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Rapid Communication

An improved procedure for rapid determination of viral RNA sequences of avian RNA viruses

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Summary

A system for rapid determination of viral RNA sequences, RDV, was improved for detection of avian RNA virus in allantoic fluids. We detected avian paramyxovirus nucleotide sequences using RDV method ver 2.0.

There have been a number of recent outbreaks of avian influenza and West Nile virus spread *via* birds. Assays based on molecular diagnostics, such as polymerase chain reaction (PCR), are now available. However, PCR is not available for unknown emerging infectious viral diseases because of the lack of nucleic acid sequence data. Therefore, a system for rapid nucleic acid sequence determination is required for newly emerging viruses spread *via* birds.

Recently, we developed two new methods for the detection of RNA viruses, one of which is based on cDNA representational difference analysis (cDNA RDA) using 96 hexanucleotides that do not prime ribosomal RNAs but prime most RNA viral genomes at the reverse transcription step [2]. However, this method requires at least one week for determination of the nucleic acid sequence. The other method is a system for rapid determination of viral RNA sequences (RDV method) [3], which uses whole-genome amplification and direct sequencing techniques. The RDV method enables a broad range of partial nucleotide sequences of viral RNA genomes to be obtained within 2 days without cloning. We succeeded in determining the nucleic acid sequences of severe acute respiratory syndrome coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants using the RDV method [3]. However, the limitation of this method is the requirement for supernatant of virus-infected cultured cells as a starting material. In the present study, we demonstrate the application of this method to avian RNA virus from allantoic fluid because many viruses spread *via* birds have been diagnosed by virus isolation using inoculation of embryonated chicken eggs. We have improved the

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previously reported RDV method (renamed RDV method ver 1.0) with regard to synthesis of cDNA, double-stranded DNA, and amplification to increase sensitivity.

A fecal sample was collected from northern pintail (*Anas acuta*) at Lake Izunuma-Uchinuma in Tohoku district, northeast Japan. The sample was placed into PBS containing antibiotics to give a 30% suspension. The suspension was centrifuged and the supernatant was inoculated into the allantoic cavities of 10-day-old chicken embryos. The eggs were incubated for 3 days. At the end of the incubation period, the allantoic fluids were taken and tested for hemagglutination activity with 0.5% chicken red blood cells. The allantoic fluids showed hemagglutination activity; however, they were not identified as Newcastle disease virus nor avian influenza A virus.

RNA was first extracted from the allantoic fluids using Isogen-LS (Nippon Gene, Tokyo, Japan) and then extracted using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). A whole transcriptome amplification system (WTA; Sigma-Aldrich, Saint Louis, MO, USA) was used to amplify viral double-stranded cDNA in accordance with the manufacturer's instructions. Instead of the Taq polymerase recommended in the kit, we used 25 µl of multiplex PCR mix 2 containing reaction buffer, 0.25 µl of multiplex PCR mix 1 containing Taq polymerase (Takara Bio Inc., Shiga, Japan), WTA master mix containing primers (Sigma-Aldrich: no information regarding their sequences), and 2.5 µl of cDNA in a total volume of 50 µl to obtain PCR products in high yield. The reaction mixture was heated at 94 °C for 60 sec, followed by 40 cycles of amplification using a Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 65 °C for 90 sec, primer extension at 72 °C for 90 sec, and denaturation at 94 °C for 30 sec. The results of preliminary experiments confirmed that this modified method, RDV ver 2.0, increased sensitivity compared with the previous protocol, RDV ver 1.0 (data not shown).

After the first cDNA library was digested with *Hae*III and ligated with a blunt *Eco*RI-*Not*I-*Bam*HI adaptor, the second cDNA library was amplified by PCR with specially designed primer sets as follows: forward primer, H1-5: 5′-AATTCGGCGGCCGCGGATCC*CC*GGCG-3′; reverse primer H9-1: 5′-AATTCGGCGGCCGCGGATCC*CC*AGGA-3′ or H9-2, -3, -4, -5, -6, -7, -8, -9, -10, -11 (described in ref. [3]) and -13 (AATTCGGCGGCCGCGGATCC*CC*AGTG) (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics). Direct sequencing was performed with the forward primer, H1-5.

Four of fifteen DNA fragments were closely related to avian paramyxovirus (APMV) type 6 designated APMV-6/duck/Taiwan/Y1/98 (accession no. AY029299) [1]. Figure 1 shows the locations of PCR fragments on the genome of APMV. To confirm that the RNA specimen contained genomic RNA of APMV, we designed primer sets corresponding to the large (L) region of APMV (approximately 1.1 kb) for reverse transcription (RT)-PCR based on the sequence deposited in GenBank (accession no. AY029299), and RT-PCR was performed using SuperScript III (Invitrogen, San Diego, CA, USA) for RT and the Expand High Fidelity PCR system (Roche, Mannheim, Germany) for PCR. We obtained a single band at 1.1 kb (data not shown) and determined the nucleic acid sequence by direct sequencing using the forward and reverse primers. The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB297681. Comparison

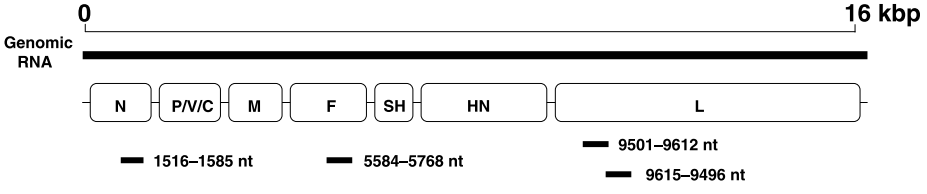

Fig. 1. Locations of PCR fragments of viral cDNAs amplified using the RDV method. The amplified PCR fragments were sequenced directly and mapped onto the avian paramyxovirus genome

of the nucleotide sequence of the L protein revealed that the isolate was closely related to APMV-6/duck/Taiwan/Y1/98, isolated in Taiwan in 1998, with 98.6% nucleotide identity.

RDV ver 2.0 is a rapid, sensitive method for the direct determination of avian viral RNA sequences in allantoic fluids inoculated with the test specimen. This method has the potential to become a standardized and qualified method for the detection of known and unknown avian RNA viruses.

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