 6 September 1983

A bovine calici-like virus and astrovirus, present in the same fecal sample from an outbreak of diarrhea, were separated from each other by calf passage. The calici-like virus (Newbury agent SRV-1) caused anorexia, diarrhea, and xylose malabsorption in gnotobiotic calves aged 17 to 60 days, whereas the bovine astrovirus was nonpathogenic in similar calves. The calici-like virus was shown to be antigenically distinct from a previously described isolate (Newbury agent SRV-2) by two-way cross-protection experiments in calves; calves immune to homologous challenge became clinically ill and excreted virus when challenged with the heterologous virus.

Viruses resembling caliciviruses have been associated with diarrhea in calves (27), pigs (1, 17), and humans (4, 13, 15, 22, 23). In calves, a calici-like virus, termed the Newbury agent, was shown to be pathogenic in gnotobiotic calves when coronaviruses and astroviruses present in the original fecal sample had been removed. From preliminary experiments, Woode and Bridger (27) suggested that antigenically different types of Newbury agent might exist; the present study examines this possibility by cross-protection experiments in calves between the original isolate, Newbury agent SRV-2, and a second isolate, Newbury agent SRV-1, which was freed of bovine astroviruses by serial passage in calves. The pathogenic effect of Newbury agent SRV-1 is also described.

Astroviruses have also been associated with diarrhea in several animal species (1, 9, 12-14, 20, 25-27), but inoculation of calves with one bovine isolate, free of other known viruses, failed to produce clinical disease (27). The present study includes infection of gnotobiotic calves with a second bovine astrovirus (SRV-1) freed from calici-like viruses.

MATERIALS AND METHODS

Viruses. Newbury agent SRV-2 was obtained from SRV sample 2 (27). Newbury agent SRV-1 and astrovirus SRV-1 were present in SRV sample 1, which originated from calves at this Institute; rotaviruses were eliminated from this sample as described previously (27).

Animal inoculation. Three-milliliter samples of bacteria-free fecal filtrates were given orally at 2 p.m. to gnotobiotic calves produced and reared as described previously (6, 8). For Newbury agent cross-protection experiments, standard inocula were prepared and stored at -70°C. In the absence of a cell culture infectivity assay, the viability of the inocula was tested in challenge control calves.

Clinical assessment. With male calves, the daily fecal output was collected with the aid of a harness and collection bag; feces were removed daily at 9 a.m. and weighed. If perineal dermatitis began to develop, the harness was removed. With female calves, a sample was obtained when feces were passed naturally. Anorexia was assessed by a comparison of feeding habits before and after inoculation.

Fecal dry matter estimations. Approximately 5 g of feces was dried to constant weight in an oven at 55°C.

δ-Xylose absorption. With each calf, absorption was measured over a period of 3 h on the day of inoculation and when fecal color change had occurred or when fecal change might have been expected to have occurred. Plasma xylose concentration was plotted against time, and the area under the curve was measured with an image analyzer (Kontron, British American Optics). Postinfection absorption was expressed as a percentage of preinfection absorption (G. A. Hall, J. C. Bridger, B. E. Brooker, K. Parsons, and E. Ormerod, submitted for publication).

Infection index. For calves in the cross-protection experiment an infection index was derived by allotting one point for each of the following parameters: daily fecal output above 500 g, a change in fecal color compared with preinoculation feces, presence of anorexia, xylose absorption within the range observed for primary infection with that agent (i.e., 4 to 57% of preinoculation level for Newbury agent SRV-1, 68 to 82% for Newbury agent SRV-2), and presence of calici-like virus particles in feces. Thus, the maximum score possible was 5.

Virus detection by electron microscopy. Three to five grams of feces was prepared by differential centrifugation as described previously (3) but with a sucrose cushion of 5 ml of 40% (wt/wt) sucrose. Preparations were negatively stained with potassium phosphotungstate (pH 6.0).

Astrovirus immunofluorescence. Bacteria-free fecal filtrates, containing astrovirus SRV-1 and SRV-2, were inoculated into calf kidney cells as described previously (27). After incubation for 24 to 48 h, acetone-fixed monolayers were stained by indirect immunofluorescence. One in ten dilutions of convalescent calf sera, taken 3 to 4 weeks after inoculation, were allowed to react for 1 h, after which they were replaced by a 1:40 dilution of fluorescein-conjugated rabbit anti-bovine immunoglobulin (Nordic Laboratories, Ltd.).

