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Predominance of G3B and G14 equine group A rotaviruses of a single VP4 serotype in Japan

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Summary. A total of 65 equine group A rotaviruses (GAR) isolated from diarrheal foals at 48 farms in Hokkaido, Japan, between 1996 (29 isolates) and 1997 (36 isolates) were characterized for their VP7 and VP4 serotypes by PCR, nucleotide sequencing, and virus neutralization (VN) tests. By PCR VP7 typing, all isolates were classified as G3 or G14, and the predominant serotype in each year was G3 (86%) in 1996 and G14 (53%) in 1997. VN tests with these 20 isolates randomly selected confirmed the specificity of PCR on the bases of complete agreement of the results in these methods (9 G3 and 11 G14), and revealed that all 9 G3 isolates were subtype G3B. There were five differing amino acid residues in three VP7 antigenic regions between subtypes G3A and G3B. Antiserum to a baculovirus recombinant that expressed P[12] VP4 neutralized all isolates and P[12] reference strains. These results suggest that genotype P[12] GAR belong to a single VP4 serotype, and that one VP4 and two VP7 serotypes (G3B and G14) of GAR were predominant in the equine population in Japan.

Introduction

Equine group A rotaviruses (GAR) are a major cause of diarrhea in foals up to 3 months of age [5, 24]. The GAR are classified into serotypes as defined by the outer capsid proteins, VP4 and VP7, which elicit independently production of

neutralizing antibodies [20, 35]. At least 14 or 15 different VP7 serotypes of GAR have been identified [10, 37]. VP4 serotyping is difficult because virus neutralization (VN) tests for serotyping using hyperimmune antisera prepared with rotavirus virions reflect mainly antigenic properties of VP7 [10]. Recently, several techniques have been used to examine VP4 serotypes among some human and animal rotavirus strains, e.g. VN tests with hyperimmune antisera prepared with baculovirus-expressed VP4 or genetic reassortants, and enzyme-linked immunosorbent assays (ELISA) with neutralizing monoclonal antibodies [8, 14, 32, 33, 39]. However, antigenic diversity within the VP4 neutralization antigens has not been clearly defined as yet. Genetic analyses of the VP4 gene with nucleotide sequencing, hybridization assays and PCR techniques have been reported as reliable surrogates for VP4 serotyping [12, 18, 23, 28, 38, 45]. With these alternative approaches, at least 21 distinct VP4 genotypes have been identified [10, 37]. However, genotypes do not always agree with serotypes [29, 30].

Six VP7 serotypes [G3 (subtypes G3A or G3B), G5, G8, G10, G13, and G14] of equine GAR have been reported to date [2–4, 6, 21, 22, 27]. Serotype G3 equine GAR have been found to be predominant worldwide [1, 2, 19, 25, 27], with the predominant subtype being G3A [1, 2]. Serotype G14 equine GAR have recently been reported [6, 7, 40], and the prevalence of this serotype in the UK and Australia was lower than that of G3 [1, 27]. The other VP7 serotypes [G5, G8, G10, and G13] in equine GAR might be uncommon because at most very few strains of these serotypes have been detected [1, 25, 27].

Three VP4 serotypes among equine GAR have been characterized by VN tests using antisera to reassortant viruses, and all G3 and G14 reference strains tested were classified into the same VP4 serotype as strain H2 of which VP4 genotype is P[12] [26]. One VP4 genotype P[12] has been predominant in equine GAR in the UK and Australia [1, 27]. These results predict that a single VP4 serotype of equine GAR is predominant in the field, but there have been no reports on direct demonstration of this speculation.

For substituted VP7 serotyping of GAR, ELISA and genetic analyses of the VP7 gene, e.g. nucleotide sequencing, hybridization assays and PCR have been described [11, 15, 16, 41, 42]. For equine GAR, ELISA, hybridization assays and restriction fragment length polymorphism (RFLP) assays of PCR products have been reported as reliable surrogates for VP7 serotyping [1, 2, 23, 27]. However, no reports on PCR VP7 typing for equine GAR have existed.

