Molecular and antigenic analyses of serotypes 8 and 10 of bovine rotaviruses in Thailand

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Antigenic and genomic properties of non-serotype 6 bovine rotaviruses isolated in Thailand and Japan were studied by cross-neutralization tests, nucleotide sequence determination of the VP7 gene, and RNA–RNA hybridization. Two Thai strains (61A and A44) were serologically related to a Japanese isolate KK3 which has been assigned to serotype 10. In contrast, strain A5 was found to be antigenically similar to human strain 69M with serotype 8 specificity, although strain A5 showed a one-way cross-reaction with serotype 6 strain NCDV. VP7 sequence analysis confirmed these results. High degrees of similarity in nucleotide and amino acid sequences (92.5 to 98.2% and 96.3 to 97.9%, respectively) were found among the VP7 genes of the four serotype 10 bovine strains (61A, A44, KK3 and B223). The VP7 amino acid sequence of strain A5 was similar to those of serotype 8 human strains (91.7% and 94.8% for strains B37 and 69M, respectively). In RNA–RNA hybridization experiments, a high level of overall relatedness was found among the three serotype 10 bovine strains (61A, A44 and KK3), and strains A5 and NCDV were also moderately related to the three serotype 10 viruses. All the bovine rotaviruses tested in this study, regardless of their serotype specificity, exhibited a moderate genetic-relatedness to strain 69M of serotype 8, and, to a lesser extent, to serotype 2 human rotavirus strains.

Introduction

Group A rotaviruses, which are the most common cause of diarrhoea in the young of a number of mammalian and avian species, include 12 serotypes defined by cross-neutralization tests (Estes & Cohen, 1989; Kapikian & Chanock, 1990). Serotypes 1, 2, 9 and 12 have been detected almost exclusively in humans so far (Hoshino et al., 1984; Clarke et al., 1987; Urasawa et al., 1990), except that porcine strains of serotype 1 or 2 specificity have been isolated recently (Bellinzoni et al., 1990). Serotype 3 strains have a broad host range and are found in humans, monkeys, horses, pigs, dogs, cats, rabbits and mice (Nishikawa et al., 1989). Serotype 4 is the cause of diarrhoea in humans and pigs, and serotype 5 strains have been isolated from horses and pigs (Hoshino et al., 1984). Serotypes 7 and 11 are restricted to avian and porcine species, respectively (Hoshino et al., 1984; Ruiz et al., 1988). Serotype 8 strains have been isolated from humans (Matsuno et al., 1985; Albert et al., 1987), and they have been detected recently in calves (Snodgrass et al., 1990). Most bovine rotaviruses (BRV) including reference strains NCDV and UK belong to serotype 6, whereas several BRV strains represented by strains B223, KK3 and V1005 were recently assigned to serotype 10 as the second serotype of BRV (Brüssow et al., 1990; Snodgrass et al., 1990).

Group A rotaviruses with genomes composed of 11 dsRNA segments, have two neutralization proteins, VP4 and VP7 (Hoshino et al., 1985; Offit & Blavat, 1986). The serotype specificity is defined largely by VP7, which is encoded by RNA segment 7, 8 or 9 depending on the strain (Estes & Cohen, 1989; Kapikian & Chanock, 1990). Precise analysis of the serotype-specific VP7 antigenic structure and worldwide epidemiological surveys on the serotype distribution of rotaviruses will provide information necessary for the development of effective vaccines.

In our previous study (Pongsuwanna et al., 1990), 23 Thai BRV strains, all which were of the subgroup I and long RNA migration pattern, did not react with a serotype 6-specific monoclonal antibody suggesting that they belong to other serotypes and that non-serotype 6 BRVs are frequent in Thailand. The nucleotide sequence of the VP7 gene of strain 61A was determined (Taniguchi et al., 1990a) and found to be similar to that...
of the B223 strain of serotype 10 (Xu et al., 1991). In the present study, by investigating the non-serotype 6 BRV strains 61A, A44, A5, and KK3, we determined antigenic and genomic relatedness among BRV and human rotavirus (HRV) strains by cross-neutralization, nucleotide sequencing of the VP7 gene and RNA-RNA hybridization.

