Comparison of Conventional Virus Isolation Technique With RT-PCR For Enterovirus Detection In Stool Specimens From Patients of Acute Flaccid Paralysis.

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Abstract

Every year Enteroviruses cause different diseases and infect millions of people. Cell culture is conventional method of choice for the detection of enteroviruses (EVs) in Acute Flaccid Paralysis (AFP) cases. In this study, we compared cell culture and polymerase chain reaction (PCR) methods for the detection of EVs in AFP suspected children below 15 years of age. A total of 789 stool samples were cultured in Rhabdomyosarcoma (RD) cell line. The specimens were tested for 5´ UTR by Pan EV Real-time PCR and VP1 PCR. A total of 109 were found positive by cell culture, 163 by RT-PCR and 152 by VP1 PCR, so increasing the percentage of identification from 13.81% to 20.65%. PCR was recorded higher sensitivity than the cell culture method. In conclusion, molecular methods were found to be useful for rapid and specific detection of EVs with higher sensitivity compared with the conventional cell culture method will make more confident for the detection of EVs.

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Introduction

The burden of global poliomyelitis which is due to circulation of wild polio virus in the community has been reduced due to regular vaccination (1-2). The wild polio virus which is the important causative agent for acute flaccid paralysis (AFP) in India has been eradicated successfully. Despite the accomplishment of eradication, the cases are still remaining in the community, especially in the state of UP and Bihar (3). The clinical manifestations of EVs range from conjunctivitis, respiratory tract infection, myocarditis, meningitis, encephalitis, and neonatal sepsis, like illness (4-7). The important neurological presentations were recorded in AFP cases, where EV is identified as a foremost etiological agent (8-9). Enterovirus(EV) classically can be identified by (i) virus isolation by cell culture followed by EV serotyping with the combination of antisera (10) (ii) isolation of the virus using the VIR- ADEN (an acronym derived from virus adsorption enumeration) and further identified by RT-PCR and partial sequencing of the VP1 protein coding region (11). However, both the procedure take 14-15 days, limiting success due to a non-cytopathic effect (CPE) and the unavailability of reference sera of emerging EVs. The new approach for typing of NPEVs based on VP1 sequences of viral protein has been developed and correlates well with serotypes (12). Rapidity in the detection of causative agents in AFP cases correlates with preventive measures in the field. RT-PCR is the most sensitive and rapid method for the detection of enteroviruses. However, cell culture is still used in clinical laboratories for diagnosis for reasons associated with cost and availability (7). Taking this into consideration the study has been planned to compare conventional and RT PCR for the diagnosis of AFP cases.

Material and methods

Study design & sample collection

This comparative study was designed and conducted at the Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS) Lucknow, India. A total 789 patients involving 432 males and 357 females from different district of Uttar Pradesh, India from May 2015 to April 2016. The two stool sample ≥ 24 hour apart collected from children (≤ 15 years), clinically suspected for AFP syndrome. Samples were stored below -70 °C for further processing as per the WHO protocol for isolation of polio virus. This protocol has some modification, the one part of stool supernatant was inoculated on Human rhabdomyosarcoma (RD) cell and other was used for direct RNA extraction. The supernatant of cells having characteristic EV like cytopathic effect (CPE). RNA was extracted from using a viral RNA isolation minikit (Qiagen, Hilden, Germany) per the manufacturer's protocol.

Virus Isolation

The conventional cell culture method was used in line with the WHO polio laboratory manual for poliovirus cell culture (7, 10, 13). RD was cultured in Eagle's minimum essential media (MEM) supplemented with 10% fetal calf serum (FCS) (Gibco), penicillin/streptomycin (10000 U/ml penicillin and 10 mg/ml streptomycin; Sigma) and L-glutamine (2 mM) (Sigma) at a constant temperature of 37 °C. All the cell culture experiments were performed in biosafety cabinet class II A2 (Bakar, USA). Cells were passaged in mid exponential phase at every 3-4 days at split ratio of 1:4 after treatment with 0.05% trypsin-EDTA solution and fed with sufficient media. Virus isolation was performed on cells supplemented with 2% FCS. The suspension of stool specimen was made into 10 mL phosphate buffer solution, 1 mL chloroform, and 1 g of glass bead. The mixture was vigorously shaken for 20 min and centrifuged at 1500× *g* for 20 min. Cell culture tubes containing RD in 1 mL of maintenance medium were inoculated with 0.2 mL of extract of stool specimen. The tubes were examined daily for evidence of cytopathic effect (CPE).

Pan EV Real time-PCR

Pan EV Real-time reverse-transcription PCR (rRT-PCR) targeting 5´ UTR was performed as per (13). RRT-PCR mixture consists of 10 µl of Tough Mix (Quanta Biosciences, Beverly, MA), 1 µl of primer-Probe mixture (forward 5´GGCCCCTGAATGCGGCTAATCC3´; reverse 5´GCGATTGTCACCATWAGCAGYCA3´; probe 5´CCGACTACTTTGGGWGTCCGTGT3´; 8 µl RNase-free water, and 1µl of template (extracted viral RNA). The thermo cycling conditions are 30 min at 50°C for the RT step and 1 min of incubation at 95°C, followed by 40 cycles of 95°C for 15 s, 50°C for 1 min, and 72°C for 5 s. (A reduced ramp rate of 25% between annealing and elongation was applied on the ABI 7500 real-time PCR system.

