

Multiplex Reverse Transcription-PCR Assay for Detection of Type A Influenza Virus plus Differentiation of Avian H7 and H9 Hemagglutinin Subtypes in Iran

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Abstract

Background: Avian influenza virus (AIV) infection is a major cause of bird and human morbidity and mortality. We aimed to evaluate a specific and sensitive multiplex RT-PCR that can simultaneously detect influenza type A viruses and differentiate the two most important subtypes of avian influenza viruses H7 and H9 subtypes.

Methods: A multiplex reverse transcriptase-polymerase chain reaction (mRT-PCR) was developed and optimized for the detection of type A influenza virus. Simultaneously avian H7 and H9 hemagglutinin subtypes was differentiated. Three sets of specific oligonucleotide primers were used in this test for type A influenza virus, H7 and H9 hemagglutinin subtypes.

Results: The mRT-PCR DNA products were visualized by gel electrophoresis and consisted of fragments of 313 bp for H7 and 428 bp for H9 hemagglutinin subtypes, and 101 bp for type A influenza virus. The common set of primers for type A influenza virus were able to amplify a 101 bp DNA band for any of the other subtypes of influenza A virus

Conclusion: The mRT-PCR assay developed in this study was found to be sensitive and specific. No specific amplification bands of the same sizes (313 and 428 bp) could be amplified for RNA of other influenza hemagglutinin subtypes, nor specific amplification bands of type A influenza (101 bp) for Influenza B, C, or other viral or bacterial pathogens tested in this study.

Keywords: *Multiplex RT-PCR, Hemagglutinin H7, H9, Influenza A, Iran*

Introduction

Influenza is a zoonotic disease, infecting a wide variety of warm-blooded animals, including birds and mammals. Influenza viruses are classified into types A, B, and C. Influenza A viruses are responsible for major disease problem in birds, as well as in humans. Infections among domestic or confined birds have been associated with a variety of disease syndromes ranging from sub-clinical mild upper respiratory disease, to acute generalized fatal disease (1, 2).

Influenza A are enveloped, two envelope glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), are the major viral antigens that induce protective antibodies following infection. Both proteins have highly variable sequences that give rise to variable antigenicity (1, 3).

Based on the antigenic properties of their two surface HA and NA glycoproteins influenza viruses classified into different subtypes. To date, 16 HA and 9 NA subtypes have been identified (4, 5). All influenza A virus subtypes have been found in aquatic and domestic birds, but only a few subtypes have been recovered from mammals and humans (2). Among 16 HA subtypes, only H5 and H7 are highly virulent in poultry. Since, there is a greater risk for these subtypes to become highly pathogenic, so it is important to identify them specifically in surveillance programs. Apart of the H5N1 influenza virus, another subtype of influenza virus, H9N2, has become panzootic in the last decade and has been isolated from different types of terrestrial poultry worldwide (6-8). Diagnosis of influenza A virus infection is routinely done by the isolation and identification of the vi-

rus. Serotyping is required to differentiate the subtypes of the AI viruses and is laborious and time-consuming. Furthermore, other tests required to determine the HA cleavage site sequence must be done to determine its potential virulence. Although single band PCR has been used to detect and differentiate subtypes, it only recognizes one specific subtype at the time. mRT-PCR has been previously reported for the detection of avian influenza virus H5 and H9 subtypes (9).

In addition, real time-RT-PCR assays for influenza virus have been developed for the detection of influenza virus types A and B and differentiation of two subtypes H5, and H7 (10).

In this study, we have described a specific and sensitive multiplex RT-PCR that can simultaneously detect influenza type A viruses and differentiate the two most important subtypes of avian influenza viruses H7 and H9 subtypes.

Materials and Methods

Virus strain

The A/turkey/England/50-92/91 (H5N1) and A/Turkey/England/7732/66(H7N7) were originally obtained from the Veterinary Laboratories Agency (VLA) in United Kingdom. Dr T Mokhtari from the National Influenza Centre, Tehran Medical Science University was kindly provided the A/ Tehran/49/2001(H1N1) and A/Tehran/82/79 (H3N2) human influenza viruses. The A/Chicken/Iran/11T/99 (H9N2) influenza whole virus was obtained from Razi vaccine and serum research institute isolated previously from outbreak among poultry in Iran. The influenza type B and C were obtained from virology Department of Technique University of Munich-Germany.

