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Thesis :

Serological and Genetical Characterization of Human and Animal Rotaviruses Prevailing in Thailand

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ABSTRACT This series of studies used antigenic and genetic analysis to examine rotavirus strains prevailing in humans and animals in Thailand. The results are summarized as follows :

1) A number of fecal specimens were collected from humans (patients with diarrhea), cattle (young calves with diarrhea) and pigs (piglets with diarrhea) in Thailand. These were screened for rotavirus by polyacrylamide gel electrophoresis (PAGE) of segmented viral genome and enzyme-linked immunosorbent assay (ELISA) with group A-specific monoclonal antibodies. Group A rotavirus-positive specimens were then examined for their subgroup and G serotype specificity using subgroup (I-and II-) specific monoclonal antibodies and G serotype (1-, 2-, 3-, 4- and occasionally 6-) specific monoclonal antibodies, respectively. G serotype specificity of the specimens untypable by ELISA was then examined by polymerase chain reaction (PCR) using serotype-specific primers, which showed very high sensitivity in serotype determination.

2) The rate of rotavirus detection in pediatric diarrhea was calculated at 36.9% on the basis of the examination of diarrhea specimens collected in Bangkok in 1991-94. Rotavirus gastroenteritis was shown to occur most frequently in infants and young children equal to or less than 2 years of age with a peak at 6-11 months of age. The male to female ratio was calculated at 1.36. Monthly distribution of the occurrence of rotavirus gastroenteritis in Bangkok indicated that the illness tends to cluster in September through January.

3) Although as a whole G serotype 1 was the most often detected in this series, the frequency of detection of individual serotypes differed greatly by year. While in the 1983-84 survey G serotype 4 was the most frequent, both G serotypes 1 and 2 dominated in the 1987-88 survey. In the following two years of 1988-89 and 1989-90, G serotype 1 predominated, while in 1990-91 survey G serotype 3, which had been detected only rarely until then, surpassed other G serotypes 1 and 2. G serotype 1 was the most frequent in 1991-92, second to G serotype 2 in 1992-93 and again predominant in 1993-94.

4) It was unexpected that all bovine rotavirus strains found in the present studies were antigenically non-G serotype 6, since most bovine rotaviruses reported until then belonged to G serotype 6. The RNA patterns of these strains were grouped into the three electropherotypes a, b and c. Sequence analysis of the VP7 gene of strain 61A (representing the electropherotype c) indicated that it does not belong to G serotype 1, 2, 3, 4, 5, 6, 8, 9, or 11.

These results formed the basis of our subsequent antigenic and genetic studies showing that while the G serotype of strains A44 (electropherotype a) and 61A is 10, that of strain A5 (electropherotype b) is 8, which is discussed in this paper.

This thesis is based for the most part on the four papers listed in the Introduction.
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5) Serotyping by ELISA showed that the majority of porcine rotavirus strains (7/13) detected in 1987 was G serotype 3. Twenty-three group A rotavirus strains detected in 1990-91 were grouped by RNA PAGE into six electropherotypes a-f. The G serotypes assigned to these by ELISA and PCR were as follows: G serotype 3 (three strains of electropherotypes a and c), G serotype 10 (14 strains of electropherotype d) and undetemined serotype (6 strains).

The presence of G serotype 10 porcine rotavirus, a finding unreported to date, has been further confirmed by serologic and genetic analysis in this laboratory (Pongsuwanna et al., submitted), which is discussed in this paper.

6) On the basis of the findings indicating the close genetic relationship among rotaviruses of humans and various animal species, the possibility of interspecies transmission and subsequent genome reassortment in the recipient host and the significance of these events in the mechanisms of evolution and diversification of rotavirus in nature are discussed.

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Key words: Group A rotavirus, Human rotavirus, Animal rotavirus, Rotavirus serotype, Rotavirus genome, ELISA, RNA-electropherotype, PCR, Nucleotide sequence

1. Introduction

Diarrheal diseases are among the leading causes of morbidity in developed countries and of both morbidity and mortality in developing countries[1]. It has been estimated that there are 3-5 billion cases of diarrhea and 5-10 million deaths associated with diarrhea per year in Asia, Africa, and Latin America[1]. Most of these deaths occur during infancy and early childhood. Since rotavirus is the single most important etiologic agent of severe diarrhea of infants and young children worldwide[2] and since approximately 30% of diarrheal cases are known to be attributed to rotavirus infection[3], it is estimated that rotavirus infection causes the deaths of 1.5-3 million infants and young children in developing countries annually.

Rotavirus, Family Reoviridae, genus Rotavirus, is a major cause of infectious gastroenteritis in the young of a variety of mammalian and avian species as well as of humans. Rotavirus virion consists of three concentric protein layers (outer capsid, inner capsid and core layers) and 11 segments of double-stranded RNA (dsRNA) as genome. Based on the antigenic property of protein capsids and the profile of genomic RNA in polyacrylamide gel electrophoresis (PAGE) in which the 11 segments are separated into discrete bands according to their molecular size, rotaviruses are classified into six distinct groups A through F. Among them, group A rotavirus is recognized as the most important from its highest prevalence and pathogenicity in humans and various animal species[2]. Strains within group A also show genome diversity demonstrated by various means of RNA analysis including PAGE of viral RNA[4]. The outermost protein layer (or outer capsid) of group A rotavirus comsists of VP4 and VP7, which have independent antigenic specificity[5,6]. VP7, which is encoded by RNA segment 7, 8, or 9 depending on the strain, determines the specificity of G serotype, the major neutralization antigen of the virus. Fourteen G serotypes have been identified to date, of which seven (G1-G4, G8, G9 and G12) have been found in human rotaviruses[7,8,9,10].

VP4, which is encoded by RNA segment 4, determines the specificity of P serotype, the minor neutralization antigen. However, since unified nomenclature for VP4 serotyping has not been established, both the term P serotype defined by a virus neutralization test and the term VP4 genotype determined by a sequence analysis of the gene have been employed for the classification of VP4 for the time being.

At least 19 VP4 genotypes have been described[11]. Besides these serotype-specific antigens, common (or cross-reactive) neutralization antigens shared by group A rotaviruses of different serotypes are also located in VP4 protein[12].

VP6, the product of RNA segment 6 and the major component of the middle concentric protein layer (or inner capsid), carries two non-neutralization antigens (group and subgroup antigens). While the group antigen is common to all group A rotaviruses, the specificity of the subgroup antigen of most group A rotaviruses is classified into either I or II.

Global surveys on the prevalence of individual rotavirus serotypes are needed before the introduction of any of the rotavirus vaccines now being developed. Further, antigenic and genetic analysis of rotavirus strains prevailing in humans and animals will provide important knowledge to understand the evolution of rotavirus and in particular the mechanism of emergence of novel strains of the virus. The series of studies described in this paper were carried out with these purposes in mind.

" List of original publications "

This thesis is based for the most part on the following four papers.

1. Pongsuwanna Y, Taniguchi K, Choonthanom M, Chiwakul M, Susansook T, Saguanwongse S, Jayavasu C, and Urasawa S. Subgroup and serotype distributions of human, bovine, and porcine rotavirus in Thailand. J Clin Microbiol 1989, 27: 1956-1960.
2. Pongsuwanna Y, Taniguchi K, Choonthanom M, Chiwakul M, Jayavasu C, Snodgrass DR, and Urasawa S. Serological and genetic characterization of bovine rotaviruses in Thailand by ELISA and RNA-RNA hybridization: Detection of numerous non-serotype 6 strains. Southeast Asian J Trop Med Public Health 1990, 21: 607-613.
3. Taniguchi K, Pongsuwanna Y, Choonthanom M, and Urasawa S. Nucleotide sequence of the VP7 gene of a bovine rotavirus (strain 61A) with different serotype specificity from serotype 6. Nucleic Acids Res 1990, 18 : 4613.
4. Pongsuwanna Y, Taniguchi K, Wakasugi F, Sulivijit Y, Chiwakul M, Warachit P, Jayavasu C and Urasawa S. Distinct yearly change of serotype distribution of human rotavirus in Thailand as determined by ELISA and PCR. Epidemiol Infect 1993, 111: 407-412.

