

Detection of avian influenza virus of H9 subtype in the feces of experimentally infected chickens by RT-PCR

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ABSTRACT

Avian Influenza (AI) is a viral respiratory disease of domestic and wild birds. In the diagnostic laboratory, it is essential to have methods for rapid detection of avian respiratory viruses. Cloacal swabs collected from chickens experimentally infected with H9 subtype AI virus, used in a reverse transcription-polymerase chain reaction (RT-PCR) assay for detection of AI. In infected animals, AI viruses are detected most frequently between days 3 and 7 post infection (p.i.). The RT-PCR assay was able to detect, at least, $10^{3.5}$ EID₅₀ of AI viruses in the allantoic fluid. The RT-PCR assay did not show any cross-reactivity with some other avian respiratory viruses. In comparison with virus isolation (VI) assay, the relative sensitivity, specificity, correlation rate and positive predictive value of the RT-PCR were 80%, 84%, 82% and 83%, respectively. The κ index of agreement between the two tests were substantial ($\kappa = 0.64$). The results proved that the RT-PCR assay could be a reliable and rapid alternative to VI assay for detection of AI viruses A H9 subtype H9 in fecal specimens.

Keywords: Avian influenza, RT-PCR, Broiler chickens

INTRODUCTION

Avian influenza (AI) is a viral respiratory disease of domestic and wild birds (Murphy *et al* 1999). Influenza A viruses of subtype H9N2 are now considered to be widespread in poultry and have demonstrated the ability to infect humans (Fedorko *et al* 2006, Liu *et al* 2003, Swayne *et al* 2003). In Iran, H9N2 subtype outbreaks have been frequently recorded since 1998 (Karimi *et al* 2004, Toroghi & Momayez 2006, Nili & Asasi 2003, Vasfi Marandi & Bozorgmehri Fard 1999).

Because mildly pathogenic AI infection in chickens does not cause any pathognomonic clinical sign, the presence of the infection should be determined by diagnostic tests (Spackman *et al* 2002). On the other hand, for laboratory diagnosis of avian respiratory viruses, it is essential to have rapid methods able to detect viruses in early stages of the infection in clinical specimens (Cattoli *et al* 2004). Virus isolation (VI) is regarded as the “gold standard” among different methods for influenza virus detection (Allwinn *et al* 2002, Booth *et al* 2006, Gavin *et al* 2003, OIE 2004). However, as a diagnostic method, VI has some important disadvantages. In this method, samples should be

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transferred quickly to the laboratory and processed immediately to avoid inactivation of the virus (Allwinn *et al* 2002). Virus culture is laborious and results are generally available in four to five days and a definitive negative diagnosis may require culture for up to 3 weeks (Aldous & Alexander 2001, Cattoli *et al* 2004, Gavin *et al* 2003, Gohm *et al* 2000). Therefore, the use of rapid, validated alternative tests for diagnosis of AI could be advisable (Cattoli *et al* 2004). Serological methods can only detect a suspected case of influenza 2-3 weeks post infection and two pair of sera samples should be collected during acute and convalescence periods of disease (Allwinn *et al* 2002). Some new rapid diagnostic kits for influenza can detect viral antigens within 10-30 min and do not require viable virus and, therefore, are less prone to bad conditions of sample storage and transportation (Allwinn *et al* 2002). But, overall, there are controversial results about the satisfactory sensitivity and specificity of these kits (Cattoli *et al* 2004, Davison *et al* 1998, Dominguez *et al* 1993, Fedorko *et al* 2006, Gavin *et al* 2003, Hermann *et al* 2001, Ryan-poirier *et al* 1992, Waner *et al* 1991, Woolcock *et al* 2005). Reverse transcription-polymerase chain reaction (RT-PCR) technology promises to revolutionize influenza diagnosis and monitoring (Swayne *et al* 2003). Compared to VI, RT-PCR assay could be completed within 1 day (Gohm *et al* 2000) and reduces the handling of infectious materials (Spackman *et al* 2002). AI virus shedding in chicken feces has been previously described (Swayne *et al* 2003). The Feces is one of the most important sources of AI virus (OIE 2004). Virus detection in feces allows the detection of AI in live domestic or wild birds. Particularly, in migratory birds, this could be an important tool for detection of latent AI virus infection and epizootiological surveys. According to the above, for detection of avian influenza, RT-PCR method is a rapid and reliable method could be done on fecal samples

and used as an alternative assay to the laborious, time consuming VI. The aim of this study was to set up a rapid, sensitive and specific RT-PCR assay for simultaneous detection and subtyping of H9 subtype of AI virus in fecal specimens. The efficiency of this RT-PCR assay was compared with standard VI assay of AI virus in embryonated chicken eggs.

