Epidemiology of Sepsis-like Illness in Young Infants: Major Role of Enterovirus and Human Parechovirus

Eveline P. de Jong, MD[1,5], Monique G.A. van den Beuken, MD[2], Erika P.M. van Elzakker, MD[3], K.C. Wolthers, MD, PhD[4], Arwen J. Sprij, MD[1], E. Lopriore, MD, PhD[5], Frans J. Walther, MD, PhD[5], and Frank Brus, MD, PhD[1]

1. Department of Paediatrics, HAGA hospital, location Juliana's Children's Hospital, The Hague, Netherlands.
2. Department of Paediatrics, Van Weel-Bethesda Ziekenhuis, Dirksland, Netherlands
3. Department of Medical Microbiology, HAGA hospital, The Hague, Netherlands.
4. Department of Medical Microbiology, Laboratory of Clinical Virology, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, Netherlands.
5. Department of Paediatrics, Division of Neonatology, Leiden University Medical Centre, Leiden, Netherlands.

Corresponding author: Eveline P. de Jong

Leiden University Medical Centre, Dept. of Paediatrics, J-6

Albinusdreef 2, 2333 ZA Leiden, the Netherlands

Tel: +31 71 526 2824 / Fax: +31 71 5248198

E-mail: evelinedejong@gmail.com

Abbreviated title: Epidemiology of Sepsis-like Illness in Young Infants

Running head: Sepsis-like Illness in Young Infants

Keywords: Human Enterovirus, Human Parechovirus, Infants, Sepsis, CSF pleocytosis.

No reprints available via authors

Support sources / funding: Viral typing was made possible through a financial gift from the HAGA-Hospital Scientific Fund.

The authors have no conflicts of interest to disclose.

Acknowledgements

We kindly thank Bernadette van Asbeck and Melissa de Ruijter, medical students, for their contribution to the data collection, Nan van Geloven, Department of Clinical Statistics at the LUMC, for her advice on statistical analysis, and Rachid Yahiaoui, microbiology analyst at the HAGA Hospital, for technical assistance with viral typing.

Background: Sepsis-like illness is a main cause for hospital admission in young infants. Our aim was to investigate incidence, epidemiology and clinical characteristics of enterovirus (EV) and human parechovirus (HPeV) infections in young infants with sepsis-like illness.

Methods: This is a prospective observational cohort study in which infants younger than 90 days of age, presenting with sepsis-like symptoms in a secondary care children's hospital, underwent a full sepsis work-up. Clinical signs and infectious indices were recorded. EV or HPeV RNA was detected by PCR in plasma and/or cerebrospinal fluid (CSF).

Results: Infants were diagnosed with EV, HPeV, fever of unknown origin or severe infection. EV and HPeV were detected in 132/353 (37%) and 52/353 (15%) of cases, respectively. EV and HPeV have distinct seasonability. Some differences in clinical signs and symptoms occurred between children with EV and HPeV infection, but were of limited clinical value. CSF pleocytosis occurred in 44% of EV positive infants, and only in 13% of those with HPeV infection.

Conclusions: EV and HPeV infections are major causes of sepsis-like illness in infants < 90 days of age. Neither clinical characteristics nor laboratory indices were predictive for EV/HPeV infection. CSF pleocytosis occurs, but not in all patients. Testing for EV and HPeV in all young infants with sepsis-like illness is strongly advised.

Introduction

Sepsis-like symptoms in children, especially in young infants (under 90 days of age) remain a diagnostic challenge for pediatricians because it is often hard to distinguish between serious bacterial infections and more benign viral infections[1 2].

In young infants enterovirus (EV) and human parechovirus (HPeV) infections are a known cause of sepsis-like illness, aseptic meningitis and febrile disease[3-6]. Numerous EV-types, specifically several serotypes of the enterovirus B species, have been associated with febrile-illness and aseptic meningitis in infants[7 8]. In HPeV-infection, type 3 (HPeV3) is the main genotype causing sepsis-like symptoms in young infants[9 10]. EV and HPeV infections can also cause serious symptoms such as cardiorespiratory instability and neurologic symptoms, leading to hospital or, in some young infants, pediatric intensive care unit admittance[11-14].

