J. Microbiol. Biotechnol. (2006), **16**(7), 1017–1025

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Generation and Segregation of Hantaviral RNA Genomic Diploid; Implications of Reassortant Generation Mechanism

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Received: May 11, 2005
Accepted: July 24, 2005

Abstract Hantaviruses possess three RNA segments of negative sense. Co-infection of closely related hantaviruses may result in generation of a progeny virus with genomic polyploidy, containing a partial or complete set of genome originated from more than one parental virus. To characterize the formation of viral genomic polyploidy, cultured Vero-E6 cells were co-infected with two closely related hantaviruses, Hantaan and Maaji, and the progeny viruses examined. The genotype of plaque-purified viruses was analyzed by a virus-specific RT-PCR. Seventy percent (67/96) of the progeny virus was categorized as Hantaan and 3.3% (2/96) was classified as Maaji, whereas 20% (21/96) was considered polyploidy as they contained both types of the S RNA segment. Most of the polyploidy progeny viruses were unstable and gave rise to either one of the parental viruses or a reassortant after several rounds of plaque purification. No recombination between the heterologous pair of S RNA was observed for those polyploid viruses during three consecutive plaque-to-plaque passages. These data suggest that the viral polyploidy formation constitutes a primary mechanism underlying the generation of a newly emerged hantavirus.

Key words: Hantavirus, diploid, genetic reassortment, Hantaan virus, Maaji virus

Hantaviruses (family *Bunyaviridae*, genus Hantavirus) are a class of emerging viruses that have been associated with two distinct human zoonoses; namely, hemorrhagic fever with renal syndrome, and hantavirus pulmonary syndrome [24, 12]. These viruses are enzootic viruses normally harbored by wild rodents, and are known to cause persistent infection in their natural hosts. They can be spread by aerosolized excreta, yet not directly by insect vectors, and human infections can occur under conditions involving close contact with the enzootic host [21].

Over 30 different types of hantavirus have thus far been distinguished, initially according to the results of serotyping via plaque reduction neutralization tests (PRNT) [16], and later confirmed and extended via polymerase chain reaction (PCR) genotyping. At least half have been shown to be of clinical relevance, and each serotype is associated with its own specific main rodent reservoir, all of which belong to the family *Muridae*. Furthermore, each serotype exhibits a distinct pattern of geographical spread [1, 25, 28].

Among the many known hantaviruses, the present study examines the Hantaan virus (HTN) and Maaji virus (MAA) [14]. HTN and MAA share 81% sequence homology in the S segment, and also exploit the same natural host, *Apodemus agrarius* [14, 15]. MAA was originally recognized from the result of its antigenicity, which is different from that of the HTN virus. The MAA nucleocapsid protein shares a common antigenic determinant with the Prospect Hill virus (PH), yet not with the nucleocapsid protein contained in HTN [13]. MAA has been isolated from both *Apodemus agrarius* mice and the acute phase sera of HFRS patients, and is considered to be a causative agent of severe HFRS in Korea, similar to HTN.

Hantaviruses are enveloped viruses with genomes consisting of three negative-sense, single-stranded RNA segments. The large (L), medium (M), and small (S) RNAs encode an RNA-dependent RNA polymerase (termed L protein), two envelope glycoproteins (G1 and G2), and nucleocapsid protein (N), respectively [6]. RNA viruses with segmented genomes carry their genomic RNA in encapsidated virions. Although it is commonly assumed that a single copy of an RNA molecule is packed into one virion, diploid RNA

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molecules have also been discovered within one virion. In cases of co-infection or super-infection, the genotypes of the resultant progeny virus strains are difficult to predict. Two RNA types are frequently discovered in the virus from one plaque, and these mixed genomes appear to persist even after several passages. However, most of these genotypes have been determined to be unstable, becoming either reassortant or parental viral strains as the result of plaque-to-plaque passage [23]. This characteristic is thought to perform a key function in the evolution, pathogenesis, and epidemiology of important pathogens, including influenza viruses, rotaviruses, and arthropod-borne orbiviruses [8, 2, 19].

Recently, Plyusnin et al. [20] reported that a recombinant virus could be generated via the transfer of an RNA into a virus-replicating cell. This breakthrough experiment was conducted via the use of type 2 Tula virus, a member of the hantavirus family. They suggested the possibility of a host being simultaneously infected with two genetically distinct variants of the same hantavirus genus, clearly creating an environment in which recombination can occur.

Accordingly, this study shows that both HTN and MAA can supply a diploid S RNA segment via co-infection, and confirms that the diploid S segments are segregated into monoploids, during plaque-to-plaque passage. This diploid pattern was detected in both M and L segments. Consequently, it would appear that the formation of polyploids facilitates viral reassortment. The present data also suggest that polyploid formation may provide a mechanism underlying the generation of newly evolved hantavirus strains.

