A porcine G9 rotavirus strain shares neutralization and VP7 phylogenetic sequence lineage 3 characteristics with contemporary human G9 rotavirus strains

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Abstract

Of five globally important VP7 (G) serotypes (G1–4 and 9) of group A rotaviruses (the single most important etiologic agents of infantile diarrhea worldwide), G9 continues to attract considerable attention because of its unique natural history. Serotype G9 rotavirus was isolated from a child with diarrhea first in the United States in 1983 and subsequently in Japan in 1985. Curiously, soon after their detection, G9 rotaviruses were not detected for about a decade in both countries and then reemerged in both countries in the mid-1990s. Unexpectedly, however, such reemerged G9 strains were distinct genetically and molecularly from those isolated in the 1980s. Thus, the origin of the reemerged G9 viruses remains an enigma. Sequence analysis has demonstrated that the G9 rotavirus VP7 gene belongs to one of at least three phylogenetic lineages: lineage 1 (strains isolated in the 1980s in the United States and Japan), lineage 2 (strains first isolated in 1986 and exclusively in India thus far), and lineage 3 (strains that emerged/reemerged in the mid-1990s). Currently, lineage 3 G9 viruses are the most frequently detected G9 strains globally. We characterized a porcine rotavirus (A2 strain) isolated in the United States that was known to belong to the P[7] genotype but had not been serotyped by neutralization. The A2 strain was found to bear serotype G9 and P9 specificities as well as NSP4 [B] and subgroup I characteristics. By VP7-specific neutralization, the porcine G9 strain was more closely related to lineage 3 viruses than to lineage 1 or 2 viruses. Furthermore, by sequence analysis, the A2 VP7 was shown to belong to lineage 3 G9. These findings raise intriguing questions regarding possible explanations for the emergence of variations among the G9 strains.

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Keywords: Rotavirus; Porcine rotavirus; Serotype G9; Neutralization characterization

Introduction

Group A rotaviruses continue to be the major etiologic agents of severe diarrhea in infants and young children worldwide and are estimated to be responsible for a median of approximately 440,000 deaths each year among children <5 years of age, predominantly in the developing countries (Parashar et al., 2003). In the United States alone, approximately 500,000 physician visits, 50,000 hospitalizations, and about 20 deaths are estimated to occur among the approximately 2.7 million children <5 years of age affected by rotavirus diarrhea yearly (Bresee et al., 1999; Glass et al., 1994; Kapikian et al., 2001; Parashar et al., 2003). Thus, the development and implementation of a safe
and effective rotavirus vaccine has been a global public health goal. Group A rotaviruses are also important causative agents of diarrhea of the young of various domestic animals including pigs (Saif et al., 1994).

Rotaviruses carry three important antigenic specificities: group, subgroup, and serotype. Based on group specificity, which is determined predominantly by VP6, rotaviruses are divided into seven groups (A to G) (Kapikian et al., 2001). Human rotavirus-associated infections are predominantly caused by group A, and less commonly by group B or C; thus, the emphasis of vaccine development has been targeted at group A rotavirus-associated disease. Subgroup specificity, which is also determined by VP6, has been used for characterizing the antigenic properties of various rotavirus isolates in epidemiologic surveys. Most human rotaviruses belong to either subgroup I or subgroup II (Kapikian et al., 2001). Typing of subgroup specificity is performed by (i) subgroup-specific monoclonal antibody-based ELISA (Greenberg et al., 1983) or (ii) sequencing or restriction fragment length polymorphism analyses of a 379-bp cDNA amplicon of the VP6 gene (Iturriza-Gomara et al., 2002a,b). A determination of genotypes of NSP4, the gene 10 product that has been reported to act as an enterotoxin (Ball et al., 1996), is performed by sequencing of the gene. Genotypes A, B, and C NSP4s have been detected in human rotaviruses.

