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Original Article

Development and Evaluation of Real-time RT-PCR Test for the Quantitative and Qualitative Recognition of Current H9N2 Subtype Avian Influenza Viruses in Iran

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ABSTRACT

Avian influenza H9N2 subtype viruses have had a great impact on Iranian industrial poultry production economy since introduction into the country. Rapid and precise identification of the viruses, as well as proper management and epidemiologic studies accompanied with other control measures in poultry industry are of the most important tools for the control and prevention of the disease. To approach this, a real time probe based assay was developed to directly detect a specific influenza virus of H9N2 subtype, which has been endemic in the country over the past two last decades. To prevent laboratory contamination events, it was decided to run a single step rRT-PCR procedure. An Iranian avian influenza virus strain of A/Iran/chicken/772/1998 H9N2 subtype was selected as the reference strain for primers and probe designing. The high agreement value of 99% indicated that the devolved real time assay for the detection of H9 subtype viruses could easily replace the conventional method of virus isolation, particularly in the investigation of viruses like national surveillance plan. The limit of detection was almost one EID50, which was the least real infectious unit that could be detected. Thus, it can be said that this sensitive assay provided provides a powerful tool to not to miss any significant viral biological activity neither in the host body nor in the environment. A high level of correlation coefficient (R2=0.998) also indicated a good correlation between cycle of threshold values and viral concentrations. It can be concluded that the real time RT-PCR could easily replace virus isolation in the detection of H9N2 influenza viruses, especially in large monitoring program. The ability to quantify the virus concentration extends the usage of this test in more accurate studies.

Keywords: Real time-PCR, Detection, Avian influenza, Comparison, H9N2, Culture

Développement Évaluation du Test RT-PCR en Temps Réel pour la Reconnaissance Quantitative et Qualitative des Virus Actuels de l'Influenza Aviaire du Sous-type H9N2 en Iran

Résumé: Les virus appartenant au sous-type H9N2 de l'influenza aviaire ont eu un impact considérable sur l'économie et production de l'industrie aviaireiranienn depuis leur introduction dans le pays. L'identification rapide et précise des virus, ainsi que des études épidémiologiques et de gestion appropriée accompagnées d'autres mesures de contrôle dans l'industrie aviaire constituent les outils les plus importants pour le contrôle et la prévention de la maladie. Pour y parvenir, un test basé sur une sonde en temps réel a été développé pour détecter directement un virus grippal spécifique du sous-type H9N2, endémique dans le pays au cours des deux dernières décennies. Pour prévenir les cas de contamination en laboratoire, la mise en place d'une

procédure de PCR en une seule étape a été privilégiée. Une souche iranienne du virus de la grippe aviaire de type A / Iran / chicken / 772/1998 H9N2 a été sélectionnée comme souche de référence pour les amorces et la conception des sondes. La valeur d'accord élevée de 99% indique que le test en temps réel décentralisé pour la détection des virus du sous-type H9 pourrait facilement remplacer la méthode conventionnelle d'isolement du virus, en particulier dans les études portant sur les virus menées dans le cadre des programmes de surveillance nationaux. La limite de détection était presque d'une EID50, représentant l'unité infectieuse minimale pouvant réellement être détectée. Ces résultats montrent donc que ce test sensible fournit un outil puissant pour la détection de toute activité virale significative, aussi bbien dans l'organisme hôte que dans l'environnement. Le niveau élevé du coefficient de corrélation (R2 = 0,998) indique également une bonne corrélation entre les valeurs du cycle de seuil et les concentrations virales. On peut en conclure que la RT-PCR en temps réel pourrait facilement remplacer les techniques d'isolement du virus dans la détection des virus de la grippe H9N2, en particulier dans les grands programmes de surveillance. La capacité de quantifier la concentration du virus est augmentée et permet donc l'utilisation de ce test pour une surveillance plus précises.

