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Rotavirus Cross-Species Pathogenicity: Molecular Characterization of a Bovine Rotavirus Pathogenic for Pigs

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Rotaviruses which cause disease in heterologous animal species have been reported but the molecular basis of cross-species infectivity and disease is not established. We report the molecular characterization of a cloned rotavirus, PP-1, which was originally obtained from cattle and which had been biologically characterized *in vivo* in two target animal species, gnotobiotic pigs and calves. In pigs, PP-1 caused severe clinical disease but in experimental calves it replicated subclinically. PP-1 was characterized as a G3 reassortant with a porcine VP4 and NSP4 but a bovine NSP1. The PP-1 VP4 had 96 to 97% deduced amino acid identity to P[7] porcine rotaviruses and P[7] specificity was confirmed with VP4-specific monoclonal antibodies. Sequence analysis of the PP-1 NSP1 showed 94 to 99.6% deduced amino acid identity to bovine rotaviruses but the NSP4 protein had 94 to 98% identity to the NSP4 genotype B porcine rotaviruses. G-typing PCR initially classified PP-1 as a G10 rotavirus but sequence analysis revealed 92 to 96% identity of the PP-1 VP7 with porcine, simian, and human G3 rotaviruses. These results, combined with the *in vivo* properties of PP-1 in the two target species, supported the concept that species-specific VP4 and NSP4, but not NSP1, are required to induce rotavirus disease, at least in calves and pigs. The results illustrate experimentally that rotaviruses circulating in one animal species can pose a risk to another by the emergence of a pathogenic reassortant rotavirus under appropriate conditions.

INTRODUCTION

Rotaviruses are enteric pathogens of a number of animal species including human, cattle, and pigs. The majority of rotaviruses are perceived as species specific and rotaviruses are commonly referred to by their species of origin. However, experimental infection of heterologous animal species has been demonstrated with naturally occurring rotaviruses (reviewed by Theil, 1990). Rotaviruses are commonly typed by their two outer capsid proteins, VP4 (P-type) and VP7 (G-type) (reviewed by Hoshino and Kapikian, 1994). Specific types predominate in natural infections of specific animal species although P- and G-types once thought to be specific for a particular animal species have been identified in other species (Das *et al.,* 1993; Gentsh *et al.,* 1993; Li *et al.,* 1994; Nakagomi *et al.,* 1994; Taniguchi *et al.,* 1994; Pongsuwanna *et al.,* 1996; Cubitt *et al.,* 2000; Iturriza-Gomara *et al.,* 2000). Six rotavirus proteins, VP3, VP4, NSP1, NSP2, VP7, and NSP4, have been associated with rotavirus virulence in mice, pigs, rats, and human (reviewed by Burke and Desselberger, 1996). Four of these proteins, VP4, VP7, NSP1, and NSP4, have shown sequence heterogeneity related to the animal species of origin although this has not always been absolute (reviewed by Hoshino and Kapikian, 1994; Xu *et al.,* 1994; Kojima *et al.,* 1996; Horie *et al.,* 1997; Ciarlet *et al.,* 2000). The molecular basis of natural rotavirus cross-species infectivity and disease is not understood but the four genes which show species-specific heterogeneity are clearly candidates for determinants of host species-specific infectivity and disease.

Early in the study of rotavirus disease, two groups reported that some bovine rotaviruses replicated and caused diarrhea in experimental pigs (Hall *et al.,* 1976; Tzipori *et al.,* 1980). One rotavirus, PP-1, was obtained from calves in an outbreak of diarrhea in the United Kingdom but caused diarrhea in experimental pigs with extensive intestinal villous atrophy before and after cloning in cell culture (Bridger and Brown, 1984; Hall *et al.,* 1976). PP-1 replicated in experimental calves but without causing clinical signs (Bridger and Pocock, 1986). Rotaviruses with heterologous VP4 genes have been associated with natural asymptomatic rotavirus infections in human (Das *et al.,* 1993; Gentsh *et al.,* 1993) and, in experimental pigs, a porcine rotavirus with a bovine-like VP4 replicated subclinically in pigs to titers equivalent to those of its virulent counterpart (Bridger *et al.,* 1992; Burke *et al.,* 1994). We hypothesized that, as the bovine rotavirus PP-1 was nonpathogenic to experimental calves but pathogenic to experimental pigs, it would have a porcine-like not bovine-like VP4 if VP4 is a deter-

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minant of virulence in specific animal species. In addition, we have characterized the PP-1 NSP1, NSP4, and VP7 proteins, which show heterogeneity depending on the animal species of origin and for which there is evidence for a role in rotavirus pathogenicity.

RESULTS

Genome profile analysis

PAGE revealed that the original bovine fecal sample from which PP-1 was derived contained more than 11 gene segments, indicating a mixture of rotaviruses (J. C. Bridger, personal observation). Rotaviruses with different genome profiles and genomic compositions emerged depending on their passage history. The G6P[5] bovine rotavirus UK was obtained by passage in cell culture (Bridger and Woode, 1975), a G3P[5] calf pathogenic rotavirus, CP-1, emerged by passage in calves (Bridger and Pocock, 1986; El-Attar *et al.,* submitted for publication), and the rotavirus PP-1 emerged by passage in pigs (Bridger and Brown, 1984). The PP-1 genome profile had the typical 4-2-3-2 pattern of gene segments of group A rotaviruses (Fig. 1). The PP-1 gene segments 1, 2, 4 (VP4), 7, 8, 9, and 11 (NSP5) differed in migration from the equivalent segments of rotavirus UK run in a parallel track. The PP-1 segment 10 (NSP4) appeared to run slightly faster than that of UK BRV. PP-1 segment 5 (NSP1) and gene 6 (VP6) migrated indistinguishably from segments 5 and 6 of rotavirus UK.

VP4 analysis

The 264 amino acids at the amino terminus of the PP-1 VP4 (representing VP8*, the interconnecting peptide and the amino terminus of VP5*) had 96 to 97% deduced amino acid (90 to 93% nucleotide) identity to the amino terminus of the porcine P[7] rotaviruses OSU, YM, BMI-1, and SW20/21 (a rotavirus obtained from pigs in the United Kingdom in 1976) but less than 66% deduced amino acid (62% nucleotide) identity to representatives of the other P-types found in pigs (the P[5] rotavirus 4S, the P[6] rotavirus Gottfried, and the P[13] rotavirus MDR-13). It had 79% amino acid (68% nucleotide) identity to the P[1] bovine rotavirus C486 and less than 62% deduced amino acid (56% nucleotide) identity to the bovine rotaviruses UK (P[5]), B223 (P[11]), and 993-83 (P[17]). These relationships were confirmed by phylogenetic analysis where the PP-1 VP4 clustered with the P[7] rotaviruses (Fig. 2a). The sequence analyses were endorsed with the P[7]-specific monoclonal antibodies 5G7 and 3G5 and the P[5]-specific monoclonal antibody C2/1. Rotaviruses PP-1, SW20/21, and OSU gave positive reactions by immunoperoxidase staining with 5G7 and 3G5, confirming their P[7] specificity, in contrast to the bovine rotavirus UK (P[5]). PP-1 and SW20/21 failed to react with the monoclonal antibody C2/1, which reacted with the P[5] rotavirus UK.

