Isolation and Detection of Echoviruses in Acute Flaccid Paralysis among children under 15 years old in different Iraqi Provinces

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الخلاصة

الشلل الرخوي الحاد (AFP) هو متلازمة سريريه تتصف بالأعراض السريعة من الوهن والضعف حيث يسبب ضعف العضلات ورخاوتها ثم تتطور الأعراض سريعا إلى الشلل الرخوي خلال بضعة أيام أو أسابيع من الإصابة وأحيانا يؤدي إلى الوفاة.

الهدف من هذه الدراسة هو عزل وتشخيص أنماط الفيروسات المعوية (NPEVs) العائدة الى النوع (ECHO viruses) من 300 نموذج براز تم جمعها من أطفال دون سن الخامسة عشر سنة ظهرت لديهم أعراض الشلل الرخوي الحاد ومن كافة المحافظات العراقية. و ثم تمييز أنماطها المختلفة باستخدام طريقتي الزرع النسيجي (خلايا RD) والأسلوب التشخيصي بالتنميط المصلي وهذه طبقا للبروتوكول المستخدم من قبل المختبرات المرجعية لمنظمة الصحة العالمية

إن أسلوب التشخيصي العام للإصابة المتوقعة بالفيروسات المعوية هو عزل هذه الفيروسات من نماذج البراز باستخدام خلايا زرعيه مناسبة مثل خلايا الـــ(RD CELLS) التي تظهر عليها التأثيرات الخلوية الناتجة من فيروس شلل الأطفال والفيروسات المعوية وان هذه الفيروسات المعزولة تخضع لطريقة التعادل (neutralization assay) وذلك لغرض التعرف على الأنماط المختلفة من الفيروسات المعزولة وتعرف هذه العمليات عموما بالتنميط المصلي (Sero-typing) .

الدراسة الحالية لمختلف المحافظات العراقية بينت إن فيروس الـــ (ECHO) هو الأعلى تكرارا في الإصابات وتم عزله من 79 حاله (26.33%) وسجلت أعلى نسبة في محافظة البصرة 21 (52.5%) وبغداد 31 (51.67%)

Abstract

Acute flaccid paralysis (AFP) is a clinical syndrome characterized by rapid onset of weakness, affecting the muscles then progressing to maximum severity within several days to weeks and sometimes may lead to death.

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The objective of this study was the isolation and detection of Non-Polio Enteroviruses, The ECHO virus from three hundred stool specimens obtained from children aged less than 15 years old showing symptoms of acute flaccid paralysis (AFP) from different Iraqi provinces and characterization the different serotypes of these viruses using both tissue culture (using RD cells) and neutralization-assay methods. This was done according to protocols applied by reference laboratories recommended by World Health Organization.

The common diagnostic approach in a suspected Enteroviruses infection is the isolation of the virus (stool samples) in susceptible cell cultures such as RD cell line this cells appearance cytopathic effect (CPE) for polio viruses and NPEVs . Virus isolation is then followed by the use of a neutralization-type assay to identify the serotype of the isolated Enterovirus, a process commonly known as serotyping.

The current study, which covered in different Iraqi provinces shows that echoviruses (ECHO) had the highest prevalence affecting 79 cases (26.33%) out of 101 NPEVs. The present study showed that the maximum frequency of ECHO virus isolation was reported in Baghdad 31 (51.67%) and Basrah 21 (52.5%)

Introduction

Echoviruses are enteroviruses (genus Enteroviruses, family *Picornaviridae*) are among the commonest viruses infecting humans all over the world. ECHO are associated with diverse clinical syndromes ranging from minor febrile illness to severe, potentially fatal conditions (e.g., aseptic meningitis, encephalitis, paralysis, acute flaccid paralysis, especially in children[1,2] myocarditis, and neonatal enteroviral sepsis and could be linked with the development of some chronic diseases (e.g., type 1 diabetes and dilated cardiomyopathy)[3,4]. Each year, an estimated 10-15 millions of symptomatic enterovirus infections occur in the United States[5]. The enteroviral group ECHO includes many serotypes :- E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-8, E-9, E-11, E-12,

