Development and Optimization of Multiplex PCR for the Identification of Different Strains of Avian Influenza Viruses in Pakistan

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ABSTRACT

The present study was conducted to develop and optimize a multiplex reverse transcriptase-polymerase chain reaction (mRT-PCR) assay for the rapid detection of type A Avian influenza (AI) viruses. The assay simultaneously differentiates avian H_5, H_7 and H_9 hemagglutinin subtypes. Specific primers were used in this assay for H_5, H_7 and H_9 heamagglutinin subtypes. The mRT-PCR DNA products were visualized by gel electrophoresis. The fragments observed were of 362bp, 699bp and 287bp for H_5, H_7 and H_9 hemagglutinin subtypes, respectively. The multiplex PCR is rapid and cost effective compared to other available assays for AI in Pakistan.

Key words: *Avian Influenza, Multiplex PCR, Virus, Pakistan*.

INTRODUCTION

Avian Influenza (AI), is a viral infection of poultry that has caused extensive losses to the commercial poultry production (Shane, 1995). The poultry industry in Pakistan is being adversely affected by many diseases, of which AI is of significant importance (Muneer et al., 2001). The high density and rearing of different age birds, nutritional deficiencies, contaminated feeds, existence of poultry farms in close proximity, free movement of stray animals, wild and domestic birds, fomites, sites which could be carrier of many microbes are believed to be the possible risk factors involved in disseminating AI in Pakistan. Influenza A viruses belong to the family of *Orthomxyoviridae*. The AI virus is an eight segmented RNA virus which codes for ten proteins including two surface glycoproteins, haemagglutinin (H) and neuraminidase (N). These two highly variable glycolproteins are being used for the classification of AI viruses into subtypes. The pathogenicity and the immunogenicity of virus is directly associated with the type of H and N antigens.

Among HA proteins, H_5 and H_7 are highly virulent in poultry. In Pakistan, H_5, H_7 and H_9 have been diagnosed in various regions during the last 2 decades. Human population has also been affected by the AI due to its zoonotic potential (Muneer et al., 2001; Naeem et al., 1999). For the detection of avian influenza, haemagglutination (HA) and haemagglutination inhibition (HAI) tests have been used for a long time. However, studies have shown that AI shows variability, diversity and a high rate of mutation, which makes diagnosis difficult (Shane, 1995; Xie et al., 2006). Several factors have been elucidated that affects hemagglutination activity of AI viruses (Hussain et al., 2008). Owing to this, the reverse transcriptase PCR (RT-PCR) assays are considered to be a helpful tool. The RT-PCR technique is also used prior to sequencing of the haemagglutinin cleavage sites of H_5 and H_7 AI viruses (Banks et al., 1998). In the present study, we have developed a Multiplex PCR assay which can simultaneously detect and differentiate between three very important subtypes of avian influenza.

MATERIALS AND METHODS

Sample Processing and Extraction

Source of Sample

Stock viruses for H_5, H_7 and H_9 were obtained from the Department of Microbiology and Quality Operation Laboratory, University of Veterinary and Animal Sciences, Lahore.

RNA extraction

Viral RNA from allantoic-amniotic fluid was extracted using Trizole based method (Chomczynski and Sacci, 1987). Briefly, 800µL of Trizole solution was added to 400µL of virus suspension sample in a 1.5 mL tube. The solution was gently mixed and incubated for 5 minutes at 25°C. Then, 200 µL of chloroform was added for phase separation and was centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube and 500µL of isopropanol was added. The tube was incubated for 30 minutes at -10°C. Later, it was centrifuged at 12,000 g for 10 minutes. Thereafter, the resulting pellet was washed with 1.0mL of 75 % ethanol, dried and again washed with 1.0 mL of 100% ethanol. The pellet was dried and re-suspended in 20µL of Diethylpyrocarbonate treated water (DEPC- H_2O). Extracted RNA was stored at -40°C until further processing.

