



UvA-DARE (Digital Academic Repository)

Clinical and molecular insights into human parechovirus infection

Benschop, K.S.M.

Publication date
2009

[Link to publication](#)

Citation for published version (APA):

Benschop, K. S. M. (2009). *Clinical and molecular insights into human parechovirus infection*.

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

4

Human Parechoviruses as an Important Viral Cause of Sepsislike Illness and Meningitis in Young Children

**K. C. Wolthers¹, K. S. M. Benschop¹, J. Schinkel¹,
R. Molenkamp¹, R. M. Bergevoet¹, I. J. B. Spijkerman³,
H. C. Kraakman⁴, and D. Pajkrt²**

Clinical Infectious Diseases 2008; 47:356-363

¹ Lab. of Clinical Virology, Dept. of Medical Microbiology, Academic Medical Center, Amsterdam.

² Dept. of Pediatric Infectious Diseases, Emma Children's Hospital, Academic Medical Center, Amsterdam.

³ Dept. of Medical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam.

⁴ Dept. of Pediatrics, Onze Lieve Vrouwe Gasthuis, Amsterdam.

Human Parechoviruses as an Important Viral Cause of Sepsislike Illness and Meningitis in Young Children

Background. Enteroviruses (EVs) belong to the family *Picornaviridae* and are a well-known cause of neonatal sepsis and viral meningitis. Human parechoviruses (HPeVs) type 1 and 2, previously named echovirus 22 and 23, have been associated with mild gastrointestinal or respiratory symptoms in young children. Six HPeV genotypes are currently known, of which HPeV3 is associated with neonatal sepsis and meningitis.

Methods. Cerebrospinal fluid samples from children aged <5 years previously tested by EV-specific polymerase chain reaction (PCR) during 2004–2006 were selected ($n=761$). Samples from 716 of those children were available for retrospective testing by HPeV-specific real-time PCR. The prevalence of EV and HPeV in these samples was compared. Data on clinical presentation of children infected with HPeV were retrospectively documented.

Results. HPeV was found in cerebrospinal fluid samples from 33 (4.6%) of the children. Yearly prevalence of HPeV in cerebrospinal fluid varied remarkably: 8.2% in 2004, 0.4% in 2005, and 5.7% in 2006. EV was detected in 14% (108 of 761 samples), with no variation in yearly prevalence. Children with HPeV in cerebrospinal fluid presented with clinical symptoms of sepsislike illness and meningitis, which led to hospitalization and antibiotic treatment.

Conclusion. EV-specific PCRs do not detect HPeVs. The addition of an HPeV-specific PCR has led to a 31% increase in detection of a viral cause of neonatal sepsis or central nervous system symptoms in children aged <5 years. HPeV can be considered to be the second cause of viral sepsis and meningitis in young children, and rapid identification of HPeV by PCR could contribute to shorter duration of both antibiotic use and hospital stay.

INTRODUCTION

Enteroviruses (EVs) belong to the family *Picornaviridae* and are a well-known cause of sepsis and meningitis in young children [1, 2]. Two former EV serotypes, known as echovirus 22 and echovirus 23, have been reclassified in the newly assigned genus *Parechovirus* of the *Picornaviridae* as human parechovirus (HPeV) types 1 and 2 [3]. New HPeV types 3–6 were recently identified in Japan, The Netherlands, and the United States

[4–7]. HPeV1 is considered to be a widely spread pathogen that affects mainly young children [8, 9]. HPeV1 infections are most commonly associated with mild gastrointestinal or respiratory symptoms. HPeV2 infections rarely occur and are associated mostly with gastrointestinal symptoms [10]. HPeV3 has been associated with more-severe disease, such as neonatal sepsis and meningitis [11, 12]. Symptoms of CNS involvement, as with encephalitis and paralysis, have been reported for HPeV1 as well [13, 14] but have been reported less frequently than have infections with EV [15] or HPeV3 [12, 16]. To date, hardly any clinical data are available on the more recently discovered HPeV types 4, 5, and 6.

