REAL-TIME RT-PCR OF HANTAAN VIRUS RNA USED FOR THE DETECTION OF VIRUS RESPONSE TO ANTIVIRAL DRUGS

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Summary. – Hantaan virus (HTN) is an important cause of hemorrhagic fever with renal syndrome (HFRS) in Korea. HTN RNA can be detected with the RT-PCR and the quantity of HTN RNA in infected cells can be measured by competitive RT-PCR. The current study used the real-time RT-PCR for the detection of viral RNA S gene in a more detailed fashion than in the previous study (Nam *et al*., *Virus Genes* **26**, 31–38, 2003). A standard curve was generated with serial 10-fold dilutions of the HTN RNA. The sensitivity of RNA detection was approximately 10 PFU of HTN. The cells infected with HTN were treated with the antiviral drugs ribavirin, zidovudine, and amantadine. 24 hrs after infection, real-time RT-PCR was used to detect the HTN RNA synthesized in the infected cells. No viral RNA was detected in the HTN-infected cells treated with antiviral drugs, but HTN RNA was detected in untreated HTN-infected cells. This finding suggested that real-time RT-PCR should be used for the detection of antiviral activity against HTN.

Key words: Hantaan virus; real-time RT-PCR; ribavirin; zidovudine; amantadine

HTN is an enveloped virus with a genome containing three RNA segments (small-S, medium-M, and large-L) of negative polarity. HTN belongs to the family *Bunyaviridae,* the genus *Hantavirus*. It is a causative agent of HFRS manifested with vascular hemorrhage and kidney dysfunction associated with acute thrombocytopenia. HFRS occurs in more than 150,000 individuals per year in Europe, China, and Korea with 4–15% lethality, what represents a serious public health concern (Lee, 1999). However, no therapeutic agents are available for therapy of HTN infection in humans.

The plaque-reduction neutralization test is widely used to detect the hantaviruses. However, there are a few non-culturable hantaviruses and consequently, this method cannot be of a general use. Immunofluorescence antibody test, ELISA, and immunoblotting are used commonly for the detection of hantaviral infections. However, these methods require virus-specific antibodies to detect hantaviral antigens (Aitichou *et al*., 2005). Recently, RT-PCR and real-time RT-PCR have been used to detect the hantaviral RNA (Puthavathana *et al*., 1992; Aitichou *et al*., 2005).

Currently, there are no antiviral drugs or agents approved by the US Food and Drug Administration for the treatment of hantavirus diseases. Only ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carboxamide) has been shown to exert an antiviral effect *in vitro* and *in vivo* (Severson *et al*., 2003; Chapman *et al*., 1999).

In this study, we set up a real-time RT-PCR to detect the hantavirus RNA S segment in the infected cells. Next, we used this method to evaluate the effects of some antiviral drugs such as zidovudine (AZT, 3'-azido-2',3'-dideoxythymidine) and amantadine (tricyclo[3.3.1.1.^{3,7}]decanal-amine hydrochloride) against HTN replication in infected cells.

In the presented experiments, HTN strain 76-118 was propagated in Vero E6 cells. To test the antiviral drugs against HTN, a human lung carcinoma cells (A549 cells) were infected with HTN at a multiplicity of infection (MOI) equaled

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Abbreviations: AZT = zidovudine; C_T = threshold cycle; HTN = Hantaan virus; HFRS = hemorrhagic fever with renal syndrome

