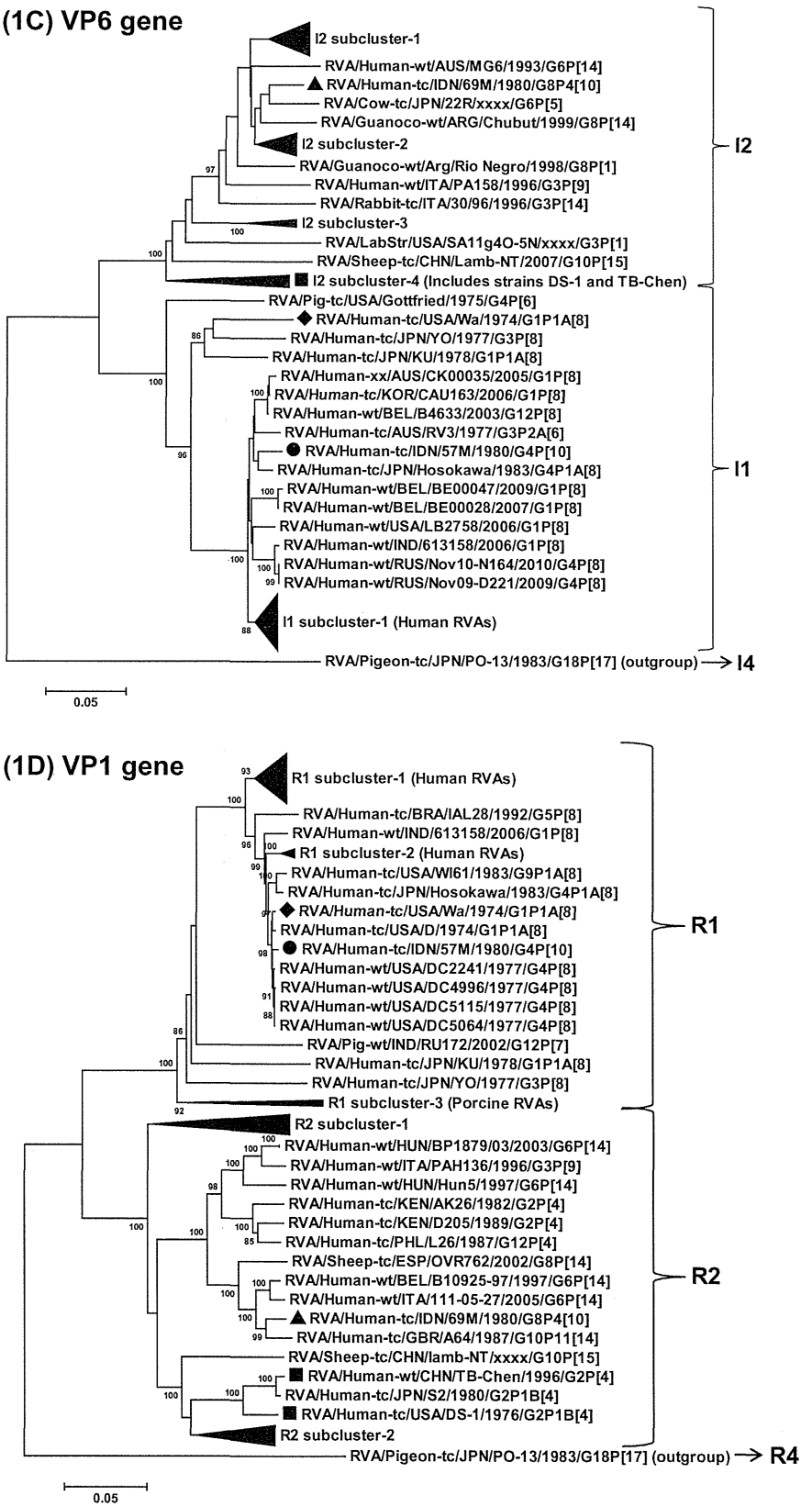

Fig. 1. (*continued*)

nucleotide sequence identities (⩽91%) with those of other DS-1-like RVAs. Phylogenetically, the NSP5 genes of strains 57M, 69M and B37 clustered together to form a distinct lineage (shown as H2b) within the DS-1-like H2 genotype (Fig. 1K).

Taken together, human G4P[10] RVA strain 57M appeared to have originated from intergenogroup reassortment events involving acquisition of 69M-like VP4, NSP3 and NSP5 genes by a co-circulating typical human Wa-like G4 RVA strain. This was

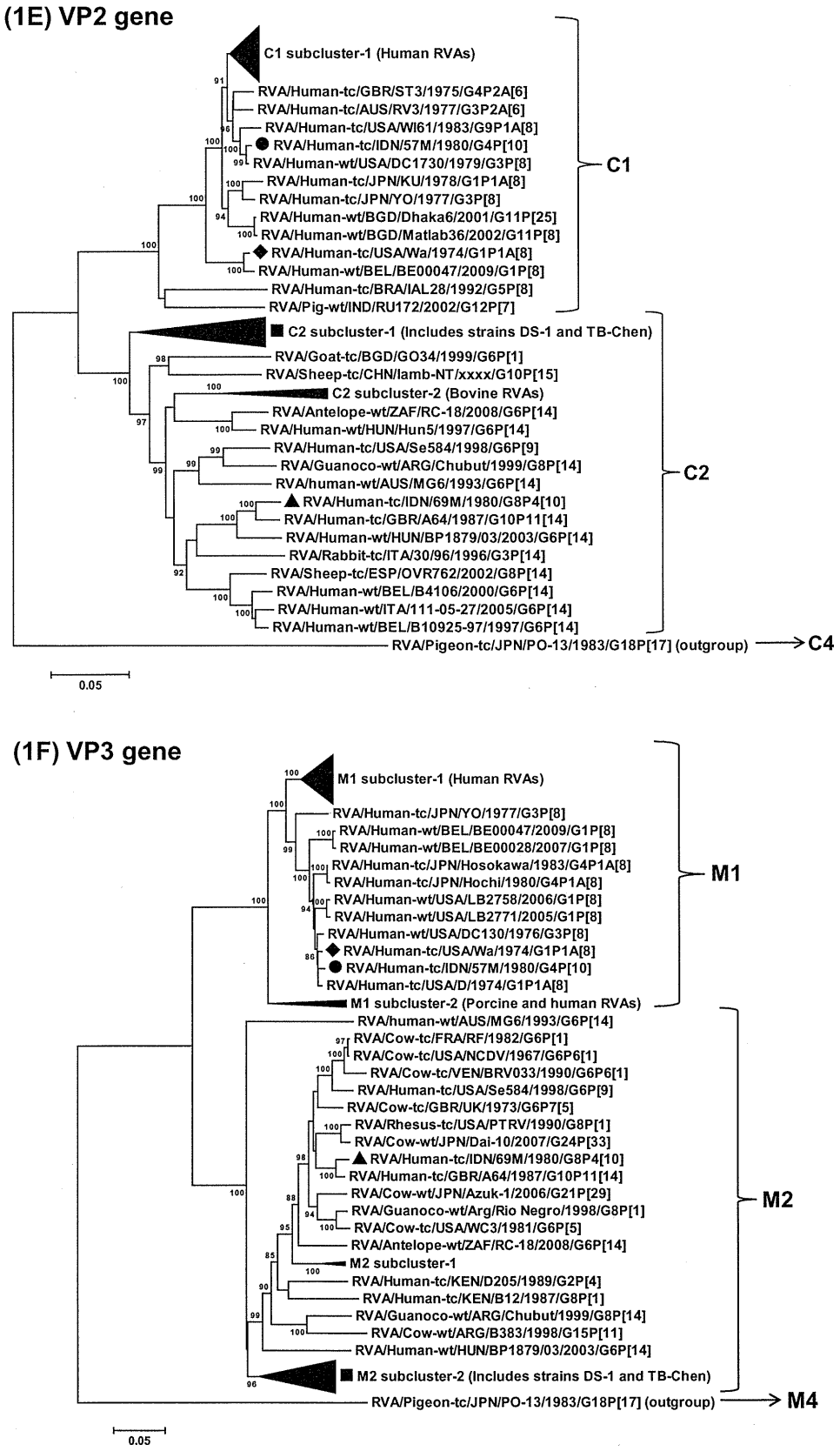

Fig. 1. (*continued*)

corroborated by the high levels of genetic relatedness observed in the VP4, NSP3 and NSP5 genes between 57M and 69M, and the detection of these strains in the same city and in the same year (Hasegawa et al., 1984). On the other hand, the VP1-VP3, VP6, NSP2 and NSP4 genes of 69M were found to be more closely related to those of artiodactyl or artiodactyl-like human P[14] strains

