Comparison of real-time PCR with PCR assays for identification of H9N2 influenza A viruses

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Abstract

Rapid detection of the influenza viruses is basic means for preventing widespread transmission. In this study a TaqMan real-time PCR was compared with PCR assay for identification of H9N2 influenza viruses. A total of 21 chicken's fecal samples were analyzed for H9N2 influenza genome. Overall, 15 and 12 samples were positive in the real-time PCR and PCR assays, respectively. The amplified virus genome was detected by real-time PCR within 2 hours compared to 3 hours with PCR assay. The results showed that the real-time PCR had a higher performance regarding the detection of influenza as compared to the PCR method.

Keys word: H_9N_2, Influenza A, PCR, RT-PCR

Introduction

Influenza viruses are segmented negative-sense single stranded RNA viruses that belong to the family *Orthomyxoviridae*. Influenza viruses are unique among respiratory viruses with their segmented genome and great antigenic diversity. Influenza viruses are classified into three distinct genus based on serologic reactions of the internal proteins, principally NP and M1 proteins: A, B and C. All avian influenza viruses are Influenza virus A or type A. Type A influenza viruses are divided into subtypes based on the antigenic relationship in the surface glycoproteins haemagglutinin(H) and neuraminidase (N). To date, 16 H subtype and 9 N subtype have been recognized. Avian influenza viruses can be divided into two distinct groups on the basis of their ability to cause diseases in the poultry. Highly pathogenic avian influenza (HPAI) and non- Highly pathogenic or mildy pathogenic avian influenza (MPAI).

H9N2 subtype of avian influenza viruses (AIV) is widespread in domestic poultry in many Asian countries (Alexander, 2007). The virus was first reported in 1998 in a layer farm in Tehran (Vasfi Marandi and Bozorgmehrifard, 1999) and also caused widespread outbreaks in commercial broiler chickens in Iran (Nili & Asasi, 2003). The H9N2 is reported as LPAI virus but has caused severe economic losses. Currently, the virus is endemic in the Iranian poultry population. Avian influenza viruses shed from infected birds in the environment and pose potential threat to human health and environment. Rapid detection of the virus is basic means for preventing widespread transmission of the disease.

A definitive diagnosis of influenza is established by isolation and identification of virus and direct detection of viral proteins or genes in specimens. The preferred method for isolation and identification of influenza viruses has been 9-11-days-old embryonating chicken eggs inoculated via the allantoic sac (Swayne, 1998) but it is time consuming and laborious assay and might not be sensitive especially when the quantity of virus is low in the samples. Molecular methods have been described that are up to 100-fold more sensitive than virus isolation procedures. This technologies promise to influenza diagnosis and monitoring. The objective of the present study was to comparison of PCR with real-time PCR (RRT-PCR) assays for identification of H9N2 influenza viruses.

Materials and methods

Challenge Virus

Avian influenza virus, A/chicken/Iran/772/1998(H9N2) passaged two times in 9 to 11-days old emberyonated chicken eggs and used as a challenge virus. The embryo infective dose (EID50) of infected allantoic fluid was calculated according to the Reed and Muench formula (Reed & Muench, 1938). The virus was diluted 10 fold in sterile phosphate buffered saline (PBS) solution to obtain concentration of 10^6 EID50/100µl.

Experimental design

At the age of 30 days, 21 one-day-old commercial broiler chicks were challenged intranasally (IN) with 100µl allantoic fluid containing 10^6EID50. Prior to challenge, all birds were tested using RRT-PCR to confirm negative to AI virus. The chickens were monitored daily for 15 days for identification of H9N2 influenza viruses. On days 3, 7 and 15 post inoculation (PI) seven chickens were randomly selected. The feces were collected separately for virus detection with RRT-PCR and PCR assay. All samples were immediately stored at -70 °C until used.

RNA isolation

RNA was extracted from 140µl of the supernatants of 10% (w/v) fecal suspensions using the QIAamp®Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol. Viral RNA was suspended in 60µl DW.