The objectives of this study were to develop PCR for equine GAR VP7 serotyping, to characterize VP4 serotypes of equine GAR by VN tests using hyperimmune antisera raised to baculovirus-expressed VP4, and to investigate VP7 and VP4 serotypes of equine GAR field isolates in Hokkaido, Japan, in 1996 and 1997.

Materials and methods

Viruses and fecal samples

The following equine, bovine, porcine and human GAR strains were used as reference viruses: H2 (G3, P[12]), FI14 (G3, P[12]), HO5 (G3), SA11 (G3, P[2]), OSU (G5, P[7]), 69M (G8,

P[10]), KK3 (G10, P[11]), L338 (G13, P[18]) and FI23 (G14, P[12]). The HO5 strain was isolated in 1982 in the same district as that of the present study [25, 43]. These reference strains were propagated in MA104 cells. Sixty nine GAR-positive fecal specimens collected from Thoroughbred foals with diarrhea in 1996–1997 in the Hidaka district of Hokkaido, Japan, were used in this study. Over 80% of Thoroughbred foals in Japan have been produced in this district. The presence of GAR in feces was determined by a latex agglutination test (Rotascreen; Microgen Bioproducts LTD) or by electron microscopy. GAR isolation from these fecal samples was conducted with MA104 cells in the presence of trypsin essentially as described by Murakami et al. [31].

Hyperimmune antisera to equine GAR

Hyperimmune antisera were prepared in guinea pigs against equine GAR 3 reference strains (HO5, L338 and FI23) and 2 isolates (designated the JE29 and JE77) that had been propagated in MA104 cells and semipurified on sucrose gradients. Hyperimmune antisera to H2 and FI14 strains were prepared in rabbits as described previously [40]. These antisera were used for VN tests.

VN tests

VN tests were performed by the fluorescent focus neutralization test as described previously [36]. Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that resulted in an 80% or greater reduction of fluorescent foci.

VP7 typing by PCR

Viral RNA was extracted from 10% fecal suspensions or virus-infected cell culture fluids using an Isogen LS kit (Wako Pure Chemical Industries). The procedure for PCR VP7 typing was similar to the method developed by Gouvea et al. for human GAR [15], which comprised three steps (Fig. 1): (i) reverse-transcriptase (RT) reaction with genomic dsRNA; (ii) the first amplification of the full-length VP7 gene with the primers Beg9 and End9 [15]; and (iii) the second amplification with VP7 typing primers. For G3, G13 and G14 typing, the primers G3E, G13F and G14D listed in Table 1 were designed with the published sequence data. The RT reaction was conducted by the following procedure. In the tube, 5 μl of RNA samples was mixed with 2 μl of dimethyl sulfoxide, heated at 95 °C for 5 min, and then quenched on ice for 5 min. Subsequently, 10 μl of 5×RT buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 5 μl of 0.1 M DTT, 10 μl of 2 mM dNTPs, 1 μl of the primer End9 (50 μM), 1 μl of ribonuclease inhibitor (RNasin; Promega), 15 μl of water, and 1 μl (200 U) of M-MLV RT (Life Technologies) were added and incubated at 37 °C for 60 min. Next, 5 μl of the RT reaction samples was used for the first amplification in the presence of the PCR mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 0.5 μM each of the End9 and Beg9 primers and 1.25 U of Taq polymerase (AmpliTaq Gold; Perkin-Elmer Applied Biosystems). The mixture was subjected to 10 min of preheating at 94 °C, 30 cycles of 1 min at 94 °C, 2 min at 45 °C, 3 min at 72 °C, and a final 7 min incubation at 72 °C. The second amplification was conducted on 1 μl of the first PCR product diluted 1:10 in the presence of the same PCR mixture except for the different primer set (0.25 μM each of G3E, G13F, G14D and End9). The second amplification was consisted of preheating at 94 °C for 10 min, 25 cycles of 1 min at 94 °C, 2 min at 42 °C, 1 min at 72 °C, and a final 7 min incubation at 72 °C. PCR products were separated by electrophoresis on 1.2% agarose gels and visualized with UV light after staining with ethidium bromide.

