A modified strategy of multiplex RT-PCR for simultaneous detection of H5, H7, and H9 subtypes of avian influenza virus based on common forward oligo

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Primary Audience: Diagnostic Laboratories, Veterinarians

SUMMARY

Avian influenza virus subtypes H_5, H_7, and H_9 related epidemics are accountable for huge losses to the poultry and avian industry in Pakistan. Well-timed and accurate diagnosis of subtype(s) concomitant with a specific epidemic allows a margin of prophylaxis to farmers through requisite vaccination. The current study was designed to develop and validate a rapid, accurate, and efficient diagnostic technique based on a modified strategy in reverse transcription PCR (RT-PCR) for simultaneous detection of avian influenza virus subtypes H_5, H_7, and H_9. Instead of 3 pairs of primers, a single common forward sense primer and 3 negative sense primers specific for 3 subtypes were used. Genotyping on 2% agarose gel sorted out specific products of desired sizes for individual and mixture samples of three subtypes.

Key words: avian influenza, multiplex, reverse transcription polymerase chain reaction (RT-PCR), subtypes, genotyping

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DESCRIPTION OF PROBLEM

Avian influenza virus comprises a major proportion of respiratory diseases and high mortality resulting in huge economic losses to the poultry industry in Pakistan. Haemagglutinin (**HA**) and Neuraminidase (**NA**) genes are major determinants of sub typing of avian influenza virus [6]. The virus can be classified into 16 subtypes based upon HA gene sequence and 9 subtypes based upon NA gene sequence [7, 17]. H_5N_1, H_7N_3, and H_9N_2 are mainstream subtypes that have been isolated from some of the most serious outbreaks in poultry species throughout the world [4, 1, 2, 15]. Avian influenza subtypes are classified into low pathogenic avian influenza (**LPAI**) and high pathogenic avian influenza (**HPAI**) virus as per severity of clinical signs and mortality rates. H_9N_2 is considered LPAI virus while H_5N_1 and H_7N_3 are

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considered HPAI virus [8]. Along with immense damages to the poultry industry, H_5 and H_7 subtypes are also reported to be involved in zoonosis [14]. During initial phases of epidemics of avian influenza virus, timely and accurate diagnosis of specific subtypes facilitates poultry farmers to adopt stringent prophylactic measures, including vaccination against the epidemic subtype.

Different techniques including ELISA, virus isolation, and virus neutralization, are being explored to diagnose avian influenza virus infections in the flocks, but routine tests are not effective enough to discriminate subtypes of the virus [9]. Use of reverse transcription-PCR (**RT-PCR**) and real-time reverse transcription PCR (**RRT-PCR**) has been found to be very effective in distinguishing 3 subtypes of the avian influenza virus simultaneously [13, 11, 5, 18, 10]. However, frequent variations in sequences of the HA gene belonging to said 3 subtypes pose much difficulty in designing a single pair of primers from the same positions of sequences to amplify 3 subtypes with differentiating product sizes. Previous studies based on these molecular techniques have used one pair of primers specific for each subtype in the case of RT-PCR, and an additional fluorescent-labeled probe for each subtype in the case of RRT-PCR. The greater the number of primers in a reaction, the greater are the chances of primer dimers, mis-priming, and non-specific products, which may compromise the authenticity of results at the diagnostic level [3]. The increased cost of diagnostic testing is also one of the disadvantages of RRT-PCR and previously practiced RT-PCR. Moreover, optimization of priming conditions and a recipe for a large number of primers in a multiplex assay in laboratory conditions are cumbersome tasks.

The present study was designed to validate a modified approach in reverse transcription and multiplex PCR by decreasing the number of primers in reaction to avoid complexity of the process and non-specific products in the results of diagnostic testing, keeping in mind the cost effectiveness of the assay as well. A total of 4 primers instead of 6 (or more) were designed and validated for authentic and efficient detection of subtypes H_5, H_7, and H_9 simultaneously.