Rotavirus antigens that can induce neutralizing antibodies have played a central role in research and development of a rotavirus vaccine because neutralizing antibodies and their type specificities appear to play an important role in protection against many viral diseases. Rotavirus outer capsid proteins VP7 and VP4 are independent neutralization and protective antigens (Greenberg et al., 1985; Hoshino et al., 1985; Offit et al., 1986) and thus a binary system of rotavirus classification for the VP7 and VP4 neutralization specificities has been established: the VP7 or G (because VP7 is a protease-sensitive) serotype (Estes, 2001; Hoshino and Kapikian, 1996; Kapikian et al., 2001). In order to circumvent the lack of readily available reagents (i.e., type-specific high titered polyclonal hyperimmune antisera) for serotyping and to enable the typing of large numbers of rotavirus field isolates, a G or P genotyping assay was developed in which “genotype-specific” primers were employed in RT-PCR (Gentsch et al., 1992; Gouvea et al., 1990). The numbers assigned for G serotypes (types determined by neutralization assay) and G genotypes (types determined by a nonserological assay) are identical, and thus a single number is used (e.g., G1). However, the numbers assigned for P serotypes and P genotypes are different; therefore, a P serotype is designated with a P followed by the assigned number, whereas the P genotype is designated by a P followed by the assigned number in brackets (e.g., P1A[8]) (Estes, 2001; Kapikian et al., 2001). If the genotype but not the serotype is known, then the P is followed by the bracketed number only. Interestingly, only 4 (G1, G2, G3 and G4) of 10 G serotypes and only one (P1A[8] and P1B[4]) of 13 P serotypes found in humans have repeatedly been reported to be of epidemiological importance throughout the world (Gentsch et al., 1996; Kapikian et al., 2001; Kirkwood and Buttery, 2003; Koshimura et al., 2000; Palombo, 1999; Santos and Hoshino, in press). Rhesus rotavirus (RRV)-based or bovine rotavirus (UK or WC3)-based reassortant vaccines have been developed and are thus designed to cover such epidemiologically important G and P types (Advisory Committee on Immunization Practices, 1999; Glass et al., 2004; Hoshino et al., 2002, 2003; Midthun et al., 1985, 1986; Offit, 2002). Four (G3, G4, G5, and G11) of 10 G types and two (P[6] and P[7]) of 8 P types detected in pigs are thought to be of epidemiologic importance (Liprandi et al., 2003; Martella et al., 2001; Saif et al., 1994).

A serotype of rotavirus outer capsid glycoprotein VP7 (G serotype) has been defined based on a criterion that has been used to establish a serotype of various viruses including polioviruses, ECHO viruses, coxsackie viruses, and rhinoviruses, namely a greater than 20-fold difference between homologous and heterologous reciprocal neutralizing antibody titers (for reviews, see Hoshino et al., 1998). Serotypes of outer capsid spike protein VP4 (P serotype) of selected human rotaviruses were established by neutralization using hyperimmune antiserum raised against each of four human rotavirus VP4 proteins expressed by baculovirus recombinants (Gorziglia et al., 1990). A criterion used to establish a P serotype in that study was an eightfold difference between homologous and heterologous reciprocal neutralizing antibody titers. A P subtype (P1A and P1B) was established by the same authors based on a fourfold difference in neutralizing antibody titers. However, today, it is possible to determine P serotype using the same criterion (>20-fold antibody difference) used for G typing (Hoshino and Kapikian, 1996).

Serotype G9 viruses that have emerged recently as the fifth globally common G type of clinical importance have several unique characteristics (Arista et al., 2004; Bok et al., 2001; Clark et al., 1987; Coulson et al., 1999; Cunliiffe et al., 2002; Das et al., 1993; Gentsch et al., 1993; Iturriza-Gomara et al., 2000; Kirkwood et al., 1993, 2002, 2003; Laird et al., 2003; Martella et al., 2003; Nakagomi and Nakagomi, 2002; Nakagomi et al., 1990; Oka et al., 2000; Ramachandran et al., 1998, 2000; Santos et al., 2001, 2003; Unicomb et al., 1995; Urasawa et al., 1992; Zhou et al., 2000, 2001, 2003). For example, (i) prior to 1983, serotype G9 viruses were not known; (ii) soon after their initial isolation in the US (in 1983) and Japan (in 1985), G9 viruses were not detected for about a decade in both countries and reappeared in the mid-1990s in both countries; (iii) unlike other globally common G serotypes (G1–G4), which occur almost exclusively in conjunction with either P1A[8] or P1B[4], G9 viruses have been detected in association with a variety of P types including P[4], P[6], P[8], P[9], P[11], and P[19]; and (iv) at least three phylogenetic sequence lineages have been reported to exist among the VP7 gene of G9 viruses. It is noteworthy that (i) lineage 1 G9 viruses that include those
isolated in the 1980s had not been detected anywhere in the world for the last 18–20 years but recently they have been reported to occur in limited numbers in Japan (Zhou et al., 2003); (ii) lineage 2 G9 viruses have been detected only from asymptomatic neonates in India, thus far; and (iii) a majority of G9 viruses that are prevalent around the world today belong to lineage 3. Thus, it has been suggested that the contemporary lineage 3 G9 strains are not direct descendants of the lineage 1 G9 strains isolated in the 1980s (Laird et al., 2003). Recently, by analyzing a total of 40 G9 strains collected in the United States or India, Laird et al. (2003) speculated that two U.S. strains (Om46 and Om67) isolated in 1997–1998 may be the distant progenies of the lineage 1 viruses since they bore VP7 gene equally divergent in sequence from lineage 1 or lineage 3 VP7 gene. It is also noteworthy that in addition to three major VP7 gene lineages, there exist a number of minor VP7 gene lineages (Laird et al., 2003; Martella et al., 2003).