**Methods**

**Virus strains.** Seventy faecal specimens were collected from young calves with diarrhoea in dairy herds in Nakon Ratchasima Province, Thailand. Twenty-three BRV strains from the faecal specimens were strains: (i) KU (serotype 1), (ii) 2, DS-1, HN-126 and 1076 (serotype 2), (iii) YO and SA11 (serotype 3), (iv) Hochi, Hosokawa, ST-3 and 57M (serotype 4), (v) OSU (serotype 5), (vi) NCDV and UK (serotype 6), (vii) Ty-1 (serotype 7), (viii) 69M and B37 (serotype 8), (ix) W161 (serotype 9); (x) KK3 (serotype 10) and (xi) L26 (serotype 12). The viruses were pretreated with 10 μg of acetylated trypsin (type V-S from bovine pancreas; Sigma) per ml, propagated in MA-104 cells in the presence of trypsin (1 μg/ml), and harvested 1 to 3 days after infection.

**Neutralization test.** This was performed by a fluorescent focus reduction method as described previously (Urasawa et al., 1984).

**RNA-RNA hybridization.** Virus in culture supernatant was pelleted by centrifugation at 100000 g for 3 h and resuspended in PBS. After treatment with fluorocarbon, the suspension was centrifuged at 100000 g for 3 h. The pellet was treated with 50 mM-EDTA at 37 °C for 30 min to convert double-shelled particles to single-shelled particles, and centrifuged on 47.5% (w/v) caesium chloride at 100000 g for 16 h. The visible virus band was pelleted by centrifugation at 100000 g for 3 h and suspended in 50 mM-Tris-HCl buffer pH 8.0.

Preparation of labelled probe and RNA-RNA hybridization were performed as described by Flores et al. (1982a, b). A 32P-labelled ssRNA probe was prepared from purified single-shelled particles by in vitro transcription in the presence of [³²P]GTP (400 Ci/mmol; Amersham) for 6 h at 42 °C followed by precipitation with 2 M-LiCl. Genomic dsRNA was prepared from the purified virus preparation by phenol–chloroform extraction and ethanol precipitation, and dissolved in 1 mM-EDTA. The 32P-labelled ssRNA probes were hybridized to the denatured genomic RNAs which had been prepared by boiling for 2 min followed by quenching on ice. Hybridization was allowed to occur at 65 °C for 16 h in a buffer containing 50 mM-Tris–HCl pH 8.0, 100 mM-NaCl and 0.1% SDS. After hybridization the RNAs were precipitated with ethanol. The hybrids were analysed on a 12.5% polyacrylamide gel with a 4% stacking gel. After observation of gels stained with ethidium bromide, they were dried and autoradiographed.

By this RNA–RNA hybridization, the origin of the RNA segments in the hybrids can not be identified exactly since the partially base-paired bands migrate aberrantly. Overall genetic relatedness between two strains was assessed by the number of the hybrids formed in the hybridization reaction.

**Nucleotide sequence analysis.** The nucleotide sequence of the VP7 gene of the BRV strains was determined by using dideoxynucleotide sequencing reactions with oligonucleotide primers, reverse transcriptase (Seikagaku Kogyo) and [³²P]dATP (3,000 Ci/mmol; Amersham) as described previously (Gorziglia et al., 1986b).

**Results**

**Antigenic characterization.**

Two Thai BRV strains, 61A and A44 had a high degree of antigenic relatedness to each other, and were not significantly neutralized by antisera against serotype 6 reference BRV strain NCDV and vice versa (Table 1). The strains 61A and A44 were closely related antigenically to KK3 of serotype 10 isolated in Japan. In contrast, another Thai BRV strain, A5, was antigenically distinct from the strains 61A, A44 and KK3. The strain A5 exhibited a high degree of antigenic relatedness to serotype 8 HRV strain 69M with a super-short RNA profile (Matsuno et al., 1985). However, a one-way antigenic relation was found between strain A5 and serotype 6 BRV strain NCDV: antisem to strain A5 neutralized strain NCDV moderately (Table 1).