MATERIALS AND METHODS

Virus strain. Reference strain of A/chicken/Iran/ZMT-101/98 (H9N2) was used for experimental infection of chickens. Intravenous pathogenicity index (IVPI) of this field strain was 0.26 as low pathogenic avian influenza (LPAI) virus (Vasfi Marandi & Bozorgmehri Fard, 2001).

Chickens. Twenty, four-weeks-old commercial broiler chickens never been exposed to avian influenza subtype H9 and had no antibodies to AI, were used in this experiment. The chickens were divided in 2 ten-bird groups including test and control groups.

Experimental design. Chickens of the test group were inoculated via oculo-nasal route with 0.2 ml of infectious allantoic fluid containing $2 \times 10^{7.5}$ EID₅₀ of the applied virus strain diluted in sterile PBS solution (PH 7.2) (day 0 of the experiment). Fecal samples were obtained daily from chickens of the test and control groups until day 10 post infection (p.i.) and stored separately at -70 °C for further analysis. All of the fecal samples collected from chickens of the test group and those collected at days 0, 5 and 10 p. i. from control group were used in VI and RT-PCR.

Fecal swabs sampling. Cotton swabs were used to prepare fecal samples from birds of the test and control groups. Each swab was placed into a tube containing 1ml PBS solution (PH 7.2) containing antibiotics (10.000 IU/ml penicillin, 1 mg/ml streptomycin sulfate, 1mg/ml gentamicin sulfate, 20 IU/ml nystatin).

Hemagglutination test (HI). Blood samples from pre-inoculation, 7th and 14th day p.i. from all chickens, were collected and sera were tested against specific antibodies to H9N2 antigen by using HI-test, according to the manual of standards for diagnostic tests and vaccines (OIE 2004).

Sensitivity and specificity of RT-PCR. The sensitivity of the RT-PCR assay was determined according to the standard method (Villages 1998). Briefly, serial dilutions of the challenge virus in sterile PBS solution ranging from 10^{-1} to 10^{-9} were prepared and inoculated into 9-day-old embryonated chicken eggs. EID₅₀ titration was determined and all dilutions were examined by the RT-PCR assay for detection of AI virus. To evaluate the specificity of the RT-PCR, allantoic fluids containing NDV (La Sota strain) and IBV (H120) were used.

Virus isolation (VI). This was performed according to the standard method (Senne 1998). Briefly, A volume of 0.2 ml of samples prepared for virus isolation, were inoculated into chorioallantoic sac of 9-11-day-old chicken embryonated eggs from healthy mycoplasma free raised chickens. Each sample was inoculated into three eggs and incubated at 37 °C for up to 6 days. Eggs were candled daily and embryos dying within 24 hr post inoculation were discarded. All other eggs with embryos dying were transferred to 4 °C for further testing. Chorioallantoic fluids (CAFs) were harvested, clarified by low speed centrifugation and tested for hemagglutinating activity (HA). All of the HA negative CAFs were inoculated for the 2nd passage.

RNA extraction. Viral RNA was extracted with the High pure viral RNA kit (Roche, Germany) according to the manufacturer's instruction with slight modification. Briefly, in a nuclease free 1.5 ml microcentrifuge tube, 400 μ l binding buffer was added to 200 μ l sample and mixed well. The mixture was left at room temperature for 10 min. The sample was transferred to a filter tube. After centrifugation for 15 s at 8000 g, 500 μ l inhibitor removal buffer was added and the microtube was

centrifuged at 8000 g for 1 min. A volume of 450 μ l washing buffer was added and the microtube was centrifuged at 8000 g for 1 min. Finally, RNA was diluted in 50 μ l elution buffer containing DEPC (diethylpyrocarbonate) by centrifugation at 8000 g for 1 min and quickly used in RT reaction.

RT reaction. For reverse transcription, 200 u M-MVLV reverse transcriptase (Fermentas), 20 u RNase inhibitor (Cinnagen), 4 μ l 5 \times RT buffer, 2 μ l 10 mM dNTPs, 2 μ l Uni-12 primer (20 pmol) and 5.5 μ l DEPC treated water, were added to 5 μ l RNA solution. The Uni-12 primer sequence was: 5' - AGC AAA AGC AGG -3' (peiris *et al* 1999). The mixture incubated at 43 °C for 1 hr and then, heated to 95 °C for 2 min and subsequently chilled on ice.

