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The Single-Step Multiplex Reverse Transcription-Polymerase Chain Reaction Assay for Detecting H5 and H7 Avian Influenza A Viruses

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Thontiravong, A., Payungporn, S., Keawcharoen, J., Chutinimitkul, S., Wattanodorn, S., Damrongwatanapokin, S., Chaisingh, A., Theamboonlers, A., Poovorawan, Y. and Oraveerakul, K. *The Single-Step Multiplex Reverse Transcription-Polymerase Chain Reaction Assay for Detecting H5 and H7 Avian Influenza A Viruses*. Tohoku J. Exp. Med., 2007, **211** (1), 75-79 —— Avian influenza (AI) A virus subtypes H5 and H7 cause severe disease in domestic poultry, including chickens and turkeys. Moreover, H5 and H7 AI A viruses can cross the species barrier from poultry to humans. In the present study, we have developed a single-step multiplex reverse transcription-polymerase chain reaction assay (RT-PCR) for detecting H5 and H7 AI A viruses. This assay was applied to the poultry isolates with the aim of establishing a surveillance method to monitor possible transmission to humans. Two subtype-specific primer sets capable of producing PCR products of 157 and 326 base pairs corresponding to AI A virus H5 and H7 subtypes, respectively, were utilized in a one-step and one-tube reaction. The single-step multiplex RT-PCR assay developed in this study was found to be specific for detecting H5 and H7 AI A viruses. No specific amplification bands were detected with total nucleic acids extracted from other influenza hemagglutinin subtypes and other viral pathogens. The sensitivity of this assay was about 10^3 RNA copies/μl. In conclusion, this novel single-step multiplex RT-PCR is a simple assay with high potential for rapid, specific and cost effective laboratory diagnosis of H5 and H7 AI A virus isolates from clinical specimens of poultry. ———— Avian influenza virus; hemagglutinin; H5 and H7 AI A viruses; single-step multiplex RT-PCR

Avian influenza (AI) is a highly contagious disease caused by type A influenza virus, a member of the *Orthomyxoviridae* family. Influenza A viruses cause major and frequently fatal disease in birds, as well as in mammals including humans (Webster et al. 1992). Influenza A viruses harbour

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a segmented genome of single-stranded negative sense RNA. They can be further subtyped based on the antigens of two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Sixteen subtypes of HA (H1-16) and nine subtypes of NA (N1-9) have been identified to date (Fouchier et al. 2005). Among 16 HA subtypes, only H5 and H7 are virulent in poultry. In contrast, only three HA subtypes (H1, H2 and H3) and two NA subtypes (N1 and N2) have commonly been found in humans (Ohrui et al. 2000; Miura et al. 2001). However, over the past few years, several subtypes of AI virus including H5N1, H7N7, H7N3 and H9N2 have directly crossed the species barrier to infect humans (Rezza 2004).

In early 2004, H5N1 influenza A virus has been reported in several Asian countries including South Korea, Vietnam, Japan, Taiwan, Laos, Cambodia, China, Pakistan, Indonesia, and Thailand. As a consequence, this virus has emerged in Thailand causing severe epidemics in the poultry industry (OIE 2006). Moreover, human cases of H5N1 virus infection were reported in Vietnam, Cambodia, Indonesia and Thailand. In Thailand, 22 confirmed human cases of AI (H5N1) infection have been reported, 14 of them were fatal (WHO 2006).

Currently, virus isolation (VI) in embryonated chicken eggs and subsequent HA and NA subtyping by serological methods constitute the standard for AI detection and subtype identification. These methods are highly accurate and sensitive for virus detection but are also laborious and time-consuming (Murayama et al. 1999).

Reverse transcription polymerase chain reaction (RT-PCR) technique was used to detect influenza A virus in throat or nasal specimens collected from humans, pigs and horses (Schorr et al. 1994). This method is not only more rapid but also more sensitive and specific than conventional procedures (Atmar et al. 1996). However, using RT-PCR technique to detect several RNA viruses individually is labor-intensive and expensive. These limitations can be overcome by applying a multiplex RT-PCR assay. The multiplex format is a significant improvement of the conventional RT-PCR technique, achieved by incorporating multiple primers that amplify RNA from several RNA viruses simultaneously in a single reaction. This assay has been used successfully for typing and subtyping influenza viruses in humans (Payungporn et al. 2004).

In this study, the development of a single-step multiplex RT-PCR for detecting AI A virus was described, with particular attention being paid to those subtypes capable of infecting humans, such as H5 and H7 AI A viruses. This method was applied on the poultry isolates with the aim of establishing a surveillance method to monitor possible transmission to humans.

