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Rapid Detection of Highly Pathogenic Avian Influenza H5N1 Virus by TaqMan Reverse Transcriptase-Polymerase Chain Reaction

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Abstract: Highly pathogenic Avian Influenza (AI) H5N1 viruses have been spreading from Asia since late 2003. Early detection and classification are paramount for control of the disease because these viruses are lethal to birds and have caused fatalities in humans. Here we describe a TaqMan Reverse Transcriptase-Polymerase Chain Reaction Assay for rapid detection of Avian Influenza virus and for H5 subtyping by targeting HA gene of AI viruses. The assay was highly sensitive than RT-PCR and virus isolation in chick embryos. In the present study all samples (field samples) which are positive for HI and RT-PCR were tested by using TaqMan Reverse Transcriptase-Polymerase Chain Reaction Assay for reconfirmation. AI viruses (H5N1) were detected from nine samples which are received from Maharashtra during Avian influenza outbreak in India in 2006. Real-Time PCR assays was also conducted for detection of viral genome in different organs of experimental infected chickens revealed presence of the virus in all organs with high virus concentration in brain, heart, intestine and cloaca. This test allows definitive confirmation of an AI virus as H5 within hours, which is crucial for rapid implementation of control measures in the event of an outbreak.

Key words: Avian Influenza, H5N1, TaqMan-RTPCR

INTRODUCTION

The widespread occurrence of High Pathogenic Avian Influenza (HPAI) H5N1 in Asia and the potential of H5N1 to cross species and infect humans pose major threats to human and animal health. The disease is best controlled in birds and rapid diagnostic capability for H5N1 diagnosis is crucial for swift index case diagnosis, facilitating timely implementation of control measures. The Real-time PCR system is a novel technique based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template (Dorak, 2001; Heine *et al*., 2007). TaqMan assays by Spackman *et al*. (2003) to detect North American Type A Influenza virus strains were adapted to increase the analytical sensitivity for detection of Highly Pathogenic Avian Influenza H5N1 strains currently circulating in Asia.

Real-time PCR is a relatively new technique, but it has been used widely for the diagnosis and study of avian pathogens (Jackwood *et al*., 2003). It also has been successfully applied in the screening of AI virus in field samples and subtyping (Lee *et al*., 2005; Spackman *et al*., 2003). The Real time TaqMan PCR assays described, in this study, have been applied to study the organ tropism of the avian influenza virus in experimentally infected chickens.

The advantages of Real-time PCR over standard Reverse Transcriptase-PCR include speed and the reduced chance of cross contamination among samples because no post-amplification sample handling is necessary. Additionally, the labeled probe used to detect the PCR product with Real-time PCR methods is target specific, providing an additional level of confirmation that the PCR product is the expected target, as compared to standard Reverse Transcriptase-PCR (Spackman *et al*., 2003).

MATERIALS AND METHODS

A total number of 295 experimental infected birds samples including field samples (field samples, H5N1 infected and also control birds samples) were used in study. A total number of 24, 3-4 weeks old SPF birds were used in Intra vinous pathogenicity index text. Birds were divided into 3 groups, in each group 8 birds were maintained in stainless steel biosafety cabinets. First and second group of birds were infected with 0.2 mL of 4HA units of 1:10 diluted Ck/India/7966/06 and Ck/India/7972/06 virus respectively. Lost group is maintained as control by infecting with distilled water. HPAI H5N1 virus isolates were derived from samples submitted to high security animal disease laboratory Bhopal (BSL-4 Lab) during the first out breaks of HPAI in India during 2006. Nine viruses were choosen for the initial evaluation of Real-time PCR tests. Viruses were re-isolated from the experimentally infected chickens with two Indian H5N1 AIV isolates (Ck/India/7966/06 and Ck/India/7972/06) is also subjected to Real-time PCR

assay. Live virus was processed in approved isolation rooms or in biosafety cabinets. Viruses were stored at -70°C or immediately added to RNA extraction buffers (QIAGEN).

Viral nucleic acid was purified from 100-200 µL samples (allantoic fluid, tracheal swabs, cloacal swabs and 10% direct tissue homogenates) by using either QIAamp Viral RNA Mini Kit or QIAamp RNeasy mini Kit (QIAGEN, Germany) according to the manufacturers protocols. Nucleic acid was eluted in 50 µL of elution buffer and 2 µL of eluant was used as template in each 25 µL Real time PCR reaction.

Primers and probes used for TaqMan real time PCR assay

H5-1F	HA_1	TGCCGGAATGGTCTTACATAGTG	Ng *et al.* (2005)
PROBE-1		FAM-AGTCCTCGCTCACTGGGC ACGGT-BHQ1	
H5-1R		GGCATTTTGGACAAATCGTCTACG	
H5-2F	HA_2	GTGGCGAGCTCCCTAGCA	Ng *et al.* (2005)
PROBE-2		FAM-TGGCAATCATGGTAGCTG GTC-TAMRA	
H5-2R		TCTGCATTGTAACGACCCATTG	

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrho-damine

Two sets of primer-probe were used in this assay as recommended by CDC, USA. Primer-probe were commercially supplied by Operon are used under study. One step RT-PCR reactions were performed using TaqMan one step RT-PCR kit by targeting the HA_1 and HA_2 junctions of the AI viruses.

