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Multiplex RT-PCR for the rapid detection of matrix protein and H_5 genes of highly pathogenic avian influenza virus

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Abstract

Avian influenza (AI) is an economic, zoonotically important disease in worldwide. It is an infectious disease of birds caused by influenza virus which also affect human beings. Outbreaks of H_5N_1 highly pathogenic avian influenza (HPAI) virus caused great economic losses to the poultry industry. Rapid typing and subtyping of HPAI viruses are desirable for taking prompt control measure to prevent the spread of the disease. Here, we develop multiplex RT-PCR technique for the rapid, simultaneous detection of Matrix protein (MP) and H_5 genes of HPAI from RNA extracted from tracheal swabs using Finnzymes Phusion™ Flash High-Fidelity PCR Master Mix (2x) based on modified Phusion Hot Start DNA Polymerase and QIAgen RT-PCR Kit. Reverse transcription and PCR were carried out with a mixture of primers specific for influenza viruses of type A subtype H_5 in a single reaction system under identical conditions. Both the PCR detection systems amplified matrix protein and H_5 genes specific 245bp and 545bp bands, respectively. However, QIAgen RT-PCR Kit required 3 hours to complete the PCR cycle, whereas Phusion Flash method required 1 hour and 15 minutes only. On the other hand, multiplex PCR was finished in one tube for both MP and H_5 genes. Therefore, it is time and cost effective. Phusion Flash method required relatively less time and cost. After validation with few more field samples, it can be used for the rapid, simultaneous detection of MP and H_5 genes of HPAI in the field condition.

Keywords: Avian influenza, pathogenic, matrix protein and H_5 genes.

Introduction

Highly pathogenic avian influenza (HPAI) is a devastating disease in poultry; it is associated with high mortality and disrupts poultry production and trade (Capua and Alexander, 2004; OIE, 2004). HPAI viruses may be transmitted from birds to humans (Alexander and Brown 2000; Koopmans *et al*., 2004),

and they are a potential source of future human influenza pandemics (Capua and Alexander, 2004). HPAI outbreaks were relatively rare until 1990 but occurred in many countries in the last decade (Capua and Alexander, 2004). In Asia, since epidemic of the HPAI H_5N_1 in Hong Kong in 1997, HPAI viruses have been isolated continuously through routine surveillance in Hong Kong (Sims *et al*., 2003; Ellis *et al*., 2004), South Korea (Tumpey *et al.*, 2002), and China (Liu *et al*., 2003; Chen *et al*., 2004 and Li *et al*., 2004). Now a day, avian influenza is considered as the number one threat for rising poultry industry in Bangladesh. According to Office International des Epizooties (OIE), Bangladesh has encountered the first outbreak of HPAI (H_5N_1) on 5th February, 2007 at Sharishabari in Jamalpur District. According to the Ministry of Health, Bangladesh has confirmed its first case of human infection with H_5N_1 avian influenza on 28th May, 2008. 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 and type A viruses are further sub-typed on the basis of antigenic differences of the surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) proteins (Easterday *et al*., 1997). So far, sixteen HA (H_1 through H_{16}) and nine NA (N_1 through N_9) subtypes have been identified (Rohm *et al*., 1996; Steinhauer and Skehel, 2002). Among the 16 HA subtypes, only H_5 and H_7 are highly virulent in poultry although all do not cause highly pathogenic avian influenza (HPAI). The rest of the viruses cause a much milder, primarily respiratory disease known as low pathogenic avian influenza (LPAI) (Alexander, 2000). Since the outbreaks of the H_5N_1 HPAI occur in Hong Kong in 1997, growing evidence has been showing that H_5N_1 viruses might directly cross the species barrier to infect humans and cause high mortality in both species (Suarez *et al*., 1998; Subbarao *et al*., 1998 and Zhou *et al*., 1999). In Asia, at the beginning of 2004, the outbreaks of the H_5N_1 HPAI caused not only tremendous economic losses to the poultry industry but also human infections and high mortality in Vietnam and Thailand (CDC. Morb., 2004; Parry, 2004).