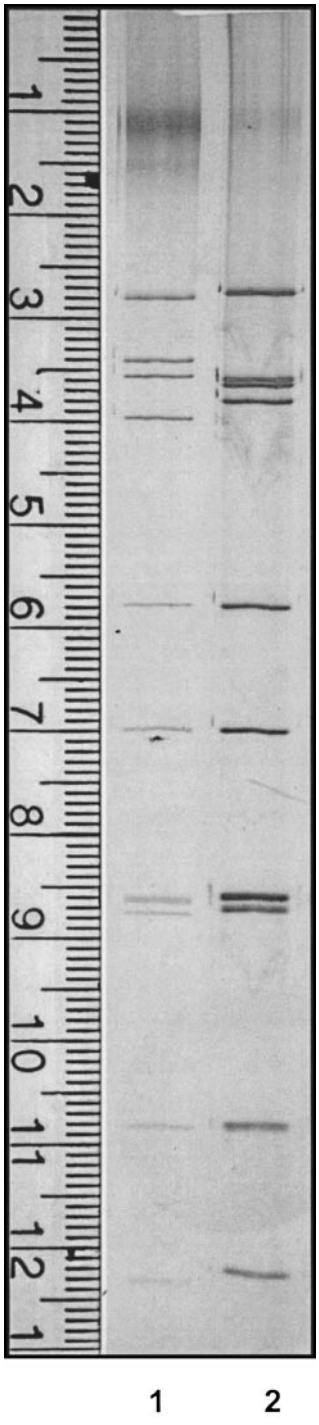

FIG. 1. Genome profile analysis of the bovine rotaviruses PP-1 and UK on 10% PAGE gels. Lane 1, the G3P[7] rotavirus PP-1; lane 2, the G6P[5] rotavirus UK. Ruler indicates migration distance in centimeters.

The PP-1 VP4 deduced amino acid sequence possessed the cysteine residues at positions 203 and 216 found in the four P[7] rotaviruses (Fig. 3). It possesed 16 proline residues conserved in the four P[7] rotaviruses but lacked the 2 proline residues found at positions 148 and 157 in most of the P[7] rotaviruses. At the putative trypsin cleavage region, PP-1 had a sequence (REIVHTR) identical to that of the P[7] porcine rotaviruses OSU, YM,

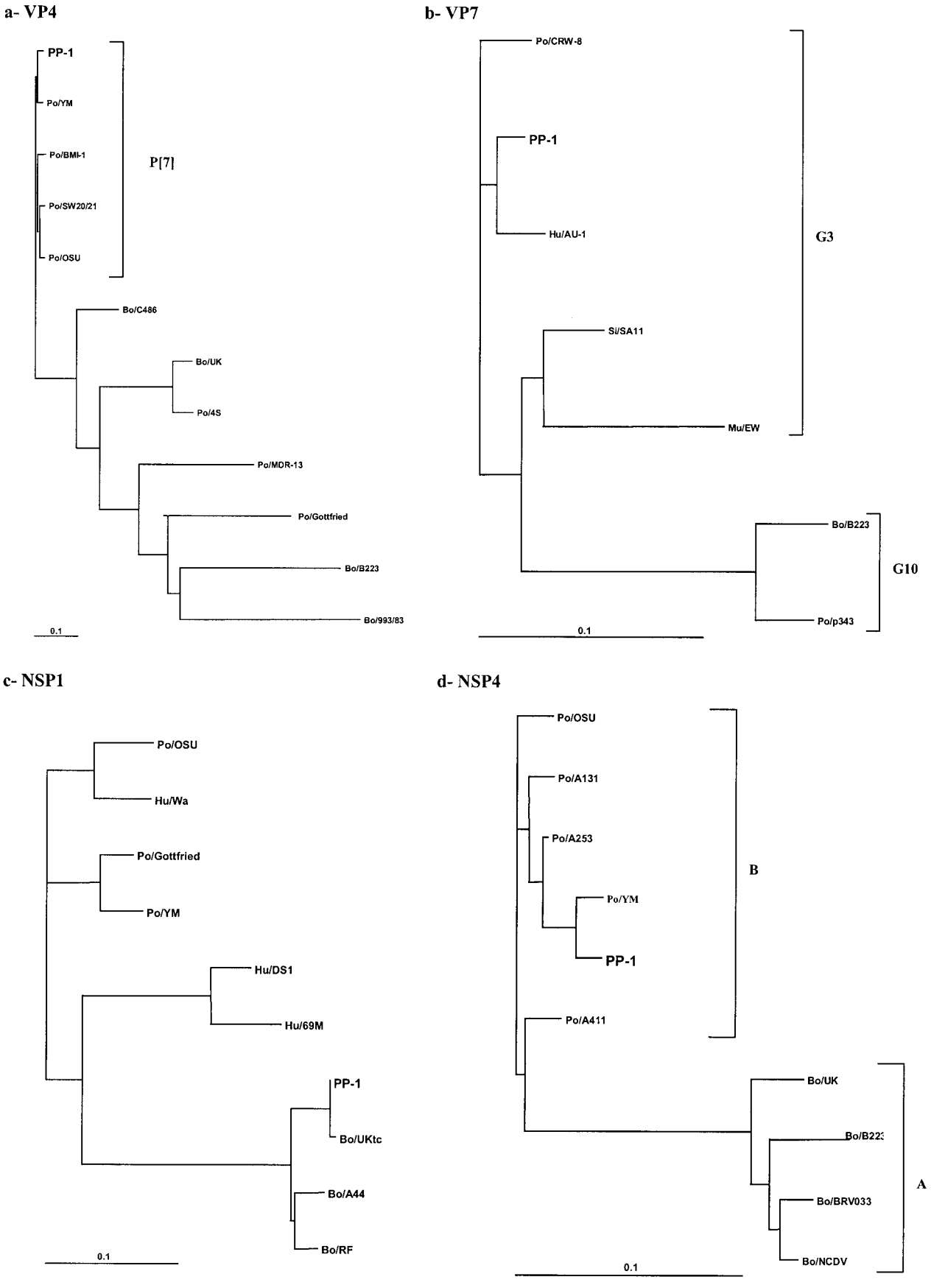

FIG. 2. Phylogenetic analyses of the PP-1 deduced amino acid sequences of (a) 264 amino acids at the amino terminus of VP4, (b) the full-length VP7, (c) 229 amino acids at the amino terminus of NSP1, and (d) full-length NSP4 using the Clustal W neighbor-joining method.