RT-PCR

The cDNA was synthesized using AccuPowder®RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's protocol. The primers are show in Table 1. Briefly, 5µl of total RNA and 20pmol of primers were used for cDNA preparation. The AccuPower PCR PreMix kit was used with a 20µl reaction mixture containing 1.5 mM MgCl2, 30 mM KCl, 10mM tris- HCl, 250µM (each) dNTP and 1U DNA Polymerase (BioNeer Corporation, South Korea). Five µl of cDNA and 10pmol of each primer were used. The reaction mixture was subjected to 35 cycles of 94ºC, 53ºC and 72ºC each for 1min, followed by a final extension at 72°C for 10min. The PCR products were resolved in 1% (w/v) agarose gel containing ethidium bromide and visualized under UV illumination.

Real time PCR

The cDNA of samples was carried out using AccuPowder® RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's protocol. Reaction was performed with a mixture of 20pmol random hexamer and 20pmol of primer (Table 1). The reaction mixture was incubated at 70°C for 5 min, then incubated at 42ºC for 60min, heated to 95ºC for 5min, cooled to 4ºC then stored at -70ºC until used. The real-time PCR primers and TaqMan probe were shown in Table 1. The primers amplified a 104bp fragment in the M1 gene of influenza A. The assays were performed on a 48-well microtitre plate of BIO-RAD MiniOpticonTM System. The reaction mixture contained 5µl of target cDNA, 1µl of each primer at concentration of 10pmol/µl, 0.6µl of the TaqMan probe at concentration of 10pmol/µl, 2.5µl of dUTP mix, 2.4µl of MgCl2 at concentration of 50mM, 0.2µl of each Uracil DNA Glycosylase (UDG) and Taq polymerase enzyme, and 2µl of x10 buffer in a final volume of 20µl. The reaction was subjected at at 50ºC for 2min, at 95ºC for 10min, and then cDNA was amplified by 40 two step cycles(15sec at 95ºC, 1min at 60ºC).

Table 1: RT-PCR and real-time PCR primer and probe sequences

Specificity	Primer/Probe	Sequence
RT-PCR	Forward	5´ CTY CAC ACA GAR CAC AAT GG 3´
	Reverse	5´ GTC ACA CTT GTT GTR TC 3´
cDNA synthesis	forward	5´TCT AAC CGA GGT CGA AAC GTA 3´
Real time PCR	Forward	5´AAG ACC AAT CCT GTC ACC TCT GA 3´
	Reverse	5´CAA AGC GTC TAC GCT GCA GTC C 3´
	probe	5´FAM TTT GTG TTC ACG CTC ACC GT TAMRA 3´

RESULTS

RT-PCR results

The RT-PCR test was performed for virus detection (Fig. 1). Of the 21 fecal samples, 12 samples were positive by RT-PCR test. The virus was detected in the fecal samples 71.4%, 85.7%, and 14.2 on days 3, 7 and 15 PI, respectively. The amplified virus genome was almost detected within 3 hours.

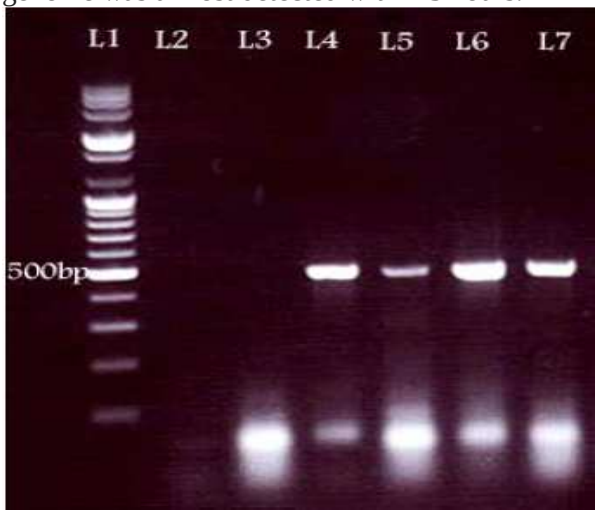

Fig.1. RT- PCR assay: Amplifying 488-bp segment of H9N2 influenza gene. Lane L1: DNA marker (100-bp), L2: negative control, L3: negative sample, L4: positive control, L5, L6, L7: positive samples.

Real time-PCR results

The RRT-PCR test was performed for virus detection (Fig. 2). Of the 21 fecal samples, 15 samples were positive by RT-PCR test. The virus was detected in the fecal samples 71.4%, 100%, and 42.8 on days 3, 7 and 15 PI, respectively. The amplified virus genome was almost detected within 2 hours.