MATERIALS AND METHODS

Cell and Viruses

Vero-E6 cells (Vero C1008: American Type Culture Collection CRL 1586. U.S.A.) were allowed to grow in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker, Walkersville, MD, U.S.A.) containing 5% fetal bovine serum (FBS) (Gibco BRL Co., U.S.A.). All the cells were grown in 5% CO_2 at 37°C. The Hantaan (prototype strain: HTN/76-118) and Maaji (MAA-I strain) viruses were originally isolated from *Apodemus agrarius* and from the sera of acute-phase HFRS patients, respectively. These viruses were then propagated in Vero-E6 under normal cell culture conditions for 6 days, and harvested. The viral stock was maintained at −70°C. The viral titer was determined via the agarose neutral red plaquing method. Confluent Vero-E6 cells grown on 6-well plates were then infected with a serially-diluted viral suspension, and covered with 0.4% agarose-DMEM. Five days after infection, the plates were overlaid with a neutral red medium, and subjected to 2 additional days of incubation to visualize the plaque.

Co-Infection

The Vero-E6 cells were allowed to grow in a T-25 flask (Corning Co., U.S.A.), and then co-infected with the HTN and MAA viruses at a multiplicity of infection (MOI) of 2. MOI normalization was then accomplished by dilution of the stock virus by more than 1×10^7 PFU/ml in DMEM. To infect the cells, the culture medium was removed and the monolayers of cells grown in the flasks were adsorbed with viruses for 2 h at 37°C, while the flask was rocked once every 20 min. A fresh medium containing 5%FBS was then added to the flasks, and the cells incubated at 37°C with 5% CO_2. Seven days after infection, the culture media were harvested and stored at −70°C for conducting a plaque formation assay.

Plaque Assay

The progeny viruses produced by co-infection were diluted and infected into a culture of Vero-E6 cells in 6-well cell culture plates. After being subjected to 2 h of incubation at 37°C, the viral solution was removed from the well, and the cells were overlaid with 3 ml/well of an overlay medium composed of a 1.5 volume of 1.2% agarose (Seakem ME) and 2 volumes of 2× DMEM containing 20% FBS. After 4 days of incubation, the cells were overlaid with 2 ml/well of a second overlay medium composed of 4.4% (v/v) neutral red (3.3 μg/ml of stock, Gibco BRL), 1 volume of dd H_2O, and 1.25 volume of 2× DMEM containing 20% FBS. After incubation overnight, the plaques were counted. The separated, visualized plaques were then randomly selected, regardless of the plaque size. The selected plaques were homogenized in DMEM and inoculated into Vero-E6 cells grown on 24-well cell culture plates. The culture media were harvested 7 days after infection, and then stored at −70°C for use as a viral stock.

RNA Extraction

To prevent cross-contamination of the RNA templates or PCR products, the viral RNAs were extracted and purified in a laminar flow biosafety cabinet. The viral RNAs were then extracted from infected Vero-E6 cells with the TRIzol reagent (Gibco BRL Co., U.S.A.), according to the manufacturer's instructions. In brief, TRIzol was administered to the cells, followed by the addition of chloroform. Thereafter, the samples were mixed, and then centrifuged at 12,000 ×*g* for 20 min at 4°C. RNA was precipitated from the aqueous phase via the addition of an equal volume of isopropanol. The RNA pellets were then washed twice with 70% ethanol, air dried, and resuspended in 100% formamide.

Specific Primers for Multiplex RT-PCR

The complete sequences of the M and S segments of HTN (strain 76-118), and partial M sequences and complete S segments of MAA (strain I) were obtained from the GenBank database. The segment sequences of each virus

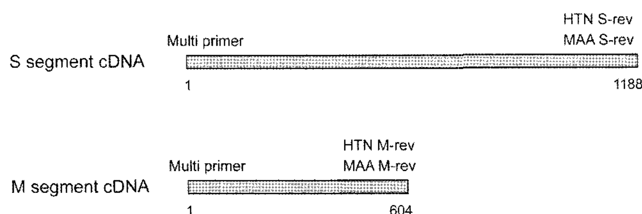
Table 1. Specific primers for multiplex RT-PCR.

