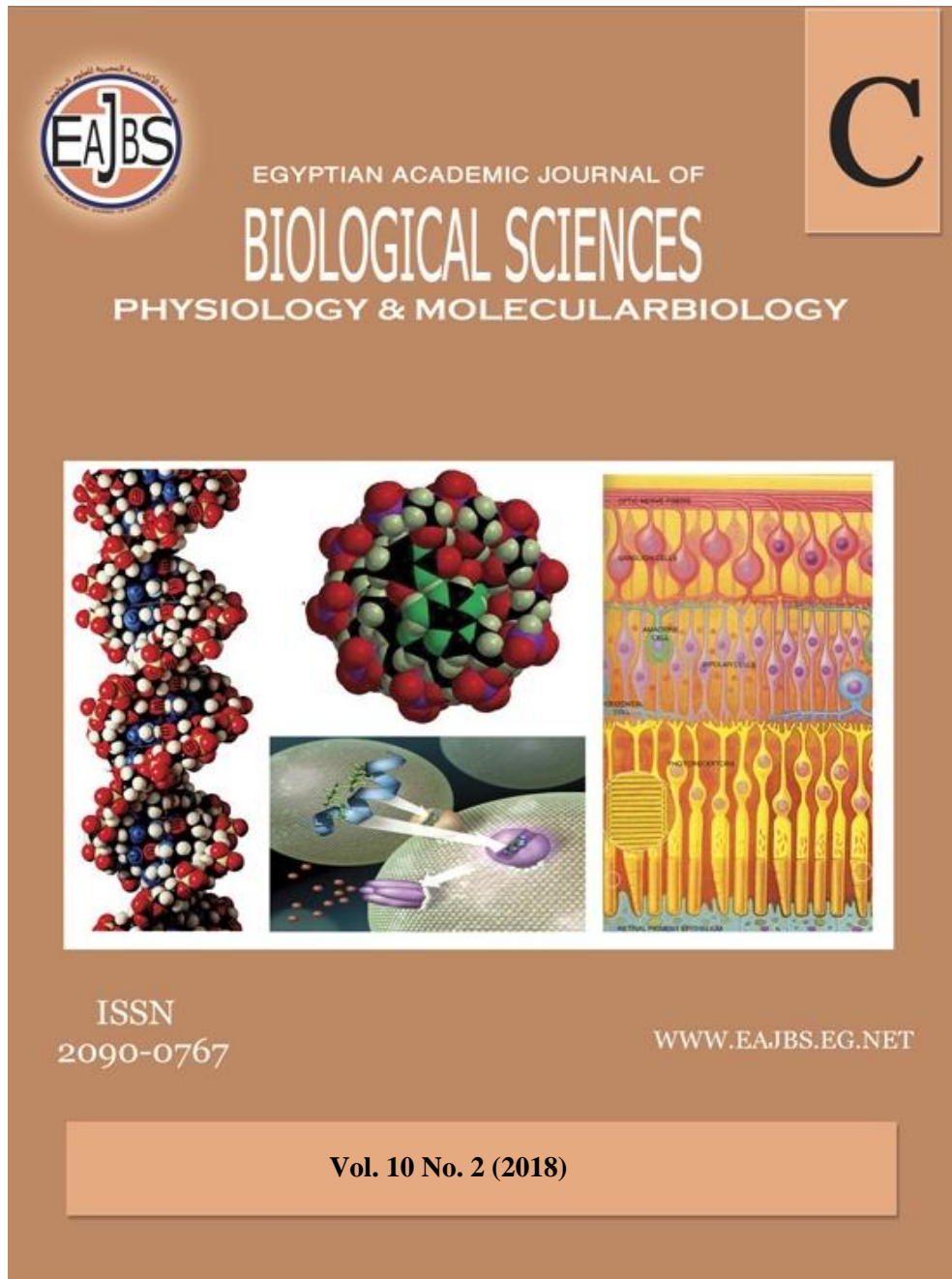


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Molecular Analysis of Human Parechovirus in Cerebrospinal Fluid of Young Infants in Albaha, Saudi Arabia

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ABSTRACT

Many studies show the involvement of Human Parechovirus (HPeV) especially HPeV3 with central nervous system (CNS) infection in young infants. The current study analysed the presence of HPeV in cerebrospinal fluid (CSF) to understand the epidemiological behaviour of parechoviruses and to examine their clinical associations in Albaha, Saudi Arabia. Real-time RT-PCR assay targeting the viral protein 1 (VP1) region was performed on RNA extracts of CSF specimens collected from young infants attending the tertiary care hospital at Albaha.