MATERIALS AND METHODS

Primer Designing

Multiple HA gene sequences of 3 selected subtypes from different geographical regions (Accession No. JN540074, FN600115, AB753217, KM076704, CY068643, HM208702, FJ839920, EU008592, AY770991, FJ577510, FJ577542, KP416740, FJ750852) were aligned using a population genetics software, "Molecular Evolutionary Genetics Analysis (**MEGA**)." Aligned sequences allowed designing of a single common positive sense primer specific to all 3 subtypes with a few adjustments for variations among sequences with Inosine. Due to the mixed pattern of homology and variations among the sequences, a total of 3 negative sense primers were designed, one specific to one subtype sequence only. Moreover, negative sense primers were designed from different positions of the sequences of 3 subtypes so that amplification products of 661 bp, 140 bp, and 276 bp corresponding to H_5, H_7, and H_9, respectively, could be obtained in distinguished manner. The designed primers are shown in Table 1 and synthesized by Genelink, Hawthorne, NY, USA. The strategy is depicted in Figure 1.

RNA Extraction

Reported samples of avian influenza virus subtypes H_5, H_7, and H_9 collected from epidemics in different geographical regions of

Table 1. Primers and their specifications.

Name	Sequence	Annealing Temperature (°C)	Product
Positive	GTAGAGGGCTATTTGGIGC	54	-
Negative-H5	TGCAAATTCTGCATTGTAAC	54	661
Negative-H7	CTGACTGGGTGCTCTTGTA	54	140
Negative-H9	CGTCTTGTATTTGGTCATCA	54	276

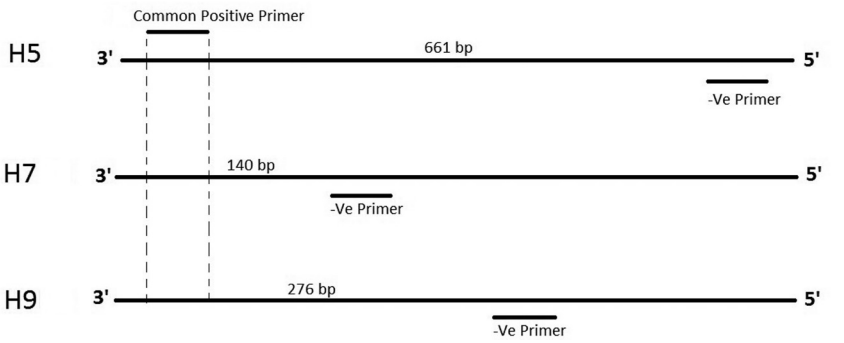

Figure 1. Schematic modified strategy of primers designing and multiplex RT-PCR.

Pakistan during the past several years were provided by Ottoman Pharma Lahore, Pakistan. After heat incubation with 2% Proteinase K (Merck, Darmstadt, Germany) for half an hour, RNA was extracted by Trizol (Merck, Darmstadt, Germany) method followed by purification with ethanol [16].

Reverse Transcription

Samples of extracted RNA were subjected to reverse transcription to develop cDNA, using common positive sense primer. The reaction mixture was 20 uL, constituted of 10 uL RNA, 4 uL 10X reverse transcription buffer, one uL (200 U) reverse transcriptase enzyme (Thermo scientific, Waltham, USA), 2 uL 10 mM dNTPs (Merck, Darmstadt, Germany), 1 uL 10 pM forward primer, and 2 uL double-distilled water. The reaction mixture was incubated in a thermal cycler at 42°C for 60 min followed by heat shock at 70°C for 10 min.

Multiplex PCR

Multiplex PCR for cDNA of samples along with individual and mixed positive controls of 3 subtypes was performed using common positive sense primer and 3 negative sense primers. Quantity of negative sense primers optimized for the recipe were 1, 0.3, and 0.15 uL H_5, H_7, and H_9 primers (10 pM each), respectively. Other ingredients of the recipe included one uL cDNA, 3 uL 10X reaction buffer, 3 uL 10 mM dNTPs, 3 U Taq DNA Polymerase (Merck, Darmstadt, Germany), and 13.55 uL PCR grade water. To avoid non-specific binding across the templates, stringent thermal cycling conditions were programmed with 30 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, and extension at 72°C for 45 s.

Genotyping

PCR products were subjected to electrophoresis on 2% agarose gel along with 50 bp gene ruler to detect size-based confirmation of amplicons of respective subtypes.