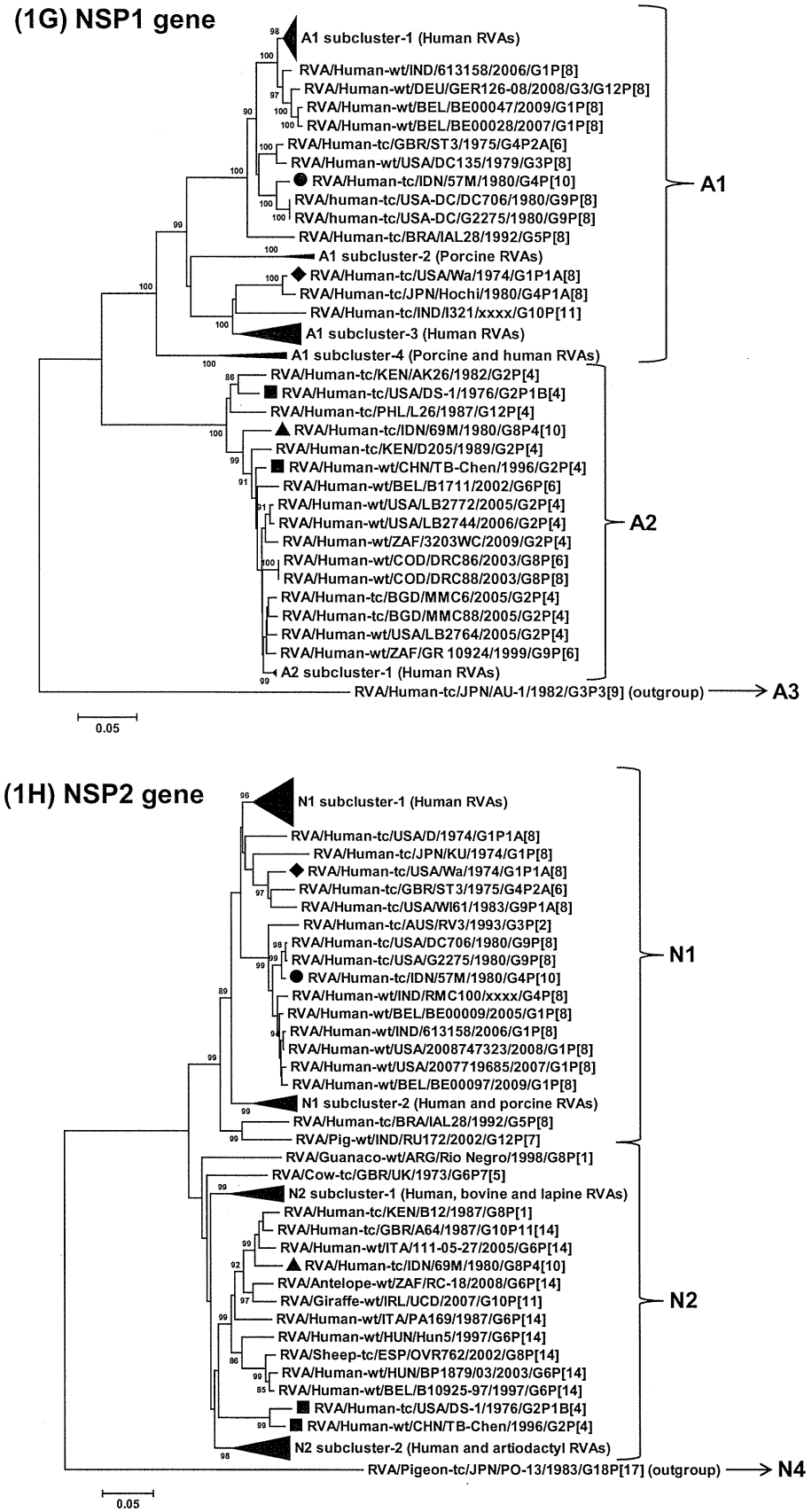

Fig. 1. (*continued*)

(Martella et al., 2010; Matthijnssens et al., 2009b) than those of other RVAs, whereas its NSP1, NSP3 and NSP5 genes were assigned to the A2, T2 and H2 genotype, respectively, that consists of human DS-1-like RVA strains (Fig. 1C–K; Supplementary Table S2).

Fig. 1. (*continued*)

Therefore, although the origin of the VP4 gene of 69M could not be established, the present analysis clearly indicated that human G8P[10] strain 69M might have originated from reassortment events involving at least the artiodactyl or artiodactyl-like human

Fig. 1. (*continued*)

strains and the typical human DS-1-like RVAs. Based on these observations, it may be hypothesized that a 69M-like animal strain was first introduced in humans, subsequently acquired its NSP3 and NSP5 genes from human DS-1-like RVAs through reassortment events, and then again reassorted with a co-circulating human Wa-like G4 RVA strain, resulting in strain 57M.

The Wa-like genotype constellation is believed to represent one of the stable RVA genetic backbones (Ghosh and Kobayashi, 2011; Matthijnssens and Van Ranst, 2012). On the other hand, the stable Wa-like genetic backbone has been found to also possess gene(s) derived from other RVA genogroups, as evident from the whole genomic analyses of strain 57M and a few other RVA strains (Matthijnssens and Van Ranst, 2012), pointing towards the complex genetic diversity of RVAs. Whole genomic analyses of a large number of human RVAs might be required to confirm the actual prevalence and stability of these intergenogroup reassortant strains under natural conditions. Our analysis of strain 69M revealed the importance of detailed phylogenetic analysis within the RVA genotypes in pinpointing the true origin of a strain, and once again exposed the vulnerability of humans to artiodactyl RVAs under poor hygienic conditions, especially in developing countries, such as Indonesia.

In conclusion, the present study provided rare evidence for intergenogroup reassortment events involving co-circulating typical human Wa-like RVAs and unusual RVAs of the DS-1-like genogroup, and revealed the presence of artiodactyl-like genes in a human P[10] strain, highlighting the complex evolutionary patterns of the P[10] RVAs. Based on the available data, the origin of the RVA P[10] VP4 gene still remains obscure. Proper detection of P[10] strains in humans and animals, and whole genomic analyses of several of these RVAs may be required to elucidate the exact origin of the P[10] genotype.

Acknowledgments

The study was supported in part by the grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant number 22406017), Health and Labour Sciences Research Grants in Research on Emerging and Re-emerging Infectious Disease (H24-shinkou-ippan-005) from the Ministry of Health, Labour and Welfare of Japan and Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (Okayama University-National Institute of Cholera and Enteric Diseases, India).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.10.021.

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Current Opinion in Virology

Genetics and reverse genetics of rotavirus

Koki Taniguchi and Satoshi Komoto

Rotavirus is a member of the family *Reoviridae*, which have genomes consisting of 10–12 double-stranded RNA segments. The functions of proteins encoded by each segment of the rotavirus genome have been studied extensively by several methods including reassortants, temperature-sensitive mutants, isolates with rearranged RNA segments, RNAi analysis, and other procedures. However, as found for most RNA viruses, the technique of reverse genetics is required for precise genotype/phenotype correlation, for the analysis of the role of specific mutation in replication process and pathogenesis, and for the development of vectors and vaccines. In 2006, we presented the first description of a reverse genetics system for rotavirus, although a helper virus and a selection system are required. Since then, two other approaches have been reported for rotavirus reverse genetics, both requiring the presence of a helper virus. A tractable, helper virus-free reverse genetics system for rotavirus has not been developed so far, in contrast to the recent developments of plasmid only-based reverse genetics systems for other members of the *Reoviridae*.

Address
Department of Virology and Parasitology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan

Corresponding author: Taniguchi, Koki (kokitani@fujita-hu.ac.jp)

Current Opinion in Virology 2012, **2**:399–407

This review comes from a themed issue on **Rotaviruses**

Edited by **Ulrich Desselberger** and **Mary K Estes**

For a complete overview see the Issue and the Editorial

Available online 30th June 2012

1879-6257/$ – see front matter,

http://dx.doi.org/10.1016/j.coviro.2012.06.001

Introduction

Extensive studies on rotavirus genetics have been carried out since the discovery of human rotavirus in 1973 [1,2]. Progress in methodology enhanced the genetic studies. This has included the cultivation of human rotavirus in cell culture, preparation of reassortants and *temperature (ts)* mutants, *in vitro* transcription involving double-layered particles (DLPs), RNA-RNA hybridization, recombinant baculovirus expression systems, the application of specific RNAi, and other procedures. When a report on *in vitro* RNA replication of rotavirus involving open cores appeared in 1994 [3], most rotavirus researchers thought that the development of rotavirus reverse genetics would follow soon. However, it took more than 10 years for a report on the successful development of such a system. In 2006, we succeeded in the establishment of a reverse genetic system of rotavirus, although it required a helper virus and a strong selection system [4••,5]. This success for rotavirus triggered the development of reverse genetics for other members of the *Reoviridae*. In 2007, a plasmid only-based reverse genetics was developed for orthoreovirus [6••], the efficiency of which was later improved further [7,8]. By applying this reverse genetics system to orthoreovirus, various new contributions were made to elucidate different steps of the viral replication cycle [9,10]. Furthermore, for bluetongue virus and African horse sickness virus of the genus *Orbivirus* in the family *Reoviridae*, the transfection of *in vitro* transcribed RNA from cDNA or of mRNA prepared *in vitro* from single-shelled core particles into cultured cells yielded infectious viruses [11,12••,13]. For rotavirus reverse genetics, two other methods were developed in 2010 [14•,15••]. However, all systems including the one developed by our group [4••] require a recombinant vaccinia virus expressing T7 RNA polymerase and particular ways to exert selection pressure. So far, a generally applicable plasmid only-based reverse genetics of rotavirus has not been developed for rotavirus, despite extensive efforts of many research groups. This is a mystery. Why is it so difficult to develop a tractable, true reverse genetics system for rotavirus?

In this review, after touching upon the classical genetics of rotavirus, we summarize the reverse genetics for multi-segmented double-strandes (ds)RNA viruses such as members of the *Birnaviridae* (2 segments), *Cystoviridae* (3 segments) and the viruses of the *Reoviridae*. Then, we describe the development of reverse genetics for rotavirus in different systems and provide a few examples of its application by site-directed mutagenesis of the rotavirus VP4 gene. Finally, we envisage the future developments of rotavirus reverse genetics.

Genetics of rotavirus

Rotavirus has a genome consisting of 11 dsRNA segments, which encode six structural proteins and six non-structural proteins. The function of each protein has been elucidated by various means such as preparation of reassortants, viruses with rearranged gene segment, and *ts* mutants, and knockdown of a given viral protein using siRNAs or intrabodies.

Reassortants

When cells are co-infected with two different but compatible rotaviruses (of the same group/species), a high

percentage of progeny viruses contain novel assortments of gene segments (reassortants). Studies involving a number of reassortants derived from two strains with different phenotypes contributed very effectively to mapping of the phenotypes to specific viral segmental gene products [16,17,18•]. For example, VP4 and NSP1 were found to determine the distinctive replication capacities in the mouse biliary tract [19]. VP3, VP4, VP7, and NSP4 were found to be associated with virulence and host range restriction [20,21].