We have recently analyzed VP7 neutralization characteristics of eight human G9 strains (2 lineage 1, one lineage 2 and 5 lineage 3) isolated in five different countries (Bangladesh, Brazil, India, Japan, and United States) and reported that (i) VP7 phylogenetic lineage-specific neutralization specificities exist, and, moreover, (ii) lineage 1 G9 strains may be the most suitable as G9-specific vaccine candidates because they induced the broadest cross-reactive antibodies that neutralized all G9 viruses tested regardless of their phylogenetic lineages (Hoshino et al., 2004). Thus, we demonstrated in that study that not only nucleotide sequence analysis but also VP7-lineage-specific serologic reagents are powerful tools to study the phylogenetic origin of the VP7 gene products of the G9 rotavirus isolates.

Ten of fifteen rotavirus G types are shared by humans and animals. Of note is the report that describes the detection of G9 viruses in pigs (in the United States and Brazil) (Paul et al., 1988; Santos et al., 1999; Winiarczyk et al., 2002) or sheep (in Scotland) (Fitzgerald et al., 1995). The purpose of this study was threefold: (i) to characterize serologically and molecularly the VP7 and VP4 of a porcine rotavirus, designated as the A2 strain, which was one of two porcine rotavirus strains in a commercial bivalent vaccine for veterinary use in the United States (Hesse et al., 1993); (ii) to analyze phylogenetic relationships of the VP7 between the porcine A2 strain and selected human G9 strains belonging to lineage 1, 2, or 3; and (iii) to determine the electropherotype, subgroup specificity, and NSP4 genotype of the A2 strain.

Results

Characterization of selected phenotypes of a porcine rotavirus A2 strain

The A2 strain exhibited an electrophoretic pattern that was distinct from that of (i) porcine rotavirus strain Gottfried (P2B[6]:G4), OSU (P9[7]:G5), or YM (P9[7]:G11) and (ii) various human G9 strains including WI61, F45, AU32, 116E, US1205, R44, R143, BD524, and INL1 (Fig. 1). The A2 strain, which had a “long” electrophoretic pattern, was found to belong to subgroup I by ELISA (data not shown).

Neutralization characteristics of the VP7 and VP4 proteins of the A2 strain

Studies were carried out to determine the serotype specificity of the A2 strain by neutralization assays in which each of 3 guinea pig hyperimmune antisera raised to the A2 virus was tested against selected prototype rotavirus strains belonging to G1–G14. The three sera were also tested against reassortant rotaviruses UK × A2 (P7[5]:G,A2) and DS-1 × A2 (P1B[4]:G,A2), each of which bore only the VP7 gene from the A2 strain and the remaining 10 genes from either the bovine rotavirus UK or human rotavirus DS-1 strain (Fig. 2). As shown in Table 1, each of the three anti-A2 antisera was shown to contain high levels of anti-A2 VP7 neutralizing antibodies. None of the 16 rotavirus strains representing the 14 established VP7 serotypes were neutralized significantly by any of the three anti-A2 antisera. Two porcine strains OSU and YM and one equine rotavirus strain L338 were neutralized to low titers, which were >20-fold less than the homotypic titer.

Fig. 1. Electrophoretic migration patterns of genomic RNAs of porcine G4 rotavirus Gottfried strain (lane 1), porcine G5 rotavirus OSU strain (lane 2), porcine G11 rotavirus YM strain (lane 3), porcine G9 rotavirus A2 strain (lane 4), human G9 rotavirus WI61 strain (lane 5), human G9 rotavirus F45 strain (lane 6), human G9 rotavirus AU32 strain (lane 7), human G9 rotavirus 116E strain (lane 8), human G9 rotavirus R44 strain (lane 9), human G9 rotavirus R143 strain (lane 10), human G9 rotavirus US1205 strain (lane 11), human G9 rotavirus INL1 strain (lane 12), and human G9 rotavirus BD524 strain (lane 13) in 10% polyacrylamide gel.
Thus, the A2 strain appeared to be a new serotype at least in this one direction. In addition, the two reassortants that contained only the VP7 of the A2 strain were each neutralized to high titer, suggesting that the response was to the VP7 and not to the VP4 of the A2 strain.

