

**EVOLUTIONARY MECHANISMS OF *ROTAVIRUS A*  
AS STUDIED FROM MOLECULAR CHARACTERISATION OF  
THE G2 VP7 GENES AND A RARE G1P[6] STRAIN ISOLATED  
IN JAPAN**

By

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## **ABSTRACT**

### **Introduction**

*Rotavirus A* (RVA), belongs to genus *Rotavirus*, family *Reoviridae*, is a major cause of acute diarrhoea in infants and young children worldwide. Its genome comprises 11 segments of double-stranded RNA that encode 6 structural viral proteins (VPs) and 6 nonstructural proteins (NSPs).

The development of nucleotide sequencing technology over the last few decades made it increasingly feasible to characterise rotavirus strains at the level of nucleotide sequence, advancing the molecular epidemiology of RVA. When it comes to the targets of molecular epidemiological studies, two kinds of strains are to be distinguished; predominant strains and uncommon ones. The former are the strains that predominate and their information is crucial to develop preventive strategies such as vaccines. The latter are uncommon or novel strains and the characterisation of such exceptional strains often provide clues to understand how RVA evolves in nature.

Over the course of my PhD studies I have addressed the questions concerning the molecular epidemiology of RVA strains by characterising two actual examples of the two kinds of strains archived in the Department of Hygiene and Molecular Epidemiology, Graduate School of Biomedical Sciences, Nagasaki University to understand the evolutionary mechanisms of RVA. The first study was concerned with the molecular characterisation of the predominant strains: G2 strains detected in Japan during 31 years before the introduction of rotavirus vaccines. The second study was concerned with the molecular characterisation of an uncommon strain: a rare super-short strain named AU19 possessing genotype G1P[6] detected in 1997 in Japan. AU19 was the first and the only strain (until very recently) in terms of either “super-short” genome pattern or P[6] genotype in Japan.

## **The aims of the study**

The objective of the first study was to examine how the VP7 genes of G2 strains evolved over a 31 year period before the introduction of rotavirus vaccines in Japan. The objective of the second study was to determine the full-genome sequence of the AU19 strain to understand how such a rare P[6] rotavirus strain with a super-short RNA pattern emerged in nature. Through these two sets of specific studies, I aimed to gain a better understanding of how RVA strains evolved under unvaccinated conditions.

## **Materials and Methods**

From archival rotavirus-positive stool specimens collected from Japanese children with diarrhoea between 1980 and 2011, 35 G2 strains were chosen, and their VP7 genes were sequenced. I also sequenced 10 genome segments of the AU19 strain (as the VP7 gene was already sequenced and published previously). Phylogenetic analysis was conducted using the MEGA 5 software package. Amino acid substitutions were analysed at positions 87, 96, 213, and 242 in the G2 VP7 genes which were previously identified to carry evolutionary importance.

## **Results**

All Japanese G2 strains evolved along the stepwise changes from lineage III, lineage I, group IV except IVa and sub-lineage IVa (lineage II was absent in Japan). As a potential next step of the evolutionary change, I observed the emergence of what I named “a nascent lineage” outside of the currently and globally predominant lineage (i.e., sub-lineage IVa). This nascent lineage contained a D96N substitution, which is the hallmark of modern G2 lineage (i.e., sub-lineage IVa) and was previously shown to get involved in the change in neutralisation specificity of G2 strains.

Full-genome sequence analysis revealed that all genome segments of AU19 were more closely related to those of porcine RVA strains than those of human RVA strains except the NSP5 gene. The NSP5 genome segment contained a rare rearranged NSP5 sequence that was typed as H2 (by the online typing tool of RotaC). It belonged to H2b sub-genotype but distantly related to the H2b sequences previously identified in human RVA strains. These observations indicated that AU19 emerged as a consequence of complex evolutionary events: interspecies transmission of a porcine rotavirus to a child coupled with acquisition of a rare rearranged NSP5 gene (H2b genotype) by genetic reassortment probably from a co-circulating human RVA strain (because the H2b genotype was known to occur only in human RVA). The finding of a rare H2b NSP5 gene of AU19 which was originated from human rotavirus made the genetic diversity of H2b genotype as diverse as that of the H2a genotype, leading to the hypothesis that super-short strains carrying H2b genotype have long been circulating unnoticed in the human population.

## **Conclusions**

Molecular characterisation of Japanese RVA strains analysed in this thesis revealed: (1) the genes of RVA strains are likely to evolve in nature through the accumulation of point mutations resulting in the emergence of a new lineage in a stepwise fashion (a lineage followed by another) as exemplified by the nascent lineage in the G2 VP7 gene; (2) interspecies transmission of animal RVA strains to humans (and subsequent genetic reassortment) is likely to have played an important evolutionary role, when succeeded in establishing a human-to-human transmission chain, as exemplified by a rare porcine-like human RVA strain AU19 (although AU19 appears to have ended in dead-end infection). However, it remains to be seen whether the accumulation of point mutations will be accelerated and interspecies reassortant strains will have a survival advantage over co-circulating human RVA strains under selective pressures imposed by wide-spread use of vaccines. Further studies are, therefore, warranted to monitor at the full-genome level if such

emerging strains will spread globally. Such studies should help to ensure the successful use of vaccines and to explain the vaccine failure if it occurs.

## **CHAPTER 1. INTRODUCTION**

*Rotavirus A*, a member of the genus *Rotavirus*, family *Reoviridae*, is a major cause of acute diarrhoea in infants and young children where rotavirus vaccines are not introduced into the national immunisation programmes (Tate et al., 2012). Molecular epidemiology of RVA is concerned with the understanding of the distribution of wild-type strains in the temporal, geographical and population's perspectives as well as with the evolution of RVA. The targets of molecular epidemiological studies are roughly divided into strains that constitute the majority (predominant strains) and uncommon strains; the former provides the basis on which preventive strategies are built and the latter provides clues to understand the evolution of RVA in nature. Two outer capsid proteins, VP7 and VP4, are independently involved in virus neutralisation and define the G type and P type, respectively, have been paid much attention because of its importance in protective immunity. Although there are 27 G types and 37 P types described in the literature, the vast majority of human RVA strains have one of the 6 major G and P type combinations; G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] (Banyai et al., 2012, Doro et al., 2014, Gentsch et al., 2005, Matthijnssens et al., 2011, Santos and Hoshino, 2005, Trojnar et al., 2013).

Since 2009 when two globally licensed vaccines were recommended to be included in national immunisation programmes by the World Health Organisation (WHO) (WHO, 2009), the concern was raised about the effectiveness of these vaccines to the G2 strains which were partly or fully heterotypic to the vaccine strains. A series of studies showed natural fluctuation of the relative frequency of circulating G2 strains and the relationship of amino acid substitutions occurring in the VP7 gene with the re-emergence or an increase in frequency of G2 strains (Antunes et al., 2009, Esteban et al., 2010, Iturriza-Gomara et al., 2009, Kirkwood et al., 2011, Luchs et al., 2011, Martinez et al., 2010, Santos and Hoshino, 2005, Snelling et al., 2011, Vieira et al., 2011). In addition, the D96N substitution in the VP7 gene was the

salient feature of G2 strains circulating in the world for the last decade, even when and where the rotavirus vaccines were introduced (Doan et al., 2011).

Studies based on G and P genotypes alone may miss differences in the genotype constellation that only occur in the internal capsid and non-structural protein genes where human rotavirus-specific genotypes are replaced with unusual or animal rotavirus-specific genotypes. The whole genome sequences studies include the remaining capsid and non-structural protein genes enabling us to study rotaviruses as a whole rather than just the outer layer of the virion (Matthijnssens et al., 2008a, Matthijnssens et al., 2011). The two prototype strains, Wa and DS-1, which represent two distinct RNA migration patterns: long and short electropherotypes, are described as G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 and G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, respectively (Matthijnssens et al., 2008a). Super-short electropherotypes may infrequently appear with the rearranged 11<sup>th</sup> genome segment making it become longer than that of short electropherotype strains (Dyall-Smith and Holmes, 1981, Kutsuzawa et al., 1982). A rare super-short human RVA strain, named AU19, was shown to carry a unique P[6] VP4 gene and it was later suspected to be originated from porcine rotavirus when some Japanese porcine RVA strains were investigated for their VP4 genes (Ahmed et al., 2007, Nakagomi et al., 1999, Teodoroff et al., 2005). Although rare/usual/minority strains may not immediately affect the public health of people as, by definition they are very few in numbers, they are likely to give us a clue to understand the evolutionary mechanisms of RVA strains under natural conditions.

Over the course of my PhD studies I have addressed the issues concerning the evolutionary mechanisms of RVA strains through molecular characterisation of two actual examples archived in the Department of Hygiene and Molecular Epidemiology. The first study was concerned with the molecular characterisation of the predominant strains: G2 strains detected in Japan during 31 years before the introduction of rotavirus vaccines. The second study was concerned with the molecular characterisation of an uncommon strain: a rare super-short strain named AU19 possessing genotype G1P[6] detected in 1997 in Japan.

AU19 was the first and only strain (until very recently) in terms of either “super-short” genome pattern or P[6] genotype.

The objective of the first study was to examine how the G2 VP7 gene possessed by RVA strains evolved over a 31 year period before the introduction of rotavirus vaccines in Japan. The objective of the second study was to determine the full-genome sequence of the AU19 strain to understand how such a rare P[6] rotavirus strain with a super-short RNA pattern emerged in nature. Through these two sets of specific studies, I aimed to have a better understanding how RVA strains evolved under unvaccinated conditions.



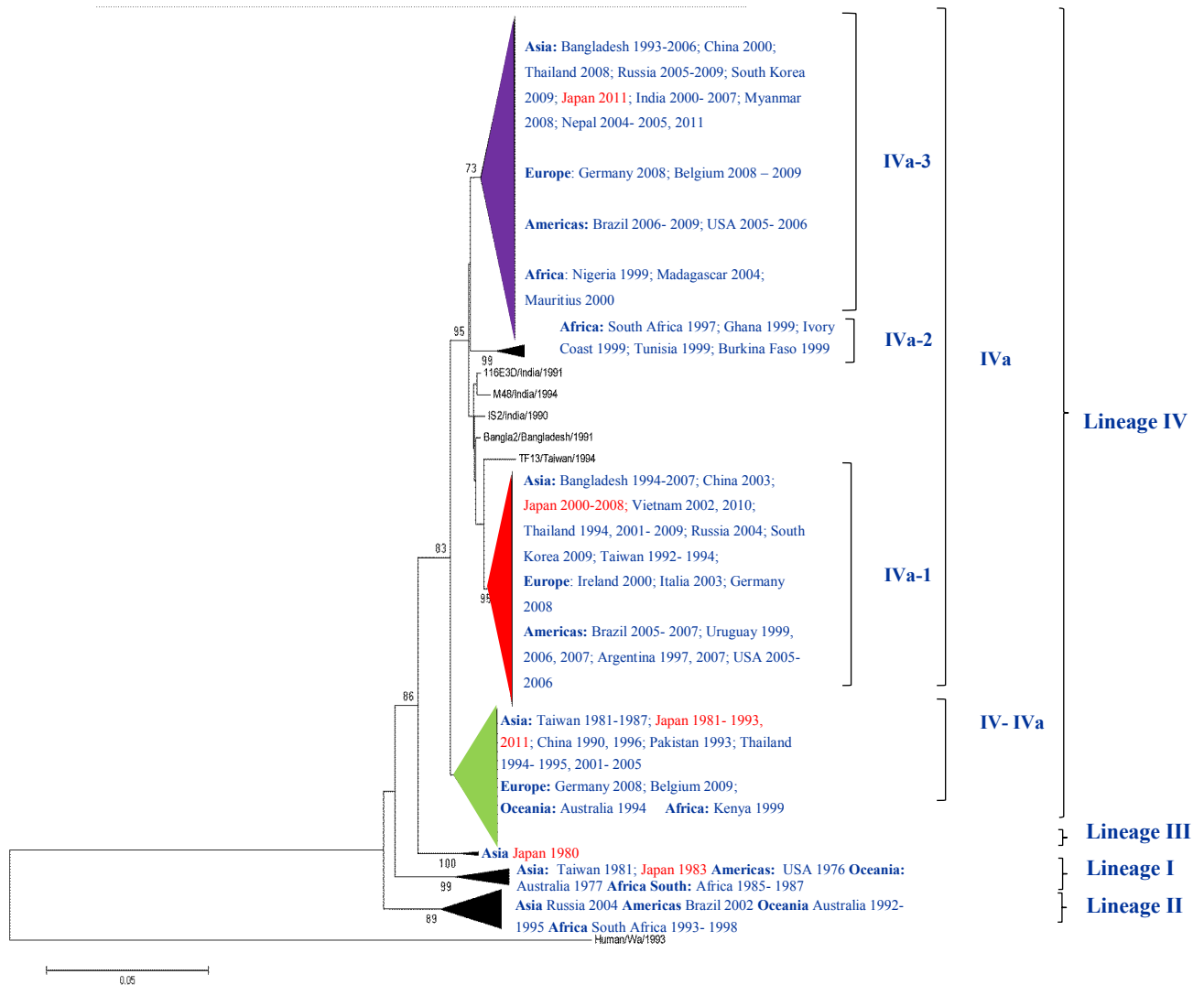
## **CHAPTER 2. RESULTS**

In this thesis, I analysed the nucleotide sequences of the VP7 genes of the 35 G2P[4] rotavirus strains collected between 1983 and 2014 and the nearly full genome sequence of a rare G1P[6] AU19 strain detected in 1997 in Japan.

### **2.1. Molecular evolution of the VP7 gene of Japanese G2 rotaviruses before vaccine introduction**

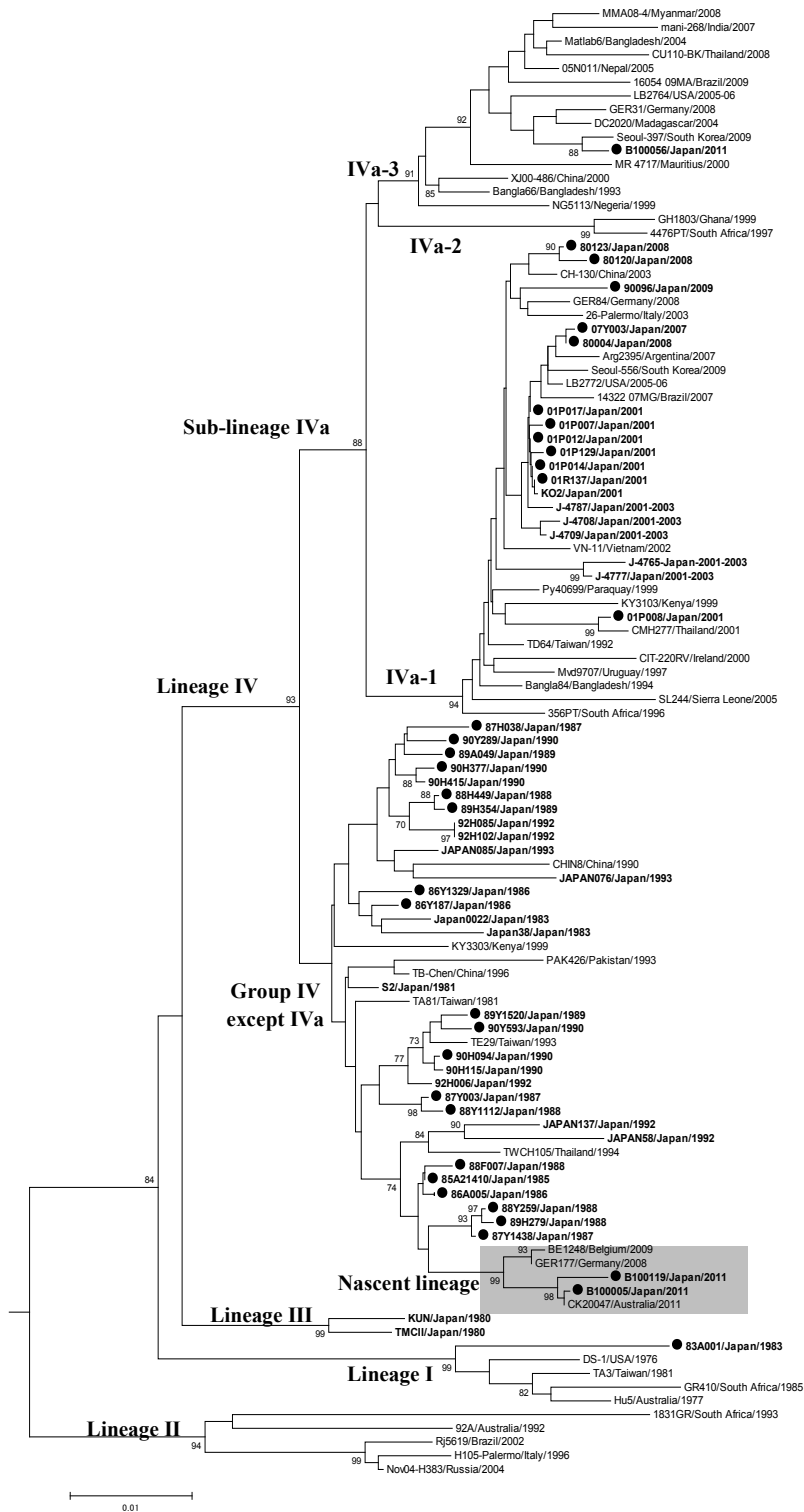
The sequences of 35 G2 rotavirus strains determined in this study were compiled with 20 Japanese G2 VP7 sequences available in the GenBank database to analyse the dataset that contained a total of 55 G2 VP7 sequences from Japanese strains detected over the period of 31 years (1980-2011). There was, however, a 7-year period (1993-2000) in which no VP7 sequence was available for G2 strains in the literature.

The phylogenetic tree was constructed using the 55 Japanese G2 VP7 nucleotide sequences along with 488 G2 sequences that were obtained from the DNA databases (Fig.2.1). Four lineages and four sub-lineages were identified in the phylogenetic tree as defined by Doan et al. (2011) as shown in Fig.2.1 where lineages and sub-lineages were collapsed to make the topological relationship clearer.



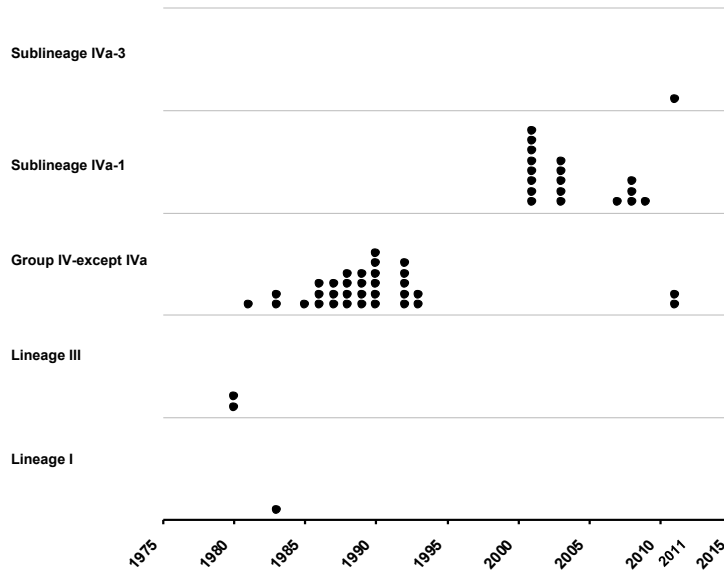
**Fig.2.1.** A phylogenetic tree based on the global G2 VP7 nucleotide sequences. The tree was constructed using the neighbor-joining method with bootstrap probabilities after 1,000 replicate trials. The lineages and sub-lineages are collapsed to make the topological relationship clearer. At the right-hand side of the figure are the lineages and sub-lineages defined by Doan et al (2011). The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

Fig.2.2 is a phylogenetic tree contained all 35 Japanese G2 VP7 sequences determined in this study (with dot) along with some G2 sequences representative of the lineages and sub-lineages defined previously (Doan et al., 2011). The Japanese G2 VP7 sequences clustered into three lineages: lineage I, III and IV. Lineage I, which the VP7 sequence of DS-1, the prototype G2 strain, belonged to, contained only one Japanese G2 strain isolated in 1983 (83A001). The nucleotide sequence identities between 83A001 and other G2 VP7 sequences in this lineage including those from Taiwanese, South African and Australian strains were >97.9%. Lineage III contained only two Japanese strains which were detected in a single year of 1980, and they were 99.9% identical in the nucleotide sequence. The vast majority of Japanese G2 strains (50/54) belonged to lineage IV, which further divided into three independent groups. Two thirds (33) of strains belonged to one group whose bootstrap value was only 62%; hence incapable of being named as sub-lineage. One third (18) of the remaining strains clustered into sub-lineage IVa-1 with a bootstrap value of 94% and the mean p-distance within this group was 0.008. Only one strain, B100056, detected in 2011 belonged to sub-lineage IVa-3.