PCR based on the highly conserved 5'untranslated region has been shown to be a rapid and sensitive method for diagnosing EV as a cause of meningitis and sepsis [2, 17, 18]. However, molecular assays for diagnosing EV will not detect HPeV, because of the lack of sequence conservation between HPeV and EV at the 5'end of the genome [19, 20]. Therefore, infections in which HPeV causes severe disease like meningitis or sepsis may be underdiagnosed, because viral culture of CSF is insensitive and culture of stool samples or throat swabs is often not performed. Therefore, the relative contribution of HPeV, compared with EV, as a causative agent of viral meningitis or sepsislike illness in children is unknown. We developed a real-time TaqMan PCR assay directed at the 5'untranslated region, to detect HPeV directly from clinical samples [21]. Here, we retrospectively studied the prevalence of HPeV in CSF samples from children obtained during 2004–2006, and we studied the clinical symptoms associated with HPeV detection in CSF.

METHODS

Clinical specimens

Since 2004, CSF samples that had been referred to the Laboratory of Clinical Virology for viral diagnostics were routinely stored at -80°C . CSF samples from children <5 years of age previously tested for EV by RT-PCR were selected (840 samples obtained from 761 children). Available complementary DNA (cDNA) samples were retrospectively tested for HPeV (793 samples obtained from 716 children).

RNA extraction

CSF samples (200 μL) were extracted, as described by Boom et al. [22], with use of 20 μL of sizefractionated silica particles in combination with 900 μL of lysis buffer L6. Samples were coextracted with 6250 copies armored

internal control RNA, corresponding to 500 copies internal control cDNA in PCR [23]. RNA was eluted in 100 μ L of Tris-EDTA buffer.

Detection of EV by 5 untranslated region RT-PCR

Forty microliters of extracted RNA was used for reverse transcription, as described elsewhere [23], with use of random hexamers (Roche Diagnostics). Twenty-five microliters of the cDNA sample was used for the EV-specific end-point PCR, as described by Beld et al. [23]. The remaining 25 μ L of cDNA sample was stored at -80°C .

Detection of HPeV by real-time PCR

Five microliters of the initial cDNA sample was used for the previously described HPeV-specific single-target real-time PCR [21]. The HPeV PCR was performed in a 25- μ L volume containing 900 nM of each primer (ParechoF31 and K30) [20], 200 nM of the HPeV-WT MGB probe (Applied Biosystems), 400 ng/ μ L of bovine alpha-caseine (lot number 17H9551; Sigma), 1x TaqMan universal PCR mastermix (Applied Biosystems), and 5 μ L of the RT reaction. The PCR was performed in an Applied Biosystems 7000 sequence analyzer, as follows: 2 min at 50°C and 10 min at 95°C , followed by 45 cycles, each consisting of 15 s at 95°C and 1 min at 60°C .

Clinical data

Data on clinical presentation of children infected with HPeV were retrospectively collected using a questionnaire. The patient's age at time of virus isolation, sex, hospital or ward of admission, and duration of hospital stay were documented. Letters of discharge and medical records were used to document data on presence and duration of fever (temperature, $>38^{\circ}\text{C}$), irritability (as judged by the examining physician), sepsislike illness (fever or hypothermia with signs of circulatory and/or respiratory dysfunction defined by tachycardia or bradycardia, low blood pressure, and decreased saturation), neurological symptoms (clinically suspected meningitis, encephalitis, seizures, or paralysis), cell count determined in sample obtained by lumbar puncture (cell count, <10 cells/ mm^3), CSF protein level (<0.35 g/L), glucose level (2.8–4.4 mmol/L), presence of other microbial pathogens in CSF, and abnormalities revealed on diagnostic imaging of the brain. In addition, symptoms of respiratory infections (rhinorrhea, cough, tachypneu, apneu, wheezing, and/or abnormalities on radiograph of the thorax), and symptoms of gastrointestinal infections (diarrhea and/or vomiting, alone or in combination with abdominal distension), rashes, use of antibiotics, and diagnosis at discharge were recorded. If the presence or

absence of a specific symptom was not clearly mentioned in the medical record or letter of discharge, the symptom information was labeled as “missing.”

Statistical analysis

Analysis was performed using SPSS for Windows, version 12.1 (SPSS). Statistical analysis was performed on the first sample available per child. The Mann-Whitney *U* test was used to compare age distribution between categorical variables. Categorical variables were analyzed by Chi² test. A *P* value <.05 was considered to be significant.

RESULTS

HPeV and EV detection in CSF samples

During 2004–2006, 761 children <5 years of age were tested, by PCR, for EV in their CSF, and 108 children had positive results (table 1). At the time of our study, samples from 716 children were still available for HPeV testing; 33 children had test results positive for HPeV. Double infections were not observed. HPeV infection and EV infection were found in very young children, as presented in table 1. Ninety-seven percent of the children infected with HPeV were <24 months of age, and 46% were neonates (age, <28 days). For children infected with EV, 95% were <24 months of age, and 50% were neonates. There was no statistical difference in age between children positive for HPeV or children positive for EV. Of note, most samples (92.5%) that had been sent to the laboratory were obtained from children <2 years of age.