Next, we determined the reciprocal neutralization pattern by examining the neutralization pattern of the A2 strain with hyperimmune antiserum raised to selected prototype rotavirus strains belonging to each of the 14 G types (Table 2). Antiserum to G1, G2, G7, G10, or G12 viruses did not neutralize the A2 virus. However, the A2 strain was neutralized (i) to high titers by antiserum to G5 (OSU) and G9 (WI61 and F45) viruses and (ii) to moderate to low titers by antiserum to G3 (P), G4 (ST3), G6 (NCDV), G8 (69M), G11 (YM), G13 (L338), and G14 (F123) viruses. These results suggested that (i) the A2 virus may be related via VP7 to either G5 or G9 and (ii) an intermediate level of cross-reactivity observed between the A2 virus and antiserum to the YM (P9[7]:G11) or L338 (P18:G13) may be due to the shared VP4 specificity.

To test these hypotheses, the A2 virus was tested against antiserum raised against selected reassortants. As shown in Table 2, the A2 virus was neutralized to high titers by antiserum raised to each of the eight single VP7 gene substitution UK reassortants (UK × WI61, UK × AU32, UK × 116E, UK × R44, UK × R143, UK × US1205, UK × INL1 or UK × BD524) each of which bore only a single VP7 gene with G9 specificity (WI61, AU32, R44, R143, US1205, INL1 or BD524) and the remaining 10 genes from bovine rotavirus UK (Hoshino et al., 2004), suggesting that the A2 virus was related to G9 viruses due to the shared VP7 specificity. In addition, the A2 virus was not neutralized by anti-DS-1 × OSU (P18[4]:G5) antiserum but was neutralized moderately by antiserum to OSU × DS-1 (P9[7]:G2), indicating that the cross-reactivity between the A2 and the OSU was due to the shared P9 VP4 specificity. The latter observation confirmed previous findings in which close genetic and antigenic relationships between the A2 VP4 and the OSU VP4 were demonstrated by RNA–RNA hybridization and neutralization (Hesse et al., 1993). Furthermore, an intermediate cross-reactivity observed between the A2 and YM (G11) or L338 (G13) was confirmed to be due to the shared VP4 specificity since (i) the A2, OSU, and YM share the same P genotype specificity (i.e., P[7]) and (ii) the OSU VP4 and L338 VP4 are highly related serotypically (Hoshino et al., unpublished observation).

Thus, the A2 strain shares VP7 neutralization specificity with serotype G9. However, as noted previously in Table 1, antiserum to A2 virus did not show any neutralizing activities against human G9 strains WI61 and AU32 and thus the A2 virus appeared to be a new serotype. This apparent contradiction was examined further as follows: (i) since in our previous study, antisera to lineage 3 G9 viruses neutralized lineage 2 and 3 viruses to high titers but exhibited marginal to very low neutralizing activities to the lineage 1 prototype G9 strain WI61 (Hoshino et al., 2004), we examined by neutralization assay three lineage 1, one lineage 2, and five lineage 3 G9 strains against antisera to the A2 or reassortant UK/C2 A2 (P7[5]:G,A2) (Table 3), suggesting that the porcine G9 A2 strain shared high VP7 antigenic specificities with lineage 2 and 3 human G9 strains.

Nucleotide sequence as well as phylogenetic analyses of the VP7, VP8*, and NSP4 genes of the A2 strain

The VP7 gene of A2 strain was 1061 bp in length, which is common in G9 VP7 genes. The A2 strain demonstrated higher nucleotide percent identity to lineage 3 than lineage 1
Table 1
Neutralization profiles of three guinea pig hyperimmune antisera raised to porcine rotavirus A2 strain toward selected prototype rotavirus strains belonging to G1–G14

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host of origin</th>
<th>Country of origin</th>
<th>Year collected</th>
<th>G type</th>
<th>P type [genotype]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch2</td>
<td>chicken</td>
<td>UK</td>
<td>1979</td>
<td>7</td>
<td></td>
<td>McNulty et al. (1980)</td>
</tr>
<tr>
<td>L338</td>
<td>equine</td>
<td>UK</td>
<td>1991</td>
<td>13</td>
<td>7[18]</td>
<td>Browning et al. (1991a)</td>
</tr>
<tr>
<td>UK × A2 reassortant</td>
<td>NA</td>
<td></td>
<td>10,240</td>
<td>20,480</td>
<td>20,480</td>
<td></td>
</tr>
<tr>
<td>DS-1 × A2 reassortant</td>
<td>NA</td>
<td></td>
<td>10,240</td>
<td>20,480</td>
<td>20,480</td>
<td></td>
</tr>
</tbody>
</table>

a Reciprocal of 60% plaque reduction neutralization antibody titer.
b A titer of <1:80 is considered background.
c Year published.
d NA = not applicable.

