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Molecular detection and phylogenetic tree of echovirus in children at Thi-Qar province Iraq

Dalal Kadim Sakr

University of Thi-Qar, College of Science, Department of Biology

*Corresponding author email: dala.sak.bio@sci.utq.edu.iq

Bushra Jabbar Hamad

University of Thi-Qar, College of Science, Department of Biology

Email: bushra.jh_bio@sci.utq.edu.iq

Abstract---The current study was conducted in Thi-Qar province, southern Iraq, A number of 100 blood samples collected from sick Children in Mohammed Al-Mousawi Hospital for Children (ages between 2 days - 3 years), males and females, during the period from November 2021 to February 2022. This study aimed to detect the presence of Echovirus RNA in patients serum by RT-PCR and compare sequences results of current study isolates with previously described isolates from other region of the world and then draw its phylogenetic tree. Based on the results recorded among 100 child have different symptoms only 9% of patients infected with Echovirus, the results also noted a significant difference in viral prevalence in babies patients. The results from the sequencing analysis indicated that three viral isolates belong to the human echovirus, out of nine positive samples for PCR analysis. The results of the viral sequencing showed that the Iraqi strain is identical to the American isolate, China's isolate and Russia's isolate, all of them have 100% sequencing identical.

Keywords---echovirus, children, PCR, phylogenetic tree, Thi Qar, Iraq.

Introduction

Every year, Enterovirus epidemics occur over the world, particularly in the Asia-Pacific area (Wiatr et al. 2020). Enterovirus infections can cause many disease from a mild fever to life-threatening conditions like myocarditis or sepsis, Hand-foot-mouth illness, acute hemorrhagic conjunctivitis, herpangina. Severe consequences are more likely to occur in infants and those with weakened immune systems. In newborns, Enterovirus is a prevalent cause of infection. Infections in infants can spread vertically before, during, or after birth,

horizontally from family members, or nosocomially in nurseries (Zhang *et al.* 2021). Echoviruses are primarily transferred via the fecal–oral route where they target the gastrointestinal (GI) epithelium (Morosky *et al.* 2019). Echovirus infections are common and often go unnoticed.

However, depending on the virus type, its effects on host protein expression, and physiological metabolism, they can be severe and life-threatening (Wang *et al.* 2020). Echovirus infections affect infants and neonates the most, with echoviruses accounting for the majority of Enterovirus infections in newborns under the age of two months, in Neonatal Intensive Care Unit (NICU) outbreaks, Echovirus infections are extremely dangerous, accounting for 15–30% of nosocomial viral infections and resulting in neonatal death in up to 25% of cases (Civardi *et al.* 2013). Detection and molecular typing of Enterovirus have been facilitated during endemic and epidemic instances in recent years, thanks to the introduction of sensitive molecular tools for the diagnosis of Enterovirus infection (Chowdhury and Chakraborty 2017).

Molecular diagnosis of EVs now relies on amplification and sequencing of portion of the VP1 capsid region for serotype identification and phylogenetic analysis, followed by amplification and sequencing of the highly conserved 5'UTR for EV detection (Singh *et al.* 2016). Because VP1 genotyping frequently correlates with viral serotype, it is increasingly being used to classify newly identified Enteroviruses instead of normal serotyping. The 3D gene, which is responsible for encoding RNA-dependent RNA polymerase, is investigated in collaboration with VP1 to unravel the complicated evolutionary history of numerous Enteroviruses (Kang *et al.* 2021). This study aimed to detect the presence of Echovirus RNA in patients' serum by RT-PCR and compare sequences of our study isolates with previously described isolates from other regions of the world and then draw its phylogenetic tree.