PCR reaction. For PCR, 1.25 u Taq DNA polymerase (Cinnagen), 2.5 μ l 10 \times PCR buffer, 0.5 μ l 10 mM dNTPs, 1 μ l sense and anti-sense primers (each) (10 pmol) and 16.5 μ l double distilled water were added to 2.5 ml cDNA mixture. The sequence of HA primers was: Sense 5' - TTG CAC CAC ACA GAG CAC AAT-3' and Anti-sense 5' - TGA TGT ATG CCC CAC ATG AA-3' (peiris *et al* 1999). The amplification protocol was: One step of denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 1 min – annealing at 50 °C for 1 min – extension at 72 °C for 1min, and one step of final extension at 72 °C for 10 min. Samples with RNase free sterile water instead of specific template used as negative controls and RNA extracted from the CAF containing challenge virus used as positive control. The 432-bp product was detected by 1.5 % agarose gel electrophoresis.

RESULTS

HI test. All of the pre-inoculation serum samples obtained from test and control groups of chickens, were AI antibody negative. Seroconversion was observed in the test group at days 7 and 14 post

inoculation (p. i.). In the control group, no seroconversion was observed at day 7 and 14 p. i. (Table 1).

Table 1. Seroconversion of the test and control chickens groups related to the day post inoculation (p.i.).

	AI subtype H9 serum antibody titration (Mean \pm SD)		
	Day 0 p.i.	Day 7 p.i.	Day 14 p.i.
Test	0	5.4 \pm 0.8 ^a	6.6 \pm 1.3 ^b
Control	0	0	0

a) CV=15%

b) CV=19.6%

Sensitivity and specificity of RT-PCR. The detection limit of the RT-PCR was determined to be approximately equivalent to $10^{3.5}$ EID₅₀. The RT-PCR did not cross-amplify Newcastle disease virus (La Sota) and infectious bronchitis virus (H120) strains.

Virus detection by virus isolation method. To evaluate AI virus detection by VI method, fecal samples from 10 different birds/days in the test group were screened by VI assay. First positive samples were seen on day 2 p.i. and the last positive sample was detected on day 10 p.i. Most positive results were detected on days 3,4,5,6 and 7 p.i. . The percentage of positive sample to total ranged from 10 to 100 % (Table 2). The fecal samples obtained from chickens of the control group were all negative in VI method.

Table 2. AIV detection by VI and RT-PCR in fecal samples from experimentally infected chickens related to the day post inoculation.

Test	Day p.i.										Total
	1	2	3	4	5	6	7	8	9	10	
VI	0/10 ^b	1/10	7/10	7/10	9/10	10/10	9/10	4/10	1/10	1/10	49/100 (49%)
RT-PCR	0/10	1/10	6/10	8/10	10/10	8/10	6/10	4/10	3/10	1/10	47/100 (47%)

a) Number of positive sample. b) Number of negative sample.

Virus detection by RT-PCR method. Fecal samples from 10 different birds/days in the test group were evaluated by RT-PCR assay, too. First positive samples were seen on day 2 p.i. and the last positive sample was detected on day 10 p.i..

Majority of positive samples were detected on days 3,4,5,6 and 7 p.i. The percentage of positive samples to total ranged from 10 to 100 % (Table 2). The fecal samples obtained from chickens of the control group, were all negative in RT-PCR method. The RT-PCR results of day 7 p.i. are showed (Figure 1).

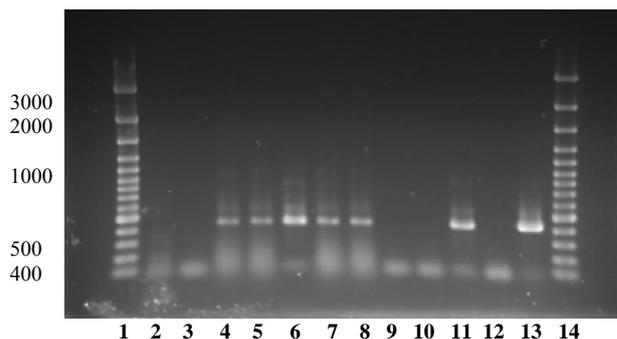


Figure 1. The RT-PCR results of 10 fecal swabs collected at day 6 post inoculation from chickens inoculated with A/Chicken/Iran/ZMT-101/98 (H9N2) avian influenza virus strain. Lane 1 and 14, 100bp marker; lane 2-11, fecal samples from chickens number 1-10, respectively; lane 12, negative control, lane 13, positive control.