**Fig.2.2.** A phylogenetic tree based on 35 Japanese G2 VP7 nucleotide sequences determined in this study (indicated in boldface with dots) together with 20 Japanese G2 VP7 nucleotide (in boldface) and representative sequences obtained from the GenBank database. A nascent lineage is highlighted in grey shadow. The tree was constructed using the neighbor-joining method with bootstrap probabilities after 1,000 replicate trials. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger. Five porcine G2 strains were used as the outgroup strains, which are not shown in the tree. Only the node where the root is located is shown.

When the lineage distribution of Japanese G2VP7 sequences was examined along the timeline from 1980 to 2011, a few observations were made. There was the co-circulation of Japanese G2 strains that belonged to three lineages: lineage I, lineage III and group IV except IVa in the early 1980s (Fig.2.3). Japanese G2 strains in lineages I and III never appeared again since the early 1980s. In contrast, G2 strains belonging to group IV except IVa continued to circulate much longer until 1993 and with an interruption of 18 years, two Japanese G2 strains (B100005, B100119) belonging to this group re-emerged in 2011 (Fig.2.3). These two Japanese strains formed a nascent lineage supported by a high bootstrap probabilities of 99% with other three G2 strains detected in Germany in 2008 (GER177-08), detected in Belgium in 2009 (BE1248) and detected in Australia in 2011 (CK20047) (Fig.2.2). Other than these two strains, after 2000, most of the rotavirus G2 strains in Japan belonged to sub-lineage IVa-1. In 2011, we noticed one strain (B100056) belonging to sub-lineage IVa-3 in Japan (Fig.2.3).



**Fig.2.3.** The distribution of G2 strains in Japan by the year of detection and the lineages to which they belonged. Each dot represents one strain.

With respect to the four amino acid positions 87, 96, 213 and 242 in the VP7 antigenic regions previously described as potential epitopes for G2 strains by Doan et al. (Doan et al., 2011), the 83A001 strain, the only Japanese G2 strain belonging to lineage I, had the same amino acid pattern with the DS-1 strain, the reference strain of lineage I: A-D-N-N at positions 87, 96, 213 and 242, respectively (Fig.2.4). Two Japanese G2 strains detected in 1980 belonging to lineage III that had two amino acid substitutions A87T and N242S; hence, these sequences had an amino acid constellation T-D-N-S (Fig.2.4). All Japanese G2 sequences belonging to group IV except IVa had the same substitution N213D and N242S from the amino acid constellation of the lineage I strains (Fig.2.4). A further amino acid substitution D96N was noted in five G2 strains in this group that were detected after 2007 and they belonged to the nascent lineage; hence two different amino acid constellations, A-D-D-S and A-N-D-S, in the group IV except IVa (Fig.2.4). The amino acid constellation of sub-lineage IVa-1 was T-N-D-S which was completely different from that of DS-1 (Fig.2.4). One peculiar exception to the rule for sub-lineage IVa-1 was a strain detected in Nara Prefecture in 2009, 90096, which contained glycine at residue 96, a very unusual amino acid at this position (Fig.2.4). Finally, in 2011, a strain belonging sub-lineage IVa-3 with the amino acid constellation T-N-D-N appeared for the first time in Japan (Fig.2.4).

				Antigenic region (aa position)			
				A	A	C	F
				(87)	(96)	(213)	(242)
DS-1	USA	1976		A	D	N	N
<b>Lineage I</b>							
DS-1	USA	1976		A	D	N	N
Hu5	Australia	1977		A	D	N	N
TA3	Taiwan	1981		A	D	N	N
83A001	Japan	1983		A	D	N	N
GR410	South Africa	1985		A	D	N	N
<b>Lineage II</b>							
92A	Australia	1992		A	D	S	S
1831GR	South Africa	1993		A	D	S	S
H105-Palermo	Italy	1996		T	D	S	S
Rj5619	Brazil	2002		T	D	N	S
Nov04-H383	Russia	2004		T	D	S	S
<b>Lineage III</b>							
KUN	Japan	1980		T	D	N	S
TMCI	Japan	1980		T	D	N	S

				Antigenic region (aa position)			
				A	A	C	F
				(87)	(96)	(213)	(242)
DS-1	USA	1976		A	D	N	N
<b>Group IV except IVa</b>							
S2	Japan	1981		A	D	D	S
TA81	Taiwan	1981		T	D	D	S
Japan022	Japan	1983		A	D	D	S
Japan038	Japan	1983		A	D	D	S
85A21410	Japan	1985		A	D	D	S
86A005	Japan	1986		A	D	D	S
86Y187	Japan	1986		A	D	D	S
86Y1329	Japan	1986		A	D	D	S
87Y003	Japan	1987		A	D	D	S
87H038	Japan	1987		A	D	D	S
87Y1438	Japan	1987		A	D	D	S
88F007	Japan	1988		A	D	D	S
88Y259	Japan	1988		A	D	D	S
88H449	Japan	1988		A	D	D	S
88Y1112	Japan	1988		A	D	D	S
89A049	Japan	1989		A	D	D	S
89H279	Japan	1989		A	D	D	S
89H354	Japan	1989		A	D	D	S
89Y1520	Japan	1989		A	D	D	S
90Y289	Japan	1990		A	D	D	S
90H377	Japan	1990		A	D	D	S
90H415	Japan	1990		A	D	D	S
CHIN8	China	1990		A	D	D	S
90H094	Japan	1990		A	D	D	S
90H115	Japan	1990		A	D	D	S
90Y593	Japan	1990		A	D	D	S
92H006	Japan	1992		A	D	D	S
92H085	Japan	1992		A	D	D	S
92H102	Japan	1992		A	D	D	S
JAPAN58	Japan	1992		A	D	D	S
JAPAN076	Japan	1993		A	D	D	S
JAPAN085	Japan	1993		A	D	D	S
JAPAN137	Japan	1992		A	D	D	S
PAK426	Pakistan	1993		A	D	D	S
TE29	Taiwan	1993		A	D	D	S
TWCH105	Thailand	1994		A	D	D	S
TB-Chen	China	1996		A	D	D	S
KY3303	Kenya	1999		A	D	D	S
<b>Nascent lineage</b>							
GER177	Germany	2008		A	N	D	S
BE1248	Belgium	2009		A	N	D	S
CK20047	Australia	2011		A	N	D	S
B100005	Japan	2011		A	N	D	S
B100119	Japan	2011		A	N	D	S

				Antigenic region (aa position)			
				A	A	C	F
				(87)	(96)	(213)	(242)
DS-1	USA	1976		A	D	N	N
<b>Sub-lineage IVa</b>							
<b>Sublineage IVa-1</b>							
TD64	Taiwan	1992		T	N	D	S
Bangla84	Bangladesh	1994		T	N	D	S
356PT	South Africa	1996		T	N	D	S
Mvd9707	Uruguay	1997		T	N	N	S
KY3103	Kenya	1999		T	N	D	S
Py40699	Paraguay	1999		T	N	D	S
CIT-220RV	Ireland	2000		T	N	N	S
KO2	Japan	2001		T	N	D	S
01P007	Japan	2001		T	N	D	S
01P008	Japan	2001		T	N	D	S
01P012	Japan	2001		T	N	D	S
01P014	Japan	2001		T	N	D	S
01P017	Japan	2001		T	N	D	S
01P129	Japan	2001		T	N	D	S
01R137	Japan	2001		T	N	D	S
CMH277	Thailand	2001		T	N	D	S
J-4708	Japan	2001-2003		T	N	D	S
J-4709	Japan	2001-2003		T	N	D	S
J-4765	Japan	2001-2003		T	N	D	S
J-4777	Japan	2001-2003		T	N	D	S
J-4787	Japan	2001-2003		T	N	D	S
VN-11	Vietnam	2002		T	N	D	S
CH-130	China	2003		T	N	D	S
26-Palermo	Italy	2003		T	N	D	S
SL244	Sierra Leone	2005		T	N	D	S
LB2772	USA	2005-2006		T	N	D	S
Arg2395	Argentina	2007		T	N	D	S
14322/07MG	Brazil	2007		T	N	D	S
07Y003	Japan	2007		T	N	D	S
80004	Japan	2008		T	N	D	S
80120	Japan	2008		T	N	D	S
80123	Japan	2008		T	N	D	S
GER84	Germany	2008		T	N	D	S
Seoul-556	South Korea	2009		T	N	D	S
90096	Japan	2009		T	G	D	S
<b>Sublineage IVa-2</b>							
GH1803	Ghana	1999		T	N	D	S
4476PT	South Africa	1997		T	N	D	S
<b>Sublineage IVa-3</b>							
Bangla66	Bangladesh	1993		T	N	D	N
NG5113	Nigeria	1999		T	N	D	N
MR 4717	Mauritius	2000		T	N	D	N
XJ00-486	China	2000		T	N	D	N
DC2020	Madagascar	2004		T	N	D	N
Matlab6	Bangladesh	2004		T	N	D	N
05N011	Nepal	2005		T	N	D	N
LB2764	USA	2005-2006		T	N	D	N
mani-268	India	2007		T	N	D	N
GER31	Germany	2008		T	N	D	N
MMA08-4	Myanmar	2008		T	N	D	N
CU110-BK	Thailand	2008		T	N	D	N
Seoul-397	South Korea	2009		T	N	D	N
16054 09MA	Brazil	2009		T	N	D	N
B100056	Japan	2011		T	N	D	N

**Fig.2.4.** The amino acid constellation at residues 87, 96, 213 and 242 in the VP7 antigenic regions of various G2 strains shown in Fig.2.2 including those detected in Japan between 1980 and 2011.

## **2.2. A rare G1P[6] super-short human rotavirus strain carrying an H2 genotype on the genetic background of a porcine rotavirus**

In this study, we determined nearly-full nucleotide sequences of the genome segments coding for VP4, VP6, VP1-VP3, and NSP1- NSP5 genes of AU19 (Table 2.1).

**Table 2.1.** The position and length of the sequence determined for each the 10 genome segments of AU19

<b>Segment</b>	<b>Positions and length of sequence</b>	<b>Accession number</b>	<b>Reference</b>
VP4	23-2312 (2289)	AB770153	This study
VP6	151-1307 (1156)	AB770154	This study
VP1	28-3245 (3217)	AB770155	This study
VP2	65-2657 (2592)	AB770156	This study
VP3	87-2541 (2454)	AB770157	This study
NSP1	198 - 1363 (1166)	AB770158	This study
NSP2	48 - 1008 (961)	AB770159	This study
NSP3	58 -948 (891)	AB770160	This study
NSP4	51 - 738 (688)	AB770161	This study
NSP5	25 - 928 (903)	AB770162	This study



In consistent with its super-short electropherotype, the entire length of NSP5 gene of AU19 was 949 nucleotides, 128 and 329 nucleotides longer than DS-1 (a short electropherotype) and Wa (a long electropherotype), respectively, although the last 24 and 20 nucleotides of 5' and 3' ends, respectively, were those of PCR primers. According to the whole genome-based genotyping system proposed by the Rotavirus Classification Working Group (RCWG) (Maes et al., 2009, Matthijnssens et al., 2008b) the genotype of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of AU19 was assigned to G1-P[6]-I5-R1-C1-M1-A8-N1-T1-E1-H2. Since I5 and A8 genotypes are exclusively carried by porcine and porcine-like human rotaviruses (Matthijnssens et al., 2008a) the genotype constellation of AU19 was compared with that of selected human and porcine rotavirus strains as well as super-short human strains (Table 2.2).

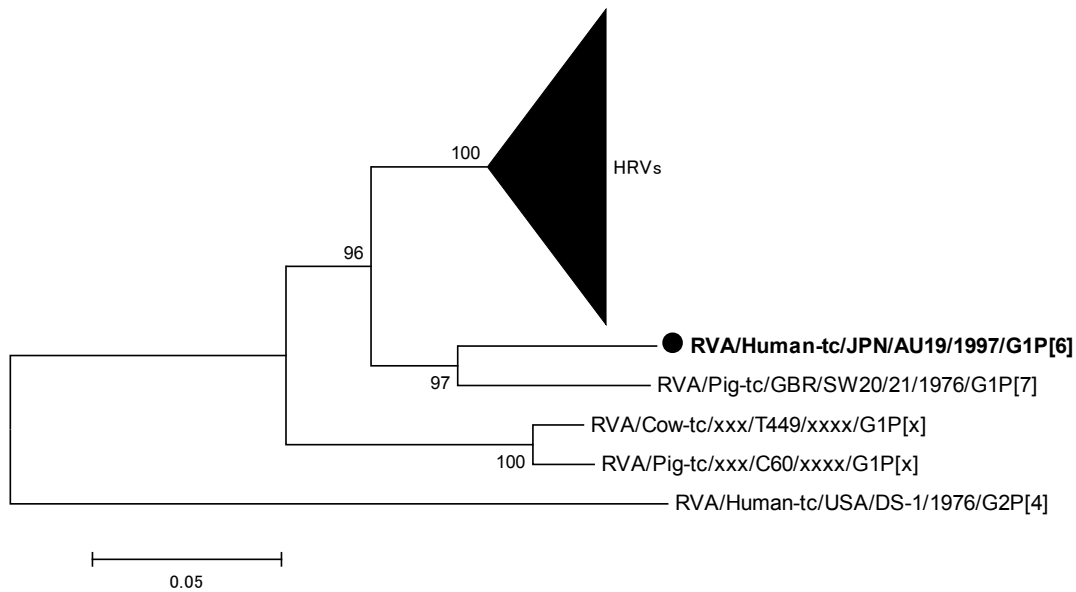
**Table 2.2.** Comparison the genotype constellation of AU19 with other human and porcine rotavirus strains including reference strains, Wa and DS-1. Strain AU19 is in bold. The genome segments that have a genotype identical to that of AU19 are highlighted in gray. The gene segment whose sequence is the closest to that of AU19 is in bold and italicized. “-” indicates that no sequence data was available in the GenBank database.

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Reference
<b>RVA/Human-tc/JPN/AU19/1997/G1P[6]</b>	<b>G1</b>	<b>P[6]</b>	<b>I5</b>	<b>R1</b>	<b>C1</b>	<b>M1</b>	<b>A8</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	<b>H2</b>	<b>This study</b>
RVA/Human-tc/USA/Wa/1974/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	Matthijnssens et al., 2008a
RVA/Pig-tc/GBR/SW20/21/1976/G1P[7]	<b>G1</b>	P[7]	-	-	-	-	-	-	-	-	-	El-Attar et al., 2001
RVA/Human-tc/USA/DS-1/1974/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	<b>H2</b>	Matthijnssens et al., 2008a
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3	Isegawa et al., 1992, Nakagomi and Kaga, 1995, Rahman et al., 2007
RVA/Pig-tc/USA/Gottfried/1975/G4P[6]	G4	<b>P[6]</b>	I1	R1	C1	M1	<b>A8</b>	<b>N1</b>	<b>T1</b>	<b>E1</b>	H1	Matthijnssens et al., 2010b
RVA/Human-tc/IDN/57M/1980/G4P[10]	G4	P10]	I1	R1	C1	M1	A1	<b>N1</b>	T2	E1	<b>H2</b>	Ghosh et al., 2013
RVA/Pig-tc/USA/OSU/1975/G5P[7]	G5	P[7]	<b>I5</b>	R1	<b>C1</b>	M1	A1	N1	<b>T1</b>	E1	H1	Matthijnssens et al., 2010b
RVA/Human-wt/JPN/Ryukyu-1120/2011/G5P[6]	G5	P[6]	<b>I5</b>	R1	C1	<b>M1</b>	A8	N1	<b>T1</b>	E1	H1	Komoto et al., 2013

RVA/Horse-tc/GBR/H-1/1975/G5P[6]	G5	P[7]	I5	<b>R1</b>	C1	M1	A8	N1	T1	E1	H1	Ghosh et al., 2012a
RVA/Human-tc/IND/69M/1980/G8P[10]	G8	P[10]	I2	R2	C2	M2	A2	N2	T2	E2	<b>H2</b>	Matthijnssens et al., 2008a
RVA/Human-tc/IDN/B37/197x/G8P[10]	G8	P[10]	-	-	-	-	-	-	-	-	<b>H2</b>	Hum et al., 1989
RVA/Pig-wt/JPN/JP3-6/2002/G9P[6]	G9	<b>P[6]</b>	-	-	-	-	-	-	-	-	-	Teodoroff et al., 2005
RVA/Human-wt/BEL/BE2001/2009/G9P[6]	G9	P[6]	<b>I5</b>	R1	C1	M1	A8	N1	T7	E1	H1	Zeller et al., 2012
RVA/Human-tc/THA/Mc323/1989/G9P[19]	G9	P[19]	I5	R1	C1	M1	A8	N1	T1	E1	H1	Ghosh et al., 2012b
RVA/Human-tc/THA/Mc345/1989/G9P[19]	G9	P[19]	I5	R1	C1	M1	A8	N1	T1	E1	H1	Ghosh et al., 2012b
RVA/Human-wt/BEL/B4106/2000/G3P14]	G3	P[14]	I2	R2	C2	M3	A9	N2	T6	E5	H3	Matthijnssens et al., 2006
RVA/Human-wt/ARG/Arg4605/2006/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T7	E1	H1	Degiussepe et al., 2013
RVA/Human-wt/IND/mani362/07/2007/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	Mukherjee et al., 2010
RVA/Human-wt/IND/mani253/07/2007/G4P[6]	G4	P[4]	I1	R1	C1	M2	A8	N1	T1	E1	H1	Mukherjee et al., 2010
RVA/Human-wt/IND/mcs/13-07/2007/G9P[6]	G9	P[6]	I1	R1	C1	M1	<b>A8</b>	N1	T1	E1	H1	Mukherjee et al., 2010
RVA/Human-wt/HUN/BP1901/1991/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T7	E1	H1	Papp et al., 2013a

Phylogenetic trees were constructed for the genes encoding the structural and non-structural proteins of AU19 (indicated with a black dot and in bold) with other human and animal (mainly porcine) rotavirus strains as references for the host species of origin. The trees were constructed using the neighbour-joining method included in the MEGA 5 software package. The trees were statistically supported by bootstrapping with 1000 replicates, and rooted with sequences of different genotypes. The actual lengths used for phylogenetic tree construction for the VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5 genes were 981, 501, 1157, 3218, 2593, 2455, 1166, 961, 891, 688, and 904 nucleotides, respectively.