RESULTS AND DISCUSSION

Upon genotyping, positive controls produced specific hypothesized results, i.e., 661 bp for subtype H_5, 140 bp for subtype H_7, and 276 bp for subtype H_9 with specific primers. Afterwards, a total number of 12, 19, and 25 field samples were tested for H_5, H_7, and H_9 subtypes, respectively. Results of some of these samples are shown in Figure 2. Newcastle disease virus, which contains the Hemagglutinin gene, was tested as a negative control for sensitivity of assay and produced no binding with the oligos. All of these samples were provided by Ottoman Pharma. Similar results were reproduced using multiplex of primers for 3 subtypes separately. The multiplex reactions comprised of a mixture of 3 subtypes gave 3 specific bands of the respective subtypes. The methodology involving single step RT-PCR for the detection of avian influenza virus subtypes has been in frequent use. Detection of avian iInfluenza virus subtypes H_5, H_7, and H_9 by RT-PCR has been reported in studies [13, 12, 11], respectively. These studies validated the detection of avian influenza virus

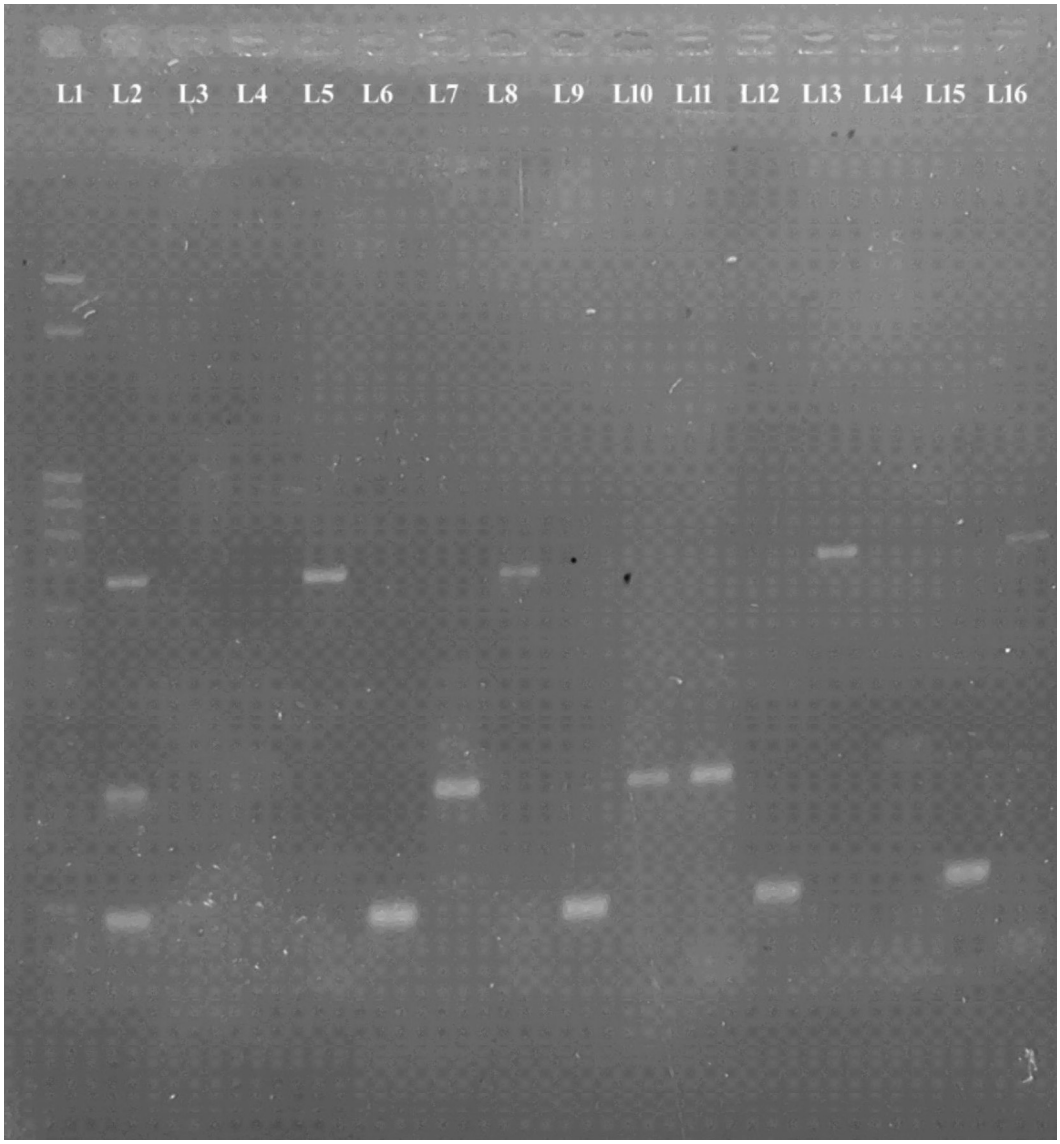

Figure 2. Specific-sized results after agarose gel electrophoresis. L1: Ladder (50 bp), L2: Positive Controls (Vaccinal Seeds of H5, H7, H9), L3: Reagent Blank, L4: NDV (Negative Control), L5; L8; L13; L16: H5 (661 bp), L7; L10; L11; L14: H9 (276 bp), L6; L9; L12; L15: H7 (140 bp).

subtypes separately. Identification of a single subtype at a time is a time-consuming and expensive option to be followed while dealing with the field samples. The current study overcame this issue by simultaneous detection of subtypes H_5, H_7, and H_9 using the multiplex-based RT-PCR technique, which was found efficacious enough to differentiate 3 subtypes on the basis of specific size-based amplification products.

The studies [5, 18] on multiplex RT-PCR for simultaneous detection of avian influenza virus subtypes H_5, H_7, and H_9 were conducted to develop differential diagnostic techniques using 3 pairs of primers, one specific for each subtype. Due to participation of a greater number of primers in reverse transcription and subsequent amplification reaction, the problems including non-specific binding, primer dimers, and cross complementarity among primers are frequently observed, which compromise the accuracy of diagnostic analysis. In the present study, the probability of occurrence of such glitches was