Material and Method

This study was carried out at the department of biology, college of science/ university of Thi-Qar, during the period from November 2021 to February 2022. All the cases (100 cases) included in this study were collected from Mohammed Al Mousawi Children's Hospital in Thi-Qar province/ Iraq. Five ml of venous blood was drawn from symptomatic children, then gel tube containing blood was centrifuged at 4000 RPM for 5 minutes to obtain serum. Then serum is kept in an Eppendorf tube at -20°C until used for RT-PCR assay. The Echovirus RNA was extracted from serum by using the Viral RNA extraction Kit (Geneaid/ Thailand). Estimation of the concentration (ng/μl) and purity (260 /280 nm) of viral RNA was done by utilizing Nanodrop spectrophotometer (THERMO/USA). Then, utilizing the First strand cDNA synthesis kit (SinaClon/ Iran) to convert the viral RNA to cDNA. The products of PCR were analyzed by 1% agarose gel electrophoresis. The PCR Master Mix (Promega/ USA) as shown in table 1 and specific designed primer (viewed in table 2) was used for detection of Echovirus based amplification of 5'UTR-AD gene.

Table 1
PCR Reaction Components and volumes

Components	Volume	Final concentration
Viral cDNA mix	10 µl	10ng-3 µg
Forward primer	1 µl	0.2
Reverse primer	1 µl	0.2
Master mix	25 µl	X1
Nuclease free water	13µl	-----
Total	50 µl	

Table 2
Sequence, O.D, molecular weight and melting temperature of the primer used to detect 5UTR-AD

Primer sequence 5'-3'	OD	MW	Tm	nmol	GC%
F-Primer CGGCCCTGAATGCGCTAA	9.6	6253	64.2	30	63
R-Primer GAAACACGGACACCCAAAGTA	6.6	6173	63.4	30	47

PCR Thermocycler cycles were performed according to the following PCR condition (as in table 3), and the conventional polymerase chain products were analyzed by 1% agarose gel electrophoresis.

Table 3
PCR program for detection UTR-A-D (according to Huston method)

CYCLE NAME	Temperature	Duration	NO. of Cycles
Initial Denaturation	95C	10 minutes	1
Denaturation	95C	30 Seconds	50
Annealing	60C	60Seconds	
Extension	72C	1 Minutes	
Final Extension	72C	5Minutes	1

The Results

Prevalence of Echovirus in Children According to RT-PCR

The current results recorded among 100 child have different symptoms only 9% of patients infected with Echovirus, and 91% were negative for viral infection (as show in fig 1). And the figure 2 show the results of the gel electrophoresis of 5-UTR-A-D gene with product size (115 bp).

