Iranian Journal of Virology 2010;4(3&4): 37-42

Original Article

Detection of Human Influenza Viruses in Nasopharyngeal Samples by RT-PCR vs Tissue Culture

Soltani Z[1,2], Hosseini M[1], Tabatabaian M[1], Bashar R[1], Shahidi M[1], Tavassoti-Kheiri M[1*]

1. Influenza Unit, Pasteur Institute of Iran, Tehran, Iran.
2. Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran.

Abstract

Background and Aims: Influenza virus is a major pathogen involved in respiratory illnesses during winter seasons. A variety of diagnostic methods have been developed to identify influenza viruses in clinical specimen.

Methods: Nasal and pharyngeal samples taken from patients were inoculated into Madin-Darby canine kidney (MOCK) cells and embryonated chicken eggs (ECEs). The culture media was assayed for hemagglutination (HA). Tissue culture supernatant and clinical specimens were used for RNA extraction, followed by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers for influenza virus typing and subtyping.

Results: 21% of the samples were positive by RT-PCR while only 8.7% and 3.5% were positive by culturing in MOCK and ECE respectively.

Conclusion: This study demonstrated that RT-PCR is more effective and sensitive than tissue culture for the diagnosis of influenza virus infection.

Keywords: MDCK cell culture; Embryonated chicken egg; Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

Introduction

Influenza is an important viral infection because of its propensity for seasonal epidemics and occasional pandemics. There are three types of influenza viruses: A, Band C. Influenza viruses A and B cause respiratory disease in human. Influenza A causes a significant health burden, because it is the one causing most of the influenza pandemics (1). Various diagnostic tests have been developed since influenza virus first characterized in 1933. These techniques are employed to confirm clinical diagnosis (2). Rapid detection of the virus is important to determine appropriate clinical intervention and to monitor these or related viruses during outbreaks or pandemic of emerging pathogens. Four methods have been routinely employed for detection of influenza virus: the virus culture, serology, immunofluorescent, and the viral nucleic acid detection (3).

Influenza virus isolation in embryonated chicken eggs was developed first by Burent et al. in 1940 (4). Even after cell cultures had been widely used, eggs continued to be the standard host for isolation of influenza viruses. In 1975, Tobita et al. (5) reported that MDCK cells are suitable for propagation of influenza viruses. Conventional laboratory diagnosis of influenza is based on the virus isolation and serological testing. Virus culture using MDCK cells is currently accepted as 'gold-standard' for laboratory diagnosis of influenza virus (1). RT -PCR, an alternative approach for rapid

***Corresponding author:** Masoumeh Tavassoti-Kheiri, PhD, Influenza Unit Pasteur Institute of Iran, Tehran, Iran.
Tel: (+98) 21 66 95 33 11
Email: mtkheiri@yahoo.com

detection of influenza viruses, was first reported in 1991 (6).
The purpose of this study was to compare RT - PCR assay for detection of human influenza viruses in patient's respiratory samples directly or after culture in MDCK and/or ECE.

Methods

Specimens

Swabs from suspected individuals (Samples for this work were provided kindly by Ekbatan Clinic; Tehran-Iran and Dr. Nikbin.). Swab was transported in a tube containing 3 ml viral transport medium (VTM) at 4خC. The tubes were centrifuged at 1157 g for 5 min. The supernatants were used for virus isolation and RNA extraction. All specimens were stored frozen at -70خC for further testing. These samples were collected from October 2005 till January 2007.
Influenza virus isolation in MDCK culture MDCK continuous cell line was used in 24 well plates to isolate influenza viruses. Cells were seeded at a concentration of $5 \times l0^6$ cells/ml in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St.Louis, MI), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), 100 IV/ml penicillin G and 100 ug/ml streptomycin. After one day, the medium from seeded well was removed gently and cells were washed with PBS. A 200 ul of sample specimen was inoculated into 2 wells of MDCK cells. The inoculums were allowed to absorb at 37خC for 60 min in a 5% CO_2 humidified incubator and then 1 ml of DMEM containing 2 ug/ml of trypsin was added to all wells. The cells were incubated at 37 DC in 5% CO_2 for 6 days. The plates were observed daily for cytopathic effect (CPE). Also, the culture medium was examined every other day for hemagglutinin activity (HA) using a 0.5 % suspension of chicken erythrocytes. Following RNA extraction RT-PCR was performed to determine type and subtype of the positive samples.