or 2 viruses (Table 4). Alignment of the VP7 deduced amino acid (aa) sequence of the A2 strain with those of other G9 human strains used in this study is shown in Fig. 3. Certain aa substitutions were shared (i) only between the A2 and lineages 2 and 3 viruses (e.g., threonine at residue 220 and asparagines at residue 242) and (ii) only between the A2 and lineage 3 viruses (e.g., threonine at residue 220 and asparagines at residue 242), suggesting a close genetic relatedness between the A2 VP7 and that of lineage 2 or 3 viruses. The A2 VP7 nucleotide sequence was shown to cluster into phylogenetic lineage 3 (Fig. 4), confirming further genetic close relatedness between them.

The highest degree of nucleotide identity of the A2 VP8* gene was 92.2% with porcine P9[7] rotavirus OSU VP8* gene, suggesting that the A2 VP4 belonged to P[7] genotype.

The NSP4 gene of A2 strain was 750 bp in length. Comparison of the deduced amino acid sequence of A2 NSP4 with published NSP4 sequences of representative rotavirus strains showed the A2 NSP4 belonged to NSP4 genotype B (96.6% aa identity versus porcine OSU NSP4[B]).

Previously, a close genetic relatedness between the porcine A2 (P[7]:G9) strain and two porcine strains OSU (P9[7]:G5) and Gottfried (P2B[6]:G4) was reported as determined by whole genome RNA–RNA hybridization (Hesse et al., 1993). In that study, nine hybrid bands were formed between the A2 and OSU (the NSP1 and VP7 genes did not hybridize) and nine hybrid bands were detected between the A2 and Gottfried (the VP4 and VP7 genes did not hybridize), demonstrating that the whole genome of the A2 virus is of porcine rotavirus origin.

Discussion

Rotavirus serotype G9 viruses continue to attract special attention as one of the globally common G serotypes (G1–4 and 9) of clinical importance. That is because the G9 viruses have a unique natural history and evolution in human communities. For example, when these strains reemerged after a decade of absence in the United States and Japan, the genetic composition and molecular characteristics of such reemerged G9 viruses were characteristically different from those of the earlier isolates. For example, (i) the G9 viruses isolated in the 1980s had a short electropherotype with P1A[8] and subgroup II specificities whereas the reemerged G9 viruses were characteristically long electropherotype with P[4], P[6], [8], [9], [11], or [19] specificity in association with subgroup I or II specificity and (ii) the VP7 gene of the earlier isolates belonged to phylogenetic lineage (lineage 1) distinct from that of the reemerged isolates (lineage 3). Thus, such findings suggested that the contemporary G9 viruses may have derived from a phylogenetic progenitor distinct from that of the G9 viruses isolated in the 1980s (Laird et al., 2003). It is of note that
### Table 3

Antigenic relationships between porcine rotavirus A2 strain and nine human G9 rotavirus strains belonging to lineage 1, 2, or 3

<table>
<thead>
<tr>
<th>G9 rotavirus strains tested against guinea pig (GP) hyperimmune antiserum</th>
<th>Antibody titera of GP antiserum to A2 or UK × A2 (P:7[5]:G:9)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Host</td>
<td>Country of origin</td>
</tr>
<tr>
<td>W161</td>
<td>Human</td>
<td>USA</td>
</tr>
<tr>
<td>F45b</td>
<td>Human</td>
<td>Japan</td>
</tr>
<tr>
<td>R44</td>
<td>Human</td>
<td>Brazil</td>
</tr>
<tr>
<td>R143</td>
<td>Human</td>
<td>Brazil</td>
</tr>
</tbody>
</table>

a Reciprocal of 60% plaque reduction neutralization antibody titer.
b E = electropherotype, L = long, S = short.
c Ikegami et al., 1987.
lineage 2 viruses that were isolated first in 1986 have not been detected outside of India. Today, the lineage 3 G9 viruses represent the fifth most common G type of clinical importance in many parts of the world (for review, see Santos and Hoshino, in press). In Australia, for example, during the 2001–2002 season, the G9 serotype was the most prevalent nationwide (40.4%) followed by G1 (38.9%) (Kirkwood et al., 2002).