E-13, E-14, E-15, E-16, E-17, E-18, E-19, E20, E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E-32, & E-33 found under the species: Human enterovirus B [6]. The enterovirus enters the human host through the gastrointestinal tract (GI) or respiratory tract. The infection can progress to CNS involvement during the major viremic phase or at a later time [7]. Antibody production in response to enteroviral infections occurs within the first 7-10 days.

Epidemiologic investigations of enteroviruses in healthy subject seem to establish the flowing points: Enteroviruses are prevalent in the stool specimens of healthy children "normal "permanent viral intestinal flora [8].these viruses were inhabited in the (GI) and are most frequently isolated before the age of four years. There are a seasonal incidence in the summer and autumn.. The percentage of enteroviruses harbored by hospital delivered newborn infants prior to being discharged from the hospital seems to be extremely low [9,10].The present study was aimed shedding light on the prevalence of ECHO viruses among AFP patient in Iraq using tissue culture and neutralization methods

Materials and Method

Specimen Collection**:**

Atotal of 300 stool specimens were included in this study. Clinical specimens were collected from children aged less than 15 years, from Iraq as well as governmental hospitals from different Iraqi governorates in the period from (October-2010) to (March - 2011) .The specimens were accompanied by an AFP notification form with details of patient personal and clinical history. All stool specimens were processed with chloroform before inoculation into RD cells and L20B cell lines from national polio laboratory of iraq (NPL/Iraq) stock held in liquid nitrogen at low passage.

The sample were prepared to stool suspension according to[11] . Extraction of the stool specimen were inoculated on RD cells performed according to[19,20,21] . Growth and maintenance media were prepared according to [12,13,14]. These media was used to keep the cell cultures in a steady state of slow cell replication during the period of virus inoculation and replication [15,22,23]

The procedure of passage describe as bellow:-

1- The L20B and RD is cell lines used in cultures usually needed for inoculation 3 times per week for AFP and environmental investigation labs

2. Growth medium was decanted from the cell culture flask and gently washed the confluent cell layer twice with Calcium and Magnesium free PBS.

3. 25% trypsin solution (or equal parts of 0.25% trypsin and 1:5000 Versene solutions) was added in PBS to the monolayer, dispersed evenly. (A volume of 0.5 ml is adequate for a 25 cm2 flask.)

4. The cells were re-suspended in 25 ml growth Medium for 75 cm^2 flask, which halted the action of the trypsin.

5. The optimum split ratio (determined by cell counting) required different concentration of cells 120.000 cells per flask for L20B and 80.000 cells per flask for RD. The split ratio became apparent as experience was gained with each culture [18].

CPE positive RD cultures

There are passages into L20B cells and incubated at 36 C°, and observed daily. This passage aims at separating polioviruses that may be present in mixtures with other enteroviruses and amplifying the titer of any polioviruses that may be present. If no CPE is obtained when L20B cultures are examined for at least 5 days, the culture will be considered negative for polioviruses.

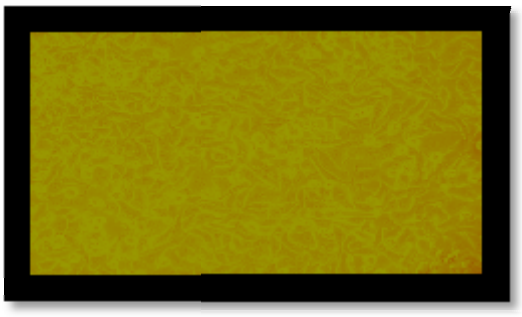
Results and discussions

In the present study, three hundred of stool samples were collected from patients were suffering from acute flaccid paralysis (AFP) Standard methods for enterovirus detection and identification are based on virus isolation on cell culture followed by stereotyping of the isolated viruses by micro neutralization assays using pools of serotype specific Antisera [26].

