Occurrence of a single type strain (G3, P[12]) of equine rotavirus in foals on a racehorse farm from 1986 to 1993

Hiromi SAKAWAKI[1], Hiroshi ISHIZAKI[1], Michihiro TAKAGI[1], Gen MASUDA[1], Koki TANIGUCHI[2], Tomoko URASAWA[2], Shozou URASAWA[2], Hitoshi GOTO[1] and Toshikazu SHIRAHATA[1]

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Abstract

Epizootiological and virological studies on RV infection of foals were done on a racehorse farm in Hokkaido, Japan from 1986 to 1993. About 200 foals were born between February and June of each year, and 23.5 to 52.9% of them developed severe diarrhea from March to September. RV strains were isolated from more than 50% of fecal specimens in each year except for 1993, and showed one predominant RNA electropherotype of the virus in all years. The G serotype and P type of all the representative strains from each year were identified as G3 and P[12] by neutralization tests and/or polymerase chain reaction assays. The strains hybridized with a probe prepared from one strain of them. The prevalent RV strains on this farm were almost identical serologically and genetically throughout the observation period. The result suggests that the occurrence of the equine RV strain with the same serotype and genotype was recognized on the farm for long time.

Key words : foals, persistence, rotavirus.

Rotavirus (RV) has been recognized as a major causal agent of acute diarrhea in the young of humans and a wide variety of animals, including horses [6,13]. It is well known that the occurrence of RV infection in young animals is affected by age, colostrum intake, antibody levels in the colostrum, stress due to animal transfer, introduction of animals to the farm, hygienic conditions on the farm, cold temperature, relative humidity and so on [13,18]. Two outer capsid proteins of group A RVs, VP4 and VP7, are involved in the production of immune responses in animals. Both proteins are independently associated with serotype specificities ; the G serotype (for glycoprotein) and P type (for protease-sensitive protein)[6,8]. At least 14 G serotypes and 20 P types have been identified in human and animal RVs.[2,4,6,21]. G3A, G3B, G5, G10, G13 and G14 for the G sero-

[1]Department of Veterinary Microbiology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan.

[2]Department of Hygiene, Sapporo Medical University School of Medicine, Sapporo 060, Japan.

Table 1. Oligonucleotide primers for G and P typing of equine rotaviruses by PCR

Primer (sence)		Sequence (5'-3')[a]	Position	Strain (Type)	PCR product (base pairs)
G typing					
C1[b]	(−)	GGTCACATCATACTTTTCTAATCTAAG	1036-1062	SA11 (G3)	
C2	(+)	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	Wa (G1)	1062
S3Eq	(+)	CTGCATGTTACCACATC	757-773	FI14 (G3)	306
S13Eq	(+)	ATATAGTACGGAACTAC	477-493	L338 (G13)	586
S14Eq	(+)	TGACTACTAATGTCGCA	671-687	FI23 (G14)	392
P typing					
PC1Eq	(−)	AAAGCTTGTGAATCATCCCA	1075-1094	L338 (P[18])	
PC2Eq	(+)	ATGGCTTCTCTTATTTACAGACAG	10-33	H2 (P[12])	1085
PS7Eq	(+)	CTTGCGCCAACTGTAGA	259-275	H1 (P[7])	836
PS12Eq	(+)	GGAACTCAAACGCTACTAC	547-565	H2 (P[12])	548
PS17Eq	(+)	AGCAACATTAGATGGTGCA	443-462	L338 (P[18])	652

[a]Nucleotide sequences were used from the following Genbank accession numbers: VP7 gene of strains FI14,[22] L338 (D00843) and FI23 (M61876), and VP4 gene of strains H1 (D16341), H2 (L04638) and L338 (D13399).
[b]The primers used were the same as previously described.[20]

type and P[7], P[12], P[18] for the P type have been found in RV isolates from foals [1-3,5,9-12,20], although G3 viruses are most prevalent in severe outbreaks of foal diarrhea world-wide [1]. However, little is known about the ecological nature of RV in foals or other young animals in a herd. This paper is concerned with the frequent occurrence of RV strains of the same G serotype and P type specifities and the same RNA electropherotype in foals raised on a racehorse farm.