The majority of the children infected with HPeV or EV were boys (70% and 62%, respectively) (table 1). When the fact that 61% of the children tested were boys was taken into account, we could not find a higher risk for boys becoming positive for either HPeV or EV (OR, 0.9; 95% CI, 0.6–1.3).

Table 1. Characteristics of children tested for HPeV and/or EV.

Characteristic	Total group (<i>n</i> =761)	HPeV pos (<i>n</i> =33 ^a)	EV pos (<i>n</i> =108)
Age, median months (IQR)	0,9 (0,3-5,0)	1,2 (0,6-2,6)	0,9 (0,4-1,8)
Sex, no. (%)			
Female (%)	294 (39)	10 (30)	41 (38)
Male (%)	467 (61)	23 (70)	67 (62)

IQR: 25-75% interquartile range.

^a33 out of 716 children tested for HPeV.

Table 2. HPeV and EV prevalence in CSF per year.

Virus(es)	Proportion (%) of virus positive-patients positive in:			
	2004	2005	2006	Total
HPeV	16/196 (8,2)	1/239 (0,4)	16/281 (5,7)	33/716 (4,6)
EV	31/216 (14,4)	37/262 (14,1)	40/283 (14,1)	108/761 (14,2)

The yearly prevalence of EV in CSF was 14% during 2004–2006 (table 2). In contrast, the yearly prevalence of HPeV in CSF varied considerably. HPeV was detected in 8.2% and 5.7% of patients during 2004 and 2006, respectively, but was detected in only 0.4% of patients during 2005. Figure 1 illustrates the monthly prevalence of HPeV and EV in CSF during 2004–2006. Yearly and seasonal distribution varied between EV and HPeV. EV infections of the CNS could be found throughout the year but were most prevalent in summer and fall, with the highest peak occurring in October 2004 (50% of patients tested positive). HPeV infections of the CNS were observed in spring, summer, and fall, with the highest peak in May 2004 (21% of patients tested positive), whereas no HPeV infections were detected in December or January of 2004, 2005, or 2006. During 2004–2006, HPeV was detected in 4.6% of the CSF samples, compared with detection of EV in 14.2% of samples (table 2). The percentage of infected children who had HPeV infection was 23.4% (33 of 141 children with either EV or HPeV infection) (table 2). By use of the HPeV-specific PCR, the percentage of

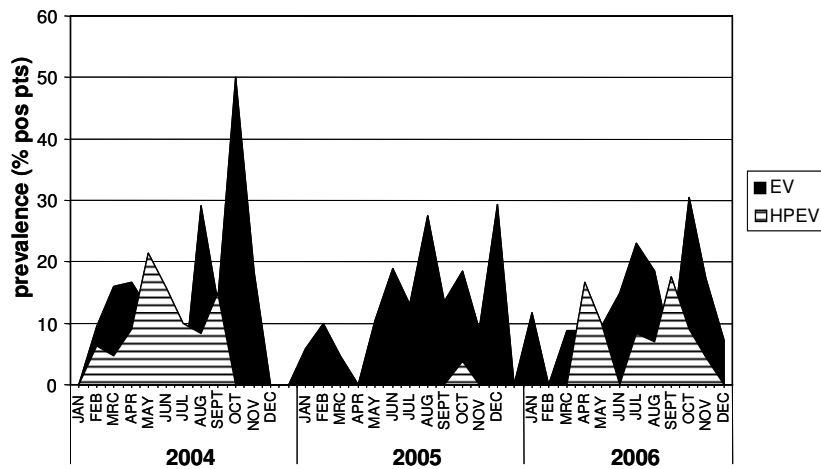


Figure 1. Monthly prevalence of HPeV and EV in CSF. The y-axis shows the monthly prevalence as percentage of positive patients per month for HPeV (hatched) and EV (black) in the total group of patients tested.

children positive for infection increased by 31% (i.e., 33 HPeV-positive children in addition to the 108 EV-positive children reported at the time of their illnesses).