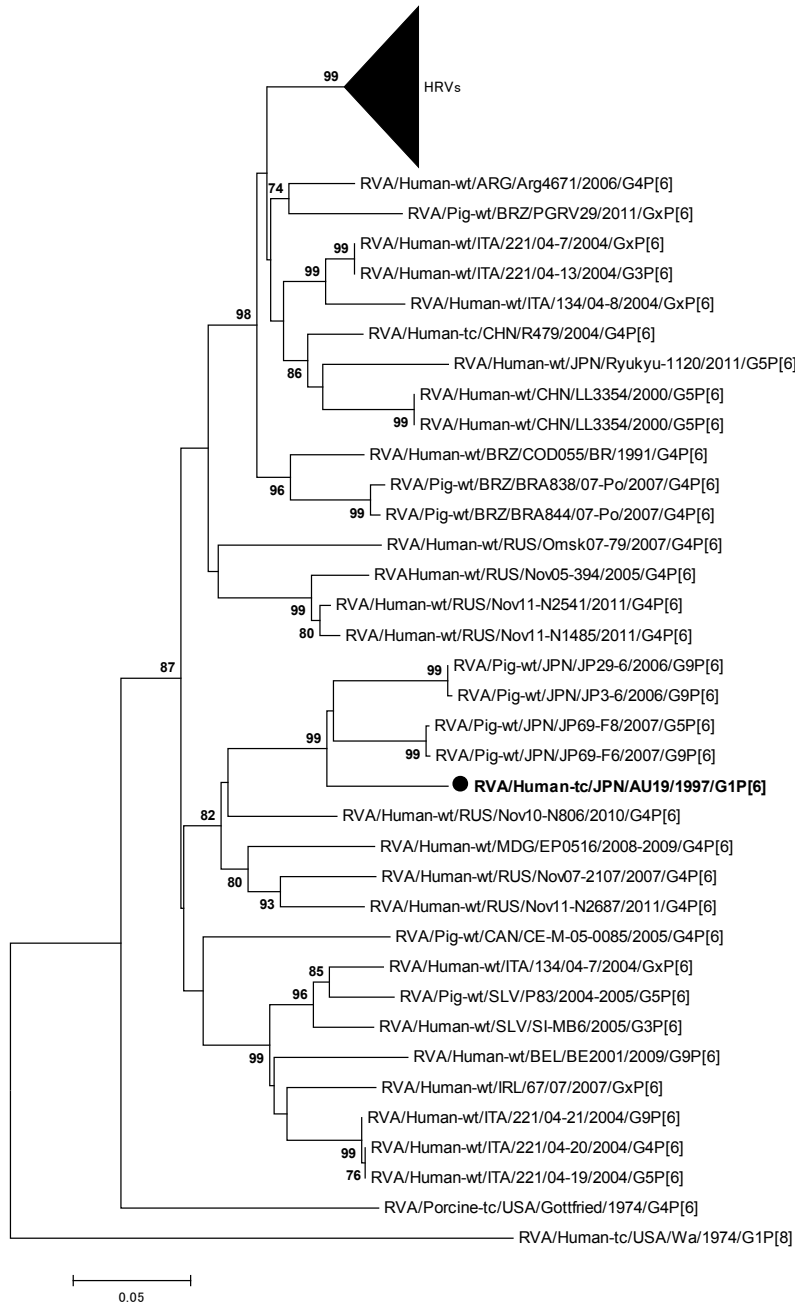
In the phylogenetic analysis, the major observations for each gene were as follows. The VP7 phylogenetic tree shown in Fig.2.5 was drawn by including the first 100 sequences that were hit when the AU19 VP7 sequence was blasted against the GenBank database. The AU19 sequence clustered together with the VP7 sequence of a G1 porcine strain SW20/21 isolated in UK in 1976 with a bootstrap probability of 97% (El-Attar et al., 2001). The sequence identity between AU19 and SW20/21 was 90.6% at the nucleotide sequence level and 95% at the amino acid sequence level. On the other hand, the lineage that contained AU19 and SW20/21 was distinct from the lineage that all other human rotavirus strains clustered into (shown as a triangle for condensation in Fig.2.5). The average sequence identity between AU19 and human rotaviruses in the aggregated lineage was 87.5% (87.2% - 88.6%) at the nucleotide level and 92.2% (91.6% - 93.1%) at the amino acid level.



**Fig.2.5.** Phylogenetic trees for the VP7 gene of AU19 (indicated with a black dot and in bold) with other human, porcine and bovine rotavirus strains as references for the host species of origin. The trees were statistically supported by bootstrapping with 1000 replicates, used the neighbor-joining method included in the MEGA 5 software package, and rooted with DS-1 sequences as outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger. An abbreviated term of HRVs is used to denote human rotaviruses.

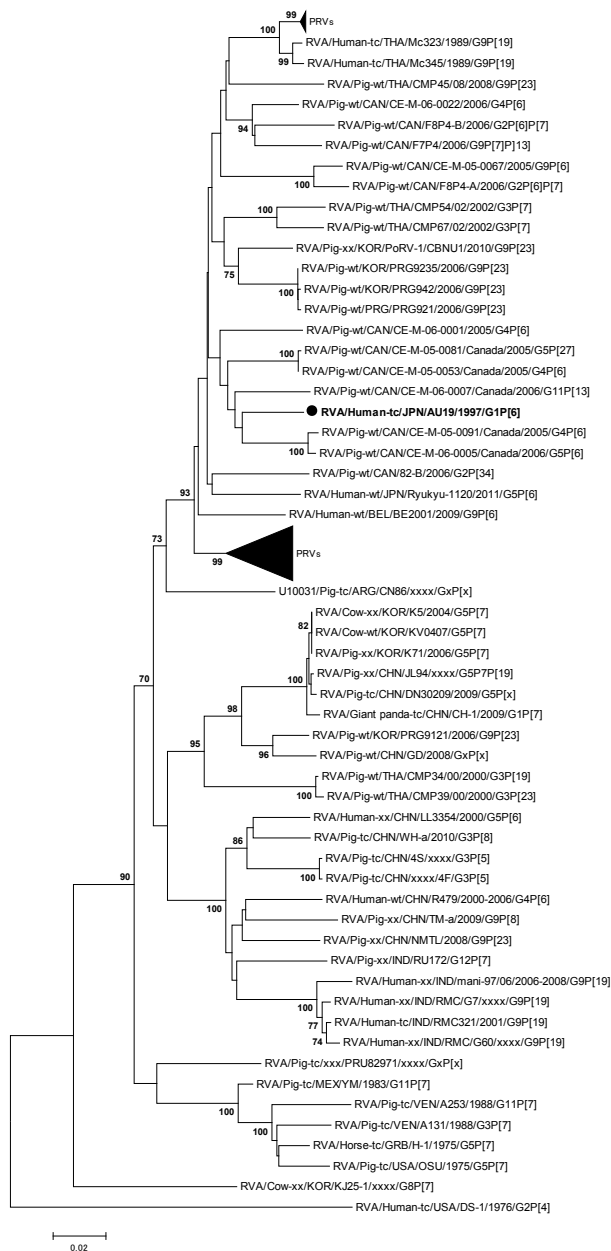
In the VP4 phylogenetic tree which the VP8 regions of the first 100 sequences that were hit when the AU19 VP4 sequence was blasted against the GenBank database, the AU19 clustered into a lineage with a 99% bootstrap probability that contained four porcine rotaviruses detected in Japan (Fig.2.6). The average sequence identity between AU19 and the four Japanese porcine rotavirus strains was 90.9% (90.8% - 91.1%) at the nucleotide level and 83.2% (82.4% - 84.3%) at the amino acid level. This lineage was distinct from the lineage that the vast majority of human P[6] strains were clustered into (shown as a triangle for condensation in Fig.2.6), and also distinct from the lineage that contained either

Ryukyu-1120 (Komoto et al., 2013) or BE2001 (Zeller et al., 2012), two human rotavirus strains that were recently shown by full-genome sequencing to be of porcine rotavirus origin.



**Fig.2.6.** Phylogenetic tree of the partial VP8 portion of the VP4 gene of AU19 (indicated with a black dot and in bold) with other human and porcine rotavirus strains. The trees were constructed by using the neighbor-joining method included in the MEGA 5 software package, rooted with Wa sequences as outgroup and statistically supported by bootstrapping with 1000 replicates. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

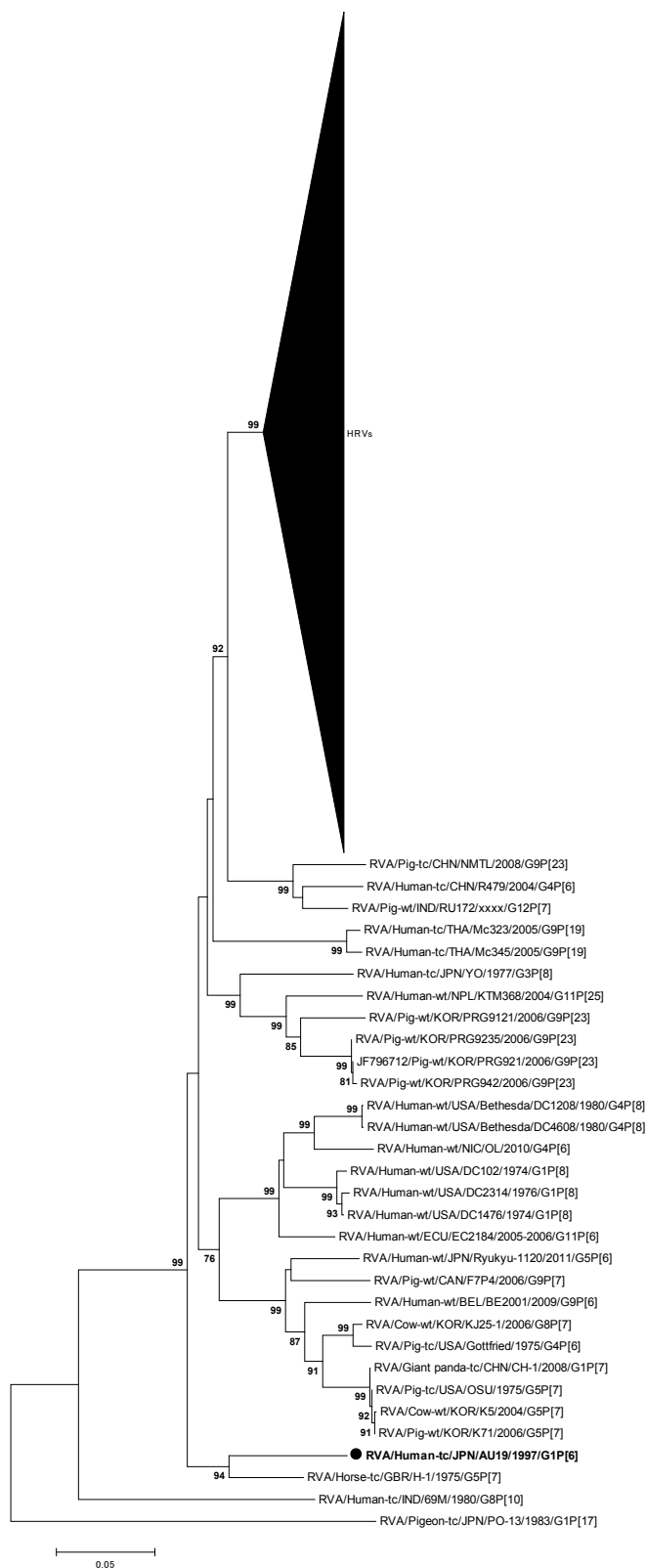
In the VP6 phylogenetic tree which all 15 sequences were included, the vast majority were porcine rotaviruses as is known that I5 genotype is specific to porcine rotaviruses (Matthijnssens et al., 2008b). AU19 clustered together with porcine-like human rotavirus strains Ryukyu-1120 (Komoto et al., 2013), BE2001 (Zeller et al., 2012) and other porcine rotaviruses in the lineage which was only marginally supported statistically with a 93% bootstrap probability (Fig.2.7).



**Fig.2.7.** Phylogenetic tree of the VP6 gene of AU19 (indicated with a black dot and in bold) with other human and porcine RVA strains. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. DS-1 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger. Abbreviated term of PRVs is used to denote porcine RVA strains.

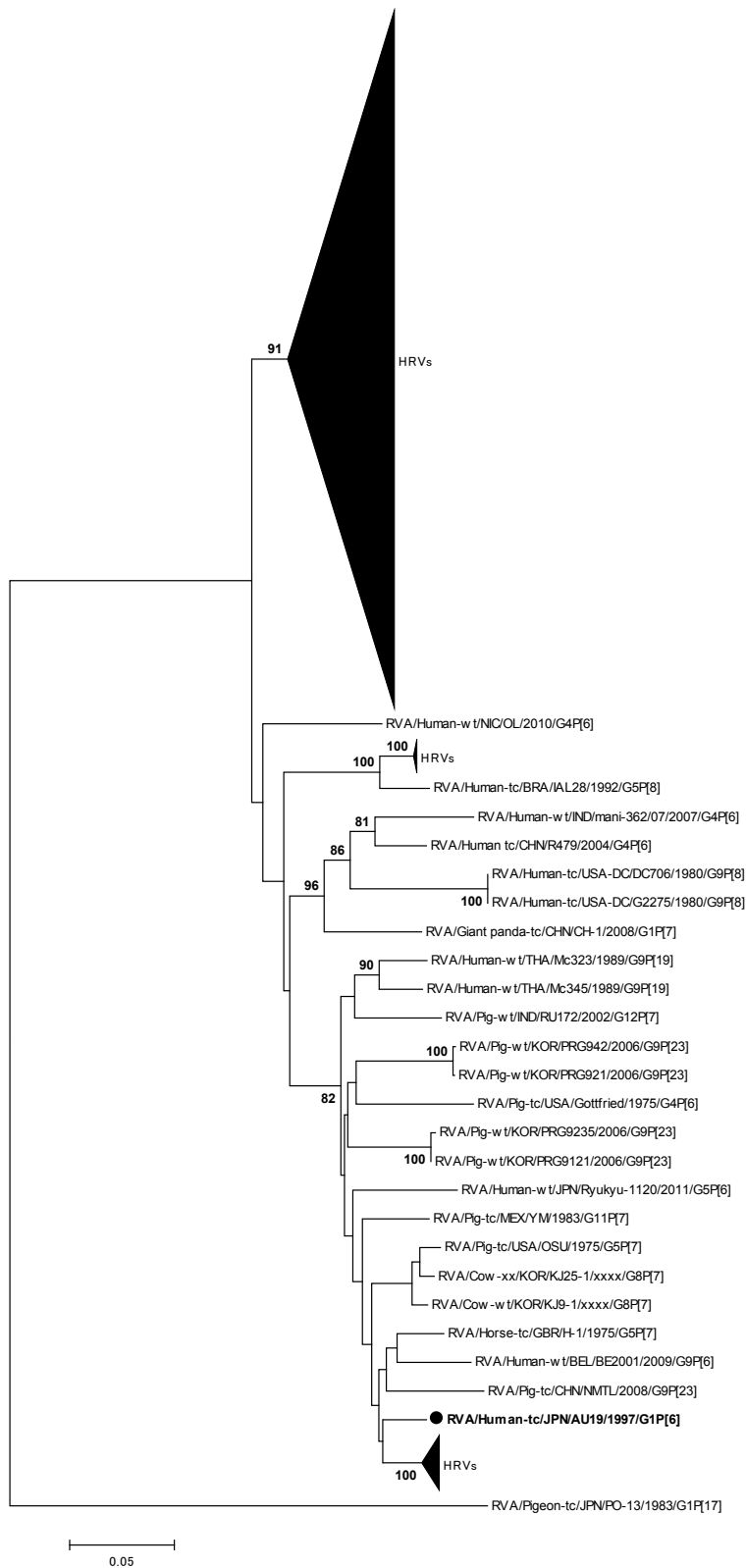
In the VP1 phylogenetic tree that contained 491 R1 sequences available in the GenBank database, the vast majority of human rotavirus clustered into one large aggregated lineage with a bootstrap probability of 99% (Fig.2.8). By contrast, while the number of PRV sequences available was much smaller, PRVs segregated into several lineages each with a significantly high bootstrap value, sometimes together with unusual porcine-like human rotavirus strains such as Ryukyu-1120 and BE2001 (Komoto et al., 2013, Zeller et al., 2012) (Fig.2.8). AU19 clustered together only with an equine rotavirus strain H-1 which was previously shown to be of porcine rotavirus origin (Ghosh et al., 2012a), although the origin of the H-1 VP1 gene was not unambiguously determined. The identity between AU19 and H-1 was calculated as 90.1% at the nucleotide sequence level and that between AU19 and other R1 strains ranged from 84.3% to 87%.





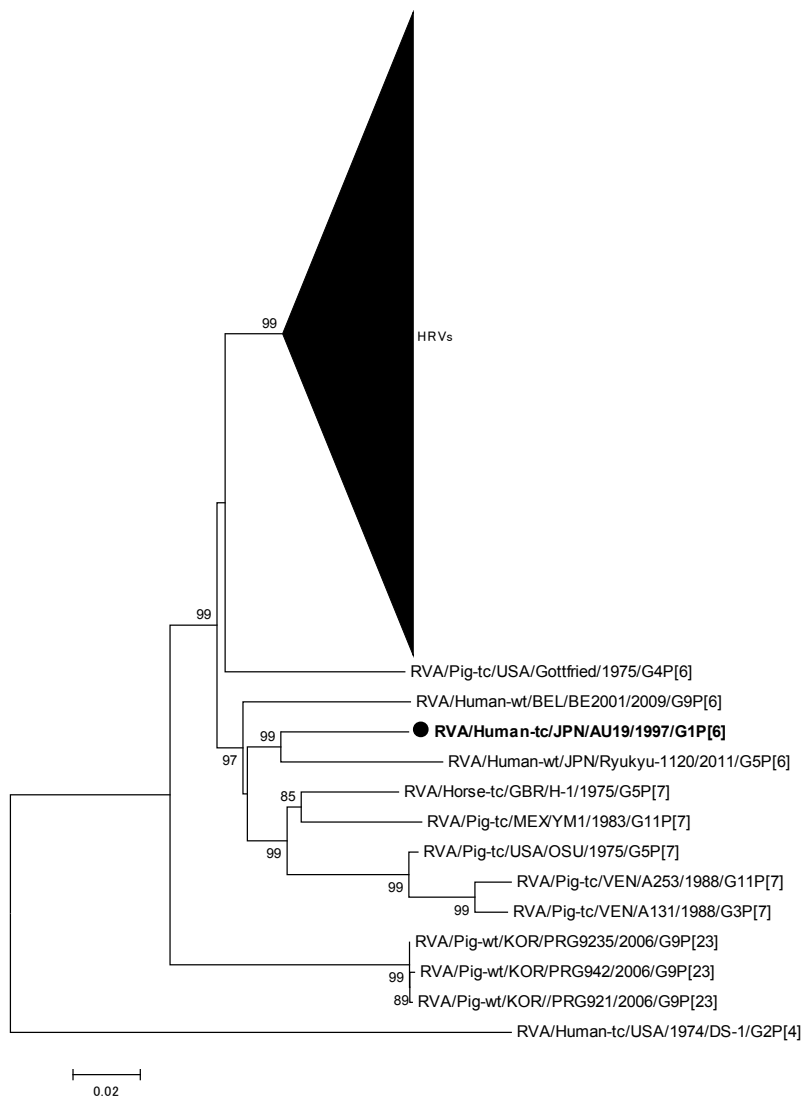
**Fig.2.8.** Phylogenetic tree of the VP1 gene of AU19 (indicated with a black dot and in bold) with other human and animal (mainly porcine) rotavirus strains. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. Pigeon rotavirus strain PO-13 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

In the VP2 phylogenetic tree that contained 352 C1 VP2 sequences available in the GenBank database, only one lineage contained porcine rotaviruses including the prototype strain OSU (Fig.2.9). To this lineage belonged AU19 together with five human rotaviruses of probable porcine origin: Ryukyu-1120, BE2001, Mc323, Mc345, and mani-362/07 (Zeller et al., 2012). In this lineage there were also 16 human rotavirus strains detected in Bethesda, USA, in 1991 (shown as a small triangle for condensation in Fig.2.9); however, it was reported that they were reassortants with rotaviruses of yet unidentified host species origin (McDonald et al., 2009). AU19 had 92.8% nucleotide identity to the rotavirus strains carrying the VP2 gene of unidentified origin, 95.6% to the porcine rotavirus OSU and 94.4% to KJ25-1.



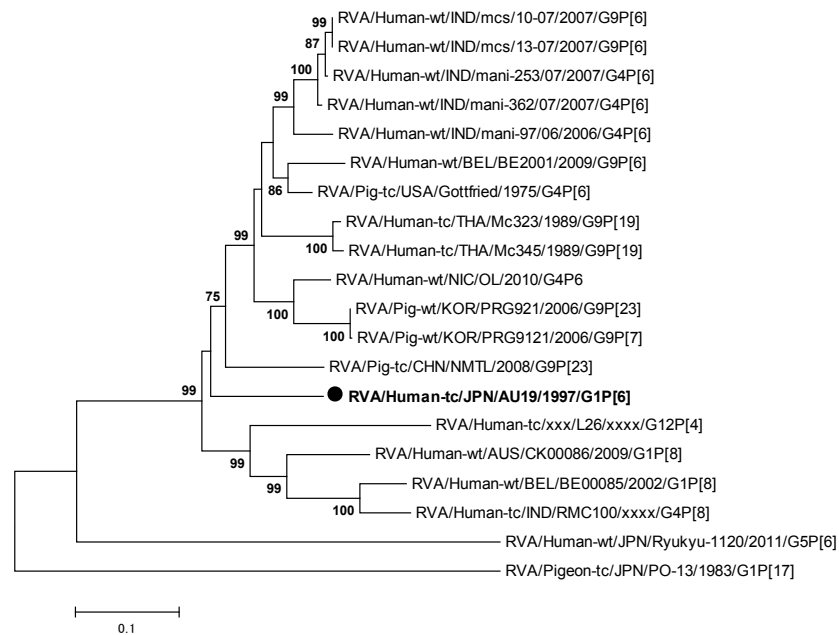
**Fig.2.9.** Phylogenetic tree of the VP2 gene of AU19 (indicated with a black dot and in bold) with other human and animal (mainly porcine) rotavirus strains as references for the host species of origin. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. Pigeon rotavirus strain PO-13 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

In the VP3 phylogenetic tree that contained 269 M1 sequences available in the GenBank database, AU19 clustered with porcine-like human strain Ryukyu-1120 (Komoto et al., 2013) with 99% of bootstrap probability and the nucleotide identity was 92% at the nucleotide sequence level (Fig.2.10). This lineage however clustered into a larger lineage with a bootstrap probability of 97% that contained porcine rotaviruses such as OSU and porcine-like equine rotavirus H-1 (Ghosh et al., 2012a). In addition, this lineage contained BE2001, a human rotavirus of probable porcine origin (Zeller et al., 2012).



