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Identification and Subtyping of Avian Influenza Viruses by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Agarose Gel Electrophoresis

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Abstract: Avian Influenza (AI) is caused by type A influenza virus belonging to the family *orthomyxoviridae*, which is classified into 16 HA and 9 NA subtypes based on two surface glycoprotein's haemagglutinin (HA) and neuraminidase (NA). In the present study we did identification and HA-subtyping of avian influenza virus by reverse transcription-PCR (RT-PCR) during the first outbreaks of AI in India during 2006. The avian influenza virus is identified by RT-PCR using a set of primers specific to the nucleoprotein (NP) gene of avian influenza virus. The HA-subtypes of avian influenza virus were determined by running with HA subtype specific primers for H5, H7 and H9 RT-PCR reactions, each using a set of primers specific to one HA-subtype. A total of 10,236 tissue / cloacal swab samples, received at the HSADL from various parts of the country, were processed for isolation of AI virus in embryonated chicken eggs. Out of these, 9 samples originating from poultry in Maharashtra (Navapur and Jalgaon) and Gujarat (Surat) states of India were found positive for H5 virus by RT-PCR. All samples received from outbreaks areas were tested by using all tree subtype specific primers(H5, H7 and H9) only H5 RT-PCR reactions was give the product of expected size, and thus the HA-subtype of the virus is determined. One sample gave the positive result with H9 subtype specific primers. The RT-PCR procedure is rapid and sensitive, and could be used for the identification and HA-subtyping of avian influenza virus in organ homogenates.

Key words: Avian influenza, H5N1, RT-PCR, India

INTRODUCTION

Influenza viruses are of 3 types A, B and C. The typing is based on antigenic differences on the nuclear and matrix proteins of the virus. Most pandemics of influenza are associated with type A. Type A is subtyped further on the basis of antigenic differences on the external glycoproteins, the Hemagglutinin (H) and the Neuraminidase (N) proteins. Molecular diagnosis of influenza virus is a challenge as the influenza viruses show extreme genetic variability. However, a number of promising molecular based techniques have been developed several in house Reverse-Transcription Polymerase Chain Reaction (RT-PCR) assay, which use nested primers to detect and subtyping of influenza viruses. These demonstrated greater sensitivity as compared to other rapid test and cell culture techniques (Taubenberger and Layne, 2001). The development of PCR technology has enabled rapid and sensitive influenza viral diagnostic test (Ellis and Zambon, 2002). RT-PCR has also been used for subtyping of influenza virus (Stockton *et al*., 1998; Wright *et al*., 1995; Poddar, 2002) by subtype specific primers. However Lee *et al*. (2001) were first to design subtype-specific primers that where able to differentiate all the known subtypes (H1-H16) of Influenza virus. With the development of molecular assays, especially different types of PCR, diagnosis and subtyping of AIV has become easy and time saving.

The RT-PCR demonstrating sensitivity for influenza was 10^3 and 10^6 greater than cell culture and ELISA, respectively. Presence of influenza virus can be confirmed by the use of RT-PCR using nucleoprotein-specific conserved primers (Altmuller *et al*., 1991). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5 or H7 specific primers (Garcia *et al*., 1998; Senne *et al*., 1996; Wood *et al*., 1995). In this study we used different subtype specific primers for identification and subtyping of avian influenza viruses isolated in India during 2006 outbreaks.

MATERIALS AND METHODS

A total number of 10,236 tissue samples, out of these 2100 samples were from migratory birds, received from various parts of the country were analyzed.

Processing of tissues: One hundred milligram of tissue was ground in 1 mL with 1X PBS in a sterile mortar and pestle, making a 10% suspension. The suspension was transferred to an eppendorf tube and centrifuged at 400 x g for 10 min to remove extraneous materials. The

supernatant was removed and after the addition of 1/10 volume of 10× antibiotic mixture, incubate at 37°C for 1 h. The inoculum was filtered with 0.45 µM filter and inoculated into 9-11 day old embryonated eggs *via* allantoic and amniotic routes. An aliquot was stored at -70°C for use if the need arise.

