Comparison of Cell Culture With RT-PCR for Enterovirus Detection in Stool Specimens From Patients With Acute Flaccid Paralysis

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Since October 2000, Iran has been declared polio-free by the World Health Organization (WHO). Despite the fact that poliomyelitis caused by polioviruses has been eliminated from Iran, the number of acute flaccid paralysis (AFP) cases has not been reduced. Therefore, it is of great importance to investigate the other viral agents that may cause AFP (mainly nonpolio enteroviruses, which play a significant role in the etiology of neurological syndromes). Some enteroviruses do not grow in the conventional cell lines that are being used for enterovirus detection. Furthermore, the virus titer is an important factor in the sensitivity of cell culture to detect the virus. The fact that cell culture is a time-consuming procedure is another reason to

find a more practical method for enterovirus detection. Therefore, a more sensitive and rapid method should be used to detect enteroviruses as efficiently as possible in the stool specimens of AFP cases. The aim of this study was to evaluate cell culture and RT-PCR in enterovirus detection. Findings have shown that RT-PCR can increase the rate of nonpolio enterovirus detection by up to 10% in comparison with cell culture. Also, the rapid detection of enteroviruses by RT-PCR can decrease both the unnecessary use of antibiotics and the costs in clinical practice. For this reason, we find that RT-PCR is a more practical technique for enterovirus detection. *J. Clin. Lab. Anal.* 21:232–236, 2007. © 2007 Wiley-Liss, Inc.

Key words: RT-PCR; cell culture; enterovirus; sensitivity; AFP

INTRODUCTION

Enteroviruses are small RNA and nonenveloped viruses that comprise a large group of immunologically distinct serotypes of viruses belonging to the Picornaviridae family. These viruses are transmitted by the oral-fecal route, replicate in high titer in the enteric tract, and are carried by the blood to target organs (1–3). There are a total of 64 immunologically distinct serotypes that infect humans. Serotypes of human enteroviruses have traditionally been classified into echoviruses, coxsackievirus groups A and B, polioviruses, and the newer numbered enteroviruses (enteroviruses 68–71). Echoviruses 22 and 23 have been reclassified as a new genus in Picornaviridae and are termed human parechoviruses 1 and 2, respectively (3).

Infections caused by most members of this group of viruses, even the most pathogenic ones, may culminate

in subclinical manifestations. They are responsible for a wide range of acute symptoms as a result of infection of the central nervous system, cardiac and skeletal muscles, pancreas, skin, and mucus membranes. A very well-known disease is paralytic poliomyelitis, which is perhaps the oldest recorded viral disease. Its viral etiology was demonstrated in the early 20th century, when epidemics of this disease increased in frequency and severity. Today, it still causes significant disability in many parts of the world (4).

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Received 25 December 2006; Accepted 22 March 2007

DOI 10.1002/jcla.20171

Published online in Wiley InterScience (www.interscience.wiley.com).

On the other hand, various studies in India have suggested that the frequency of non-polio enterovirus (NPEV) isolation from acute flaccid paralysis (AFP) cases ranges from 20% to 54%. Most AFP cases in the United States were found to be caused by NPEV in the postvaccination era. Several cases of paralysis were reported in association with enteroviruses, especially coxsackieviruses in Scotland. Paralysis has also been reported in association with coxsackieviruses B2–B6, enterovirus 71, and echovirus types 3, 4, 6, 9, 11, 19, and 22 (3).

Since October 2000, Iran has been declared polio-free by the World Health Organization (WHO). Despite the fact that poliomyelitis caused by polioviruses has been eliminated from Iran, the number of AFP cases has not been reduced. Therefore, it is of great importance to investigate the other viral agents that may cause AFP (mainly nonpolio enteroviruses, which play a significant role in the etiology of neurological syndromes) (1).

Some enteroviruses do not grow in the conventional cell lines that are being used for enterovirus detection (5). Furthermore, the virus titer is an important factor in the sensitivity of cell culture to detect the virus. The fact that cell culture is a time-consuming procedure is another reason to find a more practical method for enterovirus detection. Therefore, a more sensitive and rapid method should be used to detect enteroviruses as efficiently as possible in the stool specimens of AFP patients.

MATERIALS AND METHODS

Patients and Clinical Specimens

All of the patients studied were less than 15 years old. Their information was recorded on a standard questionnaire that included demographic details, date of onset of paralysis, and clinical presentation (e.g., fever at the onset of paralysis, symmetry of paralysis, and location of paralysis by a medical officer).

A total of 140 stool specimens from the AFP patients were collected within 14 days of paralysis onset and sent to the Virology Department of the Tehran University of Medical Sciences. The specimens were stored at -20°C until they were processed (6).