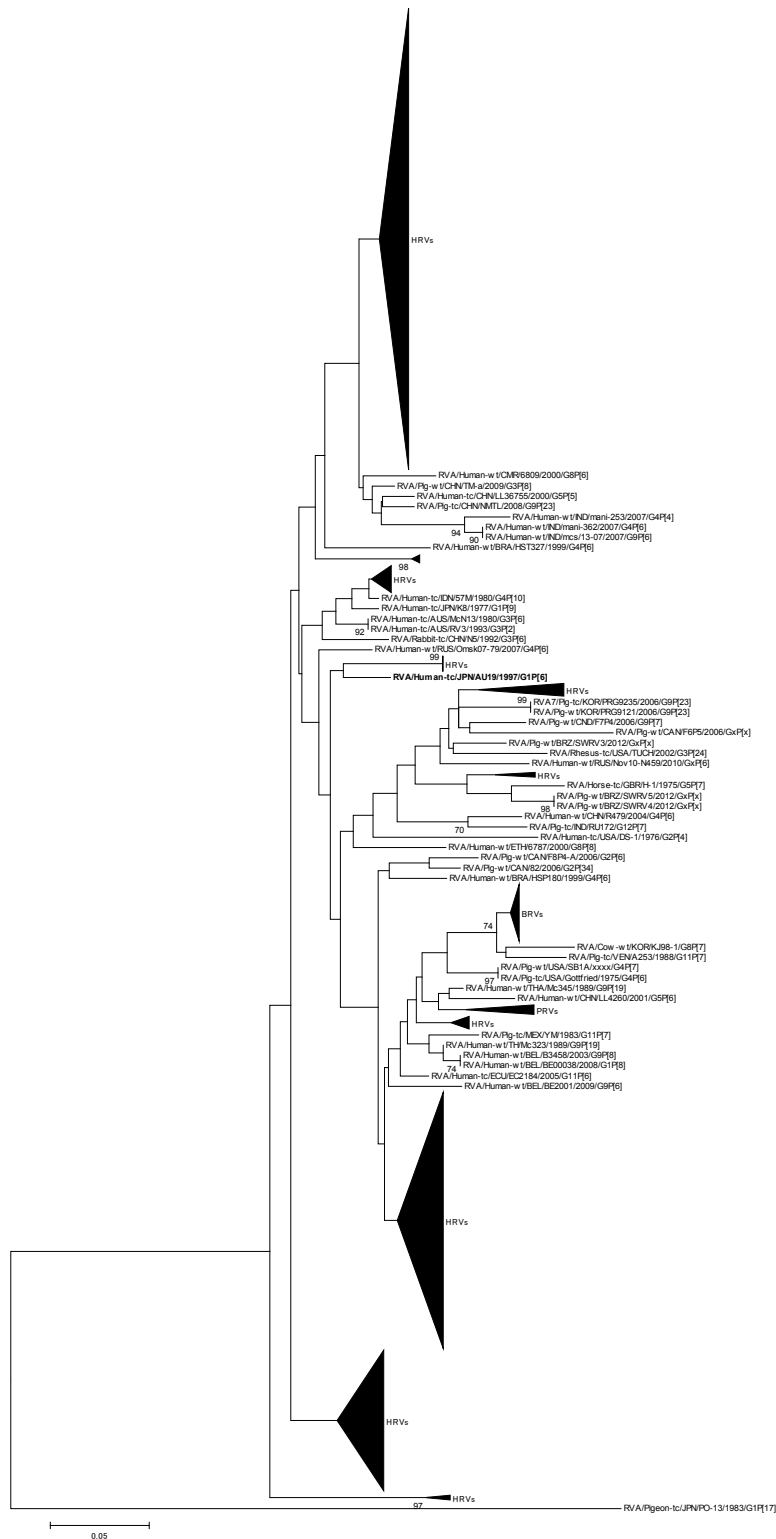
**Fig.2.10.** Phylogenetic tree of the VP3 gene of AU19 (indicated with a black dot and in bold) with other human strain, one horse strain and PRV strains. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. DS-1 strain was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

The NSP1 genotype carried by AU19 was A8, and this genotype was previously depicted as indicative of porcine rotavirus origin (Matthijnsens et al., 2008a). The AU19 NSP1 sequence was located within a single large lineage that contained porcine rotaviruses and a few human rotaviruses of probable porcine rotavirus origin such as BE2001, Mc323, Mc345, and mani-362/07 (Ghosh et al., 2012a, Mukherjee et al., 2011, Zeller et al., 2012) (Fig.2.11).



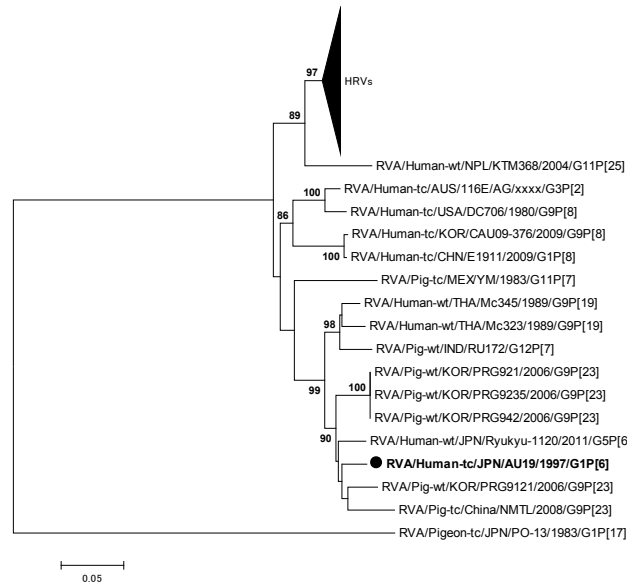
**Fig.2.11.** Phylogenetic tree of the NSP1 gene of AU19 (indicated with a black dot and in bold) with other human and porcine rotavirus strains. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. PO-13 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

In the NSP2 phylogenetic tree that contained 684 N1 NSP2 sequences available in the GenBank database, the AU19 did not cluster with any rotaviruses of either human or bovine rotavirus origin with a statistically significant bootstrap value (Fig.2.12). However, the strain that had the highest nucleotide identity (94.6%) to AU19 was super-short human rotavirus 57M.



**Fig.2.12.** Phylogenetic tree of the NSP2 gene of AU19 (indicated in bold) and other human and animal (mainly porcine) rotavirus strains as references for the host species of origin. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. PO-13 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger. Abbreviated term of BRVs is used to denote bovine rotaviruses.

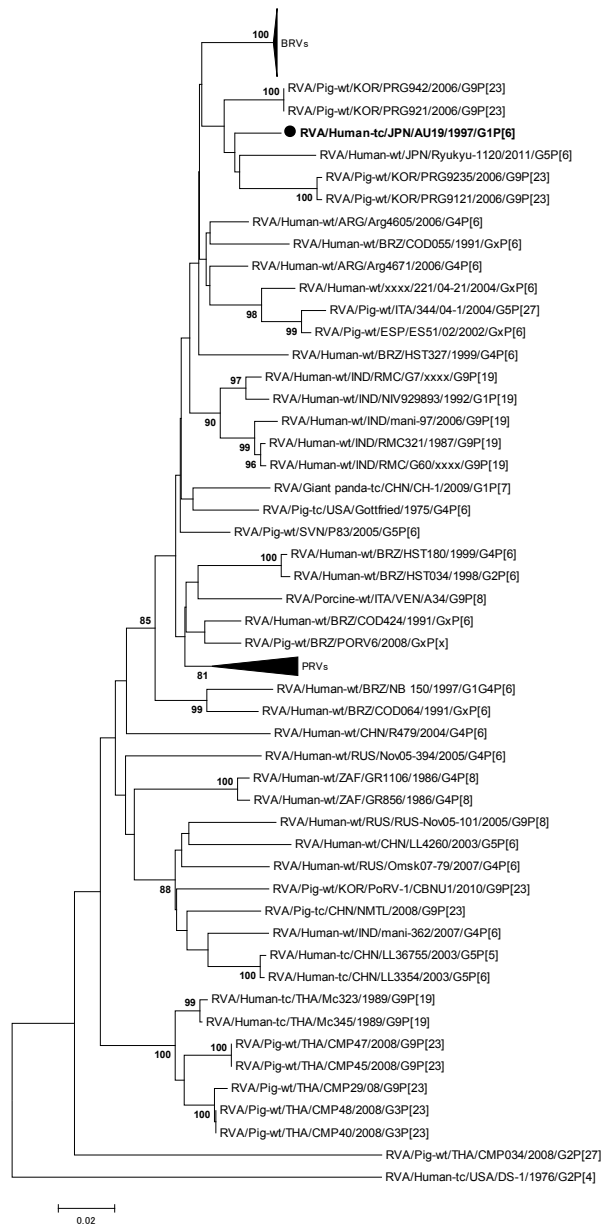
In the NSP3 phylogenetic tree which the first 100 sequences that were hit when the AU19 VP7 sequence was blasted against the GenBank database were included, the AU19 was clustered into a lineage with a 90% bootstrap probability that contained all porcine rotaviruses except Ryukyu-1120 (Fig.2.13). This lineage was distinct from the lineage that the vast majority of human T1 strains clustered into (shown as an aggregated triangle in Fig.2.13). The strains that had the highest nucleotide identity (95.4%) to AU19 were Ryukyu-1120 and three porcine strains PRG942, PRG921, PRG9235 detected in Korea.



**Fig.2.13.** Phylogenetic tree of the NSP3 gene of AU19 (indicated with a black dot and in bold) with other human and porcine rotavirus strains as references for the host species of origin. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. PO-13 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.



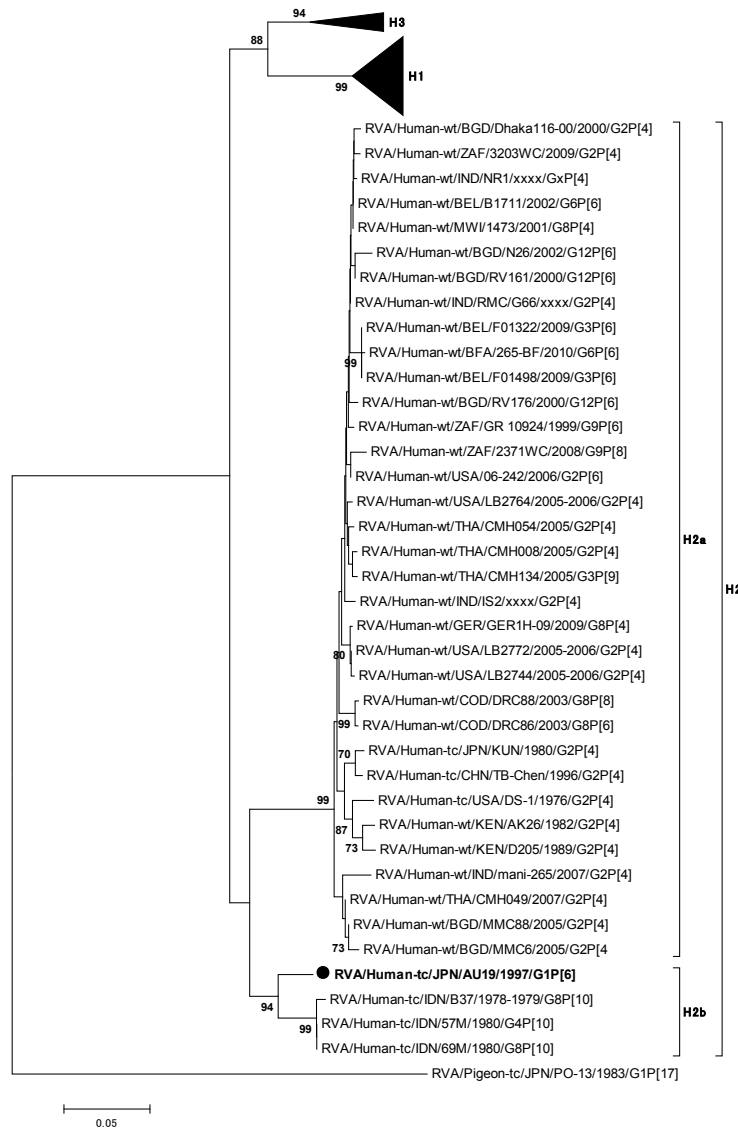
In the NSP4 phylogenetic tree which the first 100 sequences that were hit when the AU19 VP7 sequence was blasted against the GenBank database were included, the AU19 did not cluster with any rotaviruses of either human or bovine rotavirus origin with statistically significant bootstrap supports (Fig.2.14). The strains that had the highest nucleotide identity (96%) to AU19 were four porcine rotavirus strains PRG942, PRG921, PRG9235, and PRG9121 all detected in Korea, implying the porcine rotavirus origin of AU19.



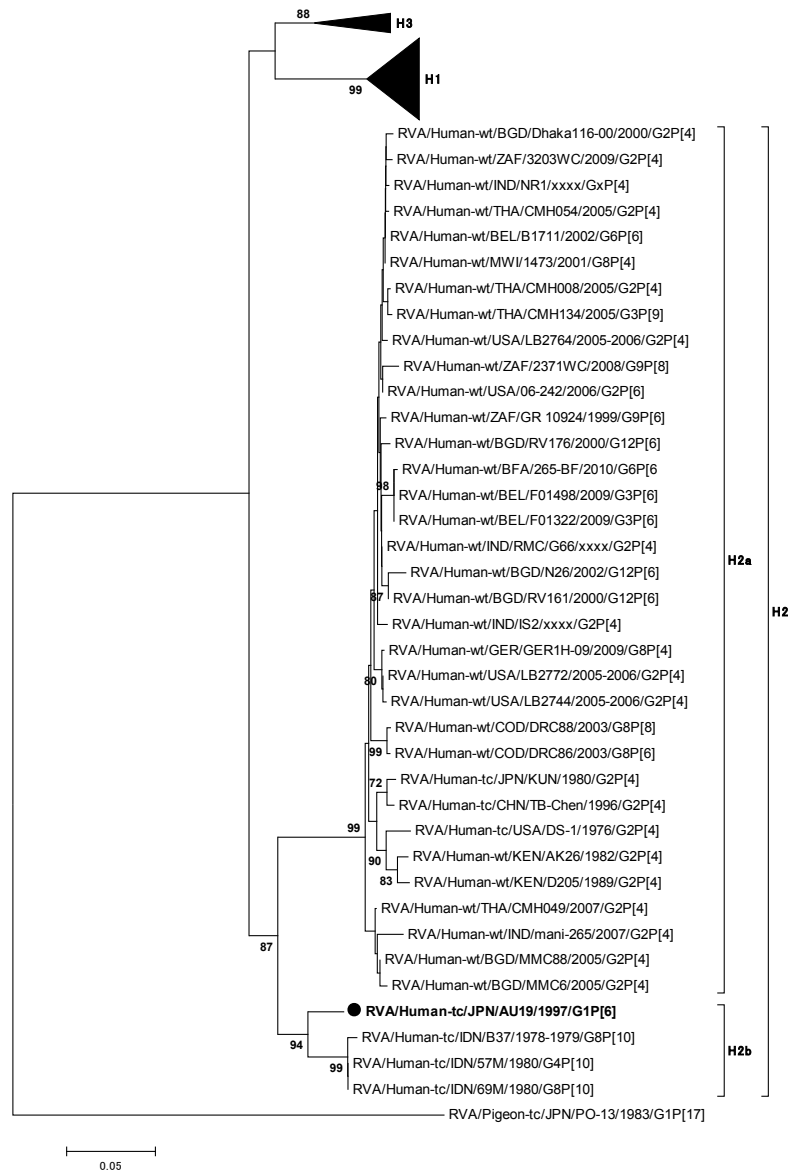
**Fig.2.14.** Phylogenetic tree of the NSP4 gene of AU19 (indicated with a black dot and in bold) with other human and animal (mainly porcine) rotavirus strains as references for the host species of origin. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package and statistically supported by bootstrapping with 1000 replicates. DS-1 was chosen as the outgroup. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

The phylogenetic tree shown in Fig.2.15 was drawn for the NSP5 sequences that belonged to H1, H2 and H3 genotypes. AU19 clustered into the sub-lineage that contained three super-short strains, 57M, B37, and 69M, with a bootstrap value of 94%. The sub-lineage that contained the four super-short strains clustered together with the lineage that contained all

short strains with a bootstrap value of 69%, which increased to 87% when the nearly-full 3' and 5' non-coding sequences were taken into calculation (Fig.2.16). Within the NSP5 genes carried by the four super-short strains, the mean pair-wise distance was 2.3% with a range from 0 to 4.6%. Similarly, within the NSP5 genes carried by short strains, the mean pair-wise distance was 1.8% with a range from 0 to 4.2%. The pair-wise distance between sequences belonging to these two lineages (between H2a and H2b) ranged from 8.3 to 10.6% with a mean of 9.2%. On the other hand, the pair-wise distance within the H1 lineage ranged from 0 to 6.9% with a mean of 2.2%, and had a bootstrap value of 99% at the branching point (Fig.2.15). Similarly, the pair-wise distance within the H3 lineage ranged from 0 to 8.5% with a mean of 4.9%, and had a bootstrap value of 94% at the branching point (Fig.2.15). The pair-wise distance between H1 and H3 sequences ranged from 10.8 to 15% with a mean of 12.3%.



**Fig.2.15.** Phylogenetic tree of the ORF of the NSP5 gene of AU19 (indicated with a black dot and in bold) with other human and porcine rotavirus strains. The trees were constructed using the neighbour-joining method included in the MEGA 5 software package with bootstrap probabilities after 1,000 replicate trials, and rooted with sequences of different genotypes. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.



**Fig.2.16.** Phylogenetic tree of the entire length of the NSP5 gene of AU19 (indicated with a black dot and in bold) with other human and porcine rotavirus strains. The trees were constructed using the neighbor-joining method included in the MEGA 5 software package with bootstrap probabilities after 1,000 replicate trials, and rooted with sequences of different genotypes. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger.

## **CHAPTER 3. DISCUSSION**

### **3.1. Molecular evolution of the VP7 gene of Japanese G2 rotaviruses before vaccine introduction:**

Molecular evolutionary analysis of the VP7 genes of Japanese G2 strains detected during the 31 year period (1980-2011) showed that the evolutionary process of the G2 VP7 genes of Japanese G2 strains occurred in parallel with that of the global G2 strains as described by Doan et al. (Doan et al., 2011). The two oldest strains detected in 1980 belonged to the lineage III, one strain detected in 1983 belonged to the lineage I to which prototype strain DS-1 belonged, the 33 strains detected between 1981 and 2011 belonged to the group IV except IVa, 18 strains detected between 2001 and 2009 belonged to the sub-lineage IVa-1, and one strain detected in 2011 belonged to the sub-lineage IVa-3. There was no Japanese G2 strain belonging to lineage II. This observation was consistent with the previous study describing that no strain in lineage II was detected in Asia except one strain reported from the Asian part of Russia (Doan et al., 2011).

The most interesting observation in this study that was different from what Doan et al. (Doan et al., 2011) concluded from their observations was the emergence of a nascent lineage outside of sub-lineage IVa and within group IV except IVa that was accompanied by the D96N substitution in the VP7 gene. Doan et al. (Doan et al., 2011) mentioned that the G2 strains in group IV except IVa was not regarded as forming a lineage due to a low bootstrap probability and that the vast majority of these strains had the A-D-D-S amino acid constellation at position 87-96-213-242, respectively. However, in 2011, two Japanese G2 strains belonging to group IV except IVa re-emerged after an 18-year interval. This emergence revealed the persistence of strains that belonged to group IV except IVa, which may be related to its genetic diversity as mentioned by Doan et al. (Doan et al., 2011). These two Japanese strains (B100005, B100119) formed a nascent lineage which was supported by a high bootstrap probability of 99% with other three G2 strains detected in Germany in 2008

(GER177-08), detected in Belgium in 2009 (BE1248) and detected in Australia in 2011 (CK20047). More importantly, this nascent lineage had the D96N substitution that was shown to be the cardinal feature of strains that belong to lineage IVa. The amino acid at position 96 was previously described as an epitope recognized by a G2-specific neutralizing monoclonal antibody (RV5:3) (Lazdins et al., 1995). Furthermore, the D96N substitution was shown to be strongly associated with the cause of the emergence of G2 strains in Taiwan, Thailand, UK and Nepal (Doan et al., 2011, Iturriza-Gomara et al., 2011, Khamrin et al., 2007, Trinh et al., 2010, Zao et al., 1999). This substitution may impart to the strains in this nascent lineage the potential to spread globally in the near future.

