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انتروویروس‌ها و پاراکوویروس‌ها در کودکان مبتلا به عفونت‌های حاد تنفسی: بررسی در اصفهان، ایران

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چکیده

مقدمه: انتروویروس‌ها (EVs) و پاراکوویروس انسانی (HPeVs) نقش حائز اهمیتی در بروز بیماری در کودکان دارند. این ویروس‌ها قادر به ایجاد طیف گسترده‌ای از بیماری‌ها هستند که در مواردی باعث بروز عوارض شدید بالینی و مرگ‌ومیر می‌شوند. همچنین روش‌های تشخیصی مناسب برای جداسازی این ویروس‌ها به‌طور معمول در آزمایشگاه بالینی در دسترس نیستند؛ ازاین‌رو اپیدمیولوژی آنها به‌صورت دقیق گزارش نشده است. این مطالعه با هدف بررسی فراوانی انتروویروس‌های انسانی و پاراکوویروس در کودکان بستری در بیمارستان انجام شد.

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مواد و روش‌ها: سواب‌های نازوفارنکس از کودکان زیر پنج سال با علائم شدید تنفسی بستری در بیمارستان جمع‌آوری شدند. برای بررسی وجود EVs و HPeVs، از روش‌های RT-PCR و Real-time PCR دو مرحله‌ای استفاده شد.

نتایج: از مجموع هشتاد سواب نازوفارنکس جداشده از کودکان با علائم ARTIS، انتروویروس از چهار بیمار جدا شد (۵ درصد). نتایج Real-time PCR برای pan-HPeVs هیچ تشخیصی از HPeVs را نشان نداد. هر چهار بیمار، نوزاد بودند. فراوانی انتروویروس و پاراکوویروس در میان جنس‌ها و گروه‌های مختلف سنی غیرمعنادار گزارش شد.

بحث و نتیجه‌گیری: انتروویروس‌ها و پاراکوویروس‌های انسانی می‌توانند باعث بیماری‌های شبه آنفلوانزا و اختلالات عصبی شوند؛ اما اطلاعات مربوط به ویژگی‌های اپیدمیولوژیک آنها بسیار محدود است. تحقیقات بیشتر دربارهٔ عفونت‌های دستگاه تنفسی ویروسی برای بهبود سلامت عمومی و جلوگیری از عوارض شدید لازم‌اند.

واژه‌های کلیدی: پاراکوویروس انسانی، انتروویروس، عفونت حاد تنفسی، ریل‌تایم پی‌سی‌آر

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Enteroviruses and Parechovirus in Children with Acute Respiratory Infections: A Survey in Isfahan, Iran

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Abstract

Introduction: Enteroviruses (E.V.s) and human parechovirus (HPeV) play an important role in childhood diseases, from mild infections to severe illnesses which cause morbidity and mortality. Since the diagnostic procedures are not routinely accessible in the clinical laboratory, the epidemiology of enterovirus and parechovirus remains ambiguous. The current study investigates the frequency of human Enteroviruses and Parechovirus in hospitalized children.

Materials and Methods: Nasopharyngeal swabs were collected from children younger than five years with severe acute respiratory symptoms admitted to the hospital and screened for E.V.s and HPeV using RT-PCR and a two-step real-time PCR.

Results: From 80 nasopharyngeal swabs, viral agents were detected in 4 (5%) hospitalized children with ARTIs. All of these positive cases were infants. The results of RT-PCR of pan-HPeV demonstrate no detection of HPeV. Four samples were positive for enterovirus

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(5%). The frequency of E.V.s and HPeV between different sexes was not significant. Also, the distribution of E.V.s and HPeV among the other age groups was not significant (P>0.05).

Discussion and Conclusion: Enteroviruses and human Parechoviruses can cause severe consequences like neurological impairments, and more importantly, the epidemiological characteristics information in this regard is limited. Therefore, further research on viral respiratory tract infections is needed to improve public health and prevent severe complications.

