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***Original Article***

# Development and Evaluation of Real-Time Reverse Transcription Polymerase Chain Reaction Test for Quantitative and Qualitative Recognition of H5 Subtype of Avian Influenza Viruses

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**ABSTRACT**

Avian influenza viruses (AIV) affect a wide range of birds and mammals, cause severe economic damage to the poultry industry, and pose a serious threat to humans. Highly pathogenic avian influenza viruses (HPAI) H5N1 were first identified in Southeast Asia in 1996 and spread to four continents over the following years. The viruses have caused high mortality in chickens and various bird species and deadly infections in humans. Multiple conventional methods have been so far introduced for the detection and identification of avian influenza viruses. Traditional virus isolation methods are gold standard protocol in AI detection; nonetheless, virus isolation in embryonating chicken eggs (ECE) is not a rapid method for the detection of influenza viruses since it is time-consuming and labor-intensive. Furthermore, the isolation of highly pathogenic viruses, such as H5, needs BSL3 laboratories. Real-Time Reverse Transcription-Polymerase Chain Reaction (RRT-PCR) is a sensitive and specific method for the detection of influenza viruses. The application of these nucleic acid-based techniques has increased our ability to identify and perform influenza virus care programs, especially in surveillance programs. The current study aimed to detect H5 subtype of avian influenza (AI) virus using fast, specific, and sensitive TaqMan RRT-PCR. Notably, single step RRT-PCR was used to prevent possible laboratory contamination. The specificity of this test was evaluated using nucleic acid extracted from several poultry pathogenic microorganisms and negative clinical specimens from AI-uninfected birds. The sensitivity analysis of the RRT-PCR assay was performed using in vitro-transcribed RNA copy and 10-fold serial dilution of standard AI virus with specific titer. The results indicated the high sensitivity of this method and the lowest detectable dilution of this method based on RNA copies and 1:10 serial dilutions of the standard virus was $10^{1.9}$ $EID_{50}$ /100.

**Keywords:** H5, Avian influenza virus, Real-time RT-PCR

**Développement et Évaluation d'un Test d'Amplification en Chaîne par Polymérase, Couplée à une Transcription Inverse pour la Reconnaissance Quantitative et Qualitative des Virus de l'influenza Aviaire du Sous-type H5**

**Résumé:** Les virus de l'influenza aviaire (AIV) affectent une large gamme d'espèces d'oiseaux et de mammifères, causant de graves dommages économiques à l'industrie de la volaille et constituant ainsi une grave menace pour l'homme. En 1996, les virus de l'influenza aviaire hautement pathogènes (HPAI) H5N1 ont été identifiés pour la première fois en Asie du Sud-Est et se sont répartis sur les quatre continents au cours des années suivantes. Les virus ont provoqué une mortalité élevée chez les poulets et diverses espèces d'oiseaux et des infections mortelles chez l'homme. Pour la détection et l'identification des virus de l'influenza aviaire,

plusieurs méthodes conventionnelles ont jusqu'à présent été utilisées.méthodes conventionnelles ont jusqu'à présent été introduites. Les méthodes traditionnelles d'isolement des virus sont un protocole de référence pour détecter de l'AI. Les méthodes traditionnelles d'isolement des virus représentent le protocole de référence pour détecterces virus. Cependant, l'isolement du virus à partir des œufs de poule embryonnésreste une méthode laborieuse et consommatrice en temps. De plus, l'isolement des virus hautement pathogènes (H5), nécessite des laboratoires BSL3. Le test d'amplification en chaîne par polymérase, couplée à une transcription inverse (RT-PCR) en temps réel est une méthode sensible et spécifique de détection des virus de l'influenza. L'application de ces techniques à base d'acide nucléique a augmenté notre capacité à identifier et à exécuter des programmes de soins pour des virus de l'influenza, en particulier dans les programmes de surveillance. La présente étude visait à détecter des virus de l'influenza aviaire du sous-type H5 à l'aide d'une RT-PCR TaqMan qui permet une étude rapide, sensible et spécifique. Notamment, pour prévenir les cas de contamination en laboratoire, la mise en place d'une procédure de PCR en une seule étape a été privilégiée. La spécificité de ce test a été évaluée à l'aide d'acide nucléique extrait de plusieurs micro-organismes pathogènes de volaille et des échantillons cliniques négatifs provenant d'oiseaux non infectés par l'AI. L'analyse de sensibilité du test RT-PCR a été réalisée en utilisant une copie d'ARN transcrit in vitro et une dilution en série de 10 fois du virus AI standard avec un titre spécifique. Les résultats ont démontré la sensibilité élevée de cette méthode et une capacité de détecter desconcentrations très faibles d'ARN dansles dilutions en série 1:10 du virus standard (jusqu'à $10^{1.9}$ $EID_{50}$ /100).
**Mots-clés:** H5, Virus de L'influenza Aviaire, PCR en Temps Réel