In this regard, it may deserve to examine the amino acid substitution at position 96 of the VP7 gene in other common human rotavirus genotypes such as G1, G3, G4 and G9. In theory, the substitutions are more likely to occur between amino acids with similar biochemical properties and are therefore less likely to affect the function of protein. However, the G2 human rotavirus strains had a substitution at position 96 that occurred between a negatively charged polar amino acid (aspartic acid) and an uncharged polar amino acid (asparagine) with the most nucleotide changes occurring between G and A at position 334 in the nucleotide sequence; hence codon changes occurring between GAT and AAT, respectively (Arista et al., 2005, Doan et al., 2011, Gomara et al., 2001, Lazdins et al., 1995, Mascarenhas et al., 2010, Page and Steele, 2004a, Page and Steele, 2004b, Trinh et al., 2010, Zao et al., 1999). Therefore, this substitution probably contributed to a major antigenic change that may have helped abrupt increase or re-emergence of G2 rotavirus strains in many locations in the world. Interestingly, this radical substitution at position 96 between asparagine and aspartic acid also observed in G3 human rotavirus strains (Dyall-Smith et al., 1986, Lazdins et al., 1995, Mackow et al., 1988, Nishikawa et al., 1989, Phan et al., 2007). The re-emergence in high frequency of G3 strains (95%) detected in Japan in 2003-2004 that was also associated with D96N substitution in antigenic site of the VP7 gene (Nishikawa et al., 1989, Phan et al., 2007). In contrast, the G4 and G9 rotavirus strains only showed

conservative substitutions at position 96: T96N in G4 strains, and T96A, T96P in G9 strains (Coulson et al., 1996, Kirkwood et al., 1993, Teodoroff et al., 2005). The re-emergence of G4 and G9 rotavirus strains associated with substitution at position 96 is unknown to date. The amino acid at position 96 of the G1 VP7 gene was conserved with glycine, except two G1 strains in Argentina that showed a G96V substitution (Barril et al., 2013).

One peculiar exception to the rule for sub-lineage IVa-1 was a strain detected in Nara Prefecture in 2009, 90096, which contained glycine at position 96. To the best of our knowledge, this is the first report of the G2 strain that had a substitution from asparagine to glycine at position 96 of the G2 VP7 protein. Thus, it is tempting to speculate that once the 90096 strain adapted to the human population, the N96G substitution would likely be fixed as it occurred in the G1 VP7 proteins.

Strains with the G2P[4] genotype have captured recent attentions because of its abrupt increase or re-emergence in many locations in the world including where the universal mass rotavirus vaccination program was implemented. In this study, we found one G2 strain (B100056) belonging to sub-lineage IVa-3 in Japan. The sub-lineage IVa-3 is a contemporary, globally dominant lineage. In Brazil, it appears that there was a shift of sub-lineage from IVa-1 to IVa-3 after rotavirus vaccine introduction (Matthijssens et al., 2011). However, in Japan, we found a G2 rotavirus strain of sub-lineage IVa-3 before rotavirus vaccine introduction. Thus, it was hypothesised that the shift from sub-lineage IVa-1 to IVa-3 was due to natural genotype variation and that it was facilitated by the introduction of rotavirus vaccine.

When the proportion of G2P[4] genotype was analysed in the context of the G and P genotype distribution in Japan, there was a significant increase of G2 strains from 7.4% in 1988-1989 to 25% in 1989-1990, then a sharp increase to 87% in 1990-1991 (Nakagomi and Nakagomi, 2009). However, the increase in frequency of G2 strains in Japan in the 1990-1991 was not associated with the D96N substitution in the antigenic sites of the VP7 gene. Changes in genotypic patterns within a population may be explained by the interplay between the



population's immunity and viral evolution. New highly infective viral strains may only disappear when populations reach herd immunity (Kobasa et al., 2004). So the sharp increase of Japanese G2 strains in 1990-1991 may be due to the population immunity.

In the viral evolutionary aspect, this study was only provided the evolution of G2VP7 genes. Thus, it would also be interesting to examine the genetic evolution of other genome segments to investigate whether viral evolution were involved in the sharp increase of Japanese G2 rotavirus strains in 1990-1991.

### **3.2. A rare G1P[6] super-short human rotavirus strain carrying an H2 genotype on the genetic background of a porcine rotavirus**

Nearly-full genome sequencing disclosed that human rotavirus strain AU19 isolated from a Japanese infant with severe gastroenteritis possessed an unusual G1- P[6]- I5- R1- C1- M1- A8- N1- T1- E1- H2 genotype constellation that has never been described before. The possession of the I5 and A8 genotypes strongly suggests that the original host of AU19 was the pig species. Since human rotaviruses carrying the Wa-like genotype constellation share the same genotype with porcine rotaviruses in several genome segments (Matthijnssens et al., 2008a), it is essential to examine what lineage AU19 clustered into to estimate the host species origin of the genome segments of AU19. Other than the VP6 and NSP1 genes for which the porcine origin was clear at the genotype level, seven genome segments of AU19 was determined most likely to be of porcine rotavirus origin: the VP7, VP4, VP1, VP2, VP3, NSP3, and NSP4 genes that carried the G1, P[6], R1, C1, M1, T1, and E1 genotypes, respectively. The host species origin of the NSP2 gene (the N1 genotype) was not determined conclusively due to the paucity of sequence information; however, the porcine rotavirus origin of this genome segment was not excluded, either. Overall, the genetic background of AU19 was of porcine rotavirus origin, and the parental porcine rotavirus was likely to have acquired the H2 NSP5 gene by genetic reassortment with a co-circulating human super-short strain when the parental virus changed its host from pigs to humans.

Pigs are often considered as the host species from which rotaviruses crossed the species barrier into humans; at least 27 strains were recorded in the literature whose genome was fully sequenced (Banyai et al., 2009a, Degiuseppe et al., 2013, Esona et al., 2009, Ghosh et al., 2012a, Heiman et al., 2008, Komoto et al., 2013, Matthijnssens et al., 2008a, Matthijnssens et al., 2011, McDonald and Patton, 2008, Mladenova et al., 2012, Mukherjee et al., 2011, Mullick et al., 2013, Papp et al., 2013a, Varghese et al., 2004, Varghese et al., 2006, Wang et al., 2010, Zeller et al., 2012). While BE2001, Ryukyu-1120 and mani362/07 (Komoto et al., 2013, Mukherjee et al., 2010, Zeller et al., 2012) are examples of direct transmission of porcine rotaviruses as a whole genome, the majority of porcine-like human rotavirus strains represent interspecies transmission involving genetic reassortment. AU19 together with strains like Arg4605, mani253/07, mcs/13-07, and BP1901 belonged to the latter category in which interspecies transmission occurred in the form of genetic reassortment (Degiuseppe et al., 2013, Mukherjee et al., 2010, Papp et al., 2013a) (Table 2.2). While there appears no clear trend regarding which genome segments preferentially derived from human rotaviruses, it is only AU19 that carried an H2 genotype among all porcine-like human rotavirus strains reported to date (Table 2.2).

The H2 genotype has never been linked to any animal rotaviruses and has so far been detected only in human rotaviruses (Fig.2.15). In addition, the H2 genotype is further divided into H2a and H2b genotypes, each of which is carried by strains possessing short or super-short electropherotypes, respectively (Ghosh et al., 2013). Both electropherotypes are known to contain longer 3' non-coding sequences than long electropherotypes, and may occur for human rotavirus strains with the H1 genotype as exemplified by BE2001 (Zeller et al., 2012) and Mc345 (Ghosh et al., 2012b) as well as human strain with the H3 genotype: B4106 (Matthijnssens et al., 2006). The mechanisms by which prolonged 3' non-coding sequences were generated are not completely understood, but at least two mechanisms have been proposed (Matsui et al., 1990). One is a duplication starting from an internal coding sequence until the near the 3' end of the gene, which explains the generation of the rearranged NSP5

gene of BE2001, Mc345, and B4106 (Kojima et al., 1996, Matthijnsens et al., 2006, Zeller et al., 2012). The other mechanism is less well understood but was proposed to involve polymerase stuttering. This latter mechanism is believed to generate short and super-short strains of the H2 genotype (Matsui et al., 1990). It has been pointed out that the NSP5 genes of DS-1 and 69M shared similar ancestral lineages (Matsui et al., 1990). However, more recently, it was reported that a small percentage of rearranged forms of the NSP5 gene can be produced in the course of acute rotavirus infection of immunocompetent children by the same mechanism as the partial duplication was generated for BE2001 (Schnepf et al., 2008). Thus, it could be possible that partial gene duplication occurred in the a shared common ancestral NSP5 gene of short and super-short strains, and random point mutations accumulated over time, making it impossible for the original sequence to be traced.

The NSP5 gene of AU19 clustered together with that of 69M, 57M and B37 with a bootstrap probability of 94% (Fig.2.15). The lineage that contained AU19 and the other three super-short human strains possessing the H2 genotype (H2b genotype) clustered with the lineage to which short strains belonged (H2a genotype) only with a marginal bootstrap probability of 69%. Inclusion of the sequence of the long 3' non-coding region into the phylogenetic analysis however increased the bootstrap value to an 87% at the branching point into the short and super-short lineages whereas it did not change the tree topology (Fig.2.16). Assuming that the H2a and H2b genotypes have a common ancestor and that the evolutionary time is roughly proportional to the genetic distance, it is worthy of pointing out that the pair-wise distance, an approximation of the genetic diversity, within the H2a genotype (0.2 - 4.4%) was as diverse as that within the H2b genotype (0-4.6%), suggesting that the time elapsed for the sequences to diversify within each sub-lineage was similar. If this speculation is correct, the observed difference in the relative frequency of short and super-short strains detected among sick children may suggest that short strains have more advantages in spreading among humans than super-short strains. An alternative possibility is that super-short strains may be maintained more frequently among asymptomatic children.

### **3.3. Evolutionary mechanisms of RVA strains through the molecular characterization studies**

Rotaviruses are a large genetically diverse population of double-stranded RNA viruses which cause severe gastroenteritis in both humans and animals (Patton, 2012). The diversity of rotavirus is generated by several mechanisms: (i) accumulation of point mutation; (ii) reassortment that lead to the novel genetic and antigenic characteristics when two strains exchange the genetic materials; (iii) direct transmission of animal strains into a human host as the whole genome; (iv) gene rearrangement (e.g., deletions, insertions and duplications) (Gentsch et al., 2005, Kirkwood, 2010).

The previous study analysed by Doan et al. confirmed that the G2VP7 gene evolved in a fashion in which new lineages emerged within the previous dominant lineage (Doan et al., 2011). In this study, the molecular evolution of Japanese G2 RVA strains during 31 year period (1980-2011) showed that the Japanese G2 VP7 genes correlated well with that of global strains. However, in this study, the new emergence of a nascent lineage was derived from the previous existing lineage which detected for the last time 18 years before. We hypothesised that during this period, strains in group IV except IVa circulated at a low frequency, accumulated point mutations over time and became emerged when acquiring the D96N substitution, a salient feature of the lineage IVa strains that have prevailed since the last decade.

AU19 strain is likely to be assumed to have emerged as a consequence of two sets of events, interspecies transmission of a porcine rotavirus to a child, coupled with acquisition of a rare rearranged NSP5 gene (H2b genotype) by genetic reassortment probably from a co-circulating human rotavirus strain. Adding AU19 strain into the H2b genotype made the genetic diversity of H2b genotypes as diverse as that of the H2a genotype, lending support to the hypothesis that super-short strains carrying H2b genotype have long been circulating unnoticed in the human population. Two rare events occurred in AU19 strain provided the

hypothesis that the AU19-like strains had been circulating in human population for a long time. The strains did not die out after being transmitted as in the case of the direct transmission from porcine into human hosts as a whole genome. AU19-like strains were likely to be adapted to the human population by the acquisition of the H2b gene through genetic reassortment and circulated in the human population.

## **CHAPTER 4. CONCLUSIONS**

The conclusions drawn from the molecular characterisation of Japanese RVA strains analysed in this thesis are twofold.

Firstly, the genes of RVA strains are likely to evolve in nature through the accumulation of point mutations resulting in the emergence of a new lineage in a stepwise fashion. While it was previously stated that the G2VP7 gene evolved in a dynamic fashion such that new lineages emerged within the previously dominant lineage, one of which became subsequently dominant (Doan, et al., 2011), a new lineage can emerge outside of currently dominant as exemplified by the nascent lineage in the G2 VP7 gene. The potential for a new lineage to spread and become dominant lineage may be associated with the acquisition of the D96N substitution. As the nascent lineage carries this hallmark substitution of D96N, close monitoring is worthy.

Secondly, interspecies transmission of animal RVA to humans (and subsequent genetic reassortment) is likely to have played an important evolutionary role, when succeeded in establishing a human-to-human transmission chain, as exemplified by a rare porcine-like human RVA strain AU19. However, the case of AU19 appears to have ended in dead-end infection rather than establishing a successful human-to-human transmission chain.

However, it remains to be seen whether the accumulation of point mutations will be accelerated and interspecies reassortant strains will have a survival advantage over co-circulating human RVA strains under selective pressures imposed by wide-spread use of vaccines. Further studies are, therefore, warranted to monitor at the full-genome level if such emerging strains will spread globally in order to ensure the successful use of vaccines and to explain the vaccine failure if it occurs.

## **CHAPTER 5: LITERATURE REVIEW**

Rotaviruses belong to the genus *Rotavirus* within the family *Reoviridae* causing the severe acute gastroenteritis in infants and young children worldwide. Rotaviruses share the common morphological and biochemical properties as described as Estes and Greenberg (Estes and Greenberg, 2013).

- The mature virus particles are about 100 nm in diameter including the spikes, relative large for a non-enveloped virus. The particles possess a triple-layered icosahedral protein capsid: an outer layer, an intermediate layer, and an inner core layer.
- The particles contain an RNA-dependent RNA polymerase and other enzymes capable of producing capped RNA transcripts.
- The virus genome contains 11 segment dsRNA in which the genetic assortment can occur during the infection with rotaviruses.
- Virus replication occurs in the cytoplasm of infected cells.
- Virus cultivation *in vitro* is facilitated by treatment with proteolytic enzymes because the outer capsid spike polypeptides are cleaved to enhance viral infectivity.

### **5.1. History of rotavirus discovery**

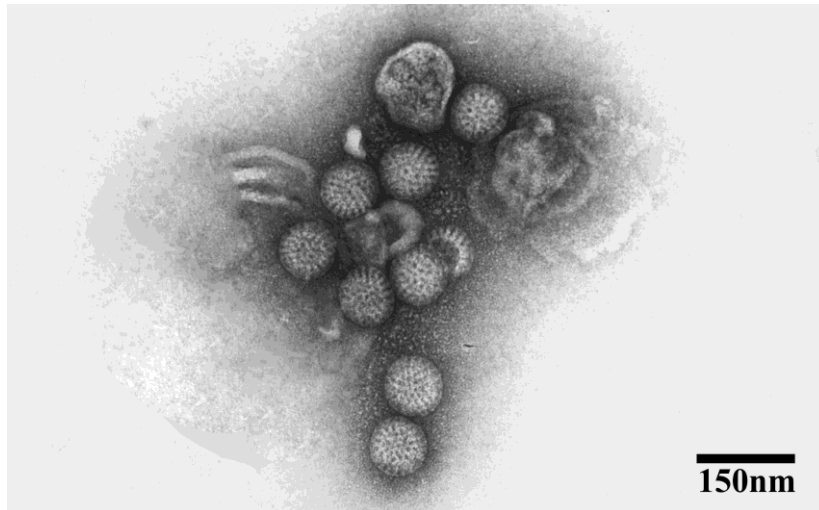
Rotavirus was detected in human from an Australian child in 1973 for the first time in electron micrograph of thin section of duodenal mucosa and later by electron microscope (EM) in faeces (Bishop et al., 1973) just 1 year after the discovery of Kapikian et al. about Norwalk virus from the gastroenteritis outbreak in 1972. Soon after the discovery of rotavirus, a number of reports about the rotavirus as the important aetiology causing gastroenteritis all over the world increased day by day. However, rotaviruses were described in animals in the 1960s which were later confirmed by sharing the morphology and the group antigen with human rotaviruses (Estes and Greenberg, 2013, Flewett et al., 1974b).

## 5.2. Description and classification

### 5.2.1. Morphology

The morphology of rotavirus particles is very distinctive with a short spokes surround the hub of the wheel (the Latin word, *rota*, means wheel). Under the cryo-electron microscopy, the complete rotavirus virion is the triple-layered particle penetrated by 132 channels and has 60 spikes on its surface (Flewett et al., 1974a, Flewett et al., 1974c).

- The smooth surface of the outer layer formed by viral protein (VP) 7 from which sixty protein spikes formed by VP4 rise out.
- The middle layer formed by VP6, the most abundant protein of the virus particles, and it specifies for rotavirus serotype.
- The innermost layer consists of VP1, VP2, and VP3 which packed the viral genome of rotavirus.

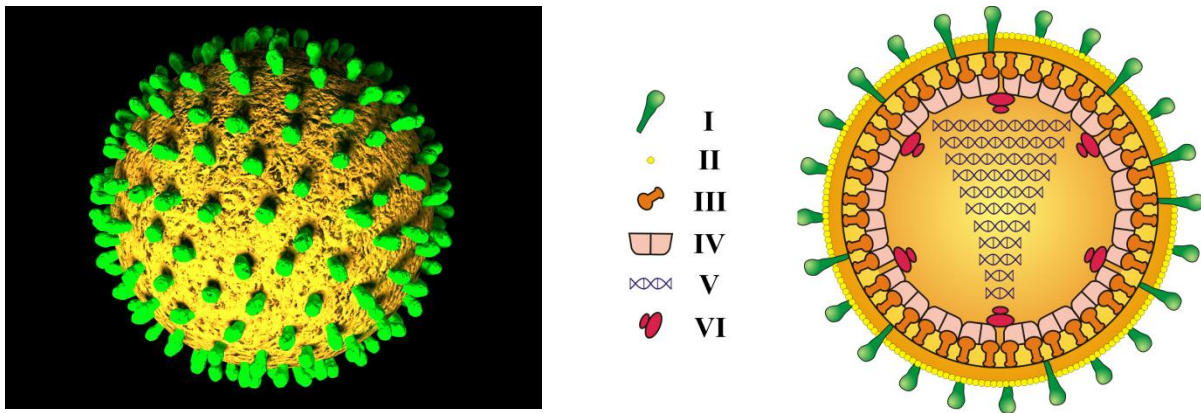


**Fig. 5.1.** Negative-stained electron micrograph of rotavirus particles in a faecal specimen. Uranyl acetate 1% stain. (Courtesy of T.T.Nguyen, National Institute of Hygiene and Epidemiology, Vietnam)



### 5.2.2. Genome and gene-coding assignments

The viral genome of 11 segments of dsRNA is surrounded by the three concentric layers of protein. The genome encodes six structural viral proteins (VPs) and six non-structural protein (NSPs), each genome segment codes for a single viral protein except NSP5 gene which codes two proteins (NSP5 and NSP6) (Estes and Greenberg, 2013).



**Fig.5.2.** Three and two dimensional structure of the mature rotavirus particle derived from cryo-electron microscopy images and computer image processing. (Courtesy of T.T.Nguyen, National Institute of Hygiene and Epidemiology, Vietnam)

RNA segments 1, 2 and 3 code for the inner core protein VP1, VP2 and VP3, respectively which plays the important roles in segment packing and RNA interaction and replicase activity (Estes and Greenberg, 2013). RNA segment 6 codes the middle layer protein, which carries specific- group and subgroup epitopes. VP7, encoded by RNA 7, 8 or 9, depending on the strain and VP4, encoded by RNA 4 formed the outer capsid, the outmost layer. VP4 is cleaved into VP8\* and VP5\* subunit by protease that is essential for the infectivity. These two proteins, VP7 and VP4, carry the epitopes that specific for the virus serotype.

The segments RNA 5, 6, 8, 9, 10 and 11 encoding for the six non- structural proteins, NSP1, NSP 2 and NSP3 (depending on the strain), NSP4, NSP5 and NSP6. These non- structural proteins have many functions on the virus replication cycle, especially in morphogenesis.