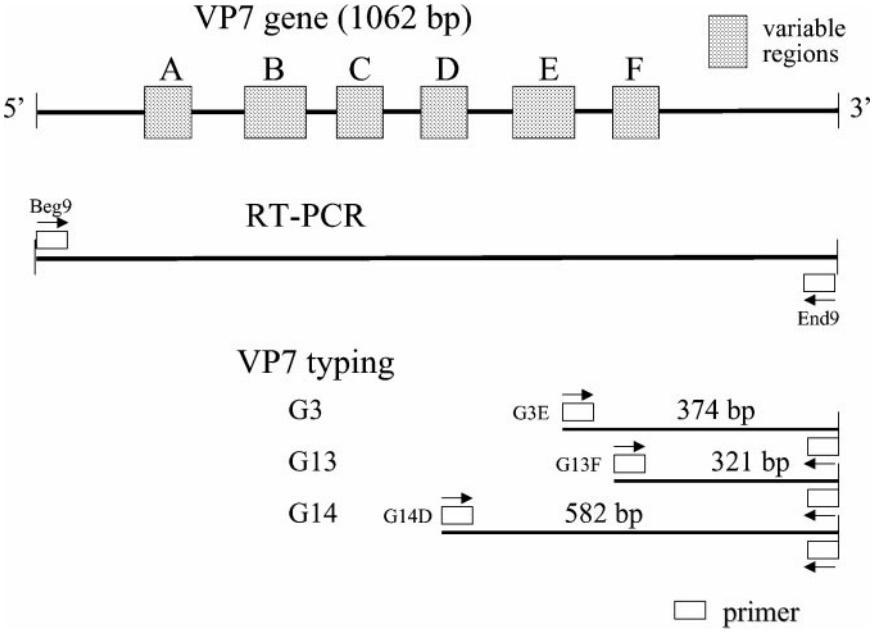

Fig. 1. Equine GAR VP7 gene. The schema shows the locations of 6 variable regions [13] and PCR typing primers, and the expected lengths of amplified products

Table 1. Oligonucleotide primers for RT-PCR VP7 typing

Name of primer	Sequence (5′-3′)	Position	Variable region[a]	Strain (VP7 serotype)	Accession no.[b]
Beg9[c]	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28		Wa (G1)	K020033
End9[c]	GGTCACATCATACAATTCTAATCTAAG	1062–1036		SA11 (G3)	K02028
G3E	CAATCGAAGAGATTGCGACAG	689–709	E	ERV316 (G3)	L49043
G13F	GGAGTAAATCACAAAATAAATCTC	742–765	F	L338 (G13)	D13549
G14D	GACGAAGCATTGCAATTA	481–498	D	FI23 (G14)	M61876

[a]Reported by Glass et al. [13]
[b]DDBJ/EMBL/GenBank accession number
[c]Reported by Gouvea et al. [15]

Nucleotide sequencing of VP7 and VP4 genes

The full length VP7 genes of the 4 isolates (JE29, JE75, JE77 and JE91) were produced by RT-PCR with the primers Beg9 and End9, and the PCR products were sequenced directly by cycle sequencing with an auto sequencer (ABI PRISM 377; Perkin-Elmer Applied Biosystems). The full length VP4 genes of the HO5 strain and 2 isolates (JE75 and JE77) were produced by RT-PCR with primers corresponding to the 5′ end sequence (VP4U; 5′-GGCTATAAAATGGCTTCTCT-3′) and complement of the 3′ end sequence (VP4L; 5′-GGTCACATCCTTCAGAAGCTAC-3′) of the H2 VP4 gene. These primer sequences were highly conserved among equine GAR. The PCR products were cloned into the pGEM-T Easy vector (Promega), and sequenced by cycle sequencing. The DDBJ/EMBL/GenBank accession numbers for the VP7 genes of the JE29, JE75, JE77 and JE91strains

and for the VP4 genes of HO5, JE75 and JE77 strains were are AB046465, AB046466, AB046467, AB046468, AB046471, AB046469 and AB046470, respectively.