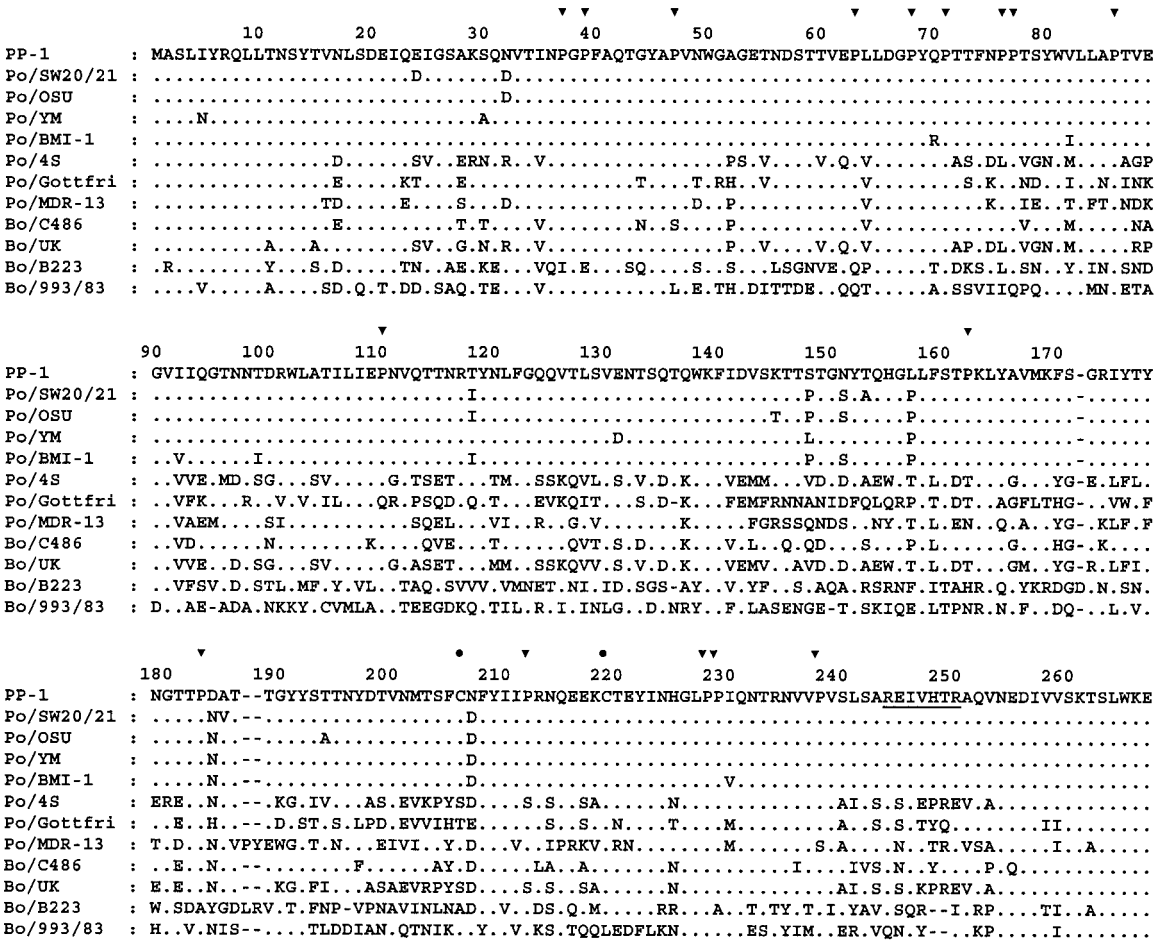

FIG. 3. Multiple sequence alignment of the 264 deduced amino acids of the rotavirus PP-1 VP4 protein, representing VP8*, the interconnecting peptide at the trypsin cleavage site (underlined), and the amino terminus of VP5*, with the P[7] porcine rotaviruses SW20/21, OSU, YM, BMI-1, the P[5], P[6], and P[13] porcine rotaviruses 4S, Gottfried, and MDR-13 and the P[1], P[5], P[11], and P[17] bovine rotaviruses C486, UK, B223, and 993/83. Accession numbers are given in the text. Dots indicate identity to PP-1. (▼) Proline residues; (●) cysteine residues. To identify PP-1 amino acid positions, allow for the 3 additional amino acids found in some strains at positions 172, 187, and 188 (indicated by dashes).

BMI-1, and SW20/21 possessing 2 arginine residues separated by 5 residues. PP-1 did not resemble the P[5] bovine rotavirus UK or the P[11] bovine rotavirus B223 but, interestingly, was identical to the P[1] bovine rotavirus A5 in all but one of its residues (RSIVHTR) and identical to the P[1] bovine rotaviruses NCDV and C486 in all but 2 residues (RNIVYTR).

VP7 analysis

Nucleotide and deduced amino acid sequence analyses of the full-length PP-1 VP7 showed that PP-1 belonged to rotavirus G type 3 with 95, 96, and 92% deduced amino acid (81 to 90% nucleotide) identities to the porcine CRW-8, human AU-1, and simian SA11 G3 rotaviruses (data not shown). It had 82 and 83% amino acid (75 and 76% nucleotide) identities to the bovine and porcine G10 rotaviruses B223 and P343 and between 75 and 87% amino acid (between 73 and 77% nucleotide) identities to the porcine C95 and SW20/21 and bovine T449 G1 rotaviruses, the human G2 rotavirus S2, the porcine G4 and G5 rotaviruses Gottfried and OSU, the G6 and G8 bovine rotaviruses UK and 678, and the G9 and G11 porcine rotaviruses ICB2185 and YM. It had 57% amino acid (64% nucleotide) identity to the G7 bovine rotavirus 993/83.