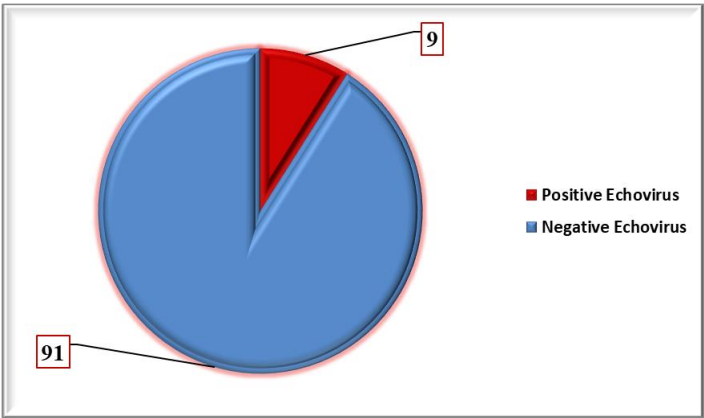


Figure 1. Echovirus in babies with different disease

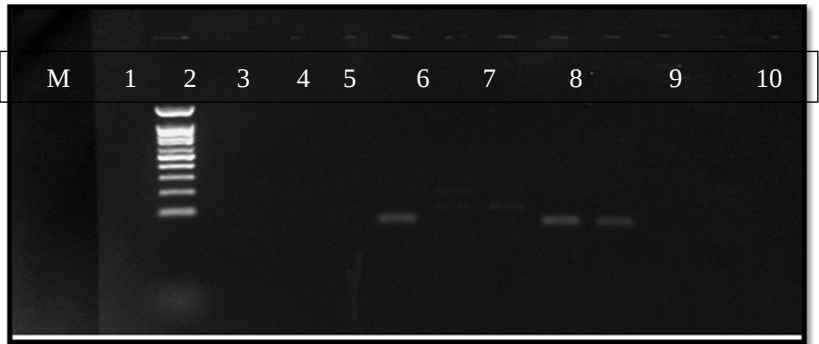


Figure 2. Agarose gel electrophoresis of 5-UTR-A-D gene with product size (115 bp). Lanes : M molecular marker (2kbp), lanes :1,2,3,4,5,7 and 8 negative , lanes :6,9 and 10 positive amplification

Sequencing Analysis results

The PCR product of isolated Echovirus from serum, was sent all positive samples (9 samples) to Macrogen Company in south Korea for sequencing of whole genome, were blasted in NCBI against standard strains of Echovirus. The sequencing analysis recorded there is non-genetic variation in three isolated Echovirus (as in table 4), and the other sample (6 sample) did not give results of the sequencing because the cDNA was damaged due to storage and transportation process.

Table 4
Percentage of compatibility and variation in the nuclear bases sequence for Echovirus isolates in the current study with the isolates previously recorded in NCBI

	Default name by NCBI	NCBI No.	Accession	Identity percentage	Country of NCBI Isolation
First Isolated Echovirus	Ouery-260671	AY302556.1		100%	USA

Second Isolated Echovirus	Ouery-48355	MK791151.1	100%	China
Third Isolated Echovirus	Ouery-42943	MN171535.1	100%	Russia

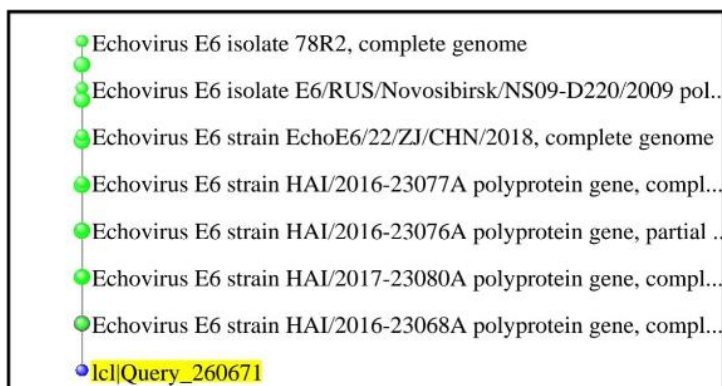


Figure 3. Phylogenetic tree analysis of first isolated Echovirus show genetic relationship with NCBI Echovirus. The phylogenetic tree was constructed in Blast of NCBI

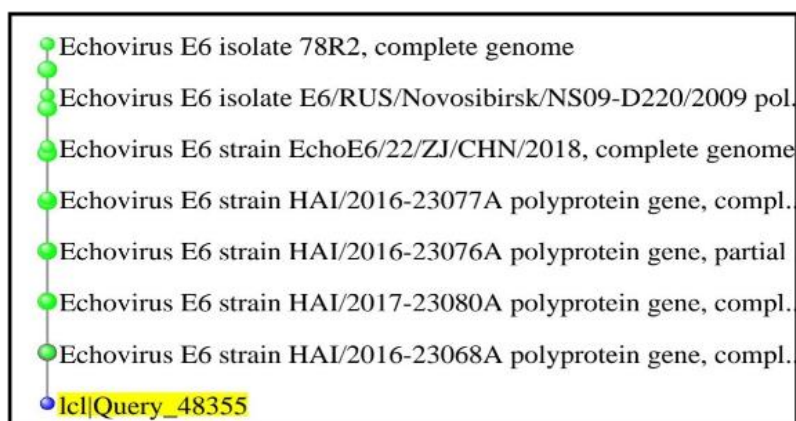


Figure 4. Phylogenetic tree analysis of second isolated Echovirus show genetic relationship with NCBI Echovirus. The phylogenetic tree was constructed in Blast of NCBI

