ABSTRACT A multiplex reverse transcription-PCR (mRT-PCR) was developed and standardized for the detection of type A influenza viruses, avian influenza virus (AIV) subtype H7, H9, and H5 hemagglutinin gene with simultaneous detection of 3 other poultry respiratory pathogens, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and infectious laryngotracheitis virus (ILTV). Seven sets of specific oligonucleotide primers were used in this study for the M gene of AIV and hemagglutinin gene of subtypes H7, H9, and H5 of AIV. Three sets of other specific oligonucleotide primers were used for the detection of avian respiratory pathogens other than AIV. The mRT-PCR DNA products were visualized by agarose gel electrophoresis and consisted of DNA fragments of 1,023 bp for M gene of AIV, 149 bp for IBV, 320 bp for NDV, and 647 bp for ILTV. The second set of primers used for m-RT-PCR of H7N3, H9N2, and H5N1 provided DNA products of 300 bp for H7, 456 bp for H5, and 808 bp for H9. The mRT-PCR products for the third format consisted of DNA fragments of 149 bp for IBV, 320 bp for NDV, 647 bp for ILTV, 300 bp for H7, 456 bp for H5, and 808 bp for H9. The mRT-PCR products for the third format consisted of DNA fragments of 149 bp for IBV, 320 bp for NDV, 647 bp for ILTV, 300 bp for H7, 456 bp for H5, and 808 bp for H9. The mRT-PCR techniques were found to be sensitive and specific for the detection of AIV and other poultry respiratory pathogens. In this present study, multiplex PCR technique has been developed to simultaneously detect and differentiate the 3 most important subtypes of AIV along with the 3 most common avian respiratory pathogens prevalent in poultry in Pakistan. Therefore, a mRT-PCR that can rapidly differentiate between these pathogens will be very important for the control of disease transmission in poultry and in humans, along with the identification of 3 of the most common respiratory pathogens often seen as mixed infections in poultry, and hence economic losses will be reduced in poultry.

Key words: avian influenza virus, multiplex reverse transcriptase polymerase chain reaction, poultry respiratory pathogen, Pakistan

INTRODUCTION

Avian influenza (AI) is a viral disease spread worldwide and is caused by influenza A viruses of the family Orthomyxoviridae. Influenza viruses are classified into 16 subtypes on the basis of the surface glycoprotein hemagglutinin and 9 subtypes on the basis of the glycoprotein neuraminidase. These 2 proteins are highly variable; therefore, a great number of AI virus (AIV) subtypes occur (Fouchier et al., 2005). Although most AIV in chickens cause mild and localized infections of the respiratory and intestinal tracts, highly pathogenic strains become dispersed throughout the body causing viraemia and produce an acute, systemic, and often fatal disease (Senne et al., 1996; Wood et al., 1996). Historically, highly pathogenic AIV of poultry belong to the H5 and H7 hemagglutinin subtypes. Because there is a greater risk for these subtypes to become highly pathogenic for humans, it is important to identify them specifically in surveillance programs (Webster and Kawaoka, 1987; Starick et al., 2000). The H9 subtype has also caused infections in poultry and humans (Cameron et al., 2000; Butt et al., 2005).

Recently, infections from AI subtypes H5, H7, and H9 have been repeatedly reported in poultry in Pakistan, especially since 2004 (Naeem et al., 2007). Unfortunately, due to very close resemblance of lesions produced by several other pathogens in poultry, it is difficult to differentiate clinical manifestations of any of the above AI infections from diseases such as infectious laryngotracheitis (ILT), Newcastle disease, and infec-
tious bronchitis. The ILT, Newcastle disease, and infectious bronchitis infections are prevalent at a rate of 10 to 15% in commercial poultry in Pakistan, despite the use of live and inactivated vaccines against such diseases. Therefore, it becomes essential to develop sensitive diagnostic techniques to differentiate between these 6 diseases. In this study, a multiplex PCR technique has been developed to simultaneously detect and differentiate the 3 most important subtypes of AIV along with the 3 most common avian respiratory pathogens prevalent in poultry in Pakistan.

**MATERIALS AND METHODS**

**Source of Viruses and Specimens**

The following listed isolates of H7N3 were used in the standardization of multiplex reverse transcription-PCR (mRT-PCR):

1. A/Chicken/Faisalabad/Pakistan/NARC-N30/05 (H7N3)
2. A/Chicken/Karachi/Pakistan/NARC-100/04 (H7N3)
3. A/Chicken/Mansehra/Pakistan/NARC-74/04 (H7N3).

The following isolates of H9N2 were used in the standardization of mRT-PCR:

1. A/Chicken/Karachi/Pakistan/NARC-4935/06 (H9N2)
2. A/Chicken/Abbotabad/Pakistan/NARC-6649/06 (H9N2)
3. A/Chicken/Islamabad/Pakistan/NARC-N240/06 (H9N2).