Primers[a]	Sequences (5'→3')	Size (bp)[b]
Multi primer	TAGTAGTAG	
HTN M-fwd	CAGTGTGATAGGTTATGTAGAATTACCCCCCG	451
HTN M-rev	TGGGACAAAGCATTTCCCTTCAATCAGG	
HTN S-fwd	GAAAAACCTTGGGAAGGAACAAGAT	937
HTN S-rev	GAAGAGCACAATAATTCTCTGGCCT	
HTN S-rev2*	GGACTGATAAAATGATGATTTCTTCCGT	
MAA M-fwd	TAGTGTGATAGGATATGTAGAGCTACCTCCTA	451
MAA M-rev	AGGGACAAAACATTTTCCTTCTATCAAA	
MAA S-fwd	CAAGAACCTTGGTAAAGAGCAAGAC	937
MAA S-rev	AAATAAGACAATAATCCTCTGATCC	
MAA S-rev2*	TGATTGGTAGAACGATGATTTTTTTCTC	

[a]Primer names are depicted as follows: Hantaan (HTN), Maaji (MAA) viral genome segments (M and S), and sense (fwd), antisense (rev).
[b]Size of Multiplex RT-PCR product.
*Used for semi-nested PCR.

were then aligned using the DNASTAR sequence alignment software (Lasergene Navigator) to identify any heterologous regions. Specific primers for the genome segments of each virus were then selected based on specific recognition of each viral M, S genome segment. The purified RNA was converted into cDNA by reverse transcriptase using a strand-specific primer set: multi primer, HTN S-rev, MAA S-rev, HTN M-rev, and MAA M-rev (Table 1, Fig. 1). One μl of the reverse transcription mixture was used as the PCR template. The PCR for HTN was conducted using the following primers: HTN S-fwd, HTN S-rev, HTN M-fwd, and HTN M-rev. Meanwhile, the PCR for MAA was conducted using the following primers: MAA S-fwd, MAA S-rev, MAA M-fwd, and MAA M-rev (Table 1, Fig. 1). Each viral genotype was analyzed by electrophoresis of the amplified DNA on an agarose gel.

A. Reverse-Transcription (RT)



B. PCR

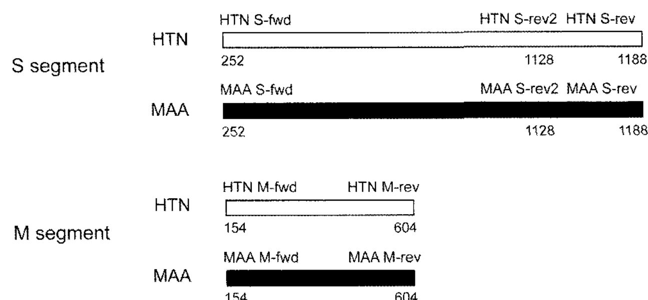


Fig. 1. Primer sets for Multiplex RT-PCR.
For the multiplex RT-PCR, eleven primers were designed to reverse-transcribe and used to amplify the S and M segments, the genomes of HTN and MAA.

Reverse Transcription

The RNA was reverse-transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.), 10 pmol sense primers for the S and M segments, 100 mM DTT, 2.5 mM dNTP, and 5× reaction buffer. The cDNA synthesis was conducted at 25°C for 10 min and 42°C for 90 min, and then terminated via the inactivation of RT by incubation at 80°C for 10 min.

Multiplex RT-PCR

The genotyping of the progeny viruses by multiplex RT-PCR requires the use of a specific primer pair for each genome segment to amplify the regions in which the two parental viruses diverge. In the present experiment, the complete sequences of the M and S segments of HTN, and partial M sequence and complete S segments of the MAA virus were aligned using the DNA Lasergene Navigator program. Each progeny virus was analyzed for the M and S genomic segments using a multiplex RT-PCR. Amplification was conducted with a GeneAmp PCR System 2700, using a temperature profile of 95°C for 5 min, 28 cycles at 95°C for 30 sec, 60°C for 30 sec, 68°C for 1 min, and a final extension step at 68°C for 10 min. The PCR products were analyzed by electrophoresis in a 0.8% agarose gel, and stained with ethidium bromide (EtBr). The TA cloning method was employed using pGEM ez-T vector (Promega Co., Madison, WI, U.S.A.) and the sequence analyzed by automatic sequencing.

Semi-Nested PCR

Semi-nested PCRs were carried out in separate tubes. Primers HTN S-fwd, HTN S-rev, MAA S-fwd, and MAA

S-rev were used for the first amplification step, as described for multiplex RT-PCR. HTN S-fwd and MAA S-rev2 or MAA S-fwd and HTN S-rev2 were used for the second amplification step. The predicted PCR and semi-nested PCR products were 877 bp.

The second-step (semi-nested) amplification was then carried out in separate tubes, the 20 μl reaction mix containing 1× Taq polymerase buffer, $MgCl_2$, dNTPs, and Taq polymerase, plus primers and 1–2 μl of the first-step reaction mix. The PCR cycles were as described for the first step.