PP-1 was initially typed as a G10 rotavirus by G-typing RT-PCR using published primers to G5, G6, G8, and G10 rotaviruses (Gouvea *et al.,* 1994) either as a pool or individually. A single amplicon of the expected 715 bp was produced with identical migration to the amplicon produced with the positive control G10 rotavirus B223 (not shown). When VP7 sequence analysis failed to confirm G10 specificity, the 715-bp amplicon was sequenced and this confirmed G3, not G10, specificity of the PP-1 VP7. The 715-bp amplicon had 93% deduced amino acid (89% nucleotide) identity to the G3 rotavirus AU-1 but only 77% amino acid (75% nucleotide) to the G10 rotavirus B223. The lack of G10 specificity was confirmed with the G10-specific monoclonal antibodies B223-N7 and B223/3, which pro-

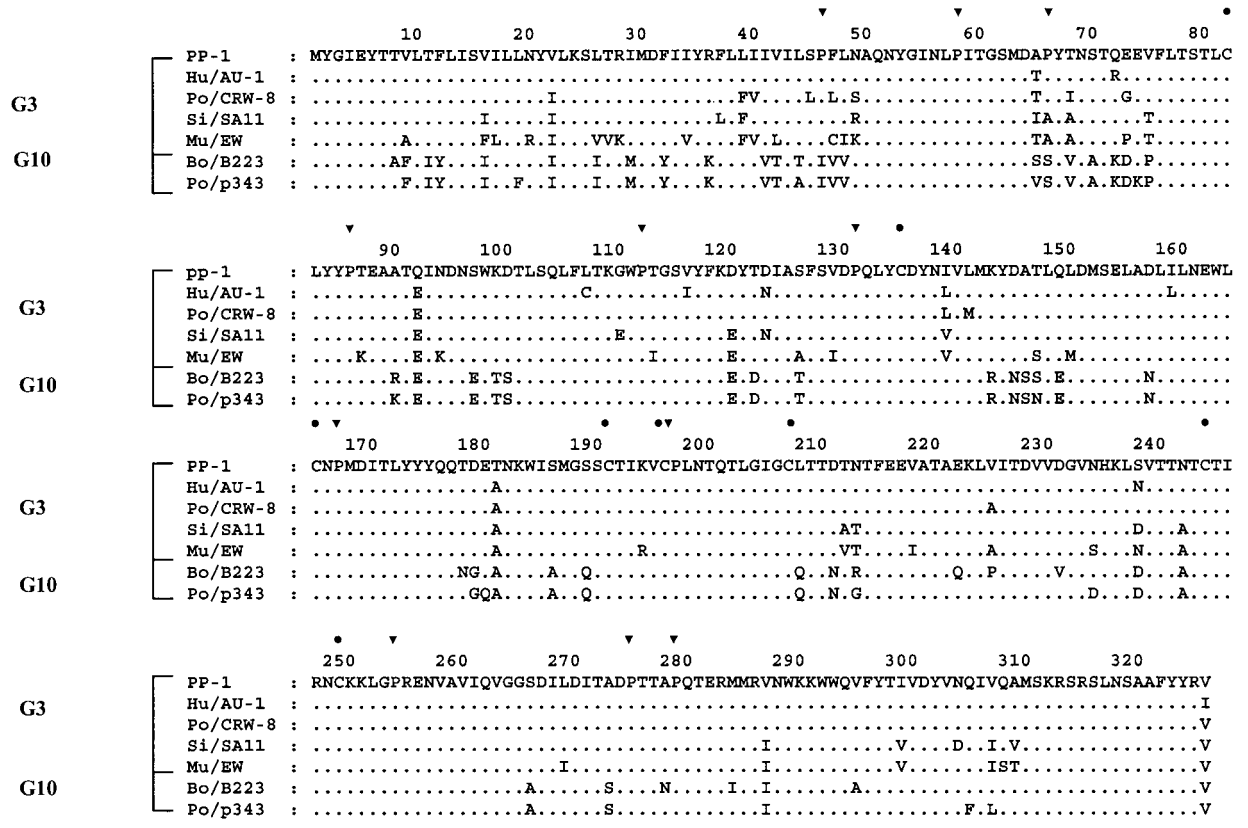

FIG. 4. Multiple sequence alignment of the deduced amino acid sequence of the PP-1 VP7 with that of human AU-1, porcine CRW-8, simian SA11, and murine EW G3 rotaviruses and the bovine B223 and porcine P343 G10 rotaviruses. Accession numbers are given in the text. Dots indicate identity to PP-1. (▼) Proline residues; (●) cysteine residues.

duced a positive immunoperoxidase reaction with MA104 cells infected with the G10 rotavirus B223 but not with cells infected with PP-1. Cells infected with PP-1 also failed to react with the G6-specific monoclonal antibody UK/7. Phylogenetic analysis of the full-length PP-1 VP7 protein confirmed that PP-1 grouped with G3, but not G10, rotaviruses (Fig. 2b).

Eight cysteine residues were identified in the PP-1 VP7, in common with other rotaviruses (Fig. 4). Eleven proline residues were identified. Nine were common to, and at the same positions as, other rotaviruses but there were proline residues also at position 46, in common with the four G3 rotaviruses AU-1, CRW-8, SA11, and EW but not the two G10 rotaviruses B223 and P343, and at position 66, in common with the porcine G3 rotavirus CRW-8 and the human G3 rotavirus AU-1. In antigenic regions B (amino acids 145 to 150) and C (amino acids 211 to 223) (reviewed by Kapikian and Chanock, 1996), the PP-1 VP7 protein was identical to the G3 porcine rotavirus CRW-8 and the G3 human rotavirus AU-1. It had one amino acid difference, glutamine (Q) to glutamic acid (E), to these two rotaviruses in region A (amino acids 87 to 99). It differed from the porcine P343 and bovine B223 G10 rotaviruses in each of the three regions by two to four amino acids.