Materials and Methods

Sources of clinical specimens

The 30 AI A virus subtype H5 ($n = 29$) and H7 ($n = 1$) specimens used in this study were obtained from several avian species, including chicken ($n = 11$), duck ($n = 16$), quail ($n = 1$), pigeon ($n = 1$), and open-billed stork ($n = 1$), during the 2004-2005 outbreak in Thailand. These specimens were isolated and provided by the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and the Department of Livestock Development, Bangkok, Thailand. The viruses were propagated in 9-day-old embryonated chicken eggs. AI A viruses and their subtypes had previously been identified by RT-PCR, nucleotide sequencing and phylogenetic analysis (Payungporn et al. 2004; Keawcharoen et al. 2005).

RNA extraction

RNA was extracted from the eggs' allantoic fluid using the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's recommendations.

Primer design

Using the nucleotide sequences available in the GenBank database, multiple sequence alignments of the H5 and H7 genes were performed using the CLUSTAL X program (version 1.8 from ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX).

H5 and H7 primers were selected from conserved regions of 50 known sequences specific for H5 and H7 AI A viruses. The primers were chosen and analyzed using the OLIGO primer design software (version 9.1) (Institute of Biotechnology, University of Helsinki,

TABLE 1. Multiplex RT-PCR primers to amplify H5 and H7 avian influenza A viruses.

Primer name	Primer sequence	Position	Amplicon length (bp)
H5-F2	5′-TGAGAAAATTCAGATCATCCCC-3′	409-430	157
H5-R1	5′-GCTCCTCTTTATTGTTGGGTATG-3′	565-543	
H7-F4	5′-AAGATCAGGTTCTTCTTTCTATG-3′	465-487	326
H7-R4	5′-TGAAAGTGAAGGTCACTGTGTCA-3′	790-768	

Finland) to ensure that they could be combined in a multiplex format under the same PCR condition. The primers used in this study are shown in Table 1.

Control for specificity test

A sample previously identified as influenza/A/chicken/Nakorn-Patom/Thailand/CU-K2/2004(H5N1) served as a control for developing the multiplex RT-PCR assay. The cDNAs of avian influenza A/chicken/Netherland/1/03 (H7N7) was provided by the Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands. The specificity of the multiplex RT-PCR assay was evaluated by cross-reaction tests performed on RNA extracts from isolates or clinical specimens with other hemagglutinin subtypes of influenza A virus (H1-15) and other viral pathogens such as Newcastle disease virus (NDV), Infectious bronchitis virus (IBV), Infectious laryngotracheitis virus (ILT), Infectious bursal disease virus (IBD), A/Fujian/411/02 (H3N2), Human respiratory syncytial virus (HRSV) and Human metapneumovirus (hMPV). The RNA of influenza A virus subtypes H1-H15 were provided by the Department of Livestock Development, Bangkok, Thailand.

RNA standard for sensitivity

The HA genes of AI A virus (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004 [H5N1] and A/chicken/Netherland/1/03 [H7N7]) were used to construct plasmid DNAs by inserting the H5 (409-543) and H7 (465-1630) genes into the pGEM-T Easy Vector (Promega, Madison, WI, USA). In vitro transcription was performed by using the RiboMAX^{TM} Large Scale RNA Production system-T7 (Promega) following the manufacturer's recommendations. Concentration and copy number of the transcribed RNAs were calculated by measuring absorbance at 260 nm. To perform sensitivity tests, the RNAs were serially diluted 10-fold, ranging from 10^{10} to 10 copies/μl.

Multiplex RT-PCR condition

RT and multiplex PCR were performed simultaneously in a single-step reaction using the AccessQuick RT-PCR System (Promega). Two sets of primers specific for the H5 and H7 genes of AI A virus were used at a final concentration of 0.5 μM each. A combination of 5.0 μl of RNA sample with a reaction mixture containing 12.5 μl of AccessQuick Master Mix, 5 unit (U) of RNase inhibitor, 5 U of AMV Reverse transcriptase, 1.0 mM $MgCl_2$ and RNase-free water was used in a final volume of 25 μl.

Thermocycling condition

Cycling conditions included a reverse transcription step at 48°C for 45 min. After an initial denaturation step at 94°C for 3 min, amplification was performed during 40 cycles including denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and extension (72°C for 30 sec), followed by final extension at 72°C for 10 min.

Detection of amplified multiplex RT-PCR products

A total of 10 μl of PCR product was added to loading buffer and run on a 2% agarose gel (TopVision^{TM} LE GQ Agarose, Fermentus, USA) at 125 V for 45 min. After electrophoresis the DNA bands were stained with ethidium bromide and visualized by UV transilluminator.

RESULTS

Interpretation of the band pattern from multiplex RT-PCR

The multiplex RT-PCR products consisted of 157 base pair (bp) for the H5 gene and 326 bp for the H7 gene were visualized by gel electrophoresis.

Specificity test

All positive specimens were subjected to nucleotide sequencing and FASTA (http://www.