Key words: Human Parechovirus, Human Enterovirus, Acute Respiratory Distress Syndrome, Childhood, Real-Time PCR

Introduction:

Acute Respiratory Tract Infections (ARTIs) are among the most frequent causes of childhood complications and fatalities worldwide (1, 2). Annually, respiratory infections cause approximately 13 million deaths in children under five worldwide, with three to six-time ARIs per year regardless of where they live or their economic situation. Meanwhile, in under-developed or developing nations, the occurrence is significantly higher and can lead to death in nearly one-third of under five-year-old children. According to the World Health Organization (WHO), respiratory infections account for about 6% of the total international disease burden (3-5). Influenza viruses (types A and B), human respiratory syncytial virus (RSV), parainfluenza viruses (PIV type 1-3), rhinoviruses (R.V.), metapneumoviruses, adenoviruses (ADV), bocaviruses, human coronaviruses (HCoV), enteroviruses, and parechoviruses are the most frequent agents which can lead to cause respiratory complication, particularly in infants and young children (6, 7).

Enteroviruses and human parechoviruses are members of *Picornaviridae* and are characterized by small (around 30 nm in diameter), non-enveloped, single-strand, and positive sense of RNA genome (approximately 7400 nucleotides). Nevertheless, most enteroviruses infections are asymptomatic. In some circumstances, they can lead to severe illnesses, namely upper respiratory illness, hand, foot, and mouth disease and herpangina, pleurodynia, aseptic meningitis, encephalitis, acute flaccid paralysis, and neonatal sepsis-like infection. Likewise, the significant number of infections with HPeV are mild and lead to respiratory and gastrointestinal diseases; however, severe clinical manifestations have been reported, including encephalitis, meningitis, myocarditis, and sepsis (8, 9).

Although the global impact of acute respiratory infection on mortality and morbidity in children under the age of five is undeniable data on the frequency of E.V. and HPeV in these cases have not been cleared in Iran. There is some research about the prevalence of E.V.s in Iran; however, HPeVs have still been undiagnosed in the routine laboratory because previous detection methods, such as cell culture, are complicated (10-14).

The present experiment investigated the prevalence and epidemiological features of E.V. and HPeV in pediatric patients to achieve a comprehensive approach to surveillance and control of acute respiratory infections.

Material and Methods:

This prospective study was carried out in Imam Hossein Hospital, the pediatric referral hospital in the center of Iran, Isfahan, taking charge of pediatric emergency care in Iran. Nasopharyngeal

swab samples were taken from children under five with acute respiratory infection symptoms between September 2020 and February 2021. The sample volume with the below formula accounts for 120 patients, but only 80 patients could be included in our study during the sampling. Written informed consent was taken from the patient's parents or legal guardians. The local ethical committee approved this study (with ID: IR.MUI.MED.REC.1397.022).

$$n = \left[\frac{Z\ 1-\frac{\alpha}{2}\ \sigma}{d}\right]^2$$

RNA Extraction and cDNA Synthesis: According to the manufacturer's instructions, RNA extraction was performed by the Jena Bioscience kit (Germany). Extracted RNA was stored at -80ºC until further analysis. RNA reverse transcription was carried out by the Jena Bioscience kit and Fermentas cDNA Synthesis Kit. In twenty microliters of the reaction mixture, there were 4 µl of 5× reaction buffer, 2 µl of mixed dNTPs (100 mM), 1 µl of Revert Aid M-Mul-V reverse transcriptase (200 U/ml, Fermentas, Germany), 2 µl random hexamer (Fermentas), 8 µl RNA template and 3µl Ribo Lock RNase inhibitor (20 U/ ml). The reaction mixture was incubated at 25℃ for 10 min, 42℃ for 60 min, followed by incubation at 70℃ for 5 min. The extracted cDNA was either used directly for PCR, stored at -20℃ for less than a week, or stored frozen at -80℃ for extended periods before PCR and Real-Time PCR analysis.

Positive/Negative Viral Control: In this experiment, the following viruses were utilized as positive controls. HPeVs (6.5 x 106 copies/L) and E.V.s (5.7 x 106 copies/L) cDNA preparations were provided by Iranian children hospitalized for acute fever and subsequently confirmed by sequence analysis. Nuclease-free water and clinical specimens positive for *E. coli* were used as negative controls, and no amplicon was detected.