Mots-clés: PCR en Temps Réel, Détection, Grippe Aviaire, Comparaison, H9N2, Culture

INTRODUCTION

Avian influenza (AI) is a viral respiratory disease or infection in wild and domestic birds caused by type A influenza viruses belonging to Orthomyxoviridae family (El Zowalaty et al., 2013). This type or genus has being increased public health significance and also causing a great economic losses in the poultry industry (Tahir et al., 2015). According to antigenic differences of two surface glycoproteins, influenza A viruses are serologically categorized and subtyped into 18 hemagglutinins (HA) (H1-H18) and 11 neuraminidases (NA) (N1-N11) subtypes (Tong et al., 2012; Tong et al., 2013). The HA protein is responsible for attachment and fusion steps which eventually leading to virus replication to take place. In addition, influenza viruses show the greatest variation in HA protein and the occurrence of new epidemics depending on the structural variations of a novel strain in this protein (Fouchier et al., 2005). Since 1998, H9N2 avian influenza virus of low pathogenicity was rapidly distributed in commercial chicken farms (Vasfi Marandi and Bozorgmehrifard, 1999). Thereafter, it has caused outbreaks in commercial broiler chickens in Iran (Nili and Asasi, 2003). Rapid and precise isolation and identification of the viruses, proper management and epidemiologic studies, good quality of vaccines, biosecurity programs, and hygienic measures in poultry industry are important in the successful control and prevention of the disease (Wright et al., 2007). Currently, many methods are being used for the detection of AI viruses. Virus isolation in embryonated specific-pathogen free (SPF) eggs is usually considered as the standard detection method (van Elden et al., 2001). However, this method is not applicable in many laboratories due to being time consuming and requiring sophisticated materials and facilities such as SPF eggs (Di Trani et al., 2006; Ellis et al., 2007). Therefore, there is always a need for highly sensitive and specific methods for the detection of AI particularly as a part of control measure (Boivin et al., 2001; Herrmann et al., 2001). Molecular methods are often designed to amplify an individual segment of a target gene (such as HA) after recognizing a specific sequence within the same gene. However, because of the continuous mutation process of AI genes and proteins, particularly HA, the sequence complementarity within the primer and probe-binding regions is often not achieved resulting in many IA viruses to remain undetected. Therefore, it is important to design primers and probes considering gene sequences of the current AI viruses in the country. The purpose of this study was to develop and evaluate a detection method for the diagnosis of AI virus subtype H9N2 viruses with high sensitivity and

specificity, particularly for current virus strains in Iran. A real-time (RT)-PCR assay using the TaqMan probe technique for the detection of H9N2 subtype was established and compared with conventional embryonated egg-based culture method in detection of these viruses.

MATERIALS AND METHODS

Virus strains. A H9N2 influenza virus strain A/chicken/Iran/772/1999 with the accession number of GQ497120.1 in GenBank of the National Center of Biotechnology Information (NCBI) was used as the reference strain to set up the test. The virus was isolated in Razi Vaccine and Serum Research Institute. The virus was propagated in 10-day-old SPF chicken embryonated egg following the method described by Office International des Epizooties (OIE). The virus titer was calculated by the equation of Reed and Muench. The various viral antigens and viruses were also used as the controls of test specificity through the establishing process.

Viral RNA extraction. Viral RNA extraction of the reference strain and all controls was accomplished by using High Pure Viral Nucleic Acid Kit (Roche, Germany) following the manufacturer's instructions. Viral RNAs were extracted from 200 μL of each specimen, which was finally collected in a final volume of 50 μL RNase-free water. The extracted viral RNAs were allocated before storing at −80 °C.

Probe and primers design. To design the real-time primers and probe, at least 100 hemagglutinin sequences were recruited from the GenBank of the National Center of Biotechnology Information (NCBI). All the selected sequences were aligned using DNASTAR software (DNASTAR, Inc, USA) to recognize conserved regions for primers and probe design (Primer 3 software, USA). For more confirmation, a comprehensive alignment analysis was performed using nucleotide BLAST (http://www. ncbi.nlm.nih. gov/). The 5 and 3 ends of the probe were labeled with FAM and TAMRA reporters, respectively (Table 1).

Table 1. Primer and probe sequences

primer/ probe	Sequence (5 ´to 3´)
Primer (For)	CCTGCTAGATCAAGTAGAG
Primer (Rev)	GAGTTGAATCCCTATCTGCA
probe	CATAGCTGGATTCATAGAAGGAGGTT

Real-Time RT-PCR. In this study, real-time RT-PCR was carried out in a Rotor Gen 3000 apparatus (Corbett, Australia) using a one-step kit (Quanti Tect Multiplex RT-PCR Kit, Germany). The first runs of the experiment were performed according to the default conditions recommended by the manufactures. Further optimizations were considered to achieve best results by the adjustment of each component including the concentration of probe and primers and different thermal conditions. The final volume of real time reaction was 25 µl consisting of 12.5µl of 10x RT-PCR buffer, 1 µl of each forward and reverse primer (100 M), 10l of probe H9PRO (3 µM), 1 µl of RT-PCR Enzyme Mix, 6.5 µl of nuclease free water, and 20l of RNA. The RT-PCR conditions ran at the following condition: RT at 50 °C for 30 min (m) and 95 °C for 15 m, followed by 45 cycles of PCR at 94 °C for 15 s and at 60 °C for 45 s.