Detection of antibodies to bovine rotavirus, bovine enteric coronavirus, and bovine parvovirus. The presence of antibodies in 1:10 dilutions of convalescent sera was examined by indirect immunofluorescence. Antigens were primary calf kidney monolayers infected with bovine rotavirus (2), bovine enteric coronavirus British strain (3), or bovine parvovirus (Haden strain).

RESULTS

Separation of astrovirus and calici-like (Newbury) agent in SRV sample 1. (i) Astrovirus SRV-1. During two serial
passages of SRV sample 1 in gnotobiotic calves, the ability to produce diarrhea was lost. By electron microscopy, only astroviruses were identified in the feces of these calves; neither calici-like viruses nor any other viruses were identified.

To date, five gnotobiotic calves aged 21 to 49 days have been inoculated orally with bovine astrovirus SRV-1 without any clinical effect, even though the virus was known to have multiplied, as shown by the development of specific antibody in convalescent sera or by the presence of astroviruses in feces. After infection, no change in the color or consistency of the feces was noted, the dry matter content being between 14 and 18%. In the two calves for which data were available, daily fecal output remained below 500 g, xylose absorption was similar on the day of inoculation and 4 days after inoculation, and anorexia was absent.

Astroviruses were first identified in feces by electron microscopy or immunofluorescence on day 2 or 3 after inoculation and were excreted for at least a further 3 to 5 days. They measured approximately 28 nm in diameter, and some showed a five- or six-pointed star on their surface (Fig. 1). In calf kidney cells stained by immunofluorescence at 24 to 48 h after inoculation single fluorescent cells were found; diffuse fluorescence occurred in the cytoplasm of infected cells, but fluorescence was also seen in two or three granules in the nuclei, as observed with astrovirus SRV-2 (27). Convalescent sera to astroviruses SRV-1 and SRV-2 at 1:10 dilution immunofluoresced similarly with both homologous and heterologous astroviruses. Astrovirus SRV-1 sera were devoid of antibodies to bovine rotavirus, thus confirming that the rotavirus originally present in SRV sample 1 had been removed (27).

(ii) Calici-like (Newbury) agent SRV-1. Three weeks after infection with astrovirus SRV-1, a calf was challenged orally with SRV sample 1. This was followed by three serial passages of the challenge feces in four calves which became clinically ill and excreted, in the feces, calici-like viruses which were detected by electron microscopy (Table 1). Neither astroviruses nor any other viruses were identified in the feces, and convalescent sera were free of antibodies to bovine astrovirus, bovine rotavirus, bovine enteric coronavirus, bovine virus diarrhea virus, feline calicivirus, and bovine parvovirus. This material was accordingly used as the Newbury agent SRV-1 inoculum for the cross-protection studies.

To date, 16 gnotobiotic calves aged 17 to 60 days have been inoculated orally with Newbury agent SRV-1, and all showed clinical signs within an incubation period of 1 to 5 days. Anorexia developed in 10 (63%) of the calves and lasted on average for 4 days. In nine calves, it was the first clinical sign, and its severity varied from reluctance to feed to total refusal.

Changes in the feces first occurred between days 2 and 5 after inoculation (mean, day 3). In all inoculated calves, the color changed from dark green or brown to light yellow or yellow-green and remained abnormal on average for 6 days (range, 4 to 13 days). Changes in consistency were variable, and 25% of calves showed no change. The feces of the remaining 12 calves became either watery and floccular or thicker, with some calves showing both types of change on successive days. These changes were reflected in the dry matter content; the highest value obtained was 30% and the lowest 5%, but overall, dry matter content was a poor indicator of Newbury agent infection. In unoinoculated calves, daily fecal excretion was in the region of 300 g. In seven calves for which data were available, daily fecal output was above 500 g for an average of 4 days (range, 1 to 11 days); peak outputs ranged from 516 to 2,150 g (mean, 1,210 g). Where anorexia was severe, the fecal output was reduced.

In all nine calves tested, xylose absorption decreased after infection; between days 3 and 5, the capacity decreased on average to 17% (range, 4 to 57%) of the preinoculation level. Five of the sixteen calves became quiet and lethargic but recovered without treatment. In the most severely affected calf, aged 49 days, blood was seen in the feces on days 15 and 16 after infection.