Influenza virus isolation in ECEs

12-day-old specific pathogen free (SPF) ECEs were examined with egg candler. The labeled eggs were placed with blunt end up into egg tray and inoculated via allantoic sac with 100 ul of clinical specimens (3 eggs per specimen). The inoculated ECEs were incubated at 35خC for 3 days, and then allantoic fluid of each egg was harvested separately to do end-point titration by hemagglutination assay with 0.5 % suspension of chicken erythrocytes.

RT-PCR

Viral RNA was extracted from 300 ul of each sample (tissue culture supernatant or direct clinical specimens) using a commercial RNX-PLVSژ solution (CinnaGen, Tehran, Iran). The viral RNA was eluted in 30 ul DEPC treated water and immediately kept at -70خC. The eDNA synthesis was performed using Superxcript" III Firststandard Synthesis Kit (Invitrogene, Carslbad, CA) according to the manufacturer's instructions.
Two sets of primers were used for this study. The first detects type A and B influenza viruses and the second differentiated HINI and H3N2 subtypes of influenza A. Primers were synthesized based on matrix protein gene and nucleoprotein gene of influenza A virus (M-A & NP-A), nucleoprotein and non-structural gene of influenza B virus (NP-B & NS-B), for typing and hemagglutinin glycoprotein gene of influenza AlHINI and *AlH3N2* viruses (HI-A and H3-A) and neuraminidase glycoprotein gene of influenza AlHINI and *AlH3N2* viruses (NI-A and N2-A), these primers were designed by Influenza unit, Pasteur institute, Iran [7].
A total 5 ul of cDNA was added to 20 ul of master mix containing 1 xPCR buffer, 1.5 mM MgCh, 0.2 mM dNTPs (Fermentas, Lithuania), 1.5 *V* Taq polymerase enzyme (CinnaGen) and 1 ul of each appropriate primers (forward and reverse) (CinnaGen). Amplification of DNA was carried out at 95خC for 5 min for reverse transcription followed by 35 cycles of denaturing at 94خC for 30 sec, annealing at 63خC for 30 sec and extension at 72خC for 30 sec. The PCR was completed with a final extension step at 72خC for 5 min.

Sensitivity of RT-PCR method

The sensitivity of detection of human influenza virus with gene specific primer sets used individually in a RT-PCR reaction was determined by analyzing amplified RNA

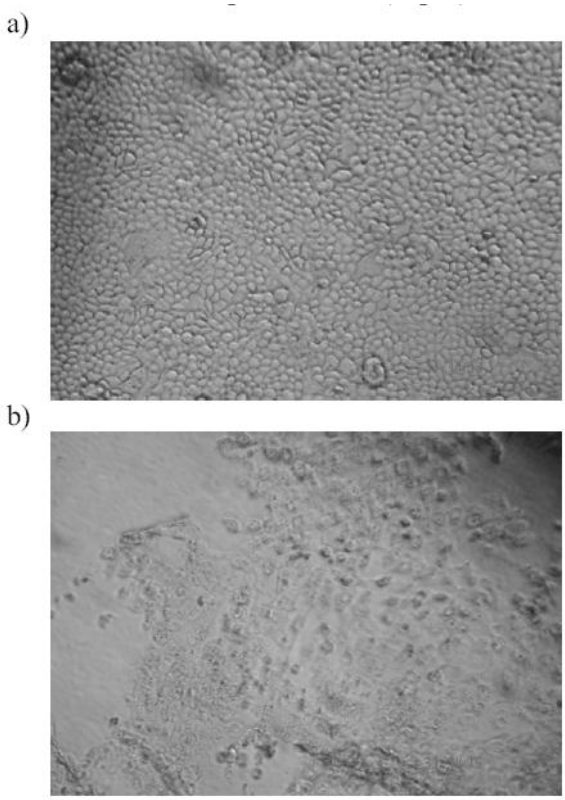

Fig. 1. Cytopathic effects (CPE) on cell culture was observed on 2nd or 3rd culture day
a) Intact MOCK b) Influenza virus inoculated MOCK.