MATERIALS AND METHODS

Animals and feces -- The racehorse farm investigated in this study consisted of 300-450 horses throughout the observation period from 1986 to 1993. On this farm, 10-35 pregnant mares were imported from the U. S. A., England and France from January to February each year. The horses were raised in 3 foal-raising locations, nearly 130 horses in each location. The first location is situated 20 km from the second location and 55 km from the third location, and the third location is situated at the seaside 40 km from the second location. Some mares moved from one location to other ones every year, but this is not the case in foals. There was crossover of employees and veterinarians between the locations each year, but was no contact after the occurrence of foal diarrhea. Fecal swabs were collected from diarrheal foals during the outbreaks of the disease each year and stored at −30 °C until use. Each specimen was used for detection and/or isolation of RV.

Viruses -- In 1986-93, 176 RV strains of RV were isolated from diarrheal feces (Table 2). Of these, 8 strains, one from each year [designated SH31 ('86), SH52 ('87), SH86 ('88), SH98 ('89), SH101 ('90), SH127 ('91), SH102 ('92) and HH90 ('93)] were selected at random from the 3 locations for well-growing in cell cultures. The 8 strains were purified through five consecutive plaque clonings or limiting dilutions and used for antigenic analysis. Strains KU (G1), S2 (G2), SA11 (G3), H2 (G3A) and FI14 (G3B)[1,3], Hochi (G4), OSU (G5), H1 (G5), NCDV (G6), UK (G6), PO13 (G7), 69M (G8), WI61 (G9), KK3 (G10), YM (G11), L26 (G12), L338 (G13), FI23 (G14) and CH3 (G14)[19,20], were employed as reference strains. The viruses were

Table 2. Annual occurrence of foal diarrhea in a race horse farm from 1986 to 1993

Years	No. diarrheal cases/No. foals born(%)	No. positive for RV detection[a] /No. tested(%)	No. positive for RV isolation[b] /No. tested(%)
1986	71/179 (39.7)	NT[c]	6/7 (85.7)
1987	89/168 (52.9)	NT	48/60 (80.0)
1988	≥32/202[d]	24/32 (75.0)	5/5 (100.0)
1989	67/201 (33.3)	26/67 (38.8)	4/4 (100.0)
1990	63/223 (28.3)	48/63 (76.2)	20/20 (100.0)
1991	60/235 (23.5)	44/60 (73.3)	32/45 (71.0)
1992	95/229 (41.5)	57/95 (60.0)	43/80 (53.8)
1993	71/213 (33.3)	45/71 (63.4)	18/52 (34.6)

[a] Rotavirus in feces was detected by latex agglutination test.
[b] Rotavirus in feces was isolated by MA-104 cell culture method.
[c] NT : not tested.
[d] Detailed data was not available.

pretreated with 10 μg/ml of acetylated bovine trypsin type III[a] for 20 minutes at 37°C and propagated in MA-104 cells in the presence of 1 μg/ml of trypsin [17].

Detection and isolation of RV -- RV detection in feces was carried out by use of the latex agglutination test[b] according to manufacturer's instructions. RV isolation was performed using MA-104 cell cultures through the 3 to 5 blind passages as described previously [17].

Neutralization test -- Serum neutralization tests were performed by the fluorescentfocus 60 %-reduction method (FFN)[22] using the reference RV strains and their rabbit hyperimmune antisera [17,21].

Polyacrylamide gel electrophoresis (PAGE) -- Electrophoresis of viral RNA was done on 10% polyacrylamide slab gels by the method of Laemmli [14] as described previously [17,21].

Polymerase chain reaction (PCR) -- By using RV dsRNA extracted with the method of Ohta and colleagues [17], PCR for G and P typing of equine RVs was carried out as described previously [23,25]. The common and type-specific primers employed for PCR are listed in Table 1.