2. Materials and Methods

2·1 Fecal specimens

2·1·1 Fecal specimens from humans

In this study the first examination on rotavirus gastroenteritis in humans was conducted using 543 stool specimens collected from infants and young children (1 day to 1 year and 7 months of age) and adults (24-48 years of age) with acute gastroenteritis who were admitted to three hospitals in Bangkok, Thailand (Children's Hospital, Prapinkhlao Hospital, and Bumrasnaradura Hospital) between June 1983 and May 1984 and between June 1987 and May 1988.

The second examination was conducted using a total of 241 stool specimens which were collected mostly from inpatients in the two hospitals in Bangkok (Children's Hospital and Charoenkrung Pracharuk Hospital) and which were found positive for group A rotavirus by PAGE of viral RNA. Of these 241 rotavirus-positive specimens 82 were obtained during the period July 1988-June 1984, 82 during July 1989-June 1990 and 77 during July 1990-May 1991.

In the third examination 659 stool specimens were collected from the two hospitals in Bangkok (Children's Hospital and Charoenkrung Pracharuk Hospital). One hundred and eleven, 192 and 356 specimens were obtained during the periods November 1991-May 1992, June 1992-May 1993 and June

1993-May 1994, respectively. An approximately 10% suspension of each specimen was made in Eagle's minimal essential medium (MEM).

2・1・2 Fecal specimens from cattle

Bovine fecal specimens used in this study were collected in 1988 and 1989. Twenty-five fecal specimens were collected from young calves with diarrhea in two dairy herds in Saraburi and Nakornrachasima Provinces in Thailand between July and December 1988. Additional 45 diarrheic fecal specimens were collected in a dairy farm in Nakornrachasima Province between July and December 1989. Ten percent fecal suspensions were made in Eagle's MEM.

2・1・3 Fecal specimens from swine

Eighteen fecal specimens were obtained from pigs with diarrhea in a herd in Rachaburi Province in October 1987. An additional 557 diarrheic fecal specimens were collected from piglets in three herds in Nakhon Pathom, Ratchaburi and Chonburi Provinces between December 1990 and July 1991. Ten percent suspensions were made in Eagle's MEM.

2・2 Propagation of rotaviruses in cell cultures

Rotaviruses in selected fecal specimens were adapted to growth in MA-104 cells (an established rhesus monkey kidney cell line) in roller tube cultures as described previously[13]. Briefly, 10% fecal suspensions in MEM were clairfied by low-speed centrifugation and filtered through 450 nm millipore filters. Virus inocula pretreated with trypsin (10 μg trypsin/ml for 30 min at 37°C) were serially passaged in serum-free culture medium containing 2 to 3 μg of trypsin/ml. After adaptation of virus to cell cultures, the virus-infected cell cultures were harvested, usually 2 to 3 days after inoculation.

Representative strains of human and animal rotaviruses entablished previously were also propagated in a similar manner and were employed in this study.

2・3 Electrophoretic pattern analysis of viral RNA (RNA electropherotyping)

Viral RNA was extracted from fecal suspension or culture fluid with disruption solution containing SDS, 2-ME, and EDTA and then with phenol-chloroform. The RNA elctropherotype of each virus was determined by PAGE and co-electrophoresis of viral RNA in 10% polyacrylamide gels (2 mm thick) for about 16 h at 18 mA at room temperature. RNA segments separated were stained with silver nitrate as described previously[14].

Basically, the screening of rotavirus in human and animal fecal specimens was carried out by the detection of RNA patterns characteristic of rotaviruses in PAGE.

2・4 Enzyme-linked immunosorbent assay (ELISA)

Human rotaviruses (HRVs) and animal rotaviruses were examined for their subgroup and G serotype specificities by ELISA, as described previously[15,16]. Eight monoclonal antibodies (MAbs) were used as capture antibodies. YO-156, reacting with the group A common antigenic epitope in the inner capsid protein VP6, and YO-2C2 (or KU-6B11), reacting with the cross-reactive neutralization epitope in the outer capsid protein VP4, were used to confirm group A rotavirus in fecal specimens. Antibodies S2-37 and YO-5, reacting with subgroup I- and subgroup II-specific epitopes in VP6, respectively were used to subgroup group A rotaviruses. Finally KU-4 (or KU-6BG), S2-2G10, YO-1E2, anid ST-2G7, which each recognize specific G serotype 1, 2, 3 and 4 neutralization epitopes in the outer capsid protein VP7, were used to serotype viruses. In some experiments IC3 antibody (donated by HB Greenberg) recognizing G serotype 6-specific epitope in VP7 was also used. The properties of these MAbs were described previously[12,15,17,18].

The wells of polyvinyl microtiter plates were coated with ascitic fluids (MAbs) diluted 1 : 10,000 in 10 mM PBS (pH 7.5) for 1 day at 4°C. After the plates were washed with PBS containing 0.05% Tween 20 (PBST), 1% bovine serum albumin (BSA) in PBST was added, and the plates were kept for

1 day at 4°C. After washing, a test sample (a mixture of 10% fecal suspension and 10% skim milk at a ratio of 3 : 1, respectively) was allowed to react in the wells overnight at 4°C. After washing, rabbit anti-HRV serum (a pool of antisera against rotavirus G serotypes 1-4) diluted 1 : 10,000 in PBST was added as a detector antibody and the plates were incubated for 1 h at 37°C. After washing, peroxidase-conjugated goat anti-rabbit IgG in PBST was added and the plates were incubated for 1 h at 37°C. After the final washing, the reaction with the substrate (o-phenylenediamine dihydrochloride) was allowed to occur for 20-30 min at room temperature and then was stopped by adding 3N sulfuric acid. Absorbance was measured at 492 nm with a microplate photometer. A virus was assigned to a serotype when the optical density (OD) value in the reaction with the MAb to that serotype exceeded 0.2 and when the OD value in the reaction with that MAb was greater than twice the value obtained with the MAb to any other serotype. The criteria for subgroup determination was essentially the same.

2•5 Neutralization test

A fluorescent focus reduction neutralization (FFN) test was performed with MA-104 cell monolayers in a 96-well microplate using representative rotavirus strains and their antisera prepared in rabbits[19]. Neutralizing antibody titer was expressed as the reciprocal of the highest serum dilution at which the fluorescent focus count was reduced by more than 60%.

2•6 G serotyping of rotavirus by polymerase chain reaction (PCR)

Extraction of rotavirus dsRNA and G serotyping by PCR were carried out following the procedure described by Taniguchi *et al.*[20] Virus in fecal suspension or culture medium was disrupted with disruption solution containing EDTA, Nonidet P-40 and proteinase K. Rotavirus dsRNA was extracted with phenol-chloroform and precipitated with ethanol. Serotyping by PCR was performed in two steps (first and second amplifications). In the first amplification, the full length VP7 gene was reverse transcribed by avian myeloblastosis virus reverse transcriptase and amplified with a pair of common primers, C1 and C2, which correspond to 3′ and 5′ ends of the gene, respectively. The second amplification was carried out using the first amplification product and a mixture of the common primer C1 and six (in human rotavirus) or three (in bovine rotavirus) different serotype-specific primers. The location of individual serotype-specific primers was fixed in such a way that they amplified the product of different molecular size depending on the serotype[20]. PCR products were separated by electrophoresis on 1% agarose gel. In each PCR run, the sample without template was included to exclude the possibility of contamination.

2•7 RNA-RNA hybridization

RNA-RNA hybridization in liquid phase was performed as described by Flores et al[21,22]. Virus particles lacking the outer capsid layer (single-shelled particles) were prepared by treatment of complete virions (double-shelled particles) with EDTA. A ^{32}P-labeled single stranded RNA (ssRNA) probe was prepared by *in vitro* transcription in the presence of purified single-shelled particles and [^{32}P] GTP. Genomic dsRNA was prepared from concentrated virus preparation by phenol extraction and ethanol precipitation. The ^{32}P-labeled ssRNA probes were hybridized to the heat-denatured genomic RNAs. The RNA hybrids precipitated with ethanol were analyzed by PAGE and autoradiography.