Primer Design

Primers were designed for H_5, H_7 and H_9 subtypes of influenza virus by using the sequence for the subtypes in Pakistan and the neighboring countries (India, Iran and China) from the Influenza Virus Database (http://influenza.psych.ac.cn/). Sequences were aligned using the Clustal W (www.clustal.org) program, and were designed using Geneious Pro (www.geneious.com). The annealing temperature for the primers was determined by in-silico PCR using Fast PCR Professional Software (PrimerDigital, Co., Helsinki, Finland).

Multiplex PCR

The PCR amplification of cDNA was carried out in a total volume of 25µL using 2X PCR master mix (Fermentas, Thermo-Scientific, Pittsburg, USA, Catalogue No. #0171). Reaction mixtures was made by adding 3.0µL cDNA, 1.5µL of each forward and reverse primer, 12µL of PCR master mix (Taq DNA-Polymerase 0.05 units/µL, 4.0mM $MgCl_2$ and 0.4mM dNTPs) and DEPC-H_2O to the final volume of reaction. The mixture was subjected to the following program using a thermocycler; primary denaturation at 94°C for 7 minutes (1 cycle), 35 cycles of denaturation at 94°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10minute (1 cycle). The annealing temperature was specific for each subtype of virus and was adjusted according to the primer used which was 50°C for H_5, 53.5°for H_7 and 51°C for H_9 (Beladi et al., 2005). The primers for H_5, H_7 and H_9 were added to the single reaction mixture and were annealed at different temperatures using a Gradient Thermocycler (ESCO, Life Sciences, USA). Multiplex RT-PCR was carried out in 50µL total reaction volume for HA gene of H_5, H_7 and H_9 subtypes. Thermocycler was programmed as; denaturation at 94°C for 7 minutes, 35 cycles of denaturation at 94°C for 1 minute and extension at 72°C for 1 minute. A temperature gradient was made from 50°C to 60°C for the annealing temperature, then final extension at 72°C for 10 minutes (Esmaeil et al., 2007).

Agarose Gel Electrophoresis

The PCR products were electrophoresed on a 1.5% agarose gel in Tris–acetate–EDTA (TAE) buffer (40mM of Tris and 2.0mM of EDTA, with a pH value of 8.0), for 45 minutes at 100 volts. The samples were loaded in the wells after mixing with loading dye (6X Loading Dye). About 1.0µL of loading dye (10mM Tris-HCl, 0.03% Bromophenol Blue, 0.03% Xylene Cyanol, 60% Glycerol and 60mM EDTA) was added to 5.0µL of PCR product. Thereafter, the gel was stained in staining solution containing Ethidium Bromide (1.0µg/mL) for 15 minutes. (Dergham et al., 2009).

Table 1 List of primer sequences used

Strain	Primer	Sequence	Primer position	Product size (bp)	Annealing temp. (°C)
H_5	Forward	CAATGACCTCTGTTACCCAG	334 → 353	362 bp	50
	Reverse	GGTTTAGTGTTGAGTGCCCAA	675 ← 695		
H_7	Forward	GGAGATCAGGATCTTCATTCTATGC	464 → 488	699 bp	53.5
	Reverse	TGTAATCTGCAGCAGTTCCCTC	1141 ← 1162		
H_9	Forward	ATCTAATCGCTCCATGGTATGG	773 → 794	287 bp	51
	Reverse	TGACCAACCTCCCTCTATGA	1040 ← 1059		

RESULTS AND DISCUSSION

Presently, we developed a Multiplex RT-PCR to detect group A avian influenza viruses and simultaneously differentiate three hemagglutinin subtypes H_5, H_7, and H_9 in a single reaction through 35 cycles of PCR. The mRT-PCR products were 362 bp for H5, 699 bp for H7and 287 bp for H9. These products were visualized by electrophoresis (Figure 1). The mRT-PCR was found to be a specific assay for type A avian influenza subtypes H_5, H_7, and H_9.