**Nucleotide sequence analysis of VP7 genes**

The nucleotide sequence of the VP7 gene and the deduced VP7 amino acid sequence of strains 61A, A44, KK3 and B223 showed a high degree of identity (92.5 to 98.2% and 96.3 to 97.9%, respectively) (Fig. 1 and Table 2). In contrast, strain A5 had a VP7 sequence distinct from those of four strains. As expected from the results of the cross-neutralization tests, the VP7 sequence of strain A5 was similar to those of serotype 8 strains 69M and B37 (94.8 and 91.7% identical at the amino acid level; Fig. 1 and Table 2). Amino acid sequences in three variable regions B, D and E (amino acid residues 87 to 101, 143 to 152 and 208 to 221, respectively) of VP7, which are considered to be the major antigenic sites (Dyall-Smith et al., 1986; Mackow et al., 1988; Taniguchi et al., 1988), were compared among BRVs and representative HRVs with different serotype specificity (Fig. 2). Among the four BRV strains (61A, A44, KK3 and B223) assigned to serotype 10, a close resemblance (94.9 to 97.4%) of the VP7 amino acid sequence was found in the three regions, but little identity (51.3 to 69.2%) with those of serotypes 1 to 12 except for serotype 7, whose sequence is not available. Similarly, a high level of identity (94.9-9%) was detected between the amino acid sequences of the three variable regions of HRV and BRV serotype 8 strains (69M, B37 and A5). Thus, the presence of serotypes 6, 8 and 10 in BRVs was confirmed by serological and genomic analyses.

**RNA-RNA hybridization**

The overall genomic relatedness among six BRV strains (61A, A44, KK3, A5, NCDV and UK) was studied by
Table 1. Antigenic characterization of BRV strains by fluorescent focus reduction neutralization assay

<table>
<thead>
<tr>
<th>Strain (Serotype)</th>
<th>KU</th>
<th>S2</th>
<th>YO</th>
<th>Hochi</th>
<th>OSU</th>
<th>NCDV</th>
<th>Ty-1</th>
<th>69M</th>
<th>W161</th>
<th>KK3</th>
<th>L26</th>
<th>61A</th>
<th>A5</th>
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<td></td>
<td>256</td>
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</table>

*Neutralizing titres are expressed as the reciprocal of the highest dilution of rabbit antiserum that reduced the fluorescent focus counts by more than 60%.
†Neutralizing titres were shown previously (Urasawa et al., 1990) and were less than 1/16 of the titre against the homologous strain.

Table 2. VP7 nucleotide and amino acid sequence identities* of seven BRV strains of serotypes 6, 8 and 10 and two HRV strains of serotype 8

<table>
<thead>
<tr>
<th>Strain (Serotype)</th>
<th>61A</th>
<th>A44</th>
<th>KK3</th>
<th>B223</th>
<th>NCDV</th>
<th>UK</th>
<th>A5</th>
<th>B37</th>
<th>69M</th>
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<td>96-6</td>
<td>96-6</td>
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<td>81-9</td>
<td>82-5</td>
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<td>96-3</td>
<td>96-3</td>
<td>82-0</td>
<td>81-9</td>
<td>82-2</td>
<td>78-2</td>
<td>80-4</td>
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<td>93-6</td>
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<td>93-6</td>
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<td>82-8</td>
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<td>79-1</td>
<td>81-3</td>
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<td>94-6</td>
<td>94-6</td>
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<td>79-4</td>
<td>81-6</td>
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<td>75-8</td>
<td>75-8</td>
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<td>76-1</td>
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<td>76-1</td>
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<td>76-0</td>
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<td>76-3</td>
<td>76-3</td>
<td>97-9</td>
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</tbody>
</table>

* Amino acid sequence identities are presented in the top right triangle, and nucleotide sequence identities are presented in the bottom left triangle.