Hyperimmune antisera to recombinant VP4

The full length HO5 (P[12]; determined by sequencing in this study) and L338 (P[18]) VP4 genes were produced by RT-PCR with primers VP4U and 5′-ATGGATCCGGTCACATC CTTCAGAAGCTAC (sequence representing a flanking *Bam*HI site and complement of the 3′ end of H2 VP4 gene). The PCR products were cloned into the pGEM-T Easy vector. VP4 genes were then excised with *Not*I and *Bam*HI, and subcloned into the *Not*I-*Bam*HI sites of the pVL1392 vector (PharMingen). Cotransfection of *Spodoptera frugiperda* (Sf9) cells with the recombinant pVL1392 and the Baculogold linearized baculovirus DNA (PharMingen) was performed as described previously [14, 33]. The expression of each HO5 and L338 VP4 in Sf9 cells infected with the baculovirus recombinant was identified by immunofluorescence [36] and Western blot analysis [44] with each hyperimmune antiserum to the homologous virus. Hyperimmune antisera were raised to lysates of Sf9 cells expressing each HO5 and L338 VP4 with Freund's adjuvant in guinea pigs.

Results

VP7 typing by PCR

The full length VP7 genes (1062 bp) were produced by the first PCR with the primers Beg9 and End9 on the reference equine GAR strains (Fig. 2). In the second PCR, the mixture of 3 sets of primers yielded the expected PCR products, 374, 321 and 582 bp, which reflected G3, G13 and G14, respectively (Fig. 2). No cross-reaction was observed for any serotype.

A total of 65 equine GAR strains were isolated from 69 rotavirus-positive feces in MA104 cells, which were designated as JE strains. Twenty-nine of these strains were derived from 27 farms in 1996, and the remaining 36 were from 24 farms in 1997. The VP7 serotype of these field strains was examined by PCR. As shown in Fig. 3, the serotype was readily detected by PCR. All field strains could be serotyped, which belonged to G3 or G14, and no cross-reaction was observed. In 1996, 25 of 29 strains (86%) from 23 of 27 farms (85%) were determined to be G3, and the remaining 4 strains (14%) from 4 farms (15%) were G14. In contrast, in 1997, 17 of 36 strains (47%) from 11 of 24 farms (46%) were G3,

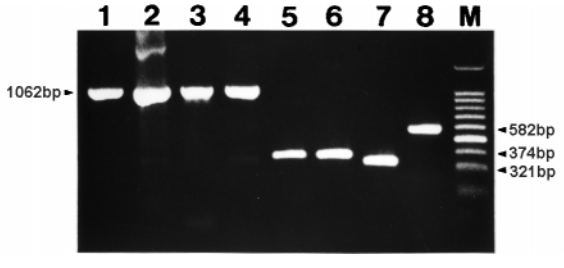

Fig. 2. PCR VP7 typing of equine GAR reference strains. *1–4* first amplification (RT-PCR); *5–8* second amplification (VP7 typing). *1*, *5* HO5 (G3); *2*, *6* H2 (G3); *3*, *7* L338 (G13); *4*, *8* FI23 (G14); *M* molecular weight marker (100 bp DNA Ladder; Takara)

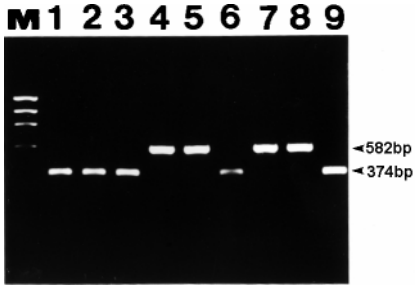

Fig. 3. PCR VP7 typing of equine GAR field strains. *1–9* were amplified products of field strains. *1–3*, *6* and *9* were determined as G3, and *4*, *5*, *7* and *8* were as G14. *M* molecular weight marker (ϕX174 *Hae* III digest; Takara)

and the remaining 19 strains (53%) from 13 farms (54%) were G14. In each year, the isolates derived from the same farm were the same VP7 serotype. The VP7 serotype was different between 1996 and 1997 in 2 of 3 farms where serotyping was conducted in both years.

Sixty GAR-positive feces from which GAR were isolated in MA104 cells were used for direct PCR typing. Fifty-eight (97%) of these feces could be typed by PCR. The results of typable feces as directly determined by PCR were in complete agreement with those of the isolates by PCR. Untypable feces showed no amplification products in the first amplification by PCR.

