



Original Research Article

Isolation and Identification of Non-Polio Enteroviruses Increasing by RT-PCR Assay Among Infected Children with AFP

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ABSTRACT

In Iraqi population, there is no previous study investigated the NPEVs in AFP which is negative in cell line. Thus, this study used the RT-PCR method to increase the enterovirus isolation especially from risky groups. A total of 1073 stool samples were tested in cell line technique to diagnosis of enterovirus (EV). The RNA genome was extracted from 90 cell suspension of positive and negative isolates for EV in cell line. These samples included positive (40), weak positive (10) and negative (40) samples to detect the EVs gene by reverse transcriptase polymerase chain reaction (RT-PCR). Results gave away on cell line that 114 (14.3%) represented as non polioenterovirus (NPEV), and 46 (5.7%) resulted as poliovirus (PV) in patient group. While 274 case of (contact/ healthy group) compromised into 33 cases (12.1%) as NPEV and 16 cases (5.8%) as PV (Incubatory carrier or healthy carrier). So the results converted from 55.5% (50/90) by cell line to 71.1% (64/90) in RT-PCR, while negative results decreased to 26 isolates (28.9%). The findings in this study confirm the reports by others and have shown that RT-PCR makes the researcher more confident in detecting viruses in a variety of samples.

Keyword: Non-Polio Enteroviruses; AFP; Cell line and Reverse transcriptase-PCR; Iraqi population

INTRODUCTION

Human enteroviruses (HEV) belonging to the family Picornaviridae, that have five accepted genera causing human infection: Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus, and

Aphthovirus. Enterovirus including the polioviruses and non-polioenterovirus (NPEV) (coxsackieviruses, echoviruses and other new EVs), which are small, non-enveloped viruses

that have a spherical shape of about 30nm in diameter with a single-stranded, positive-sense RNA genome [1, 2]. Poliovirus and NPEV are the major viral cause of neurologic disease with a known etiology in humans, including meningitis, encephalitis and acute flaccid paralysis [3-5]. The clinical picture depends on the particular EV subtype or strain, the level of viral replication, as well as host factors [6].

The differential diagnosis of AFP based on laboratory tests is one of the important factors in order to evaluate the role of Non-Polio Enteroviruses in such disorders [7]. Virus isolation in cell culture is still being the most common laboratory method for EV detection prior to neutralization or molecular typing of HEV. No single cell line is sufficient for the isolation of all serotypes; a combination of at least four different cell lines has been recommended for sufficient serotype coverage [8], due to the more than 100 different genotypes of HEV or the great variability in the VP1 region which is applicable for "molecular serotyping" [9]. There is some HEV growing slowly and results are typically only available after the patient has recovered. Hence, the RT-PCR assay is a rapid technique reach a definite diagnosis in 48 hours [10, 11].

Obtaining false negatives in cell culture can be due to the presence of slow growing Enteroviruses in stool specimens, lack of sensitivity of the cell line, low titer of the virus in the specimens and toxic factors [12]. Nevertheless, cell culture remains a primordial step for the identification of EV serotype by seroneutralisation protocols [13]. However, the reverse transcriptase-polymerase chain reaction techniques (RT-PCR) not only improve laboratories' sensitivity for detection of the virus, but are also more suitable and of course, more economic [14-16]. In our population, there is no previous study investigated the NPEVs in AFP which is negative in cell line. Thus, this study used the RT-PCR method to increase

the enterovirus isolation especially from risky groups.

MATERIALS AND METHODS

Sample collection and preparation

A total of 1073 stool samples were tested in cell line technique to diagnosis of enterovirus (Poliovirus and NPEV). Of these, 274 stool samples obtained from healthy children as a contact person with infected children. They were collected from children aged less than 15 years (5 months-7 years old) and accompanied by an AFP notification during January-May 2015. All stool samples were processed with chloroform before inoculation into RD cells and L20B cell lines from National Polio Laboratory (NPL/Iraq) stock held in liquid nitrogen at low passage (passage up to 10). Briefly, 10 ml PBS buffer was added to 1g glass beads and 1 ml chloroform, then transferred to 2g of fecal sample and shaken vigorously for 20 min. Centrifugation at 1500g in cooled centrifuge for 20 min to transfer supernatant from each sample into new tube and stored at 4°C to be inoculated in the same day or kept at -20 °C to 3 months (WHO, 2004).