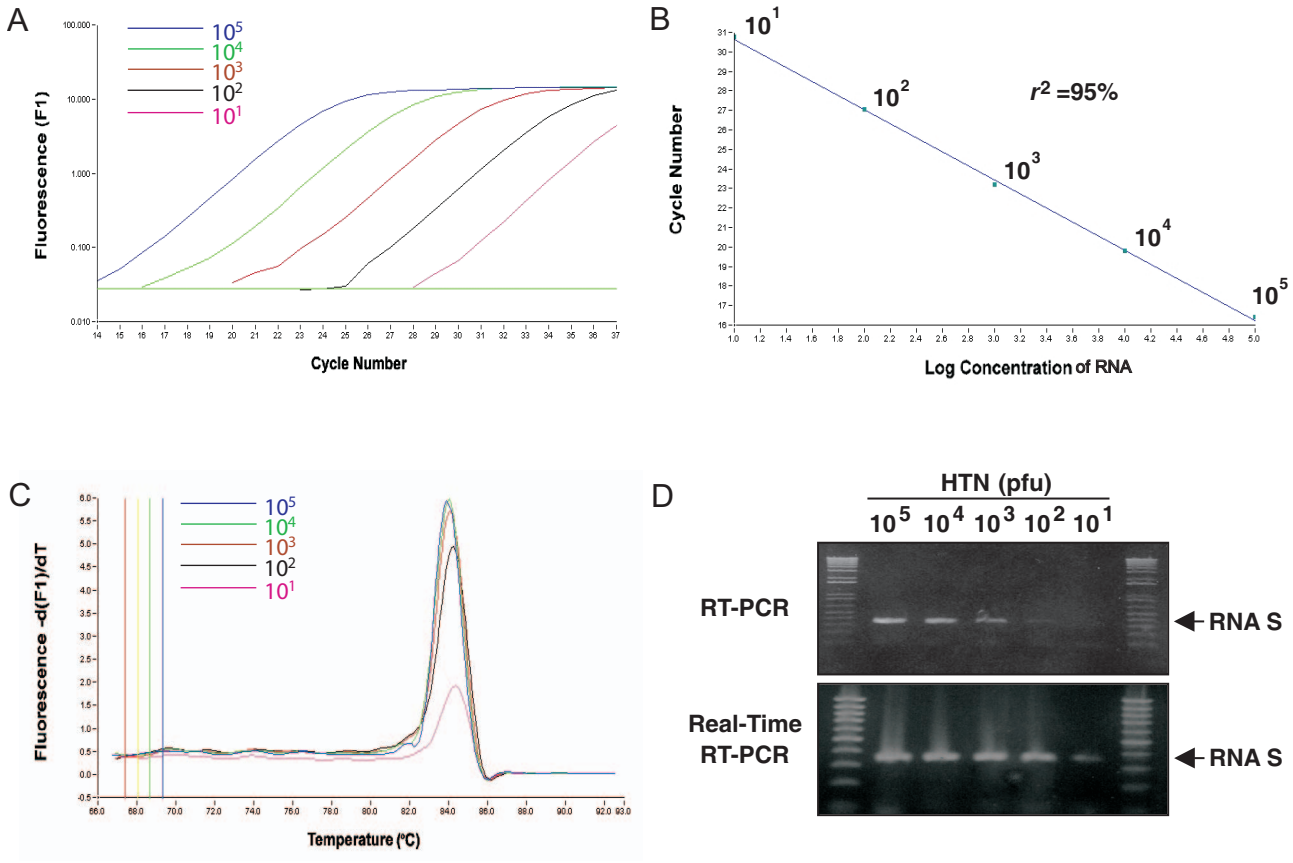

Fig. 1

Detection of HTN RNA by real-time RT-PCR

(A) Fluorescent signal produced by the amplification of HTN RNA from virus diluted from 10^5 to 10^1 PFU/reaction. (B) Regression analysis of HTN RNA from virus diluted as (A), r^2 = coefficient of correlation. (C) Melting curve of HTN RNA from virus diluted as (A). (D) Agarose gel electrophoresis of RT-PCR and real-time RT-PCR products after HTN RNA amplification using the same primer set.