Previous studies have reported a high incidence of EV and HPeV infections among febrile infants, but most were retrospective[15 16], based on laboratory[17-19] results rather than clinical presentation, did not solely focus on young infants[3] or described neonates only[6]. Only one prospective cohort that included patients up to 90 days of age was described earlier[5].

We performed a prospective observational cohort study to describe epidemiology, clinical characteristics and infectious indices of young infants with sepsis-like illness who presented at our emergency department. Our hypothesis is that EV and HPeV are a major cause of sepsis-like illness in this vulnerable group of infants up to 90 days of age and that symptoms of infants with EV or HPeV infection are not different from other infants with sepsis-like illness. Main outcome is frequency of diagnosis of EV or HPeV infection in our study population, secondary outcomes are clinical signs and symptoms and laboratory indices.

Materials and Methods

Study protocol – clinical aspects

This prospective observational cohort study was performed at the Juliana Children's Hospital, The Hague, Netherlands. All children under 90 days of age who were evaluated at our emergency department for sepsis-like symptoms between January 1, 2008 and June 30, 2012 were evaluated in this study. Sepsis-like illness was diagnosed based on age-specific criteria (see table, Supplemental Digital Content 1), which were evaluated at physical examination by the attending physician. All physicians in our hospital were trained in their use. In addition to the clinical signs and symptoms described in Supplemental Digital Content 1 (table), the following clinical parameters were collected: sex, prematurity (gestational age < 37 weeks), medical history, abnormal behavior (defined as lethargic or agitated), skin rash, oxygen saturation at presentation and duration of symptoms before presentation. If a specific symptom was not clearly noted on admittance, this item was labeled as 'missing'.

We excluded patients with signs of a localized infection, defined as clinically apparent gastro-enteritis, upper respiratory tract infection, pneumonia (clinically apparent and confirmed on chest x-ray) or abnormal analysis of urine sediment (more than five white blood cells (WBC) per microscopic field view, magnification of 40 times).

Patients with need of systemic intravenous treatment for a confirmed (with bacterial culture or HSV PCR) pathogen were assigned to the group 'severe infection'. Patients with insufficient sample size of both plasma and cerebrospinal fluid (CSF) to perform PCR for EV and HPeV were labelled as 'non-evaluable result'. To investigate whether or not these patients influenced our results, we performed additional analyses including them once in the EV or HPeV group and once in the fever of unknown origin (FUO) group.

Biochemical and microbiologic data

Children underwent blood and CSF sampling for biochemical analysis, viral analysis for EV and HPeV, and bacterial cultures. Herpes simplex virus PCR was performed on CSF.

The results for C-reactive protein, serum glucose, full blood count, and WBC differentiation were recorded. When CSF was successfully collected, CSF white and red blood cell counts, CSF glucose and protein levels were recorded in the study database. We corrected CSF WBC count for traumatic puncture if the CSF red blood cell count was > 1000 cells/μL, using a 1000:1 ratio[22]. CSF pleocytosis was defined as a CSF WBC count > 19 cells/μL for children <28 days of age, >9 cells/μL for children 28-58 days of age and >5 cell/ μL for children 59-90 days of age [23] [24].