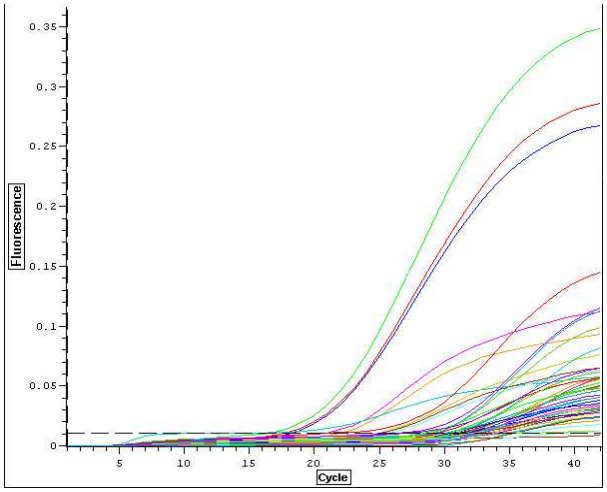

Fig.2. Real time - PCR assay: Amplifying 104-bp segment of H9N2 of influenza gene.

Discussion

Some subtypes of avian influenza viruses can transfer to humans and cause devastating pandemic. While much attention has been paid to H5 and H7 AI subtypes, the potential is also real for H9N2 (Cameron *et al.*, 2000, Matrosovich *et al.*, 2001, Wan *et al.*, 2008, Li *et al.*, 2003). Recent evidence shows that H9N2 avian influenza virus is widespread in poultry throughout Asian countries and raising a possibility that this virus would be a potential threat against human population(Choi *et al.*, 2004, Cameron et al., 2000, Guo *et al.*, 2000, Li et al., 2003, Guan *et al.*, 2000, Lee *et al.*, 2010, Aamir *et al.*, 2007)**.** This virus is now endemic in poultry industry of Iran (Nili & Asasi, 2002, Nili & Asasi, 2003). Since chickens are often reared in close proximity to human, Circulation of this virus in our country poses a serious public health threat. Several cases of transmission of H9N2 AI viruses to humans have been documented (Lin *et al.*, 2000, Peiris *et al.*, 1999, Guo *et al.*, 2001, Butt *et al.*, 2005, Saito *et al.*, 2001). This virus is regarded by the World Health Organization as a potential pandemic candidate. Rapid detection of the influenza viruses is basic means for preventing widespread transmission of infection. The molecular techniques are the rapid assays for detection of viral nucleic acid sequences. Therefore we investigated

the performance of two molecular assay can be used to diagnosis of influenza viruses.

RT-PCR methods is a useful method for detection of influenza viruses during outbreaks of infections. However, a disadvantage of RT-PCR is the time consuming, low sensitivity and specificity, compared with RRT-PCR assay.

Several real-time PCR assays for influenza A virus using SYBR green, hybridisation probes or hydrolysis probes (Taq- Man chemistry) have been described (Stone et al., 2004; Smith et al., 2003;Ward et al., 2004; Templeton et al., 2004;). In the present study, a real-time PCR assay was used using Taq-Man specific probes with primers targeting the influenza A matrix gene. The performance of this assay was compared was compared with PCR assay

The highest frequency of viral RNA detection in the fecal samples was observed by real-time PCR assay (15 samples). Predominant H9N2 influenza virus was detected by real-time PCR on days 7 PI (100%) compared to (85.7%) with PCR assay. These results indicate that the RRT- PCR had a higher sensitivity compared with PCR assay. On the other hand, the amplified virus genome was detected by real-time PCR within 2 hours compared to 3 hours with PCR assay**.** This data show that RRT- PCR technique is faster than PCR methods for detection of influenza viruses.

These results indicate that the RRT- PCR had a higher performance regarding the detection of influenza as compared to the PCR method.

References

Aamir, U., U. Wernery, N. Ilyushina and R. Webster, 2007: Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology, 361, 45-55.

Alexander, D. J., 2007: Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. Avian diseases, 51, 161-166.

Butt, K., G. J. D. Smith, H. Chen, L. Zhang, Y. Leung, K. Xu, W. Lim, R. G. Webster, K. Yuen and J. Peiris, 2005: Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. Journal of clinical microbiology, 43, 5760.

