A Reverse transcription-PCR assay for detection of type A influenza virus and differentiation of avian H7 subtype

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Abstract : Avian influenza virus (AIV) infection is a major cause of influenza mortality in birds and can cause human mortality and morbidity. Although the risk of infection with avian influenza virus (AIV) is generally low for most people, the pathogenic virus can cross the species barrier and acquires the ability to infect and be transmitted among the human population; therefore the rapid identification of the virus is of important clinical and epidemiological implication. A reverse transcriptase-polymerase chain reaction (RT-PCR) was optimized for the detection of type A influenza virus. The assay differentiates avian H7 hemagglutinin subtypes. Two sets of specific oligonucleotide primers were used in this test for type A influenza virus and H7 heamagglutinin subtypes. The RT-PCR DNA products were visualized by gel electrophoresis and consisted of fragments of 98 bp for H7 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. The RT-PCR assay developed in this study was found to be sensitive and specific. No specific amplification bands of the same sizes (98 bp) could be amplified for RNA of other influenza hemagglutinin subtypes, specific amplification bands of type A influenza (101 bp) for Influenza B, C, or other viral or bacterial pathogens was not tested in this study.

Key word:. Influenza A ❺RT-PCR ❺Hemagglutinin ❺H7 ❺Subtyping

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Introduction

Influenza is a zoonotic disease, infec-ting 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]. On the basis of the antigenic properties of their two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA) influenza viruses were classified into different subtypes. To date, 16 HA and 9 NA subtypes have been identified [3, 4]. Most of the influenza A virus subtypes have been found in wild 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. Historically, highly pathogenic avian influenza viruses of poultry belonged only to H5 and H7 hemagglutinin (HA) subtypes. Since, there is a greater risk for these subtypes to become highly pathogenic, it is important to identify them specifically in surv-eillance programs [5,6]. Diagnosis of influenza A virus infection is routinely done by the isolation and identification of the virus from nose and throat swabs with tissue culture or embryonated chicken eggs and/or of antigen detection by immunofluorescence (IF) or enzyme-linked immunosorbent assay. Serotyping is required to differentiate the subtypes of the AI viruses and is a laborious and time-consuming task. Furthermore, other tests required to determine the HA cleavage site sequence must be done to determine the potential virulence of the subtype [7]. Another approach to the rapid detection and

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identification of influenza viruses is the application of reverse transcription- PCR (RT-PCR) for influenza virus. In this study, we describe a specific and sensitive RT-PCR that can detect influenza type A viruses and differentiate the subtype of avian influenza viruses H7.

Material and Methods

Viruses

Virus strain used: A/turkey/England/50-92/91 (H5N1), A/Turkey/England/7732/66 (H7N7). A/Tehran/49/2001(H1N1), A/Tehran/82/79 (H3N2), A/Chicken/Iran/11T/99 (H9N2). The H5N1, H5N9 and H7N7 antigens were originally obtained from the Veterinary Laboratories Agency (VLA) in United Kingdom. Dr T. Mokhtari from the National Influenza Centre, Tehran Medical Science University kindly provided the H1N1 and H3N2 human influenza viruses. The A/Chicken/Iran/11T/99 (H9N2) influenza antigen was obtained from Razi Vaccine and Serum research institute which had been isolated previously from an outbreak among poultry in Iran. Haemagglutination (HA) titers of the viruses stocks was determined according to the methods as described previously [8]. Their HA titers ranged from 512 to 1024 units/ml.

RNA extraction from virus stocks

RNA purification was performed using the RNX™-Plus Kit (cinagen, Iran) as described previously [9]. Briefly 100-150 µl of viral suspension (egg-fluid, clinical specimens and water as a control) were mixed with 1 ml of RNX and left for at least 5 min at 4ºC. After the addition of 200 µl chloroform and mixing, the liquid was clarified by centrifugation at 12000 g for 15 min at 4ºC. 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 4ºC. The pellet was washed with 1 ml of 70% ethanol. Finally, RNA was disolved in 50 µl of RNase free distilled water.

Reverse transcription

An influenza virus matrix gene-specific PCR primer set used for a region conserved in all type A influenza virus matrix (M) genes as described in [9]. In addition H7-specific primer set for conserved regions of the H7 hemagglutinin gene sequences were developed and used for RNA isolation and RT-PCR detection with single primer set, Table1. The oligonucleotide primers were commercially synthesized (MWG, Germany). The 20 µl reaction mixture 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 reverse or random hexamer 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 RNase free distilled water. Reverse transcription was carried out at 42ºC for 45 min followed by incubation at 70ºC for 10 min.

Polymerase chain reaction

PCR was carried out for both genes of each subtype. The 30 µl reaction mixture contained 10 µl of cDNA product, 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 ºC for 5 min, three-steps PCR cycling protocol was used to amplify the matrix gene (M) through the 35 cycles of 94 ºC for 10 sec, 54 ºC for 10 sec and 72 ºC for 10 sec were followed by a final extension at 72 ºC for 3 min. The H7 PCR cycling conditions were the same as those for the matrix gene except that annealing and extension temperatures were used for 7 and 10 sec respectively. RT-PCR was performed with the BIOTECH-MWG thermalcycler (Germany). Negative reagent controls were included in each assay. No contamination was detected at any time.