1 with or without 10 mg/ml AZT, 1 mg/ml amantadine, or 0.1 mg/ml ribavirin as a positive control with activity against HTN. 24 hrs after infection with/without the drugs, HTN RNA was purified and real-time RT-PCR performed. The effective concentration of the antiviral drug was determined as the lowest concentration that showed no toxicity in an MTT assay (colorimetric test for measuring cellular proliferation) (data not shown). To detect HTN RNA by real-time RT-PCR, we followed the previously reported protocol (Nam *et al*., 2003). To construct a standard curve, 1×10^6 PFU/ml of HTN was serially diluted 10-fold and the RNA from 100 ml of each dilution was purified with TRIzol LS Reagent (Invitrogen). The total RNA was used as a template for a first-strand cDNA synthesis in a reaction with Random primers and SuperScript™ II reverse transcriptase (Invitrogen). Semiquantitative values were expressed by determination of the lowest threshold cycles (C_T).

Tested HTN sample was diluted to the concentration of 10^5, 10^4, 10^3, 10^2, and 10^1 PFU/reaction. Real-time RT-PCR detected approximately 10^1 PFU of HTN with a C_T value of 30.8 (Fig. 1A,B). When the C_T and PFU values were analyzed by a regression analysis, the correlation between C_T and PFU values was 95% as analyzed by Microsoft Excel to confirm linearity (Fig. 1B). Moreover, a melting curve clearly showed that only one band was amplified with this primer set (Fig. 1C). This was confirmed with RT-PCR using the same primer set (HS398S, 5'-GCATCATCGTCTATCTTACATC-3', and HS751R, 5'-ATTGTTCGATACGATCACTCC-3'), which generated the same product as the real-time RT-PCR, the single band in agarose gel electrophoresis (Fig. 1D). This means that real-time RT-PCR with this primer set specific for the RNA S segment is useful in detecting the HTN RNA with any nonspecific amplification.

In the application of this technique, we evaluated activity of available human antiviral drugs, such as AZT, amantadine, and ribavirin against the replication of HTN. AZT is a nucleoside analogue of the thymidine with added azido-group. It was the