Viruses particles, which were absent in preinoculation and early postinoculation samples, were identified in the feces on the day before the first clinical signs in 42% of calves and on the day of their appearance in 50% of calves; they continued to be excreted for a further 1 to 4 days (mean, 3 days) but were often scanty, 10 or less particles per 400 mesh grid square. They measured approximately 33 nm in diameter and had an indefinite feathery outline (Fig. 2). Dark hollows were present on the surface of particles, but they did not form clear patterns. Occasional particles which displayed a 10-spiked sphere morphology were identified.

The clinical pattern observed in the three older calves, aged 42, 49, and 60 days at the time of inoculation, was not different from that seen in calves aged 17 to 24 days.

In addition to the 16 calves described above, 5 calves aged 30 to 35 days received Newbury agent SRV-1 as a third viral infection in a gut hormone study (G. A. Hall, M. S. Ghatei, K. Parsons, S. R. Bloom, and J. C. Bridger, manuscript in preparation); these calves responded similarly.

In a farm survey of the incidence of enteric pathogens in calf diarrhea, calici-like viruses were identified in low numbers in 6 of 18 outbreaks by electron microscopy.

Cross-protection studies between Newbury agents SRV-1 and SRV-2. (i) Experimental design. Two groups of four calves were used. In group A, the effect of infection with Newbury agent SRV-1 on a subsequent infection with Newbury agent SRV-2 was examined; in group B, the reverse was done (Table 2).

In each group, three of the four calves received a primary
oral inoculation on the same day when they were between 15 and 24 days of age. Twenty (group A) or 22 days (group B) later, the immunity of these calves to homologous virus was tested by a second oral inoculation of homologous virus, the primary challenge inoculation. The influence of these previous infections on infection with heterologous virus was then examined. Fourteen (group B) or 15 (group A) days after the primary challenge inoculation, two of the three calves received heterologous virus. The third calf received homologous virus at this time. These inoculations constituted the secondary challenge inoculations.

In each group, the fourth calf, which had not been infected previously, served as a control for the heterologous secondary challenge inoculation.

(ii) Primary inoculations. The primary inoculations in groups A and B produced clinical effects in all six calves (Table 2 and Fig. 3). Newbury agent SRV-1 gave infection indices of 4 or 5 (maximum, 5), whereas in group B, infection with Newbury agent SRV-2 gave scores of 3 or 4 (maximum, 4 or 5).

The effects of Newbury agent SRV-1 infection in the three group A calves (P272, P276, and P278) were similar to those described above. With Newbury agent SRV-2, the three calves (P131, P137, and P142) aged 15 to 22 days showed clinical signs within 2 to 3 days of inoculation. The first sign was a color change of the feces to yellow which lasted for 3 to 5 days. Anorexia developed in one of three calves (P131) on day 3 after inoculation and lasted for 2 days. In the one calf (P142) for which data were available, no increase in daily fecal output was observed. The ability of the gut to absorb xylose on day 5 after infection was reduced in all three calves to 68 to 82% of that on the day of inoculation. Virus particles were identified in the feces of all three calves from day 2 or 3 after infection for 1 to 4 days.

(iii) Primary challenge inoculations (homologous challenge). Infection indices of 0 were obtained with all six calves reinoculated with homologous virus, indicating that they had become immune. In some calves, P272 and P278 in group A and P131 and P142 in group B, xylose absorption was depressed (Fig. 3) but not to within the range observed for primary infection with the homologous virus.

(iv) Secondary challenge inoculation. In previously uninfected calves (Q21 in group A and P143 in group B), the challenge inocula produced infection indices of 3 and 5, respectively, indicating that calves of this age were susceptible to infection with Newbury agents SRV-1 and SRV-2. Heterologous challenges resulted in infection indices comparable with those obtained with primary infections (Table 2), i.e., 4 or 5 in calves of group A (P276 and P278) and group B (P137 and P142). No delay in onset of clinical signs or virus excretion was observed (Fig. 3), indicating that the previous heterologous Newbury agent infections had not prevented or delayed the subsequent one. In contrast to inoculation of Q21 and P143 (primary inoculation) with Newbury agent SRV-2, an increase in daily fecal output was observed after the secondary challenge inoculation of P276 and P278; 500 g was exceeded for 5 and 3 days, respectively.