Inoculation of embryos: Examine the 9-11 day old eggs with an egg Candler, place the eggs with blunt end up into egg trays and label each egg with a specific identification number (3 eggs per specimen). Wipe the tops of the eggs with 70% ethanol and punch a small hole in the shell over the air sac. Three eggs per specimen are usually inoculated. Aspirate 1 mL of processed clinical specimen into a tuberculin syringe with a 22 gauge, 1½ inch needle. Holding the egg up to the Candler, locate the embryo and insert the needle into the hole of the egg. Using a short stabbing motion, pierce the amniotic membrane and inoculate 100 µL into the amniotic cavity. Withdraw the needle about ½ inches and inoculate 100 µL of the specimen into the allantoic cavity, remove the needle. Inoculate the 2 other eggs in the same manner with the same syringe and needle. Discard syringe into a proper safety container. Seal the holes punched in the eggs with a drop of glue. Incubate the eggs at 33-34°C for 2-3 days. Note down embryos which are dead in every 24 h by candling. The embryos that died within 24 h after inoculation were discarded. After 72 h incubation period the embryos were removed from the incubator and chilled at 4°C.

Harvesting of inoculated chicken eggs: Eggs were chilled at 4°C overnight or for 4 h before harvesting. Label one plastic tube (15 mL) for each egg with the specimen number. Clean off the top of each egg with 70% ethanol. With sterile forceps, break the shell over the air sac remove the cell membrane and push aside the allantoic membrane with the forceps. Using a 10 mL pipette, aspirate the allantoic fluid in a labeled plastic tube. Then using a syringe and needle, pierce the amniotic sac and remove as much amniotic fluid as possible. Place harvest in a separate tube, but because of the low volume of amniotic fluid obtained from each egg, it is usually necessary to combine the amniotic fluid from the three eggs inoculated per specimen. Centrifuge harvested fluids at 3000 rpm/ 5 min to remove blood and cells. The clear supernatant was aseptically transferred into sterile and labeled vials. These vials were then stored at -70°C until further use.

QIAamp Viral RNA Mini Kit (Qiagen, Germany) was used to extract viral RNA from allantoic fluids. 560 µL of the prepared buffer AVL containing carrier RNA was pipetted into a 1.5 mL microfuge tube. 140 µL of allantoic fluid was then added to the buffer AVL/carrier RNA in the microfuge tube. To mix properly pulse vortexing was done for 15 sec. Tubes were then incubated at room temperature 22-25°C for 10 min. Pulse centrifugation was done to collect drops from inside lid. To the sample 560 µl of pure ethanol was added, and mixed by pulse vortexing for 15 sec. After mixing, the eppendorf tubes were briefly centrifuged to collect drops from inside the lid. 630 µL of the solution from previous step was applied to the QIAamp spin column with collection tube. The column was placed in the centrifuge and spun at 8000 rpm for 1 min. This step was repeated for one more time. The flow through was discarded and 500 µL of buffer AW1 was added to the column. Centrifugation was done at 8000 rpm for 1 min. The QIAamp column was then transferred to a fresh collection tube and the tube containing the filtrate was discarded. Five hundred micro liter of buffer AW2 was added to the column and centrifuged at 14,000 rpm for 3 min. The QIAamp column was then carefully transferred to a fresh 1.5 mL eppendorf tube and the collection tube was discarded. Sixty micro liter of buffer AXE pre equilibrated to room temperature was added to the center of the column and incubated at room temperature (22-25°C) for 1 min. Centrifugation was performed at 8000 rpm for 1 min. The flow through collected is viral RNA and was stored at -70°C until further use.

One-step reverse transcriptase polymerase chain reaction: HA gene was amplified using QIAGEN® One Step RT-PCR) Kit (Germany) as per the protocol given below. The following reaction mixture of 25 µL (for one reaction) was prepared in a 0.5 mL tube on ice:

Component		Volume	Final concentration
5X Buffer	:	5.0 µL	1X
Q Solution	:	5.0 µL	1X
10mM dNTPs	:	1.0 µL	200 µM
5 U/µl RT Enzyme	:	1.0 µL	5 U
Primer HSAIV 1F	:	1.0 µL	20 pmol
Primer HSAIV 1R	:	1.0 µL	20 pmol
Template (RNA)	:	4.0 µL	Not applicable
Nuclease free water	:	7.0 µL	Not applicable
Total volume	:	25.0 µL	

The same reaction composition as indicated above was used for the other RT-PCR reaction with different sets of primers (WHO, 2002 and Lee *et al*., 2001).