In the G typing assay, the conditions for the first amplification using common primers C1 and C2 (20 pmol each) were as follows: reverse transcription at 42°C for 30 minutes, one cycle of 5 minutes at 94°C, 3 minutes at 43°C, and 3 minutes at 72°C, 30 cycles of 1 minute at 94°C, 1.5 minutes at 43°C, and 2 minutes at 72°C, a final cycle of 1 minute at 94°C, 1.5 minutes at 43°C, and 10 minutes at 72°C. In the second amplification, a mixture of primers (40 pmol each of primers S3Eq, S13Eq and S14Eq), which are specific to FI14, L338, and FI23 VP7 gene alleles, respectively, a common primer, C1, and a 1 : 100 dilution of the first amplification product were subjected to 30 cycles of 94°C for 1 minute, 52°C for 1.5 minutes, and 72°C for 2 minutes, and a final incubation at 72°C for 7 minutes.

In the P typing assay, PCR conditions were essentially the same as in the G typing assay except for the annealing temperature (50°C). The primers used were a pair of commom primers, PC1Eq and PC2Eq (20 pmol each) for the first amplification, and a mixture of primers (25 pmol each) PS7Eq, PS12Eq, andPS17Eq, specific to H1, H2, and L338 VP4 gene alleles, respectively.

PCR products (5 μl) were analyzed by electrophoresis on 1% agarose in Tris-acetate-EDTA buffer containing ethidium bromide (1 μg/ml).

RNA-RNA hybridization -- Liquid RNA-RNA hybridization assay using ^{32}P-labeled single-stranded RNA (ssRNA) from strain SH101 virus was performed by the method as reported previously [15,24].

RESULTS

Clinical and Epidemiological findings -- On the farm studied, many foals were born from Febru-

[a] Sigma Chemical Co, St Luis, Mont. U. S. A.
[b] Orion Diagnostica, Espoo, Finland.

ary to June each year, and they were exposed to outbreaks of endemic diarrhea throughout the observation years. The foals suffered from diarrhea at various rates year by year (Table 2). Diarrheal feces were in general stinking, buff-colored and watery. The duration of diarrhea was 2 to 5 days in many cases. Affected foals received orally Ringer's solution supplemented with 5 % glucose or solution of bovine colostral immunoglobulin (manufacture for experiment)[c] during the diarrhea. All the affected foals developed pyrexia with a temperature of 38.5 to 40.8°C. RV was detected and isolated from more than half of the fecal specimens tested in all the years except a few.

The occurrence of foal diarrhea was observed for a long period from March to September. However, the majority of the RV positive foals were recorded from May to August. More than half of the cases (46.7-100%) were observed in June and July, with the exception of low value (19.3%) in 1992. In particular, the earliest occurrence of foal diarrhea appeared to correspond closely with cloudy weather and thick fog at the seaside (the third foal-raising location) in June and July for most years. As shown in Table 3, a few foals developed diarrhea as early as one to 30 days after birth in 1987 and 1989, while many of the diarrheal foals with RV were observed on days 31-167 in the other years regardless of the location. All the RV-negative diarrheal feces which were collected from March to April in 1987 and 1989 were inoculated on Bacto-MacConkey agar for detection of *Salmonella* spp, after enriching the cultures in Bacto-Selenite Broth. The results obtained were negative in all cases.

Characterization of representative isolates — The cross-FFN tests were carried out between the 8 representative isolates and the 19 reference strains of serotypes G1 to G14. All the isolates showed antigenic relatedness to G3 strains SA11 (G3), H2 (G3A) and FI14 (G3B) (Table 4). However, the H2 (G3A) strain showed only one-way cross reactivity with antisera to the isolated strains SH31 and SH52. Conversely, antiserum to SA11 (G3) showed lower reactivity to the isolates. No cross (two-way) reactivities between the isolates and other reference strains were observed. Thus, the highest level of cross-reactivity was found between strain FI14 (G3B) and the 8 isolated strains.