Table 4
Percent nucleotide (below diagonal) and deduced amino acid (above diagonal) identity among the VP7s of porcine rotavirus A2 strain and nine human G9 rotavirus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Host</th>
<th>VP7 lineage</th>
<th>Lineage 1</th>
<th>Lineage 2</th>
<th>Lineage 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>W161</td>
<td>human</td>
<td>1</td>
<td>98.5</td>
<td>97.9</td>
<td>92.6</td>
</tr>
<tr>
<td>F45</td>
<td>human</td>
<td>1</td>
<td>99.0</td>
<td>99.4</td>
<td>92.3</td>
</tr>
<tr>
<td>AU32</td>
<td>human</td>
<td>1</td>
<td>98.1</td>
<td>99.2</td>
<td>91.7</td>
</tr>
<tr>
<td>I16E</td>
<td>human</td>
<td>2</td>
<td>89.4</td>
<td>89.6</td>
<td>89.3</td>
</tr>
<tr>
<td>R44</td>
<td>human</td>
<td>3</td>
<td>89.4</td>
<td>89.6</td>
<td>88.8</td>
</tr>
<tr>
<td>R143</td>
<td>human</td>
<td>3</td>
<td>89.3</td>
<td>88.8</td>
<td>88.5</td>
</tr>
<tr>
<td>US1205</td>
<td>human</td>
<td>3</td>
<td>89.4</td>
<td>89.8</td>
<td>88.8</td>
</tr>
<tr>
<td>INL1</td>
<td>human</td>
<td>3</td>
<td>89.1</td>
<td>88.6</td>
<td>89.9</td>
</tr>
<tr>
<td>BD524</td>
<td>human</td>
<td>3</td>
<td>87.9</td>
<td>87.5</td>
<td>87.7</td>
</tr>
<tr>
<td>A2</td>
<td>porcine</td>
<td>3</td>
<td>90.0</td>
<td>89.7</td>
<td>90.2</td>
</tr>
</tbody>
</table>

Fig. 3. Comparison of the deduced amino acid sequence of the VP7s of the porcine rotavirus A2 strain and nine serotype G9 human rotavirus strains employed in this study. Three variable regions (VR1–VR3) that are not antigenic sites are marked in black boxes. Six variable regions (VR4–VR9) and seven amino acid residues that have been demonstrated to be involved in the formation of antigenic sites (shown as letters in parentheses) through epitope mapping studies (reviewed in Kapikian et al., 2001) are marked in red boxes.
(1999) concluded that the year 1995 may mark the first appearance of the lineage 3 G9 viruses in the United States. More recently in 2003, by analyzing a total of 40 G9 viruses isolated in India or the United States, Laird et al. (2003) reported that the lineage 3 G9 viruses were detected as early as 1993 in India.

In the present study, we showed a porcine rotavirus (designated the A2 strain), which had not been VP7 serotyped previously but had been genotyped as belonging to genotype P[7] (Hesse et al., 1993), bore a serotype P9:G9, NSP4[B] and subgroup I specificities. Of note is the finding that prototype G9 human rotavirus strain WI61 (isolated in the United States in 1983) as well as two Japanese G9 strains F45 and AU32 (isolated in 1985 and 1986, respectively) were not neutralized or neutralized inefficiently by anti-A2 antisera although reciprocally anti-WI61 antiserum or anti-AU32 antiserum neutralized significantly the A2 virus (a one-way neutralization relationship). However, antisera to A2 neutralized significantly other human G9 strains tested including 116E (isolated in India, R44 and R143 (isolated in Brazil), US1205 (isolated in the US), INL1 (isolated in India), and BD524 (isolated in Bangladesh). In addition, antiserum raised to each of the latter six G9 strains neutralized the A2 virus significantly (a two-way neutralization relationship). Recently, we reported that (i) G9 viruses belonging to lineage 2 (strain 116E) or lineage 3 (strains R44, R143, US1205, INL1 and BD524) are distantly related based on the VP7 neutralization specificities to lineage 1 viruses (strains WI61, F45, and AU32), and (ii) the lineage 3 viruses may have a progenitor distinct from that of the lineage 1 viruses (Hoshino et al., 2004). The present study has shown that the VP7 of the porcine A2 strain is similar, if not identical, to that of lineage 3 human G9 strains serotypically. In addition, we have shown by sequence analysis that the A2 VP7 belongs to phylogenetic lineage 3. Furthermore, the A2 VP7 shares characteristic amino acid (aa) substitutions with lineage 3 human G9 strains. For example, aa residues 17 (valine), 208 (isoleucine), and 250 (lysine) are found only in the A2 and lineage 3 viruses. Preliminary data obtained from codon usage bias analysis of the A2 VP7 and that of human G9 viruses belonging to lineage 1–3 shows that the A2 and lineage 3 G9 viruses share common codon usages distinct from those exhibited by lineage 1 or 2 viruses (data not shown). These findings suggest that the porcine G9 virus and human G9 lineage 3 viruses may have a common ancestor or perhaps the porcine G9 virus itself may be a progenitor of contemporary lineage 3 human viruses. In this regard, it is worthy to note that two lineage 3 G9 strains (Mc323 and Mc345) isolated from patients with diarrhea in Thailand in 1989 are postulated to be naturally occurring porcine–human reassortant rotaviruses since (i) they are more closely related genetically to porcine than human rotaviruses as determined by RNA–RNA hybridization; (ii) the VP4 type (P[19]) of these strains has only been detected in pigs thus far (Kapikian et al., 2001; Okada et al., 2000); and (iii) they exhibit a long RNA electropherotype with subgroup I specificity, rare characteristics to human rotaviruses (Urasawa et al., 1992). Interestingly, lineages 2 and 3 viruses share certain common aa substitutions (e.g., aa residues 220 [threonine] and 242 [asparagines]). This indicates that the lineages 2 and 3 G9 viruses may have evolved from a common ancestor.