VP7 serotyping by VN tests

Cross VN tests were performed with 20 randomly selected field strains and the reference strains. Different serotypes were determined by a $>$20-fold difference in VN antibody titer. As shown in Table 2, strain JE29 showed two-way neutralization reactions with G3 reference strains. Antisera against strains HO5 (G3) and JE29 neutralized strains JE28, JE60, JE75, JE76, JE79, JE97, JE102 and JE105 at the similar titers to the homologous strains, and antisera against strains L338 (G13) and FI23 (G14) showed at least an 128-fold lower VN antibody titer to these strains than to the homologous strains. In contrast, strain JE77 showed a two-way neutralization reaction with the G14 FI23 strain. Antisera against strains FI23 and JE77 neutralized strains JE81, JE84, JE85, JE87, JE91, JE103, JE104, JE113, JE115 and JE116 at the similar titers to the homologous strains, and antisera against strains HO5 and L338 showed at least a 32-fold lower VN antibody titer to these strains than the homologous strains. These results indicated that strains JE28, JE29, JE60, JE75, JE76, JE79, JE97, JE102 and JE105 belonged to serotype G3, and strains JE77, JE81, JE84, JE85, JE87, JE91, JE103, JE104, JE113, JE115 and JE116 belonged to serotype G14. These serotyping results for the field strains showed complete agreement with those by PCR typing. Among G3 strains, the field strains and strain HO5 have a close relationship to strain FI14 (G3B) and a distant relation to strain H2 (G3A). Antisera to strains HO5, FI14 and JE29 showed lower VN antibody titers to strain H2 than the homologous and field strains. Antiserum against strain H2 showed 4 to 8-fold lower VN antibody titers to the HO5, FI14 and field strains than the homologous strain. These results indicated that these field strains and HO5 belonged to subtype G3B. One-way cross-neutralization was observed between strain H2 or FI14 and G14 strains [40].

Table 2. Antigenic comparison of 20 equine GAR field strains (JE28 to JE116) to reference equine and other species GAR strains by virus neutralization test

Strain (VP7 serotype)	Virus neutralization antibody titer[a] of antiserum to						
	HO5	H2	FI14	L338	FI23	JE29	JE77
HO5 (G3)	**12800**	12800	6400	<400	400	12800	<400
H2 (G3)	3200	51200	1600	400	800	1600	<400
FI14 (G3)	6400	6400	**6400**	400	400	6400	<400
L338 (G13)	<400	<400	<400	**51200**	<400	<400	<400
FI23 (G14)	<400	1600	1600	<400	**51200**	<400	6400
OSU (G5)	<400	<400	<400	<400	<400	<400	<400
69M (G8)	<400	<400	<400	400	<400	<400	<400
KK3 (G10)	<400	<400	<400	<400	<400	<400	<400
JE28	12800	6400	6400	<400	400	6400	<400
JE29	6400	6400	6400	<400	<400	**6400**	<400
JE60	6400	6400	3200	<400	<400	6400	<400
JE75	6400	6400	6400	<400	400	6400	<400
JE76	12800	6400	3200	<400	400	6400	<400
JE79	12800	6400	6400	<400	400	12800	<400
JE97	6400	6400	3200	<400	<400	12800	<400
JE102	12800	6400	3200	<400	<400	6400	<400
JE105	6400	6400	3200	<400	<400	6400	<400
JE77	<400	1600	800	<400	51200	400	**12800**
JE81	<400	800	400	<400	25600	<400	6400
JE84	<400	800	800	<400	25600	<400	12800
JE85	<400	800	400	<400	25600	<400	12800
JE87	<400	800	400	<400	51200	<400	12800
JE91	<400	–[b]	–	<400	51200	<400	12800
JE103	<400	–	–	<400	25600	<400	6400
JE104	<400	–	–	<400	25600	<400	6400
JE113	<400	–	–	<400	51200	<400	12800
JE115	<400	–	–	<400	25600	<400	6400
JE116	<400	–	–	<400	51200	<400	12800

[a]Determined as the reciprocal of the highest serum dilution which caused an 80% or greater reduction in fluorescent foci. Homologous titers are given in boldface type