Clinical characteristics of children with HPeV in CSF

Clinical data about the children who tested positive for HPeV in CSF were available for 29 children (88%; 10 girls and 19 boys). The majority of the children were born at term (23 children [79%]) and were healthy before admittance to the hospital (25 children [86%]). Twenty (69%) of the 29 children were admitted to a general hospital, and 9 (41%) were admitted to the academic hospital (2 of whom were admitted to an intensive care unit). The mean duration of hospital stay was 7 days, and 82% of the children were given antibiotics for a mean of 5.7 days (table 3). Fever was present in 97% of the children, and irritability was described by the examining pediatrician in 86% of the children. Fifteen (54%) of the children showed signs of sepsislike illness, and another 6 children (21%) received a diagnosis of sepsislike illness from the examining pediatrician (“suspected SLI”) but did

Table 3. Clinical characteristics of 29 patients with human parechovirus in CSF.

Variable	Finding
Age, months	
Mean	3.7
Median (range)	1.2 (0.2-58)
Hospital stay, days	
Mean	7.2
Median (range)	5.0 (1.0-39)
Antibiotic treatment	23/28 (82)
Duration of antibiotic treatment, days	
Mean	5.7
Median (range)	7.0 (3.0-10)
Fever	28/29 (97)
Irritability	24/28 (86)
Sepsislike illness	15/28 (54)
Suspected sepsislike illness	6/28 (21)
Meningitis	3/26 (12)
Seizures	2/28 (7)
Encephalitis	1/26 (4)
Paralysis	1/27 (4)
CSF	
Cell count, mean no. of cells/mm ³	4.6 (3.1-22)
Normal glucose level	19/25 (76)
Elevated protein level	13/21 (62)
Rash	5/29 (17)
Gastrointestinal tract symptoms	11/28 (39)
Respiratory tract symptoms	10/28 (36)

not meet our definition of sepsislike illness. The maximum cell count in CSF was 22 cells/mm³, and overall normal glucose values but elevated protein levels were found in the CSF. Meningitis was diagnosed in 3 children (12%), and encephalitis with seizures was diagnosed in 1 child (4%). In 1 child with severe meningitis, cerebral infarction determined by CT of the brain could not be explained by other causes. In 2 children, underlying disease was the most likely cause for the scored symptoms in the questionnaires. Paralysis was noted for 1 child, but that child received a diagnosis of neurological trauma after an accident. One child with preexisting developmental brain damage developed seizures. In those children, abnormalities revealed on diagnostic imaging were found as expected, in accordance with the underlying disease. Other clinical symptoms that were recorded were symptoms of gastrointestinal infections (39%), respiratory infections (36%), and rash (17%). Bacterial sepsis and meningitis were excluded on the basis of negative blood and CSF culture results. In 1 of 2 CSF cultures performed for one child, *Staphylococcus epidermidis* was found; in another child, culture of skin samples were positive for *Staphylococcus aureus*. No other pathogens were found in our study group.

DISCUSSION

Here, we show, in a retrospective analysis, that HPeV could be detected by real-time PCR in 4.6% of CSF samples obtained from children <5 years of age who were referred to our laboratory. Compared with EV infection, HPeV infection of the CNS was detected less frequently in our study, which is in agreement with previous observations [15, 24]. Children with HPeV in CSF presented mainly with clinical symptoms of sepsis, accompanied by signs of respiratory or gastrointestinal infection. CNS symptoms were reported to a lesser extent, and CSF cell counts were not significantly increased. The clinical presentation of children with HPeV in CSF described here closely resembled the clinical symptoms described for EV infection in children reported elsewhere [1, 25].

The mean duration of hospital stay for children with HPeV in CSF was 1 week, and antibiotic therapy was given to 82% of the children for at least 3 days. We demonstrated that the addition of HPeV-specific PCR increased the detection of infection in children by 31% in our laboratory. It has been shown that rapid diagnosis of EV infection by PCR can reduce hospital stay and duration of antibiotic use [26–28]; therefore, the addition of an HPeV-specific PCR could further reduce duration of antibiotic use and duration of hospital stay in children with sepsislike illness or CNS symptoms. However,

additional studies are needed to determine the effect of rapid viral diagnosis on reduction of hospital stay and reduced use of antibiotics.