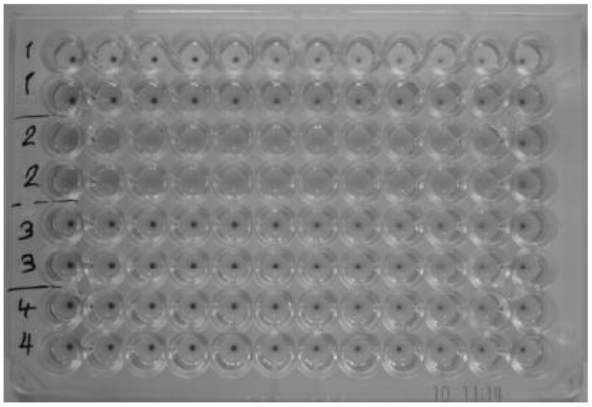

Fig. 2. Hemagglutinin assay of MOCK culture of the patient sample. Rows 1: control negative, Rows 2 the positive sample with 1024 HA titer. Rows 3 and 4 negative samples.

extracted from 1:10 serial diluted tissue culture grown virus that had been quantified in terms of TCID50.

Agarose gel electrophoresis

Ten percent of the amplified product was loaded into an agarose gel (1.5%) containing 1 ul ethidium bromide and the electrophoresis

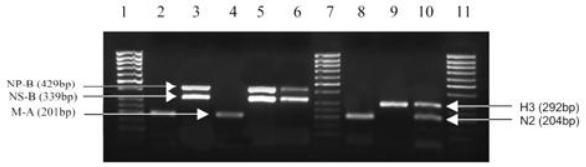

Fig. 3. Molecular typing and subtyping of Influenza viruses in a panel of cell cultured clinical isolates using RT-PCR assay. Lanes 1, 7 and 11: 50bp ladder. Lanes 2, 3, 8 and 9: positive control. Lanes 46 and 10: clinical panel.

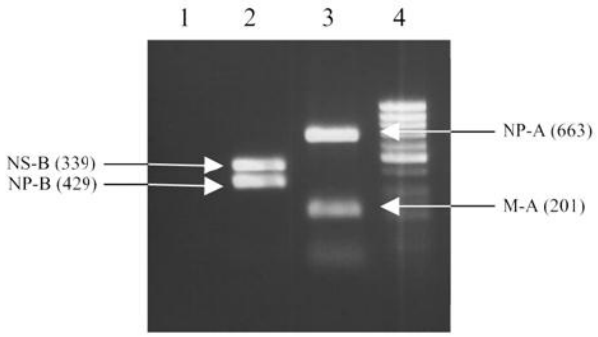

Fig. 4. RT-PCR typing with specific primers; Lane 1: negative control. Lanes 2 and 3: clinical samples. Lane 4: 50bp ladder.

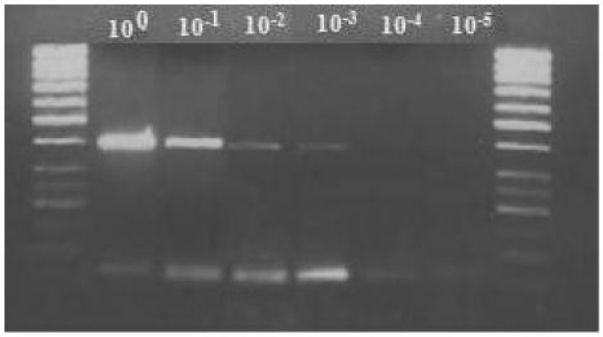

Fig. 5. Gel Electrophoresis of RT-PCR products of serial dilutions of a true positive clinical H1N1 sample. A positive sample was serially diluted (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and following RNA extraction and RT-PCR the products were loaded on Lanes 2-7; lanes 1 & 8 50 bp ladder.

was conducted in TBE (Tris-Borate and EDTA, pH 8.0) buffer. The DNA bands were visualized by UV transillumination.

Table 1. Influenza virus detection by RT-PCR from direct samples and cultured in MOCK and ECE.

Total Clinical samples = 57 Total isolates = 12			
	Influenza A virus (n = 10)		influenza B virus (n = 2)
	Positive No. (%) H1N1	Positive No. (%) H3N2	Positive No. (%)
MDCK	1(1.7)	2 (3.5)	2 (3.5)
ECE	0	2 (3.5)	0
Direct Sample (RT-PCR)	2 (16)	8 (68)	2 (16)

Results

MDCK cell culture

In total, five out of 57 samples (8.7%) were positive for influenza virus by cell culture. The culture media was tested for HA activity and following RNA extraction RT-PCR was performed to determine type and subtype of the positive samples and the results are as follow: 3 INFV A (2 H3N2 and I HINI) and 2 INFV B. An HINI sample strain which showed cytopathic effect (CPE) on MDCK cells (Fig. I), was positive in RT -PCR, but negative in HA (Fig. 2).