2•8 Sequence determination

Viral ssRNA was synthesized *in vitro* by the endogenous transcriptase present in single-shelled particles and precipitated with 2M LiCl. The nucleotide sequence of the VP7 gene of a rotavirus was determined using dideoxynucleotide sequencing reactions with a series of oligonucleotide primers (17 to 25 mers), reverse transcriptase from avian myeloblastosis virus (Seikagaku Kogyo) and [^{32}P]dATP (Amersham) as described previously[23,24]. The 3′-terminal 50 nucleotides of the RNA were determined using denatured dsRNA in 50% dimethylsulfoxide after boiling for 2 min instead of ssRNA transcript.

3. Results

3·1 Human rotavirus infections in Bangkok, Thailand

3·1·1 Characterization and epidemiology of rotavirus

a) Examination with specimens collected in 1983-84 and 1987-88

In the first investigation 543 stool specimens from inpatients with diarrhea collected in 1983-84 and 1987-88 were examined for rotavirus by PAGE of viral RNA and ELISA with group A common MAbs. Eighty-eight (16.2%) specimens were positive for group A HRV. Results for subgroup and serotype determination of these HRVs are shown in Table 1. Seventy-seven (87.5%) of 88 specimens could be determined for subgroup specificity; 20 for subgroup I and 57 for subgroup II. Eleven specimens were unable to be subgrouped. Regarding the relationship between subgroup specificity and electrophoretic pattern, subgroup I rotaviruses showed a short RNA pattern in which segments 10 and 11 migrated slowly, while subgroup II rotaviruses showed a long RNA pattern in which the two segments migrated relatively rapidly (Fig. 1).

Fifty-eight (65.9%) of 88 group A HRV-positive specimens were serotyped; 18 for G serotype 1, 17 for G serotype 2 and 23 for G serotype 4. The serotype specificity of 30, however, could not be determined; while 12 appeared not to contain enough double-shelled particles, judging from their reactivity with the commonly reactive YO-2C2 MAb which recognizes double-shelled particles, 16 appeared to contain considerable numbers of double-shelled particles. Further, 22 specimens showed clear RNA PAGE patterns. The differing serotype distributions of HRV by year will be discussed later.

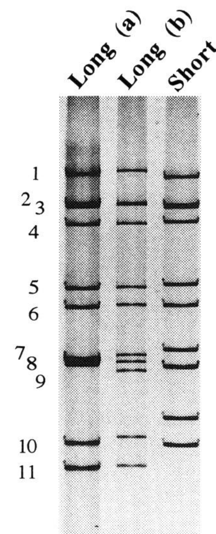


Fig. 1 Typical examples of short and long RNA patterns. Migration is from top to bottom.

b) Examination with specimens collected in 1988-91

Table 1 *Subgroup and serotype distribution of human rotavirus in Bangkok, Thailand in 1983-84 and 1987-88 as determined by ELISA*

Subgroup	Number of specimens	No. of strains with the following serotypes:				
		1	2	3	4	Not determined
(A) 1983 to 1984						
I	5	0	4	0	0	1
II	39	7	0	0	21	11
Not determined	5	0	1	0	0	4
(B) 1987 to 1988						
I	15	0	12	0	0	3
II	18	11	0	0	2	5
Not determined	6	0	0	0	0	6

The protection against rotavirus infection in infants and young children is considered to be G serotype-specific[25]. G serotyping of prevalent rotavirus strains, therefore, is important clinically and epidemiologically. Since the usefulness of PCR for G serotyping of rotavirus has been reported by Gouvea *et al.*[26] and Taniguchi *et al.*[20], the practical utility of PCR was evaluated in this study using a total of 241 group A HRV-positive specimens collected in Bangkok over a three-year period 1988-1991. First, all the specimens were subjected to G serotype determination by ELISA. The specimens left untyped were then examined for G serotype by PCR using primers specific to G serotypes 1-4, 8 and 9. The results are shown in Table 2.

The serotypes of 162 (67.2%) of 241 specimens were successfully determined by ELISA. Of the 79 whose serotype remained undetermined, 63 were examined by PCR (the remaining 16 could not be examined due to low remaining volumes). As shown in Table 2, the serotypes of 59 (93.7%) specimens were identified, so that overall, 221 (91.7%) of 241 specimens were successfully serotyped by either ELISA or PCR. The details of the serotype distribution of HRV by year will be discussed later.

By ELISA using subgroup-specific MAbs, the subgroup specificity of 241 specimens was examined. As shown in Table 2, 204 (84.6%) of them could be subgrouped: 47 were subgroup I and 157 were subgroup II. In all the specimens except four the combination of assigned subgroup and serotype specificities conformed to the generally known relationships in HRVs: subgroup I HRVs have G serotype 2 or 8 specificity, and subgroup II HRVs, serotype 1, 3, 4 or 9 specificity[2].

c) Examination with specimens collected in 1991-94

In order to obtain some basic data on rotavirus epidemiology in Bangkok, again a collection of 659 specimens was made over a three-year period 1991-94. Occurrence of rotavirus gastroenteritis was examined by sex, age and monthly distribution of the illness. Of 659 specimens, 243 (36.9%) were

Table 2 *Subgroup and serotype distribution of human rotavirus in Bangkok, Thailand between July 1988 and June 1991 as determined by ELISA and PCR*

Subgroup	Number of specimens	ELISA					PCR							
		1	2	3	4	ND	1	2	3	4	8	9	ND	NT
(A) July 1988-June 1989														
I	8	0	6	0	0	2	1	0	0	0	0	0	0	1
II	65	36	0	0	7	22	12	0	2	2	0	0	1	5
ND	9	0	0	0	0	9	3	1	0	0	0	1	0	4
Total	82	36	6	0	7	33	16	1	2	2	0	1	1	10
(B) July 1989-June 1990														
I	19	1	14	0	0	4	0	2	0	0	0	0	1	1
II	58	35	0	3	0	20	13	2	1	1	0	0	1	2
ND	5	0	2	0	0	3	0	0	1	0	0	0	0	2
Total	82	36	16	3	0	27	13	4	2	1	0	0	2	5
(C) July 1990-June 1991														
I	20	0	19	0	0	1	0	0	0	0	0	0	1	0
II	34	13	0	16	1	4	2	0	2	0	0	0	0	0
ND	23	5	0	4	0	14	3	2	8	0	0	0	0	1
Total	77	18	19	20	1	19	5	2	10	0	0	0	1	1

PCR-typing was performed for only the specimens whose serotype could not be determined by ELISA.
ND, Serotype or subgroup of the sample could not be determined.
NT, PCR could not be applied to the sample because of the shortage.

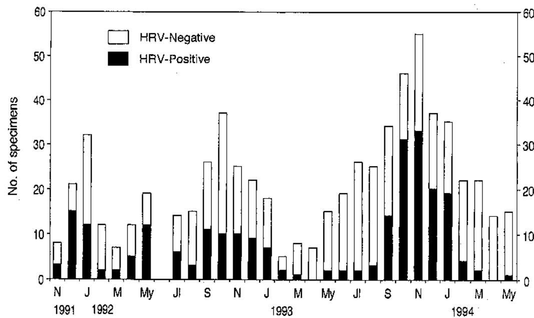


Fig. 2 Monthly distribution of rotavirus gastroenteritis patients in two hospitals in Bangkok

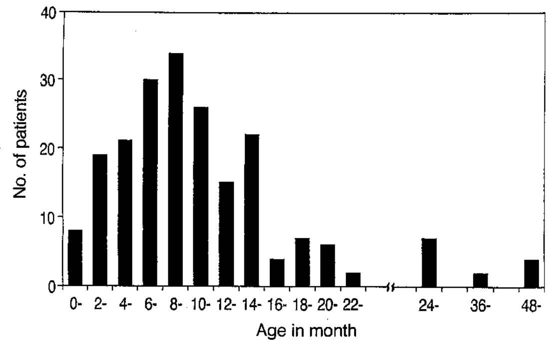


Fig. 3 Age distribution of patients with rotavirus gastroenteritis in Bangkok

positive for group A rotavirus. The monthly distribution of diarrheal illnesses is depicted in Fig. 2. In general, the occurrence of rotavirus diarrhea in Bangkok seems to have a unimodal distribution pattern with a peak in September through to January, although somewhat irregular occurrence was seen in May in 1992. The male to female ratio of rotavirus gastroenteritis was 1.36. The occurrence by age of rotavirus gastroenteritis is shown in Fig. 3. It is most frequent in infants and young children of 2 years or less with a peak at 6-11 months of age.