In PAGE of viral RNA from the 173 isolates, almost all the strains showed the same RNA electropherotype in the migration of the 11 RNA segments (data not shown), although RNA migrations of some strains (one isolate from 1992 and 2 isolates from 1993) revealed a few differences in segments 3 and 7. One isolate (strain CH3) from 1987 showed considerable differences in many segments, and this strain has been reported previously as a minor prevalent strain on this farm [19]. The strain CH3 belonged to G14 and shared VP4 genotype with equine strain H2 in the futher

Table 3. Relationship between the onset of diarrhea and the age after birth of foals

Years[a]	No. of diarrheal foals on days after birth						Average age (days)
	1-10	11-30	31-60	61-80	81-120	121-167	
1987	9 (3)[b]	15 (1)	33 (21)	15 (10)	17 (13)	0	47.9±30.7
1989	15 (0)	16 (2)	11 (2)	9 (7)	14 (13)	2 (2)	26.0±23.8
1990	0	0	6 (5)	14 (10)	31 (22)	12 (11)	92.7±24.5
1991	0	7 (2)	12 (9)	14 (13)	24 (19)	3 (1)	66.7±41.0
1992	0	3 (1)	22 (6)	13 (8)	35 (22)	22 (20)	69.8±29.6
1993	0	7 (2)	10 (2)	25 (17)	24 (21)	5 (3)	64.3±39.7

[a] No data in 1986 and 1988.
[b] Number in parenthesis shows the cases positive for RV in MA-104 cell culture method in 1987 and in latex agglutination test in other years.

[c] Shimizu Chemical Co, Hiroshima, Japan.

Table 4. Results of FFN tests with the isolates and reference RV strains against antisera of the known G serotypes

Rotavirus (G serotype)	FFN antibody titer[a] of antiserum to indicated RVs					
	SA11(3)	H2(3A)	FI14(3B)	SH31	SH52	SH102
SA11(3)	131072	4096	4096	4096	4096	8192
H2(3A)	4096	32768	1024	512	512	2048
FI14(3B)	16384	1024	16384	8192	4096	8192
SH31 [1986][b]	8192	2048	8192	32768	16384	8192
SH52 [1987]	8192	2048	8192	16384	16384	8192
SH86 [1988]	4096	2048	4096	32763	16384	8192
SH98 [1989]	8192	2048	4096	16384	8192	16384
SH101[1990]	8192	2048	4096	32768	16384	16384
SH127[1991]	8192	1024	4096	16384	16384	8192
SH102[1992]	8192	4096	4096	16384	8192	16384
HH90 [1993]	16384	4096	4096	16384	8192	8192

[a] Neutralizing titers represent reciprocals of highest dilution reducing fluorescent focus counts by more than 60%.
[b] Isolation year.

studies on the antigenic and genomic properties of the strain [20].

RNA-RNA hybridization was carried out in order to examine the overall genomic relatedness among the 8 strains isolated in 1986-93 (Fig 1). When dsRNA of the 8 isolates were subjected to hybridization with a radiolabeled total ssRNA from strain SH101 virus isolated in 1990, most hybrid bands were recognized on all the strains tested. However, there were no hybrid bands between SH101 and the genomic RNA from porcine strain OSU and avian strain PO13 which served as a negative control though the data not shown in Fig. 1.

G and P typing by PCR -- PCR assay was applied for the assignment of G and P types of the 8 representative and of all the 113 strains isolated from 1990-93 in the present study. The 8 representative strains produced the DNA fragments (306 bp) with the same size as the reference strains H2 (G3A, P[12]) and FI14 (G3B, P[12]), differing from the size of DNA fragments of strains H1 (G5, P[7]), L338 (G13, P[18]), FI23 (G14, P[12]), CH3 (G14, P[12]) and UK (G6, P[5]) (Fig 2). For the P type, DNA fragments of 548 bp (specific for