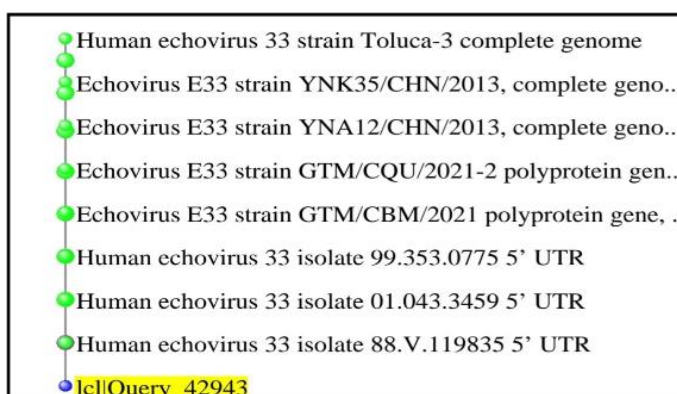


Figure 5. Phylogenetic tree analysis of third isolated Echovirus show genetic relationship with NCBI Echovirus. The phylogenetic tree was constructed in Blast of NCBI

Discussion

The current study indicates an outbreak of Echovirus in children. Although most echovirus infections are asymptomatic and usually mild, they are a public health concern, as the virus is easily transmitted via the fecal-oral route, and the virus is released from infected individuals for several weeks. The current study recorded the percentage of echovirus in present study was 9%. Where the present study disagreed with study performed by *Klement et al. (2013)* there were 538 commonly detected human Enteroviruses (HEVs) including 200 (37%) Echoviruses, was more than current study percent. Also the present study disagreed with study performed by *BUJAKI et al. (2020)*, where E9 was detected in 3.5% of 284 EV cases reported between 2010 and 2018. was less than our percent. *Walters et al., (2011)*, have reported Enterovirus RNA positivity in 31 of 373 (8.3%) neonates, infants and children with sepsis-like illness, in the Chicago area, similar to the rate in our study.

Echoviruses were also among the types detected most frequently as part of hospital-based Enterovirus surveillances in France (2000–2004) and the USA (1970–2005) (*Bubba et al. 2020*). Enteroviruses, especially E9 are an important cause of encephalitis among neonates, infants and young children in Kuwait (*Dalwai et al. 2009*). A large upsurge of E30 infections was reported in several countries in Europe during 2018 (*Broberg et al. 2018*). The co-circulation of several different groups fits better with changes in population susceptibility from birth cohort effects and a breach of a critical immunity level that controls Echovirus spread within the population (*Pons-Salort and Grassly 2018*). Echovirus cases were recorded in Thi Qar province due to the fact that the virus was a nosocomial infection, and it was likely that the virus was transmitted while children were in the children care unit or through of oral- fecal transmission, due of the lack of services available from sterile water or good sanitation services.

The results from the sequencing analysis indicated that three viral isolates belong to the human Echovirus, out of nine positive samples for PCR analysis, and the other sample (6 sample) did not give results of the sequencing because the cDNA

was damaged due to storage and transportation process, so there is no similarity to them in NCBI, except for the third isolates, which showed a perfect match. The comparison of newly sequenced isolates showed a 100% match percentage as shown, where the first isolate was identical to the American isolate registered AY302556.1, the second isolated virus was identical to China's isolate registered MK791151.1, and the third isolated virus was identical to Russia's isolate that registered MN171535. 1, all of them have 100% sequencing identical. The reasons for the emergence of Echovirus infections in Thi -Qar province indicate that the virus is endemic and is still a cause of various diseases in children.

Conclusion

The prevalence of the Echovirus was recorded at 9% in Thi-Qar province, the results from the sequencing study showed that three viral isolates belonged to the human Echovirus. A comparison of recently sequenced isolates revealed a 100% match rate, with the first isolate, the second isolate and third isolate being 100% identical to the American isolate, China's isolate registered, Russia's isolate respectively.

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