Real-Time PCR Mix (25 µL reaction) was prepared as follows:

Component		Volume
10× PCR buffer	=	2.5 µL
dNTP (20 mm)	=	1.0 µL
Mgcl (50 mm)	=	2.75 µL
Primer forward-H5-1F	=	0.9 µL (9pmol)
Primer reverse-H5-1R	=	0.6 µL (6pmol)
Probe-H5-1P	=	0.5 µL (5pmol)
Taq DNA polymerase	=	0.25 µL
Rox dye	=	0.4 µL (30nm)
RT-Enzyme	=	0.1 µL
Template (RNA)	=	2.0 µL
Nuclease free water	=	14.0 µL
Total volume	=	25 µL

A master mix containing all reagents except the template was prepared for each set of reactions and 23 µL of master mix was aliquot onto a 96 well optical reaction plate. Two micro liters of samples template was then added to each well. No template was added to Negative Control (NTC).

Same concentration of reagents was also used for second set of Primers and probe. In each test NTC and positive control were included.

The Real-Time PCR machine was programmed as follows:

Reverse transcription	50°C	30 min (1X)	
Hot start Taq activation	95°C	10 min (1X)	
Amplification			
Denaturation	95°C	30 sec	(35X)
Annealing	50°C	1 min	
Extension	72°C	30 sec	

The results of TaqMan assay were expressed in the form of software generated characteristic amplification curves. Amplification curves from positive and negative were compared to the test samples and the results are expressed as Cycle threshold (Ct) values representing the number of cycles necessary for a statistically significant rise in reporter dye emission. Ct values up to 28 were obtained from positive samples and Ct values above 30 were considered negative.

RESULTS AND DISCUSSION

Real-Time PCR assay data were generated and analyzed to calculate "threshold cycle" value (Ct value), which is inversely proportionate to the initial template concentration in the samples and dissociation temperature (Tm-temperature melting), which differentiate between specific and non-specific amplifications.

The tissue triturated (1:10 dilution) samples were directly used for viral RNA extraction. Detection of AIV genome in different organs of chickens experimentally infected with 2 Indian H5N1 AIV isolates (Ck/India/7966/06 and Ck/India/7972/06) by Real-Time PCR TaqMan assay is presented in Table 1 and 2. A typical amplification curve obtained from different organs is shown in Fig. 1 and 2. It may be seen that the Ct values varied depending on the virus concentration in the starting material from which total RNA was isolated. Less Ct values were observed in brain, heart, intestine and cloaca (Table 1 and 2), which indicates these organs were contained more number of RNA copies.

In chicken infected with H5N1 AIV isolates (Ind/Ck/7966/06 and Ind/Ck/7972/06), the virus could be detected in all the 11 organs viz, brain, trachea, lung, heart, liver, spleen, proventriculus, intestine, pancreas, kidney and cloaca. Absence of specific amplification from uninfected healthy birds from any of the organs indicates specificity of the primers and test.

Real time-PCR serves as a fast and effective alternative to virus isolation for the detection of Influenza A virus (Yuen *et al.*, 1998). Keeping this in mind Real-time PCR was used to study the tissue tropism of avian influenza viruses isolated in experimentally infected chickens. A total number of 295 experimental infected birds samples including field samples (field samples, H5N1 infected and also control birds samples) in that 110 samples were negative. All samples positive in HI are also gave

Table 1: Results of real-PCR assay with TaqMan probe-1 targeting HA_1 gene

Isolate	Brain	Trachea	Lung	Heart	Proventriculus	Liver	Spleen	Kidney	Intestine	Pancreas	Cloaca
7966/06	+	+	+	+	+	+	+	+	+	+	+
Ct values	15.35	22.72	21.02	15.07	19.52	21.73	22.70	21.75	16.03	21.82	18.36
7972/06	+	+	+	+	+	+	+	+	+	+	+
Ct values	17.67	25.47	22.73	19.05	19.18	23.82	21.02	22.03	17.67	24.71	18.70
Control	-	-	-	-	-	-	-	-	-	-	-
Ct values	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table 2: Results of real-time PCR assay with TaqMan probe-2 targeting HA_2 gene

Isolate	Brain	Trachea	Lung	Heart	Proventriculus	Liver	Spleen	Kidney	Intestine	Pancreas	Cloaca
7966/06	+	+	+	+	+	+	+	+	+	+	+
Ct values	14.97	25.90	19.22	12.51	16.92	20.18	19.44	16.35	16.39	23.25	16.89
7972/06	+	+	+	+	+	+	+	+	+	+	+
Ct values	16.19	25.87	24.70	15.71	20.80	25.27	23.86	23.95	20.44	25.61	18.38
Control	-	-	-	-	-	-	-	-	-	-	-
Ct values	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