Standard virus titers preparation and sensitivity. The median egg infectious dose (EID50) of each of the viruses used in this study was calculated according to the Reed and Muench formula. The standard curve was established on 10-fold serial consecutive concentration of viral RNA with duplicate testing from 106.2 to 101.2 EID50/100 µl of allanthoic fluids harvested from inoculated SPF eggs, by which efficiency and detection limitation (as a reflect of sensitivity) of the rRT-PCR procedure were also measured.

Specificity of real-time RT-PCR assay. The specificity of the primers and probe was evaluated on the nucleic acid extracted from different viruses and antigens. The following antigens and viruses were also used as controls of specificity of the test through establishing H5 and H7 avian influenza viruses (antigen), 793/B and Massachusetts types of infectious bronchitis virus (IBV), Newcastle disease virus (B1 strain), avian metapneumovirus, and infectious bursal disease virus (IBDV).

Agreement analysis of real-time rRT-PCR assay and virus isolation (VI). To investigate the agreement between VI and rRT-PCR assay results, 185 samples were tested by both methods. The samples of different areas of the country were previously collected in Avian Viral Research Department of Razi Vaccine and Serum Research institute as a routine diagnostic program. The VI results were confirmed by HI. The agreement was measured as a total agreement (%) and Cohen's K values (Thrusfield, 2008).

Table 2. Comparison Real-Time RT-PCR method with culture method

Number	VI/HI	Real-Time RT-PCR
147	+	+
29	-	-
9	-	+
0	+	-

RESULTS

Standard curve and limit of detection. To understand the limit of detection of the assay, avian influenza H9N2 virus strain A/chicken/Iran/772/1999 was propagated in SPF embryonated eggs and RNA was extracted from six 10-fold dilutions of the original allantoic fluid with a viral load of EID 506.2 /ml (Figure 1).

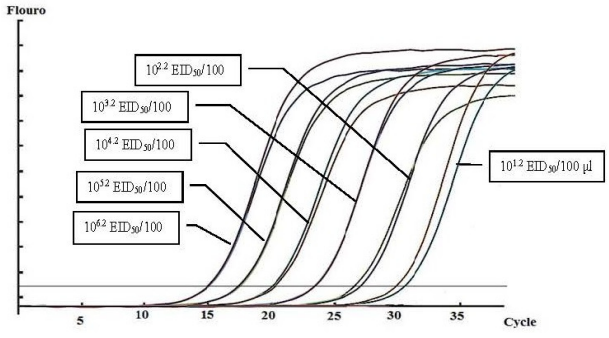

Figure 1. Amplification curves of consecutive concentration of avian viral infectious dose of H9N2 influenza strain used in this study.

Threshold cycle (Ct) values were considered as the point at which an amplifying curve crossed the threshold line. From a diagnostic point of view, the sample is known positive as its amplifying curve crosses the threshold line. The correlation coefficient (R2) was 0.99558, which reflected the consistency of the replicates. The limit of detection (senility) was EID501.2 /reaction, which reflected the lowest virus concentration detected in the assay (Figure 2).

Comparing of real time assay and virus isolation. To evaluate the accordance of real time assay and virus isolation in this study, 185 samples were tested for the detection of H9 influenza viruses by both mentioned methods. Of the 185 samples, 147 and 29 samples were positive and negative for both tests, respectively. More details of the comparison of the two tests are presented in Table 2, which showed an agreement of 93.13% for the detection of H9 influenza viruses. Furthermore, the Cohen's K coefficient was 0.82, which is a good result in terms of comparison of the relationship of the two tests.

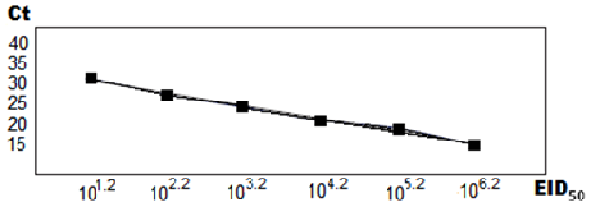

Figure 2. Standard curve generated by real-time RT-PCR. X-axis: log virus concentration; y- Axis: Ct value.