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 labor intensive in spite of its high sensitivity and specificity. The enzyme-linked immunosorbant assay (ELISA) has been applied for the rapid detection of influenza virus (Reina *et al*., 1996) although it has comparatively poor sensitivity. There is a requirement for the development of rapid and sensitive diagnostic techniques for the verification of clinical diagnosis of influenza and improvement of the quality of surveillance systems. Molecular techniques have enabled major advances in the rapidity and sensitivity of the laboratory diagnosis of viral infections (Gavin *et al*., 2003). The RT-PCR has a higher sensitivity (93%) for influenza A virus than cell culture (80%) and ELISA (62%) (Steininger *et al*., 2002). PCR-based methods for identification of subtypes of influenza A viruses (Wright *et al*., 1995), distinction between influenza type A, B and C viruses have been developed (Stockton *et al*., 1998). By the inclusion of multiple sets of primers, the multiplex RT-PCR assay offers the possibility for the molecular detection of the presence of more than one gene or genome segment in a single pathogen or in more than one pathogen in a single reaction system (Stockton *et al*., 1998; Ellis *et al*., 1997 and Fan *et al*., 1998).

Materials and Methods

Glassware and plasticware were dipped in 2% sodium hypochlorite solution for six hours and then transferred to detergent solution (Trix, Rackitt and Benckiser). After overnight soaking into the detergent solution, the glassware were cleaned by brushing and washed thoroughly in running tap water and rinsed four times in distilled water. The cleaned glassware was then dried on a bench at room temperature or in an oven at 60°C. The pipettes were soaked overnight in a sulphuric acid-dichromate cleaning solution (120 g sodium dichromate dissolved in 1000 ml tap water to which 1600 ml concentrated sulphuric acid was added). The graduated pipettes were then washed repeatedly in tap water and rinsed two to three times in distilled water. The pipettes were dried in an oven at 50°C. Before sterilization, the graduated pipettes were plugged with cotton at the neck and placed in a canister. The graduated cylinders were sealed with aluminium foil cover. The petridishes were wrapped with brown paper. This glassware was usually sterilized by dry heat at 160°C for one and half an hour in an oven. However, the bottles with plastic caps or rubber lined aluminum caps were sterilized by autoclaving for 20 mins at 121°C under 15 1bs pressure per sq. inch in an oven. During autoclaving the caps were loosely fitted on the bottles, after autoclaving the glassware were immediately dried in an oven at 60°C and the caps of the bottles were tightened after cooling. The tips were placed in a tip-dispensing box and autoclaved as above. The autoclaved tips were dried in an oven. Sterile glassware and plastic ware were preserved in dust free place. The reagent used in the experiment for the

preparation of 20% suspension from field samples was Dulbecco's Phosphate buffered saline solution. All procedures were conducted under strict aseptic condition. Utmost precautions were taken to avoid cross contamination and bacterial contamination. Hand gloves were used all time, nothing were touched in bare hands to avoid contamination with RNase from the skin. Isolation of RNA, preparation of RT-PCR reaction mix, thermocycling and analysis were performed in three separate rooms to avoid carry-over contamination. Preferably filter tips were used and ethidium bromide (mutagen) was handled accordingly. Appropriate primer sequences for RT-PCR were selected to amplify matrix (M) protein gene (Fouchier *et al.*, 2000) and H_5 hemagglutinin gene (Lee *et al.*, 2001) of AI viruses. The primers targeting the M and NP genes are common for all Type A influenza viruses, but the primers for H genes were designed for specific H_5 subtype. The primers were synthesized commercially in Germany which was a gift from Prof. H. Müller, University of Leipzig, Germany.

Results

One step RT-PCR was optimized and evaluated by the amplification with one primer pair of MP and H_5 genes respectively. Several different primers for the two primer sets were designed, but here, the best primers that presented the ideal amplification results and could be used in any other experiments have been used.