## INTRODUCTION

Influenza A viruses cause infection in different birds and some mammals. They are categorized into two distinct groups, namely low-pathogenic AI (LPAI) and highly pathogenic AI (HPAI), on the basis of their ability to cause disease in susceptible poultry. So far, all virulent viruses (HPAI) have been reported from H5 and H7subtypes; nonetheless, all of these viruses do not cause HPAI (Fouchier et al., 2005). There has been a worldwide increase in the number of reported outbreaks of HPAI in the last decades. In most of those outbreaks, viruses spread rapidly necessitating diagnostic methods for fast and accurate identification and/or subtyping for the implementation of successful outbreak control and management strategies. Various efficient methods have been developed for the detection of avian influenza virus, as well as continuous surveillance of domestic, migratory, and farmed bird. According to OIE-World Organization for Animal Health, virus isolation is recognized as the traditional gold standard method in typing and subtyping influenza A viruses. This technique is followed by NA and HA subtyping procedures (OIE, 2016). Serological tests are also used to detect influenza virus infection; nonetheless, both of these methods cannot identify influenza viruses of novel subtypes. Virus isolation needs embryonated chicken eggs (ECE) or tissue culture; therefore, it is a time-consuming and labor-intensive technique. Other diagnostic tests, such as antigen-based assays, have a relatively low sensitivity and require high amount of sample for detection. In general, these traditional methods have limitations and disadvantages; consequently, molecular-based methods have been established for accurate and early identification or control of influenza A virus infections. They have rapidly transitioned from basic research to detection and surveillance formats and are now substantial tools in the early and sensitive recognition of influenza viruses in both human and animal samples (Wang and Taubenberger, 2010). Real-time polymerase chain reaction (PCR) assay for the detection of type A, H5, and H7-specific nucleic acid sequence was described by Spackman et al. (2002). Lee and Suarez, (2004) reported that there are a good correlation between the influenza Ribonucleic acid (RNA) quantity

evaluated in quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RRT-PCR) and the EID50 determined by virus isolation in embryonated chicken eggs method. In addition, they indicated that the RRT-PCR has a high sensitivity and specificity in detection. Given the increasing role of RRT-PCR in AI detection methods as a front-line, we decided to develop and validate one-step Real-Time PCR assay for the recognition of H5Subtype Avian Influenza Virus. The TaqMan approach is used in most of real-time RT PCR assays in influenza A virus studies. In this approach, a probe will hybridize to an internal region of the final product; therefore, the highest sensitivity and specificity can be achieved during the procedure. The current study aimed to perform a TaqMan Real time RT-PCR for detecting H5 subtype of avian influenza (AI) viruses with high sensitivity and specificity.

## MATERIAL AND METHODS

**Virus and antigen.** Standard H5 virus with specified titer brought from *Padova* influenza reference laboratory, ITALY was used for the development and validation of the RRT-PCR test for H5 viruses. RNA Extraction: RNA was extracted using the "High Pure Viral Nucleic Acid Kit" (Qiagen, Germany) based on company principles. Purity and stability of the RNA were evaluated by spectrophotometry. The extracted viral RNAs were stored at -80 °c up to usage.

**Primers and probes.** Primers and probes that were used in the present study were according to previous research conducted by Coker et al. (2014). Primers and probes were sent to MWG Company, Germany, for production (Table 1).