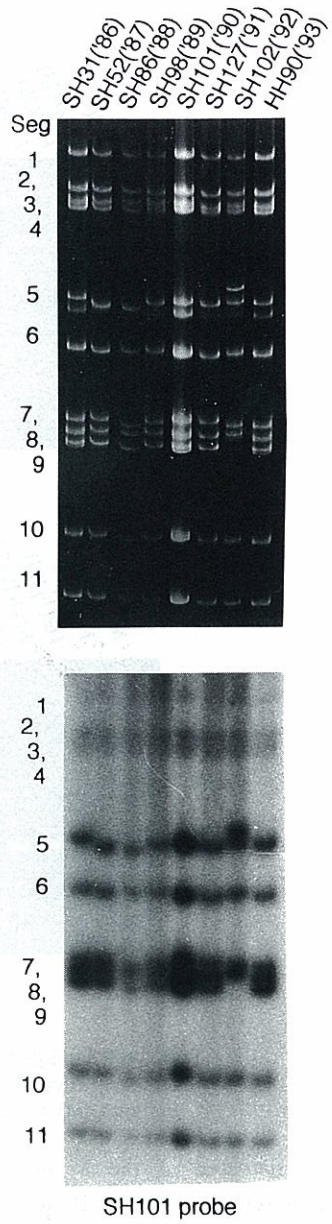

Fig. 1 Hybridization patterns between genomic RNAs from the representative 8 RV strains indicated above each lane and 32P-labeled total ssRNA from strain SH101 virus. Extra band around segment 5 appears to be the rearranged gene, although the origin of the gene has been identified. RNA segments are indicated to the left.

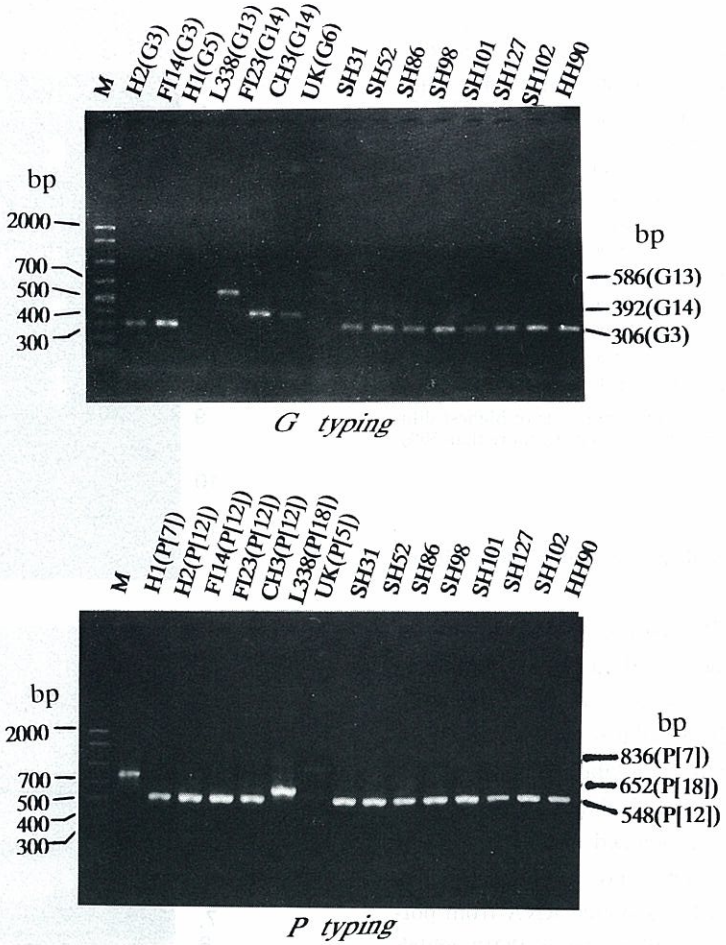

Fig. 2 G and P typings by PCR of VP7 and VP4 genes of the reference and representative RV strains indicated above each lane. Base pair markers (M) are indicated to the left

P[12]) were detected in the 8 representative strains and the reference strains H2 (G3A, P[12]), FI14 (G3B, P[12]), FI23 (G14, P[12]) and CH3 (G14, P[12]). All 113 strains except one (not amplified in the first amplification) were identified as G3 and P[12] (data not shown).