Pan-HPeV Detection: The two-step SYBR Green real-time PCR was applied to detect HPeVs. The reaction mixture consisted of 10 µl RealQ Plus 2x Master Mix Green (Ampliqon), 1 µl of each pan-HPeV primers (10 pmol) (Table 1), 6 µl of the cDNA template, and 2 µl of RNase/DNase free water and in a final volume of 20 µl. The amplification process was performed in Step One Plus Real-time PCR system instrument (ABI, USA), according to the cycling protocol: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10s and 60 °C for the 60s. Melting curve analysis was performed for each reaction.

E.V.s Detection: The PCR method, targeting E.V.s, was carried out by a specific primer (Table 1). For PCR reaction, 2 µl of cDNA was added to 25 µl of reaction mixture containing 5 µL of 5x Taq DNA polymerase buffer (sinaclon, Iran), 2 µL of dNTPs (2.5 mM/µL), 0. 5 µL of each specific primer (10 µM), and 0.1 µL of Taq DNA polymerase (5 U/µL) (Promega, Madison, WI, USA), and RNase/DNase free water. The thermal cycling program was as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 45 s, 72°C for 1 minute, and a final extension at 72 °C for 5 min (15). All the amplicons were subject to sequencing and BLAST analysis.

Table 1- PCR Primers Sequences used in the Study

Virus (Accession Number)	Target region	Sequence (5' to 3')	Amplicon size (bp)	Reference
Enterovirus	5'-NCR	F: CAAGCACTTCTGTTTCCCCGG R: ATTGTCACCATAAGCAGCCA	440	[17]
Pan-HPeV	5'-NTR	F: CTGGGGCCAAAAGCCA R: GGTACCTTCTGGGCATCCTTC	141	[18]

Statistical Analysis: Statistical analysis was performed by the SPSS software package (Version v16, IBM Corporation, Armonk, NY, USA). All results were presented as mean ± standard deviation (S.D.). One-way ANOVA plus post-hoc Tukey test or two-tailed paired t-test was used to evaluate statistical significance between samples. Statistical significance was regarded as p-values < 0.05.

Results

From September 2020 to February 2021, 80 nasopharyngeal samples were collected from children younger than five years. As clinical sampling was carried out in the fall, there wasn't any seasonal pattern distribution observed. Most admitted patients were infants (45%). Sixty-five patients (81.2%) were males with a median age of 18 months (range two months to 62 months). The age of patients varies and is grouped into three classes, 50 % of them had less than one year, 20% of patients were between one and two years, and 30% were between 2 and 5 years old. Patients' clinical and demographical data are demonstrated in Table 2.

Table 2- Patients' Clinical Data and Relation of Sex and Age with Isolated Viruses

Variable	N (%)	P value
Sex (M)	65 (81.2)	>0.05
Age (Mean ± S.D.)	23.4 ± 19 Months	
- Newborn (Up To 3 Months Old)	4 (5)	>0.05
- Infant (3-12 Months Old)	36 (45)	
- Toddler (1-5 Years Old)	40 (50)	
Signs And Symptoms		
- Fever	54 (67.5)	
- Cough	32 (40)	
- Fatigue and Lack of Energy	4 (5%)	
- Convulsion	2 (2.5)	
- Headache	2 (2.5)	
- Pharyngitis	2 (2.5)	
- Dyspnea	40 (50)	
- Wheezing	22 (27.5)	
- Stridor	8 (20)	
- Common Cold	8 (20)	
- Rhinitis	38 (47.5)	
- Nasal Congestion	10 (12.5)	
- Dysphonia	22 (27.5)	
- Laryngotracheobronchitis	38 (47.5)	
- Tachypnea	6 (7.5)	
- Sputum Cough	4 (5)	
- Vomiting	4 (5)	
- Diarrhea	4 (5)	
Treatment		
- Epinephrine	20 (25)	
- Epinephrine + Dexamethasone	18 (22.5)	
- Epinephrine + Ceftriaxone	2 (2.5)	
- Epinephrine + Acetaminophen	2 (2.5)	>0.05
- Epinephrine + Ventolin	2 (2.5)	
- Epinephrine + Methylprednisolone	2 (2.5)	
- Epinephrine+ Methylprednisolone+ Ventolin	2 (2.5)	
- Methylprednisolone + Ventolin	2 (2.5)	