RT-PCR for MP gene (QIAgen RT-PCR kit)

The presence of RNA of Type A influenza virus in the positive control was confirmed through RT-PCR for MP gene fragment. Products of 245 bp were successfully amplified. The results of amplification are presented in Fig.-1. MP protein is type- specific. Therefore, RT-PCR for Type A MP protein can detect all sub-types of Type A influenza viruses. The RT-PCR protocol used in this study for MP genes was originally developed by Fouchier *et al.* (2000). Successful amplification of MP gene fragment from the positive control sample of avian influenza virus indicates that the sample was indeed an avian influenza virus and RNA extraction from the positive control sample was successful. Subsequently, the extracted RNA was used for RT-PCR to detect H_5 gene.

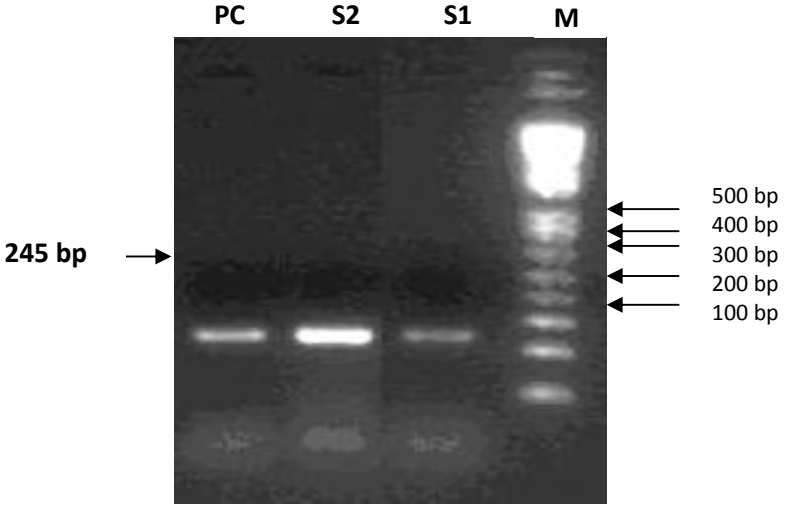

Fig.-1. Amplification of the fragment of matrix protein (MP) gene of Type A influenza virus by RT-PCR using primers designed by Fouchier *et al.* (2000). M=Marker, S1=Sample-1, S2=Sample-2, PC= Positive control.

RT-PCR for H_5 gene (QIAgen RT-PCR kit)

RNA extracted from the H_5 positive control sample was tested in RT-PCR for H_5 gene fragment. A product of expected 545 bp was successfully amplified (Fig.-2.). The RT-PCR used in this study for the amplification of H_5 gene fragment was originally developed by Lee *et al.* (2001). The protocol successfully amplified the product of desired size from the positive control sample indicating that the test can be used for identifying H_5 sub-type of avian influenza viruses.

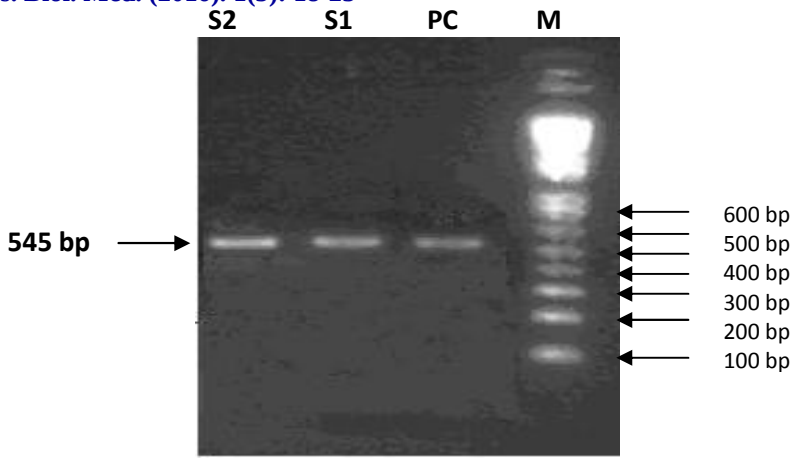

Fig.-2. Amplification of the fragment of H_5 gene by RT-PCR using primers designed by Lee *et al.* (2001). M=Marker, PC= H_5 AI positive control, S1=Sample-1, S2=Sample-2