Sensitivity, specificity and correlation rate of RT-PCR versus VI method. A total number of 39 out of 100 samples were positive and 43 were negative with both RT-PCR and VI assays. Ten negative samples with RT-PCR were found positive with VI assay. Eight positive samples with RT-PCR were negative with VI assay (Table3). None of the negative controls were positive. Chi-Squared test was used to compare the results of two tests, statistically. The relative specificity, sensitivity, correlation rate and positive predictive value of RT-PCR compared with VI were 84%, 80%, 82% and 83%, respectively. The κ index of agreement between the two tests was substantial ($\kappa = 0.64$).

Table 3. Comparison between VI and RT-PCR assays in fecal swabs of chickens experimentally infected with LPAI A/Chicken/Iran/ZMT-101/98 (H9N2).

	VI - positive	VI - negative	Total RT-PCR samples
RT-PCR positive	39	8	47
RT-PCR negative	10	43	53
Total VI samples	49	51	100

DISCUSSION

RT-PCR method has previously been used to detect influenza A virus in throat and nasal specimens collected from humans, pigs and horses (Claas *et al* 1993, Oxburgh *et al* 1999, Schorr *et al* 1994, Yamada *et al* 1991) and to detect avian respiratory viruses in clinical specimens (Fouchier *et al* 2000, Gohm *et al* 2000, Handberg *et al* 1999, Munch *et al* 2001, Starick *et al* 2000). Gohm *et al* (2000) reported that Newcastle disease virus (NDV) in clinical samples from experimentally and contact infected chickens, could be quickly, easily and reliably detected by the RT-PCR assay until the end of the experiment (day 28 p.i.) in cecal tonsils, kidneys and fecal samples and NDV detection by RT-PCR lasted longer after infection than by VI using embryonated eggs. They suggested that RT-PCR is more sensitive than VI to detect NDV neutralized by specific antibodies. In the study of Gohm *et al* (2000), the RT-PCR assay results of proventriculus and intestine were negative, although fecal samples were positive at the same time. They suggested that fecal sample is a homogenate in itself and can be used for RNA extraction of viruses excreted in feces, without further processing. It is possible that only distinct parts of the intestinal tract contain fecal excreted viruses at a given time, and therefore fecal samples passed through the whole intestine can be enriched with high amounts of the viruses. Furthermore, the viruses originating from kidney is also excreted within feces. Also, because of high bacterial load and toxic substances, it is sometimes difficult to perform virus detection in feces by VI (Gohm *et al* 2000). Therefore, RT-PCR could be a valuable alternative test for detection of avian viruses excreted in gastrointestinal tract in fecal samples. Hermann *et al* (2001) developed a nested multiplex RT-PCR for simultaneous detection and typing of influenza A. Target sequences were located in the HA gene. The efficiency of the RT-PCR assay was evaluated by comparing the results with VI on clinical specimens

collected from influenza suspected patients. Sensitivity and specificity of the RT-PCR compared with VI were 95.4 % and 86.6 %, respectively. These results are significantly higher than results of the present study. This difference could be explained by the extreme sensitive nature of nested PCR assay. However, this extreme sensitivity by itself can emerge some important problem in a diagnostic laboratory. The detection rate of this nested multiplex RT-PCR assay was clearly higher than was found in another HA gene-nested multiplex RT-PCR (Magnard *et al* 1999), but lower than reported from a study using matrix gene as the target (Wallace *et al* 1999). These results suggest that type of the target gene and length of the amplified segment may affect the sensitivity of RT-PCR. Atmar *et al* (1996) applied a RT-PCR assay for detection of influenza A virus on clinical specimens. Compared with VI, the RT-PCR assay had a sensitivity, specificity and correlation rate of 95%, 98% and 97%, respectively. Cattoli *et al* (2004) compared RT-PCR with VI on specimens collected from experimentally and naturally infected birds. On experimentally infected cases, the results indicated that VI assay could detect infectious virus in the tracheal swabs of the infected turkeys from day 3 to 10 post challenges and RT-PCR from day 3 to 12 post challenge. The duration of AI detection by VI and RT-PCR in this study is longer than results of the present study. On field samples obtained from naturally infected poultry flocks, the relative sensitivity, specificity and correlation rate of the RT-PCR assay compared to VI were 95.6%, 96.3% and 88%, respectively (Cattoli *et al* 2004). These above results are substantially higher than the present study results.