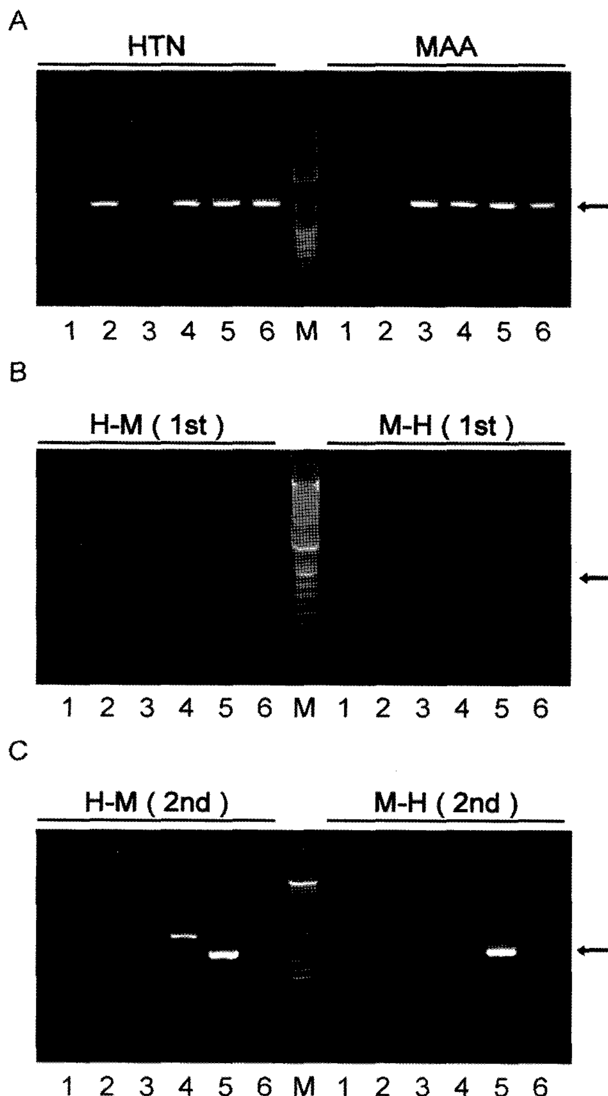


Fig. 2. Agarose gel for control experiments, amplified with specific primers for HTN and MAA.

A. PCR amplicons obtained in RT-PCR with primers HTN S-fwd and HTN S-rev or MAA S-fwd and MAA S-rev. **B.** PCR amplicons obtained in RT-PCR with primers HTN S-fwd and MAA S-rev or MAA S-fwd and HTN S-rev. **C.** PCR amplicons obtained in semi-nested PCR. **M**; molecular weight markers. Lane 1, MOCK; lane 2, HTN infected; lane 3, MAA infected; lane 4, HTN, MAA cDNA mixed; lane 5, HTN, MAA RNA mixed; lane 6, HTN, MAA co-infected.

RESULTS

Specific Primers for Multiplex RT-PCR

A multiplex RT-PCR system constitutes a set of specific, sensitive, and simple-to-use tools for the rapid differentiation of hantavirus genotypes, and also allows for the accurate diagnosis of hantavirus infection. In the present study, a multiplex RT-PCR system was established to amplify a specific genome segment of the hantaviruses. The complete sequences of the S segments of both HTN and MAA were compared using the DNASTAR alignment program to search for a sequence homology between the two viruses. Appropriate oligonucleotide primers for the multiplex RT-PCR were synthesized and applied for the detection of diploidy progeny viruses. To perform the multiplex RT-PCR, eleven primers were designed for the reverse-transcription, and used to amplify the S segments that were originated from HTN or MAA (Fig. 1). Among the eleven primers used in this study, the multi primer, HTN S-rev, and MAA S-rev were used in the reverse-transcription (Fig. 1A), whereas all the primers, with the exception of the multi primer, were used for the initial PCR or semi-nested PCR (Fig. 1B). The multi primer was also allowed to bind to the 5'-end region, and then applied to all segments of the hantavirus genome (L, M, and S). The specificities of primers with regard to discriminating the genotypes of the progeny viruses were confirmed by a multiplex RT-PCR (Fig. 2). The PCR was conducted using each specific primer and the viral cDNA of HTN or MAA as the template (Lanes 2, 3). In addition, a mixture of cDNA, the cDNA of the RNA mixture, and RNA from cells co-infected with both viruses were also used (Lanes 4, 5, and 6). Meanwhile, HTN and MAA were amplified separately, with the exception of lanes 1 and 3 and lanes 1 and 2 (Fig. 2A), respectively. An attempt was made to detect recombinants by using combination of primers, yet nothing was amplified in these trials (Fig. 2B). When the semi-nested PCR was performed, bands were detected in lanes 4 and 5 (Fig. 2C). As such, these results imply that only the S segment cDNA of HTN and MAA was amplified. An additional band was detected from the mixture of two types of cDNA or RNA, probably as a result of complex recombination.