VP1 PCR

cDNA was synthesized in a 10 µl reaction mixture containing 5 µl of RNA, 100 µM each de-oxy nucleoside triphosphate (dNTP; Roche diagnostics), 2µl of 5X reaction buffer (Invitrogen, Carlsbad, CA), 0.01 M dithiothreitol, 1 pmol each cDNA primer (primers AN32, AN33, AN34, and AN35), 20 U of RNasin (Promega Corp., Madison, WI), and 100 U of Super Script III reverse transcriptase (Invitrogen) followed by incubation at 22°C for 10 min, 45°C for 45 min, and 95°C for 5 min. A total 10µl RT reaction mixture was used in the (PCR1) (final volume, 50 µl) (PCR1), consisting of 5 µl of 10X PCR buffer (Roche Diagnostics), 200 µM each dNTP, 50 pmol each of primers 224 and 222, and 2.5 U of *Taq* DNA polymerase (Roche Diagnostics), with 40 cycles of amplification (95°C for 30 s, 42°C for 30 s, 60°C for 45 s). One microliter of the first PCR product was used as template in second PCR (PCR2) for semi-nested amplification. PCR2 contained 40 pmol each of primers AN89 and AN88, 200µM each dNTP, 5 µl of 10X Fast Start *Taq* buffer (Roche Applied Science), and 2.5 U of Fast Start *Taq* DNA polymerase (Roche Applied Science) in a final volume of 50 µl followed by incubation at 95°C for 6 min prior to 40 amplification cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 15 s.

Ethics Statement

The study protocol was approved by the Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareli Road, Lucknow.

Result

Virus detection in specimens

In order to determine a more effective method for diagnosing AFP cases, 789 stool samples (suspected from AFP) compared by using different identification techniques: cell culture, Real time PCR and VP1 PCR.A total of 109 out of 789 specimens (13.81%) were found to be positive for enterovirus from cell culture, 163 (20.65%) were found positive from Pan Enterovirus Real time PCR, and 152 (19.26%) were found positive from VP1 PCR.

Clinical findings and epidemiology

The highest rate of AFP infection occurred in children from 1 year to 5 years of age. The positivity of AFP infection decreases with increase in age. Of the 163 total positive AFP cases, 93 were from Males and 70 were from females resulting in a sex ratio of 1.3:1. Clinical findings showed that 16 (9.8%) of the 163 patients had residual paralysis (Table 1).

Gender and age distribution

The ages of the AFP positive population begin from 1 month to15 years, and their distribution is as follows: 1 Month – 1Year (27.82%), 1y-5y (28.97%), 6y-10y (13.36%) and 11y-15y (11.49%) respectively (Table 1).

Table 1 Clinical and Demographic distribution of AFP cases

Age Distribution (%)	Median Age	Gender M/F	Fever No. (%) M/F	Vomiting No. M/F	Diarrhea No. M/F	Asymmetrical Paralysis No. M/F	Residual Pralysis No. M/F	Sample Tested	Positive (%)M/F
0-1 (14.57)	0.8	61/54	41/38 (68.69)	11/14	8/5	21/18	3/1	115	19/13 (27.82)
1-5 (35.86)	3.2	162/121	102/98 (70.67)	45/61	17/23	48/41	6/6	283	45/37 (28.97)
6-10 (27.50)	7.9	111/106	78/64 (65.43)	38/45	12/8	23/19	0/0	217	18/11 (13.36)
11-15 (22.05)	12.8	98/76	43/36 (45.40)	39/21	14/9	14/9	0/0	174	11/9 (11.49)

M –Male; F- Female

Seasonal distribution

However, AFP infection was reported throughout the year during the study period, variation in the rate of detection month wise analysis of the AFP revealed a low period of transmission during winter (December, January and February) and a high period of transmission from May 2015 to November 2015 with a peak from July to November 2010 (Figure 1).

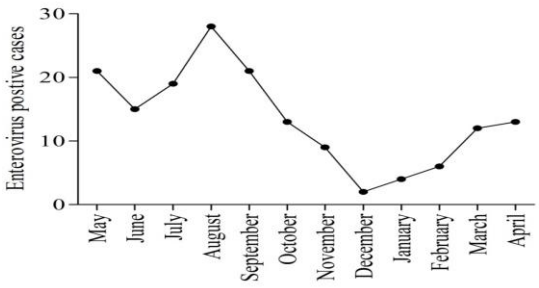

Figure 1 Seasonal distribution of positive cases

Geographical distribution of NPEV-associated AFP cases.

The geographic distribution of the AFP positive cases reflected their circulation in 24 of the 49 districts of Uttar Pradesh. Distribution was however spread from the northern through the central to the eastern belts of the UP state (Figure 2). Lucknow region, followed by the Hardoi, Unnao, Kheri, Sitapur, Raebareli and Sultanpur region located in the middle part of the state saw more positive cases with the least from the, Gorakhpur,Deoria, Basti, Gonda, Balrampur, Maharajganj, Kushinagar and Varanasi region in the eastern part of the state.

