The pathogenicity of two porcine rotaviruses differing in their \textit{in vitro} growth characteristics and genes 4

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The pathogenicity of two rotavirus variants, 4F and 4S, obtained following adaptation to cell culture of rotavirus from a diarrhoeic pig in China, was compared by serial passage in 24 gnotobiotic piglets. The rotavirus variants have markedly different growth characteristics \textit{in vitro}, and their genome profiles differ only in the relative migration of genes 4. Both cell culture-grown variants replicated to an equal extent in gnotobiotic piglets and neither caused disease, although weight gain was slightly affected in piglets inoculated with the 4F variant. During five serial pig-to-pig passages, variant 4F became highly pathogenic at the fourth and fifth passages, causing severe diarrhoea and weight loss, and premature death in two animals. Piglets inoculated with rotavirus variant 4S remained healthy during all passages although weight gain was slightly affected. Mean duration and peak infectivity titres of virus shedding were similar for both variants. Thus, variant 4F, which grew slowly and produced small plaques \textit{in vitro} and had the faster migrating gene 4, was pathogenic in pigs, whereas variant 4S was apathogenic.

Rotaviruses are the major aetiological agents of acute infantile gastroenteritis (Kapikian & Chanock, 1990) and are responsible for approximately 20% of diarrhoea-associated deaths in children under 5 years in developing countries (de Zoysa & Feachem, 1985). Rotaviruses are also an important cause of diarrhoea in farm animals and are frequently recognized pathogens (Bridger, 1980), although many natural rotavirus infections in man, cattle and pigs are subclinical (Kapikian & Chanock, 1990; Bridger, 1990). Bovine rotaviruses that replicate without causing disease have been identified in experimental calves (Tzipori \textit{et al.}, 1981; Bridger & Pocock, 1986; Bridger & Oldham, 1987), and it appears that rotaviruses of low virulence could account for some natural subclinical infections.

The rotavirus genome is composed of 11 segments of dsRNA with known coding assignments (Estes & Cohen, 1989). The fourth gene encodes VP4, a nonglycosylated structural protein which has neutralization-specific epitopes (Hoshino \textit{et al.}, 1985) and haemagglutinating activity in some strains (Kalica & Greenberg, 1983). Proteolytic cleavage of VP4 is associated with enhancement of plaque formation (Estes \textit{et al.}, 1981), and VP4 is an important determinant of \textit{in vitro} growth and plaque characteristics (Greenberg \textit{et al.}, 1983). VP4 segregates with virulence in mice (Offit \textit{et al.}, 1986), and different VP4 genes were observed in rotaviruses from symptomatic and asymptomatic neonatal human infections (Gorziglia \textit{et al.}, 1986, 1988; Flores \textit{et al.}, 1986).

Rotavirus from the faeces of a Chinese pig with diarrhoea has been adapted to growth in cell culture (Ni, 1986). From this, two variants have been purified by plaquing and limiting dilution techniques (Haddow \textit{et al.}, 1989). The variants differ notably in the electrophoretic migration of gene 4, whereas the other 10 genes comigrate. The two variants also differ markedly in their growth kinetics and plaque formation in MA104 cells. Variant 4F (fast migrating gene 4) grows slowly and produces only small plaques after 10 to 12 days (Haddow \textit{et al.}, 1989), whereas variant 4S (slow migrating gene 4) grows faster and to higher titres and produces large plaques 4 to 5 days after infection.

As the fourth gene has been associated with rotavirus virulence and pathogenicity, we investigated the \textit{in vitro} growth and pathogenic properties of variants 4F and 4S in gnotobiotic piglets after inoculation of cell culture-grown virus and during serial pig-to-pig passage.

Rotavirus variants 4F and 4S (Ni, 1986; Haddow \textit{et al.}, 1989) were propagated in the continuous rhesus monkey kidney cell line MA104 in the presence of 0.5 µg/ml trypsin (Sigma type IX). The viral infectivity of

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Table 1. Outcome of inoculation of gnotobiotic piglets with rotavirus variants 4F and 4S

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose (log&lt;sub&gt;10&lt;/sub&gt; TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
<th>Passage in pigs</th>
<th>No. of pigs</th>
<th>With diarrhoea</th>
<th>With weight loss or failure to gain weight</th>
<th>With virus detected in faeces (ELISA)</th>
<th>Peak infectivity in faeces (log&lt;sub&gt;10&lt;/sub&gt; f.f.f.u/ml)</th>
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<tbody>
<tr>
<td>4F</td>
<td>5-8</td>
<td>1st</td>
<td>2*</td>
<td>19</td>
<td>2</td>
<td>7</td>
<td>6-3; 5-9</td>
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<td>1st</td>
<td>2</td>
<td>20</td>
<td>4</td>
<td>10</td>
<td>5-6; 6-7</td>
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<td>6-0; 6-6</td>
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<tr>
<td></td>
<td>6-7</td>
<td>3rd</td>
<td>2</td>
<td>19</td>
<td>5</td>
<td>10</td>
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<tr>
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<td>2†</td>
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<td>8</td>
<td>6</td>
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<td>2†</td>
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<td>12</td>
<td>7</td>
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<td>25</td>
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<td>Controls</td>
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<td></td>
<td>13</td>
<td>123</td>
<td>0</td>
<td>5</td>
<td>ND§</td>
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</table>

* One piglet died on day 5 p.i. from an unrelated cause (umbilical hernia).
† One piglet was killed on day 6 p.i. due to severe diarrhoea and dehydration.
‡ One piglet was killed on day 7 p.i. due to severe diarrhoea and dehydration.
§ ND, Not determined.