Application of standardized RT-PCR on experimental specimens (QIAgen RT-PCR kit)

To see whether the standardized RT-PCR procedure could be used for the identification and subtyping of avian influenza viruses, we conducted RT-PCR amplification of a suspected Experimental Specimens collected from BLRI, Savar. After RT-PCR using MP primers of avian influenza virus amplification of 245 bp fragment was observed (Fig.-3). RT-PCR for H_5 gene was successfully amplified with a band of 545 bp fragment from the Experimental Specimens (Fig.-4).

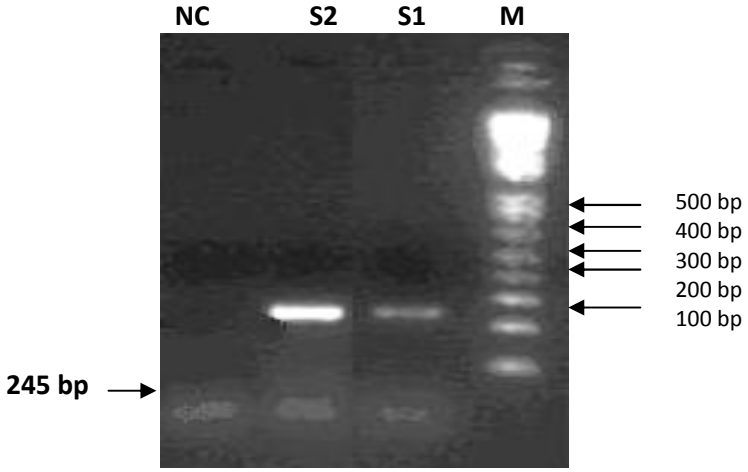

Fig.-3. Amplification of the fragment of MP gene by RT-PCR from Experimental Specimens. M=Marker, NC=Negative control, S1=Sample-1, S2=Sample-2

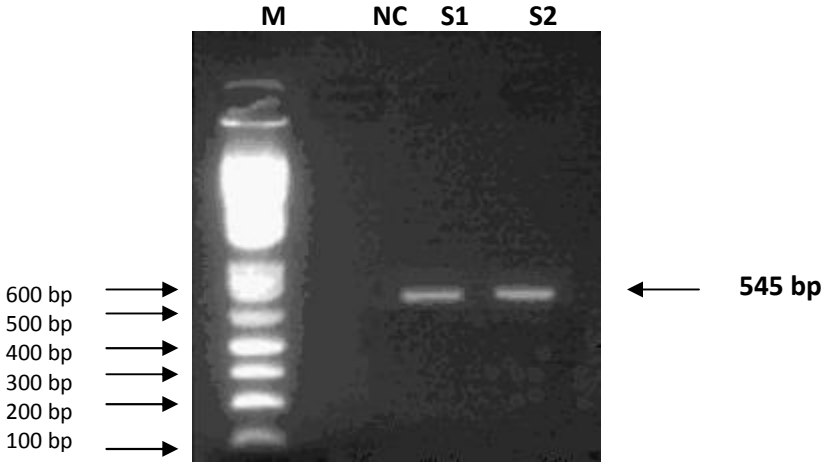

Fig.-4. Amplification of the fragment of H_5 gene by RT-PCR from Experimental Specimens. M=Marker, NC=Negative control, S1=Sample-1, S2=Sample-2

Multiplex RT-PCR for MP and H_5 genes simultaneously using QIAgen one-step RT-PCR enzyme mix

Multiplex RT-PCR for MP and H_5 genes simultaneously, were optimized by modulating the concentration of the reaction buffer, the annealing and the extension temperatures. DNA fragments for the influenza type A and subtype H_5 gene were amplified simultaneously in a single step multiplex format (Fig.-5.) One band of 245 bp for MP gene and another band of 545 bp for H_5 gene were observed for each sample. Total time required for this protocol was 3 hours.