Specimen Preparation

The stool specimens were subjected to chloroform pretreatment before inoculation to cell culture and genome extraction for RT-PCR (6). In addition to removing bacteria and fungi, this method removes potentially cytotoxic lipids and dissociates virus aggregates (6).

Cell Cultures

According to the instructions given by the WHO, two cell lines were used. These cell lines were human rhabdomyosarcoma (RD) and L20B (a mouse cell line “L-cell” that was genetically engineered to express the human poliovirus receptor) in test tubes containing 2 mL of Dulbecco’s minimum essential medium (DMEM).

A total of 200 μL of inoculum per tube was used. The inoculated tubes were then incubated in a roller at 37°C for a period of 1–7 days, until a complete cytopathic effect (CPE) was observed under an inverted microscope. Uninfected cells from each of the two cell lines were used as the negative control (4,6).

Neutralization

Neutralization method with mixed, equine antisera pools (provided by the WHO) was used to type the clinical isolates, following the available instructions. In brief, high-titered polyclonal antisera were used and mixed with approximately 100 TCID₅₀ of the virus isolate of unknown identity. A back titration isolate was included in each assay, which allowed the titer of the virus actually present in a sample to be calculated. The serum/virus mixtures were incubated for 1 hr at 37°C . Subsequently, a suspension of cells was added to the microtiter plates, which were examined daily for the presence of CPE for up to 5 days. The antiserum that prevented the development of CPE indicated the identity of the virus (4,6).

RNA Extraction and RT-PCR

RNA extraction was performed using RNX solution (Cinagen Co., Tehran, Iran) based on the manufacturer’s procedure (7). RT-PCR was carried out using Pan-enterovirus primers for enterovirus detection based on the WHO manual (6). This set of primers produces a product of 114 base pairs and was designed to detect and amplify a genome segment present at the 5′-UTR of the enterovirus genomes.

The PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) based on WHO procedures (6).

RT-PCR amplification was performed: one cycle of reverse transcription at 42°C for 20 min, one cycle of denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, and elongation at 70°C for 45 sec followed by one cycle of elongation at 70°C for 7 min. The reaction mixtures were then held at 4°C . Amplification products were analyzed in 10% polyacrylamide gels, ethidium-bromide stained, and examined under a UV DNA transilluminator (6).

Pan-Enterovirus primer	5'-ACACGGACACCCAAAG TAGTCGGTTCC-3' 5'-TCCGGCCCCTGAATGC GGCTAATCC-3'	114 base pairs
Pan-Poliiovirus primer	5'-TTIAIIGCRTGICCRTRTT-3' 5'-CIIAITCIMGITTYGAY ATG-3'	79 base pairs

M = A and C, R = A and G, Y = C and T, I = Inosine (6).

All of the samples identified by neutralization as poliovirus types 1, 2, and 3 were examined by the method of RT-PCR using primers containing Sabin 1, 2, and 3 to differentiate between Sabin-like strain and wild-Poliiovirus. The same cycling conditions were used with all sets of primers (Pan-enterovirus, Pan-polio, and Sabin 1–3) (6).

Sabin 1 primer	5'-TCCACTGGCTTCAGTGTT-3' 5'-AGGTCAGATGCTTGAAAGC-3'	97 base pairs
Sabin 2 primer	5'-CGGCTTTGTGTGTCAGGC-3' 5'-CCGTGAAGGGATTACTAAA-3'	71 base pairs
Sabin 3 primer	5'-AGTATCAGGTAAGCTATCC-3' 5'-AGGGCGCCCTAACTTTG-3'	53 base pairs

RESULTS

Cell Culture Analysis

A total of 140 fecal samples collected from the AFP patients were analyzed by cell culture. Overall, 18 of the 140 samples (12.8%) exhibited CPE. The polio type 1, 2, and 3 Sabin-like viruses were isolated in two, two, and four specimens, respectively. Also, the echoviruses 6, 7, 13, and 20 were isolated in two, four, two, and two specimens, respectively (Table 1).