NSP1 analysis

The 229 deduced amino acids at the amino terminus of the PP-1 NSP1 had 99.6% deduced amino acid identity to the amino terminus of the bovine rotavirus UK differing from the bovine rotavirus UK by only one amino acid, threonine (T) to asparagine (N) at position 127 (represented by one nucleotide difference from AAT in PP-1 to ACT in UK) (Fig. 5). It had 94% deduced amino acid (87% nucleotide) identity to the bovine rotaviruses RF and A44 but only 69 to 72% amino acid (69 to 70% nucleotide) identity to the three porcine rotaviruses OSU, Gottfried, and YM and 66 to 70% amino acid (70 to 73% nucleotide) identity to the human rotaviruses Wa, DS1, and 69M which clustered with porcine rotaviruses (Kojima *et al.,* 1996). This grouping was confirmed by analysis of 229 amino acids at the carboxyl end of the PP-1 NSP1 (data not shown). Phylogenetic analysis of 229 deduced amino acids at the amino-terminal end of the NSP1s of bovine rotaviruses UK, RF, and A44 and porcine rotaviruses OSU, Gottfried, and YM confirmed that the NSP1s of the bovine and porcine rotaviruses sequenced to date segregated into different groups, as found by the full-length NSP1 sequence analysis of Kojima *et al.* (1996) (Fig. 2c). The PP-1 NSP1 grouped with the bovine, not porcine, rotaviruses. The PP-1 NSP1 fragment had seven proline

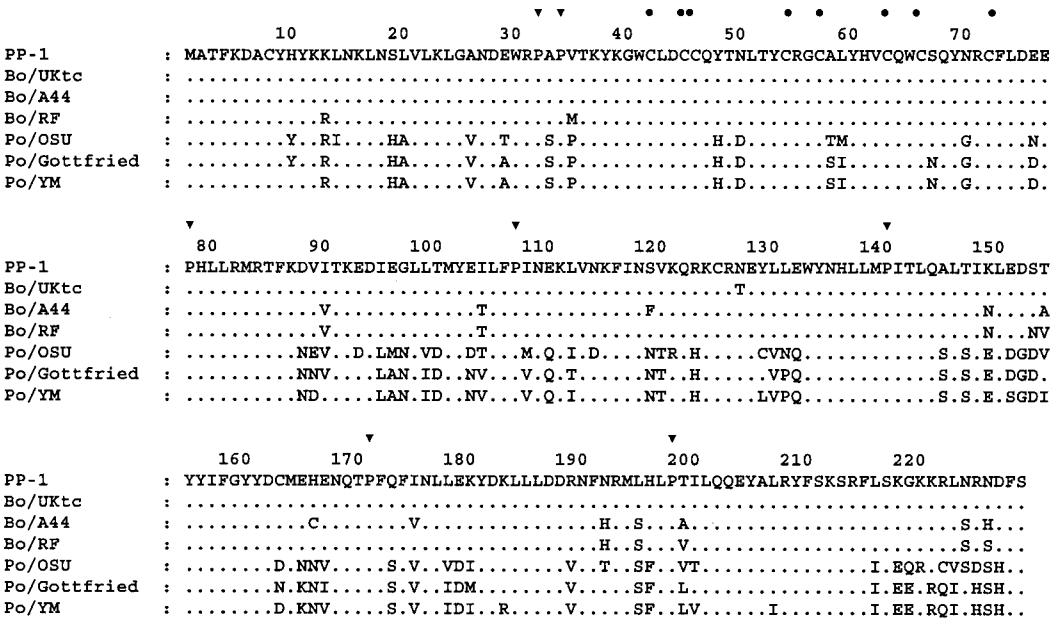

FIG. 5. Multiple sequence alignment of the deduced 229-amino-acid sequence at the amino-terminal end of the PP-1 NSP1 protein with that of the bovine rotaviruses UKtc, A44, and RF and the porcine rotaviruses OSU, Gottfried, and YM. Accession numbers are given in the text. Dots indicate identity to PP-1. (▼) Proline residues; (●) cysteine residues.

residues which were conserved in the three bovine rotaviruses UK, RF, and A44 and the three porcine rotaviruses OSU, Gottfried, and YM (Fig. 5). The porcine rotaviruses had an additional proline (P) residue at position 35 where PP-1 and the bovine rotaviruses UK and A44 had a valine (V) residue and the bovine rotavirus RF had a methionine (M) residue. In the cysteine-rich region, between amino acids 42 and 72, PP-1 was identical to the bovine rotaviruses but differed from the porcine rotaviruses.

NSP4 analysis

The PP-1 NSP4 was closely related to NSP4s from porcine rotaviruses and belonged to the proposed genotype B (Kirkwood and Palombo, 1997; Ciarlet *et al.,* 2000). It had 94 to 98% amino acid (82 to 90% nucleotide) identity to the genotype B porcine rotaviruses OSU, YM, A253, A411, and A131 and 92 to 97% amino acid (88 to 90% nucleotide) identity to genotype B human rotaviruses Wa, AU32, ST3, M37, RV4, and VA70. In contrast, it had 82 to 85% amino acid (77 to 80% nucleotide) identity to the genotype A bovine rotaviruses UK, NCDV, B223, and BRV033 and 82 to 84% amino acid (78 to 80% nucleotide) identity to the genotype A human rotaviruses, KUN, E210, 1076, S2, and RV5. Phylogenetic analysis of the deduced amino acid sequence of the PP-1 NSP4 confirmed its classification with the genotype B porcine and human rotaviruses, not with the genotype A bovine rotaviruses (Fig. 2d).

The PP-1 NSP4 had cysteine residues at amino acids 63 and 71 in common with all rotavirus NSP4s sequenced to date excluding murine rotaviruses (Fig. 6). It had potential N-linked glycosylation sites located at amino acids 8 and 18 which were identical to all other rotavirus NSP4s but at position 19 it had an aspartic acid (D) residue in common with genotype B but not genotype A. The PP-1 NSP4 had 6 proline residues at positions 28, 34, 52, 138, 165, and 168. Four (at positions 28, 52, 165, and 168) have been found in all genotype A and B rotavirus NSP4s sequenced to date but the proline residue at position 138 has been commonly found in genotype B NSP4s but not in the 4 bovine genotype A rotaviruses. At position 97, the PP-1 NSP4 had a glutamine (Q) residue in common with the other genotype B rotaviruses, whereas the genotype A rotaviruses had a lysine (K) residue. In the putative VP4-binding region (amino acids 112 to 148), the PP-1 protein was identical to the genotype B porcine rotavirus YM. It differed from the other 4 genotype B porcine rotaviruses by 1 to 4 amino acids, differed from the 6 human genotype B rotaviruses by 2 to 5 amino acids, but differed from the 9 genotype A human and bovine rotaviruses by 6 to 10 amino acids. At amino acid 148, PP-1 had a phenylalanine (F) residue, in common with all 11 genotype B rotaviruses, whereas the 10 genotype A rotaviruses had isoleucine (I) at this position. In the enterotoxin peptide region (amino acids 114 to 135), the PP-1 protein was identical to the genotype B porcine rotaviruses YM, A411, A131, and A253 but differed from the genotype B porcine rotavirus OSU by 1 amino acid, valine (V) to alanine (A), at position 135. It differed from the genotype A bovine rotaviruses NCDV and BRV033 by 1 amino acid at position 135, valine (V) to methionine (M), and differed from the genotype A bovine rotaviruses B223 and UK by 2 amino acids, including differences at position 135.