The two calves which received homologous virus as the secondary challenge virus (P272 in group A and P131 in group B) gave infection indices of 0, indicating that immunity to homologous virus was present at the time of challenge.

DISCUSSION

A calici-like virus, Newbury agent SRV-1, separated from bovine rotaviruses and astroviruses, was shown to cause clinical disease in experimentally infected calves and to be antigenically distinct from a morphologically similar bovine calicivirus described previously (27). The bovine astrovirus was nonpathogenic in 21- to 49-day-old calves and shared some common antigens with the previously described bovine astrovirus.

The pathogenic effect of Newbury agent SRV-1 in gnotobiotic calves was similar to severity to that of a strain of bovine rotavirus used in this laboratory. It had similar effects in calves aged 17 to 24 days and in those aged more than 40 days. In the present study, infection with Newbury agent SRV-1 appeared to cause more severe changes than that induced by Newbury agent SRV-2. In six infections by Newbury agent SRV-2 in immune animals (P131, P137, P142, and Q21 as primary inoculation and P276 and P278 as secondary challenge inoculations), the feces of all six calves changed to a lighter color for 3 to 5 days, but in only two animals out of the four tested did daily fecal output rise above 500 g. Anorexia, which was evident in two of five calves tested, was of short duration (1 of 2 days), and xylose absorption was less severely depressed than after infection with Newbury agent SRV-1. In a previous study, however, in which xylose absorption was the only quantitative param-

### TABLE 1. Passage of Newbury agent SRV-1 in gnotobiotic calves

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>Serial passage</th>
<th>Age (days)</th>
<th>Clinical effect</th>
<th>Calicivirus in feces by EM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M193</td>
<td>First</td>
<td>22</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>M208</td>
<td>Second</td>
<td>22</td>
<td>+</td>
<td>(2–4)</td>
</tr>
<tr>
<td>M205</td>
<td>Third</td>
<td>60</td>
<td>+</td>
<td>(1)</td>
</tr>
<tr>
<td>P56</td>
<td>Third</td>
<td>22</td>
<td>+</td>
<td>(2–5)</td>
</tr>
</tbody>
</table>

* No astrovirus was found in feces by electron microscopy or immunofluorescence. No astrovirus antibody was found in convalescent serum (M205 was not tested).

* EM. Electron microscopy; numbers in parentheses are days after infection on which virus was detected. NT, Not tested.
TABLE 2. Summary of experimental design and results

<table>
<thead>
<tr>
<th>Calf</th>
<th>Primary inoculation</th>
<th>Primary challenge inoculation</th>
<th>Secondary challenge inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculum</td>
<td>Infection index</td>
<td>Inoculum</td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P272</td>
<td>NA-1</td>
<td>5</td>
<td>NA-1</td>
</tr>
<tr>
<td>P276</td>
<td>NA-1</td>
<td>4</td>
<td>NA-1</td>
</tr>
<tr>
<td>P278</td>
<td>NA-1</td>
<td>5</td>
<td>NA-1</td>
</tr>
<tr>
<td>Q21</td>
<td>None</td>
<td>NR</td>
<td>None</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P131</td>
<td>NA-2</td>
<td>4*</td>
<td>NA-2</td>
</tr>
<tr>
<td>P137</td>
<td>NA-2</td>
<td>3*</td>
<td>NA-2</td>
</tr>
<tr>
<td>P142</td>
<td>NA-2</td>
<td>3</td>
<td>NA-2</td>
</tr>
<tr>
<td>P143</td>
<td>None</td>
<td>NR</td>
<td>None</td>
</tr>
</tbody>
</table>

* NA-1, Newbury agent SRV-1; NA-2, Newbury agent SRV-2; NR, not recorded. For method of scoring see text; maximum possible score, 5.

b Data on daily fecal output or anorexia (Q21) were not available; maximum possible score, 4.