The reactions were carried out in a thermal cycler (EPPEDORF, HYBAID, USA) as per the following program:

Step I	Reverse Transcription	:	50°C for 30 min (1 cycle)
Step II	Initial denaturation	:	95°C for 15 min (1 cycle)
Step III	Denaturation	:	94°C for 30 sec
	Annealing of primer	:	50°C for 30 sec (35 cycles)
	Extension	:	72°C for 40 sec
Step IV	Final extension	:	72°C for 10 min (1 cycle)

Similar, reaction and cycling conditions were used for RT-PCR amplification with both WHO and Lee primers. Two Steps RT-PCR - Synthesis of cDNA (Fermentas, USA) RevertAid™H Minus First Strand cDNA Synthesis

kit was used for cDNA synthesis. The following reaction mixture of 20 µl (for one reaction) was prepared in a tube on ice:

Template RNA	:	4.0 µL
Primer oligo(dt) (0.5 µg/µL)	:	1.0 µL
DEPC-treated water	:	7.0 µL

Above components were mixed gently by tapping and spun briefly for 3-5 sec in a centrifuge. The mixture was incubated at 70°C for 5 min and immediately chilled on ice for 5 min and centrifuged briefly. Place the tube on ice and added the following components,

5X reaction buffer	:	4.0 µL 10 mM dNTPs
	:	2.0 µL Ribolock™
Ribonuclease inhibitor (20 U/µL)	:	1.0 µL

Mixed gently, centrifuged briefly and incubated at 37°C for 5 min,

Add RevertAid™ H Minus M-Mulv RT (200 u/µL)	:	1.0 µL
Total volume	:	20.0 µL

Incubated the mixture at 42°C for 60 min. stop the reaction by heating at 70°C for 10 min. Then the reaction was chilled on ice for 5 min and stored in -40°C for further use.

Polymerase chain reaction (PCR):
HA gene was amplified using TaqPCRx DNA polymerase, Recombinant (Invitrogen, USA) as per the protocol given below:
Following components were added to a 0.5 mL microcentrifuge tube:

Component	Volume	final concentration
10 µ buffer	: 2.5 µL	1X
50 mM $MgCl_2$	: 2.0 µL	4mM
10 mM dNTPs	: 0.5 µL	0.2 mM each
Taq DNA polymerase Enzyme (5 U/µl)	: 0.25 µL	1.25 units
Primer HSAIV 1F	: 1.0 µL	20 pmol
Primer HSAIV 1R	: 1.0 µL	20 pmol
Template (cDNA)	: 1.0 µL	not applicable
Nuclease free water	: 16.3 µL	not applicable
Total volume	: 25.0 µL	

The same reaction composition as indicated above was used for the other PCR reaction with different sets of primers (WHO, 2002 and Lee *et al*., 2001, Table 1).
The reactions were carried out in a thermal cycler (EPPENDORF, HYBAID, USA) as per the following program:

Step I	Initial denaturation	:	94°C for 5 min (1 cycle)
Step II	Denaturation	:	94°C for 30 sec
	Annealing	:	50°C for 30 sec (35 cycles)
	Extension	:	72°C for 30 sec
Step III	Final extension	:	72°C for 10 min (1 cycle)

Agarose Gel Electrophoresis: The HA gene RT-PCR products were subjected to agarose gel electrophoresis using submarine electrophoresis system (Thermo Orion). Agarose gel (1.5% w/v) was prepared in 1× TAE was melted in microwave oven and ethidium bromide was added to a final concentration of 0.5 µg/mL. It was poured in a gel casting tray with comb and then allowed to solidify at 10°C for 20 min. Ten micro liter of each PCR product was mixed with 2 µL of 6x loading dye and loaded in to the well. Along with the products a 100 bp DNA molecular size marker (Fermentas, USA) was run. Electrophoresis was carried out at 10 Volts/cm and 500 Amp, for 1 hr. After the run, the gel was observed under long range UV light.

RESULTS AND DISCUSSION

The RT-PCR was conducted for all the samples which are received from outbreak areas and also samples which gave HA and HI activity with reference serum. RNA was extracted from the amnioallantoic fluids of avian influenza virus inoculated chicken embryos. A total of nine samples, were tested positive RT-PCR with H5 subtype specific primers. Positive bands of 219 bp (Fig. 2) and 545 bp (Fig. 1) with WHO (2002) and Lee primers (obtained from USA), respectively were observed in the agarose gel. Samples gave positive results by both the one-step RT-PCR and two-step PCR methods (QIAGEN® one step RT-PCR kit, Germany and Ivitrogen PCR kit USA). No amplification with H9 (HSAIVH9F and HSAIVH9R) or H7 (HSAIVH7F and HSAIVH7R) subtypes specific primers was noticed in any of the samples. The RT-PCR was also conducted for one sample (5844/05) which gave positive reaction with H9 specific reference serum. With H9 AIV subtype specific primers (HSAIVH9F

Table 1: Sequences of oligonucleotides used in the study

Name	Gene	Sequence (5'-3')	Reference
H5-1F	HA	GCCATTCCACAACATACACCC	WHO, 2002
H5-3R		CTCCCCTGCTCATTGCTATG	
H5-F	HA	ACACATGCYCARGACATACT	Lee *et al*., 2001
H5-R		CTYTGRTTYAGTGTTGATGT	
HSAIVH9F	HA	TCAAGACGCC CAATACACAAATAA	Lee *et al*., 2001
HSAIVH9R		TAGTCCTGGCCAACCTCCTTCT	