Not Significant: P-value > 0.05

No HPeV could be detected in this study; the samples' cycle of threshold (Ct) is shown in Fig. 1. The frequency of E.V.s by RT-PCR was 5% (4/80). And there wasn't any significant relationship between age and sex with E.V. The analysis of 4 positive samples confirmed the presence of E.V. (Coxsackievirus A4) (Table 3). The majority of children had a fever (67.5%), as well as dyspnea (50%), and

laryngotracheobronchitis (47.5%). Children aged under one represented significantly more dysphonia (72.7%), laryngotracheobronchitis (52.6%), and rhinitis (47.4%). However, in older children, wheezing (72.7%) and cough (62.5%) were much more prevalent. Nonspecific neurological complications, including fatigue, convulsion, and headache, were observed in 10% of participants. Meanwhile, there weren't any specific neurological signs and symptoms recorded. Death was reported in 2 patients (2.5%), but there wasn't seen any correlation between the treatment and the severity of infections. The frequency of E.V.s and HPeV between different sexes was not significant. Also, the distribution of E.V.s and HPeV among the different age groups was not significant (P>0.05).

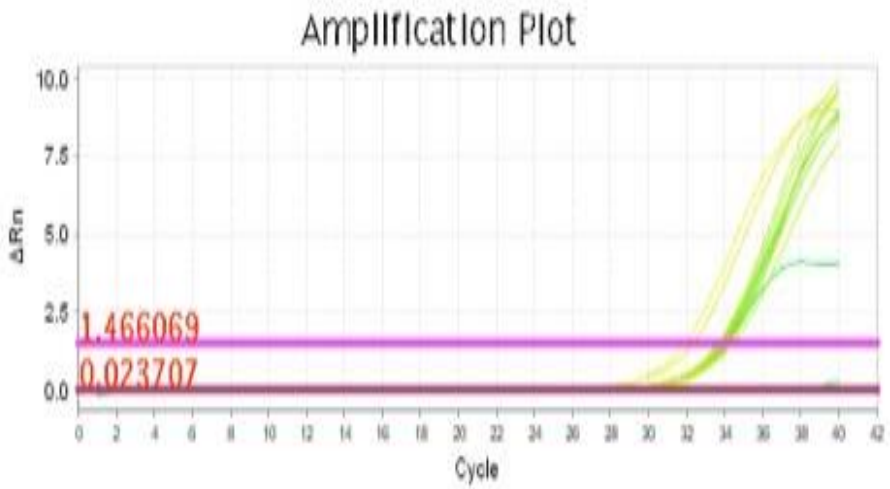

Fig. 1- The Amplification Plot of Samples in Real-time PCR

Table 3- Clinical Manifestation of Positive Samples

Viral agent	Sex	Age (month)	Signs and Symptoms	Treatment
Coxsackievirus A4	Male	8	Fever, Cough, Rhinitis, Wheezing	Epinephrine
Coxsackievirus A4	Male	12	Fever, Sneezing, Nasal Congestion, Rhinitis, Dysphonia, Stridor, Laryngotracheobronchitis	Epinephrine Dexamethasone
Coxsackievirus A4	Male	10	Fever, Cough, Nasal Congestion, Rhinitis, Stridor, Wheezing	Epinephrine Dexamethasone
Coxsackievirus A4	Male	6	Fever, Cough, Sneezing, Diarrhea, Laryngotracheobronchitis	Epinephrine

Discussion and Conclusions

Parechovirus and Enteroviruses are recognized as the two threatened and frequent pathogens that lead to some complications and fatality throughout childhood globally (16). In this research, 2.5% of participants tested positive for E.V. (Coxsackievirus A4). All PCR-positive patients were male, and this finding is consistent with previous research; however, there wasn't any significant contribution in this regard (17-19). In Tehran, Rahimi et al. (2013) discovered HPeV 1 infections in 64/148 (43.24%) young infants with aseptic meningitis. Also, in Makvandi et al.'s study, it was reported to be 5 %. According to various studies, the prevalence of HPeV3 is substantially more related to sepsis disease than HPeV1(20, 21). Although the sample time in our survey was short, Makvandi et al. reported that HPeV emerged at a rate of 3% in winter and 2% in spring. However, Rahimi et al. discovered HPeV 1 in Tehran, Iran, throughout the seasons.