Inocula was assayed by observing the fluorescence on MA104 cells of serial 10-fold dilutions and expressed as TCID<sub>50</sub>/ml (Bridger & Brown, 1981). Infectivity of faeces was tested by fluorescent focus formation on MA104 cells and expressed as fluorescent focus-forming units (f.f.f.u.)/ml (Beards & Flewett, 1984). Plaque assays were carried out using MA104 cell monolayers under a 0.4% agarose overlay containing 0.5 µg/ml trypsin and Eagle's MEM. Plaque visualization was by glutaraldehyde fixation followed by Giemsa staining. Rotavirus in faeces was detected by a VP6-specific ELISA (Beards et al., 1984). Rotavirus subgroups and serotypes were determined according to the procedures of Greenberg et al. (1982) and Beards (1987). Both variants were of subgroup I and serotype 3.

Rotavirus dsRNA was extracted from tissue culture supernatants by incubation with 1% SDS at 37 °C for 30 min followed by phenol–chloroform extraction and ethanol precipitation at -20 °C in the presence of 0.3 M-sodium acetate. Rotavirus dsRNA was extracted from 10% suspensions of faeces in TBS with 15 mM-CaCl<sub>2</sub> as described above except that extraction with 1 vol. of Arcton (1,1,2,-trichlorotrifluoroethane) preceded the phenol–chloroform extraction. PAGE was carried out as described by Rodger & Holmes (1979) with 3% stacking and 10% resolving gels, except that no SDS was included. Silver staining was carried out as described by Herring et al. (1982).

Seven-day-old gnotobiotic piglets, derived by hysterotomy, were inoculated orally with various doses of rotavirus variants 4F or 4S. The different passages were conducted in different litters, and piglets housed singly or in pairs in isolators were allocated randomly to the different treatment groups. Piglets were shown to be free of rotavirus antibody before inoculation by an indirect immunofluorescence test (Bridger & Brown, 1981). From 2 days before inoculation, piglets were weighed daily before feeding and monitored for clinical signs of infection (diarrhoea, anorexia, change in demeanour and dehydration). Faecal specimens were collected daily. Uninfected control piglets were in part littermates of the infected animals and in part from other litters.

Diarrhoea did not occur after inoculation of 10<sup>4.8</sup> to 10<sup>7.4</sup> TCID<sub>50</sub> of either cell culture-grown virus variant (Table 1) and weight gain was only slightly affected (Fig. 1a). However, piglets inoculated with variant 4F had significantly more days (6/39) with failure to gain weight than piglets inoculated with variant 4S (1/42; chi-square test P<0.05) or the controls (5/123; P<0.05). Virus excretion was similar for the two variants with regard to duration; variant 4F was excreted for 17/39 days and variant 4S for 19/42 days. The mean excretion period for variant 4F was 4-3 days (range 3 to 5 days) whereas that for variant 4S was 4-8 days (range 4 to 7 days). Mean peak titres of rotavirus shed in the faeces of infected animals were 6.13 ± 0.48 log<sub>10</sub> TCID<sub>50</sub> for the 4F variant and 5.95 ± 0.79 log<sub>10</sub> TCID<sub>50</sub> for the 4S variant, and thus not significantly different (t-test, P>0.05). Virus excretion began on day 2 or 3 after inoculation with either variant.
Serial pig-to-pig passage revealed clear differences in the pathogenicity of the two variants (Table 1; Fig. 1b). None of the eight piglets inoculated with variant 4S developed diarrhoea at any time, whereas piglets inoculated with similar doses of the 4F variant had diarrhoea for increasingly higher numbers of observation days during the second to fifth passages (2/30 days for passage 2 to 12/21 days for passage 5). Diarrhoea developed between days 2 and 4 post-inoculation (p.i.) at the third and fourth passages, whereas at the fifth passage explosive watery diarrhoea developed the day following inoculation. One piglet at the fourth and fifth passages was killed on days 6 and 7 p.i., respectively, due to severe diarrhoea and dehydration. Anorexia was observed rarely but the two piglets at the fifth passage were seen to vomit. The increasing severity of diarrhoea during the pig-to-pig passage of the 4F variant was accompanied by increasing levels of weight loss or failure to gain weight; during passages 4 and 5, piglets infected with the 4F variant had significantly more days with failure to gain weight (13/37) than animals infected with the 4S variant (6/45; P < 0.05). The number of days with failure to gain weight in piglets during passages 2 and 3 of the 4S variant was not different from the corresponding value for the passages of the 4F variant. There was no progression in the failure to gain weight in the later passages of the 4S variant, but the number of days with failure to gain weight during passages 2 to 5 (11/99) was significantly higher than that of control piglets (5/123; P < 0.05). Weight loss was severe in animals inoculated with the 4F variant at the fourth and fifth passages (9 to 21% of body weight), whereas there was only a slight interruption to weight gain in the animals inoculated with the 4S variant (Fig. 1b).