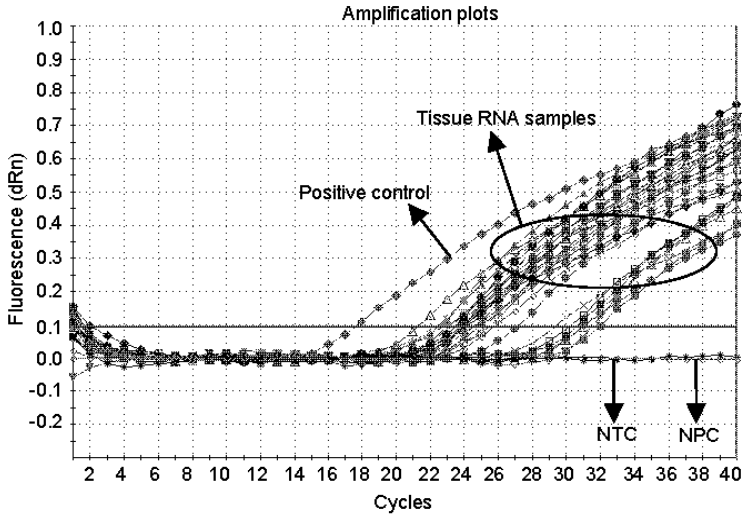

Fig. 1: A typical amplification curve of the HA Gene probe-1 Amplicons, (NTC = No Template control) (NPC = No Probe Control)

the positive results in Real time-PCR. Thus, Real-time PCR results showed 100% correlation with virus isolation in embryonated chicken eggs.

In the present study, TaqMan Real-time PCR was conducted. Recombinant *Taq* polymerase with the property of getting activated only in the event of being exposed to 95°C for 10 min was used in the Real-time PCR assay to achieve optimum specificity in target amplification. All the RNA samples obtained from direct tissue materials were subjected to 35 cycles amplification of 124 and 112 bp of HA_1 and HA_2 regions, corresponding to HPAI and LPAI, respectively. Amplification and accumulation of copies of target sequence as determined by the gene specific primers was monitored in real time at 520 nm using the ds-DNA binding property of fluorescent dye (emission maximum 497 nm, excitement maximum 520 nm) and Ct (threshold cycle) values were calculated. Ct value was inversely proportional to the initial target sequence concentration in 2 µL of RNA preparations used as template. Payungporn *et al.* (2006) also noticed similar results.

The H5 and H7 specific probe sets each have a detection limit of 100 fg of target RNA or approximately 10^3 to 10^4 gene copies (Spackman *et al.*, 2002). In present study TaqMan quantitative assay was conducted by using 2 sets of primers and dual-labeled fluorescent probes that specifically target 2 different regions of the HA gene of H5N1 virus. Our data prove that the assay is specific for H5 subtype and capable of detecting H5 RNA samples obtained from experimentally infected birds. Ng *et al.* (2005) detected and quantified H5 RNA in clinical samples obtained from patients during different outbreaks. Similar results were also obtained by Spackman *et al.* (2002).

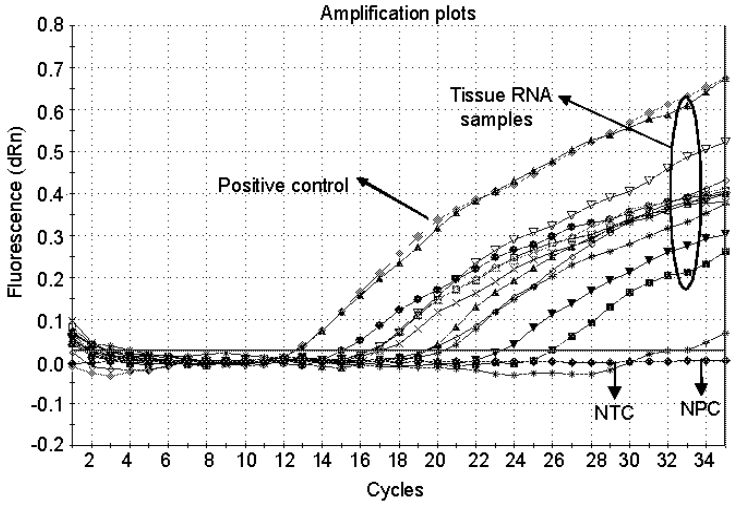

Fig. 2: A typical amplification curve of the HA Gene probe-2 Amplicons, (NTC = No Template control) (NPC = No Probe Control)

The results of tissue tropism obtained by Real-time PCR were same as those of virus isolation in 9-11 days old embryonated chicken eggs and RT-PCR studies. Thus, it can be conclude that Real-time PCR is a trustworthy alternative to virus isolation in embryonated chicken eggs and RT-PCR. This further strengthens the claim of Spackman *et al.* (2002), who concluded in a study conducted by them, that Real-time PCR result is a reliable alternative to virus isolation in embryonated chicken eggs.

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