The following isolates of H5N1 were used in the standardization of mRT-PCR:

1. A/Chicken/Peshawar/Pakistan/NARC-2517/06 (H5N1)
2. A/Chicken/Jehlum/Pakistan/NARC-N353/07 (H5N1)
3. A/Chicken/Islamabad/Pakistan/NARC-N240/06 (H5N1).

The above-mentioned viruses were obtained from the repository of the National Reference Laboratory for Poultry Diseases, National Agricultural Research Center, Islamabad, Pakistan. The viruses procured from the repository in a lyophilized form were propagated in 9-d-old embryonated chicken eggs up to 3 passages and tested using hemagglutination test (Beard, 1980; Swayne et al., 1998). After the standardization of the test, the technique was used for the detection of routine field cases of AI.

Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and ILT virus (ILT) were extracted from live freeze-dried vaccines (Nobilis, Intervet International B.V., Boxmeer, the Netherlands) for the standardization procedure. The few examples of field isolates of NDV, IBV, and ILTV identified by mRT-PCR are listed below:

1. Chicken/Rawalpindi/Pakistan/NARC-303/05 (IBV M-41)
2. Chicken/Karachi/Pakistan/NARC-2344/06 (IBV M-41)
3. Chicken/Rawalpindi/Pakistan/NARC-378/05 (NDV)
4. Chicken/Karachi/Pakistan/NARC-1045/07 (NDV)
5. Chicken/Faisalabad/Pakistan/NARC-N334/06 (ILT)

The tissues selected for mRT-PCR included trachea, lungs, cecal tonsils, and spleen from the diseased chickens showing respiratory tract infection. The samples were pooled and processed for RNA-DNA extraction.

**Extraction of RNA and DNA**

Viral RNA from the viral samples (tissue homogenates, allantoic fluids, and vaccines) was extracted using QIAamp Viral RNA Mini Kit, according to instructions of the manufacturer (52906, Qiagen Inc., Valencia, CA).

Deoxyribonucleic acid from ILTV was extracted using Easy DNA Kit (Invitrogen, Carlsbad, CA) following protocol 7 of the manufacturer. The concentrations of RNA or DNA were determined using a Bio-Photometer (Eppendorf, Hamburg, Germany).

**Primer Designs and Selection**

Four sets of primers that specifically amplify type A influenza virus (M gene) and the H gene of H7, H9, and H5 subtypes of AIV are listed in Table 1. Three sets of primers for IBV, ILTV, and NDV are listed in Table 2. The sequence of the primers for H7, H9, and H5 was obtained from already published data in literature and synthesized by Operon Biotechnologies (Huntsville, AL). Primer sequences for IBV were obtained from published data (Callison et al., 2006), and primer sequences for ILTV and NDV were obtained from published data (Pang et al., 2002). The primers were aliquoted to a final concentration of 100 pmol/μL and stored at −20°C until further use.

**Optimization of mRT-PCR**

The mRT-PCR employed in the present work consisted of a 1-step procedure, which included reverse transcription (RT) and PCR amplification in a single step. For this purpose, an RT-PCR kit (Invitrogen) was used. Following the protocol of the manufacturer, the
mRT-PCR was performed in 50-μL volumes, in which the reaction mixture according to the manufacturer contained 25 μL of 2× Reaction Mix consisting of a buffer system for RT and PCR amplification, optimal Mg++ concentration, deoxyribonucleotide triphosphates, and stabilizers. Template RNA in various dilutions, which were optimized after several mRT-PCR trials, were added in 1-μL amounts per reaction mixture. One microliter of forward and reverse primers of influenza A, H7, H9, H5, IBV, ILTV, and NDV was added in an optimized concentration of 100 pmol/μL and 2.5 μL of RT/Platinum Taq Mix was added. This amount was optimized after several trials, and it contained a mixture of Superscript II Reverse Transcriptase and Platinum Taq DNA Polymerase for optimal cDNA synthesis and PCR amplification. Nuclease-free water was added to bring the final volume to 50 μL.

The mRT-PCR was performed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). The cycling protocol consisted of cDNA synthesis at 45°C for 45 min, an initial denaturation at 96°C for 5 min, then 40 cycles that each consisted of denaturing at 95°C for 1 min, annealing at 53°C for 5 min, and extension at 70°C for 1 min, followed by a final extension at 72°C for 10 min and final storage at 4°C. A negative control did not contain template cDNA and consisted of PCR master mix, all sets of primers, and nuclease-free water.