Despite a progression to pathogenicity of one of the variants during serial pig-to-pig passage, duration and peak infectivity levels of virus excretion did not differ significantly. Variant 4F was excreted for 38/86 days whereas variant 4S was excreted for 44/99 days (P > 0.05); the mean excretion period for 4F was 4.8 days (range 3 to 6 days) whereas that for 4S was 5.5 days (range 5 to 7 days). The mean peak infectivity for 4F was $6.78 \pm 0.50 \log_{10} \text{TCID}_{50}$ and that for 4S was $6.2 \pm 1.06 \log_{10} \text{TCID}_{50}$. There were no differences in the duration of shedding or peak infectivity during the different passages of the 4F variant as pathogenicity increased. However, a difference was apparent in the onset of virus excretion for the two variants. Excretion of variant 4S began 2 days p.i. in all but one piglet, which excreted virus for the first time on day 3 p.i. Excretion of variant 4F began 1 (four pigs) or 2 days p.i. (four pigs). Both piglets infected with the 4F variant at the fifth passage excreted virus for the first time 1 day p.i., as diarrhoea commenced.

The expected RNA profiles were found in all passages and showed no differences in any of the segments from passage to passage (Fig. 2). At passage 4, the 4S and 4F variants were re-plaqued and had retained the differences in plaquing (results not shown); the 4S variant produced large plaques after 4 to 5 days whereas 4F produced small plaques after 8 to 10 days (Haddow et al., 1989).

The fourth rotavirus gene has been implicated in rotavirus pathogenicity by others; differences have been identified in the fourth genes of rotaviruses from symptomatic and asymptomatic children (Gorzigilia et al., 1986, 1988; Flores et al., 1986) and the fourth gene has also been associated with differences of virulence in mice (Offit et al., 1986). In addition, Kantharidis et al. (1988) and Lopez et al. (1991) have speculated that gene 4 determines rotavirus host specificity.

The genes 4 of rotavirus variants 4F and 4S have been cloned and sequenced and show major differences (Burke, M. A. McCrae, J. C. Bridger & U. Desselberger, unpublished results). The data presented here suggest a possible association between differences in pathogenicity and differences in genes 4 of the variants, but differences in the other 10 genes, which all co-migrated on gels, cannot be ruled out. Cloning and sequencing of some of these genes is in progress.

It has been observed that genes besides the one encoding VP4, notably the VP7 gene, may influence biological characteristics in vitro (Chen et al., 1989). Recent data have demonstrated that the interaction of different VP4 and VP7 molecules on the virus particle can change the presentation of neutralization-specific epitopes (Chen et al., 1992) and the stability of virus particles (Burns et al., 1989). It has also been suggested that virus spread and pathogenicity in mice depends on genes 5 and 9 as well as gene 4 (R. L. Broome, P. T. Vo,

Fig. 1. (a) Weight gain of piglets inoculated with the variants grown in cell culture. (b) Weight gain of piglets inoculated with the variants at the fourth pig-to-pig passage. Variant 4F, •; variant 4S, +; control piglets, *.
Differences in pathogenicity have been observed previously between two bovine rotaviruses (Bridger & Pocock, 1986) which differ in plaque formation in vitro (Pocock, 1990). Both apathogenic porcine and bovine rotaviruses produce larger plaques than virulent strains. In vivo, the pathogenic bovine rotavirus colonized a greater area of the small intestine than the apathogenic virus, indicating that it is able to spread more effectively. Other properties which correlate with pathogenicity are the greater ability to damage enterocytes and preferential infection of the upper small intestine (Bridger et al., 1992). Studies are required to determine whether the same properties are associated with the pathogenicity of porcine rotavirus variants 4F and 4S.

Variant 4S multiplied without causing overt clinical signs at all passage levels and yet weight gain was affected. Similarly, rotavirus infection was associated with decreased weight gain but not with diarrhoea in an epidemiological study in intensively managed Danish pig herds (Svensmark et al., 1989). Thus, rotavirus infection may have a significant economic effect even where diarrhoea is not evident.

These experiments have provided an example of a dramatic change to pathogenicity of a cell culture-adapted rotavirus (variant 4F) after only four to five serial pig-to-pig passages. Sequencing of genes 4 of 4F variants before and after the change in pathogenicity will show whether changes in these VP4 genes have occurred.

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References