Virus detection

Each stool suspension was injected in healthy monolayer of RD cells were grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and then it providing with maintenance medium supplemented with 2% fetal bovine serum. The inoculated tubes were placed to incubator at 36°C for 7 days and examined daily for the specific enterovirus cytopathic effects (CPE) of rounded, refractile cells detaching from the surface of tubes. The cells with CPE up to 75% were harvested and stored at -20°C, whereas those negative results were re-passaged on to another RD cells. Positive isolates on RD cells were passaged on L20B cells to identify polioviruses [18, 19]. Samples that showed CPE on L20B were confirmed with international laboratory for

WHO to differentiate between wild and vaccine polioviruses, while the sample grown only RD cell detected as NPEV.

RNA extraction

RNA genome was extracted from 90 cell suspension of positive and negative isolates for EV in cell line. These samples included positive (40), weak positive (10) and negative (40) samples to detect the EVs gene comparison with cell culture efficiency. The QIAamp Viral RNA Mini kit used according to kit instructions with the RNA elution in 40µl.

Gene amplification

One step RT-PCR was performed for EV detection based on 5'NTR amplification. A forward primer (5'-CCCTGAATGCGGCTAATCC-3', positions 456 to 474), and reverse primer (5'-ATTGTCACCATAAGCAGCCA-3', positions 582 to 601) were used [20]. After optimization of the RT-PCR condition, all samples assayed in a 25 µl reaction mixture containing 8µl of template RNA, 1 µl of enzyme mix and 5 µl of buffer 5x (QIAGEN one step RT-PCR), 0.6 µM conc. of each forward and reverse primers for EV, 1 µl of MgCl₂, 400 µM conc. of each dNTP (1 µl) and DEPC (Diethylpyrocarbonate). The RT-PCR amplification protocol was done as follows: 30 min at 50 °C for reverse transcription reaction, followed by 15 min at 95°C for initial Taq NA polymerase activation, and 35 cycles of 30 s at 95°C, 1 min at 48°C and 1 min at 70 °C then final extension 5 min at 70 °C. The amplicon visualized with ethidium bromide staining on 1% agarose to detect the specific product size 144-149 base pair.

Statistical analysis

All data were tabulated and analyzed using the SPSS IBM version 20. All values were calculated according to the positive results as percentages (%). Differences between study groups and assays were analyzed by cross-tab and person chi-square (X²) test. A value of P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Identification of NPEVs in Cell Line

In all stool samples of acute flaccid paralysis (AFP) cases only 14.3% (114/799) and 12.1% (33/274) of healthy group (contact) were positive for NPEV when it differentiated on monolayer of two cell lines (Fig.1) called Human Rhabdomyosarcoma cell (RD) and L-cell (L20B). The RD cell line was recommended to use and to isolate EVs from patients suffering from AFP [21, 22], while L20B was used to differentiate non polio enteroviruses (NPEV) from polio virus (PV) [21]. In the current study, NPEVs reported more than Poliovirus within the study groups, but there is no statistical difference of viral presence between patients and healthy groups (X²=0.27, P>0.05). These NPEVs isolated positive results of the contacts may indicated that children have infected from the patients without being pathogenized with these viruses (sub clinical infection/ a symptomatic carrier of virus) due to non-specific defense mechanisms like Age, Sex, Race, Genetics or Nutritional status or due to specific defense mechanisms by their immune system that may covered on the virus either naturally or artificially [23]. Also it may represent a latent threat in this group to induce a clinical AFP picture in future [15].

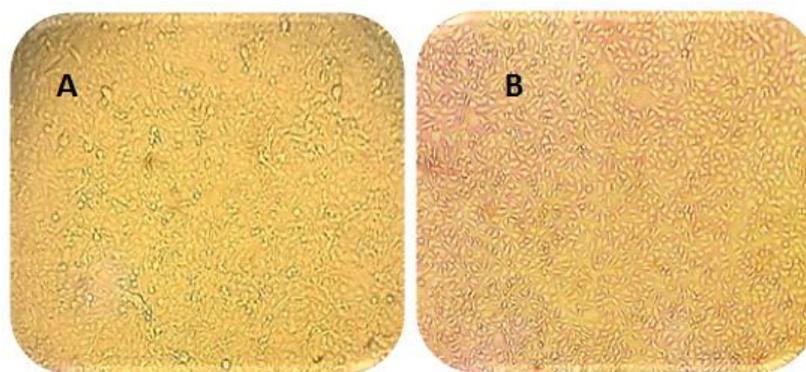


Fig. 1: the cell line control of (A): RD and (B): L20B

According to the results on RD cell lines, 209 cases showed positive result as enteroviruses that compromised to 160 cases in patient group and 49 cases in contact group. These positive cases gave away on L20B cell line that 114 (14.3%) negative result represented as NPEV, and 46 (5.7%) positive resulted as polio virus.

While 274 case of (contact/ healthy group) compromised into 33 case (12.1%) as NPEV and 16 case (5.8%) as PV (Incubatory carrier or healthy carrier) and 225 case (82.1%) as negative result (healthy persons) without any virus recorded when cultured on RD and L20B cell lines, as shown in table 1.