None of the samples analysed showed positivity for HPeVs presence. Suboptimal biological sample, the seasonal pattern of HPeVs infections and use of CSF only as the biological specimen might be some plausible reasons for the negative finding. Present study is the first such attempt in Saudi Arabia and thus it is pertinent that more stringent future studies using biological specimen of varying origins must be conducted to analyse the presence of HPeVs associated with asymptomatic infections or mild disease to severe disease symptoms in neonates and infants especially under the age of 3 months, before ruling out Human Parechovirus (HPeV) presence.

INTRODUCTION

Immaturity of the immune system in early childhood renders infants susceptible to the range of infections including life-threatening ones. Especially lower respiratory tract infections and sepsis amongst neonates and children aged less than 2 years cause the large number of mortality, globally (Shah & Robinson, 2014) (Carville et al, 2007; Schrag & Schuchat, 2005) (Lawn et al, 2006). Causes of sudden unexplained infant death include accidental suffocation, sudden infant death syndrome, many maltreatment syndromes along with Human parechoviral infections (Shapiro-Mendoza et al, 2006).

Human parechoviruses (HPeVs) cause a range of ailments including mild diarrhea, sepsis and meningitis, affecting largely young infants and children. They are nonenveloped, positive-sense, single-stranded RNA viruses, in family *Picornaviridae*. Historically, HPeVs are classified within the *Enterovirus* (EV) genus with HPeV types 1 and 2 known as echovirus 22 and echovirus 23, respectively. The reclassification was based on the sequence analysis that demonstrated genetic and biologic differences between echovirus 22 and EV genus members (Hyypia et al, 1992). So far, 16 HPeV genotypes have been characterized (Stanway & Hyypia, 1999) (www.picornaviridae.com/parechovirus/hpev/hpev.htm).

While the association of HPeV1 and HPeV3 is established with asymptomatic infections (Boivin et al, 2005; Mizuta et al, 2012; Stanway et al, 2000), clinical association of remaining HPeVs remains to be explored. HPeV1 and HPeV2 cause mild gastrointestinal and respiratory symptoms (Benschop et al, 2008; Harvala et al, 2008), whereas HPeV3 causes serious sepsis-like syndrome, meningitis, encephalitis, and hepatitis in neonates and young infants (Boivin et al, 2005). Most HPeV infections are asymptomatic or associated with mild respiratory and/or gastrointestinal symptoms (Stanway et al, 2000), however, lot remains to be elucidated about the characteristics of HPeV3 infection as well as those of other HPeV types (Abzug, 2004; Drexler et al, 2009; Harvala et al, 2008; Renaud et al, 2011; Sedmak et al, 2010; Selvarangan et al, 2011; Verboon-Maciolek et al, 2008). CNS infections in very young children and infants are difficult to diagnose as a wide range of causative agents can be involved including *group B streptococci* (subtypes III), *Escherichia coli* (carrying the K1 antigen), *Listeria monocytogenes* (serotype IVb).

While *Neisseria meningitidis* (meningococcus) and *Streptococcus pneumoniae* (serotypes 6, 9, 14, 18 and 23) and *Haemophilus influenzae* type B are generally causative agents in older children.

Among viruses, enteroviruses, herpes simplex virus (generally type 2), varicella zoster virus (known for causing chickenpox and shingles), mumps virus, HIV, LCMV and herpes simplex virus type 2 are generally the agents of CNS infections. Additionally, it is pertinent to examine the involvement of HPeV and Toscana virus in CNS infections in infants and very young children. HPeV largely causes aseptic meningitis in neonates and young infants (de Crom et al, 2016; Grist et al, 1978; Khetsuriani et al, 2006; Sawyer, 2002). Infections may deteriorate into non-paralytic aseptic meningitis (1-2% of cases) or to poliomyelitis (0.1–1% of cases) (Cristanziano et al, 2015).