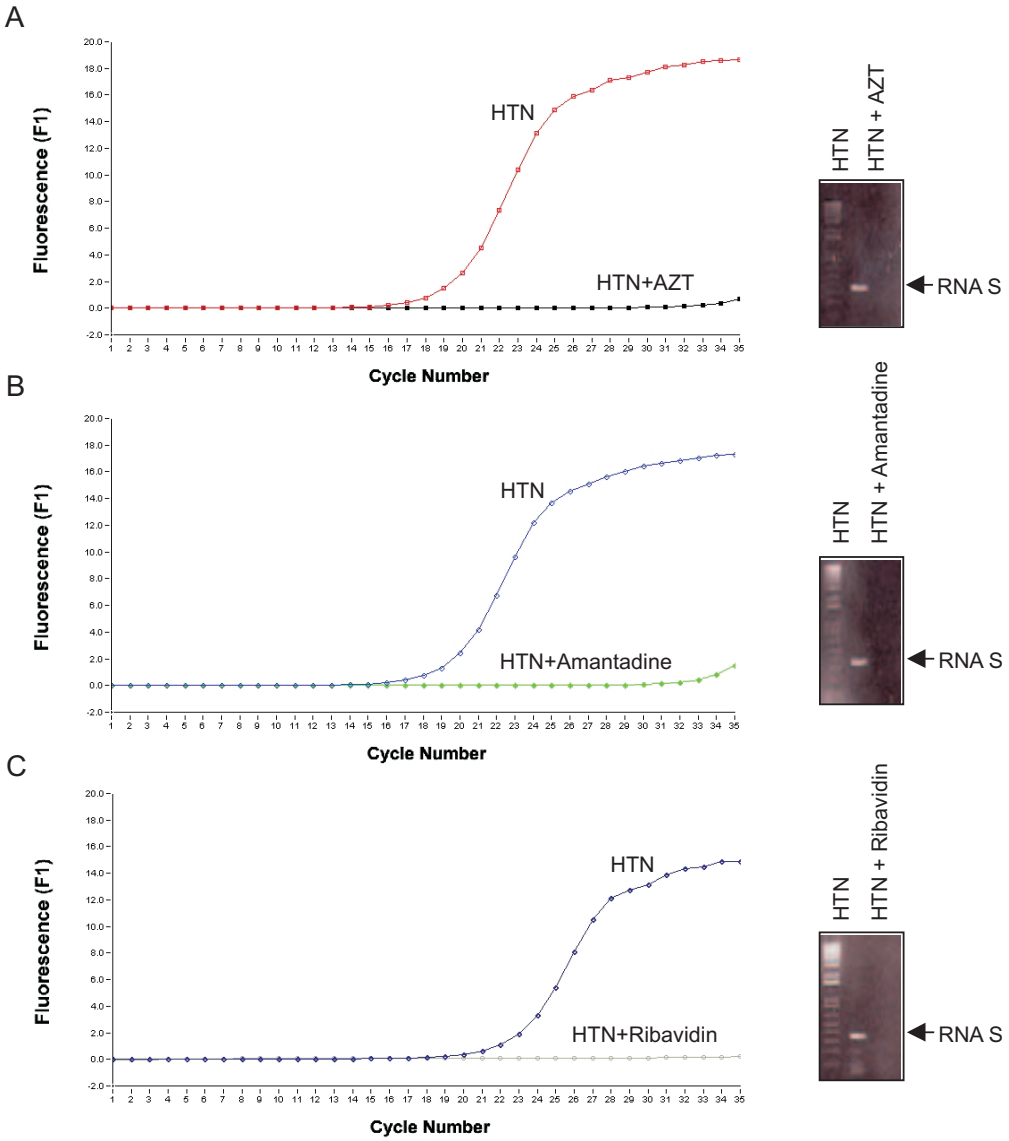

Fig. 2

Effects of AZT (A), amantadine (B), and ribavirin (C) on replication of HTN detected by real-time RT-PCR

HTN-infected A549 cells were examined 24 hrs after infection. Agarose gel electrophoresis of the real-time RT-PCR products (right panels A,B,C).

first effective treatment for AIDS patients. AZT interferes with the replication of HIV and other viruses. It incorporates into a replicating DNA strand in place of thymidine, what prevents further replication of the DNA (Samuels, 2006). Amantadine is a tricyclic amine that was used for therapy of influenza A infection. It blocks the function of Influenza A virus matrix protein M2, which is required for the internalization of the virus by endocytosis (Lim *et al*., 2005). Only ribavirin has been shown to have an antiviral activity against hantavirus-derived diseases and at the same time has a broad antiviral spectrum against both DNA and RNA viruses. The mechanism of its antiviral activity seems to involve interference with the 5'-capping of mRNA, inhibition of the viral polymerase, and induction of an error catastrophe (Sun *et al*., 2007).

The serially diluted drugs were used for determination of the lowest concentration of the drug required for prevention of HTN replication. The A549 cells were infected with HTN at a MOI = 1 and simultaneously treated with 10 mg/ml AZT, 1 mg/ml amantadine, and 0.1 mg/ml ribavirin. Ribavirin served as a positive control with known antiviral activity against HTN replication. 24 hrs after infection, all antiviral drugs completely blocked HTN replication in A549 cells. The antiviral effect of AZT and amantadine were similar to that of ribavirin (Fig. 2). These data suggested that these drugs could be useful in the

treatment of HTN-induced diseases. As far as we know, this is the first report about AZT and amantadine as antiviral drugs against HTN replication. So far, these drugs have been used for treatment of other viral diseases in humans and therefore, they could be easily applicable to HTN-induced diseases. However, further elaborate studies are required to confirm this assumption.

Taken together**,** we have developed a real-time RT-PCR to detect HTN RNA using the SYBR Green I dye method. We supposed that this HTN RNA detection method could be applied for the screening of antiviral activity of drugs on HTN replication.

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