curtailed by decreasing the number of primers in the reactions. A single common positive primer was used for reverse transcription of all 3 avian influenza virus subtypes (H_5, H_7, and H_9) to elude non-specific binding. Multiplex PCR was carried out using the same common positive primer and 3 negative primers, one specific to a complementary cDNA template of one subtype only. The strategy showed much reduced non-specific binding of the primers, which was further refined to nullity by increasing the annealing temperature of the multiplex reaction.

A one-step real-time PCR assay for detection of avian influenza virus subtypes H_5, H_7, and H_9 was performed in the investigation [10] to amplify the target regions of 3 virus subtypes using 3 pairs of primers and 3 fluorescent dye-labeled probes. This technique detects quantity of specific genome template(s) based upon the signals produced by fluorescent dye released from subtype-specific probes due to 5′ exonuclease activity of DNA polymerase. However, probability of hybridization of fluorescently labeled probes on the non-specific subtype template due to a presence of high-sequence homology among 3 subtypes, which can produce false positive signal data; increased cost and complexity of the methodology are some of the shortcomings. The present study, in comparison, was designed to provide ease in every step of operation by limiting the number of primers and using a qualitative technique of detection instead of quantitative, hence reducing complexity and the cost of reactions significantly. Moreover, the clarity of the specific-sized bands observed by the naked eye under UV light after agarose gel genotyping is a more authentic, convenient, and cheaper option than relying on the data, which may contain false positive signals, as far as the disease diagnostics is concerned.

CONCLUSIONS AND APPLICATIONS

1. Reduction in probability of non-specific binding at the reverse transcription step due to involvement of a single common positive primer for 3 avian influenza virus subtypes H_5, H_7, and H_9.
2. Application of multiplex amplification of target cDNA of 3 avian influenza virus subtypes using a limited number of primers (4 instead of 6) in PCR, hence refraining amplification of non-specific products exponentially.
3. Practical validation of novel strategy for cost-effective, authentic, and rapid detection of avian influenza virus subtypes H_5, H_7, and H_9.
4. The strategy can also be applied for identification of other viruses having multiple subtypes/strains.

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