Results for subgroup and G serotype determination of the 243 group A rotavirus-positive specimens are shown in Table 3. As in the previous study, specimens which were untypable by ELISA were then subjected to PCR. In all, 213 (87.7%) of the 243 specimens were assigned for serotypes by either

Table 3 *Subgroup and serotype distribution of human rotavirus in Bangkok, Thailand between Nov. 1991 and May 1994 as determined by ELISA and PCR*

Subgroup	No. of specimens	No. serotyped by			G serotype distribution					ND
		ELISA	PCR	Total	1	2	3	4	9	
(A) Nov. 1991-May 1992										
I	10	5	3	8	0	6	0	0	2	2
II	19	15	1	16	16	0	0	0	0	3
ND	22	5	5	10	9	0	0	0	1	12
Total	51	25	9	34	25	6	0	0	3	17
(B) June1992-May 1993										
I	38	24	12	36	0	36	0	0	0	2
II	3	2	1	3	1	0	0	2	0	0
ND	20	6	6	12	7	4	0	1	0	8
Total	61	32	19	51	8	40	0	3	0	10
(C) June 1993-May 1994										
I	0	0	0	0	0	0	0	0	0	0
II	52	42	10	52	52	0	0	0	0	0
ND	79	24	52	76	75	0	0	1	0	3
Total	131	66	62	128	127	0	0	1	0	3

RCR-typing was performed for only the specimens whose serotype could not be determined by ELISA.
ND, Serotype or subgroup of the sample could not be determined.

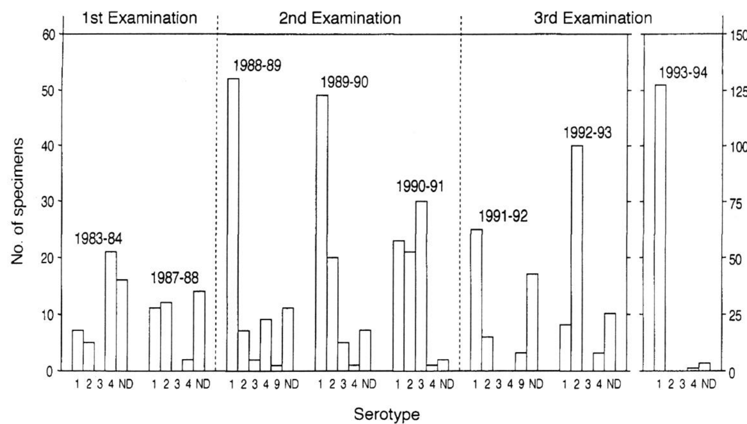


Fig. 4 Serotype distribution of human rotavirus in Bangkok, Thailand by year

ELISA or PCR. All except two showed generally known combinations of subgroup and serotype antigens mentioned above. Of note, two strains collected in 1991-92 showed subgroup I, G serotype 9. The details of the serotype distribution by year will be described below.

3•1•2 Serotype distribution of HRV by year

The yearly frequency of individual HRV serotypes prevailing in Bangkok is chronologically arranged in Fig. 4. In 1983-84, G serotype 4 was the most prevalent, while in 1987-88, both G serotypes 1 and 2 were dominant serotypes. G serotype 1 predominated over the two-year period 1988-90. In 1990-91 it was replaced by G serotype 3, which had not been detected at all or detected only in very low frequency in the preceding years. G serotype 1 was predominant in 1991-92 and 1993-94, being replaced by G serotype 2 in 1992-93. The other serotypes vanished almost completely in 1993-94. Besides these four major serotypes, it is worthy of note that one and three strains with G serotype 9 specificity were detected in 1988-89 and 1991-92, respectively.

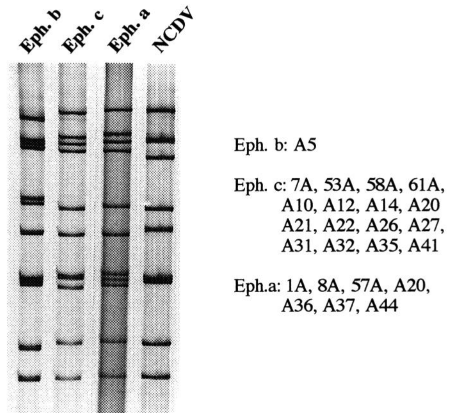


Fig. 5 RNA electropherotypes of BRV isolates in Thailand and a reference strain NCDV. Migration is from top to bottom.

3•2 Animal rotavirus prevailing in Thailand

Rotaviruses from the young of two animal species, calves and piglets, were examined in this study.

3•2•1 Bovine rotaviruses

A total of 70 fecal specimens obtained in 1988 and 1989 were subjected to subgrouping and serotyping by ELISA with MAbs and RNA PAGE analysis. Sixty-two specimens were found rotavirus-positive by ELISA with group A-common MAb (YO-156). All the strains whose subgroup specificity was determined belonged to subgroup I, as reported on the bovine rotaviruses (BRV) isolated worldwide[2]. None of the four MAbs each specific to G serotypes 1, 2, 3 and 4 reacted with BRVs as expected. It was unexpected, however, that G serotype 6-specific MAb (IC3) would react with none of these Thai BRVs, since most BRVs which had been reported until then were G serotype 6[27]. The results

obtained from selected fecal specimens are shown in Table 4.

RNA PAGE analysis showed that all the BRVs had long RNA electrophoretic patterns (RNA electropherotypes). In order to compare the RNA electropherotypes in detail and to further characterize the antigenic and genetic properties of these viruses, 30 virus-containing specimens were adapted to growth in MA104 cells. Twenty-three strains were isolated. While the RNA electropherotypes of these strains isolated in Thailand were different from that of strain NCDV, the representative of G serotype 6 BRV, they were grouped into three different patterns, a (7 strains), b (one strain) and c (16 strains). The electropherotypes of these three groups and that of strain NCDV are shown in Fig. 5.

Overall genomic relatedness between rotavirus strains can be evaluated by RNA-RNA hybridization. When ^{32}P-RNA probe of NCDV was reacted with the genomic RNAs from three Thai BRVs (53A, 58A and 61A), three hybrid bands corresponding to segments 5, 6 and either one of 7-9 complex were observed (Fig. 6). On the other hand, labeled RNA probe of strain 53A hybridized to all the corresponding segments from the three Thai strains, although some of the larger RNA segments were not clearly defined. In contrast, 53A RNA probe produced three hybrid bands when it reacted with NCDV genomic RNA (Fig. 7). Thus, the three Thai BRV strains serologically distinct from NCDV representing G serotype 6 were found to differ in overall genomic relatedness from NCDV.