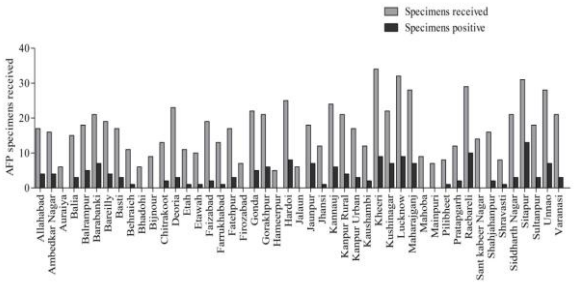

Figure 2 Geographical distribution of AFP cases in state of Uttar Pradesh

Efficacy of detection methods used in this study

The sensitivity of RT-PCR, VP1 PCR and cell culture is as 100%, 93.25% and 66.87% respectively (Table 2). And their specificity of all methods is 100%. In case of cell culture and VP1 conventional PCR the overall 94.5% agreement have been observed with calculated kappa value of 0.80 whereas it slightly decreased in case of real time PCR (93.1% agreement; kappa 0.76) (Table 3).

Table 2 Detection efficiency of different methods used for Enterovirus in AFP cases

	RT-PCR	VP1-PCR	Cell culture
Sensitivity	100.00% (97.76% to 100.00%)	93.25% (88.25% to 96.58%)	66.87% (59.08% to 74.04%)
Specificity	100.00% (99.41% to 100.00%)	100.00% (99.42% to 100.00%)	100.00% (99.41% to 100.00%)
Positive Predictive Value	100.00%	100.00%	100.00%
Negative Predictive Value	100.00%	98.30% (97.04% to 99.03%)	92.06% (90.31% to 93.51%)
Accuracy	100.00%	98.62%	93.16% (91.16% to 94.82%)

Table 3 Agreements between the results of the cell culture and Enterovirus RT-PCR assay

		Pan-Enterovirus real time PCR assay		Total	Enterovirus VP1 PCR assay		Total
		Negative	Positive		Negative	Positive	
Cell culture method	Negative	626	54	680	637	43	680
	Positive	0	109	109	0	109	109
	Total	626	163	789	637	152	789
		Overall agreement = 93.1%; Kappa = 0.762;			*Overall agreement = 94.3%; Kappa = 0.804;*		

Discussion

When conducting a study for detection of Enterovirus in AFP, it is important to consider the advantages and disadvantages of the detection methods used. In this study, we aimed to elucidate on comparing cell culture and PCR techniques for the detection of Enteroviruses isolated from AFP suspected cases. In this study we also evaluated the sensitivity and efficiency of cell culture and RT-PCR for enterovirus detection in stool specimens. Cell culture was recognized as the "gold standard" for laboratory diagnosis of enteroviruses. However, Enteroviruses replicates and produce a cytopathic effect (CPE) usually requires 2 to 14 days or sometimes more days, varying with the specimen source and with the concentration of virus in the specimen, as well as culture requires viable virus (14). 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. 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(15)Rapid diagnosis of enteroviruses and other infections caused by these viruses decreases both the unnecessary use of antibiotics and the costs in clinical practice(16)The RT-PCR assay is also able to detect enterovirus strains that do not grow in conventional cell lines (17) 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. 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 (18) Cell culture sensitivity may be affected by toxicity of the samples and inappropriate transport or storage of live organisms.

To the best of our knowledge, this is the first study from India on North Indian pediatric population about the comparative detection of Enteroviruses by cell culture and PCR techniques. Studies that compared the PCR and cell culture techniques by using clinical specimens have shown that, despite the advantages of detecting viral RNA by PCR, such as speed, sensitivity, ability to detect nonviable viruses and potential elimination of the toxic effects of the specimen, there were a large number of positive samples through the PCR method (19) We found that, on comparing cell culture with PCR, the detection rates for enteroviruses in stool specimens obtained from pediatric population with AFP was 13.81% and 20.65% respectively, which was significantly greater by PCR technique. PCR technique was found to be more reliable and useful for rapid diagnosis and is a highly sensitive and specific tool to detect enterovirus in AFP patients as compared to cell culture. It can be used to determine whether the virus is reactivated and shed without causing pathogenic effects.

After the comparison of the positivity of enterovirus by the cell culture and PCR, we found that 109 (13.81%) samples were positive cell culture, 163 (20.65%) by RT-PCR and 152 (19.26%) by positive from VP1 PCR. The present data indicated that PCR represents a significant improvement over culture. The sensitivity and specificity of the test were excellent at 100%, 93.25% and 66.87% respectively. Comparable agreements have also been observed in all the techniques.

So we can conclude that RT-PCR is a reasonably sensitive and highly specific method for

diagnosis of enteroviruses in AFP. The use of PCR should provide the resources to perform accurate epidemiological studies as well as to investigate the immune response to infection and to study potential antiviral therapeutics.

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