Rearrangement

Rearrangement denotes alterations of considerable portions of the sequence within single genome segments, sometimes in the form of deletions, and often as partial duplication. Several variants with rearranged segments and altered phenotypes have been isolated [22]. Since the virus with a rearranged gene 5 (encoding NSP1) with an abrogated open reading frame of only 40 amino acids grows well in cell cultures and induces diarrhea in mice, NSP1 was found not to be essential for virus growth *in vitro* and *in vivo* [23].

Temperature-sensitive mutants

Various *ts* mutants have been prepared by treatment of rotavirus with mutagenic chemicals. Rotavirus *ts* mutant groups (A to G, J and K) have now been mapped to 9 of the 11 rotavirus genome segments [18•], and the amino acid mutations on each protein have been identified. The two reassortment groups H and I will be assigned to the remaining segments encoding NSP3 and NSP4 in the near future. Greenberg *et al.* [24] first identified VP4 being the hemagglutinin and the virus protein that is responsible for noncultivatability of human rotavirus by using *ts* mutants.

Intrabodies

Intracellular antibodies or intrabodies have great potential as protein knockout strategies for intracellular antigens. Expression of intracellular antibody to NSP5 was found to largely reduce the assembly of viroplasms, cellular cytopathic effect and the titer of infectious viral progeny [25].

siRNAs

The highly specific and efficient inhibition of viral gene expression by siRNAs offers the potential to elucidate the function of a given viral protein, first analyzed for VP4 [26]. By RNAi analysis NSP5 was found to have a pleiotropic effect on virus replication [27]. The siRNA approach showed that NSP5 is essential for the assembly of viroplasms that are associated with the utilization of lipid droplet components for rotavirus replication [28]. Silencing of NSP4 was found to have unexpected global consequences for virus replication as well as protein localization in infected cells [29,30]. NSP4 knockdown dramatically increased levels of viral transcription and inhibited formation of packaged virus particles [29]. By NSP4 silencing, it was confirmed that NSP4 is the main protein responsible for the changes in Ca^{2+} homeostasis in cultured cells [31]. In other studies, silencing NSP4 downregulated VP7 and VP4, and resulted in a decreased incidence of biliary atresia [32].

Thus, the major functions of each protein were analyzed by the various methods described above. However, analyses involving forward genetics have limitations such as the inability to identify the precise functional region in the genome by using virions with genomes that have arbitrary substitutions, insertions, or deletions.

Reverse genetics is a powerful technique for studying precisely the function of each segment and its protein product in an otherwise unchanged genetic background and for identifying the regions of the segments important for RNA transcription, replication and packaging.

Reverse genetics of dsRNA viruses with a segmented genome

There are many viruses with multi-segmented dsRNA as a genome. Since the packaging mechanism of multiple dsRNA segments is unknown, genetic modification has been notoriously difficult. Since the success for bacteriophages ϕ6 and ϕ8 with a genome of three dsRNA segments [33–40], however, much progress has been made on the reverse genetics for multi-segmented dsRNA viruses.

Bacteriophages ϕ6 and ϕ8

Bacteriophages ϕ6 and ϕ8 are members of the family *Cystoviridae*, which have three dsRNA segments: L, M, and S. In 1990, Olkkonen *et al.* [33] developed a reverse genetics system. They prepared cDNA-derived single-stranded (ss) RNA of M segment by using a plasmid encoding M segment under the control of T7 RNA polymerase promoter. After mixing with natural L and S ssRNAs, the cDNA-derived M ssRNA was subjected to *in vitro* packaging-replication system that has been developed by Gottlieb *et al.* [34]. After coating on outer shell protein P8, the nucleocapsids were infected onto *Pseudomonas phaseolicola* spheroplasts, yielding infectious bacteriophage ϕ6. Onodera *et al.* [35] modified this first system. They prepared a replication-incompetent ϕ6 derivative containing the L segment with a deletion by the *in vitro* packaging-replication system. The *P. phaseolicola* expressing the intact ϕ6 L segment ssRNA transcribed by SP6 RNA polymerase was then infected with the replication-incompetent ϕ6 derivative. As a result, they could obtain the reverted infectious ϕ6 that possess the functional cDNA-derived L segment. Later, Onodera *et al.* could prepare novel engineered viable viruses that possess genomic segments joined together so that the number of the segment can be only one or two [36].

Finally, it is now possible to produce infectious virions by electroporating three plasmids encoding L, M and S segment, respectively, under T7 or SP6 RNA polymerase promoter into the spheroplasts expressing T7 or SP6 RNA polymerase [40].

Birnavirus

Infectious bursal disease virus (IBDV) and infectious pancreatic necrosis virus (IPNV) are members of the *Birnaviridae*, and have a genome consisting of two dsRNA segments. By transfection of mRNA prepared *in vitro* from cores or ssRNA prepared *in vitro* from cDNA clones into Vero cells, a cell line from African green monkey kidney epithelial cells, infectious IBDV and IPNV were recovered [41,42]. Later, QM5 cells were infected with chicken pox virus expressing T7 RNA polymerase, and two transcription plasmids encoding each of the two RNA segments of IBDV were transfected, and an infectious IBDV derived entirely from cDNA was recovered [43].

Orthoreovirus

The basis for reverse genetics for ortho reovirus with a genome of 10 dsRNA segments was the discovery in 1990 that ortho reovirus RNA is infectious [44]. This system is unique and complicated. A lysate of rabbit reticulocyte in which ssRNA has been translated, and ssRNA and/or dsRNA from serotype 3 ortho reovirus (ST3) were transfected into L929 cells, and then the cells were infected with serotype 2 ortho reovirus (ST2). On day 5 after the infection, plaques of ST3 virus were recovered, since ST2 virus needs at least 12 days for plaque formation. By this and its modified method, packaging signals were identified [45] and a foreign CAT gene was incorporated into the genome [46]. However, these methods are delicate and tricky to handle, and have not been reproduced in other laboratories. In 2007, a plasmid only-based reverse genetics system not requiring a helper virus nor a selection system, was developed for orthoreovirus [6••]. In this system, transcription of each gene segment located downstream of the T7 RNA polymerase promoter is driven by T7 RNA polymerase, supplied transiently by recombinant vaccinia virus (rDIs-T7pol) or by cells that constitutively express the enzyme [7]. This excellent reverse genetics system has been developed for two prototype orthoreovirus strains, type 1 Lang and type 3 Dearing. For the first generation rescue system, each cDNA for 10 genes was inserted into a separate plasmid. In the second-generation system, the efficiency of virus recovery was enhanced by engineering the cDNAs of multiple segments into single plasmids to reduce the number of plasmids to be transfected from 10 to 4 [7]. Furthermore, the use of baby hamster kidney cells that express T7 RNA polymerase (BHK-T7) eliminated potential biosafety concerns associated with the use of recombinant vaccinia virus [7,8]. The orthoreovirus reverse genetics has been applied to the analysis of viral protein functions [9,10]. Specific mutations in the attachment protein σ1 have been introduced by reverse genetics [9]. Since orthoreoviruses induce cell death and apoptosis in tumor cells, but not in healthy non-transformed cells [47], reverse genetics for orthoreovirus may be useful for generating new orthoreovirus-derived oncolytic agents and vaccines. Furthermore, it is possible to introduce different exogenous peptides, up to 40 amino acids long, at the carboxyl-terminal end of the σ1 outer capsid protein [48].

Orbivirus

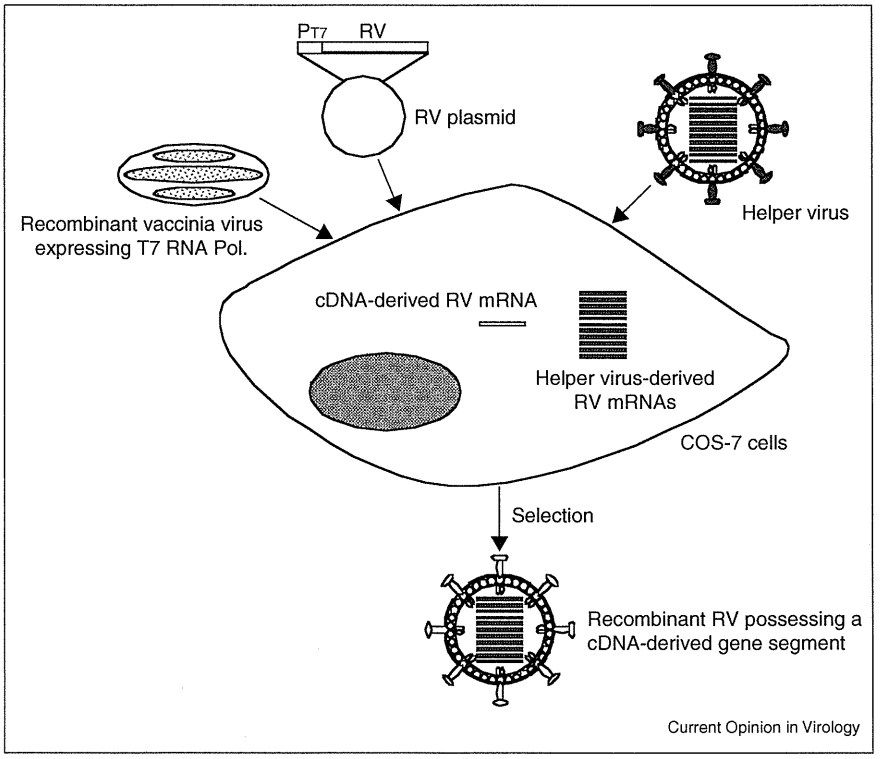
For bluetongue virus belonging to the *Orbivirus* genus of the *Reoviridae*, it became possible to recover infectious bluetongue virus by transfection of permissive cells with the complete set of 10 purified viral mRNAs derived *in vitro* from transcribing cores [11] or *in vitro* synthesized T7 transcripts using cDNA templates [12••]. Also, for African horse sickness virus, another member of the *Orbivirus* genus, a similar system has been developed [13]. Recently, a cell-free system for reconstituting functional subcore and core structures with proteins and ssRNAs was developed for bluetongue virus [49].