The samples of patients were cultured on monolayer of human cell lines called RD cell line (figure:-1) . This cell line was recommended to use and to isolate EVs from patients suffering from AFP[27, 28]. RD cell lines were first cultured in tissue culture

tubes before being inoculated with sample extraction. This kind of cell line multiplied in growth media (MEM with 10% FBS) and when the cells attached to surface of falcon and formed a confluent monolayer [16].When the multiplication of cells stopped and ultimately the monolayer cell line of RD was performed nearly confluent (2-3 days). The effect of virus can be seen by CPE that shown on cell line. Figure (2) shows the primary effect of virus on cell line and that happened during (1-3) days after inoculating cell line with sample extraction that has EVs.

The frequency of ECHO virus isolation in Iraqi provinces was shown in Table (1) . the present study showed that the maximum frequency of ECHO virus isolation was reported in Basrah and Baghdad but in north Iraqi provinces (Duhok, Erbil and Sulymania) the virus was not isolated. That confirming the highest incidence with this virus was in middle and south provinces. That can be explained by the high contamination of drinking water with this virus in middle and south provinces. The another reason for this may be relating with the high temperature of middle and south Iraqi provinces as compare to north Iraqi provinces as the high incidence of this virus related with elevating of temperature [4] .That is only the incidence of paralysis that caused by EVs found more in the tropical countries such as India [29].



Figure(1). RD cell growth(monolayer) on polystyrene plate (400x)

Figure (2):- RD cell line effected with virus(CPE)(400 x).

Table (1). Number and percentages of ECOH virus positive cases in patients and healthy control groups over different Iraqi provinces.

Provinces.	Echo positive case in test group	
	Number	Percentage %
Baghdad	31	51.67
Basrah	21	52.5
Babil	3	30
Anbar	1	10
Diala	2	20
Duhok	0	0
Erbil	0	0
Kerbala	1	10
Missan	3	30
Muthana	1	10
Najaf	3	30
Ninewah	1	5
Qadysia	3	15
Sulymania	0	0
Salahdeen	1	10
Tamim	0	0
Theqar	4	20
Wasit	4	20
Total	79 (26.33%)	

Serotyping of ECHO viruses

Further sub classification was done in this study to classify ECHO virus in to sub groups (ECHO 7, ECHO 30, ECHO 14, ECHO 6, ECHO 13, ECHO 25, ECHO 27 and ECHO 3). The results is shown in table (2). The highest frequency was found

with ECOH 7 followed by ECHO 30 while the lowest frequency was observed with ECHO 3.

Significant number of NPEVs infections in patient groups was detected as compared with healthy control group ($p<0.01$).

Table (2):- different serotypes of ECHO viruses distributed among patients

ECHO serotype	Patient group	
	Number	Percentage
ECHO 7	36	45.56
ECHO 30	18	22.78
ECHO 14	7	8.86
ECHO 6	6	7.59
ECHO 13	5	6.32
ECHO 11	4	5.06
ECHO 27	2	2.53
ECHO 3	1	1.26
Total	79	100

Conclusions

1-Significant relation between the infection with ECHO viruses and Acute Flaccid Paralysis among children under 15 years old.

2-Children with age under 15 years old were getting a higher rate of infection with NPEVs because of poor hygienic habits and lack of prior immunity.

3- . The incidence of ECHO viruses in Iraqi provinces was shown with the high frequency of in Baghdad and Basra provinces.

4- High temperature of middle and south Iraqi provinces as compare to north Iraqi provinces lead to high incidence of NPEVs type (ECHO viruses).

5-Because EVs were excreted from the intestine for several weeks after infection, it is most frequently isolated from the stool samples. These stool samples are the most suitable materials for isolation of EVs confirming WHO criteria .

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