Table 1. Primer and probe sequences

| primer /probe | **Sequence (5´ to 3´)** |
|---|---|
| Primer (For) | TTATTCAACAGTGGCGAG |
| Primer (Rev) | CCAG(T)AAAGATAGACCAGC |
| probe | CCCTAGCACTGGCAATCATG |

**Real-time reverse transcription polymerase chain reaction.** Effective components in RT-PCRs include probe and primer concentration, different materials, and thermal conditions that need to be optimized. Therefore, we performed the multifarious tests to determine optimum concentration for probe and primers, as well as optimal thermal cycles. Accordingly, the first runs of the experiment were conducted according to the default conditions recommended by the manufactures. Nonetheless, further optimizations were considered to achieve the best results by the adjustment of each of above-mentioned components. The specificity of the primer/probes was investigated on a diverse spectrum of pathogen nucleic acids that may be naturally present in avian origin samples, such as Massachusset and 793B strains of Infectious Bronchitis virus, vaccinal and wild strain of Newcastle disease virus, pneumovirus, infectious bursal disease, and chicken infectious anemia viruses (*department of viral poultry diseases, Razi Vaccine and Serum Research Institute, Karaj, Iran*). On the other hand, the sensitivity of the assay was determined using in vitro-transcribed RNA copies and 10-fold serial dilutions of the standard influenza A virus (*Padova* influenza reference laboratory, *Italy)*. In the present study, real-time RT-PCR was run in a Rotor Gen 3000 apparatus (Corbett, Australia) using a one-step kit (Quanti Tect Multiplex RT-PCR Kit, Germany). The Final volume of real time reactions was 25 µl consisting of 12.5µl 2x QuantiTect Probe RT-PCR Master Mix, 0/8µl of each forward and reverse primers (10 Pmol/ml), 0/4µl of probe (10 Pmol/ml), 0/5 µl of RT-PCR Enzyme Mix, 7.5µl of nuclease free water, and 2µl of RNA. The RRT-PCR was performed in the following conditions: RT at 50 °c for 30min and 95 °c for 15 min, followed by 45 PCR cycles at 94 °c in 15 sec then 60 °c for 45 sec.

## RESULTS

**Sensivity and limit of detection.** To figure out detection limit of the assay, we used standard H5 virus with specified titer from *Padova* influenza reference laboratory, *Italy*. Threshold cycle (Ct) values were regarded as the point at which an amplifying curve

crossed the threshold line. From diagnostic point of view, the sample was recognized as positive since its amplifying curve crossed the threshold line. The limit of detection (sensibility) was $10^{1.9}$ $EID_{50}/100$ which reflected the lowest virus concentration detected in the assay (Figure 1).

**Specificity.** The specificity of the primer/probes was investigated on a diverse spectrum of nucleic acids from pathogens that may be naturally present in avian origin samples, as well as a negative population of clinical specimens derived from AI-uninfected birds. None of those mentioned viruses nucleic acids were amplified in the present study using the H5 primers and probe.

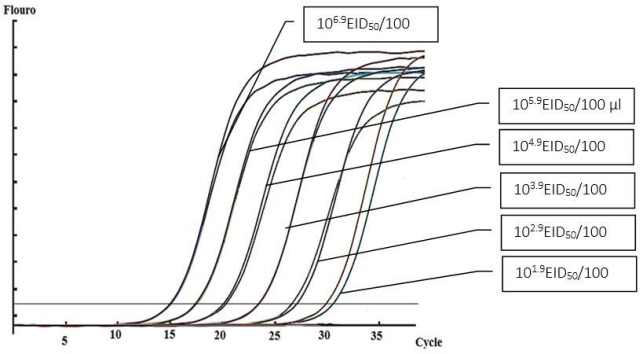


**Figure 1.** Amplification curves of consecutive concentration of viral infectious dose avian of H5 influenza strain used in this study.