Three sets of mRT-PCR were carried out; the first set, referred to as trivalent mRT-PCR, used primers of H7, H9, and H5 with template RNA of the specific hemagglutinin subtypes utilizing the optimal conditions mentioned above for mRT-PCR. A second set of mRT-PCR, called tetravalent mRT-PCR, was developed using primers for influenza A, NDV, IBV, and ILTV with template RNA-DNA of these specific viruses utilizing the optimal conditions of mRT-PCR. The third set of mRT-PCR, referred to as hexavalent mRT-PCR, was developed using primers of H7, H9, H5, NDV, IBV, and ILTV with the specific template RNA-DNA with hexavalent amplifications being carried out using the optimized conditions.

### Detection of Amplified Nucleic Acid Products of mRT-PCR

The DNA amplicons were visualized using 2% agarose gels with ethidium bromide with standard 1-kb DNA markers (Invitrogen) at 120 V for 20 min. Gels were photographed using a gel documentation system (Poddar, 2002).

### Sensitivity and Specificity of mRT-PCR

The mRT-PCR was tested using other avian pathogens that produce similar clinical signs or that can be present in mixed infections with AI subtypes (Table 1). To determine the ability of the mRT-PCR assay to detect and differentiate 3 subtypes of AIV, H7, H9, and H5, and NDV, IBV, and ILTV in the same reaction, we used a mixture of DNA-RNA concentrations

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligonucleotide sequence of primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIV</td>
<td>M-WSN F: 5′-GAA GGT AGA TAT TGA AAG ATG-3′</td>
<td>1,023</td>
</tr>
<tr>
<td></td>
<td>M-1023 R: 5′-GAA ACA AGG TAG TTT TTT ACT C-3′</td>
<td></td>
</tr>
<tr>
<td>Subtype H7</td>
<td>H7-397 F: 5′-ACA TAC AGT GGG ATA AGA ACC-3′</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>H7-391 R: 5′-TCT CTT GGA AGA ATT AG-3′</td>
<td></td>
</tr>
<tr>
<td>Subtype H9</td>
<td>H9-1 F: 5′-AGC AAA AGC AGG GGA AYW WC-3′</td>
<td>808</td>
</tr>
<tr>
<td></td>
<td>H9-808 R: 5′-CCA TAC CAT GGG GCA ATT AG-3′</td>
<td></td>
</tr>
<tr>
<td>Subtype H5</td>
<td>H5-1: 5′-ACT ATG AAG AAT TGA AAC TTT CTA TCC T-3′</td>
<td>456</td>
</tr>
<tr>
<td></td>
<td>H5-2: 5′-GCA ATG AAA TTT CCA TTA CTC TC-3′</td>
<td></td>
</tr>
</tbody>
</table>

### Table 1. Multiplex reverse transcription-PCR primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligonucleotide sequence of primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBV (up)</td>
<td>5′-GCT TTT GAG CCT AGC GTG-3′</td>
<td>149</td>
</tr>
<tr>
<td>IBV (down)</td>
<td>5′-GCC ATG TTG CCA CTG TCT ATT-3′</td>
<td></td>
</tr>
<tr>
<td>NDV (up)</td>
<td>5′-GGG GGA GGT TGG CAG CAT T-3′</td>
<td>320</td>
</tr>
<tr>
<td>NDV (down)</td>
<td>5′-GTC AAC ATA TAC ACC TCA TC-3′</td>
<td></td>
</tr>
<tr>
<td>ILTV (up)</td>
<td>5′-AGT ACT GCT CCG ACT TCC-3′</td>
<td>647</td>
</tr>
<tr>
<td>ILTV (down)</td>
<td>5′-CGT TGG AGG TAG GTG GTA-3′</td>
<td></td>
</tr>
</tbody>
</table>

1References: Callison et al. (2006; IBV) and Pang et al. (2002; NDV, ILTV).
2IBV = infectious bronchitis virus; NDV = Newcastle disease virus; ILTV = infectious laryngotracheitis virus.
ranging from 500 ng to 10 fg of DNA-RNA in various combinations of 3 subtypes of AIV and 3 other respiratory pathogens.

Sensitivity of the mRT-PCR for the detection of these 6 respiratory pathogens was determined by making 10-fold serial dilutions of 100 ng of each respiratory pathogen as template RNA-DNA; mRT-PCR of these dilutions was done in the thermocycler using the optimized program.

Specificity of mRT-PCR was determined by examining the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV, and ILTV. Primers specific for AIV, H7, H9, and H5 were added to the multiplex format for the amplification of AIV, H7, H9, and H5 to check their reactivity if any to NDV, IBV, and ILTV. Similarly, in the multiplex format, NDV-, IBV-, and ILTV-specific primers were added in the multiplex format to check if they reacted-amplified AIV, H7, H9, and H5 RNA.