Some researchers have reported that RT-PCR results did not correlate as well with VI, as some samples were positive by only one method (Munch *et al* 2001, Spackman *et al* 2002). It seems that differences in the detection rate of AI virus between VI and RT-PCR assays can probably be explained,

at least in part, by what the assays are detecting. RT-PCR, conversely to VI, is able to detect viruses inactivated during transfer or by disinfectants present in environmental samples. It was estimated that noninfectious particles constitute as much as 90 % of some virus preparation (Hirst *et al* 1973). Additionally, all influenza virus isolates may not be readily adapt and replicate to detectable titers in embryonated chicken eggs within two passages (Spackman *et al* 2002). However, false positive results due to cross contamination can cause some samples to be RT-PCR positive and VI negative (Hermann *et al* 2001). On the other hand, some factors may adversely affect the sensitivity of RT-PCR versus VI and inhibit detecting the presence of AI virus. Koch (2003) and Wilde *et al* (1990) reported RT-PCR inhibitory substances in some tissue or fecal samples. Single step RT-PCR method is supposed to be less sensitive than a two-step RT-PCR method (Nakamura *et al* 1993).

In addition, the nature of sample could have great effect on the results of RT-PCR assay. In the field studies, swab samples are generally taken from living birds and organ samples are taken from dead birds. Extracted RNA may be degraded more rapidly in organ samples containing higher levels of RNase, so that swab samples may yield better results. Alternatively, choosing a suitable RNA extraction method could assure the extraction of pure RNA (Spackman *et al* 2002) and decrease the risk of RNA degradation (Horimoto & Kawaoka 1995). Overall, these subjects could explain the varying statistical indices between different studies.

The sensitivity and specificity of PCR-based methods are most critically determined by the choice of primers (Fouchier *et al* 2000), particularly for AI viruses which have a high frequency of variations in the surface glycoprotein genes (Swayne *et al* 2003). Negative and positive controls should be included in the RT-PCR protocol to refuse the possibility of false results (Gohm *et al* 2000, Hermann *et al* 2001). The primers used in the

present study had been chosen from highly conserved area of the viral HA gene by Peiris *et al* (2003). In their study, the sensitivity of RT-PCR assay for detection of H9 subtype of avian influenza using the same primers was about 63 TCID₅₀. These primers had successfully amplified mammalian and avian H9N2 isolates and had not cross-amplified any other human or avian influenza virus subtypes (Peiris *et al* 2003). The sequence of these specific primers set was evaluated by BLAST software in order to assure its annealing efficiency. Regardless of the method chosen for sensitivity determination, it is difficult to ascertain the minimum number of target viruses needed for a detectable PCR product (Hermann *et al* 2001). We recorded a lower analytical sensitivity ($10^{3.5}$ EID₅₀) for the RT-PCR compared with other reports (Cattoli *et al* 2004, Fouchier *et al* 2000, Hermann *et al* 2001). This might be due to the different materials and modified protocols applied in the present study, to different virus strain and subtype tested or to the nature and quality of the samples. The recorded relative sensitivity, specificity and correlation rate in the present study were a few less than those of similar reports (Atmar *et al* 1996, Boivin *et al* 2001, Cattoli *et al* 2004, Hermann *et al* 2001, Steininger *et al* 2002, Taubenberger *et al* 2001). In many instances, field studies reported better relative sensitivity and specificity than experimental studies. It is suggested that the birds submitted for necropsy to a diagnostic laboratory, usually represent birds with the most clinical signs and presumably with the most virus shedding. In contrast, testing of every bird in an animal experiment does not skew samples toward birds with the most virus (Woolcock *et al* 2005). The true specificity of the RT-PCR in the present study and similar reports is probably higher than that was observed. This could be related to a loss of viability of the virus during sample transportation or storage. Serological evidences can confirm that if one case or flock detected positive by RT-PCR, has been

truly infected. The presence of influenza virus in RT-PCR products can be proved by some other assays such as sequencing of amplified product, restriction fragment length polymorphism (RFLP), hybridization, etc.

In conclusion, the RT-PCR assay reported in this study can be used for simultaneous detection and subtyping of AI virus of H9 subtype in fecal sample, as a rapid, sensitive and specific alternative to VI. This assay may be a reliable method for screening AI infected live domestic or wild birds and monitoring commercial flock to detect H9 subtype in early stages of infection. However, monitoring HA gene sequence of new H9 isolates for the detection of probable genetic variations in the primers annealing sites is essential to assure the satisfactory efficiency of the RT-PCR in detection of new isolates.

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