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DIAGNOSIS OF NON-POLIO ENTEROVIRAL INFECTIONS IN SEPTIC NEONATES BY POLYMERASE CHAIN REACTION ASSAYS

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ABSTRACT: Objective: The purpose of this study was to investigate the prevalence of non-polio enteroviral infections by quantitative and qualitative PCR assays in neonates 3-30 days old with clinical diagnosis of sepsis. **Methods:** The patients included all the neonates aged 3-30 days admitted with diagnosis of neonatal sepsis. Blood specimens, throat swabs, and in 60 cases CSF specimens were tested for Enteroviruses (EVs), using a quantitative RT-PCR assay. Alternate RT-PCR assays separated polio and non-polio enteroviruses. Demographic data including age, sex, type of feeding and gestational age were obtained. **Results:** As PCR results revealed, of the 177 neonates with clinical diagnosis of sepsis, one patient (0.55%) was positive for non-polio EVs in all of throat, serum and CSF samples. In 3 patients (1.7%), the results were positive for non-polio EVs in both throat and serum, while in 15 (8.45%) others only throat samples were positive. Sex, age and gestational age had no significant relationship with the prevalence of the infections statistically ($P > 0.05$). The prevalence of non-polio EV infections ranged from 6.1% in spring to 15.4% in fall with a significantly increased rate in patients with ill contacts within the family or others ($P = 0.05$) and inversely related with birth weight ($P = 0.02$). **Conclusion:** Non-polio EV infections are an important cause of sepsis-like illnesses in neonates. Real-time PCR can serve as a rapid and specific method for the diagnosis of non-polio EVs in neonates suspected to sepsis.

INTRODUCTION: Enteroviruses are the most important member of picornaviridae that includes at least 11 species, based on VP1, VP2, VP4 and/or P1 genomic region sequencing. Non-polio EVs belong to enterovirus B species, generally¹.

Human non-polio enteroviruses are important causes of infections in young infants, especially in the summer and fall months²⁻⁴.

Non-polio enteroviruses are traditionally classified into coxsackieviruses A, coxsackieviruses B, echoviruses and numbered enteroviruses, based on serotyping assays. These viruses are usually transmitted horizontally through fecal-oral, but oral-oral (respiratory) and occasionally vertical (prenatal infections) routes are other means of infection.

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Clinical manifestations range from non-specific febrile illnesses to more severe diseases such as meningitis, meningoencephalitis, severe hepatitis and myocarditis. The majority of the infected individuals can self-limit this infection without any specific medication.^{2, 3, 5-7}

The incidence of non-polio EVs infections in febrile infants and neonates suspected to sepsis has been reported to range from 3% to 50%^{3, 8-11}. Because of its possible bacterial origin, fever in young infants and neonates demands a complete assessment, including blood and urine culture, lumbar puncture and use of antibiotics. This all might be unnecessary if rapid diagnosis of EVs infection could be made.

There are different diagnostic lab methods to detect EVs worldwide, some of which are cumbersome, time consuming and not available easily. Although cell culture is a highly specific test for the diagnosis of EVs, it is time consuming and takes 2-3 weeks, has a limited sensitivity and cannot detect all EVs¹².

It has been shown that molecular techniques such as polymerase chain reaction (PCR) are more sensitive and rapid in detecting enteroviral genomes¹²⁻¹⁶. In contrast to classical PCR, real-time PCR is more rapid with less contamination risk^{15, 17-20}.

We studied the contribution of EVs in neonates with 3-30 days of age, who presented with clinical diagnosis of neonatal sepsis, using a quantitative real-time RT-PCR assay. Then, by using a conventional RT-PCR test, non-polio viral infections were differentiated from polio ones which might be present as the result of OPV vaccination in the neonates which is done routinely in our country.