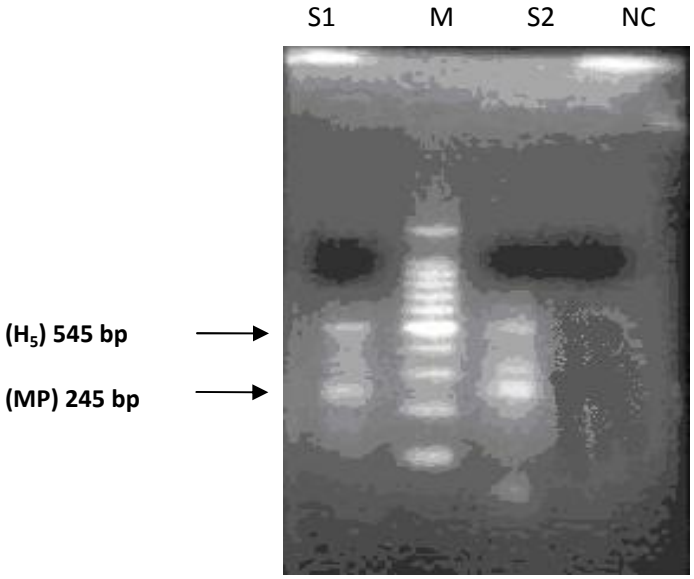

Fig.-5. Multiplex RT-PCR results of Experimental Specimens using QIAgen one-step RT-PCR Enzyme Mix. M=Marker, NC=Negative control, S1=Sample-1, S2=Sample-2

Multiplex RT-PCR for MP and H_5 genes simultaneously using Phusion Flash High-Fidelity PCR Master Mix

The conditions of the multiplex RT-PCR for MP and H_5 genes simultaneously, were optimized by modulating the concentration of the reaction buffer, the annealing and the extension temperatures. The results of the DNA fragments for the influenza type A and subtype H_5 specific gene fragments amplified simultaneously in a single step multiplex format are shown in Fig.-6. An amplified product of expected band of 245 bp for MP gene and another band of 545 bp for H_5 gene were observed for each sample. Total time required for this protocol was 1 hour and 15 minutes. Positive results of the Experimental Specimens with RT-PCR for MP gene and H_5 gene fragment indicate that the flock from which the samples were collected had been suffering from highly pathogenic avian influenza (HPAI) of H_5 subtype.

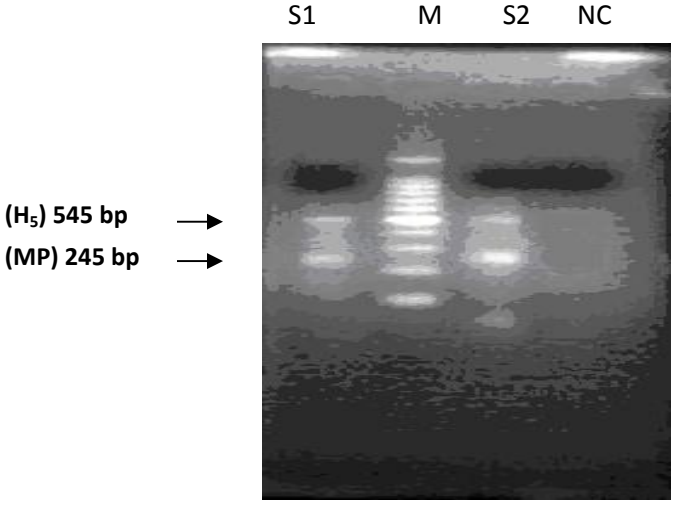

Fig.-6. Multiplex RT-PCR results of Experimental Specimens using Phusion Flash High-Fidelity PCR Master Mix. M=Marker, NC=Negative control, S1=Sample-1, S2=Sample-2