Only a few reports have been published on the epidemiology of HPeVs, and these are all based on HPeV detection by cell culture. HPeV infections are considered to be widely prevalent, mainly affecting children [7–10, 16, 29]. For HPeV1, it has been shown that a minority of patients show signs of CNS involvement [9, 15]. Recent studies of HPeV3 all show an association with more-severe disease than HPeV1 infection - that is, neonatal sepsis, meningitis, and paralysis [4, 7, 12, 16]. In our study, HPeV was not isolated by cell culture; it was detected directly from CSF samples by real-time PCR through use of primers and probes validated for all 6 known genotypes [21]. Previously, 1 other study detected HPeV directly from CSF samples by real-time PCR, reporting that 1% of the samples were positive for HPeV [24]. In that study, CSF samples that were negative for meningococcal PCR or were negative in cell culture but that were suspected to be positive for viral meningitis were selected without age restriction, decreasing the likelihood of finding HPeV. In addition, Corless et al. [24] might have missed HPeVs, because the probe of their real-time PCR, which was described in 2002, shows mismatches with the newer HPeV types 3–6.

We showed that the yearly prevalence of HPeV in CSF varied remarkably, in contrast to EV, for which the yearly prevalence in CSF was stable during 2004–2006. The difference in yearly prevalence of HPeV could be attributable to absence of HPeV circulation during 2005. However, we cannot exclude the possibility that our HPeV-specific PCR was not able to detect an unidentified HPeV that could have been circulating in 2005. Both possibilities seem unlikely, because HPeV could be detected in feces samples from 2005 [30]. However, different HPeV genotypes might circulate in different years or seasons, as has been suggested elsewhere [7, 10, 12, 29]. Thus, it could be anticipated that HPeV types able to infect the CNS - presumably HPeV3 - circulate only in specific years. Whether the HPeVs found in the CSF will be predominantly HPeV3 needs to be further elucidated. Genotyping of HPeV can be done by sequencing of the VP1 gene [6, 12], but this has been performed only on HPeV isolates obtained from cell culture. The combination of small volumes of CSF obtained from young children and insensitivity of the technique limited the possibility of genotyping in our study.

There are other potential limitations to our study. First, the samples selection is not random but biased, from a pediatric population referred for virological testing. To circumvent this bias, we compared HPeV prevalence with that of EV, the most important viral cause of neonatal sepsis and meningitis.

Another potential bias the inability to test 45 of our samples for HPeV (29 EV-positive and 16 EV-negative samples). Exclusion of these samples from the analysis would thus underestimate the EV prevalence. If the 29 EV-positive samples were considered to be HPeV negative, the HPeV prevalence in the total study group would vary between 4.3% (if all 16 EV-negative samples were HPeV negative) and 6.4% (if all 16 EV-negative samples were HPeV positive). Therefore, the HPeV prevalence of 4.6% could be a slight over- or underestimation of the real prevalence in our study group. Despite these limitations, we conclude that HPeV is another important cause of viral sepsis and meningitis in young children that has frequently been undetected. PCR, together with a sensitive molecular typing method, will elucidate further the epidemiology of HPeV relative to clinical symptoms and genotypes. HPeV-specific PCR should be included in viral diagnostic testing for CSF samples and needs to be further evaluated for use in other clinical samples, such as blood, throat swabs and feces.

REFERENCES

1. **Rotbart HA, McCracken GH Jr, Whitley RJ, et al.** Clinical significance of enteroviruses in serious summer febrile illnesses of children. *Pediatr Infect Dis J* **1999**; 18:869–74.
2. **Verboon-Maciolek MA, Nijhuis M, van Loon AM, et al.** Diagnosis of enterovirus infection in the first 2 months of life by real-time polymerase chain reaction. *Clin Infect Dis* **2003**; 37:1–6.
3. **King AMQ, Brown F, Christian P, et al.** Picornaviridae. In: Van Regenmortel MHV, Fauquet CM, Calisher CH, et al., eds. *Virus taxonomy: classification and nomenclature of viruses. Seventh Report of the ICTV.* New York: Academic Press, **2000**; 657–73.
4. **Ito M, Yamashita T, Tsuzuki H, Takeda N, Sakae K.** Isolation and identification of a novel human parechovirus. *J Gen Virol* **2004**; 85: 391–8.
5. **Benschop KSM, Schinkel J, Luken ME, et al.** Fourth human Parechovirus serotype. *Emerg Infect Dis* **2006**; 12:1572–5.
6. **Al-Sunaidi M, Williams CH, Hughes PJ, Schnurr DP, Stanway G.** Analysis of a new human parechovirus allows the definition of parechovirus types and the identification of RNA structural domains. *J Virol* **2007**;81:1013–21.
7. **Watanabe K, Oie M, Higuchi M, Nishikawa M, Fujii M.** Isolation and characterization of novel human parechovirus from clinical samples. *Emerg Infect Dis* **2007**; 13:889–95.