RT-PCR was able to detect INFV in 7 out of 52 negative samples in cell culture system (6 were positive for H3N2 and 1 was positive for HINl). All samples that were positive for INFV by cell culture also gave positive results in RT -PCR. In total, 12 (21%) of the 57 clinical samples were positive for influenza virus by RT-PCR; 10 for INFV A including 8 H3N2 and 2 HINI and also 2 for influenza B (Table 1) (Fig. 3 and 4).

ECE virus culture

In the 57 samples, the rate of isolation in ECE system was (3.5%) detecting 2 INFV A (H3N2). RT-PCR was able to detect INFV in 10 out of 55 negative samples in ECEs. All samples that were positive for INFV by ECEs also gave positive results in R T - PCR.

Direct RT-PCR on nasopharyngeal samples

In order to detect cases of false-positive and falsenegative results, RT-PCR was performed with a new RNA extract for all nasopharyngeal samples. In addition to 5 positive cell cultured specimens, seven more samples tested positive doing direct RT-PCR.

Sensitivity of the RT-PCR assay

The RT-PCR products were loaded on agarose gel and intensities of the DNA bands decreased with increased dilution of the virus in the sample. Stock virus diluted up to 10^{-4} was detectable for influenza type and subtype genes. The sensitivity of detection was about 10-30 TCID50 (Fig 5).

Discussion

Laboratory diagnosis of influenza has become a cornerstone of prevention, containment surveillance, and treatment of the associated illnesses. Emergence of the novel strains of the virus has extended the role of the laboratory to include isolation and sub-typing of the viruses for disease surveillance and vaccine development (8, 9)

In this study, we compared three different diagnostic methods for detection of INFVs on nasopharyngeal samples. We noted that RT -PCR identified more positive samples than culture methods as previously reported (10-13). Virus isolation in cell culture and embryonated chicken egg missed 58% & 83% of influenza virus-positive samples respectively. In addition, once viral RNA is extracted from the specimen, it can be used in RT -PCR not only to identify type, but also subtype of the virus. Therefore, this is a rapid and sensitive assay suitable to diagnose the disease especially in pandemics.

MDCK cell line was chosen for isolation of influenza virus based on a previous study (14), in which different cell lines were compared for isolation of influenza A virus. The sensitivity of the MDCK cell line was greater than the sensitivity of the Vero and MRC-5 cell lines and as a result, the MDCK cell line was

recommended for the isolation of influenza viruses (5). From 1975 on, MDCK cells were more frequently used for isolation of influenza A viruses after it was found that adding trypsin to stimulate the growth of influenza A viruses enables many influenza virus strains to form plaques with high efficiency (5). In addition to embryonated eggs, MDCK cells in culture medium containing trypsin are now considered a valuable system for isolation of these viruses from clinical specimens (15, 16). It has been reported that certain human influenza isolates do not grow in embryonated chicken egg as well as in cell culture (17).

Although influenza virus culture has been considered for diagnosis for a long time but it often takes 5 to 10 days to perform, thus this method is time consuming. In addition it may miss some positive samples. Therefore, it was necessary to establish a more suitable test. In some national surveillance schemes, the benefit of utilizing RT-PCR has been demonstrated successfully (18). In Portugal, a comparison of multiplex RT-PCR for detection of influenza viruses with culturing, enzyme immunoassay (EIA) and serology was performed during surveillance over seven influenza seasons from 1992 to 1999 (19). More samples were found to be positive by RT -PCR than by any of the other methods used.

It was shown that annual outbreak of influenza activity in Scotland was monitored by a community-based surveillance scheme from 2000-2001 by molecular detection alone. This decision was made following the results of a comparison of RT-PCR with culturing and serology, which had been reported (20).

Since isolation of the virus in embryonated chicken egg provides high quantities of virus, reference laboratories utilize this culture system to enable the production of virus stocks for epidemiological monitoring.

In present study, only 3.5 % of the samples were positive using embryonated chicken egg and 8.7% were positive using MDCK culture; while about 21 % positive samples were detected using RT-PCR.