In order to further confirm the unique antigenic property of the Thai strains, the VP7 gene of 61A was sequenced and the nucleotide and deduced amino acid sequences obtained were compared with those of VP7 genes of previously established strains representing 9 different G serotypes whose nucleotide sequences had already been reported. The nucleotide and deduced amino acid sequences of 61A (Fig. 8)

Table 4 *Reactivity patterns of several MAbs with representative Thai BRV strains as determined by ELISA*

Virus strain	ELISA results[a] with				Ascribed subgroup	Ascribed serotype	Electro-pherotype
	Anti-VP6			Anti-VP7[b]			
	YO-156 (Common)	S2-37 (Subgroup I)	YO-5 (Subgroup II)	IC3 (G serotype 6)			
53A	995	≧2,000	282	46	I	non-6	C
58A	1,401	≧2,000	141	32	I	non-6	C
61A[c]	1,050	1,210	109	55	I	non-6	C
A1	1,000	1,614	90	76	I	non-6	A
A5	687	607	59	48	I	non-6	B
A7	362	433	58	42	I	non-6	C
A10	771	1,514	0	27	I	non-6	C
A20	1,569	1,656	70	224	I	non-6	C
A21	340	1,483	54	63	I	non-6	C
A26	1,345	1,460	11	61	I	non-6	C
A31	1,341	1,488	100	27	I	non-6	C
A36	1,117	1,413	63	25	I	non-6	A
A37	960	1,336	64	50	I	non-6	A
Control NCDV[b]	1,833	≧2,000	151	1,255	I	6	D

[a] The data are shown as the optical density at 492 nm (×1,000) in a well. An optical density value of over 300 was considered to show a positive reaction.

[b] None of the four anti-VP7 MAbs specific to G serotypes 1-4 reacted with any of the BRVs tested (data not shown).

[c] Virus-infected cultured fluids were used. Fecal suspensions were employed for the other strains.

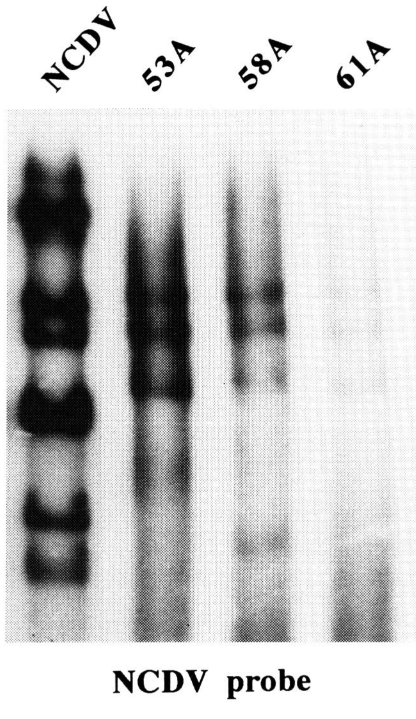
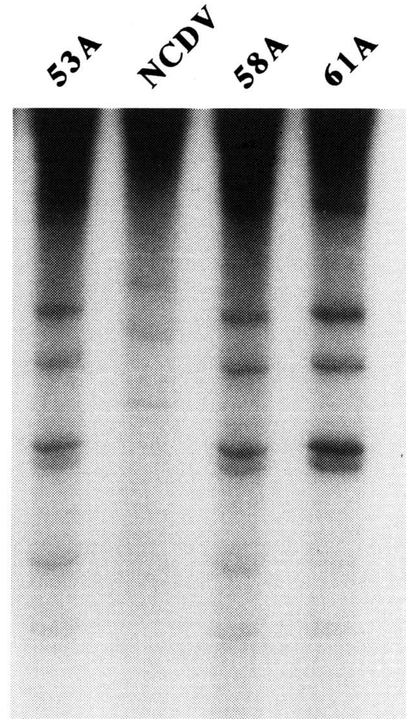


Fig. 6 Hybridization of a labeled ssRNA probe of strain NCDV to genomic RNAs of strains NCDV, 53A, 58A and 61A



53A probe

Fig. 7 Hybridization of a labeled ssRNA probe of strain 53A to genomic RNAs of strains 53A, 58A, 61A, and NCDV

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GGCTTTAAAAGCGAGAATTTCCGTTTGGCTAGCGGATAGCACCTTTTAATGTATGGTATTGAATATACCACATTCCTAATCTACTTAATATCAATTATA  99
                                                 M  Y  G  I  E  Y  T  T  F  L  I  Y  L  I  S  I  I   17

TTATTTAATTACATATTAAAAAGTATAACTAGAATGATGGACTACATAATTTATAAATTTTTGCTTATAGTCACAATCGCTTCAATCGTCGTTAATGCC 198
 L  F  N  Y  I  L  K  S  I  T  R  M  M  D  Y  I  I  Y  K  F  L  L  I  V  T  I  A  S  I  V  V  N  A   50

CAGAATTACGGCATCAATTTGCCAATAACCGGATCGATGGATGCGTCATATGTGAATGCTACTAAAGATAAGCCATTTCTAACATCAACATTATGTTTA 297
 Q  N  Y  G  I  N  L  P  I  T  G  S  M  D  A  S  Y  V  N  A  T  K  D  K  P  F  L  T  S  T  L  C  L   83

TACTATCCAACAGAAGCTAGAACAGAAATAAACGACAATGAGTGGACGAGTACGTTGTCACAGCTGTTCTTAACAAAGGGATGGCCGACTGGATCCGTA 396
 Y  Y  P  T  E  A  R  T  E  I  N  D  N  E  W  T  S  T  L  S  Q  L  F  L  T  K  G  W  P  T  G  S  V  116

TACTTTAAAGAATATGATGATATAGCTACTTTTTCAGTAGACCCACAACTGTATTGTGACTATAACATAGTTCTAATGAGATACAACTCAAGTCTAGAA 495
 Y  F  K  E  Y  D  D  I  A  T  F  S  V  D  P  Q  L  Y  C  D  Y  N  I  V  L  M  R  Y  N  S  S  L  E  149

CTTGATATGTCGGAATTGGCAAATCTAATATTGAATGAATGGCTGTGTAATCCAATGGATATTACATTGTATTATTATCAACAAAACGGACAGGCAAAC 594
 L  D  M  S  E  L  A  N  L  I  L  N  E  W  L  C  N  P  M  D  I  T  L  Y  Y  Y  Q  Q  N  G  Q  A  N  182

AAATGGATAGCGATGGGACAATCATGTACAATAAAAGTGTGTCCATTGAATACCCAAACACTAGGAATAGGATGTCAGACTACAAATACTAGAACGTTT 693
 K  W  I  A  M  G  Q  S  C  T  I  K  V  C  P  L  N  T  Q  T  L  G  I  G  C  Q  T  T  N  T  R  T  F  215

GAAGAAGTTGCAACGGCTGAAAAATTGGTAATTACTGATGTAGTTGACGGCGTAAATCATAAGTTGGATGTTACTACTGCGACTTGTACTATAAGAAAT 792
 E  E  V  A  T  A  E  K  L  V  I  T  D  V  V  D  G  V  N  H  K  L  D  V  T  T  A  T  C  T  I  R  N  248

TGCAAGAAATTAGGACCAAGAGAAAATGTAGCAGTTATACAAGTGGGTGGTGCTGATATCCTTGATATAACATCTGATCCGACGACTGCTCCACAAACT 891
 C  K  K  L  G  P  R  E  N  V  A  V  I  Q  V  G  G  A  D  I  L  D  I  T  S  D  P  T  T  A  P  Q  T  281

GAACGGATGATGCGAATAAATTGGAAAAAATGGTGGCAAGTGTTCTACACTATAGTTGATTACGTAAATCAAATTGTGCAAGCAATGTCCAAAAGGTCC 990
 E  R  M  M  R  I  N  W  K  K  W  W  Q  V  F  Y  T  I  V  D  Y  V  N  Q  I  V  Q  A  M  S  K  R  S  314

AGATCACTGAATTCAGCAGCATTTTATTATAGAGTGTAGATATTGTGTAGATTAGCAATGTATGATGTGACC 1062
 R  S  L  N  S  A  A  F  Y  Y  R  V  326
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Fig. 8 Complete nucleotide sequence and deduced amino acid sequence of the VP7 gene of bovine rotavirus strain 61A

showed low homologies (73.4 to 77.8% and 74.3 to 84.9%, respectively) to those of the strains with G serotype 1, 2, 3, 4, 5, 6, 8, 9 or 11 specificity. Especially, a comparison of amino acid sequences in variable regions B (amino acids 87-101), D (143-152), and E (208-221), which are associated with serotype specificity, between strain 61A and the strains with the known serotypes disclosed a large difference (51 to 67% homology). Thus, the prevalence of non-serotype 6 BRVs in Thailand was proven.