FIG. 3. Results of cross-protection studies in two groups of calves. NA-1, Newbury agent SRV-1; NA-2, Newbury agent SRV-2; dotted vertical arrows, day and type of inoculum; ■, days on which Newbury agent particles were found in the feces; □, days on which the feces were of abnormal color; □, days on which Newbury agent particles were found in the feces; [ ], days on which xylose absorption was measured and the percentage remaining postinfection; —, daily fecal output; *, anorexia not recorded. Excluding daily fecal output, absence of data indicates that no abnormalities or virus were seen.

eter measured, Newbury agent SRV-2 appeared to have a similar pathogenicity to that found here for Newbury agent SRV-1 (27). This disparity between the two studies requires further investigation but should await the development of techniques to quantitate the dose of virus given.

The pathogenicity of Newbury agents contrasted with the lack of pathogenicity of astrovirus SRV-1 in calves of similar age. Whether astrovirus SRV-1 is pathogenic in younger calves is not known, but a 3-day-old calf inoculated orally with astrovirus SRV-2 showed no clinical signs, even though astroviruses were excreted in the feces from days 2 to 7 after inoculation (J. C. Bridger, unpublished observations). Some decrease in villus height to crypt depth ratio was observed, however, with this isolate (27). In other animal species astroviruses have been associated with clinical illness, and a mild diarrhea was produced in experimentally infected 1 to 3-day-old lambs (19, 20).

Morphologically, bovine astroviruses SRV-1 and SRV-2 were indistinguishable and, by immunofluorescence, shared common antigens. The two viruses do not appear to be antigenically identical, however, as they failed to cross-
protect and can be distinguished in neutralization tests (J. C. Bridger and E. Ormerod, unpublished observations). The pattern of immunofluorescence obtained in calf kidney cell cultures is similar to that obtained with rotaviruses, with the exception that fluorescent granules are often found in the nucleus. This is a property of both bovine astroviruses SRV-1 and SRV-2 but was not found in cell cultures infected with human astroviruses (11). Astroviruses from different animal species appear to be distinct, as immunofluorescence was not observed when cell cultures infected with bovine astroviruses were stained with convalescent sera to human or ovine astroviruses. Also, human and bovine astroviruses failed to infect gnotobiotic piglets, as shown by the absence of antibody in convalescent piglet sera (J. C. Bridger, personal observation).

The lack of reciprocal cross-protection between the two calici-like viruses was in contrast to the protection observed after the homologous challenges (the primary challenge inoculations). The lack of cross-protection contrasted also with the one-way cross-protection observed in preliminary experiments when the calici-like virus preparations contained astroviruses (27). It might be possible that the one-way protection was due to interference between astroviruses and calici-like viruses, but we have no direct evidence for this.

Apart from a recent report from the United States of a calici-like virus from dairy calves with a persistent respiratory tract problem (18), bovine caliciviruses, calici-like viruses, and astroviruses have not been recognized outside the British calf population. The prevalence of Newbury agent in naturally occurring disease is likely to be underestimated because of the short excretion period and the low numbers of particles present in diarrheic feces. Bovine astroviruses are also a common inhabitant of the normal bovine enteric tract and, on three farms in Southern England, 60 to 100% of calves excreted bovine astroviruses in the feces within the first 5 weeks of life (E. Ormerod, personal communication).

Caliciviruses and calici-like viruses have also been implicated as causes of diarrhea in humans, and antigenically different types have been identified by immune electron microscopy and cross-challenge studies in human volunteers (5, 7, 24, 27, 28). The morphology of the virus described in the present paper more closely resembled that of calici-viruses than the previously described Newbury agent SRV-2 (27), although its morphology was not as characteristic as that described for some human enteric caliciviruses (4, 13, 15, 16, 22) or for the porcine enteric calicivirus (1, 17). Although Newbury agent SRV-1 particles displaying a clear one-plus-six arrangement of surface depressions were not identified, some particles exhibited a 10-spiked sphere morphology and dark surface hollows, typical of caliciviruses (reviewed by Studdert [21], suggesting that this agent is a calicivirus. It seems possible that 30- to 40-nm diameter viruses exist in a range of morphologies, for those with an irregular reticulate surface pattern, i.e., Newbury agent SRV-2, the human Otofuke agent (23), and the human Sapporo agent (10), to those with clear typical calicivirus morphology.

ACKNOWLEDGMENTS

We thank several members of the gnotobiotic unit for their conscientious work, without which this study would have been impossible, E. Ormerod and A. Collins for testing sera for antibody to feline calicivirus and bovine virus diarrhea virus, D. Reynolds for collection of some of the field material, and K. Parsons for plasma xylose estimations.