[b]Not tested

Nucleotide sequences of VP7 and VP4 genes

The VP7 gene of the field strains JE29 (G3), JE75 (G3), JE77 (G14) and JE91 (G14) contained one open reading frame encoding a polypeptide of 326 amino acids (aa). The deduced aa sequences of the VP7 genes from these isolates were compared with those of the published corresponding genes from 10 equine GAR strains HO5, H2, ERV316, FI14, FI23, CH3, ERV47, FR4, FR8, and L338 [1, 6, 7, 34, 40, 43]. The aa identities between the field strains in the same VP7 serotype were very high (99.7% in G3 and 99.4% in G14). Among the same VP7 serotype, the aa identities were 92.0 to 99.7%. In contrast, the aa identities

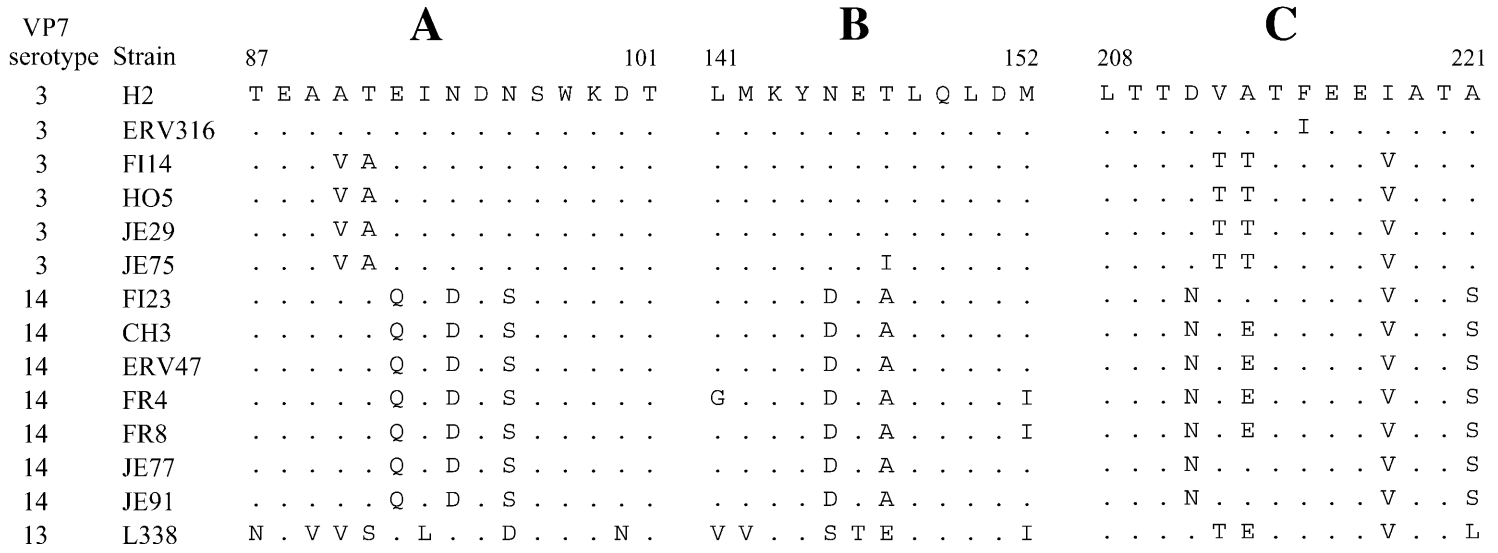

Fig. 4. Comparison of the deduced amino acid (aa) sequences in three antigenic regions (**A**, aa 87 to 101; **B**, aa 141 to 152; **C**, aa 208 to 221) [9] of VP7 among equine GAR G3 and G13 and G14 strains. The VP7 nucleotide sequences of H2, ERV316, FI14, HO5, FI23, CH3, ERV47, FR4, FR8, and L338 were previously published [1, 3, 6, 7, 34, 39, 42], and those of JE29, JE75, JE77, and JE91 were reported in the present study

were 77.0 to 88.7% among the different VP7 serotypes. When compared between subtypes G3A (H2 and ERV316) and G3B (JE29, JE75, HO5 and FI14), the VP7 aa identities were 94.5 to 96.0%. In contrast, the aa identities were 98.2 to 99.7% in the same G3 subtype strains.