Cameron, K., V. Gregory, J. Banks, I. Brown, D. Alexander, A. Hay and Y. Lin, 2000: H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology, 278, 36-41.

Choi, Y., H. Ozaki, R. Webby, R. Webster, J. Peiris, L. Poon, C. Butt, Y. Leung and Y. Guan, 2004: Continuing evolution of H9N2 influenza viruses in Southeastern China. Journal of virology, 78, 8609.

Guan, Y., K. Shortridge, S. Krauss, P. Chin, K. Dyrting, T. Ellis, R. Webster and M. Peiris, 2000: H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. Journal of virology, 74, 9372.

Guo, Y., J. Dong, M. Wang, Y. Zhang, J. Guo and K. Wu, 2001: Characterization of hemagglutinin gene of influenza A virus subtype H9N2. Chinese medical journal, 114, 76.

Guo, Y., S. Krauss, D. Senne, I. Mo, K. Lo, X. Xiong, M. Norwood, K. Shortridge, R. Webster and Y. Guan, 2000: Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology, 267, 279-288.

Lee, D. C. W., C. K. P. Mok, A. H. Y. Law, M. Peiris and A. S. Y. Lau, 2010: Short report Differential replication of avian influenza H9N2 viruses in human alveolar epithelial A549 cells. Virology Journal, 7, 71.

Li, K., K. Xu, J. Peiris, L. Poon, K. Yu, K. Yuen, K. Shortridge, R. Webster and Y. Guan, 2003: Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? Journal of virology, 77, 6988.

Lin, Y., M. Shaw, V. Gregory, K. Cameron, W. Lim, A. Klimov, K. Subbarao, Y. Guan, S. Krauss and K. Shortridge, 2000: Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proceedings of the National Academy of Sciences, 97, 9654.

Matrosovich, M. N., S. Krauss and R. G. Webster, 2001: H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology, 281, 156-162.

Nili, H. and K. Asasi, 2002: Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathology, 31, 247-252.

Nili, H. and K. Asasi, 2003: Avian influenza (H9N2) outbreak in Iran. Avian diseases, 47, 828-831.

Peiris, M., K. Yuen, C. Leung, K. Chan, P. Ip, R. Lai, W. Orr and K. Shortridge, 1999: Human infection with influenza H9N2. The Lancet, 354, 916-917.

Reed, L. J. and H. Muench, 1938: A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology, 27, 493-497.

Saito, T., W. Lim, T. Suzuki, Y. Suzuki, H. Kida, S. I. Nishimura and M. Tashiro, 2001: Characterization of a human H9N2 influenza virus isolated in Hong Kong. Vaccine, 20, 125-133.

Smith, A.B., Mock, V., Melear, R., Colarusso, P., Willis, D.E., 2003. Rapid detection of influenza A and B viruses in clinical specimens by Light Cycler real time RT-PCR. J. Clin. Virol. 28 (1), 51–58.

Stone, B., Burrows, J., Schepetiuk, S., Higgins, G., Hampson, A., Shaw, R., Kok, T., 2004. Rapid detection and simultaneous subtype differentiation of influenza A viruses by real time PCR. J. Virol. Meth. 117 (2), 103–112.

Swayne, D. E., D. A. Senne, and C. W. Beard. 1998. Influenza. In D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed (eds.). Isolation and Identification of Avian Pathogens, 4th ed. American Association of Avian Pathologist: Kennett Square, PA, 150—155.

Templeton, K.E., Scheltinga, S.A., Beersma, M.F., Kroes, A.C., Claas, E.C., 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus and parainfluenza viruses 1, 2, 3 and 4. J. Clin. Microbiol. 42 (4), 1564–1569.

Vasfi Marandi, M and Bozorgmehrifard, MH (1999). An outbreak of non-highly pathogenic avian influenza in chickens in Iran. Proceedings 61st meeting of world veterinary association. France. CD-ROM.

Wan, H., E. M. Sorrell, H. Song, M. J. Hossain, G. Ramirez-Nieto, I. Monne, J. Stevens, G. Cattoli, I. Capua and L. M. Chen, 2008: Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One, 3, e2923.

Ward, C.L., Dempsey, M.H., Ring, C.J.A., Kempson, R.E., Zhang, L., Gor, D., Snowden, B.W., Tisdale, M., 2004. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J. Clin. Virol. 29 (3), 179–188.