Generation of Diploid S Segments

To test the co-infection model for the generation of polyploidy (Fig. 3), the Vero-E6 cells were co-infected with 2 MOI (PFU/cell) of the HTN and MAA viruses. After 7 days, the supernatants were harvested and subjected to plaque assays. To analyze the genetic constellation of the progeny virus present in the plaques, the progeny viruses were isolated from the viral plaques, and amplified by infection of fresh culture of Vero-E6 cells. Viral RNAs were extracted from the propagated progeny viruses and their genotypes analyzed by multiplex RT-PCR, using virus-

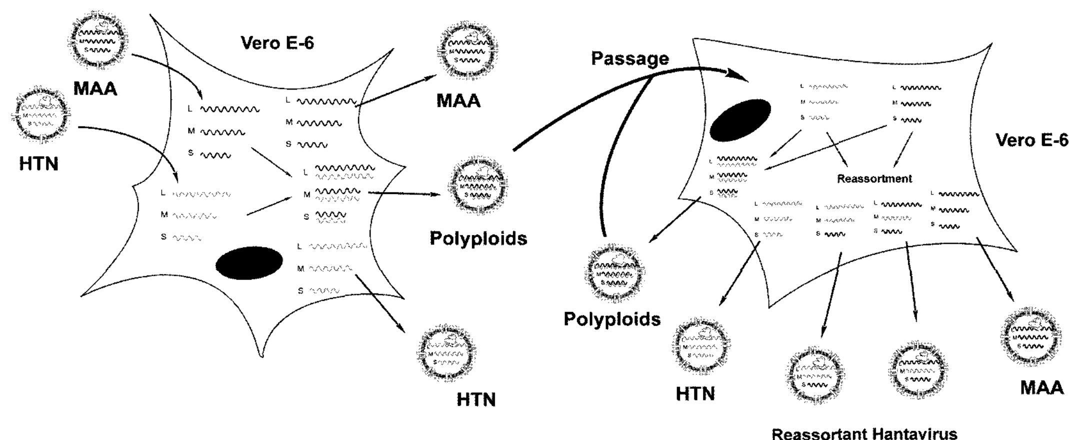


Fig. 3. A co-infection model for the generation of polyploidy and reassortant.
Vero-E6 cells were co-infected with HTN and MAA viruses. Among the progeny viruses, the viruses with polyploid RNA genome were isolated and amplified by the following infection of fresh culture of Vero-E6 cells. After passage, the progeny viruses were separated into various types such as HTN, MAA, polyploidy, and reassortant by the patterns of RNA genome. The progeny viruses of polyploidy were followed with repeated passages.

specific primers. Sixty-seven (70%) of the 96 progeny plaques were determined to harbor the HTN S segment, whereas only 2 (3.3%) harbored the MAA S segment. However, no recombination within the S segment was detected among the 96 progeny plaques. Interestingly, 21 (20%) of the progeny plaques contained the S segments of both HTN and MAA. The amplified DNA was examined with regard to that of the parental viruses by RFLP, and the sequences analyzed. As a result, it was confirmed that the viruses harbored two types of S segment.

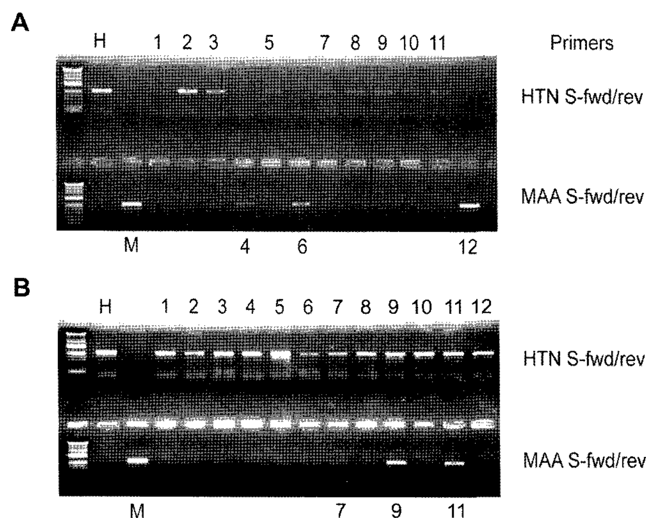


Fig. 4. Agarose gel of multiplex RT-PCR products from **passage 1**, amplified with primers specific for HTN or MAA.
A and **B** show S segment genotypes of progenies originated from two diploid viruses, respectively. Upper parts of both gels show HTN PCR amplicons obtained in RT-PCR with primers HTN S-fwd and HTN S-rev, whereas lower parts show MAA PCR amplicons obtained in RT-PCR with primers MAA S-fwd and MAA S-rev. **H**, HTN controls. **M**, MAA controls.