RNA extraction from virus stocks

RNA purification was performed using the RNXTM-Plus Kit (Sinagen, Iran) as described previously (9). Briefly 100-150 µl of viral suspension (egg-fluid, clinical specimens and water control) were mixed with 1 ml of RNXTM and left for at least 5 min at 4 °C. After the addition of 200 µl chloroform and mixing, the liquid was clarified by centrifugation at

12000 g for 15 min at 4 °C. The supernatant was transferred to a new tube and mixed with an equal volume of isopropanol followed by centrifugation at 12,000 g for 15 min at 4 °C. The pellet was washed with 1 ml of 70% ethanol. Finally, RNA was eluted in 50 µl of 1 mM RNase free EDTA.

Reverse transcription

An influenza virus matrix gene-specific primer set used for a region conserved in all type A influenza virus matrix (M) genes as described previously (9). In addition, H7 and H9-specific primer sets were used for detection of conserved regions from the H7 and H9 hemagglutinin gene sequences in RT-PCR reaction.

The oligonucleotide primers were commercially synthesized (MWG, Germany) (Table 1). The 20 µl reaction for each gene (M and HA) contained 5 µl of extracted RNA, 4 µl of 5X RT-buffer, 2 µl dNTP (2.5 mM of each dNTPs), 1 µl (10 pmol/µl) of forward primer, 0.5 µl (40 unit/µl) of RNase inhibitor and 1 µl (40 unit/µl) of M- muLV reverse transcriptase and 6.5 µl of water. Reverse transcription was carried out at 42 °C for 45 min followed by incubation at 70 °C for 10 min.

PCR

PCR was carried out for both genes of each subtype. The 30 µl reaction mixture contained 10 µl of cDNA, 0.5 µl (2.5 mM of each dNTPs), 1 µl (10 pmol) of each primer, 3 µl of 10X PCR-buffer, 1 µl (10 mM) MgCl₂, 0.25 µl (5 unit/µl) of Taq DNA polymerase and 13.25 µl of water. Wide ranges of cycling conditions were tested. After initial denaturation at 94 °C for 5 min, three-step PCR cycling protocol was used to amplify the matrix gene (M) through the 35 cycles of 94 °C for 10 S, 54 °C for 10 S and 72 °C for 10 S were followed by a final extension at 72 °C for 3 min. A three-step cycling protocol was used for the H7 and H9-specific PCR as follows: 94 °C for 30 S, 54 °C for 30 S and 72 °C for 40 S for 35 cycles. Different controls were included in each assay.

Multiplex RT-PCR

Multiplex RT-PCR was carried out in 50 µl reaction for M and HA genes of H7 and H9 sub-

types, cycling conditions were the same as those for the H7 and H9-specific PCR.

Sequence analysis

The PCR products were analyzed by sequencing (MWG, Germany). The products were cleaned using a PCR purification kit (Fermentas, USA). Sequencing was performed in both the sense and anti-sense direction using MF and MR primers for the M gene and the RT-PCR primers H7F,H7R (H7) and H9F,H9R (H9), respectively for the HA

gene. Sequencing data were aligned with M and HA influenza sequences from NCBI database to assess homology.

Detection of PCR product by gel electrophoresis

Ten µl of the PCR products were analyzed by electrophoresis, using 1.5% agarose gel. Amplified products were visualized by ultraviolet light transillumination after staining with 0.1 µg/ml ethidium bromide. A 100 base pair ladder was used as a molecular weight marker.

Table 1: The oligonucleotides used

Specificity	Primers	Sequences	Location	Size
Influenza A virus	MF	AGATGAGCCTTCTGACCGAGGTCG	24-47	101
	MR	TGCAAAAACATCTTCAAGTCTCTG	124-100	
Avian H7	H7F	ATCTTCATTCTATGCAGAG	453-471	313
	H7R	ACTGAAAGTAACTGTGTCA	765-746	
Avian H9	H9F	TTGCTCCACACAGAGCACAAT	164-184	428
	H9R	TGATGTATGCCCCACATGAA	591-572	

Results

To identify all subtypes of influenza A viruses by RT-PCR, we used two primers based on consensus sequences of M genes of Influenza viruses as target (9).

A 101 bp fragment of the M gene from a series of different influenza A strains (H1, H3, H5, H7 and H9) as demonstrated in Fig.1 was amplified. Sensitivity of the M RT-PCR was tested by serial ten-fold dilution of virus pools. The specificity of the primers for the detection of influenza A was examined using RNA from other human and avian viral species. The matrix RT-PCR detected influenza A virus not only in avian strains but also in other animal species including human strains (H1N1 and H3N2) RNA isolated from influenza B and C viruses did not yield amplified products. The HA RT-PCR (primer H7F, H7R) for the detection of H7 influenza A subtype was amplified. A sharp band of the expected size (313 bp) was obtained from H7 strains. No PCR product was amplified from non-H7 influenza subtypes (data not shown). The HA RT-PCR amplification product (primer H9F, H9R) for the H9 influenza

A subtypes was 428 bp (Fig. 2). HA sequence of H7 and H9 subtype origin verified by sequencing (not shown).