Sequence analysis

The PCR products were analyzed by sequencing (MWG, Germany). As a first step, the products were cleaned using a PCR purification kit (fermentas, USA). Sequencing was performed in both the sense and anti-sense direction with primers MF and MR for the M gene and the RT-PCR primers H7F,H7R (H7) 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

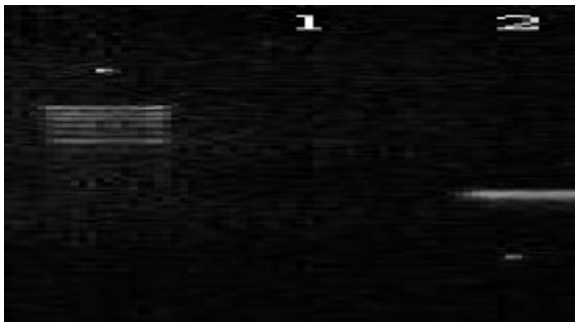

Fig. 1. The M RT-PCR was applied to a panel of avian influenza strains represent-ting various subtypes. Ten µl of PCR product was applied on a 2% gel in 1-7 as follows: 1. H_2O control, 2. H1N1, 3. H3N2, 4. H5N1, 5. H5N9, 6. H7N7, and 7. H9N2.

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Ten µl of the PCR products were analyzed by electrophoresis, using a 2% agarose gel in 1x TBE buffer. Amplified products were visualized by ultraviolet light transillumination followed by staining with 0.1 µg/ml ethidium bromide. A 50 base pair ladder was used as a molecular weight marker.

Table 1: The oligonucleotides used

Specificity	Primers	Sequences	Location	Size
Influenza A virus	MF MR	AGA TGA GTC TTC TAA CCG AGG TCG TGC AAA AAC ATC TTC AAG TCT CTG	24-47 124-100	101
Avian H7	H7F H7R	ATT GGA CAC GAG ACG CAA TG TTC TGA GTC CGC AAG ATC TAT TG	1283-1302 1380-1367	98

Results

The M RT-PCR (primer MF, MR) was evaluated using several influenza A reference strains. We showed that the RT-PCR is able to amplify the M gene from a series of different avian influenza strains (H1, H3, H5, H7 and H9) as demonstrated in Fig.1.

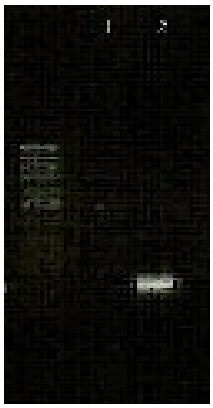

Fig. 2. RT-PCR amplification of 98 bp fragment of the H7 HA genes. Ten µl of PCR product was applied on a 2% gel in 1-2 as follows: 1 H_20 control, 2 H7N7.

To test the sensitivity of the M RT-PCR serial ten-fold dilution of virus pools of known titer were RNA purified and PCR amplified. One avian influenza A virus antigen (H7N7) was tested namely with 50% end-point dilution of 10^{-4}.

The specificity of the primers for the detection of influenza A was examined by including RNA from other viruses infecting human and avian species. Thus, RNA isolated from influenza B and C viruses did not yield amplified products. The M RT-PCR did not only detect influenza A virus from avian strains but when applied to virus strain from other species influenza A virus were also detected. Thus, influenza A was detected by the M RT-PCR from the human strains (H1N1 and H3N2).

The HA RT-PCR (primer H7F, H7R) for the detection of H7 influenza A subtypes amplified a band of 98 bp (Fig. 2). A sharp band of the expected size obtained from H7 strains tested and no PCR product was amplified from non-H7 influenza subtypes (not shown).

Discussion

Highly pathogenic avian influenza caused by certain subtypes of influenza A virus in animal population particularly chickens poses a challenging global human public health risk. Laboratory identification of influenza A virus infection is commonly carried out by direct antigen detection, isolation in cell culture or detection of influenza specific RNA by reverse transcriptase chain reaction [10]. Identification of influenza viruses by conventional culture methods can take up to 14 days and the difficulties of recovery infectious virus from clinical samples are enhanced when we include the sample traveling problem which may take from 24 hrs to 5 days 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 [11]. The value of PCR assays for the detection and surveillance of influenza viruses in clinical material has been clearly demonstrated [12]. However, these assays have been designed specifically for the diagnosis and surveillance of human influenza viruses and there is an additional need for rapid tests to detect viruses originating from non human hosts [13]. We have shown that the M gene RT-PCR is sensitive and specific method for the detection of influenza A viruses of different subtypes, of human, avian and swine origin [14].

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We have also reported the subtyping of influenza A (H5 and H9 subtypes by mRT-PCR) [9, 15] but this is the first report to optimize RT-PCR for detection of influenza A virus along with H7 subtype in Iran.

We have developed RT-PCR assays for the detection of the type A influenza viruses as a rapid alternative to virus isolation (VI) in embryonating chicken eggs and subtyping by HI test. By the method described we can detect not only live virus and also virus that has we been inactivated during shipping or by disinfectants (which may be present in environmental samples). Additionally, all influenza viruses may not readily adapt to grow detectable titers in embryonating chicken eggs. RT-PCR is less expensive than virus isolated, and importantly, results are available much faster.

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