Segregation of Diploids Through Passage

In order to determine whether the diploid hantaviruses are stable in the Vero-E6 cells, S genome RNA of progeny viruses were examined. Two plaques were selected among

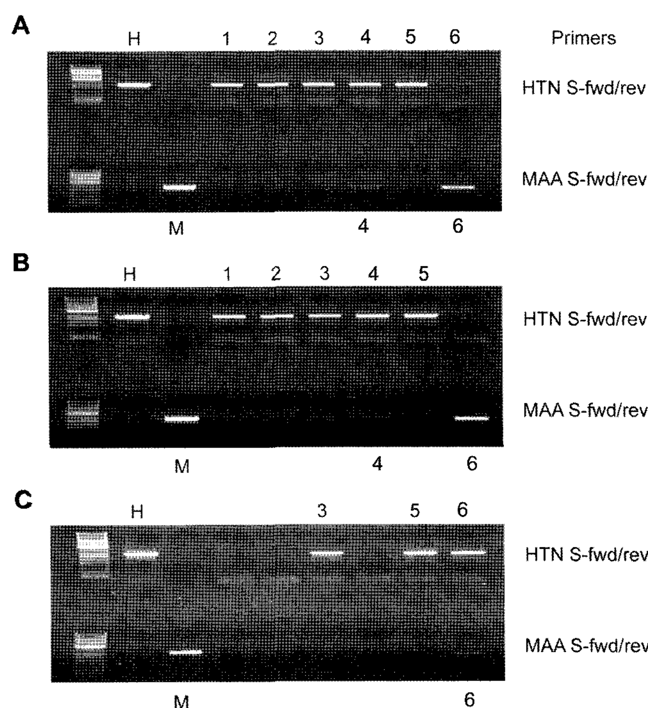


Fig. 5. Agarose gel of multiplex RT-PCR products from **passage 2**, amplified with primers specific for HTN and MAA.
A, **B**, and **C** show S segment genotypes of progenies originated from diploid viruses in lanes 7, 9, and 11 in Fig. 4B, respectively. Upper parts of both gels show HTN PCR amplicons obtained in RT-PCR with primers HTN S-fwd and HTN S-rev, whereas lower parts show MAA PCR amplicons obtained in RT-PCR with primers MAA S-fwd and MAA S-rev. **H**, HTN controls. **M**, MAA controls.

the diploid progeny viruses, and after appropriate dilution, re-plaqued in Vero-E6 cells. Analysis indicated that the progeny viruses derived from one plaque were all segregated into monoploid S segments (Fig. 4A), whereas progeny viruses from the other plaque have either segregated or maintained their diploid genome (Fig. 4B, Lanes 7, 9, and 11). These results indicate that the diploid virus is unstable during passage.

Whether the diploid virus described above undergoes segregation of diploid genome during multiple rounds of plaque-to-plaque passage was characterized (Fig. 4B, Lanes 7, 9, and 11). Some of the diploid viruses segregated into monoploid viruses with the S segment of either HTN or MAA, but other viruses remained in diploid, harboring S segments from both HTN and MAA (Fig. 5). However, after the third passage, all the progeny viruses segregated into monoploid viruses (Fig. 6). Thus, the diploid configuration could not be maintained, and the viruses eventually revert to monoploidy.

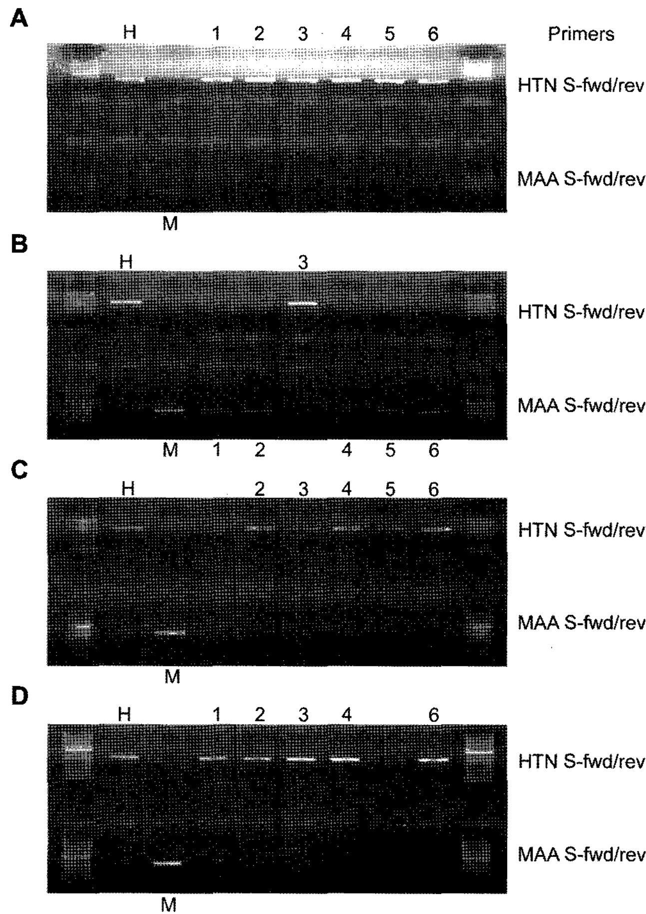


Fig. 6. Agarose gel of multiplex RT-PCR products from **passage 3**, amplified with primers specific for HTN or MAA.
A–D show S segment genotypes of progenies originated from diploid viruses in lanes 4 and 6 of Fig. 5A, lane 4 of Fig. 5B, and lane 6 of Fig. 5C, respectively. Upper parts of both gels show HTN PCR amplicons obtained in RT-PCR with primers HTN S-fwd and HTN S-rev, whereas lower parts show MAA PCR amplicons obtained in RT-PCR with primers MAA S-fwd and MAA S-rev. **H**, HTN controls. **M**, MAA controls.