RT-PCR Analysis

RT-PCR was performed on all specimens. Overall, 32 of the 140 samples (22.8%) were positive for enterovirus (Table 1, Fig. 1). In a second test, to verify whether the 14 enterovirus-positive samples contained polio-

TABLE 1. Comparison of enterovirus detection results by cell culture and RT-PCR in stool specimens

Methods	RT-PCR (%)		Total (%)
	Positive	Negative	
Cell culture			
Positive	18 (12.8)	0 (0)	18 (12.8)
Negative	14 (10)	108 (77.2)	122 (87.2)
Total	32 (22.8)	108 (77.2)	140 (100)

viruses, an RT-PCR using Pan-poliovirus primer was performed. None of the enterovirus PCR-positive samples showed the presence of poliovirus-specific amplicons. The numbers and types of polioviruses detected by RT-PCR were the same as those obtained by cell culture (the number of detected polioviruses with both methods, cell culture, and RT PCR, was eight). There were 14 more (10%) nonpolio enteroviruses detected by RT-PCR.

DISCUSSION

When conducting a study for detection of enteroviruses, it is important to consider the advantages and disadvantages of the detection methods used. In this study we evaluated the sensitivity and efficiency of cell culture and RT-PCR for enterovirus detection in stool specimens. Using cell culture, 18 of the 140 specimens (12.8%) were positive for enteroviruses. RT-PCR using a pair of Pan-enterovirus primers was able to detect enteroviruses in 32 of 140 specimens (22.8%). Therefore, RT-PCR was able to increase the rate of enterovirus detection up to 10% in comparison with cell culture. Although the main way of enterovirus diagnosis is virus isolation in cell culture, which has been the most widely used method for diagnosis of enterovirus infections, and it also makes serotyping of the isolated enterovirus possible, earlier studies have also shown the advantages of RT-PCR for diagnosing enterovirus infections (8–10). In agreement with those reports, this study shows that the sensitivity of RT-PCR is superior to that of virus cultivation for detecting enteroviruses in stool samples. For instance, only 12.8% of all of the AFP cases reported in this study were positively diagnosed for enteroviruses by virus isolation. The low sensitivity of cell culture may be due to a low concentration of infectious viruses in stool or the presence of enterovirus serotypes (especially coxsackievirus group A) that grow poorly or not at all in tissue culture (5,11). Cell-culture sensitivity may be affected by toxicity of the samples and inappropriate transport or storage of live organisms, which does favor less viral infectivity but not necessarily detection by RT-PCR (12). Furthermore, the turnaround time for cell culture is usually 5–7 days for positive results and 10 days for negative results (13), while for RT-PCR this time is reduced to 2 days.

In other surveys, RT-PCR has been applied to detect enteroviruses in stool specimens. In this study we found that RT-PCR is the most suitable method for detection of enteroviruses and should be used as a method of choice for laboratory diagnosis. Although it is sufficient in most clinical cases to know that the infection is caused by enteroviruses, further identification of the virus strains, usually determined by conventional

Fig. 1. Direct detection of enterovirus RNA in cell-culture-negative stool specimens. A-7, C-7, D-7, and F-7 show typical enterovirus group-specific RT-PCR-positive products (114bp) for cell-culture-negative specimens (E-7: extraction negative control; G-7: extraction positive control). Gels are 10% polyacrylamide, ethidium bromide stained.

serotyping, may sometimes be needed for epidemiological purposes or to select proper treatment (e.g., by identifying polioviruses or determining which antiviral drugs to use in future treatment). Typing by the neutralization assay includes an additional passage in cell culture that makes the method laborious and time-consuming. However, typing of enteroviruses can also be carried out using molecular methods that take advantage of amplification and analysis of the VP1 capsid protein gene sequences, which is a rapid and precise method for accomplishing this purpose (14). Rapid diagnosis of enteroviruses and other infections caused by these viruses decreases both the unnecessary use of antibiotics and the costs in clinical practice (15). The RT-PCR assay is also able to detect enterovirus strains that do not grow in conventional cell lines. Simultaneous identification of the enterovirus from a single sample makes analysis of large numbers of samples and various sample types possible. Because picornavirus drugs are under development and clinical evaluation, the need for rapid, specific diagnostic procedures may significantly increase in the near future (16).

These findings support earlier studies that suggested RT-PCR is more sensitive than cell culture (17,18); however, most of the clinical samples used in previous studies were cerebrospinal fluid. Only a limited number of studies have evaluated the sensitivity and specificity of RT-PCR compared to cell culture for the detection of enteroviruses using other samples (17). In this study it was shown that RT-PCR is a more sensitive and rapid method for detecting enteroviruses in stool specimens.

ACKNOWLEDGMENTS

We thank the Center for Disease Control and Management, Ministry of Health, Islamic Republic

of Iran (especially Dr. Mohammad Mehdi Gouya, Dr. Abdol Reza Esteghamati, Dr. Taha Moosavi, and Mr. Abbas Ali Babaie). We also thank the personnel of the School of Public Health and Institute of Public Health Research, Tehran University of Medical Science, and National Polio Laboratory.

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