However, HPeVs and E.V. may

resemble influenza-like illnesses and croup respiratory disease. Limited information on their epidemiological characteristics is available. The findings of this study regarding no detection of HPeV are similar to some previous reports, including Reckziegel et al., who studied the prevalence of respiratory viral agents in the immunocompromised and immunocompetent cases with ARTIs. They reported 0 of 37 (0%) immunocompromised children and only one immunocompetent toddler with IA-V infection of 98 (1%) (22). In addition, Bierbaum et al. detected only 1 out of these eight samples tested positive for Parechovirus, which represented the low prevalence of this virus by using a multiplex real-time PCR assay in Germany (23). A surveillance study was conducted in a hospital in China between 2009 and 2015 by Zhang et al. In this research, 10,212 children suffered from acute respiratory infection, acute diarrhea, and hand, foot, and mouth disease. HPeV prevalence among acute respiratory infections was 3.43%. Their study determined the wide variety of genotypes in which three dominant genotypes (HPeV1, HPeV3, and HPeV4) had occurred in different seasonal peaks (24). In another study conducted on nasopharyngeal swabs from 637 hospitalized pediatrics, only 3% of samples were infected with HPeV. The majority of the HPeV positive samples (75%) were genotype 3 (HPeV3). Benschop et al. (2007) reported HPeV infection in CSF of 20 children (3.8%) and all of the patients had severe conditions including sepsis and meningitis (25). In Makvandi et al.'s study in Iran (Ahvaz), the prevalence of HPeV and E.V. was reported at 5% and 35 %, respectively, which was a higher prevalence than our study, which could be for different times of collection and different geographical region (12).

Furthermore, 95% of positive cases were under three years (26). In Iran, Aghamirmohammadali et al. reported that of the 206 patients under five years of age, no HPeV was detected, which was in line with the results of this study (14). The frequent HPeV genotypes in cases with ARTIs in our country are still not identified. However, in a study by Rahimi et al., 43.24% of young children with aseptic meningitis were infected by HPeV genotype-1 (27). However, the HPeV prevalence in experiments should be carefully compared because the prevalence is influenced remarkably respecting the demographical characteristic of patients, the detection methods, sampling season, and residential areas.

We reported a frequency of 5% enterovirus in the nasopharyngeal secretion of children. The prevalence of EV D68 was evaluated at 10.5% among 322 children in the study conducted by Mozhgani et al. in Tehran, Iran (11). The incidence of E.V. at 5% in children with ARTIs was lower than in the former investigations in Norway (28). Accordingly, in children infected with human respiratory syncytial virus (HRSV) or influenza virus (IV), bacterial infections are less frequent than in those without a viral infection (29-31). Local determination of specific viral infections' epidemiology will betterment the treatment guidelines.

Our study has some limitations, including a small sample size and a short sampling period. Although there were not any effective antiviral treatments on the market against E.V.s and HPeVs, detection would help manage patients by supporting treatment to prevent severe neurological complications. Nucleic acid-based procedures would provide a better understanding of the epidemiology of viruses.

Although the prevalence of human Parechovirus is low, the threat of this viral agent cannot be deniable, especially in young children. Additionally, enteroviral

infections in children are frequent. Both agents can result in life-lasting complications, more importantly, neurological impairments. On the other hand, there isn't any treatment on the market for these viruses. Therefore, it is vital to prevent these infections by improving public health. Additionally, more study is needed to enhance understanding of the epidemiological features of viral respiratory infections.

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Conflicts of Interest/Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

ShM contributed to organizing and supervising the whole study and was responsible for the funding acquisition. S.S. and AM conducted the experiments. AM and MKN drafted the manuscript. ShM and BNE mainly contributed to designing the experiments and data analysis. All authors contributed to the article and approved the final manuscript.

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