DISCUSSION

Avian influenza a H9N2 influenza viruses have had a great impact on the Iranian industrial poultry production economy since its introduction in 1998 into the country (Vasfi Marandi and Bozorgmehrifard, 1999; Nili and Asasi, 2003). Despite low pathogenicity and wide use of vaccination against H9N2 influenza viruses, sometimes the associated mortality is high, especially in broiler farms (Vasfi Marandi and Bozorgmehrifard, 1999; Nili and Asasi, 2003; Kariminejhad and Mehrabanpour, 2012). That is the reason that makes the detection of H9N2 viruses very important and useful (Hablolvarid, 2016). Virus isolation (VI) in eggs is generally accepted as the most

dependable method for the detection of influenza viruses. However, this method is time consuming and needs facilities that are not available in many laboratories (Di Trani et al., 2006; Ellis et al., 2007). In recent years, molecular tests have been verified as conceivable tools for diagnostic purposes in viral and bacterial fields. It has been proven that molecular methods are very precious tools in tracking and monitoring of viral agents. This study dealt with the necessity and process of developing a sensitive, swift, and relatively economic molecular method for the detection of H9N2 influenza viruses, especially for large-scale screening through surveillance programs. A real time probe base assay was developed to directly detect a specific influenza virus instead of general detection of influenza A viruses, which have been endemic in the country over the two last decades. To prevent laboratory contamination events, it was decided to run a single step rRT-PCR procedure (Di Trani et al., 2006). Due to the lack of proof reading trait of RNA dependent RNA polymerase of Influenza A viruses, circulation and continuous replication and accompanying selective pressure result in a great variation in genes and proteins, especially in two antigenic glycoproteins of HA and NA of these viruses (Tong et al., 2013). This phenomenon also includes the H9 subtype viruses as they are phylogenically divided into two distinct lineages of Eurasian and North American (Alexander, 2003, 2007). This divergence could be the cause of false negative results of any primer-based molecular method if it would not be considered in choosing the reference strain in the primers and probe designing. To avoid this, in the current study an Iranian avian influenza virus strain of A/Iran/chicken/772/1998 H9N2 was selected as the reference strain. The probe and primers were picked up based on conserved regions on the HA gene. To determine whether the developed assay could be used as an alternative method for virus isolation, the agreement of the two methods was analyzed. The high agreement value of 99% indicated that the developed real time assay for the detection of H9 subtype viruses could easily replace the conventional method of virus isolation, particularly in mass investigation like the national surveillance plan. However, considering the potential of change in influenza H9 viruses or probable introduction of new strains into the country, the ability of the assay in the detection of current virus strain(s) must be continuously assessed (Ben Shabat et al., 2010). The limit of detection and standard curve results indicated the ability of real time PCR in quantifying H9 viruses. Many studies of developing a real time PCR assay for virus quantification were carried out based on the measurement of viral particles or proteins weight; however, quantifying these two values might not be a direct reflection of biological ability of H9 viruses in infecting the host. In this study, it was determined to develop a real time RT-PCR assay to measure the biological activity of H9 influenza viruses in infecting a certain host as the infectious dose of 50, which has been traditionally used for this purpose. The limit of detection was almost one EID50, which was the least real infectious unit that could be detected. Therefore, it can be said that this sensitive assay provides a powerful tool not missing any significant viral biological activity neither in the host body nor in the environment. A high level of correlation coefficient ($R2 = 0.998$) also indicated a good correlation between Ct values and viral concentrations. It means that the infectious biological activity of the virus can be computed in any medium, live or non-live. This kind of assay could be very useful in vaccine efficacy control of so-called killed viral vaccines. Furthermore, measuring of virus concentration in environment might shed a light on the level of risk imposed on poultry farms (Hoffmann et al., 2007). Although the applicability of real time PCR assay in measuring of viral load could be theoretically extended, it must be noted that the reality of usage should be examined first.

Regarding all the addressed advantages of such a sensitive and specific assay, it can be concluded that real time RT-PCR could easily replace virus isolation in the detection of H9N2 influenza viruses, especially in large monitoring programs. The ability of the assay

in quantifying virus concentration extends its usage in more accurate quantitating studies.

Ethics

I 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.

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