The multiplex RT-PCR was tested for its specificity for viral targets (influenza A H7 and H9) by adding each of the influenza virus primer pairs. No mispriming was observed when primer sets were present with both influenza A H7 and H9 template. A product of the expected size was obtained for each viral template by the multiplex RT-PCR with primer sets present (Fig. 3). The specific products clearly were separated and identified on a 1.5% agarose gel. The multiplex RT-PCR was performed on both avian and tracheal swabs for detection of H7 and H9 subtype. 29 of 98 (29%) cloacal swabs and 11 of 49 (22%) tracheal swab samples contained influenza A virus H9 subtype. No detectable PCR products were seen for influenza A virus H7 subtype. Nucleic acid extraction and multiplex RT-PCR amplification from 26 human clinical samples (nasopharyngeal aspirate or nose and throat swabs) did not show any PCR product.

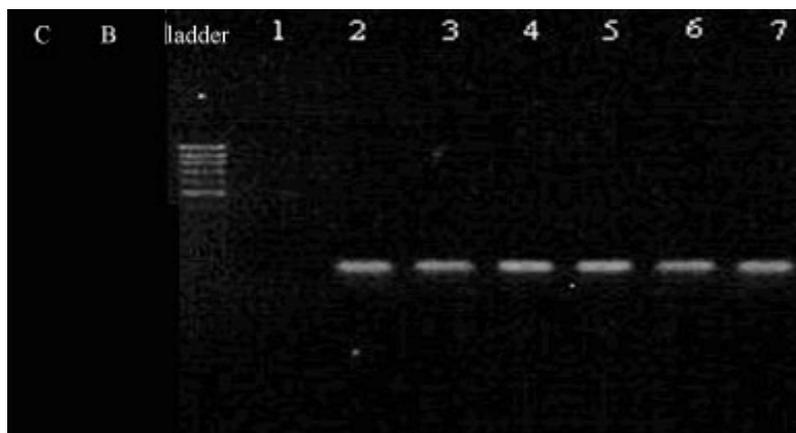


Fig. 1: Identification of Influenza type A virus by RT-PCR. The M gene RT-PCR was applied to a panel of influenza A strains. Lane M, size markers (100 bp ladder, Sinagen, Iran) lane 1, negative control and lanes 2-7, ten µl of RT-PCR products obtained by using specific primers for M genes of the viruses H1N1, H3N2, H5N1, H5N9, H7N7, and H9N2. The size of RT-PCR M gene product is 101 bp. Lane B: influenza B and lane C: influenza C virus

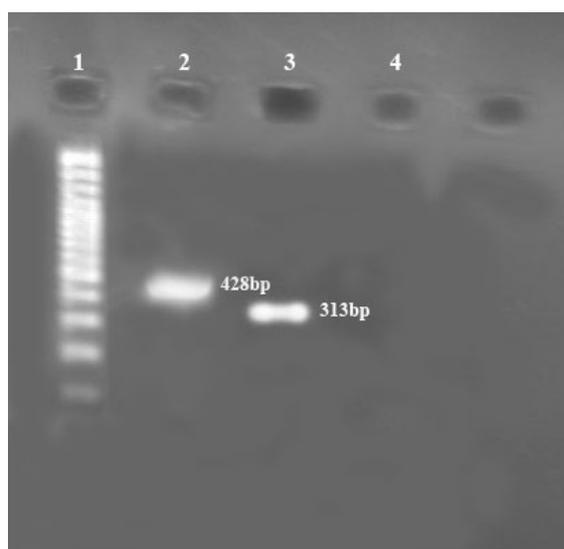


Fig. 2: Identification and subtyping of avian influenza viruses

Lane 1, size markers (100 bp ladder, Sinagen, Iran). lanes 2, ten µl of RT-PCR products obtained by using specific primers for HA genes of the H9N2 and lane 3 ten µl of RT-PCR products obtained using specific primers for HA genes of the H7N7 viruses product and lane 4, negative control was applied on a 2% gel. The sizes of RT-PCR HA gene product was indicated

