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Development of multiplex reverse transcription polymerase chain reaction (RT-PCR) for simultaneous detection of matrix, haemagglutinin and neuraminidase genes of H5N1 avian influenza virus

R. Majumder, E. H. Chowdhury*, R. Parvin, J. A. Begum, M. Giasuddin[1] and M. R. Islam
Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Abstract

Influenza A virus, subtype H5N1 causes a fatal disease in domestic poultry and could spread directly from poultry to humans. The aim of this study was to develop a multiplex reverse transcription polymerase chain reaction (mRT-PCR) for simultaneous detection of Type A influenza virus-specific matrix protein (M) gene as well as H5 and N1 genes of highly pathogenic avian influenza (HPAI) viruses. Finnzymes Phusion-Flash High-Fidelity PCR Master Mix (Finnzymes Oy, Finland) and Qiagen one-step RT-PCR enzyme mix (Qiagen, Germany) were used in a one-step RT-PCR. RNA was extracted from two known positive samples using Qiagen RNA extraction kit. RT-PCR was carried out with a mixture of primers specific for the Type A influenza virus matrix protein (M), and H5 and N1 genes of H5N1 HPAI viruses in a single reaction system. The mRT-PCR cDNA products were visualized by gel electrophoresis. The mRT-PCR yielded fragments of 245 bp for M, 545 bp for H5 and 343 bp for N1 genes of HPAI virus, which were clearly distinguishable. The mRT-PCR using the Finnzymes Phusion-Flash High-Fidelity PCR Master Mix (Finnzymes Oy, Finland) with Qiagen one-step RT-PCR Enzyme Mix (Qiagen, Germany) required only one hour and 20 minutes. (*Bangl. vet*. 2011. Vol. 28, No. 2, 55 – 59)

Introduction

Highly pathogenic avian influenza (HPAI) is a disease of poultry with high mortality: it disrupts poultry production and trade (Capua and Alexander, 2004). HPAI viruses may be transmitted from birds to humans (Alexander and Brown 2000; Koopmans *et al*., 2004), and are a potential source of human influenza pandemics (Capua and Alexander, 2004). Influenza viruses are divided into type A, B and C on the basis of antigenic differences in the nuclear and matrix proteins of the virus: type A are sub-typed on the basis of antigenic differences of the surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA) proteins (Easterday *et al*., 1997). So far, 16 HA (H1 - H16) and nine NA (N1 - N9) subtypes have been identified (Steinhauer and Skehel, 2002). All highly pathogenic avian influenza (HPAI) viruses belong to H5 or H7 subtype, although some H5 or H7 strains of wild birds may not be

[1]Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh
*Corresponding author:- *E-mail*: emdad001@yahoo.com

highly pathogenic for poultry at the initial stage. The rest of the subtypes cause a much milder primarily respiratory disease known as low pathogenic avian influenza (LPAI) (Alexander, 2000). Since the outbreaks of the H5N1 HPAI in Hong Kong in 1997, evidence suggests that H5N1 viruses might infect humans and cause high mortality (Suarez *et al*., 1998; Subbarao *et al*., 1998; Zhou *et al*., 1999). In Asia, at the beginning of 2004, the outbreaks of the H5N1 HPAI caused tremendous economic losses to the poultry industry and human infection, with high mortality in Vietnam and Thailand. Conventional laboratory diagnosis of avian influenza (AI) is based on virus isolation in tissue culture or embryonated chicken eggs. However, it is time-consuming and labour intensive, but has high sensitivity and specificity. Reverse transcription polymerase chain reaction (RT-PCR) is now routinely used for diagnosis of avian influenza viruses. Usually RT-PCR for matrix protein (M) or nucleoprotein (NP) gene is used for initial screening for Type A influenza viruses. The positive samples are subjected to RT-PCR for H5 and N1 genes for sub-typing. Multiplex RT-PCR (mRT-PCR) assay offers the possibility for the molecular detection of more than one gene or genome segment of a single pathogen or of more than one pathogen in a single reaction (Ellis *et al*., 1997; Stockton *et al*., 1998). In the present study, a multiplex RT-PCR method was developed for rapid simultaneous detection of MP, H5 and N1 genes of H5N1 viruses from positive clinical samples of chickens.

Materials and Methods

Two H5N1-positive isolates, A/Chicken/Bangladesh/BL84T/2007 and A/Chicken/ Bangladesh/BL-343T/2008, were used in this study. RNA was extracted from positive clinical samples using Qiagen RNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Appropriate primer sequences for RT-PCR were selected to amplify Type A influenza virus-specific matrix (M) protein gene (Fouchier *et al*., 2000), H5 haemagglutinin gene (Lee *et al*., 2001) and N1 neuraminidase gene (http://www.who.int/csr/disease/avian_influenza/guidelines/labtests/en/index.html) of AI viruses. Primer details are given in Table 1.

One-step RT-PCR for MP, H5 and N1 genes was performed separately using Finnzymes Phusion-Flash High-Fidelity PCR Master Mix (Finnzymes Oy, Finland) and QIAGEN RT-PCR Enzyme Mix (Qiagen, Germany) in 20 µl volumes, in which the reaction mixture contained 10 µl Phusion-Flash Master Mix, 1 µl Qiagen RT-PCR Enzyme Mix, 0.5 µl of each primer, 5 µl of RNA template and RNase-free water to make 20 µl. The thermal profile of the RT-PCR reaction for the amplification of all three genes was: 50°C for 30 min, 98°C for 30 sec, 35 cycles of 98°C for 10 sec, 52°C for 30 sec and 72°C for 30 sec, followed by 72°C for 10 min in a thermocycler. RT-PCR was performed individually for MP, H5 and N1 genes as well as simultaneously for all three genes in the same reaction tube. Then the RT-PCR products were analysed by agarose gel (1.5%) electrophoresis and visualized by UV trans-illumination.