MATERIALS AND METHODS: This study, performed prospectively from July 2013 through July 2014, aimed to investigate the prevalence of non-polio EVs infections in neonates suspected to sepsis. Overall, 177 neonates of 3-30 days of age were studied in Nemazi hospital in Shiraz, south of Iran. The data for the study were collected by history taking and physical examination of the neonates, reviewing their health records, interviewing with their parents or family members

and taking laboratory tests such as PCR on respective multiple specimens. Informed consents were obtained from the parents of the patients entered into the study.

In one year period, all the 3-30 day old neonates with a clinical diagnosis of sepsis defined by the presence of 2 or more of the following criteria entered into the study. Inclusion criteria were: temperature instability, respiratory signs (tachypnea, apnea, cyanosis, wheezing and nasal flaring), neurological signs (convulsion, hypertonia, hypotonia, agitation, lethargy, poor neonatal reflexes), gastrointestinal signs (poor feeding, diarrhea, vomiting, abdominal distention), cardiovascular signs (tachycardia, bradycardia, hypotension), rash (petechia, purpura...), jaundice and palor. Exclusion criteria were: age less than 3 days and over 30 days and patients affected by conditions such as congenital heart diseases, metabolic and neurologic diseases, urinary tract infection and bacterial meningitis during the investigation. Blood culture was done for them.

Information about each patient was recorded in a special form containing general features such as full name, age, sex, file number, type of feeding, history of ill contact and other specific information such as duration of hospitalization, gestational age, birth weight, type of birth, antibiotic use and presence or absence of fever, respiratory and gastrointestinal signs, poor feeding, jaundice and impaired tissue perfusion and also laboratory data of the patients including: CRP, CBC, CSF (smear, culture and analysis), results of blood and urine culture, blood sugar, CXR findings and results of PCR tests.

Specimen collection: Blood and throat specimens were obtained from all the 177 neonates enrolled in the current study, and CSF specimens only from 60 neonates. Throat swab specimens were obtained from the patients using sterile Dacron swabs and were placed in 300 µl viral transport media. 2-5 ml blood was drained in special collection tubes and sera samples were separated and stored at -70°C. CSF specimens were obtained in 0.5-1 ml polypropylene collection tubes. All the specimens (throat, serum and CSF) were kept at -70°C until the PCR assay was performed.

Viral genome isolation: Totally, 200 µl of each 177 obtained swab specimens and sera as well as 60 CSF samples stored at -70°C were subjected to RNA extraction using Invitek kit (Berlin-Germany), according to the guideline described.

TaqMan Real-Time PCR assay for EVs RNA detection: In the first step, a TaqMan real-time PCR assay was set-up using superscript TM III platinum one-step master mix (Invitrogen- USA) and designed specific primers and probe (Table 1 A). This is a one-step quantitative RT- PCR test that detects and quantifies the viral RNA using Applied Biosystem 7500. The sensitivity of the test

is high and it can detect as low as 10 copy number of the virus per 1 mL of the specimen ²¹.

Each 25 µl of reaction contained 5 µl of extracted RNA (corresponding to 5 µl of clinical specimen), 12.5 µl one-step master mixes, 1 µl of each primer (15 pmol) and probe (5 pmol). Thermocycling condition was as follows: 50°C for 15 minutes (cDNA synthesis step), 95°C for 2 minutes and then, 95°C for 15 seconds and 60°C for 1 minute (40cycle). To promote the accuracy of our study, we repeated the step of EVs detection on CSF and blood specimens by Primer design kit (Life Technologies, Carlsbad, CA, USA).

TABLE 1: OLIGONUCLEOTIDE SEQUENCES USED IN A) Real-time PCR assay

Name	Seq.
NC-forward	5'-CCGGCCCCTGAATGC-3'
NC-reverse	5'-CACCGGATGGCCAATCCA-3'
NC-probe	5'-FAM-AACCGACTACTTTGGGTGTCCGTGTTTC-TAMRA-3'

B) Conventional RT-PCR test

Primer Name	Sense seq.	Antisense seq.	Product length
Ent1/Ent2	5'-ATTGTCACCATAAGCAGCCA-3'	5'-CTCCGGCCCCTGAATGCGGCTAAT-3'	154 bp
PVPCR2/2A	5'-GTCAATGATCACAACCCAC-3'	5'-AAGAGGTCTCTATTCCACAT-3'	290 bp

Duplex RT-PCR assay for differentiation of polio and non-polio EVs: Following the assessment of general EVs infection in 3 types of specimens by the qRT-PCR, all the positive samples were reassessed by a duplex RT-PCR test in an effort to differentiate polio from non- polio EVs.