The rapid and accurate diagnosis of AI virus is important factor for prevention of disease and protection against economic losses in poultry industry (Thontiravong et al., 2007). Over the years, virus isolation and various seroassays have been used to detect these viruses, but both these methods have some drawbacks. Virus isolation is a time consuming, while serological tests, like antigen detections, are less sensitive and specific. In the recent years, RT-PCR has been used as a tool for detection of AI viruses but it detects nucleic acid of only one specific virus subtype (Payungporn et al., 2004).

The mRT-PCR developed here was able to detect type A influenza virus hemagglutinin subtypes from H_5, H_7 and H_9 in one single reaction. Use of this assay will help to reduce the economic losses in poultry associated with an AI virus outbreak. This mRT-PCR is a quick, efficient and cost effective method, and it may be useful in diagnosis, screening and surveillance of poultry. Primers were taken from the already published data (Lee et al., 2001), but these sets of primer failed to amplify the target site as the genetic makeup of virus varies from region to

region. As AI viruses are RNA viruses, there is high rate of mutation in the virus because of no proof reading of the RNA replication (Domengo et al., 1996). For this reason, another set of primers was designed for the subtypes by retrieving Influenza virus genome date available for Pakistan and neighboring countries (India, Iran and China) from the Influenza Virus Database. These primers were tested individually with the specific subtype and there annealing temperature was determined using in-silico PCR. The temperatures determined were between 50 to 55°C.

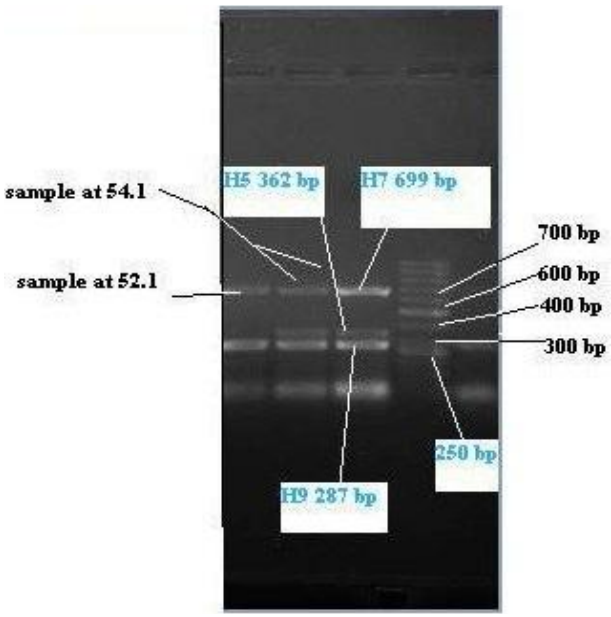

Figure 1 Gel electrophoresis of Virus Stocks of H_5, H_7 and H_9

The amplicon size for H_5, H_7 and H_9 were 362 bp, 699 bp and 287 bp. A similar study was conducted by Xie et al. (2006) using different set of primers. The mRT-PCR products were 860bp for H_5, 634bp for H_7 and 488bp for H_9. In another study (Chaharaein et al., 2009) using subtypes H_5, H_7 and H_9 of AI virus, the PCR product size was found to be 499bp, 409bp and 221bp for H_5, H_7 and H_9 respectively. The primers used were designed against the conserved and specific regions of HA gene of the virus, and annealing was at 60°C. The primers used in both the researches were different from the present study. The primer positioning, size, sequence and annealing temperature were also different due to which bands size was different in the present study. The amplification was seen in all the studies in a single reaction. Boonsuk et al. (2008) developed mRT-PCR using subtypes H_1, H_3 and H_5 of AI virus. The amplicon size was 362 bp, 112 bp and 188 bp for H_1, H_3 and H_5. The primers were amplified at a temperature of 58°C. The difference was due to the different subtypes used and the primer positioning.

In conclusion, this optimized Multiplex RT-PCR assay may be used for rapid detection of AI viruses in biological samples in Pakistan.

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