Table 1. Properties of the Primers for MP, H5 and N1 genes

Primer	Target gene	Primer Sequence*	Reference	Product size
M52CF	MP	5'-CTT CTA ACC GAG GTC GAA ACG-3'	Fouchier *et al.* (2000)	245 bp
M253R		5'-AGG GCA TTT TGG ACA AAK CGT CTA-3'		
H5-155F	H5	5'-ACA CAT GCY CAR GAC ATA CT-3'	Lee *et al.* (2001)	545 bp
H5-699R		5'-CTY TGR TTY AGT GTT GAT GT-3'		
N1-580-607F	N1	5'-TGA AGT ACA ATG GCA TAA TAA CWG ACA C-3'	http://www.who.int/csr/disease/avian_influenza/guidelines/labtests/en/index.html	343 bp
N1-891-918R		5'-CAC TGC ATA TAT ATC CTA TTT GAT ACT CC-3'		

*Inclusions of degenerate nucleotides are indicated in bold. Codes for mixed bases position: R=A/G, K=G/T, Y=C/T

Results and Discussion

The RT-PCR positively amplified 245, 545 and 343 bp fragment for MP, H5 and N1 genes, respectively in individual reactions (Fig. 1).

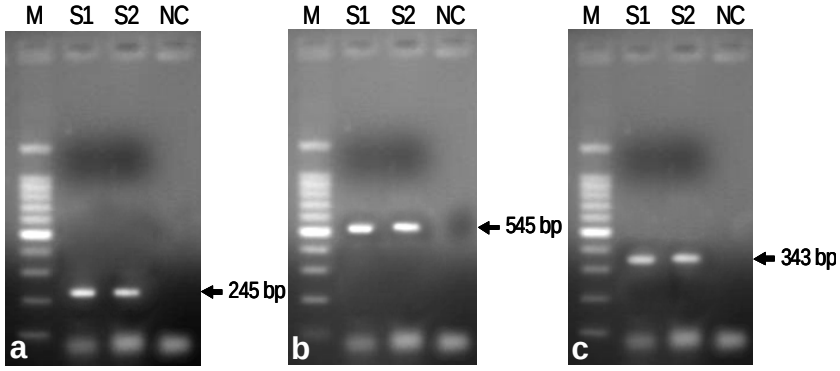

Fig. 1. Amplification of 245 bp fragment of Type A influenza virus-specific matrix protein (M) gene (a), 545 bp fragment of H5 gene (b) and 343 bp fragment of N1 gene of H5N1 HPAI viruses in separate RT-PCR reactions. M = Marker, S1 = Sample 1, S2 = Sample 2, NC = Negative Control.

The multiplex RT-PCR successfully amplified Type A influenza virus-specific M gene, as well as H5 and N1 gene fragments of H5N1 HPAI viruses simultaneously in a single reaction (Fig. 2). The positive amplification bands at 245 bp for MP gene, 343 bp for N1 gene and 545 bp for H5 gene were clearly separated. The multiplex RT-PCR assay using Phusion-Flash High-Fidelity PCR Master Mix and Qiagen RT-PCR Enzyme Mix required only 1 hour and 20 min for RT-PCR cycling. Multiplex RT-PCR

for M or NP, H5 and N1 gene of HPAI viruses has been reported (Payungporn *et al*., 2004; Wei *et al*., 2006), but requires much more time (about 3 hours). The multiplex RT-PCR will allow rapid and simultaneous detection and sub-typing of H5N1 avian influenza viruses.

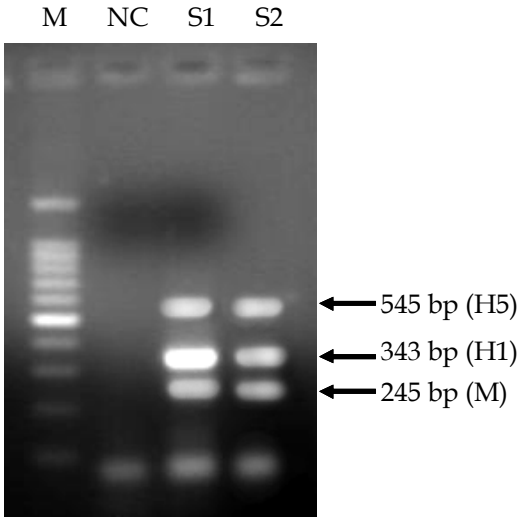

Fig. 2. Simultaneous amplification of matrix protein (M) gene of Type A influenza virus, H5 gene and N1 gene by multiplex RT-PCR. M = Marker, NC = Negative control, S1 = Sample 1, S2 = Sample 2

Conclusions

A rapid single-step multiplex RT-PCR using Phusion-Flash High-Fidelity PCR Master Mix and Qiagen RT-PCR Enzyme mix has been developed. The test can detect simultaneously Type A influenza virus-specific matrix protein (M) gene and H5 and N1 genes of HPAI influenza viruses.

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