Reverse genetics for rotavirus

Use of neutralizing antibodies

A major breakthrough in genetic engineering of rotaviruses occurred in 2006 when we pioneered a reverse genetics system that enables the generation of infectious rotaviruses containing a cDNA-derived gene segment [4••]. This rotavirus system is based on helper virus-driven reverse genetics (Figure 1), which was originally developed for influenza viruses [50–52]. This system is based on the cloned full-length VP4 gene of simian rotavirus strain SA11 (G3P[2]), flanked by the T7 RNA polymerase promoter and hepatitis delta virus (HDV) ribozyme, and followed by the T7 RNA polymerase terminator (Figure 2A). The resulting plasmid was transfected into COS-7 cells that had been infected with recombinant vaccinia virus (rDIs-T7pol) to provide T7 RNA polymerase. Each of the authentic 5′ cap and 3′ polyA tail-lacking structures of rotavirus mRNA ends had to be obtained with vaccinia virus-encoded capping activity and HDV ribozyme sequences, respectively. The transfected cells were superinfected with human rotavirus strain KU (G1P[8]) as a helper virus. Recombinant rotavirus possessing a cDNA-derived SA11 VP4 gene (P[2]) segment was rescued by P[8] specific neutralizing antibody selection against the KU helper virus. In addition to the recombinant virus having the authentic VP4 gene, three more recombinant rotaviruses, into which silent mutation(s) had been introduced as gene markers in the VP4 genome, were soon generated using this method (Figure 2A). Hence, the feasibility of this rotavirus reverse genetics system was confirmed. The keys for the development of the rotavirus reverse genetics system are the use of specific anti-VP4 neutralizing monoclonal antibodies [53] and the use of recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol) that is non-pathogenic to mammalian cells [54•].

Figure 1

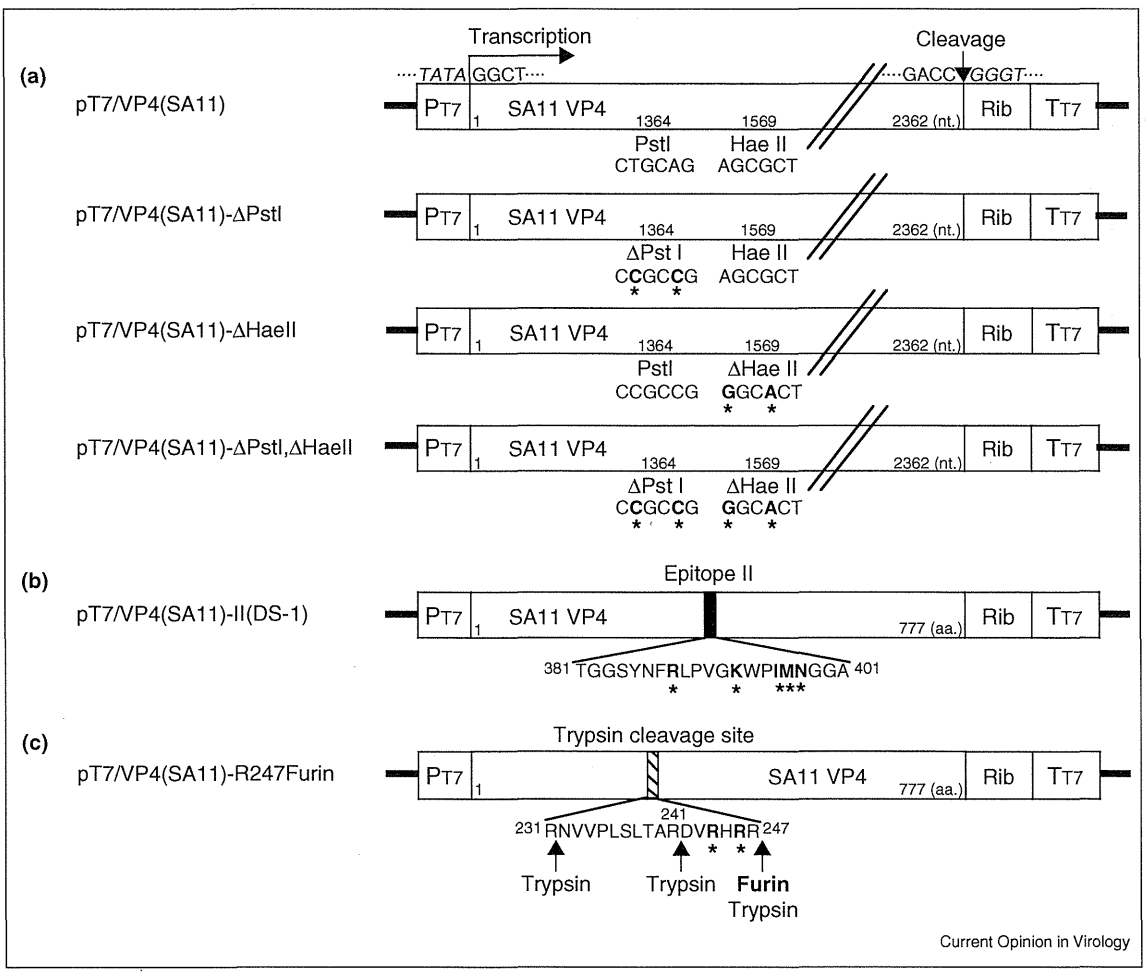


Reverse genetics system for rotavirus (RV). A plasmid containing a RV cDNA gene insert is transfected into COS-7 cells that have been infected with a recombinant vaccinia virus expressing T7 RNA polymerase. Cells are then infected with a helper virus. Intracellular transcription by T7 RNA polymerase yields artificial RV mRNA, which is packaged into progeny virions. The generated recombinant RV is rescued from the background of helper viruses by applying strong selection systems.

With this method, recombinant rotaviruses with artificial amino acid substitutions were generated. Site-specific mutations were introduced into one of the three cross-reactive neutralization epitopes of VP4, which resulted in the engineering of recombinant rotavirus expressing chimeric VP4 on its surface [55••]. The rescued virus contains SA11 (G3P[2])-based VP4, in which a cross-reactive neutralization epitope on VP5* was replaced by the corresponding sequence of a different P-type DS-1 (human rotavirus strain, G2P[4]) (Figure 2B). Serological analyses with a panel of anti-VP4 and anti-VP7-neutralizing monoclonal antibodies revealed that the engineered virus carried a novel antigenic mosaic of cross-reactive neutralization epitopes on its VP4 surface. Clearly, this approach to construct chimeric rotaviruses will potentially lead to a new generation of effective vaccine candidates against rotavirus disease.

The spike proteins of many enveloped viruses are initially synthesized as inactive precursors. Therefore, proteolytic cleavage of precursor spike proteins at mono-basic or multi-basic cleavage site(s) by cellular proteases is absolutely required to convert them into an active state and to render the virions infectious. The mono-basic cleavage sites and multi-basic cleavage sites are readily cleaved by exogenous trypsin-like proteases and endogenous furin-like proteases, respectively. VP4, the spike protein of non-enveloped rotavirus, resembles the precursor spike proteins of enveloped viruses because they exhibit substantial structural and functional similarities. VP4, as an inactive precursor, is cleaved at mono-basic cleavage sites by trypsin into VP5* and VP8* as active states, resulting in activation of rotavirus infectivity [56]. To examine the possibility that modification of the VP4 trypsin cleavage site to a furin-sensitive state would allow engineering of a viable rotavirus that can perform multicycle replication without trypsin, we generated and characterized recombinant rotavirus expressing a mutant VP4 that can be cleaved by furin-like proteases as well as trypsin [57] (Figure 2C). Unexpectedly, this VP4 mutant could not undergo multicycle replication without an exogenous protease, although the mutant VP4s on virions were efficiently cleaved by furin-like proteases. Since nascent viruses containing already cleaved VP4 were significantly constrained within the cells, it was suggested that intracellular cleavage of VP4 by furin may be disadvantageous for rotavirus infectivity, possibly owing to an inefficient virus release process. This reverse genetics approach may become valuable for the understanding of the molecular basis

Figure 2



Schematic representation of the SA11 virus-based transcription plasmids encoding the full-length VP4 gene. **(A)** The wild-type and mutated VP4 genes of SA11 were cloned between the T7 RNA polymerase and HDV ribozyme, followed by the T7 RNA polymerase terminator. The numbers indicate the nucleotide positions in the SA11 VP4 sequence. **(B and C)** Manipulations of the VP4 gene by means of amino acid mutations (positions are indicated by bold letters and asterisks below the sequences) were carried out in pT7/VP4(SA11). The mutant plasmid pT7/VP4(SA11)-II(DS-1) contains five amino acid mutations within the epitope II sequence **(B)**. The mutant plasmid pT7/VP4(SA11)-R247Furin possesses two amino acid mutations within the trypsin cleavage site that create a furin cleavage site at the R247 position **(C)**. The numbers indicate the amino acid positions in the SA11 VP4 sequence. PT7, Rib, and TT7 denote the T7 RNA polymerase promoter, HDV ribozyme, and T7 RNA polymerase terminator, respectively.