In the first step, cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis kit (Fermentas company-Finland). To differentiate non-polio cDNA of EVs from polio ones , two specific primer pairs that were chosen from 5'-NCR EVs and VP1-2A specific region of polio EVs, previously described by Chezzi, were used [22, 23]. In the second step, cDNA was amplified using 1U Taq DNA polymerase, 1x Taq PCR buffer, 10 nmol dNTPs, 1.5 mM Mgcl2, 20 pmol of each primer pair (Ent1-Ent2 for pan-EV PCR and VP1-2A for polio viruses, specifically) and 5µl of RT product.

The 50 µl reaction of PCR was performed as follows: 95°C for 5 min for initial denaturation followed by 30 cycles of DNA amplification: annealing at 56 °C for 45 Sec, extension at 72 °C

for 45 sec; and a final extension at 72 °C for 7 min. Positive samples demonstrated a 145 bp band with or without a 290bp segment in 2% agarose gel ²³.

Having performed PCR tests, we recorded the results and other patients' data in special forms.

2-5. Statistical analysis: SPSS software was used for data entry, storing and data analysis. Chi-square test was used for the analysis of continuous variables, and T-test and Mann-Whitney test for other variables. A P value <0.05 was set as the level of significance.

3. RESULTS: A total of 177 neonates aged 3-30 days were enrolled in the study. Their demographic and clinical characteristics are shown in **Table 2**. As mentioned earlier, these were the neonates with no other explanation for their illnesses except for sepsis.

The results of PCR assay for polio and non-polio EVs are shown in **Table 3** and **Fig.1**. All neonates had specimens from serum and throat and only in 60 neonates from CSF. A total of 19 neonates (10.7%) were found to have a non-polio EV infection.

Three patients (1.7%) had positive serum and throat swab PCR assays for non-polio EVs, one patient (0.55%) had positive CSF, serum and throat swab, and 15 other patients (8.45%) had only positive

throat specimens (**Table 3**). In 26.6% (4 cases) of non-polio EV samples, concurrent polio EV infection was identified by conventional RT-PCR.

TABLE 2: CHARACTERISTICS OF 177 NEONATES 3-30 DAYS OLD, ADMITTED TO HOSPITAL WITH A CLINICAL DIAGNOSIS OF SEPSIS.

Characteristic	Enterovirus Positive (n=19)	Enterovirus Negative (n=158)	P-value
Gestational age, mean weeks (range)	37 (32-40)	37.3 (30-41)	0.73
Birth weight, mean g (range)	2605 (1150-3600)	2950 (1550-4450)	0.02
Age at onset, mean days (range)	10.8 (3-26)	12.7 (3-28)	0.268
Sex, male/female (ratio)	8/11 (0.73)	89/69 (1.3)	0.33
Ill contacts, no. of patients (percent)	13 (68.4)	68 (43)	0.05
Type of feeding, no. of patients (percent)			
Breast	16 (84.2)	113 (71.5)	0.68
Formula	1 (5.3)	13 (8.3)	
Both	2 (10.5)	32 (20.2)	
Season, no. of patients (percent)			
spring	3 (6.1)	46 (93.9)	0.71
summer	3 (7.5)	37 (92.5)	
fall	6 (15.4)	33 (84.6)	
winter	7 (14.3)	42 (85.7)	

TABLE 3: THE RESULTS OF PCR ASSAY IN SERUM, CSF AND THROAT SPECIMENS OF THE NEONATES 3-30 DAYS OLD, ADMITTED TO HOSPITAL WITH A CLINICAL DIAGNOSIS OF SEPSIS.