Collected groups	No. (%) of sample tested in T.C. for EVs detection in RD cell and for NPEV detection in L20B cell in number and percentage			
	RD cell line		L20B cell line	
	-ve	+ve (PV+NPEV)	-ve (NPEV)	+ve (PV)
Patient group 799 case	639 (80%)	160 (20%)	114 (14.3%)	46 (5.7%)
contact group 274 case	225 (82.1%)	49(17.9%)	33 (12.1%)	16 (5.8%)
Total 1073 case	864 (80.5%)	209 (19.5%)	147 (13.7%)	62 (5.8%)

Therefore, this study pointed out the significant reveal of NPEVs than Poliovirus infections in both patient ($X^2=28.9$, $P<0.001$) and healthy groups ($X^2=5.9$, $P<0.05$).

Moreover, these NPEV positive cases included the weak positive (Fig 2A), which explained may other EV serotypes no growing or slowly in common cell lines used for diagnosis or lack of cell sensitivity to low viral load of these clinical samples or to the characteristic of NPEV strains [15, 24].

Furthermore, there is a 60 days follow- up test used to record if the paralysis in the patients still found after sixty day (risky group) from the

onset of paralysis or it had gone. Remarkably, this study reported that 27.2% (31/114) of NPEV patients who still suffered from residual paralysis after sixty day follow- up the onset of paralysis. Because the factors that affect transmission of human enterovirus and lead to incidence of HEVs infections particularly among infants and children, like overcrowding, poor levels of hygiene, bad water quality and poor sewage handling facilities, altogether gathered in the affected different area of Iraq, also India which recorded residual paralysis cases due the same factors that reported with other study [18]. Laxmivandana *et al.*, [25] reported that

NPEV positivity was high in AFP cases with residual paralysis as compared to recovered cases, which matched with this study result. Meanwhile, negative results for EVs when cultured on RD cell line among AFP that explained the another causes may not be viral cases it either be Guillain–Barré Syndrome (GBS) [26], traumatic neuritis (intra muscular injection in the motor neuron lead to neuron

trauma then paralysis in the injected leg) [27], hypokalemia (deficiency of the calcium in bones lead to paralysis) [28] bacterial infection (*Campylobacter jejuni*, *Clostridium botulinum*, *Corynebacterium diphtheriae*, *Treponema pallidum*, and *Clostridium tetani*) [29, 30], or Reptiles-snake venom (Cobra, krait, mamba, Australian elapid and sea snake) [7].

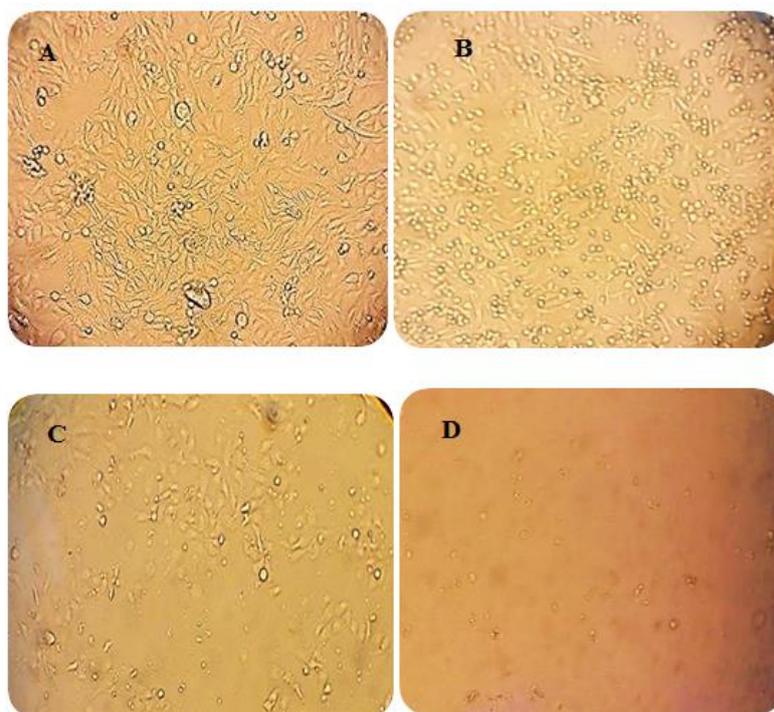


Fig. 2: Cell line growth of RD cells show the cytopathic effect of entroviruses on the cell line, filed A represent: CPE +1 which refer to the weak positive results after two passages, B: CPE +2, C: CPE+3 and D: CPE+4