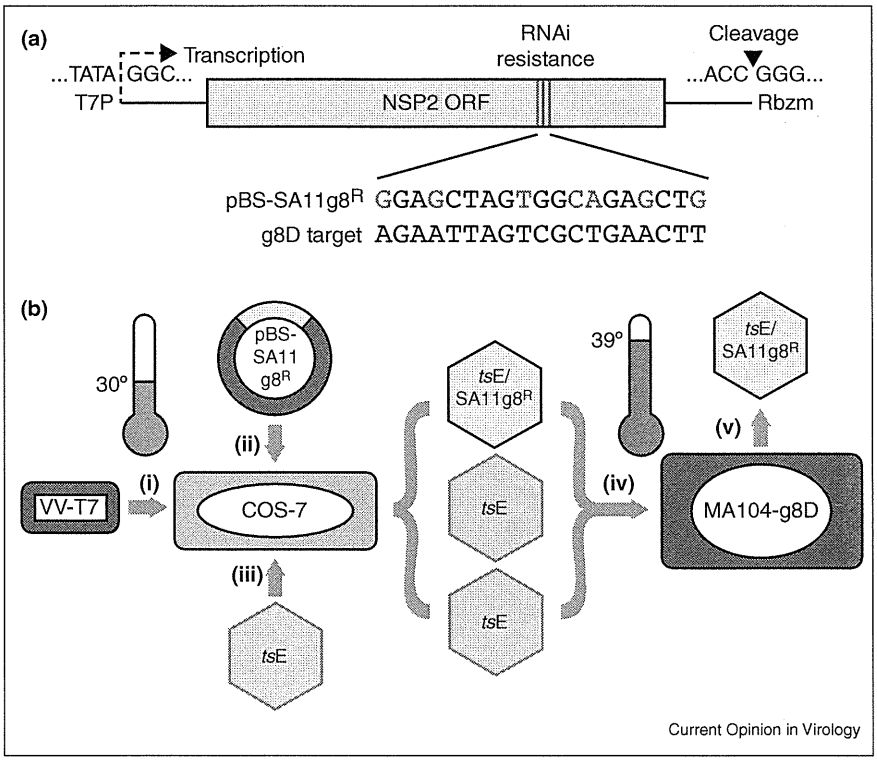
of the significance of VP4 and VP7 for rotavirus replication and pathogenesis.

VP4 possesses three conserved mono-basic residues at its trypsin cleavage site (R231, R241 and R247) (Figure 2C). Although only R247 is assumed to be required for the activation of infectivity with conventional techniques, the strict conservation of the three residues suggests that the presence of these residues may play an important role during infection. This possibility was evaluated by generating recombinant rotaviruses with trypsin cleavage site mutations using reverse genetics, and the role of each arginine residue in rotavirus infectivity will be demonstrated in the near future [Komoto and Taniguchi, unpublished data].

The present method is applied only for the VP4 gene, but can be extended to the VP7 gene. The SA11-L2 clone has little dependency on trypsin for its growth [58]. On transfection of an artificial SA11-L2 VP7 gene in the presence of neutralizing monoclonal antibodies specific to VP7 (G1-specific) as a selection pressure and the use of a KU-SA11 reassortant whose VP4 gene only is derived from SA11 as a helper virus, recombinant viruses with the VP4 and VP7 genes, both of which are derived from SA11-L2, independent of trypsin would be isolated.

Furthermore, if a marker protein such as GFP can be expressed in this system, the use of the IRES sequence to prepare a bicistronic segment (GFP and NSP protein) will also have the potential as a modified reverse genetics

Figure 3



Procedure for generation and recovery of gene 8 recombinant virus using dual selection [15••]. **(A)** Schematic representation of the SA11 virus-based transcription plasmids encoding the full-length gene 8. **(B)** Procedure for reverse genetics under dual selection. COS-7 cells are infected with rDIs-T7pol (VV-T7) **(i)**, and transfected with the recombinant gene 8 plasmid (pBS-SA11g8^R) **(ii)** before infection with the tsE helper virus at 30°C **(iii)**. The recovered virus stock may have the tsE/SA11g8^R virus as well as tsE helper virus. Passage in MA104-g8D at 39°C **(iv)** permits efficient isolation of the tsE/SA11 g8^R virus **(v)**.
From [15••], with permission of the authors and the publisher.

technique permitting the selection of fluorescing cells by FACS or any other method, since rotavirus particles have the ability to pack the extra nucleotides of 1900–2400 nucleotides [[59], Chen *et al.*, unpublished data]. In particular, NSP1 is found to be non-essential for rotavirus growth in cultured cells or in mice [23], and the NSP1-coding region might be replaced by the GFP gene.

Use of *ts* mutant

Trask *et al.* [15••] developed reverse genetics protocol in which a mutant SA11 rotavirus encoding a *ts* defect in the NSP2 protein and RNAi-mediated degradation of NSP2 mRNA are employed. They prepared a plasmid pBS-SA11g8 that has an SA11 NSP2-coding gene 8 cDNA with a T7 RNA polymerase promoter sequence at the 5′ end and a modified HDV ribozyme and T7 RNA polymerase terminator at the 3′ end (Figure 3A). Seven mutations were introduced into the SA11g8 cDNA in the region targeted by the g8D siRNA to generate plasmid pBS-SA11g8^R. COS-7 cells were infected with the T7 RNA polymerase-expressing rDIs-T7pol and then transfected with the pBS-SA11g8^R plasmid. The cells were then infected with rotavirus *ts*E as a helper virus and incubated at 30°C. Virus in the cell lysates was passaged at 39°C in MA104 cells that express g8D siRNA to select for progeny virus with the recombinant gene 8 (Figure 3B). In this system, the *ts* mutant is the much more critical selection mechanism than the use of siRNA. The utility of this reverse genetics system was evaluated by obtaining recombinants expressing chimeric NSP2 proteins.

This dual selection with *ts* mutants and RNAi can theoretically be extended to other genes, since rotavirus *ts* mutation groups have been mapped to 9 segments [18•], except for gene segments encoding NSP3 and NSP4. For this approach to be successful, the *ts* mutants must be very tight.

Use of rearranged gene

Troupin *et al.* [14•] reported a reverse genetics system for rotavirus based on the preferential packaging of rearranged RNA segments. They introduced an *in vitro*-engineered gene 7 with rearrangement encoding a modified NSP3 protein into an infectious rotavirus. In this system, they also used COS-7 cells infected with rDIs-T7pol for providing T7 RNA polymerase and a bovine

rotavirus RF as a helper virus, but no additional selective pressure. This system can theoretically be applied to other genes (genes 5–11) for which rearrangements have been described. The preferential packaging of the rearranged RNA segments might be caused by the duplication of packaging signals or secondary structures in rearranged segments [60].

Plasmid only-based

Extensive studies aiming at establishing a plasmid only-based rotavirus reverse genetics system have been performed without success so far. Why is it so difficult to develop plasmid only-based rotavirus reverse genetics in contrast to the successes with orthoreoviruses and orbiviruses? In our laboratory we could readily confirm the excellent efficiency of the orthoreovirus reverse genetics system [6••] by using a set of 10 plasmids, each containing the cDNA of one segment, and L929 cells provided by Dr. Dermody. As a result, we recognized that there are no problems with reagents and techniques used for rotavirus in our laboratory. Rotaviruses have one more RNA segment than orthoreovirus. Rotavirus infection depends on the presence of trypsin more than orthoreovirus. The ratio of the number of virus particles per infectious unit is much lower in rotavirus [61]. It will be the problem of the efficiency of the recovery of infectious rotavirus particle. There might be some modifications necessary for the success of plasmid only-based rotavirus reverse genetics such as preparation of plasmids having multiple gene inserts in order to reduce the number of plasmids for transfection, use of rotavirus strain whose dependency on trypsin is low, use of cells that express some of the rotavirus proteins and T7 RNA polymerase, or other procedures. We have examined some of them. We employed a set of 11 transcription plasmids encoding each gene segment from some animal and human rotavirus strains. The use of various cell-lines susceptible (COS-7, 293T, MA104, CV-1, HT-29, MDCK and Vero cells) and refractory (BHK, CHO and L929 cells) to rotavirus infection were examined. We used the cells that constitutively express T7 RNA polymerase. Inclusion of eukaryotic expression plasmids encoding rotavirus proteins was also examined. However, these approaches did not lead to success so far. Further persevering in investigations will be required for the success of the plasmid only-rotavirus reverse genetics.

Conclusion

Since our early success in rotavirus reverse genetics, progress has been slow. There are three different systems for rotavirus reverse genetics so far, whose reported target gene segments are VP4, NSP2 and NSP3, respectively. Theoretically, they can be extended to more gene segments. In addition, the efficiency of obtaining infectious rotavirus with a segment having undergone site-directed mutagenesis should be improved. Ultimately, as for other members of the *Reoviridae*, plasmid only-based reverse genetics for rotavirus should be developed by extensive examinations of variables that will affect the recovery efficiency of infectious virus.

Once a plasmid only-based reverse genetics system has been developed, the procedure will help to solve problems such as the precise mechanism of virus entry into cells and virus export from cells, control mechanism of packaging of 11 RNA segments into particles, mechanism of protein and vesicle trafficking, pathway of developing viremia/antigenemia, identification of the region responsible for pathogenesis and attenuation, and many others.

Acknowledgements

This work was supported partly by a grant for the Strategic Research Foundation Grant-aided Project for Private Universities from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and by Grants-in Aid from the Ministry of Health, Labour and Welfare, Japan.