Extraction of RNA from Allantoic Fluid:

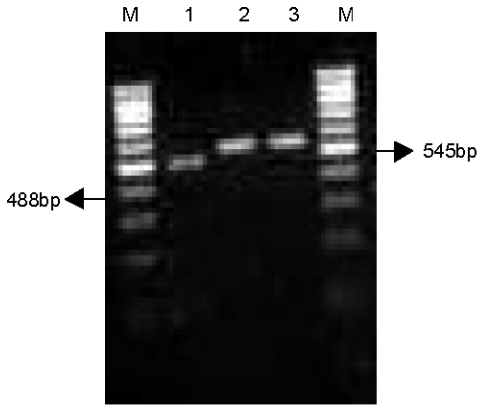

Fig. 1: Plasmid PCR amplicons for one H9N1 and 2 H5N1 virus isolates M: 100 Base pair ladder (Fermentas); Lane 1: 5844/05 (H9N1) isolate (488 bp); Lane 2: 7966/06 (H5N1) isolate (545 bp); Lane 3: 7972/06 (H5N1) isolate (545 bp)

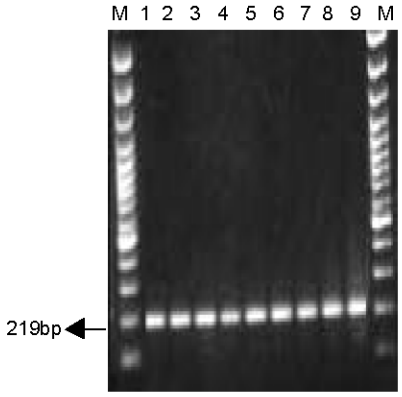

Fig. 2: Subtyping by RT-PCR with WHO (219 bp) primers M: 100 Base pair ladder (Fermentas); Lane 1-9: Positive field samples

and HSAIVH9R) this sample could amplify of 488 bp (Fig. 1) of HA gene both by one-step RT-PCR and two-step PCR (QIAGEN® one step RT-PCR kit, Germany and Invitrogen PCR kit USA), and no amplification with H5 (WHO and Lee) or H7 (HSAIVH7F and HSAIVH7R) subtype specific primers. In each test one positive (RNA extracted from reference antigen) and one negative (RNA extracted from water) control were included.

The use of RT-PCR for the detection of influenza viruses is not new. RT-PCR amplification of influenza virus RNA as a diagnostic assay was first reported in 1991 (Zhang and Evans, 1991). Since then several strategies of RT-PCR have been used to detect influenza A viruses (Cherian *et al*., 1994; Atmar *et al*., 1996). RT-PCR also finds its use in effective subtyping of influenza A viruses using subtype-specific primers (Wright *et al*., 1995; Stockton *et al*., 1998). PCR serves as a fast and effective alternative to virus isolation for the detection of influenza A viruses (Yuen *et al*., 1998) and for subtyping of the influenza A viruses (Lee *et al*., 2001). Another advantage of subtyping of the virus by PCR is that sequence analysis of the PCR product, followed by sequence comparison and phylogenetic analysis, could provide important information on the origin of the avian influenza virus (Lee *et al*., 2001).

Many a time only one test may not reliable and it necessary to be conformed by highly sensitive test like PCR. A successful PCR amplification relies on a good primer design. Lee *et al*. (2001) designed subtype-specific primers, based on conserved sequences in a single HA subtype, using the sequence information that the derived amino acid sequences of HA gene varies among different subtypes from 20-74% but variation within subtypes was only 0-9%. Two primer sets (488 bp for H9 and 545 bp for H5) reported by Lee *et al*. (2001) and one set from WHO (219 bp for H5) all targeting HA gene of AIV were used for subtyping in this study. A total number of 32 samples were tested by RT-PCR, including 24 samples which gave positive in HI. All 24 samples gave positive in RT-PCR, but 4 samples gave positive in RT-PCR but they are negative in HI. Thus PCR showed 86% correlation as compare with the virus isolation in chicken embryos. This because of PCR is sensitive compare to HI and also time gap between sample collections to processing in lab. In this time the chances of virus death is more, so there is no live virus to grow in chicken embryos, but PCR is able to detect viral genome. The 16 F primer, for HA gene reported earlier (Hoffmann *et al*., 2001) was used for synthesis of cDNA template.

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