8. **Joki-Korpela P, Hyypia T.** Parechoviruses, a novel group of human picornaviruses. *Ann Med* **2001**; 33:466–71.
9. **Stanway G, Joki-Korpela P, Hyypia T.** Human parechoviruses—biology and clinical significance. *Rev Med Virol* **2000**; 10:57–69.
10. **Ehrnst A, Eriksson M.** Echovirus type 23 observed as a nosocomial infection in infants. *Scand J Infect Dis* **1996**; 28:205–6.
11. **Boivin G, Abed Y, Boucher FD.** Human parechovirus 3 and neonatal infections. *Emerg Infect Dis* **2005**; 11:103–5.
12. **Benschop KS, Schinkel J, Minnaar RP, et al.** Human parechovirus infections in Dutch children and the association between serotype and disease severity. *Clin Infect Dis* **2006**; 42:204–10.
13. **Koskiniemi M, Paetau R, Linnavuori K.** Severe encephalitis associated with disseminated echovirus 22 infection. *Scand J Infect Dis* **1989**; 21: 463–6.
14. **Figuerola JP, Ashley D, King D, Hull B.** An outbreak of acute flaccid paralysis in Jamaica associated with echovirus type 22. *J Med Virol* **1989**; 29:315–9.
15. **Grist NR, Bell EJ, Assaad F.** Enteroviruses in human disease. *Prog Med Virol* **1978**; 24:114–57.
16. **Abed Y, Boivin G.** Human parechovirus infections in Canada. *Emerg Infect Dis* **2006**; 12:969–75.
17. **Rotbart HA, Romero JR.** Laboratory diagnosis of enteroviral infections. In: Robart H, ed. *Human enterovirus infections*. Washington, DC:ASM Press, **1995**; 401–18.
18. **Romero JR.** Reverse-transcription polymerase chain reaction detection of the enteroviruses. *Arch Pathol Lab Med* **1999**; 123:1161–9.
19. **Hyypia T, Auvinen P, Maaronen M.** Polymerase chain reaction for human picornaviruses. *J Gen Virol* **1989**; 70:3261–8.
20. **Oberste MS, Maher K, Pallansch MA.** Specific detection of echoviruses 22 and 23 in cell culture supernatants by RT-PCR. *J Med Virol* **1999**; 58: 178–81.
21. **Benschop K, Molenkamp R, van der Ham A, Wolthers K, Beld M.** Rapid detection of human parechoviruses in clinical samples by realtime PCR. *J Clin Virol* **2008**; 41:69–74.
22. **Boom R, Sol CJ, Salimans MM, et al.** Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* **1990**; 28:495–503.
23. **Beld M, Minnaar R, Weel J, et al.** Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. *J Clin Microbiol* **2004**; 42:3059–64.

24. **Corless CE, Guiver M, Borrow R, et al.** Development and evaluation of a “real-time” RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. *J Med Virol* 2002; 67: 555–62.
25. **Verboon-Maciolek MA, Krediet TG, Gerards LJ, Fleer A, van Loon TM.** Clinical and epidemiologic characteristics of viral infections in a neonatal intensive care unit during a 12-year period. *Pediatr Infect Dis J* 2005; 24:901–4.
26. **Robinson CC, Willis M, Meagher A, et al.** Impact of rapid polymerase chain reaction results on management of pediatric patients with enteroviral meningitis. *Pediatr Infect Dis J* 2002; 21:283–6.
27. **Spicher VM, Berclaz PY, Cheseaux JJ, et al.** Detection of enteroviruses in the cerebrospinal fluid by polymerase chain reaction: prospective study of impact on the management of hospitalized children. *Clin Pediatr (Phila)* 2000; 39:203–8.
28. **Michos AG, Syriopoulou VP, Hadjichristodoulou C, et al.** Aseptic meningitis in children: analysis of 506 cases. *PLoS ONE* 2007; 2:e674.
29. Ehrnst A, Eriksson M. Epidemiological features of type 22 echovirus infection. *Scand J Infect Dis* 1993; 25:275–81.
30. **Benschop KS, Thomas X, Serpenti C, et al.** Molecular detection of human parechoviruses and enteroviruses in young children. Program and abstracts of the Third European Congress of Virology (Nurnberg). Nurnberg: Eurovirology, 2007:91.