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Vaccine 30 (2012) 4661–4669

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Randomized placebo-controlled clinical trial of immunoglobulin Y as adjunct to standard supportive therapy for rotavirus-associated diarrhea among pediatric patients

Shofiqur Rahman[a,*], Kyoko Higo-Moriguchi[b], Khaing Win Htun[c], Koki Taniguchi[b], Faustino C. Icatlo Jr.[d], Takao Tsuji[e], Yoshikatsu Kodama[a], Sa Van Nguyen[a], Kouji Umeda[a], Htun Naing Oo[c], Yi Yi Myint[c], Than Htut[f], Swe Swe Myint[f], Kyaw Thura[f], Hlaing Myat Thu[g], Ni Nengah Dwi Fatmawati[h], Keiji Oguma[h]

[a] *Immunology Research Institute in Gifu, EW Nutrition Japan, 839-7 Sano, Gifu 501-1101, Japan*
[b] *Department of Virology and Parasitology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan*
[c] *Department of Medical Research, Central Myanmar, Myanmar*
[d] *Meisen Pharmaceuticals, Inc., Quezon City, Philippines*
[e] *Department of Microbiology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan*
[f] *Defense Services Obstetrics, Gynaecology and Children Hospital, Central Myanmar, Myanmar*
[g] *Department of Medical Research, Lower Myanmar, Myanmar*
[h] *Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Japan*

ARTICLE INFO

Article history:
Received 31 December 2011
Received in revised form 20 April 2012
Accepted 25 April 2012
Available online 8 May 2012

Keywords:
Rotavirus
Pediatric diarrhea
IgY
Clinical trial

ABSTRACT

This study aims to evaluate the effect of hyperimmune immunoglobulin Y (IgY) against human rotavirus (HRV) among pediatric patients receiving standard supportive treatment for rotavirus-associated diarrhea mostly with an enteric non-cholera co-pathogen in a hospital setting. Two natural HRV reassortant clinical strains ATCC VR 2273 and ATCC VR 2274 were used as mixed immunizing antigens in poultry hens to generate anti-HRV IgY (Rotamix IgY). The Rotamix IgY was used in laboratory and clinical studies against control or placebo IgY. The control or placebo IgY was prepared using tissue culture medium from mock-infected MA104 cell line as antigen for poultry immunization. In vitro, Rotamix IgY exhibited multi-serotypic cross neutralization activities along with synergistic effects against major global serotypes G1, G2, G3, G4 and other human or animal rotavirus strains when compared with mono-specific IgY. Suckling mice (ICR strain) pre-treated orally once with Rotamix IgY and then challenged with rotavirus 3 h later showed a significant dose-dependent reduction in frequency ($p < 0.05$) and duration ($p < 0.05$) of diarrhea compared to placebo IgY-treated mice. Out of 114 children aged between 3 and 14 months and with diarrhea upon admission in a Myanmar hospital, 54 dehydrated and rotavirus-positive children were randomized into Rotamix IgY group and placebo IgY group. Of these, only 52 children had complete data with $n = 26$ children per study group. Ninety-two percent of patients in each of these groups were positive for co-infecting enteric non-cholera pathogen and all patients received standard supportive therapy for diarrhea. The patients were monitored for volume and duration of oral rehydration fluid (ORF) and intravenous fluid (IVF) intake, daily stool frequency and overall duration of diarrhea, and frequency and duration of rotavirus shedding. Compared to placebo IgY group, the Rotamix IgY group had statistically significant reduction in mean ORF intake ($p = 0.004$), mean duration of intravenous fluid administration ($p = 0.03$), mean duration of diarrhea from day of admission ($p < 0.01$) and mean duration of rotavirus clearance from stool from day of admission ($p = 0.05$). Overall, our novel approach using oral Rotamix IgY for rotavirus-infected children mostly with non-cholera enteric pathogen co-infection appears to be a promising, safe and effective adjunct to management of acute diarrhea in pediatric patients.

1. Introduction

Rotavirus is the leading single etiologic agent of severe diarrhea among infants and young children worldwide during the first five years of life. It is responsible for over 500,000 deaths in infants and young children mostly in developing countries each year which

* Corresponding author. Tel.: +81 58 235 7303, fax: +81 58 235 7505.
E-mail addresses: rahman@ew-nutrition.co.jp, shofiq66@yahoo.com (S. Rahman).

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http://dx.doi.org/10.1016/j.vaccine.2012.04.091

Table 1
Rotavirus strains used in this study.

Strain	Serotype
Human origin:	
HRV 408	Natural reassortant G3 P[?]
HRV 248	Natural reassortant G4 P[4]
Wa	G1 P[8]
KU	G1 P[8]
M37	G1 P[6]
S2	G2 P[4]
1076	G2 P[6]
YO	G3 P[8]
HK	G4 P[8]
Horse origin: HO-5	G3 P[12]
Cow origin: Shimane	G6 P[5]
Pig origin: S-80	G1 P[7]

P[?] means P genotype is unknown.

represent approximately 5% of all deaths [1]. Rotaviruses have also been implicated as causative agents of outbreaks of diarrhea occurring in nursing homes [2], among travelers [3], in day-care centers [4], and adults suffering from a variety of immunodeficiency conditions [5,6]. Among the 24 G types and 33 P types of group A rotaviruses classified so far [7], 5 G types (G1, G2, G3, G4, and G9) and 3 P types (P[4], P[6], P[8]) account for most of the G/P types of human rotaviruses detected globally. Four common G-P combinations (G1, G3, and G4 with P[8] and G2 with P[4]) are of principal epidemiologic importance being responsible for approximately 96 percent of rotavirus infections worldwide [8–11], although their relative proportions may vary by year and region. It follows therefore that any proposed immunologic intervention measures must provide good protection against these four epidemiologically significant HRV serotypes.

Conventional treatment for rotavirus diarrhea is non-specific, largely symptomatic and involves fluid and electrolyte replacement and maintenance of nutrition. The use of current vaccine regimens poses inherent limitations due to variable degrees of efficacy [12], and high cost. On the other hand, passive immunotherapy using orally administered hyperimmune chicken immunoglobulins (IgY) has been reported with various degrees of success against infectious diseases of viral, bacterial, fungal and protozoal origin in both humans and animals [13–15]. There have been reports on the experimental use of rotavirus specific IgYs in cow [16], cat [17] and mice [18,19] with promising results. So far there has been only one report of a randomized placebo-controlled clinical trial in children using IgY against rotavirus [20]. The investigators in aforesaid trial used four different strains of rotavirus to produce IgY but they did not determine the dose of IgY in terms of neutralization titer as well as the possible range of cross-reactivities of the IgY with known rotavirus serotypes. In the present study we mapped the IgY cross-reactivities against an array of clinically important human and animal strains currently circulating worldwide and defined the titer of IgY administered to rotavirus-infected patients for possible application as a general adjunct to standard supportive therapy for acute rotavirus-related diarrhea among pediatric patients.

2. Materials and methods

2.1. Viruses and cell lines

The viruses used in this study were reassortant type II subgroup human rotavirus (HRV) strains HRV 408 (ATCC 2273), HRV 248 (ATCC 2274), Wa(G1P[8]), S2(G2P[4]), YO(G3P[8]), and HK(G4P[8]) originated from human, HO-5 (G3P[12], horse), shimane (G6P[5], cow), and S-80 (G1P[7], pig) (Table 1). Rhesus monkey kidney cell line MA-104 (ATCC CRL-2378) cells were used to propagate all the above rotaviruses. MA-104 cells were maintained by using Eagle's Minimal Essential Medium (EMEM, Nissui, Japan) with Earles' salts, supplemented with 10% fetal bovine serum (FBS) and incubate at 37 °C, 5% carbon dioxide.

2.2. Preparation of Rotamix IgY and placebo IgY

Reassortant strains HRV 408 and HRV 248 were isolated from stools of 9- and 17-months old children respectively from Bangladesh [21]. These two reassortant human rotaviruses were used as antigens for the production of anti-HRV IgY according to the methods described previously [22]. Prior to their use as immunizing antigen, the above rotavirus strains were inactivated using 0.3% formalin at 37 °C for 24 h. To generate IgY, 18-week-old Hy-Line hens were immunized by intramuscular injection of an emulsified mixture of inactivated human rotavirus either as single-strain or mixed-strain emulsions. Eggs laid by the immunized hens between 3 and 10 weeks after immunization were harvested and egg yolk was isolated, pooled and processed into powder form in accordance with a method described previously [23]. The egg yolk powder from mixed vaccination with two rotavirus strains HRV 408 and HRV 248 was designated as "Rotamix IgY". Placebo IgY powder was prepared by the same method from the eggs of hens immunized using as antigen the tissue culture medium of mock-infected MA-104 cell monolayer. For in vitro and in vivo mouse studies, Rotamix IgY and placebo IgY were partially purified from egg yolk by chloroform extraction and ammonium sulfate precipitation [24]. The antigen and antibody protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA).

2.3. Virus quantitation and fluorescent focus (FF) reduction assay on IgY

Virus titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) method [16]. Briefly, tissue culture supernatants were assayed for virus infectivity on MA-104 cells based on end-point dilution of samples with cytopathic effect in a 10-fold sample dilution series. For the virus neutralization assay, fluorescent focus (FF) reduction method was employed [25]. In this assay, MA 104 cells were plated onto 96-well tissue culture plate with EMEM containing Earle's salts and non-essential amino acids and supplemented with L-glutamine, sodium pyruvate, and 5% fetal bovine serum. All rotavirus cultures were maintained with the medium containing 10 μg/ml trypsin. Cells were incubated at 37 °C with 5% CO_2 tension for 24 h or until a confluent monolayer was formed. Different dilutions of Rotamix IgY in phosphate buffered saline were prepared with final concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/ml. Each dilution was mixed with an equal volume of 12 different rotavirus strains (248, 408, Wa, KU, M37, S2, 1076, YO, HK, HO-5, Shimane and S-80) at a dilution that yielded about 150–200 FF units per 0.025 ml per well and the mixtures were allowed to react at 37 °C for 1 h. About 50 μl aliquots of the IgY-virus mixtures were dispensed onto MA104 monolayers in 96-well microplate and incubated for 1 h at 37 °C. 100 μl of fresh EMEM was added, followed by 16–18 h cultivation at 37 °C, 5% CO_2. Fixation in cold (−80 °C) methanol, and reaction with first and second antibodies, were performed as described previously [26]. Neutralizing antibody titer was expressed as the reciprocal of the highest IgY dilution that reduced the FF count by >50%. The mean FF reduction titer of IgY was calculated from 3 independent assays.