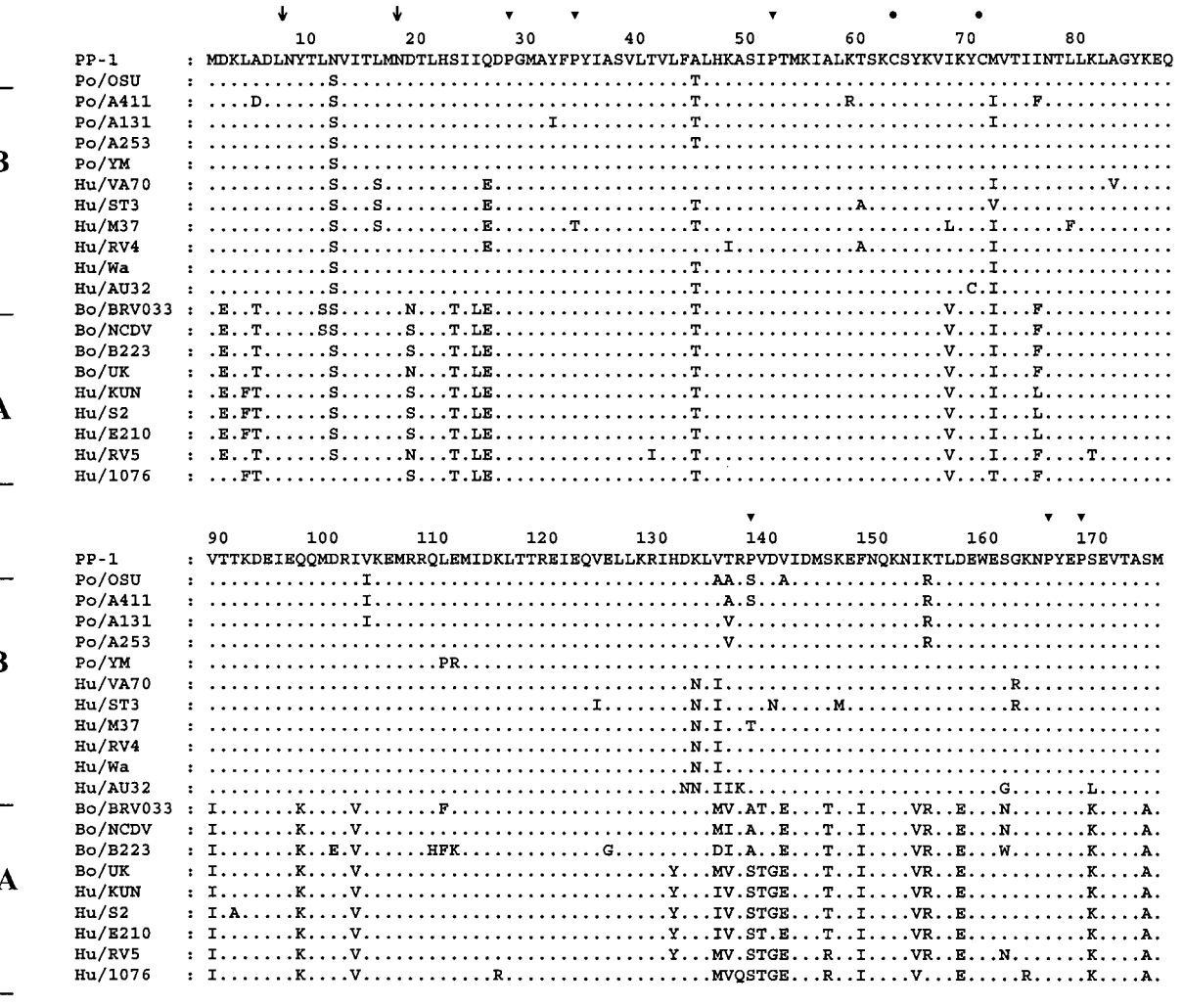

FIG. 6. Multiple sequence alignment of the deduced amino acid sequence of the PP-1 NSP4 protein with that of porcine, bovine, and human rotaviruses grouped in genotypes A and B (labeled on the left). Accession numbers are given in the text. Dots indicate identity to PP-1. Potential N-linked glycosylation sites are shown by ↓. (▼) Proline residues; (●) cysteine residues.

DISCUSSION

Rotavirus PP-1, which was pathogenic to pigs but replicated without causing disease in calves (Hall *et al.,* 1976; Bridger and Brown, 1984; Bridger and Pocock, 1986), was shown to be a bovine–porcine rotavirus reassortant. It had a porcine VP4 and NSP4 and a bovine NSP1 and was of G3 specificity, a G-type characterized previously from several animal species, excluding cattle (reviewed by Hoshino and Kapikian, 1994). To our knowledge, this is the first example of the molecular characterization of a rotavirus which has been characterized experimentally *in vivo* in two target animal species and which has not been produced in the laboratory by reassortment. The finding that the bovine rotavirus PP-1 had a porcine VP4 endorsed the hypothesis that specific VP4s influence rotavirus pathogenicity in a specific animal species. In the present study, possession of a porcine P[7] VP4 was not only associated with pathogenicity in experimental pigs (Bridger and Brown, 1984) but with subclinical replication in experimental calves (Bridger and Pocock, 1986). In a previous study in experimental pigs with the porcine rotavirus 4S, possession of a heterologous bovine VP4 was also associated with subclinical infection even though 4S replicated to the same titers in pigs as the virulent porcine rotavirus 4F (Bridger *et al.,* 1992; Burke *et al.,* 1994). Rotaviruses with heterologous VP4s have been identified in naturally acquired infections in human where possession of a heterologous VP4 has often been linked to asymptomatic natural infections (Gorziglia *et al.,* 1990; Das *et al.,* 1993; Gentsh *et al.,* 1993; Nakagomi *et al.,* 1993, 1994; Steele *et al.,* 1993; Santos *et al.,* 1994; Taniguchi *et al.,* 1994; Pongsuwanna *et al.,* 1996; Li *et al.,* 1996; Okada *et al.,* 2000). However, experiments in pigs and mice using experimentally produced reassortants have produced evidence both for and against a role of VP4 as a virulence determinant in specific animal species (Offit *et al.,* 1986; Broome *et al.,* 1993; Hoshino *et al.,* 1995; Bridger *et al.,* 1998b).

