Comparative Analysis Of Real Time RT-PCR And Virus Isolation For Detection And Subtyping Of A(H1N1)Pdm09 Influenza Virus

A Vontas, E Plakokefalos, V Krikelis, A Manouras

Citation

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Abstract

A comparative study for the detection and identification of A(H1N1)pdm09 influenza viruses was carried-out on respiratory samples collected during the period 2009-2011 when influenza activity in central Greece was dominated by this virus subtype. We evaluated 1500 nasal and pharyngeal swabs for the presence of A(H1N1)pdm09 influenza by virus culture in Madin-Darby Canine Kidney (MDCK) cells and by real-time RT-PCR assay (rt-RT-PCR). By cell culture, A(H1N1)pdm09 viruses were isolated and identified by hemagglutination-inhibition (HAI) in 387 samples (26%), while by rt-RT-PCR, A(H1N1)pdm09 RNA sequences were detected and identified in 510 samples (34%). There was found a 100% correlation of virus type and subtype, following testing by both methods. Using cell culture as the gold standard, rt-RT-PCR had a 100% sensitivity and detected an additional 123 (8%) A(H1N1)pdm09 viruses in the samples evaluated. The rt-RT-PCR assay, for the detection and subtyping of A(H1N1)pdm09 viruses during pandemic influenza activity in central Greece, provided a rapid, sensitive, and low-cost method for the screening and diagnosis of influenza in respiratory samples.

INTRODUCTION

Influenza viruses can cause severe acute and contagious respiratory illness leading to complications and increased mortality among individuals belonging to high-risk groups. It is estimated that each year influenza is linked to approximately 500,000 deaths, worldwide (1,2). The ability of influenza viruses to evade the host defense mechanisms is based on frequent mutation of its RNA genome and reassortment events that lead to new viral subtypes. The application of sensitive and specific laboratory methods with high diagnostic value is crucial for the timely detection of circulating influenza viral species and the control of influenza outbreaks including pandemic influenza (3).

Real-time quantitative RT-PCR protocols are among the most widely used methods in the diagnosis of influenza infection since they are performed to screen large number of samples with maximum sensitivity and specificity even when viral antigens are denatured (4-6). Moreover, rt-RT-PCR has been evaluated during seasonal and pandemic influenza activity in influenza diagnosis and surveillance laboratories (7).

In the present preliminary study we assessed the application of real-time RT-PCR in the screening and subtyping of A(H1N1)pdm09 influenza viruses circulating in central Greece during the period 2009-2011. Also, we compared these results with those of virus propagation and isolation in MDCK cells, in the same samples, in order to determine the sensitivities and performance of both diagnostic methods.

MATERIALS AND METHODS

Clinical samples

Nasal and pharyngeal swabs were collected during the influenza seasons 2009-2011 from non-vaccinated individuals of all ages presenting influenza like illness (ILI), as described in the World Health Organization (WHO) guidelines (8). Symptoms include fever (>38.50C), myalgia, cough, sore throat, headache, and general malaise. Sampling took place 2-3 days following the onset of symptoms at the University Hospital of Larissa, Thessaly, Greece. Swabs were placed and transported in 2.5 ml virus transport

medium (24,9 gr bacto-tryptose, 5 gr gelatin, 0,5 gr streptomycin, 500,000 U penicilin and 50 mg fungizone per liter). Samples were examined the same day or stored at 700C for longer period.

Seasonal influenza A(H1N1)

Selected influenza A(H1N1) viral isolates of known titer collected in previous seasons were included in this study to determine the sensitivity and specificity of rt-RT-PCR and virus isolation.

Real-time-RT-PCR

Viral RNA was extracted from 250ml of clinical sample or cell culture supernatant with TRIzol (Gibco BRL, Life Technologies, NY, USA), according to manufacturer's recommendations, in the presence of 20mg glycogen, molecular biology grade, (Boehringer, Germany). Real-time RT-PCR was performed in a 7500 Applied Biosystem thermocycler at a total reaction volume of 25μl (including 6.5μl RNA). Primer pairs for influenza type A, subtype H1N1 and internal control were added in each master mix. Primer and probe sequences and rt-RT-PCR reaction conditions were previously described (9).

Virus isolation and identification

Nasal and pharyngeal swabs in virus transport medium were transferred into microtubes, centrifuged at 1200rpm for 5 min. and 125μl of the supernatant was inoculated into confluent and permissive MDCK, which are sensitive to infection by human influenza viruses including A(H1N1)pdm09. The cell culture medium contained Dulbecco's Modified Eagle medium (D-MEM), 10% fetal calf serum (Gibco), 100 IU/ml penicillin and 100 mg/ml streptomycin and 1% trypsin (Sigma, USA). MDCK cultures were incubated 370C for up to 10 days and were observed daily for the appearance of cytopathic effect (CPE). The Hemagglutination test (HA) was performed on culture supernatants using 0.5% chicken red blood cells and an HA titer of 1/32 or higher was used for viral strain identification with the Hemagglutination-inhibition method (HAI) as previously described (9). Antisera for virus identification in the HAI test were provided by the WHO and used according to standard protocol (8).

RESULTS AND DISCUSSION

Samples included in this study were collected during the two-year period 2009- 2011 when influenza activity was significantly increased in the region of Thessaly in central Greece as was reported by sentinel physicians and number of samples examined. We screened a total of 1500 respiratory samples by rt-RT-PCR and virus isolation in MDCK cells in order to evaluate the sensitivity of each method in the detection and subtyping of A(H1N1)pdm09 influenza viruses. By rt-RT-PCR there were detected 510 positive samples (34%) while by virus isolation we identified 387 influenza isolates (26%). There was a 100% correlation found in the virus type and subtype by rt-RT-PCR and virus isolation. Using virus isolation as the gold standard rt-RT-PCR provided 100% sensitivity while there were 123 positive by rt-RT-PCR that were found negative by virus isolation. However, both methods had 100% sensitivity in detecting and identifying seasonal influenza A (H1N1) viruses. Based on these preliminary results we can assume that the quality of the collected specimen, virus titer in the original clinical sample and following propagation in cell culture both can act as parameters in determining the sensitivity and specificity of diagnostic method and especially virus isolation, since an intact virus is required. Both assays performed with 100% when archived seasonal influenza A (H1N1) viruses were tested. Based on these preliminary results, our laboratory is currently undertaking the evaluation of modified RT-PCR assay for the detection of influenza and other respiratory viruses contributing to improved diagnosis of seasonal and pandemic influenza activity and the timely administration of influenza antivirals.

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Author Information

A. Vontas
Department of Medical Laboratories, Technological Educational Institute of Thessaly
Larissa, Greece

E. Plakokefalos
Department of Medical Laboratories, Technological Educational Institute of Thessaly
Larissa, Greece
plakoke@yahoo.com

V. Krikelis
Department of Medical Laboratories, Technological Educational Institute of Thessaly
Larissa, Greece

A. Manouras
Department of Medical Laboratories, Technological Educational Institute of Thessaly
Larissa, Greece