PCR	Sample	No. (Percent of total)
Positive	Throat & Serum & CSF	1 (0.55)
	Throat & Serum	3 (1.7)
	Throat	15 (8.45)
	Total	19 (10.7)
Negative	-	158 (89.3)
Total	-	177 (100)

Fig. 1 differentiation non-polio EVs (145bp) from polio ones* (145bp and 290bp) by a duplex PCR test with primer pairs Ent1/ENT2 and PVPCR2/2A. Line 1 is a 100 bp gene ruler, lines 3,

5 and 8 non-polio EVs samples. Lines 2 and 4 are polio EVs and line 6 and 7 are Sabin vaccine as control positive.

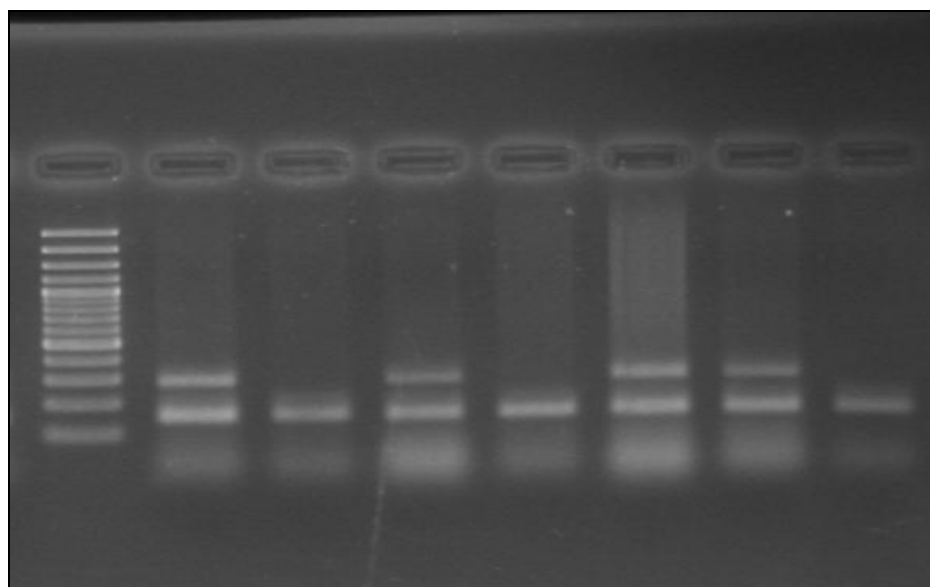


FIG. 1: DIFFERENTIATION NON-POLIO EVS (145bp) FROM POLIO ONES *

*positive control for polio EVs were live-attenuated vaccine.

The maximum viral load among EVs positive patients belonged to swab sample of a pan-EVs infected patient that was 68644 copy/ml of transport medium.

Data showed that non-polio EV infections were more common in neonates with lower birth weights ($p=0.02$) and also more common in those who had ill contacts ($p=0.05$).

Table 3 demonstrates that no statistically significant difference was found between neonates with EVs infection and those without it with respect to mean age at onset of illness, sex, type of feeding, mean gestational age and seasonality, although according to our data it seems that EVs infections are more common in younger neonates, males (73%) and cold seasons (fall=15.4% and winter=14.3%).

DISCUSSION: Acute severe illnesses in neonates and infants less than 3 months of age are usually considered to be a bacterial sepsis. The affected ones are hospitalized and treated with parenterally administered antibiotics pending the result of blood culture. However, a study showed that 90% of such cases in this age group are the result of viral infections⁵.