Detection of NPEVs from cell suspension by RT-PCR Method

From the patient group, only 90 cell suspensions selected as a study group from different Iraqi provinces for molecular assay. These suspensions included 40 clear positive NPEV isolates (with CPE from 2+ to 4+), 10 weak positive NPEV isolates (with CPE 1+ after two to three passages) and 40 negative isolates without any CPE noticed on RD cell line. Results of all positive isolates (50) in cell line was showed 100% match with RT-PCR by using of

specific primer for the highly conserved sequences in the 5' non-coding region (NCR) with 144-149 bp (Fig. 3). Moreover, it detected the weak positive cases quickly like other positive sample, and 35% (14/40) of negative isolates showed positive result with RT-PCR. Furthermore, the molecular method performed as a rapid and sensitive way for increasing EVs detection in this study, so the total of positive result converted from 55.5% (50/90) by T.C. to 71.1% (64/90) in RT-PCR, while negative results decreased to 26 isolates (28.9%). Hence, the

total of AFP cases caused by NPEVs converted patient group (Fig. 4).
from 114 (14.3%) to 128 (16.02%) among

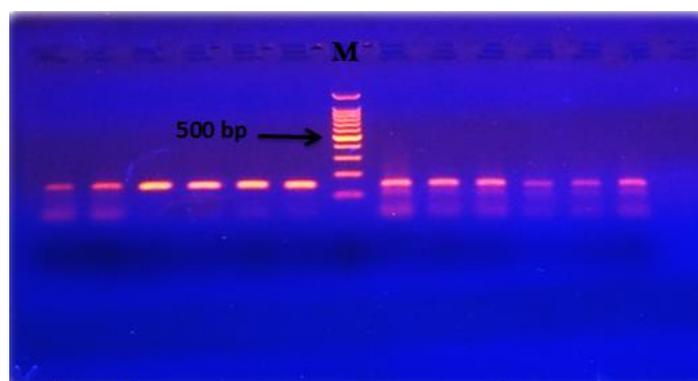


Fig. 3: Amplification of partial enterovirus genome at 5'NCR with 144-149 bp. Agarose gel 1%, M: Marker ranged 1000-100 bp

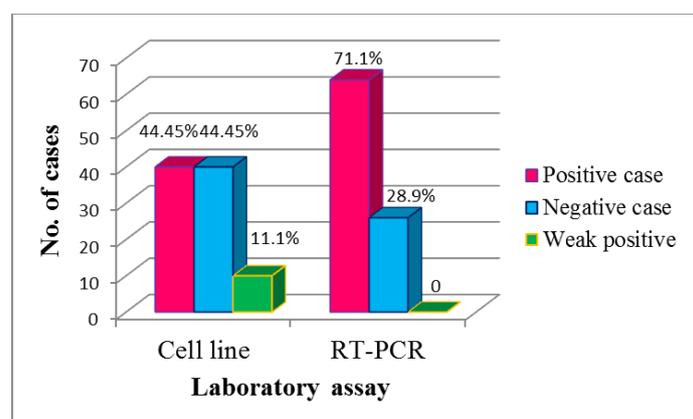


Figure 4: Comparison between the efficiency of cell line and RT-PCR techniques for NPEVs detection among study group

These positively included 25 isolates with residual paralysis constituted negative, weak and clear positive in 2, 3 and 20 isolates, respectively.

The statistical analysis determined that the RT-PCR-based method have highly significant difference ($\chi^2 = 18.73$; $P < 0.001$) in compared with the cell line method for NPEVs detection, like other previous studies for the detection of EVs using both cell line and RT-PCR which displayed that the results of RT-PCR are more accurate [31, 32].

Also Abbasian *et al.*, (2011) observed that 24% of stool samples isolated by using 5'NCR RT-PCR while cell lines isolated just 14.4% of these

samples. These results were matched with this current result in which is indicated that the RT-PCR based method is more accurate, rapid, sensitive and more economic than cell line based method in enterovirus detection. On the other hand, the weak positive isolates take up more than two passages within 10 days to give a result take up maximum two days by using RT-PCR to get the same results, plus some positive results that showed false negative results on cell culture were detected accurately and quickly by RT-PCR, because there is five

types of cell line used to overcome all the enterovirus strains, and some strains have no

growing in the laboratory used cell lines, RD and L20B cell lines at all, and need another cell line type to grow and show the CPE, beside to lack of sensitivity of the cell line, low titer of the virus in the specimens and toxic factors [33, 34]. While RT-PCR detection based on the 5'NCR which found in all the enterovirus strains and that offered specific direct detection as mentioned by other studies [15, 35, 36].

CONCLUSION

The findings in this study confirm the reports by others and have shown that RT-PCR makes the researcher more confident in detecting viruses in a variety of samples.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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