Incidence of HPeV types is variable worldwide. Early studies show HPeV types 1-6 incidence globally, while HPeV genotypes 7-16 have only been reported scarcely. Prevalence of HPeV7, 8, 9 and 12 have been reported from South America and Asia in children with gastrointestinal symptoms or asymptomatic infection (Alam et al, 2015; Drexler et al, 2009; Li et al, 2009; Moore et al, 2015; Oberste et al, 2013; Zhang et al, 2011; Zhong et al, 2013). Similarly, Genotypes 10, 11, 13, 14 and 15 have been found in samples from children with gastroenteritis conditions or asymptomatic children in Asia (Alam et al, 2015; Kim Pham et al, 2010; Oberste et al, 2013).

The incidence shows a seasonal pattern in temperate climates, with different types cocirculating simultaneously (Kolehmainen et al, 2012). The incidence of HPeV infection in Saudi Arabia is not known so far, as this infection generally go unnoticed, and

more often underexplored (Benschop et al, 2008).

Early study show an infection rate of more than 90 % with at least one HPeV type among the children of the age of 2 years and below (Harvala et al, 2010; Khatami et al, 2015; Tauriainen et al, 2007). However, HPeV infections are uncommon in older children (Esposito et al, 2014).

Aims and Objectives:

In the current study, the presence of HPeV infection was assessed during June 2016- May 2017 in CSF fluid obtained from young infants. The primary goal of this study was to obtain a deeper understanding of the epidemiological behaviour of parechoviruses and to examine their clinical associations in Albaha, Saudi Arabia, with an aim to investigate the connection of HPeV to severe infections in infants.

MATERIALS AND METHODS

Ethical Approval: Ethical approval was obtained from Deanship of Scientific Research, Albaha University.

Cerebrospinal Fluid Samples From Hospitalised Children: 45 CSF samples from 1- to 60-week-old children with a request for microbiological analysis and with no finding of a causative agent, except for EV, were collected for HPeV analysis.

Reference Sequence Material From Databases: The phylogenetic analysis in this study was intended to be compared with reference sequences and to other published sequences available in the GenBank database (www.ncbi.nlm.nih.gov/genbank) of the National Center for Biotechnology Information (NCBI).

METHODS:

RNA Extraction: Viral RNA was extracted from CSF samples for RT-PCR-based detection analyses. Extraction from CSF samples was performed using a QIAamp Viral RNA kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA samples were stored at -70°C.

One- and Two-Step Real-Time RT-PCR :

HPeV RNA detection included real-time PCR protocols with one step and two steps. The cDNA synthesis for the two-step reaction was performed in a 40 µl reaction containing 10 µl of RNA template, 8 µl of reaction buffer, 50 pmol of HPeV-specific primer, 20 nmol of dNTP, 4 units of Recombinant RNasin® RNase inhibitor and 20 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA).

The PCR step was performed using a Maxima qPCR master mix kit (Thermo Scientific, Rockford, IL, USA) in a 25 µl reaction, containing 5 µl of cDNA product, 300 nM of primers, and 200 nM of probe.

HPeV Typing PCR :

An RT-PCR targeted to the almost complete VP1-sequence area of the HPeV genome was used for genotyping HPeV-positive findings. The typing was performed using viral RNA directly extracted from the target sample. The positive PCR fragments observed by agarose gel electrophoresis were to be directed to sequencing using the same primers used in the typing RT-PCR. Sequencing reactions would be performed by MACROGEN® (Seoul, Korea) (Table 1-3).

Table 1: Primers for detection/ screening

S. No.	Name	Sequence
1.	HPeV- BR Forward primer	5'-GTGCCTCTGGGGCCAAAAG-3'
2.	HPeV- BR Reverse primer	5'-TCAGATCCATAGTGTCTGCTTGTAC-3'
3.	HPeV- Probe	5'-FAM- CGAAGGATGCCCAGAAGGTACCCGT- TAMRA -3'

Table 2: Degenerate primers

S. No.	Name	Sequence
1.	HPeV- VP1f Forward	5'- ATTCRTGGGGYTCMCARATGG -3'
2.	HPeV- VP1rev Reverse	5'- AATATCCTTAGCAATDGTYTCACARTT- 3'