RNA–RNA hybridization experiments. The 61A probe hybridized well to the genomic RNA from the other serotype 10 strains A44 or KK3; 10 or 11 hybrid bands were observed (Fig. 3). Similar hybridization patterns were observed among strains 61A, A44 and KK3 when the labelled probe of A44 or KK3 was employed (data not shown). In contrast, a weak level of hybridization as shown by the formation of five or six hybrid bands was observed between the 61A probe and genomic RNA from the serotype 8 strain A5, or the serotype 6 strains NCDV and UK. In tests of hybridization between the A5 probe and genomic RNAs of the five BRV strains (61A, A44, KK3, NCDV and UK), only three to six hybrid bands were formed (Fig. 4). Thus, strains 61A, A44 and KK3 were found to have a high level of genomic relatedness to one another whereas they are related by a lesser extent to strain A5, NCDV or UK.

The overall genomic relatedness between the six BRV strains and representative HRV strains of serotypes 1, 2, 3, 4, 8 and 9 was also examined by cross-hybridization. Hybridizations using NCDV (serotype 6), A5 (serotype 8) or 61A (serotype 10) probes showed a moderate degree of genomic relatedness of these BRVs, regardless of their difference in serotype specificity, to serotype 8 HRV strains and, to a lesser extent, to serotype 2 HRV strains (Fig. 5 and 6). This finding was confirmed by the reciprocal hybridization using DS-1 (serotype 2) or 69M (serotype 8) probes (Fig. 7 and 8).
Serotype 8 and 10 bovine rotaviruses

Fig. 1. Alignment of nucleotide sequences of VP7 genes of serotype 6, 8 and 10 BRV strains and serotype 8 HRV strains. Bases identical to 61A in serotype 6 and 10 BRV strains and to A5 in serotype 8 HRV strains are indicated by dashes.

Fig. 2. Comparison of VP7 amino acid sequences in three antigenic regions of strain 61A with those of rotavirus strains of different serotype. The VP7 sequences of strains A5, KK3 and A44 were determined in this study, whereas others were obtained from the literature: 61A (Taniguchi et al., 1990a); KU (Taniguchi et al., 1988); D, S2, DS-1, ST-3, VA70 (Green et al., 1987); SA11 (Both et al., 1983); YO (Nishikawa et al., 1989); OSU (Gorziglia et al., 1986a); NCDV (Glass et al., 1985); UK (Ellemen et al., 1983); 69M and WI61 (Green et al., 1989); B37 (Hum et al., 1989); B223 (Xu et al., 1991); YM (Ruiz et al., 1988); L26 (Taniguchi et al., 1990b). Amino acids identical to those of 61A are indicated by dashes.

Discussion

Recent serological characterizations of BRV strains have indicated that serotype 10 strains as well as serotype 6 strains are distributed in cattle world-wide, as represented by strain B223 in the U.S.A., strains V1005 and E4046 in Germany, and strain KK3 in Japan (Murakami et al., 1983; Woode et al., 1983; Bellinzoni et al., 1989; Briüssow et al., 1990). The presence of serotype 8 BRV strains was also shown in Scotland (Bellinzoni et al., 1990). The present study identified the presence of serotype 8 and 10 BRVs in Thailand by cross-neutralization and sequencing of VP7 genes. Our cross-neutralization data did not necessarily match the serotyping criteria which define two viruses to be serotypically distinct when a greater than 20-fold
difference in titres between homologous and heterologous reciprocal neutralization reactions is found (Hoshino et al., 1984). A one-way cross-reaction was observed between strains A5 and NCDV: only an eightfold difference was found in neutralizing titres of anti-A5 serum to NCDV and A5, the reciprocal cross being 64-fold different. Such one-way cross-reactions have been reported in many pairs of rotavirus strains.
Serotype 8 and 10 bovine rotaviruses

Fig. 7. Hybridization of a labelled ssRNA probe of strain 69M to genomic RNAs of strains 69M (lane 1), 57M (lane 2), A5 (lane 3), NCDV (lane 4), 61A (lane 5), A44 (lane 6), KK3 (lane 7) and UK (lane 8).