In conclusion, this study has described that detection of influenza viruses by molecular methods have an important role to characterize circulating influenza strains. The data presented in this paper demonstrated that the RT-PCR method is more suitable for detection of human INFVs from clinical samples, and the use of RT-PCR is more sensitive and rapid than other established methods.

Acknowledgements

This study was supported by Grant No.295 from the Iranian Molecular Medicine Network Pasteur, Institute of Iran.

References

1. Gavin P, Thomson R. Review of Rapid Diagnostic Tests for Influenza. Clin. Appl. Immuno Review. 2003;4: 151-172

2. Kamps BS, Hoffinann C, Preiser W. Influenza Report, Laboratory Findings. 2006.

3. Matthey S, Nicholson D, Ruhs S, Alden B, Knock M, Schultz K, Schmuecker A. Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. J Clin Microbiol.1992;24(4):677-9

4. Burnet F. Influenza virus infections of the chick embryo by the amniotic route. Aust. 1 Exp. Biol. 1940;18:353-360

5. Tobita K, Sugiura A, Enornote C, Furuyama M. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med Microbiol Immunol. 1975;162: 9-14.

6. Zhang WD, Evans DH. Detection and identification of human influenza vimses by the polymerase chain reaction. J. Virol. Methods. 1991;33:165-189.

7. Shahidi, M. Thesis for master of science degree, Establishment of Heterogenecity of Determination of Influenza virus using RT-PCR.

8. Peltola V, Reunanen T, Ziegler T, Silvennoinen H, Heikkinen T. Accuracy of clinical diagnosis of influenza in outpatient children. Clin. Infect Dis. 2005;41:1198-200.

9. Pachucki C. Rapid test for influenza. Curr. Infect Dis. Rep. 2005;7:187-92.

10. Bovin G, Hardy l, Kress A. Evaluatiui of a rapid optical immunoassay for influenza viruses (FLU OIA test) in comparison with cell culture and reverse transcription-PCR. J. Clin. Microbiol. 2001;39:730-732.
11. Church DL, Davies HD, Mitton C, Semeniuk H, Logue M, .Maxwell C, Donaldson. C. clinical and economic evaluation of rapid influenza A virus testing in nursing homes in Calgary, Canada. Clin. Infect. Dis. 2002;34:790-795.
12. Kawakami, C., Shimizu, H., Watanabe, S., Saikusa, M., Munemura, T., Mitamura, K., Sugaya, N., and Imai, M~ 2001 Evaluation of immunochromatography method for rapid detection of influenza A and B viruses. Kansenshogaku Zasshi. 75:792-799.
13. Kehl SC, Henrickson K, Hua W, Fan J. Evaluation of the Hexaplex assay for detection of influenza vims A by cytospin-euhanced immunofluorescence and membrane enzyme-linked immunosorbent assay. 1 Clin. Microbiol. 2001;38:429-430.
14. Reina, J., Fernandez-Baca, V., Balanco, 1., Munar, M. Comparision of Madin-Darby canine kidney cells (MDCK) with a green monkey continuous cell line (Vero) and human lung embryonated cells (MRC-5) in the isolation of influenza A virus from nasopharyngeal aspirates by shell vial culture. J Clin. Microbial. 1997;35: 1900-190l.
15. Brown H, Done SH, Spencer Y, Cooley WA, Harris PA, Alexander D. Pathogenicity of a swine influenza HINI virus antigenic ally distinguishable from classical and European strains. Vet. Rec. 1993;132:598602.
16. Gourreau JM, Kaiser C, Valette M, Douglas AR, Labie L, Ayrnard M. Isolation of two HIN2 influenza viruses from swine in France. Arch. Virol. 1994;135:365-382.
17. Carman S, Stansfield C, Weber J, Bildfell R, Van Dreumel T. H3N2 influenza A virus recovered from a neonatal pig in Ontario. Can Vet J. 1999;40:889890
18. Ellis lS, Fleming DM, Zambon MC. Multiplex reverse transcription- PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996, J Clin. Microbiol. 1997;35(8): 2076-2082
19. Rebelo-de-Andrade H, Zambon MC. Different diagnostic methods for detection on influenza epidemics, Epidemiology and infection. 2000;124(3):515-522
20. Carmon WF, Walker r, Mclnyre S, Noone A, Christie P, Millar 1. Rapid virological surveillance of community influenza infection in genera practice, British Medical Journal. 2000;321:736-737.