Although rotavirus strain surveillance in the pig population has been performed in various parts of the world, the detection of G9 porcine rotaviruses has been reported only in the United States and Brazil thus far (Paul et al., 1988; Santos et al., 1999; Winiarczyk et al., 2002). In the United States, the detection of four porcine G9 viruses has been reported (Paul et al., 1988; Winiarczyk et al., 2002). We wanted to include such viruses in the present study; however, unfortunately, these viruses were no longer available for further characterization. It will be interesting to continue strain surveillance to determine the prevalence of G9 viruses in the pig population in the United States as well as in other parts of the world. It is noteworthy that the A2 strain of porcine G9 virus characterized in this study was isolated in the late 1970s to the early 1980s, which makes the A2 strain (lineage 3 virus) the first G9 rotavirus ever isolated. It is of interest also to note that the detection of the first G9 human rotaviruses (lineage 1 virus) was reported in the United States in 1983. Currently, we are examining selected archival stool samples from children with diarrhea collected in Washington, DC, in the 1970s and 1980s to investigate the distribution of lineage 1 or 3 G9 viruses.

Approximately 2 decades ago, based on the Jennerian principle, rhesus monkey rotavirus (RRV, MMU18006 strain) was developed as a human rotavirus candidate vaccine (Kapikian et al., 1985, 2001). That was because (i) RRV was similar, if not identical in a two-way fashion, to human G3 rotaviruses (Hoshino et al., 1984), (ii) it bore an attenuation phenotype in humans, and (iii) it grew to high titer in primary simian cell cultures (Kapikian et al., 1985). Although the porcine rotavirus A2 strain could possibly
provide an attenuation phenotype in humans, unlike RRV, it would not be an ideal G9 vaccine candidate, because, as we have shown in this study, antiserum to the A2 virus does not neutralize lineage 1 G9 viruses efficiently. Thus, in selecting a vaccine candidate, care must be exercised to select a strain with the broadest available reactivity to extant serotypes.

Nucleotide sequence accession numbers

Sequence data reported in this work have been deposited in the GenBank database under accession numbers AB180972 (A2, VP7 gene), AB180977 (A2, VP8* gene), and AB180978 (A2, NSP4 gene).

Materials and methods

Virus isolation, cell cultures, culture medium, subgroup assay, neutralization assay, hyperimmune antiserum, and polyacrylamide gel electrophoresis