## DISCUSSION

Molecular diagnostic tests used for avian influenza detection and the virus characterization have undergone remarkable development in the past few years. These molecular methods are useful tools for surveillance programs and in outbreaks when their early detection is vital. The present experiment revealed that real time RT-PCR is a suitable replacement for the recognition of highly pathogenic influenza viruses by virus isolation methods. As evidenced by the results, high sensitivity with $10^{1.9}$ detection limit was obtained. Moreover, excellent results were achieved in specific and sensitive detection of H5 subtype. The repeatability of the H5 RRT-PCR was evaluated using three different concentrations (high, medium, and low) of viral subtype. The coefficients of variation within runs (intra-assay variability) were within the range of 0.12-2.64%. In addition, inter assay variability index was reported to be within the range of 2.76-4.06%. In the study performed by Slomka et al. (2007), a RRT–PCR was established for the detection of Eurasian H5 virus isolates. Specificity was assessed by testing representative isolates from all other AI virus subtypes, non-AI avian pathogens, as well as a negative population of clinical specimens derived from AI-uninfected wild birds and poultry. Notably, all were negative by H5 Eurasian RRT-PCR. They demonstrated that, H5 Eurasian RRT-PCR was invaluable in H5 outbreak diagnosis and management due to its rapidity and high degree of sensitivity and specificity. Pasick (2008) reported the protocol of H5 and H7 detection and primer/probe sets of avian influenza reference laboratory, Southeast Asia branch, OIE. The test had a detection limit of $10^3$ to $10^4$ gene copies. The original H5 assay was able to detect only North American and Eurasian H5 viruses; therefore, molecular assay underwent some modifications to detect Eurasian H5N1 (Slomka et al., 2007). Parmley et al. (2008) used RRT-PCR by h5 and M primers for Wild Bird Influenza Survey in Canada in 2005. In the mentioned study, RRT-PCR detected matrix protein (M1) gene sequence of influenza A virus in 37% and H5 sequence in 5% of 4,268 wild ducks samples. In the study conduected by Elvinger et al. (2007), the analysis of data from the low pathogenic AI H7N2 outbreak in Virginia in 2002 showed diagnostic sensitivity of the RRT-PCR of 85.1%. In addition, RRT-PCR relative to VI diagnostic sensitivity and the diagnostic specificity relative to virus isolation method was 98.9%. Heine et al. (2007) described a one-step RRT-PCR assay using Taq Man probe for the rapid detection of M1 protein of all AI viruses and H5 hemagglutinin gene of the Eurasian lineage of influenza, including the identification of Eurasian lineage H5N1. The assays were sensitive and quantitative over a $10^5$-$10^6$ linear range and detected all of the tested AI viruses. Consequently, RRT-PCR tests can be used for the sensitive and certain detection of viruses, such as H5

AI virus, within hours which is vital for fast outbreak control measures**.** Hoffmann et al. (2007) described another RRT-PCR procedure method which was designed to detect cleavage site of HPAIV H5N1 of the Qinghai lineage. Monne et al. (2008) described a new one-step RRT-PCR procedure for the simultaneous detection of H5, H7, and H9 subtypes in avian origin clinical samples. They determined the sensitivity of the RRT-PCR assay by using in vitro-transcribed RNA and 10-fold serial dilutions of titrated AI viruses. The obtained sensitivity level and limits of detection ranged from $10^{1.6}$ to $10^{3}$ RNA copies and from $10^{1}$ 50% egg infectious dose ($EID_{50}$)/100 µl to $10^{2.74}$ $EID_{50}$/100 µl with titrated viruses. Regarding the detection of the partial H5 gene segment, Abdelwhab et al. (2010) described a modified protocol from a previous RRT-PCR protocol for Egyptian H5N1 divergent viruses. The detection limit of 10 mean embryo infective dose ($EID_{50}$) was found in the mentioned study. An RRT-PCR method was tested by Kim et al. (2013) for the detection of H5 and H7 matrix genes from field samples obtained through AI surveillance in South Korea over the last four years. Zhang et al. (2017) developed a RRT-PCR assay for the detection of H5 - AIV based on HA gene of H5 AIV and NP gene of AIV. They successfully established a Taqman-MGB method. The specificity of the test was evaluated by using H1-H11 subtype AIVs nucleic acids and some other avian pathogens that may be naturally present in avian samples, including NDV, IBDV, IBV and MDV. Test sensitivity was determined using serial dilution mixture of HA and NP plasmids as quantitative standards for the calculation of copy numbers. In H5 detection tests, the detection limit of different clades was from 0.021 to 0.1 based on $EID_{50}$.

As a conclusion, the one-step RRT-PCR method that was run and set up in the current study had high sensitivity and specificity. Our RRT-PCR may provide a rapid and accurate method for monitoring or detection of HPAI samples in large volume. RRT-PCR protocol developed for the detection of H5 subtype is believed to be applicable in surveillance or monitoring programs in live poultry markets, poultry industry, and wild bird population. Furthermore, we can conveniently use this protocol for rapid detection and identification of the pathogen in HPAI outbreaks.

## Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Authors' Contribution

Study concept and design: Mirzaei, S.G.; Shoushtari, A.; Noori, A.
Acquisition of data: Mirzaei, S.G.
Analysis and interpretation of data: Mirzaei, S.G.,
Drafting of the manuscript: Mirzaei, S.G.
Critical revision of the manuscript for important intellectual content: Mirzaei, S.G., Shoushtari, A., Noori, A.
Statistical analysis: Mirzaei, S.G., Shoushtari, A., Noori, A.
Administrative, technical, and material support: Mirzaei, S.G., Shoushtari, A.

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