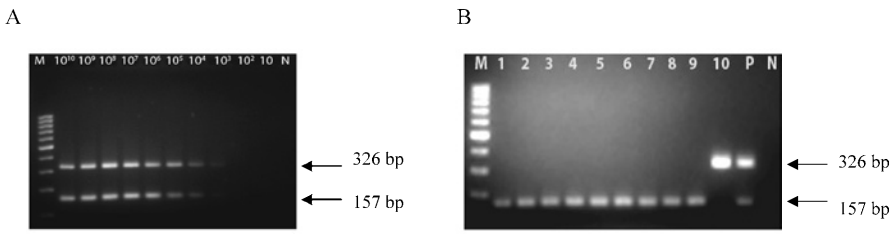

Fig. 1. The products of multiplex RT-PCR band patterns for H5 (157 bp) and H7 (326 bp) AI A viruses. The sensitivity of multiplex RT-PCR was evaluated by 10-fold serial dilutions ranging from 10^{10} to 10 copies/μl of the RNA standard (A). RNA dilutions were as indicated on the top of the lanes. Detection of H5 and H7 AI A viruses isolates from clinical poultry specimens (B). Lanes 1-9; Isolates positive for H5 AI A viruses, Lane 10: Isolate positive for H7 AI A viruses, Lane P: Positive control, Lane N: Negative control, Lane M: 100-bp ladder.

ebi.ac.uk/fasta33/nucleotide.html) search to confirm the H5 and H7 subtype detection. The specificity of this assay was evaluated by cross reaction tests. Multiplex primers showed no cross-reactivity to total nucleic acids extracted from different viruses as described above. In addition, this assay showed no reactivity to other hemagglutinin subtypes of AI A virus (H1-H15). No significant false positive bands were observed in any of the samples tested.

Sensitivity test

The sensitivity of the multiplex RT-PCR assay to detect H5 and H7 was determined using 10-fold serial dilutions of the in vitro transcribed RNAs of H5 and H7. The DNA bands were visible at RNA standard dilutions as low as 10^3 copies/μl (Fig. 1A).

Detection of target viruses by multiplex RT-PCR

We then tested a total of 30 AI A isolates from clinical specimens of poultry by multiplex RT-PCR with specific primer sets. The results showed that H5 AI A virus was identified in 29 specimens and H7 AI A virus in one. The results were shown for only 10 specimens in Fig. 1B.

Discussion

AI A virus subtypes H5 and H7 cause severe disease in domestic poultry, including chickens, turkeys and humans (Rezza 2004). Therefore, rapid detection of these subtypes is particularly important for early diagnosis, prevention of disease transmission to humans and protection against economic loss in the poultry industry. Over the past decades, virus isolation and serology were the methods used to detect these viruses. However, VI can be laborious and time-consuming, whereas rapid antigen detections are less sensitive and specific than VI. Although RT-PCR has also been used to detect these pathogens, it only allows for detecting the nucleic acid of one specific pathogen at a time. Multiplex RT-PCR is advantageous because it allows for detecting and distinguishing between multiple pathogens applying one test. Multiplex RT-PCR has the added benefits of being cost effective, time saving, specific and sensitive; furthermore, it has been used successfully for typing and subtyping influenza viruses (Payungporn et al. 2004).

In the present study, the single-step multiplex RT-PCR for detecting H5 and H7 AI A viruses was developed. The primer set selection for multiplex RT-PCR was based on the sizes of the amplicons generated. These primers produced two different PCR products of 157 and 326 bp for H5 and H7 AI A viruses, respectively. Since the present method has been carried out in a single-tube and single-step process (RT-PCR), the risk of carry-over contamination was considerably reduced.

The primer sets used in this study target the hemagglutinin gene, which has the highest rate of evolution of any of AI A virus genes (Webster et al. 1992). Therefore, it may be necessary to

re-evaluate the primer sequences to accommodate for the genetic variation of influenza A viruses.

The specificity of this assay was evaluated with multiple samples of RNA and DNA from different viruses as described above. The selected primers did not cross react with any of those samples. Moreover, this assay shows no cross-reactivity to other hemagglutinin subtypes of AI A virus (H1-15). The sensitivity of this assay was evaluated by subjecting serial dilutions of H5 and H7 in vitro transcribed RNAs to multiplex RT-PCR amplification. Using dilutions as low as 10^3 copies/μl, we could still clearly distinguish between PCR products. Furthermore, this technique can be used for human specimens as well.

In conlusion, we have developed the multiplex RT-PCR assay for simultaneously detecting H5 and H7 AI A virus isolates from clinical poultry specimens. This assay provides a simple, powerful and easily applicable method for rapid, specific and cost-effective laboratory diagnosis of H5 and H7 AI A virus isolates from clinical poultry specimens.

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