EV and HPeV detection and genotyping

PCR was performed on plasma and CSF to detect EV or HPeV RNA. RNA was extracted from 200 μL of plasma and/or 50-200 μL of CSF with the Nuclisens easyMAG system (Biomerieux, Boxtel, Netherlands). The manufacturer's protocol (Generic 1.0) was followed using easyMAG specific reagents. A fixed amount of Phocine Distemper Virus served as an internal control and was added to each sample prior to RNA extraction. Extracted RNA was used for Reverse Transcription PCR to synthesize copy DNA and PCR was performed with the ABI 7500 Real Time PCR system (Applied Biosystems, USA): 10 minutes at 95 °C, followed by 45 cycles of 15 sec at 95°C, and 1 minute at 60°C. Primers and probes for amplification and detection of EV and HPeV were located in the highly conserved 5'end of the genome. Modifications were made to previously described probes and primers to also detect HPeV 3[25-27]. Probes were adjusted to

VIC-TTACCTRCGGGTACCTTCTGGGCATCCTT-TAMRA and VIC-CCCCAGATCAGATCC-MGB and primers were adjusted to TGCAAACACTAGTTGTAAGGCCC, TGCAGACACTAGTTGTAAGGCCC, TGCAAACACTAGTTGTATGGCCC (forward primers) and TTGGCCCACTAGACGTTTTTTAA, TTGGCCCGCTAGACGTTTTTTAA, GTTTGGCCCACTAGACGTTTTT (revers primers).

All PCR runs had a mixture of an EV and HPeV strain as a positive control and nuclease free water as a negative control.

A positive diagnosis for infection with EV or HPeV was made on a positive PCR in either plasma or CSF (or both).

EV and HPeV positive plasma and CSF samples were genotyped in one batch after completion of the study period if enough material was left. EV typing was performed as previously described with modifications[28]. In short, two PCR's were run (EV-A and EV-B) for which 6 µL of input RNA was used. The original protocol was adjusted to perform a semi-nested PCR instead of a single PCR[19]. PCR-1 was performed using primers (EV-A OS 2268 + EV-A OAS 3109 and EV-B OS 2324 + EV-B OAS 3505) for 1 hour at 43°C, followed by 2 x 20 cycles at 53 and 55°C, 15 minutes at 72°C, and 2 minutes at 94°C. Thereafter, 3 x 40 cycles were performed at 94°C, 50°C and 68°C, followed by 5 minutes at 68°C. One µL of this fluid was then transferred to PCR-2 with primers (EV-A OS 2268 + EV-A IAS 3016 and EV-B OS 2324 + EV-B IAS 3477). This was processed in 3 x 30 cycles (18 min at 94°C, 21 min at 55°C and 90 min at 72°C), followed by 5 min at 72°C. Fluid of PCR-2 (5-10 µL) was loaded on an agarose gel, if positive (band visible of 750bp (EV-A) or 1150bp (EV-B)), a standard BDT sequence reaction was performed using primers for PCR-2[28].

HPeV typing was performed as previously described by Harvala et al[19]. One modification was made; we used a different OS primer (HPeV OS-R-2162; TCMACWTGGATGAGGAARAC instead of the original primer HPeV OS-2090) in PCR-1.

Statistical analysis

SPSS was used for data management (PASW statistics version 17.0) and statistical analysis (IBM SPSS statistics version 23.0). Data were checked for normality before analysis, using descriptive statistics and histograms with z-scores for skewness and kurtosis. Categorical data are shown as absolute number/total (percentage) and numerical data as median (interquartile range).

P-values <0.05 were considered to indicate statistical significance, in subgroup analyses we considered p-values <0.01 statistically significant. Mann-Whitney-U tests and Kruskal Wallis tests were used for numerical data and Fisher's Exact tests for categorical data. Binary logistic regression analysis was performed to assess the relationship between the occurrence of EV or HPEV infection or FUO (dependent variable) and clinical characteristics or laboratory parameters (independent variables).

The data described in our study were derived from our standard of care. No extra interventions were conducted for study purposes only. Therefore, no explicit informed consent from parents was warranted for this study. The personal data of our patients were protected. The study was approved by the regional medical ethics committee.

Results

During the study period 362 infants with sepsis-like illness were included. Nine infants (2%) could not be diagnosed due to insufficient sample volume of either plasma or CSF to perform EV

and HPeV PCR. The additional analyses to investigate the influence of these non-evaluable patients to our cohort showed no change in our results (data not shown).