**Table 5.1.** Rotavirus proteins and genome structure of rotavirus

<b>Genome segment</b>	<b>Protein product</b>	<b>Function</b>	<b>Name of genotypes</b>	<b>Number of genotypes</b>
1	VP1	RNA-dependent RNA polymerase, ss RNA binding, complex with VP3	RNA-dependent RNA polymerase	R1-R9
2	VP2	RNA binding, required for replicase activity of VP1	Core protein	C1 - C9
3	VP3	Guanylttransferase, methyltransferase, ss RNA binding, complex with VP1	Methyltransferase	M1 - M8
4	VP4	P-type neutralisation antigen, viral attachment, homotrimer, protease-enhanced infectivity, virulence, putative fusion region	Protease sensitive	P[1] – P[37]
5	NSP1	Interferon antagonist, putative viral E3 ligase, RNA binding	Interferon Antagonist	A1-A16
6	VP6	Subgroup antigen, trimer, protection, required for transcription	Inner capsid	I1-I16
7	NSP3	Acidic dimer, binds 3' end of viral mRNAs, cellular eIF4G, Hsp90, surrogate of PABP, inhibits host	Translation enhancer	T1-T12

		translation		
8	NSP2	Basic, octamer, RNA binding, NTPase, NDP kinase	NTPase	N1-N9
9	VP7	G-type neutralisation antigen, glycoprotein calcium dependent trimer	Glycosylated	G1-G27
10	NSP4	RER transmembrane glycoprotein, viroporin, intracellular receptor for DLPs, role in morphogenesis of TLPs, interacts with viroplasms and autophagy pathway, modulates intracellular calcium and RNA replication, enterotoxin secreted from cells, virulence	Enterotoxin	E1-E14
11	NSP5	Basic, phosphoprotein, RNA binding, protein kinase, forms viroplasms with NSP2, interacts with VP2 and NSP6	PHosphoprotein	H1-H11
	NSP6	Interact with NSP5, present in viroplasms and most virus strains, RNA binding		

ssRNA: single stranded RNA; mRNA, messenger RNA; PABP, poly(A)-binding protein; NTPase, nucleoside triphosphate; NDP, nucleoside diphosphate; RNPase, RNA triphosphate; RER, rough endoplasmic reticulum; DLP, double layered particle; TLP, triple layered particle.

### 5.2.3. Classification of rotavirus:

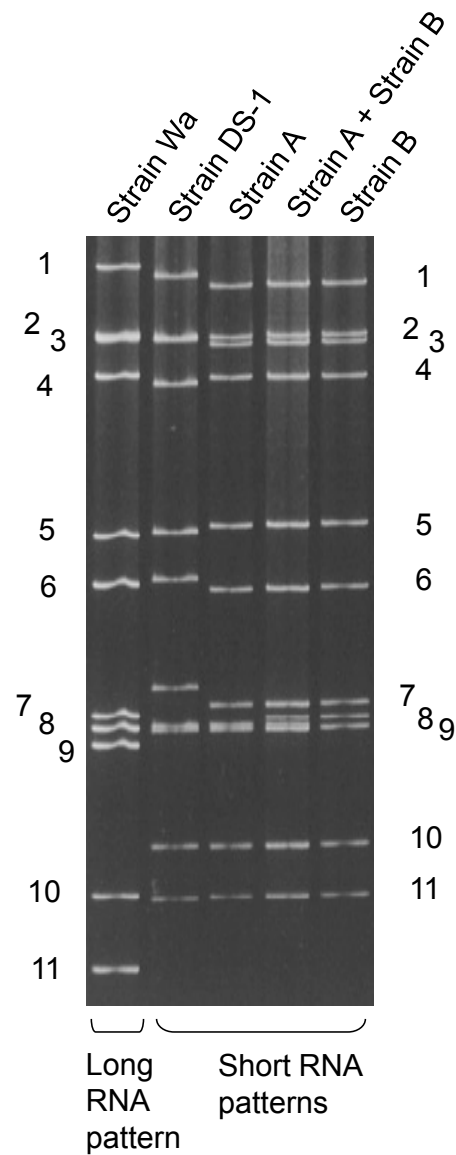
Rotaviruses have been classified into seven groups from A to G (now classified taxonomically as *Rotavirus A*, *Rotavirus B*, *Rotavirus C*, etc. (RVA, RVB, RVC, etc) based on the antigenic properties of VP6 protein (Ball, 2005, Kohli et al., 1992). Among them, only groups A, B and C showed the evidence to associate with the human illness whereas group D, E, F and G commonly detected in animals (Ball, 2005, Martella et al., 2010). RVA, or *Rotavirus A*, is responsible for the vast majority of human rotavirus infections, especially children under 5 years of age. Group B rotavirus, or *Rotavirus B*, was detected was the aetiology of the diarrhoea outbreak in China, India and Bangladesh, Nepal (Ahmed et al., 2004, Alam et al., 2013, Hung et al., 1987, Malik et al., 2011, Nakata et al., 1987, Sanekata et al., 2003). Group C rotavirus, or *Rotavirus C*, also causes occasional diarrhoea in children (Estes and Kapikian, 2007, Mitui et al., 2009).

In RVA, rotaviruses were classified by (i) antigenic VP6, VP7 and VP4 into subgroup, G serotypes and P serotypes, respectively; (ii) genomic RNA electrophoretic patterns into electropherotypes; (iii) RNA- RNA hybridization patterns into genogroups and nucleotide sequence of genome segment into genotypes.

VP6 protein, the most abundance protein, containing antigenic epitopes is the target for serological methods such as immunofluorescent, enzyme-linked immunosorbent assay (ELISA), and immune electron microscope (IEM) (Estes and Greenberg, 2013). Based on the antigenic epitopes of VP6 protein, RVA strains are classified into subgroups I and II, I+ II, and nonI/ nonII but human rotaviruses A mainly belonged to subgroup I and II (Estes and Greenberg, 2013).

The dsRNA can be extracted and separated easily by polyacrylamide gel electrophoresis. The different types from polyacrylamide gel electrophoresis are called “electropherotype” with the “long”, “short” and super-short pattern. The various minor

differences in the migration of RNA segments have been detected and used in epidemiological study. The two prototype strains Wa and DS-1 represent two distinct RNA migration patterns: the long electropherotype with faster-migrating 10th and 11th genome segments and the short electropherotype with slower-migrating 10th and 11th genome segments (Kutsuzawa et al., 1982). What actually happened in short electropherotype, however, is the rearrangement of the 11th genome segment (coding for NSP5) by which this genome segment acquired an extra few hundred nucleotide insertion at their 3' non-coding region; hence migrating slower than the original 10th genome segment (coding for NSP4) which in turn becomes the 11th genome segment as a consequence (Dyall-Smith and Holmes, 1981). Rotavirus strains, albeit infrequently, may show super-short electropherotypes in which the rearranged 11th genome segment becomes even longer than that of short electropherotype strains.

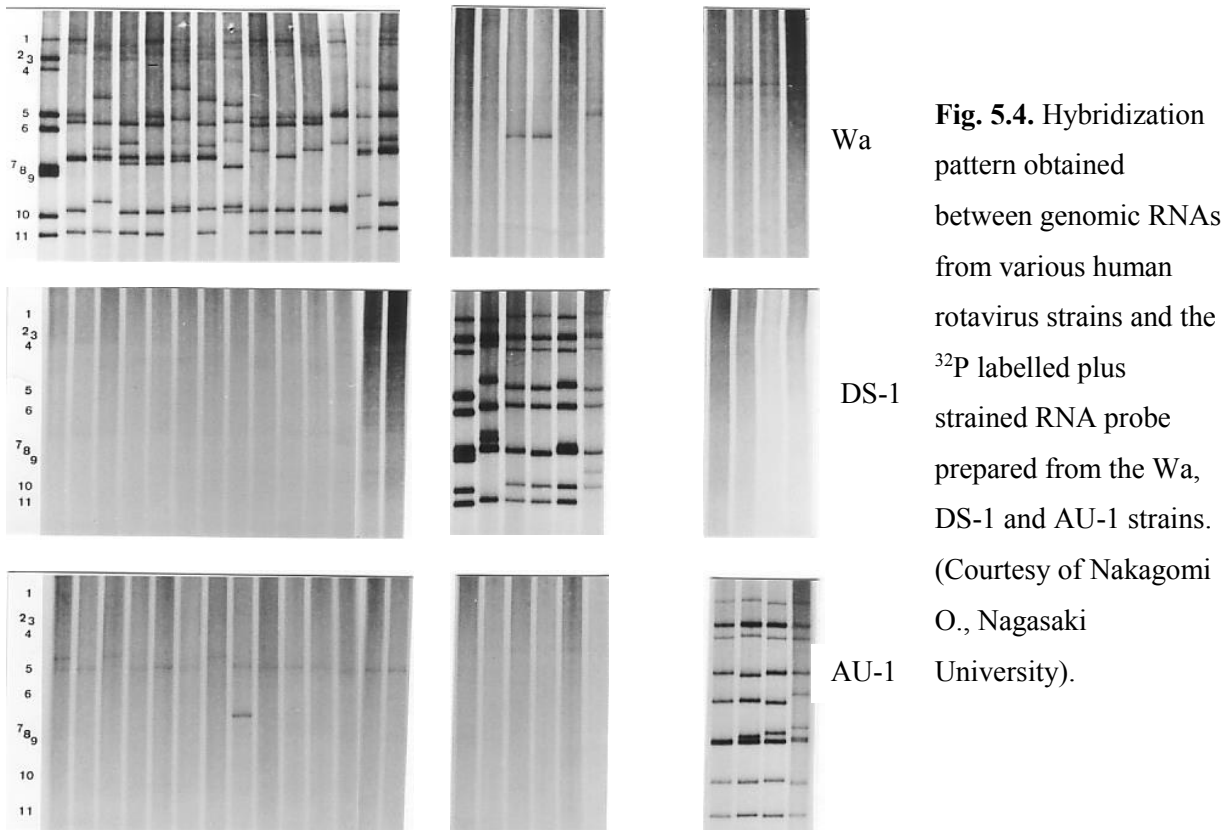


**Fig. 5.3.** Separation of rotavirus genomic RNA into 11 bands by polyacrylamide gel electrophoresis. Two RNA patterns, long and short, are represented by prototype strains Wa and DS-1, respectively. Strain A and B, both of short electropherotype patterns, differ only in the migration profile of RNA segment 8. (Courtesy of Nakagomi O, Nagasaki University).

VP7 and VP4 carry neutralisation- specific epitopes are the targets of neutralizing antibody that define the virus serotype. VP7 is a glycosylated protein and the serotypes determined by this protein are termed G serotypes. Cleaved by protease, the VP4 determines the serotypes which are termed P serotypes. With the development of molecular biological techniques, serological assay are now being replaced by molecular typing and the term “rotavirus genotyping” came out. While there is a perfect correlation between G serotypes and G genotypes, there were some difference between G serotype and G genotype. Therefore, P genotype was being designated within a spare bracket in order to differentiate with P serotype. There are now 27 G and 37 P genotypes currently ratified by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2009, Matthijnssens et al., 2011, Trojnar et al., 2013) and they can produce a numerous of G and P combinations (Nakagomi and Nakagomi, 2009). There are at least 74 G-P combination in the pre vaccine licensure period (Gentsch et al., 2005, Santos and Hoshino, 2005) and an additional 11 new G-P combinations found in human RVA strains including new antigen combination from sporadic cases for the 2007-2012 period, the post vaccine licensure period (Doro et al., 2014). However, the common combinations were limited to G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] (Banyai et al., 2012, Doro et al., 2014, Gentsch et al., 2005, Kawai et al., 2012, Santos and Hoshino, 2005).

Another classification of rotavirus is defined by RNA- RNA hybridization with probes prepared from the prototype strains. From the first two genogroups, the Wa and DS-1 (Flores et al., 1982), after 6 years, AU-1 genogroup was added (Nakagomi et al., 1989). The strains in the same genogroup of human rotaviruses contain the corresponding genes (in which the Wa strain is the prototype, for examples) share extensive homology, but this degree of homology is not shared with strains belonging to the other genogroup (in which the DS-1 strain is the prototype) and reversely. Interestingly, the Wa and DS-1 genogroup correspond respectively to two distinct electropherotypes of viral RNA: long patterns (characterised by their faster-moving 10th and 11th gene segments) and short patterns (slower-moving 10th and 11th gene

segments) (Nakagomi et al., 1988). Furthermore, short and long patterns were believed to be associated with subgroup I or II specificity, respectively until the identification of the AU-1 strain that belongs to subgroup I yet has a long RNA pattern. Super-short pattern infrequently detected showed the rearranged 11<sup>th</sup> genome segment even longer than that of short pattern.



G and P classification might miss the differences in the genotype constellation that only occur in the internal capsid and non-structural protein genes where human rotavirus-specific genotypes are replaced with unusual or animal rotavirus-specific genotypes. As more rotavirus strains were sequenced for the entire genome segments, there was an increasing need for expanding a similar classification system for every genome segment; thus, the RCWG was organised, and it adopted the classification system in which the genome of individual rotavirus strains is given the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-



Tx-Ex-Hx (“x” representing the genotype number) for denoting the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes, respectively (Matthijnssens et al., 2008a, Matthijnssens et al., 2011, Matthijnssens et al., 2008b). The three major genotype constellations are described as: G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (Wa genogroup), G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (DS-1 genogroup) and G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3 (AU-1 genogroup) (Matthijnssens et al., 2011, Matthijnssens et al., 2008b). The numbers of 11 genome segments were listed in Table.5.1

**Table 5.2.** Full-genome constellations of some human and animal RVA strains. “-” indicates that the genotypes is unknown. Interspecies transmitted RVA strains were not listed here as they are listed in Table 5.3. Blue, red, yellow and purple shades are used for segments belonging to the lineage constellation of the ancestor Wa, DS-1, AU-1 and PO-13 strains, respectively. Grey shades indicate other genotypes.

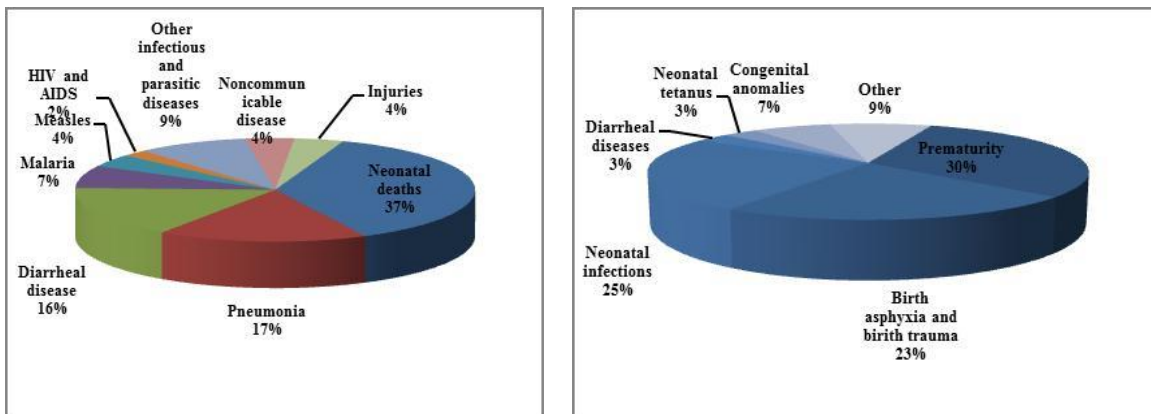
		VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
KU	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
DS-1	Human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
KUN	Human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
AU605	Human	G2	P[4]	I2	R2	C2	M2	A2	N1	T2	E2	H2
NT015	Human	G1	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
AU-1	Human	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
WC3	Bovine	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Gottfried	Porcine	G4	P[6]	I5	R1	C1	M1	A8	N1	T1	E1	H1
OSU	Porcine	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
PO-13	Avian	G18	P[17]	I4	R4	C4	M4	A4	N4	T4	E4	H4
RRV	Simian	G3	P[3]	I2	R2	C3	M3	A9	-	T3	E3	H6
FRV-1	Feline	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3

FRV-64	Feline	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
02V00-002G3	Chicken	G19	P[17]	I4	R4	C4	M4	A4	N4	T4	E4	H4
10V01-12H5	Pheasant	G23	P[37]	I4	R4	C4	M4	A16	N4	T4	E4	H4

### 5.3. Epidemiology

#### 5.3.1. The global burden of rotavirus

Diarrhoea is one the most important issues causing high morbidity and mortality in children, especially are under 5 years old. In 2003, among 6 causes which attributed to 73% deaths in children younger than 5 years old, diarrhoea was the secondary, accounted for 18% (including 17% deaths in children 1- 59 months and 3% in neonatal deaths), just after pneumonia (19%) (Bryce et al., 2005).



**Fig.5.5.** Worldwide mortality in neonates and children under 5 years old (adapted from (Parashar et al., 2003). Left panel. Worldwide causes of neonatal deaths; Right panel. Worldwide causes of death in children under 5 years of age

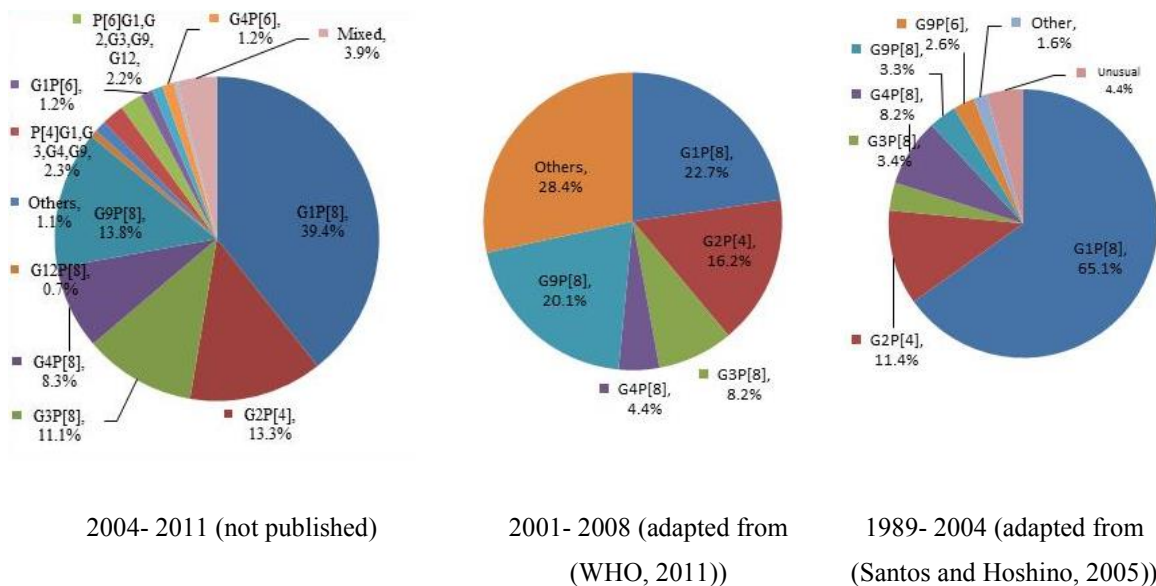
Among all diarrhoeal causes, rotavirus is the leading cause of severe gastroenteritis in infants and young children worldwide (Parashar et al., 2009, Tate et al., 2011). According to the data from WHO rotavirus surveillance network, the medians detection of rotavirus in

hospitalised children were 40% in 2010 and 39% in the period from 2000- 2004 (WHO, 2011). Of estimated 1,566,000 childhood diarrhoea deaths in 2002, rotavirus caused approximately 611,000 childhood deaths (IQR 454,000- 705,000) and more than 80% of all rotavirus-related deaths were estimated to occur in low-income countries of South Asia and sub-Saharan Africa (Parashar et al., 2006). Worldwide in 2008, diarrhoea attributable to rotavirus infection resulted in 453 000 deaths (95% CI 420 000–494 000) in children younger than 5 years old and 37% of deaths attributable to diarrhoea and 5% of all deaths in children younger than 5 years old (Tate et al., 2011).