2.4. Suckling mouse experiments

All procedures that involved animals were approved by the Institutional Animal Care and Use Committee of the Immunology Research Institute in Gifu, Japan. Pregnant, rotavirus-negative CD-1 SPF mice (Charles River Japan, Inc., Kanagawa, Japan) were housed

individually in an environmentally controlled animal facility until parturition. Environmental factors were closely monitored daily and maintained within the recommended range for the animals (18–23 °C, 40–75% humidity, and photoperiod of 12 h each of light and dark cycles). Mice were maintained on normal pellet diet, water was provided ad libitum and beddings and cages were changed once a week.

Four-day old suckling mice born after the one-week acclimatization period were randomized into several experimental groups ($n = 10$/each group). The challenge dose for HRV 248 and 408 strain were $10^{7.7}$ $TCID_{50}$ and $10^{7.5}$ $TCID_{50}$ respectively. They were given as 25-μl oral dose of rotavirus suspension after a 21-h fast. Rotamix IgY or placebo IgY in phosphate-buffered saline was given once as a single dose 3 h prior to challenge. For this pre-challenge IgY treatment, four different doses of Rotamix IgY were tested: 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL. For control mice, 5 mg/ml of placebo IgY was used. At any time point after challenge, neither Rotamix IgY or placebo IgY was given to any mouse in the experiment. After challenge, the mice were observed daily for 3 days and were checked every 4 h daily for signs of diarrhea which is determined as loose stools on gentle palpation of the abdomen or as feces-smeared tail during the 3 days of the experiment. Stool samples were retrieved by gentle palpation of the abdomen. Stool consistency was evaluated on a five-point scale as follows: 0, normal, solid and black; 1, soft brown; 2, liquid brown; 3, soft yellow; 4, liquid yellow. Mice showing scores of 3 or higher were considered as having diarrhea. The outcome measures used were frequency of diarrhea (determined as the percentage of mice with diarrhea on a daily basis) and duration of diarrhea (determined as number of days with diarrhea).

2.5. Preparation of placebo and Rotamix IgY in sachets for oral administration to infants

Rotamix IgY or placebo IgY were mixed with maltitol and banana flavor and dispensed into sachets. One sachet contains 0.5 g anti-HRV IgY, 1.48 g maltitol and 0.02 g banana favor (total of 2 g/sachet). The protein content of the Rotamix IgY and placebo IgY preparations, were 7.5% and 7.8% respectively, with an IgY content of 20% (w/w). The fat, carbohydrate, ash and moisture contents of both IgY preparations were 12%, 5%, 0.8%, and 5% respectively.

2.6. Study design and population

The research protocol followed in this clinical trial has been approved by the Ethical Committee on Medical Research Involving Human Subjects of the Department of Medical Research (Central Myanmar). A double-blind, placebo-controlled trial of rotavirus-specific IgYs was conducted in Myanmar during the rotavirus epidemic season from January to March, 2011 in the Pediatric Infectious Disease Wards of the Defense Services Obstetrics, Gynaecology and Children's Hospital, in collaboration with the Department of Medical Research, Central Myanmar Ministry of Health. Infants and children of both sexes aged between 2 and 36 months who were brought to the above hospital with history of acute watery diarrhea and dehydration were entered in the study. Children with severe malnutrition, respiratory infections, systemic infection and a history of bloody or mucoid diarrhea were excluded. Selected children were taken to the study ward and observed for 4 h during which rehydration with oral rehydration fluids (ORF) or intravenous fluids (IVF) was performed while their stools were screened for rotavirus antigen by the commercial Dipstick 'Eiken' Rota kit (SA Scientific, USA). Those with positive results were finally enrolled in the study after obtaining informed consent from their parents/guardians.

Table 2
Medications given to Rotamix IgY and placebo IgY groups as routinely prescribed by attending physician.

Treatments	No. of patient	
	Rotamix IgY group	Placebo group
Antibiotics		
Ampicillin	1	1
Metronidazole	25	23
Cotrimoxazole	10	9
Amikacin	2	3
Gentamycin	11	9
Cefotaxine	0	1
Ceftriazone	0	1
Chloramphenicol	0	1
Vitamins/minerals		
Astymin C	2	4
Folic acid	12	12
Zinc	7	10
Becozinc	1	1
Burplex	3	1
Probiotics		
Biovita:	4	3
Lactobacillus helveticus R0052		
Bifidobacterium longum R0175		
Bioflor: *Saccharomyces boulardii*	0	1
Medilac-S: *Streptococcus faecium*	4	1
Bacillus subtilis		

2.7. Treatment protocol

Out of 114 children screened, 54 children qualified for the study based on above-mentioned criteria with 2 children being dropped from the study later due to incomplete data. The selected children were divided into two groups by randomization through alternate assignment of the placebo IgY and Rotamix IgY sachets labeled A and B respectively to patients according to the order in which they were admitted at the hospital for confinement. The appearance of the sachets was identical except for the A or B label but the enroller (field scientist) did not know which of the sachets was placebo or Rotamix IgY. One sachet of the Rotamix or placebo IgYs was administered four times daily for 8 consecutive days in addition to rehydration therapy. The attending clinicians monitored other possible untoward reactions to Rotamix IgY. Under the supervision of pediatricians who did not know the group status of any of the study children or infants, the ongoing water loss was replaced by equivalent amount of ORF but intravenous fluid solution was used if ORF therapy failed to compensate for ongoing water loss. The IVF was discontinued as soon as dehydration was corrected and oral rehydration was resumed. Routine medical treatments given to participants in the form of antimicrobials or supplementary vitamins/probiotics were noted and recorded for all patients in both groups (Table 2). Exclusively breast-fed infants continued to receive mothers' milk while weaned children received a milk-based formula in addition to breast milk. Non-breastfed children were given milk formula or semisolid and solid foods appropriate for their age and food habits. The solid or semi-solid food is usually rice-based and may contain non-fermented milk or vegetable materials. There were no prebiotics or probiotics in any of the home-made diet for infants.

2.8. Clinical assessments and laboratory investigations

Baseline physical, clinical and microbiological assessments were made on the first visit at day 0. At this time, all selected patients passed a thorough physical examination and assessment of hydration by physicians who managed the subsequent ORF and IVF administrations while recording stool frequency and duration of

Table 3
In vitro cross neutralization activity of anti-HRV IgY preparations with human and animal rotavirus strains as determined by FF reduction assay.

IgY samples	Neutralization titer/0.1 ml IgY[a] against different human rotavirus strains											
	408	248	Wa	KU	M37	S2	1076	YO	HK	HO-5	Shimane	S-80
Anti-408	5120	2560	10240	5120	10240	2560	>40960	40960	10240	5120	<20	1280
Anti-248	2560	>40960	5120	5120	5120	5120	>40960	20480	40960	1280	<20	640
Rotamix	10240	>40960	20480	20480	20480	10240	>40960	>40960	>40960	5120	<20	2560
Control	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

[a] IgY titer is expressed as dilution factor of 1 g hyperimmunized IgY powder that reduced the fluorescent focus (FF) count by >50% in the FF reduction assay. Results are presented as the mean of three independent experiments.

diarrhea daily until day 8. Fecal samples were collected for stool culture to detect viral and bacterial infection using standard direct and enrichment enteric media as described elsewhere [27]. Detailed identification of bacterial serotypes using serology was not done. Stool specimens were obtained daily for 8 days to assess the duration of viral shedding. Detection of rotavirus in stool during the study was made by ELISA method [28] as described below. Technicians of the diagnostic laboratory were not aware of the group each patient belonged to. The code for the sachets was broken only when all the laboratory and clinical datas were compiled for data analysis. The randomized group assignment of patients resulted in Rotamix IgY and placebo IgY groups with demographic and co-pathogen infection profiles as shown in Tables 3 and 4 respectively. The patient exclusion criteria followed in this study (severe malnutrition, respiratory infections, systemic infection and a history of bloody or mucoid diarrhea) may have excluded *Vibrio cholera*-infected children in these groups.

2.9. Detection of rotavirus antigen in stool

A total of 416 stool specimens were collected from 52 patients at different time points (days 1–8) post-treatment and stored at −20 °C. Ten percent fecal suspensions were prepared in phosphate buffered saline (PBS), clarified by centrifugation (1000 × *g* for 5 min) and analyzed by ELISA as described previously [28] with some modifications. Briefly, polyvinyl 96-well microtiter plates (Nalgene Nunc International, Rochester, NY) were coated with 1:10,000 dilution of a monoclonal antibody (Mab) YO-156 and incubated overnight at 4 °C. The MAb YO-156 (IgG2a) is highly reactive with VP6, a common epitope of all group A rotaviruses that have been examined to date [29]. After a routine 3 times wash with PBS-Tween 20 (PBST), the wells were incubated with 1% bovine serum albumin overnight at 4 °C and washed as before. A mixture of 10% stool suspension (375 μl) and 10% skim milk (125 μl) was then incubated in the wells overnight at 4 °C. After a routine PBST wash, 50 μL of anti-human rotavirus hyperimmune rabbit serum diluted 1:5000 with PBST that contained 2.5% skim milk were added to each well. The plate was incubated at 37 °C for 1.5 h. After a routine PBST wash, wells were incubated with 1:5000 dilution of peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratory, Inc, West Grove, PA) at 37 °C for 1.5 h. After a 4× PBST wash, substrate was then added and MAb bound to rotavirus VP6 antigen in test samples were detected by a micro-ELISA reader (EAR400; SLT-Lab instruments, Salzburg, Austria) at 492-nm. To establish an appropriate cutoff value, we tested 22 fecal samples collected from control or placebo subjects prior to treatment. The optical density (OD) of 0.13 was used as the cut-off value for a positive result.