Discussions

Several laboratory methods, including the immunofluorescence, enzyme immunoassay (Slemons and Brugh, 1997; Davison *et al.,* 1998; Selleck *et al.,* 2003, 2003a; Cattoli *et al.,* 2004), RT-PCR and TaqMan PCR (Di-Trani *et al.*, 2006), for the rapid detection of influenza viruses have been reported during the past years. PCR assays have been designed for either Type A only (Pisareva *et al*., 1992; Cherian *et al*., 1994), or for distinguishing between Type A, B or C viruses (Claas *et al*., 1992; Claas *et al*., 1993) or indeed for the diagnosis of human influenza. Subtype specific primers have been designed to detect single subtypes of Type A influenza viruses (Bressoud *et al*., 1990) or to differentiate between subtypes of HAs (Lee *et al.*, 2001; Yamada *et al.*, 1991; Zhang *et al*., 1991) and NAs (Zhang *et al*., 1991). The study aimed at the establishment of a rapid method for the screening or detection of not only the MP genes, the relatively highly conserved gene in all Type A viruses (Lamb and Krug, 1996) but also the H_5 HA in a single reaction.

In the present study, a multiplex RT-PCR has been designed to amplify two genome segments simultaneously in a single step to detect type A and subtype H_5 influenza viruses using the MP and H_5 specific primers. Conventional methods for the subtyping of influenza viruses require the expansion of the viruses in tissue culture or embryonated eggs, followed by subtyping with serological methods (HI and NI test). This demands considerable efforts and might take a week or more to obtain the results. Here, two one step RT-PCR assays using both the QIAGEN one-step RT-PCR Enzyme Mix and the Phusion Flash High-Fidelity PCR Master Mix separately have been designed. It was found that the multiplex assay using QIAGEN one-step RT-PCR Enzyme Mix requires about 5-hours turn-around time from RNA isolation to gel documentation to make it appropriate for the rapid testing of clinical specimens. In the case of uniplex RT-PCR for MP and H_5 genes, it required about ±10 (5+5) hours. Whereas, the multiplex assay using Phusion Flash High-Fidelity PCR Master Mix requires only about 3-hours turn-around time for the same. If required, sequence analysis of RT-PCR products, followed by sequence comparison and phylogenetic analysis, could be conducted so as to provide more valuable information such as the origin of the AI viruses identified. The primers of the MP and H_5 gene, designed for the multiplex RT-PCR assay, were first examined separately to ascertain their usage for amplification together in a multiplex reaction under similar conditions. All primers were designed to ensure that the final reaction products could easily be differentiated on the basis of their size in a 1.5% agarose gel. To amplify the desired products, the multiplex RT-PCR conditions required optimization. (Henegariu *et al*., 1997) suggested that increasing the amount of primers for the weak loci and decreasing the amount for the strong loci should overcome the phenomenon of uneven amplification with some barely visible products.

Conclusions

This method is purposed to detect H_5 influenza viruses of all birds and humans from clinical specimens. The amplification outcome with this multiplex RT-PCR method could be explained only when the negative control is reasonable. The high sensitivity and specificity observed with the use of the single step multiplex reverse transcription polymerase chain reaction using Phusion Flash High-Fidelity PCR Master Mix described here suggest that the method could be of potential value in the rapid detection of MP and H_5 genes of influenza viruses in clinics. The specificity and sensitivity of the multiplex RT-PCR established here should be further validated and evaluated on a larger quantity of clinical specimens and compared with that of the conventional virus isolation method using embryonated chicken eggs. This work is in progress and the preliminary results show the potential application of this system for the rapid detection of MP and H_5 genes of influenza viruses.

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