DISCUSSION

All the strains tested by FFN and/or PCR assays in this study, were identified as type G3, P[12], and showed the same electropherotype in PAGE of viral RNA. Furthermore, the 8 representative strains, one obtained from each year, showed a higher degree of overall genomic related from one another in hybridization assay of viral dsRNA. In our neutralization tests, the 8 isolated strains showed a strong cross-reactivity with strain FI14 (G3B), but relatively

weak or one- way cross-reactivity with strain H2 (G3A). These results suggest that the 8 isolates in this study are ascribed to G3B. It is of interest to examine the reactivity of G3A- and G3B-specific monoclonal antibodies with the 8 isolates, since the classification of G3A and G3B was originally performed by using the specific monoclonal antibodies. Anti - SA11 hyperimmune serum showed relatively lower reactivity (1 : 4,096 -16,384) to the 8 equine strains compared with the homologous titer of 1 : 131,072. This might be explained by antigenic drift in the neutralization epitopes on VP7, by manifestation of VP4 antigenicity, or by altered display of VP7 epitopes due to the VP4 expression. Our results on the antigenic relatedness among strains SA11, H2, and FI14 was different, to some extent, from the previous results shown by Hoshino et al.[9-11]. Although precise reason for the discrepancy is unkown, the difference in immunized animal, immunization schedule, or purification procedures for the preparation of immunogens might reflect the results.

In the present study, diarrheal foals with RV were recognized in 2 to 4 months after birth in a large number of cases. A change in season might have influenced the occurrence of diarrhea in some cases. It is well-known that adequate administration of colostral milk is of major importance for protection against several digestive infections in newborn animals, because of the presence of local and passive immunity. A high incidence of RV diarrhea has been recognized among 4- to 8-month-old children and one-month- old piglets whose maternal antibodies showed a marked decrease [7,26].

The present results also strongly suggest that the widely prevalent strains on this farm were almost identical serologically and genetically throughout the observation period from 1986 to 1993. A similar finding was described as the prevalence of predominat RV strain (G3) in horses during two years [1]. In this study, it was thought that the horses might have been exposed many times to invasion of RV strains, because many pregnant mares were imported from abroad every year and some horses were transported from the farm to the race track or vice versa many times. However, no appearence of serologic and genomic variants of RV was observed on the farm for eight long years, except that a minor prevalent RV strain (G14, P[12]) was recognized in 1987 [19,20]. These results indicate the possibility that other type RV strains did not invade or could not remain for a long time among the horse population in which a singlepredominant RV strain (G3, P[12]) prevailed on the farm. Systemic sequence analysis of the strains isolated in each year would be useful for testing this possibility. Literature dealing with the observation of foal diarrhea with RV over a long term is scanty ; thus, the present findings have an interesting bearing on the ecological feature of equine RVs.

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一軽種馬牧場における子馬の単一型馬ロタウイルス(G3,P[12])の感染

阪脇廣美[1]・石崎　宏[1]・高木道浩[1]
増田　玄[1]・谷口孝喜[2]・浦沢佾子[2]
浦沢正三[2]・後藤　仁[1]・白幡敏一[1]

[1]帯広畜産大学家畜微生物学教室
(帯広市稲田町)
[2]札幌医科大学衛生学教室
(札幌市中央区南1条西17丁目)

摘　　要

子馬の集団下痢症の主因となるロタウイルスの伝播と持続の様相を明らかにするため，1986～93年間に北海道の一軽種馬牧場で集団発生した下痢症子馬の糞便からウイルス分離を試み，各流行年のウイルス株の諸性状について比較した。本牧場では，毎年2～6月に200頭前後の子馬が出生し，このうち23.9～52.9%の子馬で，3～9月に亘って下痢発症が観察された。これらの下痢便から，1993年を除き，いずれの年度も50%以上と高率にロタウイルスが検出あるいは分離され，分離ウイルス株のRNA泳動像は全て同一パターンを示した。各年度の代表分離ウイルス株は，中和試験とPCR法による型別でいずれもG3，P[12]に属し，RNA-RNA hybridizationにおいても一分離株由来のプローブとの間でよくハイブリッドを形成した。すなわち，本牧場の流行ウイルス株は観察期間を通じて血清学的並びに遺伝学的にほぼ同一であり，この成績から本馬集団では長期間に亘って単一型の馬ロタウイルスによる持続感染が推察された。

帯広研報 **20**(1997)：103～111