### ***5.3.2. Molecular epidemiology of human RVA***

Before 2005, the five most common G and P combinations were G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] represented over 92% of the strains analysed globally (Gentsch et al., 2005, Santos and Hoshino, 2005). After 2005, I reviewed the global distribution of G and P genotype and noticed that, from 2005 to 2011, G1P[8] genotype was still the most prevalent one which accounted for more than one third of all combinations (39.4%). The other four common combinations were G9P[8], G2P[4], G3P[8] and G4P[8] (15.5%, 14.2%, 12.6% and 5.1%, respectively). The five common combinations accounted for 87% of all G and P combinations. The other combinations including several novel G types and P types comprised about the rest of 13% of the total. Global distribution of G2 RVA strains did not change much over time, from 14.1% before 2004 to 15.3% after that. However, there was a big change in G9 RVA strains frequency, from 5.8% through the end of 2001 and 5% before 2004 (Gentsch et al., 2005, Laird et al., 2003, Santos and Hoshino, 2005) and increased to 19.1% in the period 2005 - 2011 and became the secondary common genotype after G1 genotype. In the future, G9 strains may need to be included in the vaccine because of the quick global spread (Tcheremenskaia et al., 2007). The first G12 strain isolated in Philippine in 1987. After 10 year unable to detect, it increased rapidly worldwide, from 0.1% before 2004 to 1.5% after that. Recently, in the post vaccine licensure surveillance, G12P[8] strains were accounted for 2.7% of all specimens collected in the countries where rotavirus vaccines were introduced.

G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] were still the most five common G and P combination (31.2%; 13.0%, 10.7%, 4.0% and 10.2%, respectively). Together with G12P[8] strains (Doro et al., 2014), these six G and P combination strains were found in 73% of the samples or 87% of the specimens when non-typeable strains and mixed-infections were removed.

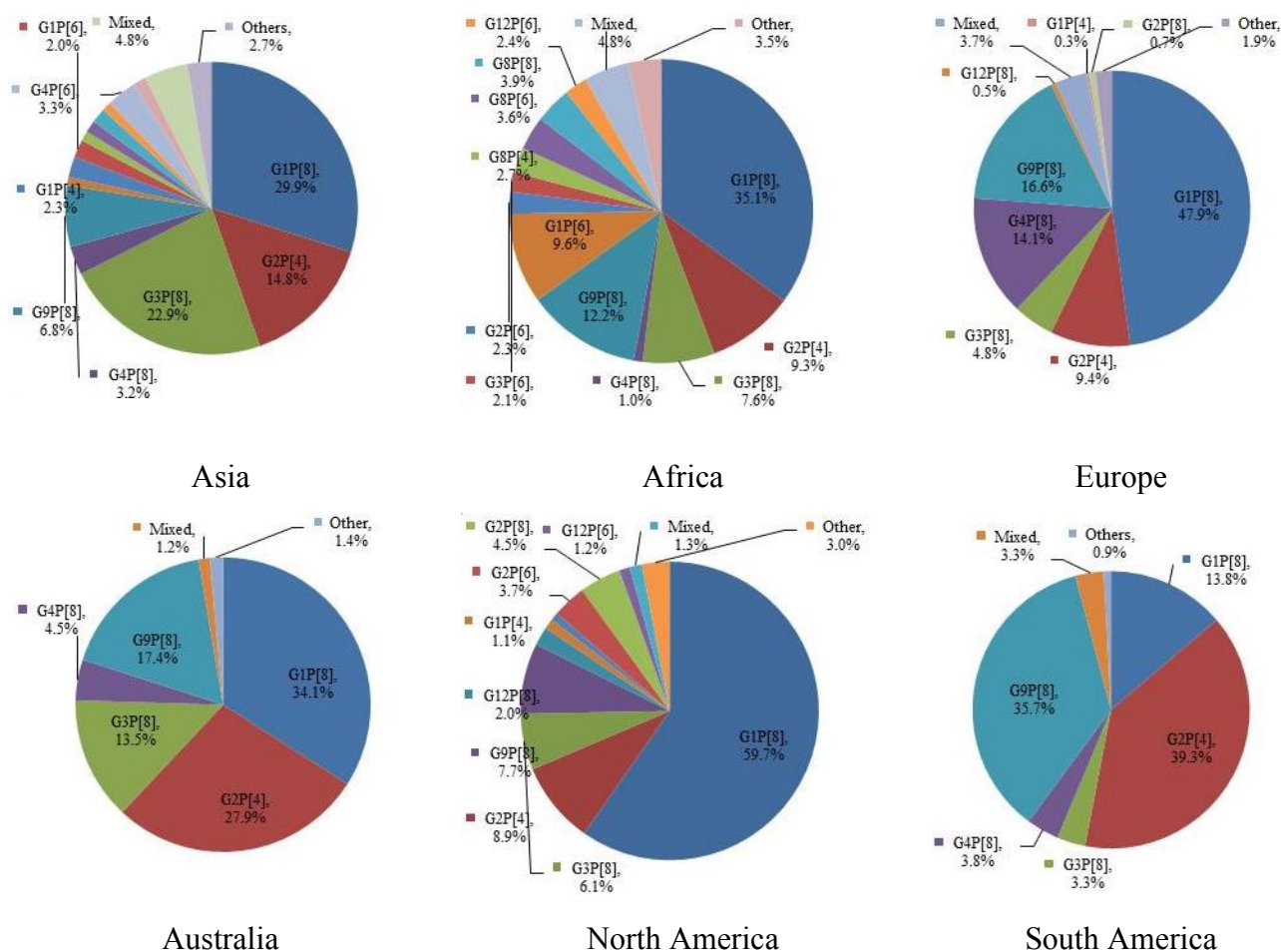


**Fig.5.6.** Global distribution of G-P combinations of RVA strains in diferent time periods.

The genotype distributions were favourable patterns in each continent and country. In Europe and North America, G1P[8] was predominant (47.9% and 59.7%, respectively), In Australia and South America, the highest frequency was G2P[4] genotype (27.9% and 39.3%, respectively) and that in Asia was G3P[8] genotype. In Africa, although G1P[8] strains were predominant, other combinations such as G9P[8], the frequency of G1P[6] strains were higher than in other continents. After vaccine introduction, predominance circulation of G1P[8] strains was found in most of regions except Americas where G2P[4] strains were relatively more commonly reported (Doro et al., 2014).

Before 2005, in Africa, the relative frequency of G8 was as high as that o the global common G3 or G3; P[6] represented for almost one-third of all P types identified; RVA strains bearing unusal combinations were accounted for 27% of rotavirus infection (Santos and Hoshino, 2005). After vaccine introduction in Africa, P[6] RVA strains continued to circulated at marked prevalence (Doro et al., 2014).

In our review, G1 genotype strains accounted for 43%, followed by G9 genotype strains (17.0%), G2 (15.3%), G3 (12.6%) and G4 (9.5%). These five common rotavirus G types comprised about 97% of the total. G2 genotype became the most predominant genotype in South America (two times higher than that in North America and nearly 3 times higher than that of global distribution). In Australia where two kinds of rotavirus vaccine was used in different regions, the follow-up study after vaccination showed that in the states using RotaTeq, G1P[8] was predominant, followed by G2P[4] while in the states using Rotarix, G2P[4] was predominant followed by G1P[8] (Kirkwood et al., 2011, Kirkwood et al., 2009, Snelling et al., 2011). On the other hand, G2P[4] genotype in Europe is nearly same in frequency before and after 2004 (Santos and Hoshino, 2005) and similar in the vaccine and non-vaccine regions (Antunes et al., 2009). These data supported a strong evidence of the fluctuant circulation of G2P[4] rather than vaccine induced selection (Iturriza-Gomara et al., 2009). In South America, especially Brazil, G2P[4] became the most predominant genotype or circulated as the only genotype that could find after the introduction of Rotarix vaccine (Borges et al., 2011, Correia et al., 2010, Gomez et al., 2011, Luchs et al., 2011, Vieira et al., 2011). However, several studies indicated that G2P[4] and G2P[NT] was not homogeneous throughout Brazil (Leite et al., 2008). In addition, G2P[4] was also the predominant genotype in the neighbouring countries where rotavirus was not available (Esteban et al., 2010, Martinez et al., 2010). Taking together with the high efficacy of Rotarix against G2P[4] (77%) (Justino et al., 2011) showed the natural fluctuation of rotavirus G2P[4] (Vieira et al., 2011).



**Fig.5.7.** Distribution of G-P combinations of human RVA strains by continents/sub-continents, 2005 - 2011 (unpublished)

### 5.3.3. Rotavirus transmission

Rotaviruses are transmitted by the faecal-oral route. The circumstance whether rotaviruses are transmitted also by the respiratory route was not well proven yet. However, there were some evidences for this circumstance are: the rapid acquisition of rotavirus antibodies in the first few years of life, regardless of hygiene standards; a few large

outbreaks in which a faecal - oral spread could not be documented; the occurrence of respiratory symptoms in a proportion of patients with rotavirus gastroenteritis.

The resistance to physical inactivation (along with the large number of viral particles shed in faeces) and long-life outside of the host may contribute to the efficient and rapid transmissions of the human rotaviruses. Although rotaviruses have been detected in raw or treated sewage, it is unlikely that contaminated water (which is very important in RVA outbreaks) plays an important role in transmission of RVA strains.

High relative humidity (about 80%) results to a rapid loss in human rotavirus infectivity. Effective disinfection of contaminated material and careful hand-washing constitute important measures to contain rotavirus infection, especially in hospital and school.

#### **5.4. Immunity**

Rotaviruses often cause symptomatic infection in children from 3 months to 2 years of age. Severe diarrhoea likely occurs in the children from 3 to 24 months of age than other age group. In infants, natural rotavirus infection confers protection against subsequent infections and this protection accumulates after new infection and help to reduce the severity of the diarrhoea (Velazquez et al., 1996).

The mechanisms responsible for immunity to rotavirus infection and illness are not completely understood, especially in humans. However, some observations suggested that antibodies in the lumen of the small intestine were the primary determinant of resistance to rotaviruses, whereas circulating serum antibodies failed to protect (Estes and Kapikian, 2007). After a mucosal humoral and cell-mediated immune response, viruses are cleared within 1 week. Rotavirus-specific immunoglobulin A (IgA) antibodies on the enteric mucosal surface are thought to mediate protective immunity. Infection with one serotype provides serotype-specific (homotypic) protection, and repeated infections lead to partial cross- serotype (heterotypic) protection (Cunliffe and Nakagomi, 2007).



### **5.5. Clinical feature:**

The incubation period of rotavirus diarrhoeal illness has been estimated to be less than 48 hours. The incubation period of the illness under the experiment conditions was 1 to 4 days (Estes and Kapikian, 2007).

Fever, vomiting and watery diarrhoea are the most common symptoms observed in the infected children (Cunliffe and Nakagomi, 2007). These symptoms normally last within one week. The temperature is 37.9°C or above. Rotavirus diarrhoea starts later than vomiting but lasts longer (Rodriguez et al., 1977). Death occurs within 1 or 2 days of onset of symptoms. The major factor causing death is believed to be dehydration and electrolyte imbalance and seizures is a contribution factor (Carlson et al., 1978). Rotavirus can produce a chronic symptomatic infection in immunodeficient children. During chronic infection, rotavirus can undergo rearrangements in its genome, leading to abnormal electrophoretic migration patterns of rotavirus RNA (Desselberger, 1996, Hundley et al., 1987). Because rotaviruses are also found in the organ outside the intestine, such as respiratory tract, liver, central nervous system and kidney, the symptoms of these organs infections are recorded in the rotavirus infection children. There is limited evidence to show that the severity of the illness is related to the serotype.

The clinical manifestations of rotavirus illnesses are not typical enough to diagnose on this basis alone. Therefore, the diagnosis needs to base on the laboratory results including detection of virus appearance and serological response. Stool specimens collected during the first four days after the onset is sufficient for virus detection but stool specimen can be collected up to 3 weeks after the onset depending on the duration of symptoms. Electron microscope that visualizes the virus particle directly has the advantage of high specificity because of the distinct morphologic appearance. ELISA assay which detect rotavirus antigen can solve the disadvantages of electron microscopy because of high sensitivity, simple requirement in equipment as well as availability of commercial kits. There are also some other techniques for virus detection such as counter-electro-

osmophoresis, gel electrophoresis, reverse passive hemagglutination assay, latex agglutination, dot hybridization assay and chromatography. However, RT-PCR and realtime RT-PCR becomes the most applicable and common techniques used in rotavirus diagnosis, which is 100,000 times more sensitive than the standard electropherotype method and 5,000 times more sensitive than hybridization.

To measure a serological response to rotavirus infection, there are a variety of techniques such as immunological EM, complement fixation (CF) tests, immunofluorescent assay, immune adherence hemagglutination assay, ELISA to detect antibody, neutralisation, hemagglutination inhibition, inhibition of reserve passive hemagglutination, enzyme – linked immunospot (ELISPOT) assay and ummuno-cyto-chemical staining assay (Ishida et al., 1997, Kapikian, 1993, Shaw et al., 1992).

Until now, there are not many effective treatment methods for most virus infections in general and rotavirus infection is no exception. The priority treatment is replacement the fluid and electrolytes lost by vomiting and diarrhoea. Intravenous fluid showed the high efficacy in treating dehydration from diarrhoea for many years and oral fluid replacement therapy also useful in the place that intravenous fluid is unavailable. A variety of formulation for oral rehydration salt (ORS) solutions has been developed and showed the high efficacy in the treatment of dehydration due to rotavirus diarrhoea. Glucose- based, sucrose- based and rice- based solutions are compared to each other to see the effect and all of them show the great archive in decreasing the stool output, increasing the absorption and retention of fluid and electrolytes.

Human milk, containing rotavirus antibodies, has been used successfully for treatment of children who are immune-deficient with chronic rotavirus infection and illness (Davidson, 1996, Losonsky et al., 1985, Saulsbury et al., 1980).

## **5.6. Prevention and control**

### **5.6.1. Rotavirus vaccines**

Realising the burden of rotavirus on the medical care and society, scientists have been exerted to produce an effective and safe vaccine to protect human, especially children, from severe diarrhoea caused by rotavirus. After the first licensed vaccine RotaShield™ was withdrawn because of a side effect, many clinical trials for the new vaccine were conducted with the hope on finding an ideal vaccine. Two globally-licensed rotavirus vaccines, Rotarix™ (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq™ (Merck & Co., Whitehouse Station, USA) have been recommended by the WHO for inclusion into national immunization programmes (WHO, 2009). RotaRix is a monovalent vaccine containing human G1P[8] strain (RIX4414) (Vesikari et al., 2004). RotaTeq is a pentavalent vaccine that comprises 4 reassortant rotaviruses express the VP7 protein (G1, G2, G3 or G4) from the human rotavirus parents and the attachment protein P7[5] from bovine rotavirus parent strain; the fifth reassortant virus expresses the attachment protein P1A[8] from the human rotavirus parent strain and the outer capsid protein G6 from the bovine rotavirus parent strain (Desselberger, 2005). The vaccine trials and observational studies showed that Rotarix and RotaTeq were found to be safe, highly immunogenic and elicit both homotypic and heterotypic protections (Breiman et al., 2012, Kawamura et al., 2011, Li et al., 2014, Linhares et al., 2011, Phua et al., 2009, Steele et al., 2012, Tregnaghi et al., 2011, Velasquez et al., 2014, Vesikari et al., 2010, Vesikari et al., 2007, Vesikari et al., 2006). These two vaccines also have shown their impacts on the reduction of mortality and morbidity due to severe diarrhoea (Bar-Zeev et al., 2015, Esparza-Aguilar et al., 2014, Gurgel et al., 2014, Macartney et al., 2011, Plosker, 2010, Richardson et al., 2011, Tate and Parashar, 2011).

It is also known that the efficacy of either vaccine in developing countries is consistently lower than in developed countries; the efficacy of RotaTeq against severe rotavirus gastroenteritis during the first year of life was 51.0% in Asia and 64.2% in Africa

(Armah et al., 2010, Zaman et al., 2010). Recently, a monovalent vaccines derived from the human neonatal strain 116E was developed in India (Rotavac, Bhara Biotech International). The vaccine strain was natural reassortant strain G9P[11] with P[11] gene from bovine RVA strain (Bhandari et al., 2014a, Bhandari et al., 2014b). Another live-attenuated vaccine (Rotavin-M1) containing a human G1P[8] rotavirus strain (KH0118-2003) was also developed in Vietnam (POLYVAC) (Dang et al., 2012).

While post-licensure studies detected a small and statically significant risk of intussusception associated with the administration of Rotarix, the conclusion is given that Rotarix is safe to administer to infants between 6- 12 weeks of age for the first dose and by 24 weeks of age for the second dose. However, the authors strongly discourage the delayed administration of the first dose between 13 to 20 weeks of age, which is allowed without any warning (Nakagomi, 2011).

#### **5.6.2. *Passive immunisation***

After many experiments, passive immunisation has been shown to be effective in preventing rotavirus illness in animals (Ebina, 1996, Hammarstrom, 1999, Sarker et al., 1998). The studies carried out in human have not had results yet. However, passive immunisation is not practical for protection against rotavirus illness in humans, except for some special circumstances such.

#### **5.7. Antigenic and genetic diversity of G2 VP7 genes**

The VP7 protein is an abundant capsid protein and is usually encoded by genome segment 9 (Estes and Cohen, 1989). The nucleotide sequence predicts an open reading frame of 326 amino acids (Kozak, 1986). Nine variable regions (VRs) have previously been described that are homologous between the same G type but highly different across the specific serotypes (Gorziglia et al., 1990, Green et al., 1987, Nishikawa et al., 1989). Six of these regions are regarded as major antigenic regions (A-F): A (amino acid (aa) 87- 100), B (aa 141-150), C (aa 208-224), D (aa 291), E (aa 190) and F (aa 235-242) (Coulson and

Kirkwood, 1991, Dyall-Smith et al., 1986, Estes and Cohen, 1989, Glass et al., 1985, Hoshino et al., 1994, Kirkwood et al., 1993, Lazdins et al., 1995, Mackow et al., 1988, Taniguchi et al., 1988).

With the development of the advanced technologies in biology, the antigenic epitopes are mapped in the neutralisation domains of the VP7 proteins based on the escape mutations selected by various neutralising monoclonal antibodies (mAbs) that recognise VP7 using the crystal structure of the rhesus rotavirus (RRV, G3) (Aoki et al., 2009, Dormitzer et al., 2004). These residues cluster into neutralisation domains 7-1a, 7-1b and 7-2.

	7-1a															7-1b							7-2									
Name of strains	87	91	94	96	97	98	99	100	104	123	125	129	130	291		201	211	212	213	238	242		143	145	146	147	148	190	217	221	264	
SA11/G3	T	T	N	N	S	W	K	D	Q	N	A	V	D	K		Q	D	A	T	D	A		K	D	A	T	L	S	E	A	G	
Wa/G1	.	.	.	G	D	.	.	.	.	.	V	.	.	.		.	N	V	D	N	T		.	.	Q	S	.	.	M	N	.	
DS-1/G2	A	N	S	D	E	.	E	N	.	D	N	.	N	.		.	.	V	N	N	N		R	.	N	.	S	D	I	S	.	
KUN/G3	.	N	S	D	E	.	E	N	.	D	T	M	N	.		.	.	V	N	N	S		R	.	N	.	S	D	I	S	.	
AU-1/G3	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	T	N	N	N		.	.	.	.	.	.	.	.	.	
YO/G3	.	.	.	.	.	E	.	.	.	.	.	.	.	.		.	.	T	N	N	N		.	.	.	.	.	.	.	.	.	
95H115/G9	.	.	G	T	E	.	.	.	.	D	.	I	.	.		.	N	T	A	.	N		.	.	S	.	.	.	.	S	.	
L26/G12	N	.	T	P	D	.	T	H	.	D	.	.	N	.		.	.	V	A	N	N		Q	Q	N	S	.	.	.	.	.	
T152/G12	S	.	T	P	D	.	T	N	.	D	.	.	.	.		.	.	V	.	N	N		Q	Q	N	S	.	.	.	.	.	
Rotarix/G1	.	.	.	G	E	.	.	.	.	S	V	.	.	.		.	N	V	D	N	T		.	.	Q	N	.	.	M	N	.	
RotaTeq-SC2-9/G2	A	N	S	D	E	.	E	N	.	D	T	M	N	.		.	.	V	S	N	S		R	.	N	.	S	D	I	S	.	
RotaTeq-WI79-9/G1	.	.	.	G	D	.	.	.	.	S	V	.	.	.		.	N	V	D	N	T		.	.	Q	S	.	.	M	N	.	

**Fig.5.8.** Amino acid alignment of several human rotavirus A strains, simian strain (SA11) and vaccine strains in the neutralisation domains of VP7 gene. The numbering of amino acid residues is based on the RRV sequence.