Table 4
Baseline characteristics of the study children upon admission prior to IgY treatment.*

Characteristics	Test (n = 26)	Placebo (n = 26)
Male:female	13:13	17:09
Resident (urban:rural)	16:10	12:14
Age (months)	13.8 ± 10.6†	13.5 ± 6.3
Weight (lb)	17.5 ± 4.5	18.8 ± 3.1
Breast-feeding frequency (number/day)	7.1 ± 7.0	7.8 ± 6.7
Temperature (°F)	100.5 ± 1.5	100.4 ± 1.8
Fever rate (%)	22/26 (85%)	21/26 (81%)
Fluid intake (ml/day):		
ORS and others supplement	767.3 ± 538.6	1005.4 ± 628.0
Intravenous fluid (IVF)	345.0 ± 347	592.3 ± 491.3
Diarrhea duration (h)	69.6 ± 33.6	74.4 ± 38.4
Stool frequency (number/day)	9.2 ± 5.6	8.5 ± 7.3

* Values are not statistically significant between groups.
† Mean ± SD.

2.10. Data analysis

All data are presented as the means ± standard deviations (SD). The proportion of mice with diarrhea between groups and rotavirus shedding among patients in test and placebo groups, were analyzed by Chi-square test. The comparison of ORF intake and stool frequency among the study children between test and placebo groups were compared by the Student's *t*-test. Probability (p) of ≤0.05 was defined as statistically significant.

3. Results

3.1. FF reduction titer of Rotamix IgY and placebo IgY

The reactivity of the anti-HRV IgY is shown in Table 3. The neutralization titers of Rotamix IgY, as measured by FF reduction assay against the HRV strain 408 and 248 serotypes were 10,240 and 40,960 respectively. All 3 test IgY samples cross-reacted with all rotavirus strains tested except for the cow strain Shimane but the Rotamix IgY showed higher cross-reactivity against strains 408, Wa, KU, M37, S2, and S-80 compared to the 2 monovalent IgY samples (Table 3). Placebo IgY showed no reaction with all serological strains (neutralization titer was <20).

3.2. Suckling mouse experiments

There was no mortality in both placebo and test mouse groups. Feeding mice with Rotamix IgY prevented diarrhea in a dose-dependent manner. Among mice challenged with HRV 248, feeding higher doses of Rotamix IgY (5 mg/ml or 2.5 mg/ml) significantly reduced the frequency of diarrhea among test mice on day 2 (1/10 vs. 5/10 among placebo mice) and day 3 (1/10 vs. 7/10 among placebo mice) (p = 0.006). Feeding with the lowest dose had the same effect on the frequency of diarrhea as the placebo group on day 3 (70% for 0.625 mg/ml compared to 70% for controls, $p > 0.05$) (Fig. 1). Among mice challenged with HRV 408, feeding of 5 or 2.5 mg/ml Rotamix IgY had a significantly reduced frequency of diarrhea in the test group on days 2 and 3 ($p \leq 0.02$). Feeding with the lowest dose had the same effect on the frequency of diarrhea as the placebo group on day 3 (90% for 0.625 mg/ml compared to 80% for placebo, $p > 0.05$) (Fig. 1). No clinical symptoms other than diarrhea were observed in any of the mouse groups.

Table 5
Infection status among study children as revealed by stool microbiological tests upon admission to hospital.

Organisms co-infecting with human rotavirus	Rotamix IgY, $n=26$ (%)	Placebo IgY, $n=26$ (%)	Total, $n=52$ (%)
Escherichia coli	10 (38.5)	8 (31)	18 (35)
Shigella dysenteriae	1 (4)	6 (23)	7 (14)
Shigella sonnei	2 (8)	2 (8)	4 (8)
Shigella flexneri	1 (4)	1 (4)	2 (4)
Proteus vulgaris	4 (16)	1 (4)	5 (10)
Staphylococcus aureus	3 (12)	0	3 (6)
Salmonella paratyphi A	1 (4)	1 (4)	2 (4)
Klebsiella aerogenes	1 (4)	1 (4)	2 (4)
Yersinia enterocolytica	0	2 (8)	2 (4)
Aeromonas hydrophila	1 (4)	0	1 (2)
Fungal origin	0	2 (8)	2 (4)
No concurrent infection	2 (8)	2 (8)	4 (8)

3.3. Baseline characteristics of study population

Patients admitted to the hospital were screened and randomly assigned to Rotamix IgY or placebo group according to flow diagram in Fig. 2. Table 4 presents the baseline characteristics of patients upon admission. The demographic and clinical background of subjects in the test and placebo groups were generally comparable. The mean ± SD of duration of diarrhea before enrollment in the study was 69.6 ± 33.6 and 74.4 ± 38.4 h for children in the test and placebo groups, respectively. The mean ± SD of stool frequency (number/day) before enrollment in the study was 9.2 ± 5.6 versus 8.5 ± 7.3 for children in the test and placebo groups, respectively. The mean of oral fluid intake (ml/day) was 767.3 and 1005.4 ml for children in the test and placebo groups, respectively.

Upon admission, 48 children (24 in test and control groups) showed mixed infection with one or more co-pathogens in their stool culture (18 *Escherichia coli*, 13 *Shigella* spp, 5 *Proteus* spp., 2 *Salmonella* spp., 8 multiple pathogens, and 2 others with fungal infection (Table 5).

3.4. Effect of anti-HRV IgY on study outcome

Fig. 3 shows the comparative data of the oral rehydration fluids (ORF) intake between Rotamix IgY and placebo IgY groups on a daily basis for 8 days. There was statistically lower volume of fluid intake among Rotamix IgY children on days 2 ($p=0.001$), 6 ($p=0.04$) and 8 ($p=0.02$). Average ORF intake during the 8 days of observation was significantly less in the Rotamix IgY treated group (Rotamix IgY vs. placebo: 699.3 ± 111.1 vs. 919.1 ± 171.31, $p=0.004$).

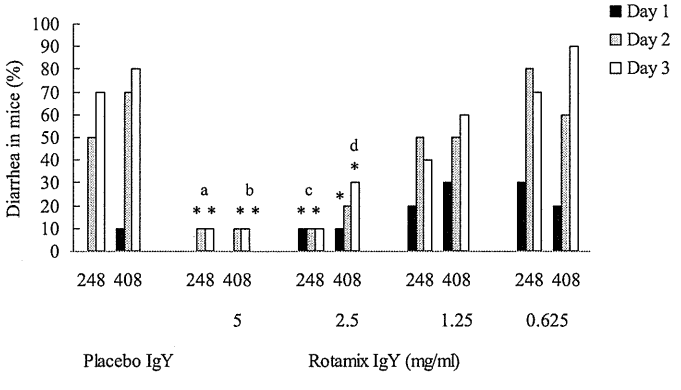

Fig. 1. Preventive effect of orally administrated different doses of Rotamix IgY against HRV 248 and HRV 408-induced diarrhea in suckling mice. *: Significant difference between placebo and Rotamix IgY groups ($p \leq 0.05$, Chi-square test). a: Rotamix IgY 5 mg/ml vs. control: Day 2: $\chi^2=3.8$; $p=0.05$; Day 3: $\chi^2=7.5$; $p=0.006$. b: Rotamix IgY 5 mg/ml vs. control: Day 2: $\chi^2=7.5$; $p=0.006$; Day 3: $\chi^2=9.9$; $p=0.002$. c: Rotamix IgY 2.5 mg/ml vs. control: Day 2: $\chi^2=3.8$; $p=0.05$; Day 3: $\chi^2=7.5$; $p=0.006$. d: Rotamix IgY 2.5 mg/ml vs. control: Day 2: $\chi^2=5.1$; $p=0.02$; Day 3: $\chi^2=5.1$; $p=0.02$.

The mean duration of IVF administration was 5 days for the Rotamix IgY group and 8 days for the placebo IgY group. Comparing day-to-day results, the Rotamix IgY group showed significantly less IVF administration only on day 1 ($p=0.03$, data not shown) of intervention. From days 2 to 8, the difference in daily mean volume (ml/day) between the two groups is not statistically significant (77.4 ± 121.4 vs. 93.3 ± 196.7 ml/day for test and placebo groups respectively, $p=0.42$).

There was a decreasing trend in daily frequency of diarrhea among children in the Rotamix group (Fig. 4) but the difference from placebo IgY group was statistically significant only on day 2 ($p=0.03$) and day 3 ($p=0.05$). The overall difference in mean duration of diarrhea (test vs. placebo: 135.3 ± 42.0 vs. 185.5 ± 41.7 h respectively) was also statistically significant ($p=0.01$) (data not shown in figure).

All pediatric patients received the usual medical treatment according to the nature of mixed infection as judged by attending physicians. These consisted of several antimicrobials, vitamin/minerals and probiotics (Table 2). Overall, both test group and placebo group were similar in terms of the kind of antimicrobials and supportive vitamins and probiotics given during the term of the study.

At the time of discharge, no stools from patients in the test group showed rotavirus shedding, while 4 patients had rotavirus positive stool in the placebo group (statistically significant ($\chi^2=4.3$; $p=0.04$). The frequency of rotavirus shedding was significantly higher in children treated with placebo IgY than in those treated with Rotamix IgY on day 3 ($\chi^2=7.7$, $p=0.005$) and on days 6–8 ($\chi^2=5.9$, $p=0.02$) (Fig. 5). The difference in mean duration of rotavirus excretion between the two groups is statistically significant (3.0 ± 1.6 vs. 4.2 ± 2.9 days for test vs. placebo group respectively, $p=0.05$).

Table 6 shows a summary of key observations and the total outcomes from day of admission of the patient to the hospital for confinement until day 8. Moreover, Rotamix IgY was not associated with any adverse clinical event after oral dosing for 8 consecutive days among infants and children with pre-existing diarrhea and dehydration.

4. Discussion

The serotypic diversity among rotaviruses is due to genetic reassortments arising from interspecies transmission and/or mixed infection. Reassortant serotypes are clinically important in as much as they cause severe forms of diarrhea among children worldwide. In this study, two natural unique human rotavirus reassortant clinical isolates ATCC VR 2273 (HRV 408) and ATCC VR 2274 (HRV 248) were selected and used to prepare the Rotamix IgY powder produced by mixing the above serotypes as antigens. These two viruses