The finding that PP-1 had a NSP1 which was almost identical to the NSP1 of the bovine rotavirus UK was not surprising as the bovine rotavirus UK was isolated from the same fecal sample as PP-1 but by passage in cell culture (Bridger and Woode, 1975). The finding that PP-1 replicated to titers of over 10^7 TCID/g feces (Bridger and Brown, 1984) and caused disease in pigs, whereas the bovine rotavirus UK failed to replicate in pigs, supported the hypothesis that NSP1 is not a determinant of host species-specific replication and disease, at least between bovine and porcine rotaviruses. This hypothesis was supported by the previous observation in which the NSP1 of the bovine rotavirus UK replaced the NSP1 of the porcine rotavirus SW20/21 (Bridger *et al.,* 1998a). Replication in pigs was unaffected. However, others have implicated NSP1 in rotavirus pathogenicity in mice (Broome *et al.,* 1993), indicating that there may be differences between some animal species.

The results of the present study supported the hypothesis that NSP4 has a role in host species-specific infectivity and disease because PP-1 had a porcine not bovine NSP4 and was pathogenic to pigs but not calves. It is noteworthy that the NSP4s from bovine and porcine rotaviruses sequenced to date segregated into two genotypes, A and B, suggesting biological differences between the NSP4s of the two species (Ciarlet *et al.,* 2000). Some, although not all, studies have identified NSP4 as a viral enterotoxin with the region spanning amino acids 114 to 135 playing a critical role in rotavirus pathogenesis in experimental mice (Ball *et al.,* 1996; Angel *et al.,* 1998). The present results did not obviously support a role for this region in rotavirus host species-specific virulence as there were no consistent amino acid differences between the porcine rotaviruses and PP-1 in genotype B and the bovine rotaviruses in genotype A. Whether NSP4 has a direct role in rotavirus host species-specific virulence requires further investigation but it should be noted that villous atrophy was a dominant pathological feature of PP-1 infection in experimental piglets (Hall *et al.,* 1976) but neither NSP4 nor the NSP4 114-135 enterotoxin peptide caused villous atrophy in experimental mice.

Characterization of PP-1 as a G3 rotavirus was unremarkable for a pig pathogen. G3 rotaviruses have been identified in pigs previously and have been found in the largest number of animal species (reviewed by Hoshino and Kapikian, 1994). Amplicons with G3-specific primers have been reported from cattle using RT-PCR (Hussein *et al.,* 1993; de Verdier Klingenberg *et al.,* 1999) but a bovine G3 rotavirus has not been characterized until recently (El-Attar *et al.,* submitted for publication). The finding that PP-1 was initially identified as a G10 rotavirus by RT-PCR using published primers (Gouvea *et al.,* 1994) was explained by the similarity of the PP-1 VP7 sequence in the G10 primer binding region (nucleotide positions 697 to 714, 5′-GAAGTTGCAACAGCTGAA-3′) and the G10 typing primer ET10 (the reverse complement of which is 5′-GAAGTCGCAACGGCTGAA-3′), which differed by only two nucleotides in the middle of the primer. Gouvea *et al.* (1994) did not attempt to diagnose bovine G3 rotaviruses but, clearly, the possible misdiagnosis of G10 and G3 in cattle rotaviruses needs to be addressed.

TABLE 1

Comparison of the Biological and Molecular Properties of the Pig Pathogenic Rotavirus PP-1 to the Calf Pathogenic Rotavirus CP-1

	Rotavirus	
Property	PP-1	CP-1
Replication in pigs	+[a,b]	−[a]
Pathogenic to pigs	+[a,b]	−[a]
Replication in calves	+[c]	+[c]
Pathogenic to calves	−[c]	+[c]
VP4 (P-type)	Porcine (P[7])	Bovine (P[5])[d]
VP7 (G-type)	G3	G3[d]
NSP1	Bovine	Bovine[d]
NSP4 (genotype)	Porcine (B)	Bovine (A)[d]

[a] Bridger and Brown (1984).
[b] Hall *et al.* (1976).
[c] Bridger and Pocock (1986).
[d] L. El-Attar, W. Dhaliwal, M. Iturriza-Gomura, and J. C. Bridger (submitted for publication).

The results from the present study support the opinion expressed by Gouvea and Brantly (1995) that rotaviruses exist as populations of reassortants from which "new" rotaviruses may emerge under appropriate conditions. The condition under which PP-1 emerged was passage of a bovine fecal sample in gnotobiotic pigs to produce, or reveal, a reassortant with a constellation of genes which allowed replication with disease in pigs but subclinical replication in calves. It appears that the original bovine fecal sample, which contained more than one genome profile, contained at least P[5] and P[7] VP4s, G3 and G6 VP7s, a bovine NSP1, and bovine and porcine NSP4 genes. Serial passage in cell cultures yielded the reference G6P[5] bovine rotavirus UK (Bridger and Woode, 1975; Bridger and Brown, 1984; reviewed by Hoshino and Kapikian, 1994), serial passage in gnotobiotic calves yielded the calf pathogenic G3P[5] rotavirus CP-1 (Bridger and Pocock, 1986; El-Attar *et al.,* submitted for publication), and passage in pigs yielded the pig pathogenic G3P[7] PP-1 (present report). The differences in host species infectivity and disease between rotaviruses CP-1 and PP-1 correlated with differences in their VP4 and NSP4s but not their VP7 and NSP1s (Table 1). The present study illustrates experimentally that rotaviruses circulating in one animal species can pose a risk to another by the emergence of a pathogenic rotavirus under appropriate conditions. The extent to which new pathogenic gene combinations occur in nature is at present unknown but naturally occurring rotavirus reassortants have been commonly reported (Das *et al.,* 1993; Gentsch *et al.,* 1993; Gorziglia *et al.,* 1990; Jagannath *et*

al., 2000; Kojima *et al.,* 1996; Li *et al.,* 1994, 1996; Nakagomi *et al.,* 1994; Okada *et al.,* 2000; Pongsuwanna *et al.,* 1996; Santos *et al.,* 1999; Taniguchi *et al.,* 1994).

MATERIALS AND METHODS

Viruses

Bovine rotavirus PP-1 was obtained from an outbreak of calf diarrhea in the United Kingdom in 1973 (Woode *et al.,* 1974). A fecal filtrate was inoculated orally into gnotobiotic piglets and serially passaged in pigs (Hall *et al.,* 1976). Severe diarrhea and villus stunting occurred at the first pig passage. The virus was cloned from the pig feces in MA104 cells and infectivity and pathogenicity for pigs were reassessed (Bridger and Brown, 1984). Its infectivity and pathogenicity for gnotobiotic calves were also determined (Bridger and Pocock, 1986). The G6P[5] bovine rotavirus UK was isolated in the United Kingdom from the same fecal sample as PP-1 but by passage in primary calf kidney cells (Bridger and Woode, 1975) and it has been used widely as a reference bovine rotavirus. The G1 porcine rotavirus SW20/21 was isolated in the United Kingdom in 1976 from a different location than PP-1 and UK and its antigenicity and pathogenicity were characterized (Woode *et al.,* 1976; Bridger and Brown, 1984). Its VP4 had 98% deduced amino acid (98% nucleotide) identity to the P[7] rotavirus (W. Dhaliwal and J. C. Bridger, unpublished observation). Reference rotaviruses were the porcine G5P[7] rotavirus OSU, the bovine rotavirus G10P[11] B223, and the bovine G8P[5] rotavirus 678.