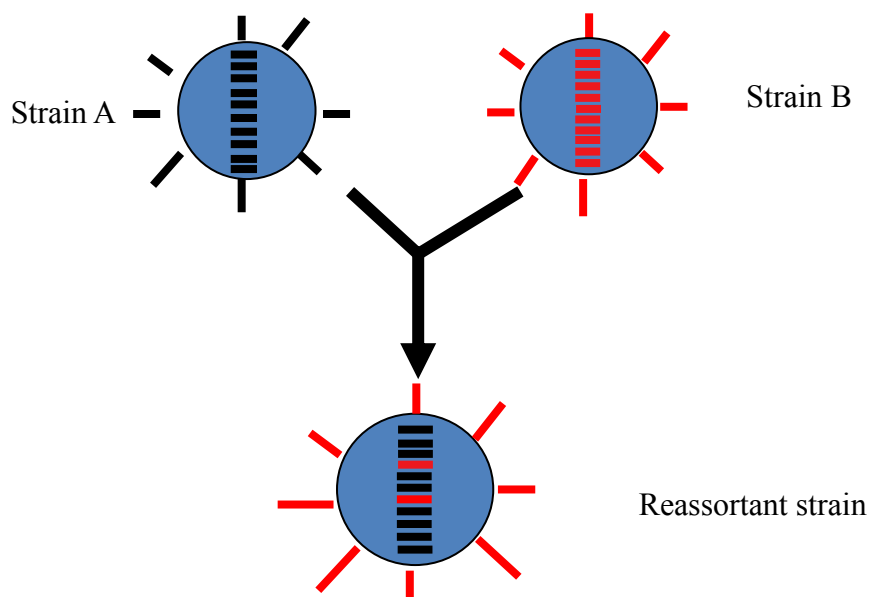
The VP7 sequence analysis of escaped mutants selected by G2 serotype-specific mAbs revealed that mutations at residue 94, 190, 201, 213 and 291 involved in virus neutralisation (Aoki et al., 2009, Dunn et al., 1993, Dyall-Smith et al., 1986, Higo-Moriguchi et al., 2004, Lazdins et al., 1995, Morita et al., 1988, Raj et al., 1992). In addition, the nucleotide sequence analysis of VP7 of G2 rotaviruses revealed that the point mutations across the VP7 gene are related to the existence of 4 lineages: A87T, D96N, N213D, N242S (Doan et al., 2011). This study also showed that the G2 VP7 genes evolved in the dynamic fashion in which a new lineage emerged within the dominant lineage and replaced that to become the dominant lineage (Doan et al., 2011). The average time

between lineage divergence events in the G2 phylogenetic history is calculated to be 7 years (Dennis et al., 2014). The mutation rates of G2 VP7 gene ( $1.45 \times 10^{-3}$ /site/year) is similar to that of G9 ( $1.87 \times 10^{-3}$ /site/year) strains and G12 ( $1.66 \times 10^{-3}$ /site/year) strains, which spread globally in less than a decade (Dennis et al., 2014, Matthijnssens et al., 2010a). The gradual acquisition of point mutations in the VP7 gene has facilitated the expansion of G2 diversity during the last century (Dennis et al., 2014).

### **5.8. Interspecies transmission of porcine rotaviruses to human hosts**

Rotaviruses are one of the viruses having a broad host range including mammalian and avian species. Although it is thought that host species of rotaviruses are restricted, an increasing number of novel rotavirus strains including several genotypes detected in humans which are believed to be of animal origin suggesting that interspecies transmission is one of the key factors for generating rotavirus diversity from the evolutionary context. Some of the most predominant genotypes (G3, G9 and G12) detected in humans are proven to be of animal rotavirus origin (Nakagomi and Nakagomi, 1989, Ghosh et al., 2006, Hoshino et al., 2005, Martella et al., 2010, Matthijnssens et al., 2006, Nakagomi et al., 1989, Teodoroff et al., 2005, Tsugawa and Hoshino, 2008). The epidemiological importance of these genotypes in the human population suggests that after crossing the host species barrier, they were adapted to the human hosts and then successfully spread globally.

Rotaviruses are transmitted from animals to humans either as a whole virion or by genetic reassortment during co-infection (Nakagomi and Nakagomi, 1993, Nakagomi and Nakagomi, 2002). The reassortant formation is probably the most efficient when the co-infecting strains are closely related to each other since the genome segments or the proteins they encode must function interchangeably. There is no reassortment between *Rotavirus* and *Reovirus*, both of which belong to *Reoviridae*. Similarly, no reassortment has been reported between rotaviruses belonging to different rotavirus species to date.



**Fig.5.9.** Reassortant process by which a new rotavirus strain was created. The reassortant strain carries the genome segment 4 and 7 from strain A and the remaining segments from strain B during the co-infection.

A number of studies provided evidence that direct transmission from RVA strains of ruminant or carnivore origin to humans are common (Banyai et al., 2009b, Doan et al., 2013, Nakagomi and Nakagomi, 1991, Nakagomi and Nakagomi, 1989, Nakagomi et al., 1989, Nakagomi and Nakagomi, 2000, Shirane and Nakagomi, 1994, Tsugawa and Hoshino, 2008, Wu et al., 2012). On the other hand, reports describing direct transmission of porcine rotaviruses to humans are less frequently found probably because the close and intermingled genetic relationships between human and porcine RVA strains (Papp et al., 2013a) make it sometimes difficult to clearly distinguish the true origin of the genome segment. The number of strains resulted from the direct transmission from porcine RVA strains is much smaller than that resulted from genetic reassortment. While BE2001,

Ryukyu-1120 and mani326/2007 were examples of direct transmission of porcine RVA strains as a whole genome, the majority of porcine-like human RVA strains involve in genetic reassortment (Table 5.3). RVA strains that carry human RVA genes in their porcine RVA genome may more transmissible in humans, thus, can be more readily circulated in patients causing gastroenteritis (Dong et al., 2013, Mladenova et al., 2012, Papp et al., 2013a, Papp et al., 2013b).



**Table 5.3.** Human rotavirus strains transmitted from pigs by direct transmission or genetic reassortment and the origin of each genome segment. Blue, red shades indicate genome segments originated from human and porcine RVA strains. Grey shades indicate genome segments which the origins are indeterminable whether the origin is of porcine or human rotaviruses.

Strain	Place of detection	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Reference
GX54	China	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	Dong et al., 2013
CAU12-2	Korea	G11	P[25]	I12	R1	C1	M1	A1	N1	T1	E1	H1	Jeong et al., 2014
BP1901	Hungary	G4	P[6]	I1	R1	C1	M1	A1	N1	T7	E1	H1	Papp et al., 2013a
BP271	Hungary	G4	P[6]	I1	R1	C1	M1	A8	N1	T7	E1	H1	Papp et al., 2013a
Mc323	Thailand	G9	P[19]	I5	R1	C1	M1	A8	N1	T1	E1	H1	Ghosh et al., 2012b
BE2001	Belgium	G9	P[6]	I5	R1	C1	M1	A8	N1	T7	E1	H1	Zeller et al., 2012
Ryukyu1120	Japan	G5	P[6]	I5	R1	C1	M1	A8	N1	T1	E1	H1	Komoto et al., 2013
EC2184	Ecuador	G11	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	Banyai et al., 2009a
RMC321	India	G9	P[19]	I5	R1	C1	M1	A8	N1	T1	E1	H1	Varghese et al., 2006
Arg4605	Argentina	G4	P[6]	I1	R1	C1	M1	A8	N1	T7	E1	H1	Degiuseppe et al., 2013
Arg4671	Argentina	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	Degiuseppe et al., 2013
IAL28	Brazil	G5	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	Heiman et al., 2008
Mani-97/06	India	G9	P[19]	I5	R1	C1	M1	A8	N1	T1	E1	H1	Mukherjee et al., 2011
Mani-253/07	India	G4	P[4]	I1	R1	C1	M2	A8	N1	T1	E1	H1	Mukherjee et al., 2011

Mani-362/07	India	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	Mukherjee et al., 2011
mcs/13-07	India	G9	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	Mukherjee et al., 2011
R479	China	G4	P[6]	I5	R1	C1	M1	A1	N1	T7	E1	H1	Wang et al., 2010
KTM368	Nepal	G11	P[25]	I12	R1	C1	M1	A1	N1	T1	E1	H1	Matthijnssens et al., 2010b

## **CHAPTER 6: MATERIALS AND METHODS**

### **6.1. Specimens**

All the specimens analysed in this thesis were collected from children who admitted to hospitals for acute diarrhoea in Japan in the pre-vaccination era.

In the first study, a total 35 G2 rotavirus strains that were analysed for their VP7 genes were in the collection of the archival stool specimens obtained from children with acute gastroenteritis in Akita or Yamagata Prefectures (two neighbouring prefectures in northern Japan) between 1983 and 2007, and the stool specimens obtained during the official surveillance activities at the Nara Prefectural Institute for Hygiene and Environment, Nara prefecture, Japan between 2008 and 2011.

In the second study, the AU19 strain was originally detected in Akita, Japan in 1997 from an infant with severe acute gastroenteritis (Nakagomi et al., 1999).

### **6.2. RNA extraction and genome segment amplification**

The genomic RNAs which were extracted from a 10% suspension or cell culture fluid by using the QIAamp Viral RNA Mini Kit (QIAGEN Sciences, Germantown, MD, USA) according to the manufacturer's instruction, and then reverse transcribed to cDNA by using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen).

The cDNAs were used for the PCR reactions to amplify the genome segments using specific primers and the AccessQuick RT-PCR system (Promega Corporation, Madison, WI, USA). The VP7 genes of the Japanese G2 strains and the AU19 strain were amplified according to the method of Gouvea et al. (Gouvea et al., 1990) or G2VP7-14F (5'- AGA ATT TCC GTC TGG CTA G - 3') and G2VP7-998R (5'- AAT GAT CTT GAC CGT TTG

GAC -3') (designed for this study). The other genome segments of the AU19 strain were amplified with the specific primers described in Table 6.1.

**Table 6.1.** The primers used to amplify the genome segments of the AU19

Genome segment	Primer name	Primer sequence	Reference
VP1	GEN_VP1Fb	5'-GGC TAT TAA AGC TRT ACA ATG GGG AAG-3'	Matthijnsens et al., 2008a
	GEN_VP1Rb	5'-GGT CAC ATC TAA GCG YTC TAA TCT TG-3'	Matthijnsens et al., 2008a
VP2	GEN_VP2Fc	5'-GGC TAT TAA AGG YTC AAT GGC GTA CAG-3'	Matthijnsens et al., 2008a
	GEN_VP2Rbc	5'-GTC ATA TCT CCA CAR TGG GGT TGG-3'	Matthijnsens et al., 2008a
VP3	M1V3 – 22F	5'-AGT GCG TTT TAC CTC TGA TGG – 3'	This study
	M1V3_2591R	5'-GGT CAC ATC GTG ACT AGT GTG TTA-3'	This study
VP4	BegG4	5'-TGT ATC ATA CGG CTA TAA AAT G-3'	Isegawa et al., 1992
	EndG4	5'-TAA GGT TAT GTA TGG TCA CAT C-3'	Isegawa et al., 1992
VP6	GEN_VP6F	5'-GGC TTT WAA ACG AAG TCT TC-3'	Matthijnsens et al., 2008a
	GEN_VP6R	5'-GGT CAC ATC CTC TCA CT- 3'	Matthijnsens et al., 2008a
NSP1	GEN_NSP1F	5'-GGC TTT TTT TTA TGA AAA GTC TTG-3'	Matthijnsens et al., 2008a
	GEN_NSP1R	5'-GGT CAC ATT TTA TGC TGC C-3'	Matthijnsens et al., 2008a

NSP2	GEN_NSP2F	5'-GGC TTT TAA AGC GTC TCA G-3'	Matthijnsens et al., 2008a
	GEN_NSP2R	5'-GGT CAC ATA AGC GCT TTC-3'	Matthijnsens et al., 2008a
NSP3	GEN_NSP3F	5'-GGC TTT TAA TGC TTT TCA GTG-3'	Matthijnsens et al., 2008a
	GEN_NSP3R	5'-ACA TAA CGC CCC TAT AGC-3'	Matthijnsens et al., 2008a
NSP4	GEN_NSP4F	5'-GGC TTT TAA AAG TTC TGT TCC-3'	Matthijnsens et al., 2008a
	GEN_NSP4R	5'-GGW YAC RYT AAG ACC RTT CC-3'	Matthijnsens et al., 2008a
NSP5	Beg11	5'-GGC TTT TAA AGC GCT ACA GTG ATG-3'	Giambiagi et al., 1994
	End11	5'-GGT CAC AAA ACG GGA GTG GG -3'	This study

All the primers are located at the 5' and 3' ends of the respective gene segments, except for primer GEN\_VP2\_Rbc, which starts at nucleotide 2, primer M1V3\_22F, which starts at nucleotide 22. Degenerate bases: R=A/G, W=A/T, Y=C/T.

### 6.3. Nucleotide sequence

The amplified products were then purified using an ExoSAP-IT purification kit (USB Products, Affymetrix, Cleveland, OH, USA) according to the manufacturer's instructions. Nucleotide sequencing reactions were performed by fluorescent dideoxy chain termination chemistry using the BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA), and then purified by using Sephadex. The nucleotide sequences were determined using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems).

#### **6.4. Phylogenetic analysis**

The nucleotide sequences thus obtained were aligned using the Megalign program in the Lasergene 8 software package (DNASTar, Inc. Madison, WI, USA). Calculation of nucleotide sequence identities and phylogenetic analysis were performed by using MEGA 5 (Tamura et al., 2011). The multiple sequence alignment was carried out using the CLUSTALW program, and the genetic distances between sequences were calculated by the Kimura two-parameter method. A phylogenetic tree was then constructed by using the neighbor-joining method. The bootstrap probability at a branching point was calculated with 1,000 pseudo-replicate datasets. Four amino acids at positions 87, 96, 213 and 242 in the antigenic region of VP7 were chosen to define amino acid constellation.

In the first study, we compiled the G2 VP7 nucleotide sequences that were determined in this study with those available in the DNA database by using the BLAST program (<http://blast.ncbi.nlm.nih.gov/>) with the DS-1 VP7 sequence as a query sequence. We increased the “Max target sequences option to 2000 to confirm to obtain all the BLAST hits. We retrieved more than 1000 sequences to include all possible G2 VP7 sequences, but used only sequences that had at least 615 nucleotides in length, spanning positions 307 and 921 (corresponding to amino acid residues from 87 to 291). We removed either sequences of reassortant vaccine strains nor animal rotavirus strains. To distinguish sequences of rotavirus G2 strains with those of the other rotavirus strains, we preliminarily reconstructed a phylogenetic tree with more than 1000 sequences. Porcine G2-like strains are known to have the highest sequence similarities to human G2 strains among all RVA strains reported. We obtained 509 nucleotide sequences of human G2 strains of which 21 sequences were from human G2 strains detected in Japan. Among these Japanese human G2 sequences, the AU605 was rotavirus strain culture-adapted from stool specimen 86A005, were used in this study. Thus, we excluded the AU605 sequence from the collection. Finally, a total 543 human G2 sequences of which 35 G2 sequences determined in this study, 20 Japanese human G2

sequences from GenBank database and 488 other human G2 sequences from GenBank database were used for this study.

In the second study, the genotype of each genome segment of AU19 was determined by using the RotaC2.0 automated genotyping tool for Rotavirus A (Maes et al., 2009). The phylogenetic trees were drawn by including the closest 100 sequences that were hit when the AU19 gene sequences was blasted against the GenBank database (VP7, VP4, NSP3, NSP4) or all sequences in the same genotype (VP1, VP2, VP3, NSP1, NSP2, NSP5).

### 6.5. Nucleotide sequence accession numbers

The sequences (n=35) of the VP7 genes were submitted to the DDJB/GenBank/EMBL databases and were assigned accession numbers AB821311-AB821345 (Table 2.1).

The nucleotide sequences for the VP4, VP6, VP1-VP3, and NSP1-NSP5 genes of AU19 were deposited in the DDJB/GenBank/EMBL databases under accession numbers AB770153 – AB770162 (Table 6.2).

**Table 6.2.** The accession number, year of detection and place where the strains were detected.

Strain	Year of detection	Place of detection	Accession number
83A001	1983	Akita Prefecture	AB770153
85A21140	1985	Akita Prefecture	AB770154
86A005	1986	Akita Prefecture	AB770155
86Y1329	1986	Yamagata Prefecture	AB770156
86Y187	1986	Yamagata Prefecture	AB770157
87Y1438	1987	Yamagata Prefecture	AB770158
87Y003	1987	Yamagata Prefecture	AB770159
87H038	1987	Akita Prefecture	AB770160
88F007	1988	Akita Prefecture	AB770161

88Y259	1988	Yamagata Prefecture	AB770162
88H449	1988	Akita Prefecture	AB770163
88Y1112	1988	Yamagata Prefecture	AB770164
89H354	1989	Akita Prefecture	AB770165
89A049	1989	Akita Prefecture	AB770166
89Y1520	1989	Yamagata Prefecture	AB770167
89H279	1989	Akita Prefecture	AB770168
90H377	1990	Akita Prefecture	AB770169
90Y289	1990	Yamagata Prefecture	AB770170
90Y593	1990	Yamagata Prefecture	AB770171
90H094	1990	Akita Prefecture	AB770172
01R137	2001	Akita Prefecture	AB770173
01P12	2001	Akita Prefecture	AB770174
01P7	2001	Akita Prefecture	AB770175
01P129	2001	Akita Prefecture	AB770176
01P17	2001	Akita Prefecture	AB770177
01P14	2001	Akita Prefecture	AB770178
01P8	2001	Akita Prefecture	AB770179
07Y03	2007	Yamagata Prefecture	AB770180
80004	2008	Nara Prefecture	AB770181
80120	2008	Nara Prefecture	AB770182
80123	2008	Nara Prefecture	AB770183
90096	2008	Nara Prefecture	AB770184
B100005	2011	Nara Prefecture	AB770185
B100056	2011	Nara Prefecture	AB770186
B100119	2011	Nara Prefecture	AB770187



## **ADDENDA**

### **(At the time of thesis submission)**

Since the submission of two papers on which this thesis was prepared (10 May, 2013, and 1 July, 2013, respectively), more than two years have passed during which there is remarkable progress that has significant implication on the questions I addressed in the AU19 paper; one by myself and another by other investigators.

The most common VP7 genotype of human RVA is G1, but G1 is rarely detected in porcine strains. To understand the evolutionary implications of AU19, I sequenced the VP7 genes of three Japanese G1 porcine strains, two isolated in 1980 (PRV2, S80B) and one in 2001 (Kyusyu-14), and performed phylogenetic and *in-silico* structural analyses. All three VP7 sequences clustered into lineage VI, which was 14.2% divergent from human G1 VP7 sequences belonging to lineages I-V. The VP7 genes of PRV2 and AU19 were 98% identical, strengthening the porcine RVA origin of AU19. The phylogenetic tree showed human G1 VP7 genes were originated from porcine G1 VP7 genes. The time of their most recent common ancestor was 1948, and human and porcine RVA strains evolved along independent pathways. *In-silico* structural analyses identified seven amino acid residues within the known neutralisation epitopes that show differences in electric charges or shape between porcine and human G1 strains. When compared with much divergent porcine G1 VP7 lineages, monophyletic, less divergent human G1 VP7 lineages support the hypothesis that all human G1 VP7 genes originated from a rare event of a porcine RVA transmitting to humans which was followed by successful adaptation to the human host. By contrast, AU19 represents interspecies transmission that terminated in dead-end infection.

Another significant observation was made by Nagai, et al. and published recently in *Vet Microbiol* (176: 250-256, 2015). The paper showed evidence that three porcine RVA strains detected in Japan in 2014 had the H2b genotype in their NSP5 genes and super-short RNA patterns. The observation by Nagai et al. prompted me to re-consider the conclusion I

drew that AU19 was resulted from interspecies transmission of a porcine RVA strain to a child coupled with acquisition of an H2 gene by genetic reassortment with co-circulating human RVA strains. The circulation of porcine RVA carrying H2b does not require to hypothesise the acquisition of a rare H2b gene by genetic reassortment with human RVA. The acquisition of an H2b gene, in turn, requires to hypothesise persistent human-to-human transmission of a precursor of AU19 in order to allow reassortment to occur. As such rare events are not necessary to hypothesise, it is more likely that AU19 was a fully porcine RVA at the time of jumping to infect a human. This scenario also agrees better with the observation that AU19 terminated in dead-end infection.

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1. Do LP, Nakagomi T, Doan YH, Kitahori Y, Nakagomi O. Molecular evolution of the VP7 gene of Japanese G2 rotaviruses before vaccine introduction. Archives of Virology 152: 315-319, 2014 **DOI: 10.1007/s00705-013-1804-6**
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