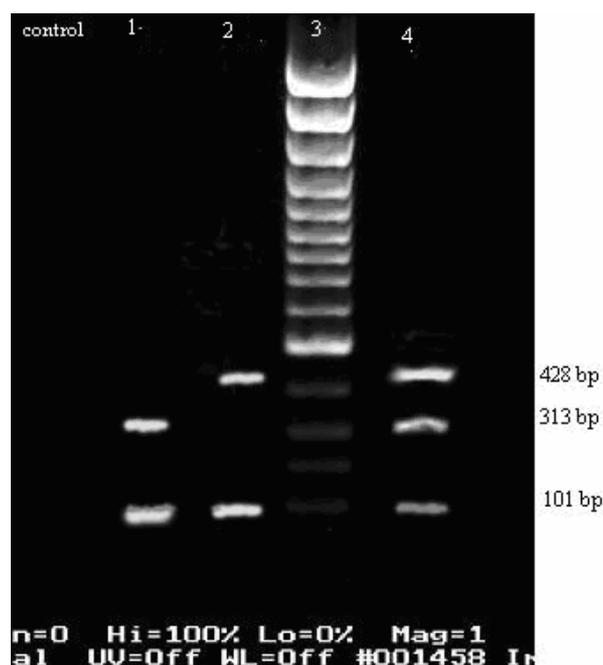


Fig. 3: Subtyping of avian influenza viruses. Lane 1, ten µl of Multiplex RT-PCR products obtained by using specific primers for HA and M genes of the H7N7 virus. Lane 2, ten µl of Multiplex RT-PCR products obtained by using specific primers to HA and M genes of the H9N2 virus. Lane 3, size markers (100 bp ladder, Sinagen, Iran). Lane 4, ten µl of Multiplex RT-PCR products obtained by using specific primers for HA and M genes of the H7N7 and H9N2 viruses. The sizes of RT-PCR HA gene products are indicated

Discussion

Highly pathogenic avian influenza caused by certain subtypes of influenza A virus in animal population particularly chickens poses a concerning global human public health risk. Laboratory identification of influenza A virus infection is commonly carried out by direct antigen detection such as Hemagglutination test (HAT), isolation in cell culture or detection of influenza specific RNA by reverse transcriptase chain reaction (11, 12).

Isolation and identification of influenza viruses by conventional culture methods can take up to 14 d and the difficulties of recovery infectious virus from clinical samples are enhanced when we include the sample traveling problem which may take from 24 h to 5 d sometimes. So developing methods based on RT-PCR strategy may improve the diagnostic yield when infectious virus is likely to have been inactivated due as prolonged incubation at in appropriate condition (13).

We have developed the multiplex RT-PCR method capable of detecting type A influenza virus hemagglutinin with simultaneous identification and differentiation of the two very important hemagglutinin subtypes H7 and H9. Since these 2 hemagglutinin subtypes recently have been the cause of human infection, therefore mRT-PCR is based the diagnosis of type influenza as well as subtypes H7 and H9 in a single reaction. Which can be achieved with in a single working day (significantly faster than diagnosis of infection by clinical methods) can play an important role in controlling disease transmission in poultry and human (14, 15).

However, several investigations have previously reported on RT-PCR assay for detection of influenza virus in clinical samples. We also have reported the subtyping of influenza A (H5 and H9 subtypes by mRT-PCR) but this is the first report to optimize mRT-PCR for simultaneous detection of influenza A virus along with H7 an H9 subtypes (9, 16).

The multiplex RT-PCR was performed on both avian and tracheal swabs for detection of H9 and H7 subtype. 29 of 98 (29%) cloacal swabs and 11 of 49 (22%) tracheal swab samples con-

tained influenza A virus H9 subtype. We found by RT-PCR that 40 out of the 127 (31%) swab samples contained avian influenza and all of them were H9 subtypes. These samples were collected from chickens of 14 different farms in Iran in 2002-2004. This finding suggests a current endemic of H9 avian influenza virus in the chicken population of Iran. In fact, we have isolated 40 avian influenza viruses from chicken in Iran in 2002-2004.

Therefore, the multiplex RT-PCR can be a reliable and accurate method for large-scale screening and detection of type A influenza virus and the avian H7 and H9 subtypes simultaneously. The Hong Kong incident marks the acceptance of the role of avian hosts as a source for pandemic Influenza viruses, especially since H9N2 was credited as providing the replicating genes for the H5N1, which has been isolated in Hong Kong from poultry, pigs and humans (12, 17, 18).

In conclusion, our newly designed mRT-PCR assay for detection of type A influenza virus and the avian H7 and H9 subtypes in less expensive than virus isolation (VI), importantly result are available much faster and more sensitive. This assay provides a rapid and feasible alternative to VI in embryonating chicken egg and subtyping by HI.

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