Comparison of the aa sequences of three antigenic regions (A, aa 87 to 101; B, aa 141 to 152; C, aa 208 to 221) of VP7 is shown in Fig. 4. Seven residues of the G3 strains differed from those of the G14 strains at positions 92 (Q → E), 94 (D → N), 96 (S → N), 144 (D → N), 146 (A → T or I), 211 (N → D), and 221 (S → A). Among the G3 strains, five residues of G3B strains JE29, JE75, HO5 and FI14 differed from those of the G3A strains H2 and ERV316 at positions 90 (A → V), 91 (A → T), 212 (T → V), 213 (T → A), and 218 (V → I).

The VP4 genes of strain HO5 and the field strains JE75 and JE77 contained a long open reading frame encoding a polypeptide of 776 aa. The deduced aa sequences of these VP4 genes were compared with those of the published corresponding genes from GAR strains. The VP4 aa identities between strain HO5, JE75 or JE77, and the published P[12] strain H2, FI14, FI23 or CH3 (these DDBJ/EMBL/GenBank accession numbers are D13397, D13398, D16342 and D25228, respectively) were very high (≥95.7%), In contrast, the aa identities of these 3 strains were 58.0–86.6% compared to those of the published other VP4 genotype strains NCDV, SA11, RRV, L26, UK, Gottfried, OSU, HI, KU, K8, 69M, KK3, MDR13, PA169, Lp14, Eb, 998/83, L338, 4F and EHP (these DDBJ/EMBL/GenBank accession numbers are M63267, X14204, M18736,

Table 3. Antigenic relationship among equine and other species GAR VP4

Strain	VP4 genotype	Amino acid sequence identity (%) with VP4 of		Virus neutralization antibody titer of hyperimmune antisera against expressed VP4 of	
		HO5	L338	HO5	L338
HO5[a]	12		77.9	1280	<20
H2	12	95.8	77.1	640	<20
FI23	12	98.5	77.9	640	<20
FI14	12	97.0	77.2	640	<20
JE75[a]	12	99.1	78.1	1280	<20
JE77[a]	12	98.7	78.0	1280	<20
L338	18	77.9		<20	1280
69M	10	86.3	78.6	<20	<20
OSU	7	78.1	78.9	<20	<20
KK3	11	58.1	56.1	<20	<20
SA11	2	82.6	80.3	<20	<20

The VP4 nucleotide sequences used were from the following accession numbers; H2 [D13397], FI23[D16342], FI14[D13398], L338[D13399], 69 M[D14367], OSU[X14190], KK3[L07887], SA11[X14204]

[a]The VP4 nucleotide sequences and genotypes were determined in the present study

M58292, M22306, M33516, X14190, M21014, D90260, M60600, D14367, L07887, L20874, L11599, U08419, D16352, D13399, X57319 and U08424, respectively). These results indicated that strains HO5, JE75 and JE77 could be classified into VP4 genotype P[12].

VP4 serotyping by VN tests using antisera to recombinant VP4

Antiserum to the recombinant HO5 VP4 neutralized the homologous virus and all of the P[12] reference strains at titers of 640–1280 (Table 3). It also neutralized all 65 field strains at titers of 640–1280. However, it failed to neutralize GAR strains representing other VP4 genotypes (P[18], P[10], P[7], P[11] and P[2]) (Table 3). Antiserum to the recombinant L338 VP4 neutralized only the homologous virus (Table 3).

Discussion

The prevalence of equine GAR VP7 serotypes has been investigated by VN test, ELISA, hybridization assays, and RFLP assays of PCR products [1, 2, 19, 25, 27]. However, VN tests are time consuming and not suitable for handling a large number of samples. In addition, 16–33% GAR-positive feces were untypable in the latter 3 methods [1, 2, 27]. PCR typing assays, which are based on examination of the serotype-specific nucleotide sequences of VP7 gene, have been reported for human, bovine and porcine GAR VP7 serotyping as powerful and quick methods [15, 16, 42]. The PCR typing developed in the present study is very specific and sensitive because the results of this method and VN tests agreed completely, and 97% of GAR-positive feces could be typed directly by the PCR. Semi-nested PCR for VP7 typing in the present method may have higher sensitivity than that of the previous RFLP assays of PCR products [42]. To our knowledge, this is the first report on the development of PCR VP7 typing against equine GAR. The PCR VP7 typing assay is useful for the investigation of equine GAR VP7 serotypes, allowing the screening of large numbers of samples.