3•2•2 Porcine rotaviruses

Eighteen diarrheic fecal specimens collected in 1987 were examined. Of these, 13 were found to be positive for group A rotavirus by ELISA and RNA PAGE. All the porcine rotaviruses (PRVs) showed subgroup I specificity and long RNA electropherotype. While four distinct G serotypes 3, 4, 5 and 11 had been identified in PRVs until 1990[28,29,30], seven of these 13 were identified as G serotype 3 in ELISA with G serotype 1- to 4-specific MAbs as shown in Table 5. MAbs specific to G serotypes 5 and 11 were not available and 6 strains were left untyped.

In order to further investigate the serologic and genomic properties of PRVs prevailing in Thailand, diarrheic fecal specimens were again collected from piglets. When a total of 557 specimens were subjected to RNA PAGE, 26 were found to contain rotavirus. Of these, 23 showed RNA profile characteristic of group A rotavirus, one, that of group B and two, that of group C (Fig. 9).

RNA patterns of 23 group A rotaviruses were compared with one another by co-electrophoresis, and they were grouped into 6 RNA electropherotypes, a (single strain). b (2 strains), c (2 strains), d (14 strains), e (2 strains), and f (2 strains) (Fig. 10). All showed a long RNA pattern.

Subgroup and serotype specificities of the 23 group A rotavirus strains were examined by ELISA. All the strains were found to react only with subgroup I-specific MAbs. Serotype detemination was incomplete because of the use of only G serotype 1-to 4-specific MAbs in this test. While the G serotype of 20 strains remained undetermined, three strains were assigned for G serotype 3 because of their selective reactivity with G serotype 3-specific MAbs (data not shown).

For assigning the G serotype to the strains which were left untyped by ELISA, serotyping by PCR was conducted using primers specific to G serotypes 1-4, 5, 6, 8, 9, 10 and 11. Three strains belonging to electropherotypes a and c were shown to be G serotype 3, in accord with the ELISA result. Fourteen strains belonging to electropherotype d were found to be G serotype 10 and the remaining 6 were left still undetermined.

4 Discussion

4•1 Human rotaviruses

In the examination of specimens collected in 1991-94, 243 were positive for rotavirus. Omitting 36 specimens of which the patient's age was unavailable, the age distribution of patients from whom speci-

Table 5 *ELISA results of selected PRV strains with serotype 3 specificity*

Fecal specimen	ELISA results[a] with				
	KU-6BG (G serotype 1)	S2-2G10 (G serotype 2)	YO-1E2 (G serotype 3)	ST-2G7 (G serotype 4)	YO-2C2 (Common)
21A	163	264	1,516	260	1,100
22A	261	209	1,506	206	1,238
37A	160	193	496	64	516
38A	138	149	409	62	500

[a] The data are shown as the optical density at 492 nm (×1,000) in a well.

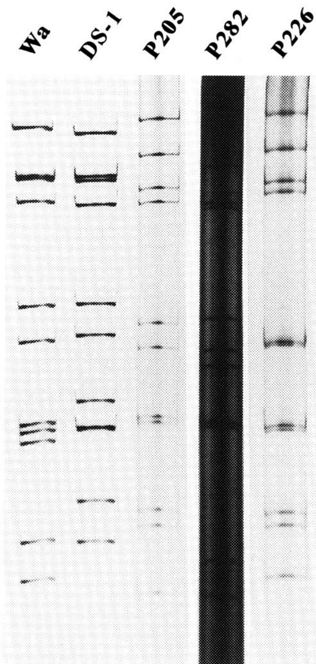

Fig. 9 RNA profile of porcine group B (P205 and P226) and group C (P282) rotaviruses detected in Thailand.
Two group A human rotavirus strains (Wa and DS-1) are included as controls.

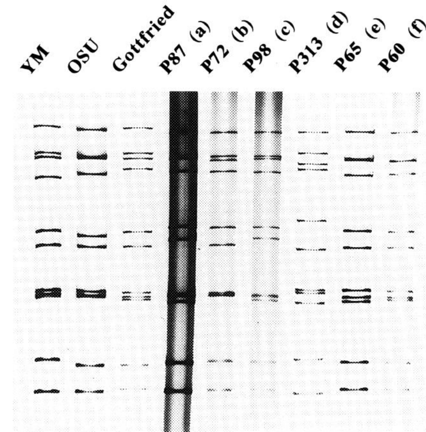

Fig. 10 Six RNA electropherotypes (a-f) in group A PRVs detected in Thailand.
YM, OSU and Gottfried are reference PRV strains.

mens were obtained was computed (Fig. 2). The number of patients ≦2 years of age composed 93.6%. The <1 year of age composed 66.9%; more were 6-11 months of age (43.6%) than were 0-5 months (23.3%). Rotavirus diarrhea tends to cluster during cooler months of the year (September-January).

Chiba *et al.* clearly indicated that the protection against rotavirus infection in infants and young children is G serotype-specific[31]. Field trials using rhesus rotavirus vaccine with G serotype 3 specificity showed failure to protect against rotavirus illness due to heterotypic strains[25]. Therefore, extensive epidemiologic studies on HRV serotypes in developing countries are required before the introduction of rotavirus vaccine. In this series of studies, serotype determination of rotavirus was carried out by an ELISA method which is cost-effective yet less sensitive and a sensitive PCR method in combination. Failure to serotype considerable number of specimens in ELISA may be due to i) lack of sufficient number of double-shelled particles in specimens, ii) the presence in specimens of substance(s) nonspecifically reactive to a certain MAb (mouse ascitic fluid), iii) limited reactivity of the MAb itself to variant strains of that serotype, iv) cases of mixed infection with viruses of different G serotypes, and rarely, v) the presence of a strain with a new G serotype. Approximately 83% of specimens untypable in ELISA were successfully serotyped in PCR.

In general, G serotype 1 was the most frequently detected serotype and was predominant in 1988-89, 1989-90, 1991-92, and 1993-94. The prevalence of other G serotypes, however, showed a remarkable yearly fluctuation: G serotype 2 was dominant in 1987-88 and 1992-93, G serotype 3 in 1990-91, and G serotype 4 in 1983-84. A similar tendency in yearly change of dominant serotype in Bangkok is also

reported by Pipittajan *et al*[32]. Distinct variations in the serotype frequencies with region and year have been reported in other countries[16,33,34,35]. Thus, all of the four G serotypes must be considered epidemiologically important and should be essential components to the rotavirus vaccine to be developed in the near future.

The presence of two types of antigenic and genomic association in HRVs, namely, subgroup I-G serotype 2 or 8-short RNA electropherotype or subgroup II-G serotype 1, 3, 4 or 9-long RNA electropherotype, has been repeatedly confirmed by many investigators. In this series of studies, several specimens which did not conform with this association were found. Detailed antigenic and genomic analysis of these strains including isolation in cell culture, RNA-RNA hybridization and sequencing of genes is required to elucidate this phenomenon. In this respect the detection of four G serotype 9 strains, particularly two of these having unusual properties (subgroup I-G serotype 9-long RNA electropherotype) detected in Bangkok in 1991-92 attracts our attention, since human strains with similar properties isolated in Chiang Mai in 1989 were found to be possible reassortant strains derived from porcine rotavirus[36], as will be discussed later.

4·2 Bovine rotaviruses

Until 1989 G serotype 6, represented by strains NCDV and UK, was the sole established serotype of BRV. Although there were a few reports indicating the possible presence of a G serotype(s) other than 6, the possibility had not been thoroughly pursued[37,38,39,40]. In this study, using MAb specific to G serotype 6, a high prevalence of non-G serotype 6 strains in cattle in Thailand was demonstrated. A genomic difference between the Thai bovine strains and G serotype 6 strain NCDV was clearly demonstrated by RNA-RNA hybridization and sequence analysis of the VP7 gene.

It is of note that in this study the non-serotype 6 strains were classified into three electropherotypes a, b and c based on the PAGE analysis of genomic RNA.