Reassortment Between HTN and MAA

To determine whether genetic reassortment occurs between HTN for MAA, the M segments of the progeny viruses were screened by PCR. Primers specific for the two types were used, and the amplified products were examined by RFLP. At passage 0, it was determined that the diploid M segments were in the same pattern as that of S segment. Interestingly, in the M segments, the quantity of diploids was greater than that in the S segments, yet all these diploid forms segregated in the next passage. The initial two viruses of S diploidy were originally diploid in their M segments (Fig. 7). After one passage, the progeny viruses exhibited monoploidy in the M segment of HTN, yet no MAA M segment was detected thereafter, whereas the S segments were maintained in a diploid form until the next passage.

Those progeny viruses then segregated completely into a monoploid form, becoming revertants or reassortants. Here, the reassortants were determined to be progeny viruses that harbored the HTN M segment and MAA S segment. When progeny viruses from the 96 plaques at passage 0 were analyzed, only two were reassortants. However,

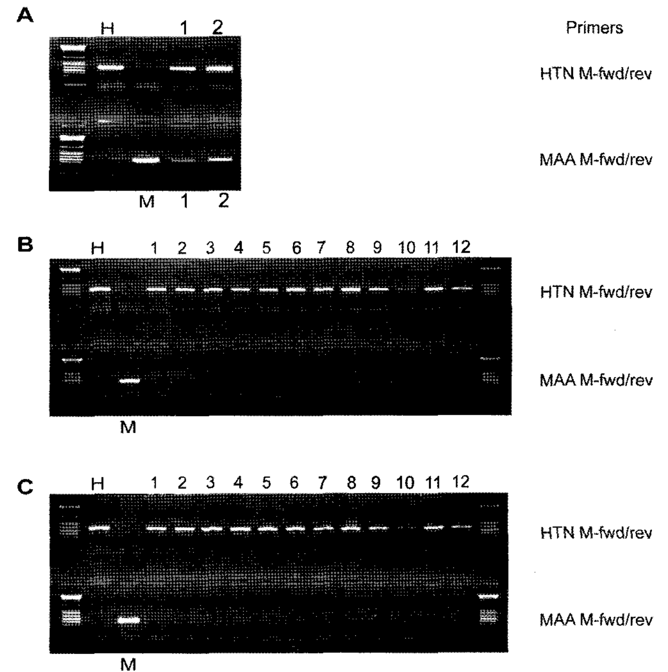


Fig. 7. Agarose gel of multiplex RT-PCR products from **passage 0, 1** amplified with M segment primers for HTN or MAA (**A–C**).
A. M segment of Initial 2 progeny virus; **B**, **C**, M segment of passage 1 progenies. Upper parts of both gels show HTN PCR amplicons obtained in RT-PCR with primers HTN M-fwd and HTN M-rev, whereas lower parts show MAA PCR amplicons obtained in RT-PCR with primers MAA M-fwd and MAA M-rev. **H**, HTN controls. **M**, MAA controls.

among the diploid viruses, reassortants were observed in 3 progeny plaques (10%) after the first passage, 2 progeny plaques (11.1%) after the second passage, and 5 progeny plaques (20.8%) after the third passage. Therefore, the quantity of reassortants was higher in the progeny of the diploid virus than in the progeny virus from co-infection.

DISCUSSION

Genetic diversity due to gene segment reassortment during the co-infection of cultured cells has already been described in many RNA-containing viruses whose genomes consist of discontinuous genes. Such viruses include members of the following families: *Arenaviridae*, *Birnaviridae*, *Bunyaviridae*, *Orthomyxoviridae*, and *Reoviridae* [18]. In addition to reassortment in the cell culture, evidence supports the possibility of genome reassortment by segmented RNA viruses during the co-infection of host organisms. The best described paradigm for genetic reassortment in nature is the antigenic shift observed in the type A influenza virus, which has been attributed to the reassortment of gene segments encoding viral hemagglutinin and neuraminidase.