Epidemiology

The remaining 353 infants were diagnosed as: EV-infection (n=132 (37%)), HPeV infection (n=52 (15%)), fever of unknown origin (FUO) (n=162 (46%)) and severe infection (n=7 (2%)). Details of the recruitment and diagnoses of our cohort and the causative pathogens of infants in the 'severe infection' group are given in figure 1.

Figure 2 shows the seasonal distribution of the different diagnoses. During summer, there is a yearly peak of EV infection and biannual peak of HPeV infections in even years. During winter 2009 an increase in FUO occurred during the influenza A (H1N1) pandemic in our country. Bacterial infections occurred with a low incidence throughout the study period.

Viral genotyping

Enough material was available to perform genotyping in 35/184 EV or HPeV positive infants (19%). Genotyping was possible for 23/132 (17%) EV positive patients, of whom 22 were enterovirus-B positive (CV-B1, CV-B2, CV-B4, CV-B5, E-6, E-7, E-9, E-11, E-18, E-25, and E-30) and 1 enterovirus-A (CV-A16) positive. This infant presented with sepsis-like illness, was EV positive in plasma and negative in CSF, and did not develop any signs of hand-foot-mouth disease during its hospital stay. Supplemental Digital Content 2 (table) shows the details of EV genotyping. HPeV-3 was found in all of the HPeV positive samples that were genotyped (12/52 (23%)).

Clinical and biochemical parameters

Clinical symptoms, vital parameters and infectious parameters of our study population are presented in Table 1 and 2. No statistically significant differences occurred between the serious bacterial infections group and the FUO and 'EV or HPeV' group.

Comparing the EV or HPeV group to the FUO group showed that infants in the EV or HPeV positive group were more often less than 28 days of age ($p=0.003$) and showed statistically significant, but only slightly higher heart and breathing frequencies at presentation compared to patients in the FUO group. There also was a difference in behavior ($p=0.006$) and children with EV or HPeV had somewhat lower infectious parameters ($p<0.01$). Comparing EV/HPeV positive infants to those in the FUO group, logistic regression showed differences in age-group (0-28 days or 29-90 days) (OR 0.243 (95% CI 0.101-0.584)), plasma WBC count (OR 0.743 (95% CI 0.601-0.919)) and CSF WBC count (OR 1.009 (95% CI 1.002-1.015).

Table 2 compares between EV and HPeV positive infants. HPeV positive infants have a lower rate of CSF pleocytosis (8%) than EV positive infants (43%) ($p=0.000$) and have somewhat lower infectious indices than infants with an EV infection.

No children were transferred to a pediatric intensive care unit and none died. All children visited our outpatient clinic 4-6 weeks after hospital admittance. None of them showed any physical abnormalities at this follow-up visit.

Discussion

We describe a high incidence of EV and HPeV infection in the largest prospective cohort study among infants 0-90 days of age to date. This adds to describing the epidemiology, clinical and

laboratory signs, and symptoms of sepsis-like illness in young infants, especially those with an EV or HPeV infection.

Several laboratory-based and retrospective studies have identified EV and HPeV as an important cause of sepsis and/or meningitis in young infants [9 10 29-31]. Rittichier et al. showed, in a prospective study, an incidence of 20% of EV infection in young infants with fever who underwent a full sepsis work-up[5]. We find a higher incidence of EV infection (36%), this may be due to our selection of a population with a higher risk, as we only included those infants with sepsis-like illness instead of all infants with fever. Cabrerizo et al. recently reported an incidence of 38% for EV infection and 11% for HPeV infection in neonates with fever, sepsis or meningitis[6]. We describe similar incidences, but in a population up to 90 days of age, instead of only neonates, adding to the importance of EV and HPeV testing in this group.

In contrast, in previous laboratory-based reports, the incidence of EV and HPeV infections was much lower[10 12 30]. For example, Wolthers et al. detected EV in 14% and HPeV in 4.6% of cerebrospinal fluid (CSF) samples of young children (median age 1 month) with sepsis-like illness and meningitis during a 3-year period[30]. The lower frequencies found in this study may be attributed to the retrospective analysis of randomly submitted samples instead of samples taken prospectively in a selected patient group, as well as the difference in age groups. Also, we tested both plasma and CSF, instead of CSF only.