Fig. 8. Hybridization of a labelled ssRNA probe of strain DS-1 to genomic RNAs of strains DS-1 (lane 1), KU (lane 2), YO (lane 3), 69M (lane 4), A5 (lane 5), 61A (lane 6), KK3 (lane 7) and NCDV (lane 8).

(Bridger & Brown, 1984; Hoshino et al., 1984; Nagesha & Holmes, 1988). The one-way crosses have been explained by antigenic drift in the neutralization antigenic epitopes on VP7 or by manifestation of VP4 antigenicity. Since our preliminary data on VP4 sequencing of strains 61A, KK3 and A5 imply that VP4 of strain 61A is UK-like, VP4 of A5 is NCDV-like, and VP4 of KK3 is neither UK- nor NCDV-like (K. Taniguchi et al., unpublished data), the one-way cross-reaction between strains A5 and NCDV may be due to the similarity between the VP4s of the two strains.

Comparative analysis of the VP7 amino acid sequences of various rotavirus strains with different serotype specificity has revealed that VP7 has six serotype-specific regions, designated A to F (amino acids 39 to 50, 87 to 101, 120 to 130, 143 to 152, 208 to 221, and 233 to 242; Glass et al., 1985; Green et al., 1987). Amino acid sequences in these regions are well conserved among strains of the same serotype but differ considerably among strains belonging to different serotypes. Each serotype has a characteristic nucleotide sequence in the six regions of VP7. The association of serotype specificity with the amino acid sequence in the VP7 variable regions was also confirmed for the BRV strains under investigation. On the basis of this association, we have developed a polymerase chain reaction method using oligonucleotides specific to each serotype for serotyping BRVs (K. Taniguchi et al., unpublished) and a large-scale epidemiological survey on the distribution of different BRV serotypes is being performed at present.

Although strain A5 was expected to be closely related to serotype 8 HRV strains, all the BRV strains, regardless of their difference in serotype specificity (serotype 6, 8 or 10), exhibited a similar moderate degree of overall genomic relatedness to serotype 8 HRV strains 69M and B37, as shown also by Oshima et al. (1990) with strain NCDV and two Japanese serotype 10 BRV strains. These results might suggest that BRV strains and serotype 8 HRVs could have originated from a common ancestor, or that serotype 8 HRVs were derived from bovine strains by genomic reassortment in nature. Genomic relatedness, although not high, was found also between HRV serotype 2 and BRV strains, in contrast to the report by Matsuda & Nakagomi (1989) who detected little genomic relatedness between bovine serotypes 6 and 10 and human serotypes 1 to 4. Furthermore, serotype 12 HRV strain L26 with subgroup I specificity appears to be, to some extent, genomically related to BRV strains (K. Taniguchi et al., unpublished results). These results suggest a consistent moderate overall genomic relatedness of BRV strains to subgroup I HRV strains with different serotype specificity.

A considerable difficulty in adapting serotype 10 strains to cell culture has been reported (Snodgrass et al., 1990). In our experience, however, the growth of serotype 10 BRV strains in cell culture was very efficient. Since VP4 has been shown to be a determinant of growth restriction and virulence of rotaviruses (Greenberg et al., 1983; Offit & Blavat, 1986), this discrepancy might be ascribed to the difference in VP4 of various serotype 10 strains as suggested by the different reactivity patterns of non-serotype 6 BRV strains with several anti-VP4
neutralizing monoclonal antibodies (Taniguchi et al., 1987; Pongswuanna et al., 1990). VP4 sequence comparisons will eventually provide an explanation.

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References


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