Other evidence suggests that reassortment may play an important role in the evolution, pathogenesis, and epidemiology of arthropod-borne members of the *Bunyaviridae* family [3, 5, 7, 9]. Several studies have reported that high frequency genetic reassortment can occur between these viruses. Genetic reassortment has also been detected in cases in which arthropods or tissue culture cells have been co-infected with viruses that belong to the same serogroup [11, 4]. In addition, various studies have reported that genetic reassortment can occur among viruses, including members of the *Bunyaviridae* family. For instance, the frequency with which reassortants were observed in the progeny of mammalian cells co-infected with La Crosse encephalitis and the Snowshoe Hare virus was 51% [27]. Meanwhile, another study reported 64% reassortment in the viral progeny from mosquitoes that had been dually infected with two distinct strains of the Rift Valley fever virus [26]. However, reassortment has not been observed between members of distinct serogroups within a genus [3, 4, 27].

Little data are currently available regarding the nature of hantaviruses, which undergo genetic reassortment. In two recently published studies, the results of a genetic analysis of SinNombre (SN) viral RNA sequences from humans and rodents in Nevada and eastern California indicated that genetic reassortment between SN variants sometimes occurs in the natural environment [10, 17]. Furthermore, genetic analyses of SN progeny RNA sequences in cases of co-infection with SN variants implied that genetic reassortment between SN genetic variants had occurred *in vitro* [22].

The current study concerns the behavior of laboratory-induced diploid viruses constructed from homologous hantaviruses. A multiplex RT-PCR was utilized to facilitate the selection of reassortants among progeny viruses. The present method was based on the exploitation of a few differences in the nucleotides harbored by HTN and MAA [15, 14]. As the PCR products from each segment of HTN and MAA were different, they could be used to determine the genotypes of the progeny viruses. Unfortunately, the sequence of the MAA L segment has yet to be established. Thus, assessment of reassortant progeny was made based on the genotypes of the S and M segments.

Despite the fact that the cells were infected with equal amounts of virus, among the 96 progeny plaques from co-infection of HTN and MAA, 67% of the progeny viruses harbored HTN S genome segments, and only 2% harbored the S genome of MAA. This appeared to be the result of a higher replication rate of the HTN virus than that of the MAA virus. Several bands were also detected that exhibited the S segments of both HTN and MAA. Diploid viruses harboring the S segments of both HTN and MAA represented 23% of the progeny viruses from the mixed infection (21/96) and 14% of the progeny viruses from subsequent passages (10/72). However, these viruses were found to be unstable and reverted to either reassortant or parental virus within 3 consecutive plaque-to-plaque passages.

The M segments of the progeny viruses were screened by PCR, to confirm if genetic reassortment had occurred between HTN and MAA. It was found that two types of diploid hantaviruses with diploid S segment had not been contaminated. Specific primers were used and the amplified product was subjected to RFLP analysis. As a result, it was ascertained that the number of M diploidy was greater than the number of S diploidy at passage 0, and M segment diploids segregated much more rapidly than the S segment diploids. It is also worth noting that some progeny viruses harbored the M segment of HTN and S segment of MAA, whereas other progeny viruses were diploid in either M or S segments. This does not represent an experimental error. Rather, it indicates that in polyploidy, two kinds of viral genome coexist. The two types of plaque selected for the S segment experiment were believed to be the result of viruses that were in a diploid state with regard to both the S and M segments. However, after the first and second passages, only S segments were observed in a diploid and monoploid state, whereas after the third passage, all had been segregated and become monoploid.

When examining the 96 plaques at passage 0 via a plaque assay, only two (2%) reassortants were found. Among the 30 progeny viruses, that were selected and subjected to plaque-to-plaque passages, only 3 (10%) were found after the first passage, 2 (11.1%) out of 18 viruses were detected after the second passage, and 5 (20.8%) out

of 24 detected after the third passage. These were all determined to be progeny viruses harboring the M segment of HTN and S segment of MAA, indicating that the parent virus and diploid virus generated more reassortants in the first stage when two similar viruses were used to co-infect the host. Some of the genes in the diploid viruses that are segregated afterward, as time goes by, are returned to revertants with the gene of parent virus along the passage. Finally, some of the viruses were shown to become reassortants.

In addition, recombination can occur in cases when two types of RNA co-exist during reverse-transcription. In the control experiments, when two kinds of RNA coexisted, the PCR products appeared to be recombinant. Nonetheless, although the diploid viruses harboring both segments may have an increased possibility of forming an S recombinant virus as an intermediate, no recombinants were detected in the present experiment. Diploid viruses have been shown to carry both the HTN S segment and the MAA S segment, thereby increasing the possibility that a recombinant virus will form. Thus, since it is believed that an encapsidated form of RNA during replication lowers the recombination frequency compared with serving the nascent RNA, future studies will attempt to isolate a recombinant virus from the progeny of S diploid viruses.

Acknowledgments

This work was supported by the Korea Center for Disease Control and Prevention (0412-BM01-716-0001) and the Korea Research Foundation (E00109).

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