Table 3: Primers for typing PCR

S. No.	Name	Sequence
1.	VP1f_Seq1_1	ATTCGTGGGGTTCACAGATGG
2.	VP1f_Seq1_2	ATTCGTGGGGTTCACAAATGG
3.	VP1f_Seq1_3	ATTCGTGGGGTTCACAGATGG
4.	VP1f_Seq1_4	ATTCGTGGGGTTCACAAATGG
5.	VP1f_Seq1_5	ATTCGTGGGGCTCACAGATGG
6.	VP1f_Seq1_6	ATTCGTGGGGCTCACAAATGG
7.	VP1f_Seq1_7	ATTCGTGGGGCTCACAGATGG
8.	VP1f_Seq1_8	ATTCGTGGGGCTCACAAATGG
9.	VP1f_Seq1_9	ATTCATGGGGTTCACAGATGG
10.	VP1f_Seq1_10	ATTCATGGGGTTCACAAATGG
11.	VP1f_Seq1_11	ATTCATGGGGTTCACAGATGG
12.	VP1f_Seq1_12	ATTCATGGGGTTCACAAATGG
13.	VP1f_Seq1_13	ATTCATGGGGCTCACAGATGG
14.	VP1f_Seq1_14	ATTCATGGGGCTCACAAATGG
15.	VP1f_Seq1_15	ATTCATGGGGCTCACAGATGG
16.	VP1f_Seq1_16	ATTCATGGGGCTCACAAATGG
17.	VP1rev_Seq2_1	AATATCCTTAGCAATGGTTTCACAGTT
18.	VP1rev_Seq2_2	AATATCCTTAGCAATGGTTTCACAATT
19.	VP1rev_Seq2_3	AATATCCTTAGCAATGGTCTCACAGTT
20.	VP1rev_Seq2_4	AATATCCTTAGCAATGGTCTACAATT
21.	VP1rev_Seq2_5	AATATCCTTAGCAATTGTTTCACAGTT
22.	VP1rev_Seq2_6	AATATCCTTAGCAATTGTTTCACAATT
23.	VP1rev_Seq2_7	AATATCCTTAGCAATTGTCTCACAGTT
24.	VP1rev_Seq2_8	AATATCCTTAGCAATTGTCTACAATT
25.	VP1rev_Seq2_9	AATATCCTTAGCAATAGTTTCACAGTT
26.	VP1rev_Seq2_10	AATATCCTTAGCAATAGTTTCACAATT
27.	VP1rev_Seq2_11	AATATCCTTAGCAATAGTCTCACAGTT
28.	VP1rev_Seq2_12	AATATCCTTAGCAATAGTCTACAATT

Sequence Analysis and Phylogeny:

The data from genotyping the VP1 sequences would be compared with reference sequences

using BLAST (Altschul et al, 1990; Thompson et al, 1994) and aligned using the CLUSTALW tool (Thompson et al, 1994)

RESULTS AND DISCUSSION

The *Parechovirus* genus including human pathogens HPeVs is the relatively new genus of viruses. The HPeVs circulation among human populations is increasingly common but is restricted to the young infants and children presenting asymptotically or with mild clinical symptoms. However, HPeVs presence

can easily progress to severe infections in neonates. The current study aimed to analyse the HPeVs presence in CSF samples of infants and describe their involvement in associated paediatric diseases at Albaha, Saudi Arabia.

CSF samples were collected from the infants having general characteristics as follows (Table 4).

Table 4: General characteristics of the infants

1.	Age (Days)	18±10.15
2.	Temperature	39±0.36 °C
3.	Respiratory rate	50±11.14 breaths per minute
4.	Heart rate	161±25.36 beats per minute
5.	Leukocyte count	6± 1.79 10 ⁹ cells/L
6.	Neutrophils	46±22.14 %
7.	Band forms	5±5.0%
8.	Lymphocytes	37±21.28 %
9.	Haemoglobin level	130±29.57 g/L
10.	Platelet count	220±67.57 10 ⁹ cells/L
11.	C- reactive protein	5± 5.57 mg/L
12.	Glucose level	3±0.21 mmol/L
13.	Protein level	1±0.15 g/L

These infants were born after the full term, and hospitalized for fever of unknown origin with irritability but without any respiratory or gastrointestinal complications. The family members may have had episodes of recent common ailments like a cough, fever, sinusitis and/ or upper respiratory tract infection. CSF analysis showed erythrocytes without leukocytes. All of them had subsequent episodes of oxygen desaturation with the sharp increase in temperature requiring ICU admission and oxygen administration.