Nucleotide sequencing of VP4, VP7, NSP1, and NSP4 genes

ds-RNA was extracted by treatment with phenol–chloroform or by treatment with guanidinium thiocyanate as described by Boom *et al.* (1990). For VP4 gene sequencing, a VP4 cDNA fragment of 811 nucleotides encoding VP8*, the connecting peptide, and the amino terminus of VP5* was produced by RT-PCR using forward (EBH1, 5′-GGT ACC CGG GAT CCG CGG CTA TAA AAT GGC TTC GC-3′) and reverse (RVA4-1, 5′-AGA TCT CGA GCG CTG CAG TAT ATT GCA TTT CTT TCC A-3′) primers which were based on nucleotides 1–19 and 793–811 (underlined) of the UK bovine rotavirus VP4 gene plus added cloning sites. The cDNA was cloned into pGEM-3Z (Promega Corp.) after digestion with *Pst*I and *Bam*HI restriction enzymes and the plasmid was transfected into competent XL1-Blue *Escherichia coli* cells (Stratagene). PP-1 VP7 and NSP4 genes were reverse-transcribed using hexamer primers. The VP7 gene was amplified by PCR using the forward and reverse VP7 gene-specific primers Beg9, End9, End9(UK), and CRW-8 primers described previously by Gouvea *et al.* (1993). The NSP4 gene was amplified using the reverse primer 5′-CATA/C/GGA/CC/TGCAGTC/TACTTC-3′ and a pool of two forward primers, 5′-ATGGAAAAGTTTCCGACCTC-3′ and 5′-GGCTTTTAAAAGTTCTGTTT-3′, modified from Jagannath *et al.* (2000). The NSP1 gene was reverse-transcribed using a gene-specific primer representing the 3′ end of NSP1 (5′-GGTCACATTTTATGCTGCCTA-3′) and amplified using the same primer and a forward primer representing the 5′ end (5′-GGCCGGCTTTTTTTATGA-3′) (modified from Tian *et al.,* 1993). VP7, NSP1, and NSP4 amplicons were sequenced directly from PCR products which were gel-purified using a Qiagen Quick kit. Nucleotide sequences were determined by cycle sequencing with an A.L.F. automated sequencer (Pharmacia) or on an ABI 3700 sequencing system (MWG-Biotech). For the VP4 gene, two or three gene clones from two PCR products were sequenced in both forward and reverse directions. For the VP7, NSP1, and NSP4 genes, two PCR amplicons were sequenced for each gene in both forward and reverse directions. Consensus sequences were compared using the DNAstar or NCBI BLAST sequence analysis programs. Multiple sequence alignments were performed using either the NCBI or the EBI Clustal X or Clustal W programs. Phyogenetic analyses were conducted using the Clustal W neighbor-joining method.

G-typing RT-PCR

For G-typing RT-PCR, viral RNA was extracted from cell culture fluids using the method of Boom *et al.* (1990). G-typing RT-PCR was conducted as described by Gouvea *et al.* (1994) with G5-, G6-, G8-, and G10-specific primers and rotaviruses OSU, UK, 678, and B223 as reference G5, G6, G8, and G10 rotaviruses, respectively. A G5-G6-G8-G10 primer pool or individual G-type-specific primers were used. Amplicons were electrophoresed on 2% agarose gels and visualized by short-wave UV light after being stained with ethidium bromide.

VP4 and VP7 typing with monoclonal antibodies

VP8*-specific monoclonal antibodies 5G7 and 3G5 raised to the G5P[7] rotavirus OSU (Liprandi *et al.,* 1991) were kindly supplied by F. Liprandi, IVIC, Caracas, Venezuela. A VP4-specific monoclonal antibody, C2/1, reactive to the bovine UK rotavirus G6P[5] was kindly supplied by D. H. Pocock (Pocock, 1990). The VP7-specific monoclonal antibody UK/7 raised to the G6[P5] bovine rotavirus UK was supplied by D. Snodgrass and the VP-7 monoclonal antibodies B223-N7 and B223/3 raised to the G10P[11] bovine rotavirus B223 were supplied by D. Snodgrass and G. N. Woode. MA104 cells were infected overnight, fixed, and stained with monoclonal antibodies at a 1:500 dilution followed by a 1:200 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO). Color was developed with 3,3-diaminobenzidene tetrahydrochloride tablets (Kem-en-Tec, Copenhagen, Denmark).

Genome profile analysis by PAGE

Rotavirus genome profiles were determined on vertical 10% polyacrylamide gels as described previously (Bridger *et al.,* 1998a).

Accession numbers

The VP4 gene sequences used were OSU (X13190), YM (M63231), BMI-1 (L07887), 4S (L10358), Gottfried (M33516), MDR-13 (L07886), C486 (Y00127), UK (M22306), B223 (M92986), and 999/83 (D16352). The VP7 gene sequences used were C95 (L24165), T449 (M92651), S2 (M11164), CRW-8 (edited from Huang *et al.,* 1989), AU-1 (D86271), SA11 (X66158), EW (U08430), Gottfried (X06386), OSU (X04613), UK (X00896), 993/83 (X98869), 678 (L20883), B223 (X57852), ICB2185 (AF192267), P343 (edited from Pongsuwanna *et al.,* 1996), and YM (M23194). The NSP1 gene sequences used were UKtc (Z12108), RF (M22308), B223 (Z12105), A44 (U23726), OSU (U08432), Gottfried (Uo8431), YM (D38154), Wa (L18943), DS1 (L18954), and 69M (Z32552). The NSP4 gene sequences used were UK (K03384), NCDV (X06806), B223 (AF144805), BRV033 (AF144804), OSU (D88831), A253 (AF144797), YM (X69485), A131 (AF144798), A411 (AF144799), Wa (AF093199), AU32 (D88830), KUN (D88829), ST3 (U59110), M37 (U59109), RV4 (U59108), E210 (U59107), 1076 (U59105), S2 (U59104), VA70 (U83798), and RV5 (U59103).

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