Table 1 summarizes the rotavirus strains employed in this study, which represented G1–G14 specificity. A porcine rotavirus A2 strain was isolated from a commercial (Ambico) bivalent live oral rotavirus vaccine for veterinary use as the rotavirus A2 strain was isolated from a commercial (Ambico) study, which represented G1–G14 specificity. A porcine polyacrylamide gel electrophoresis assay, neutralization assay, hyperimmune antiserum, and plaque-forming-units per 250 μl of the virus as described previously (Hoshino et al., 1998). Agarose (SeaKem ME, BMA, Rockland, ME) was used as a solidifying reagent in the overlay medium. Hyperimmune antiserum to each rotavirus strain or reassortant was raised in specific pathogen free guinea pigs (Charles River, Wilmington, MA) which were free of rotavirus neutralizing antibodies (titer <1:20 vs. AU32) as determined by PRN assay. Sera were inactivated before use by heating at 56 °C for 30 min. Rotavirus immunogens were prepared as described previously (Hoshino et al., 2004; Wyatt et al., 1982). Rotavirus genomic double-stranded (ds) RNAs were extracted with a mixture of phenol/chloroform/isoamyl alcohol (25:24:1 v/v, GIBCO Invitrogen Corp., Carlsbad, CA), precipitated with ethanol and analyzed in a 10% polyacrylamide gel as reported previously (Jones et al., 2003).

Construction, identification, and characterization of single VP7 gene substitution rotavirus reassortants

Since the interaction of VP4–VP7 outer capsid proteins of rotavirus has been reported to affect the expression of selected phenotypes of one or both proteins (Chen et al., 1992; Pesavento et al., 2003), we constructed reassortants UK × A2 (P7[5]:G,A2) and DS-1 × A2 (P1B[4]:G,A2). Roller tube cultures of primary AGMK cells were coinfected at a multiplicity of infection of approximately one with the porcine rotavirus A2 strain and the reassortant rotavirus D × UK. When approximately 75% of the infected cells exhibited cytopathic effects, the cultures were frozen and thawed once and the lysate was plated onto MA104 cells in a six-well plate (Costar, Corning Inc., Corning, NY) in the presence of G1-specific VP7 neutralizing mAb 2C9 (Shaw et al., 1985) for selection of the desired UK × A2 (P7[5]:G,A2) reassortant. The desired single VP7 gene substitution reassortant was selected and identified and then plaque purified three times. The plaque purified UK × A2 reassortant was then used to generate the DS-1 × A2 (P1B[4]:G,A2) reassortant. A cell culture lysate coinfected with the UK × A2 reassortant and the DS-1 virus was plated onto MA104 cells in a six-well plate in the presence of G2-specific VP7 neutralizing mAb S2-2G10 (Taniguchi et al., 1987) for selection of the desired single VP7 gene substitution reassortant DS-1 × A2 (P1B[4]:G,A2). The origin of genes of each reassortant was identified by polyacrylamide gel electrophoresis (PAGE) of its genomic dsRNAs (Kalica et al., 1978; Rodger and Holmes, 1979). Genomic RNAs were electrophoresed at 13 mA for 15 h and the resulting migration patterns were visualized by staining of gel with silver nitrate. The origin of certain genes that was not able to be determined with certainty by PAGE was studied further by constant denaturant gel electrophoresis as previously described (Jones et al., 2003). Hyperimmune guinea pig antiserum to each rotavirus strain or reassortant was analyzed for VP7- and VP4-specific antibodies to selected human and animal rotavirus strains or reassortants by 60% PRN assay.
Nucleotide sequence analysis of the VP7, VP8*, and NSP4 genes of porcine A2 strain and phylogenetic analysis

A full-length cDNA of the VP7 or NSP4 gene, or 876 base pairs (bp) of the VP4 fragment of the A2 strain was amplified using primer pair Beg9 and End9 (Gouvea et al., 1990) (for VP7 gene), Wa10F (5′ GGCTTTTTAAAAGGTCT GTTC 3′) and Wa10R (5′ GGTCACACTAAGACCATTCC 3′) (for the NSP4 gene), or con2 and con3 (Gentsch et al., 1992) (for 876-bp fragment of VP4). The amplified product from RT-PCR was separated by electrophoresis in a 1% agarose gel, recovered from the gel, purified with a Wizard SV gel and PCR clean-up kit (Promega) as indicated by the manufacturer, and cloned into the pCR2.1-TOPO vector (Invitrogen). The recombinant plasmids were introduced into E. coli TOP 10 strain (Invitrogen) and recovered using QIAprep spin miniprep kit (Qiagen). Two independent cDNA clones with the desired VP7 gene, VP8* gene, or NSP4 gene insert were sequenced at least twice using the BigDye terminator cycle sequencing kit (Applied Biosystems) with M13 forward and reverse primers and the ABI PRISM 310 automated DNA sequencer. Sequence alignment systems) with PRISM 310 automated DNA sequencer. Sequence alignment systems) with M13 forward and reverse primers and the ABI PRISM 310 automated DNA sequencer. Sequence alignment systems) with M13 forward and reverse primers and the ABI PRISM 310 automated DNA sequencer. Sequence alignment

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