None of the samples collected showed positivity for HPeVs presence despite early reports showing HPeVs association with a range of conditions including gastroenteritis, respiratory illness, meningitis, transient paralysis, and severe neonatal viral sepsis globally viz Japan (Ito et al, 2004), Canada (Abed & Boivin, 2006), the Netherlands (van der Sanden et al, 2008), Norway (Tapia et al, 2010), Scotland (Harvala et al, 2008).

The suboptimal biological sample is one plausible reason for the current negative finding in the light of an early report showing the presence of antibodies against HPeV1 in children aged less than one year establishing its absolute seroconversion during the early months of life (Stanway et al, 2000).

The second reason might be a seasonal pattern of HPeVs infections similar to that of human enteroviruses, occurring more frequently in one season than the other (Chieochansin et al, 2011; Fischer et al, 2014). Since the present study is first of its kind in Saudi Arabia, it is hard to confer which seasonal pattern HPeVs might be following in the country.

The third reason might be the use of CSF only as the biological specimen. Serum, stool, middle-ear fluid, and nasopharyngeal aspirate samples should be used in addition to cerebrospinal fluid in future studies.

HPeV infections in the first years of life can be associated with a wide variety of clinical presentations like Sepsis-like

illness and CNS infections (Sainato et al, 2011), Respiratory tract infections (Pajkrt et al, 2009), Dermatological manifestations (Shoji et al, 2013), gastroenteritis (de Crom et al, 2016), myalgia (Mizuta et al, 2012), hemophagocytic lymphohistiocytosis (Yeom et al, 2016), myocarditis (Wildenbeest et al, 2013), Hepatitis with coagulopathy syndrome (Levorson et al, 2009), Reye syndrome (Watanabe et al, 2007).

Thus it can be concluded that more stringent future studies using biological specimen of varying origins should be conducted to analyse the presence of HPeVs associated with asymptomatic infections or mild disease to severe disease symptoms in neonates and infants especially under the age of 3 months.

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Conflict of interest: The author declares no conflict of interest.

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ARABIC SUMMARY

**التحليل الجزيئي لفيروس الباريكو البشري في السائل الدماغي الشوكي للرضع الصغار في
الباحة ، المملكة العربية السعودية****شايع المالكي**

قسم طب المختبرات ، كلية العلوم الطبية التطبيقية ، جامعة الباحة ، الباحة ، المملكة العربية السعودية
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تظهر العديد من الدراسات أن فيروسات الباريكو (HPeV) خاصة النوع النمطي الثالث (HPeV3) لها علاقة في كثير من الأحيان بعدوى الجهاز العصبي المركزي (CNS) خصوصا عند الرضع الصغار. وعليه فقد حللت الدراسة الحالية وجود فيروسات الباريكو (HPeV) في سائل الحبل الشوكي (CSF) لفهم السلوك الوبائي لفيروسات الباريكو وفحص ارتباطاتها السريرية بعدوى الجهاز العصبي المركزي في منطقة الباحة في المملكة العربية السعودية. وعليه فقد تم إجراء اختبار RT-PCR مستهدفين جين بروتين الكبسيدة (VP1) وذلك لعينات (CSF) تم جمعها من أطفال رضع سُجلوا بمستشفيات تخصصية بمنطقة الباحة بتشخيص إلتهاب السحايا بسبب فيروسات غير محددة. ولم تُظهر أياً من العينات التي تم تحليلها نتيجة إيجابية لوجود فيروسات الباريكو بكافة أنواعها و لا حتى النوع النمطي الثالث من تلك الفيروسات. وحيث أن الدراسة الحالية هي أول محاولة من هذا القبيل في المملكة العربية السعودية فإنه من الضروري إجراء دراسات مستقبلية أشمل باستخدام عينات بيولوجية متعددة لتحقيق من مدى علاقة فيروسات الباريكو وخصوصا النوع النمطي الثالث لعدوى الجهاز العصبي المركزي عند الأطفال الرضع