Later, in order to further characterize these strains, the authors selected three strains, A44, A5 and 61A, representing the electropherotypes a, b and c, respectively, and analyzed their antigenic and genomic properties[41]. By cross-neutralization reaction the two Thai strains A44 and 61A were found to be serologically related to a Japanese isolate KK3[38] to which G serotype 10 was assigned in 1990[42,43]. Strain A5 was antigenically related to human strain 69M with G serotype 8 specificity. Sequence analysis of the VP7 genes confirmed these results. RNA-RNA hybridization experiments revealed a high degree of overall relatedness among the three G serotype 10 bovine strains (A44, 61A and KK3) and a moderate degree of genetic relatedness between the G serotype 10 bovine strains and the strains A5 and NCDV. All the bovine strains, regardless of their serotype specificity, showed a moderate genetic relatendness to strain 69M of G serotype 8[41].

Recent examinations on BRV isolates[42,43] have indicated that G serotype 10 strains in addition to G serotype 6 strains are distributed in cattle worldwide, as represented by the isolates B223 in U. S. A.[37], KK3 in Japan[38] and V1005 in Germany[44]. The presence of G serotype 8 BRV strains was also reported in Scotland in 1990[43]. In the present series, the prevalence of G serotype 8 and 10 BRVs was demonstrated in Thailand.

4·3 Porcine rotavirus

In porcine rotavirus (PRV), G serotype 4 and 5, represented by the Gottfried and OSU strains, resplctively, were identified in 1984[45]. In 1988, two porcine rotaviruses (strains MDR-13 and CRW-8) antigenically related to human G serotype 3 were reported from Australia[29]. In our first report in 1989 on PRVs the authors revealed the prevalence of G serotype 3 strains in Thailand. These findings suggest the worldwide distribution of G serotype 3 rotavirus in the pig population. G serotype 11 PRV (strain YM) was reported from Mexico in 1989[30].

RNA PAGE of fecal specimens in the second investigation detected 26 rotavirus-positive specimens, 23 strains of group A, 1 strain of putative group B and 2 strains of putative group C rotavirus. Twenty-three group A rotaviruses were classified by RNA PAGE analysis into 6 electropherotypes a-f. By serotyping with ELISA and PCR, 3 strains showing electropherotypes a and c were found to be G serotype 3. Fourteen strains of electropherotype d were assigned for G serotype 10.

Since G serotype 10 rotavirus has not been reported in pigs, confirmation of the result seemed necessary. One representative strain P343 was adapted to cell culture and the antiserum to strain P343 was prepared in rabbit. Cross-neutralization tests carried out using prototype G serotype 1-14 strains and their respective antisera confirmed that strain P343 belongs to G serotype 10, although 20 fold or greater difference was not always observed between homologous and heterologous reactions (Yaowapa *et al.*, submitted). In addition, sequence analysis showed that VP7 gene of P343 was highly homologous to those of G serotype 10 bovine and human rotaviruses (85.8 to 97.8% identity at nucleotide sequence and 93.6 to 97.6% identity at amino acid sequence). Of note, an RNA-RNA hybridization experiment showed that P343 strain, while lacking genomic relatedness to other representative porcine strains (Gottfried, OSU and YM), was highly related to bovine strains, especially strain 61A (G serotype 10) (Yaowapa *et al.*, submitted).

Group B and C rotaviruses have been detected in cows, pigs and humans[46]. While group C rotaviruses from humans and animals have been successfully cultivated in cell culture, the isolation of group B rotavirus has not succeeded yet. Rotaviruses having RNA patterns characteristic of group B and C rotaviruses in PAGE were detected in feces of piglets in this study. Although this seems to be the first report describing the detection of these viruses in Thai piglets, the significance and distribution of these 'atypical' rotaviruses shoud be investigated further by large-scale studies.

4·4 Relationship among rotaviruses of humans and animals

The relationship among human and various animal rotaviruses has been discussed by several investigators. Since overall gene homology among rotaviruses derived from different animal species (even when they belong to the same serotype) is generally low compared with the homology observed among viruses from the same animal species[47], close genetic relationship, if present, among strains from different animals can best be demonstrated by RNA-RNA hybridization tests.

A close genetic relationship between human and feline rotaviruses was reported first by Nakagomi and Nakagomi[48]. In RNA-RNA hybridization, human rotavirus strain AU-1 (having subgroup I-G serotype 3 antigen and a long RNA electropherotype) isolated in Japan was shown to be very closely related to a feline rotavirus strain FRV-1 (having the same properties) obtained from a cat in Japan. On the other hand, another human rotavirus strain Ro1845 isolated in Israel (which belongs to a group genetically different from that of strain AU-1) was shown to be closely related to another feline rotavirus strain Cat97 isolated in Australia[49]. In addition, the latter two strains were found to be genetically related to the canine strain RS15.

Overall genomic relatedness was also observed between equine and porcine rotaviruses[50]. The unique equine rotavirus strain H1 with G serotype 5 specificity (which is a common serotype among porcine rotaviruses) related very closely to porcine rotavirus strains Gottfried (G serotype 4), OSU (G serotype 5) and YM (G serotype 11).

A close genetic relationship between human and bovine or porcine rotaviruses was reported by Urasawa *et al*[36]. Strain Mc35, a rare human rotavirus isolated in Chiang Mai, Thailand (having subgroup I-G serotype 10 antigen and a long electropherotype) was shown by RNA-RNA hybridization to be more closely related to bovine rotaviruses (strains A44, 61A and A5 isolated in Thailand) than to any strain of human rotaviruses. Human rotavirus strain Mc323 (having subgroup I-G serotype 9

antigen and a long RNA electropherotype), also isolated in Chiang Mai, was more related genetically to porcine rotaviruses (strains Gottfried and OSU) than to any human rotavirus strains. Two human rotavirus strains from Bangkok with the same properties described in the present paper may be of a similar nature. Furthermore, close genomic relatedness of human rotavirus strains isolated from asymptomatically infected neonates in India (having subgroup I, G serotype 10 and a long electropherotype) to the bovine rotavirus strain B223 (having the same properties) was also reported by Das *et al.*[51]

These results give strong evidence for transmission of rotavirus between humans and animals and between various species of animals. Such interspecies-transmitted strains might rarely become adapted to long-term growth in the new host. Alternatively, due to the segmented nature of the rotavirus genome, a reassortant virus could occur by simultaneous infection of the host with the endogenous and the transmitted strains, and it might acquire the ability for steady growth. It is worthy of particular attention that such possible interspecies-transmitted strains or reassortant strains isolated from humans generally have subgroup I specificity and a long RNA electropherotype as described above.

In this series of studies, the authors demonstrated the predominance of G serotype 10 rotaviruses in both bovine and porcine populations in Thailand. Further, it should be emphasized that strain P343, the prevalent porcine rotavirus strain in Thailand, was very highly related genetically to strain 61A, the prevalent bovine rotavirus strain. In this respect, the high genetic similarity between bovine isolate 61A and the Thai human isolate Mc35 with the same G serotype specificity mentioned above arouses interest. These results strongly indicate that interspecies transmission of rotavirus is occurring between calves and piglets in Thailand. Further, it is likely that ecological conditions in Thailand that G serotype 10 bovine rotavirus predominates in cattle favored its transmission to humans and led to the emergence of a possible reassortant human rotavirus strain (like Mc35) with G serotype 10 specificity.

Emergence of new virus strains by interspecies transmission and genome reassortment of virus is very important in understanding the mechanism of evolution and diversification of rotavirus in nature. In this regard, continued studies of both human and animal rotaviruses are needed in developing countries where ecological circumstances in which people come in close contact with various animals facilitate the interspecies transmission of rotavirus.