Non-polio EVs are one of the most common causes of fever and sepsis-like illnesses leading to hospitalization of neonates and children (20-40%)^{9, 24-26}. There is no detailed study related to the incidence of such infections in neonatal sepsis-like illnesses in Iran, but it has been reported by different studies to range from 3% to 58% in other countries^{4, 8-10, 24-31}. EVs are responsible for 33-65% of acute febrile illnesses and 55-65% of hospitalizations for suspected sepsis in infants during the summer and fall in the USA, and 25% year round³².

There are different methods to detect EVs such as viral culture, serology and PCR which improve diagnostic capability, significantly. This study describes a reliable and sensitive technique for the detection of pan-EVs infection and differentiation of polio EVs from non-polio ones and can be performed within a few hours^{20, 32-35}. Availability of pan-EV PCR can help the recognition of infants with EVs infection and can expand the current

understanding of the epidemiology and consequences of EVs infections in this age group.

In the present study, performed on 177 neonates of 3-30 days of age with primary diagnosis of sepsis, the prevalence of non-polio EVs infections was 10.7%. This result is consistent with other studies and our study was among the few ones conducted merely on this age range and the diagnostic method was PCR. For example, in a study by Resenlew and his colleagues in Finland, the diagnostic method was viral isolation by culture and the study of Verboon-Maciolet in Netherlands conducted on infants less than 60 days old using PCR method. In Khetsuriani's study in the USA between 1983 and 2003 on non-polio EVs infections, 11.4% of infections were in neonates.

In the current study, as in many other reports, neonatal EVs infections were detected throughout the year and a seasonal variation was found with a higher incidence in fall (15.4%) and winter (14.3%) months, although the difference was not statistically significant due to small number of positive patients^{3, 8, 28}. This seasonal difference may be explained by different climatic conditions in different parts of the world.

Meanwhile, the incidence of EV infection was inversely related with birth weight ($P=0.02$) while in some other reports the incidence was greater in neonates with higher birth weight^{26, 31}. The reason for such a difference can be different criteria for selecting the patients and different methodology. Also, in our study as in many other reports, most patients had ill contacts suspicious of having viral infections in the family or living place ($P=0.05$).

Although statistically not confirmed, and in accordance with some previous studies^{4, 28}, our data showed that non-polio EV infections are more predominant in male gender. However, in the study of Byington and colleagues using PCR for detection of EVs, there was no sex difference⁹.

Breast feeding is one of the factors that reduce the risk of enteroviral infections³⁶.

In this study, there was no statistically significant difference between neonates with and without non-polio EV infections with respect to age at onset of disease, type of feeding and gestational age,

although it seems that infection may be more common in younger neonates.

Given that breast feeding is common in some countries including Iran, and prevalence of EV infections in adults and children is different throughout the world and transfer of antibodies to the fetus or neonate through placenta or breast feeding can have a protective effect on the neonate, these can explain the difference in the incidence of such infections in neonates and small infants in different societies.

In our study, only one patient who was suspected of meningitis had evidence of CNS involvement (EVs PCR positive in CSF, serum and throat swab simultaneously), although there was no CSF pleocytosis. So it is not reasonable to compare this result statistically with other reports due to limited number of positive cases and different age ranges of the patients.

One of the important features of this study was that the sole presence of clinical signs of sepsis, irrespective of the presence or absence of fever, was enough for entering the study because many septic neonates may not be febrile and may even be hypothermic. In addition, we used high accuracy real-time PCR and took multiple specimens (blood, throat, CSF) from patients that collectively can increase the sensitivity and reliability of the test and the study.

The present study has also limitations that can affect the results including limited number of enrolled patients and being carried out in a single hospital center that virtually cannot be representative of the whole population in the region. Also, to differentiate polio from non-polio EVs, additional duplex RT-PCR tests were performed that increased the cost of the study and prolonged the time to gain the results.

CONCLUSION: Febrile neonates admitted to a hospital with diagnosis of sepsis are very likely to be infected with an EV and real-time PCR offers a tool for rapid diagnosis and can help physicians to limit the use and continuation of antibiotics in ill neonates and to decrease the duration and cost of hospitalization.

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