Several reports have indicated that VP7 serotype G3 was predominant in equine GAR throughout the world [1, 2, 19, 25, 27]. Recently, serotype G14 strains have been isolated in the UK, US, Japan, Venezuela and Australia [1, 6, 7, 27, 40]. The prevalence of this serotype has been found to be about 10% in the UK and Australia [1, 27], but that in other countries has remained unknown. In Japan, Imagawa et al. [25] have revealed that 134 of 136 equine GAR strains isolated from 1981 to 1991 in the same area as the present study belonged to G3, with no G14 strain being isolated. Therefore, G14 GAR might have emerged and recently have spread in the Hidaka region of Hokkaido, Japan, because G14 isolates were the most prevalent in 1997 in the present study. This information is very important because the possibility exists that G14 equine GAR also become the most prevalent in equine populations of other countries. Our study presented that the isolates showed the same VP7 serotype in each farm in each year. This result suggests that equine GAR might mainly be spread to foals by horizontal transmission from one strain in each farm.

Subtype G3A of GAR has been the most prevalent in the UK and Australia [1, 2]. However, in the present study, the nine G3 isolates selected randomly were classified as subtype G3B, suggesting that subtype G3B strains were predominant among G3 equine GAR in the Hidaka district of Japan. The reason for this difference is unknown, but it may be due to recent imports of Thoroughbred horses to Japan being primarily from the US, not the UK or Australia. It would thus be interesting to determine the current predominant G3 subtype of equine GAR in the US. A high degree of VP7 aa sequence divergence located in 6 to 9 discrete regions has been observed among different VP7 serotypes [13, 17, 34]. Of these regions, at least three (A to C) have been shown to be involved in serotype-specific neutralization epitopes [9]. In the present study, the comparison of aa sequences of these regions revealed that 5 positions differed between G3A and G3B strains. One or several of these aa differences may reflect the antigenic diversity constituting subtypes among equine G3 strains. In addition, the VP7 aa identities in the same G3 subtype strains were higher than those between different G3 subtype strains. These results suggest that sequence analysis could predict the equine G3 subtype without antigenic analysis.

Isa and Snodgrass [26] have reported that three VP4 serotypes were present among equine GAR by using antisera to reassortant viruses, and all G3 and G14 reference strains tested belonged to the same VP4 serotype as strain H2 (P[12] of VP4 genotype). Browning and Begg [1] and Isa et al. [27] have reported that the VP4 genotype of all G3 and G14 equine GAR field strains was P[12]. In the present study, we demonstrated that all field strains belonged to a single VP4 serotype, which is the same VP4 serotype as that of P[12] GAR. These results confirmed that P[12] equine GAR belong to a single VP4 serotype that is predominant in the equine population. Overall, the results of the present study and those of previous reports [1, 23, 27, 45] can conclude that equine GAR consist primarily of two VP7 serotypes (G3 and G14) and one VP4 serotype, and that equine isolates possessing other VP7 and VP4 serotypes represent rare cross-species infections.

Li et al. [30] have reported that the human strain 69 M (P[10]) and equine strain H2 (P[12]) share the same VP4 serotype based on the data showing that hyperimmune antiserum against recombinant baculovirus-expressed 69 M VP4 neutralized strain H2 at a high titer. Isa and Snodgrass [26] have reported that hyperimmune antiserum against a reassortant virus possessing H2 VP4 neutralized strain 69 M at a high titer. By contrast, in the present study, hyperimmune antiserum against recombinant baculovirus-expressed HO5 VP4, which is the same VP4 genotype and serotype as strain H2, did not neutralize strain 69 M. These results suggest that minor variation of neutralizing epitopes in VP4 may exist between strains H2 and HO5, which reflected the difference of VP4 antigenic relationships against strain 69 M.

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