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集成論文：

タイ国に浸淫しているヒトおよび動物ロタウイルスの血清学的並びに遺伝学的研究

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ロタウイルスは，外殻蛋白（VP4とVP7)，内殻蛋白（VP6）およびコア蛋白（VP1，2，3）の3蛋白層と内在する11分節の二本鎖ウイルスRNAより構成される．その分類はウイルス蛋白の抗原性あるいは分節RNAのポリアクリルアミドゲル上での電気泳動パターン(RNA-electropherotype)に基づいて行われている．VP6にはA群ウイルスに共通の群抗原（group A antigen）と亜群抗原（Subgroup antigen）I又はIIが存在する．主要な感染防御抗原であるVP7には中和反応で区別される少なくとも14のG血清型(G serotype)が確認されており，もう一方の外殻蛋白VP4にはその血清型から11のP血清型（P serotype)が，遺伝子の塩基配列から少なくとも19のVP4-genotypeが報告されている．この内ヒトウイルスでは7種類のG血清型と4種類のP血清型が知られている．

本研究の目的はタイ国においてヒト，ウシ，およびブタの下痢症例から多数の便検体を集め，一部培養細胞による分離実験を行い，A群ロタウイルスのVP7蛋白についてその抗原型及び一部の遺伝子の塩基配列を決定することにより，タイ国で流行しているA群ロタウイルスの種類，ヒト－動物間のウイルス伝播の可能性とこれによる新たなロタウイルス出現の可能性について検討することである．

研究方法

(1)便材料：ヒト下痢便検体は1983-84年および1987-88年に合計543検体，1988-91年に241検体，1991-94年に659検体を採取し実験に供した．ウシ便材料は1988年に25検体，1989年に45検体を採取し実験に用いた．ブタ便材料は1988年に18検体，1990-91年に採取した557検体を用いた．(2)便中ウイルスの培養細胞への馴化：10％糞便上清をMA-104細胞に接種してウイルス分離を行った．(3) RNA electropherotyping：ウイルスRNAをSDS，2-MEおよびEDTA処理後フェノール法により抽出し，10％ポリアクリルアミドゲルで電気泳動（PAGE）を行い，銀染色の後PAGEパターンを検討した．(4)酵素抗体法(ELISA法)によるウイルス抗原型の同定：VP6，VP4およびVP7に対する特異モノクロナール抗体を用いるELISA法により，群，亜群，G血清型を決定した．(5)中和抗体価の測定：蛍光抗体法を用い，感染を示す蛍光フォーカスを60％以上減少させる最高血清希釈倍数の逆数で中和抗体価を表した．(6) PCR法によるG血清型の決定：ELISA法によるG血清型別が不可能な便材料について，3′側および5′側の共通プライマーを用いるPCR法によりVP7全遺伝子を増幅し，これを鋳型として3′側共通プライマーと各血清型特異的5′側プライマーを用いて2回目のPCRを行った．最終産物を1％アガロースゲルで電気泳動を行いG血清型を同定した（プライマーは，増幅産物の分子量が各血清型により異なるように設定された)．(7) RNA-RNAハイブリダイゼーション：EDTA処理で外殻蛋白を除いた一重殻粒子を用いて*in vitro*で^{32}Pラベル一本鎖RNAプローブを作成し，これを検体ウイルスから抽出し熱処理した一本鎖ウイルスRNAと反応させ形成されたRNA－RNAハイブリッドをエタノール沈殿し，PAGE後RNAの相同性を検討した．(8) VP7遺伝子の塩基配列決定：EDTA処理で得た一重殻粒子を用いて*in vitro*でmRNAを合成した．mRNAに相補的なオリゴヌクレオチドを作成し，primer extension法で塩基配列を決定した．

結果とまとめ

1. タイ国，バンコクにおけるヒトのロタウイルス感染　1) 分離ロタウイルスの性状：1983-84年および1987-88年の第一回採取材料による調査では，下痢便材料543例中PAGEあるいはELISAでA群陽性は88例（16.2％）で，77例（87.5％）で亜群が決定された（亜群 I：20例，II：57例)．G血清型は58例（54.9％）で同定された（G1：18，G2：17，G4：23)

が，多数の型未決定株が残された．1988-91年の研究では，G血清型の決定率を高めるために，ELISA法で決定不可能な検体にはPCR法によるG血清型別を行った．ELISA法で241例中162例（67.2%），PCR法を行った63例中59例（93.7%）で血清型が同定された結果，最終的に241例中221例（91.7%）でG血清型が決定された．以上の基礎的研究法を基に1991-94年には，性別，年齢別および月別発生状況調査を加えて，659検体を採取して研究を進めた．A群陽性は243例（36.9%）で，その月別分布を見ると，年間を通して患者発生が見られるものの，9月-1月の比較的低温の期間に集中し，タイ国でも温帯諸国同様ロタウイルスが冬期間に流行していることが示された．ロタウイルス陽性患児の性比は1.36で男児で高く，年齢分布では，その殆どが乳児で6から11ケ月にピークを示した．ELISA法およびPCR法を併用したG血清型決定率は，243例中213例（87.7%）であった．2）A群ヒトロタウイルスG血清型の経年変動：1983-84年にはG4血清型が優勢で，1987-88年にはG1型およびG2型が共存し，1988-89年と，1989-90年の2年間はG1型が，1990-91年にはG3型に変わり，1991-92年には再びG1型が現れ，一年後に再びG2型が優勢になり，1993－94年には完全にG1型に置き換わる，という著明な年次変化が見られた．

2．タイ国における動物のロタウイルス　1）仔ウシロタウイルス：1988年および1989年に採取した70例中62例がELISA法でA群と決定され，それらは全てウシウイルスの特徴を示す亜群Iに属したが，ヒトウイルスの代表的なVP7血清型（G1，2，3，4）を示す例あるいは従来ウシの血清型として知られていたG6血清型を示す例は1例も認められなかった．これらのウイルスは，RNA-PAGEパターンから3タイプに分けられた．最も多数例を示したcタイプの代表61A株および次いで多いaタイプの代表A44株のVP 7遺伝子の塩基配列を決定した結果，共にG血清型10と判明した．パターンbのA5株は極く稀にヒトから分離されていたG血清型8の抗原性を有しながら，RNA-PAGEパターンなどヒトG血清型8ウイルスとは明らかに相異していた．2)仔ブタロタウイルス：1987年の18検体中13例がELISAおよびRNA-PAGEパターンからA群，亜群I，Long RNA electropherotypeで，G血清型の決定された7株全てがG3と同定された．1990年までに報告されていたブタウイルスのG血清型（G3，4，5，11）の多様性は本調査では認められなかった．そこで再度1990-91年に557例の下痢便を採取し検討した．RNA-PAGEでスクリーニングを行い26例でロタウイルス陽性，うち23例がA群と決定され，さらにB群ウイルスが1例，C群ウイルスが2例検出された．A群23例の中，ELISA-およびPCR-血清型別の結果，3株がG3，14株がG10と決定された．G血清型10のブタウイルスについては過去に報告がないが，その血清型特異性は交叉中和試験およびVP7遺伝子の塩基配列から確認された．

3．ヒトおよび動物ロタウイルスの関連性　通常，同一動物種由来のロタウイルスは，RNA-RNAハイブリダイゼーションの結果に基づく遺伝子の総体的類似性により複数種類のgenogroupに類別されている．従って，同じ種からの分離ウイルスは，種特有の何れかのgenogroupに分類される一方，宿主動物が異なるウイルス間では，類似性は極めて低いため，本法によりウイルスが由来した動物種を推定し得る．本研究において，中和反応およびVP7遺伝子の塩基配列からG10と同定されたタイ国のブタロタウイルス流行株を代表するP343株は，RNA-RNAハイブリダイゼーションにおいて既知の代表的ブタウイルスとは反応せず，同じくG10と同定されたタイ国のウシ由来流行株61A株を含むウシロタウイルスと高度の反応性を示したことから，本来ウシ由来のウイルスと考えられた．

11本の分節RNAを有するロタウイルスでは，*in vitro*および*in vivo*の実験系で同一種内あるいは異種ウイルス間で容易に遺伝子組み替えウイルスが形成されることが知られている．本研究の結果は，動物間あるいは動物とヒトが密接な接触機会を有するタイ国のような自然環境における種の障壁を超えたロタウイルスの伝播と，その結果としての遺伝子組み替えウイルス出現の可能性を示唆していると考えられる．