RESULTS

Throughout the development of mRT-PCR, various modifications were made to the annealing temperature, extension time, cycle quantity, primer concentration, and template dilutions. The multiplex PCR products consisted of 300 bp for H7, 808 bp for H9, 456 bp for H5, 149 bp for IBV, 320 bp for NDV, and 647 bp for ILTV and were visualized by gel electrophoresis (Figure 1, 2, and 3). The standardized test was employed to examine various field cases. The sensitivity of mRT-PCR, depicting the limit of the method by visualization of PCR-amplified DNA products, was 1 ng for the hemagglutinin subtypes of AIV (H7, H9, and H5) and type A AIV. The detection limit was 100 pg for IBV, NDV, and ILTV. No spurious PCR amplification reactions were observed in the detection of AIV subtypes H7, H9, and H5 and other respiratory pathogens with various concentrations of DNA and RNA mixtures. All negative controls were negative. Negative controls had no template RNA-DNA and just the primers and buffering system provided by the manufacturer in the kit were added (SuperScript One-Step RT-PCR with Platinum Taq, 10928-042, Invitrogen).

Specificity of mRT-PCR was determined by examining the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV, and ILTV. The specificity of the primers used to detect amplified DNA was hence determined because no specific amplification bands of the same sizes (300, 808, and 456 bp) could be amplified for RNA-DNA of other poultry respiratory pathogens (NDV, IBV, and ILTV), and likewise, no amplification bands of the sizes (320, 149, and 647 bp) could be amplified for RNA for AIV.

Figure 1. Agarose gel electrophoresis of trivalent multiplex reverse transcription-PCR-amplified products from purified RNA of known avian influenza subtypes. Lane 1 = molecular size marker; lane 2 = H7N3, H5N1, and H9N2 subtypes of avian influenza virus; lane 3 = PCR reagent buffer as a negative control; lane 4 = H7N3, 300 bp; lane 5 = H9N2, 808 bp; lane 6 = H5N1, 456 bp.

Figure 2. Agarose gel electrophoresis of tetravalent multiplex reverse transcription-PCR-amplified products from purified RNA and DNA of known avian influenza subtypes and other avian respiratory pathogens. Lane 1 = molecular size marker; lane 2 = avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV); lane 3 = PCR reagent buffer as a negative control; lane 4 = AIV, 1,023 bp; lane 5 = NDV, 320 bp; lane 6 = IBV, 149 bp; lane 7 = ILTV, 647 bp.
DISCUSSION

The mRT-PCR was developed in 3 formats to detect and differentially diagnose in a single reaction influenza A; AI hemagglutinin subtypes H7, H9, and H5; and 3 respiratory pathogens, NDV, IBV, and ILTV.

Influenza infection is diagnosed by virus isolation and identification or serological tests. However, virus isolation and identification is tedious and time-consuming and serological tests are less practical if mass vaccination is practiced (Reina et al., 1996). This situation does exist in Pakistan where apart from the prevalence of H7, H9, and H5 in the field, the presence of NDV, IBV, and ILTV is well documented. This makes it very difficult to diagnose any respiratory infection of poultry under field conditions. More recently, PCR assays have been developed for many respiratory viruses, allowing detection of small amounts of viral nucleic acid in clinical samples. In the so-called multiplex format, PCR assays have been designed to amplify more than one viral respiratory target in the same PCR test (Ellis et al., 1997; Fan et al., 1998; Osiowy, 1998; Grondahl et al., 1999; Liolios et al., 2001; Xie et al., 2006).

Therefore, a mRT-PCR which can rapidly differentiate between AIV subtypes H7, H9, and H5 and NDV, IBV, and ILT will be very important for the control of disease transmission in poultry and in humans along with the identification of 3 of the most common respiratory pathogens often seen as mixed infections in poultry. Economic losses will be reduced in poultry associated with and AIV outbreak with the use of this assay. Conventional techniques such as RT-PCR, used to detect viral RNA, are time-consuming and perhaps even less sensitive. Although we did not compare the assay developed and reported here with the conventional technique of RT-PCR, it can be safely assumed that this assay is superior to conventional techniques because viral RNA can be directly detected in clinical and field samples in a few hours; therefore, it is less time-consuming as compared with other diagnostic methods. This mRT-PCR may be used in diagnosis, screening, and surveillance of poultry. This assay has the added benefit of being less time-consuming and using a single-step procedure for PCR with the use of fewer reagents and the simultaneous detection of AIV with other respiratory poultry pathogens. This assay has been developed specially for the developing countries where it is very difficult to buy very expensive laboratory instruments like quantitative real-time PCR machines, which are frequently used in developed countries for the detection of viral nucleic acids.

Future work is needed to expand the panel of viral pathogens detected by such rapid molecular methods to eventually circumvent the need for viral cultures. Also, future evaluation of the multiplex PCR assay is warranted.

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REFERENCES