We made minor modifications to PCR methods described previously[25-27], to also detect HPeV3. With this method, detection rate was similar to or higher than previous reports, and genotyping showed only HPeV3, confirming the accuracy of the adjustments.

In Europe HPeV3 occurs in a biannual cycle with a peak in even-numbered years [30 32]. We also detected a biannual cycle, but with a much higher incidence of HPeV3 in infants with sepsis-like

illness. In our population, the incidence of HPeV in epidemic years increased sharply to 19% in 2008, 26% in 2010 and 27% in 2012, and in non-epidemic years dropped to about 2% (both in 2009 and 2011), but HPeV was never completely absent. The higher detection rate of HPeV, which is presumably mainly HPeV3, is most probably due to the heightened awareness of HPeV as a pathogen and subsequent implementation of HPeV3 specific PCR methods just before our study period[32].

Because of low sample volumes viral typing was possible only in part of our population, we were able to type 23/132 (17%) of the EV and 12/52 (23%) of the HPeV positive infants.

As expected, in the 23 EV positive patients we found a wide variety of genotypes, all but one were Enterovirus B genotypes. E-5, 6, 11 and 30, and CV-B5, B4 and B1 have been reported to cause sepsis-like illness in young infants[16 29 33 34]. However this is the first description of E-7, 18 and 25, and CV-B4 to cause sepsis-like illness in infants.

In addition, we are the first to report a CV-A16 related to sepsis-like illness in infants[35]. CV-A16 has caused outbreaks of hand-foot-mouth disease[36] and rare complications, such as aseptic meningitis or pulmonary edema have been described in Asia[37]. It has also been described as a rare cause of fatal infection in infants, with only 4 cases described worldwide[38-41].

All of the typed HPeV positive infants were HPeV3 positive. This is concordant with previous reports that describe HPeV3 as a main pathogen for sepsis-like illness and aseptic meningitis in young infants[9]. Although we could only test 23% of our study population, we only found HPeV3 and therefore consider this the main HPeV type causing illness in our patient group. Other HPeV types have not been found in our population, these have been described to cause different symptoms[42 43].

Although differences between infants with EV or HPeV and those with FUO (table 1 and 2) were statistically significant, most likely due to the large sample size of our population, they have a very limited clinical value because the differences of the variables are small and overlapping. It is interesting to notice, however that the infectious indices of HPeV infected infants are somewhat lower than those of infants with EV infection. The clinical presentation of infants with EV and HPeV infection was similar, as has been reported previously[11].

In our study we show that although pleocytosis is uncommon in HPeV infection (8%) compared to EV infection (43%), it is not absent. Recently, Cabrerizo et al. described 32 EV positive and 9 HPeV positive neonates and found no CSF pleocytosis in those with an HPeV infection. EV positive patients showed pleocytosis in 19/32 (59%) of cases[6]. Yun et al. showed that EV meningitis occurred without pleocytosis in 68% of neonates. Absence of CSF pleocytosis was associated with a younger age and a shorter time period between onset of disease and lumbar puncture[44]. In accordance with this study, we evaluated a group of very young patients, in whom a lumbar puncture was performed shortly after onset of disease (median, 0.5 days), and find a low number of pleocytosis and high incidence of EV or HPeV (tables 1 and 2). Several studies have reported EV and HPeV positive children without CSF pleocytosis who developed neonatal seizures or cerebral white matter abnormalities[13 14 30]. More research is required to elucidate whether or not CSF pleocytosis is associated with severity of disease, cerebral white matter involvement and neurologic sequelae in children with EV and HPeV infections. Testing for EV and HPeV, even in absence of pleocytosis, should be considered standard of care.

Our study has its limitations. We only tested for the presence of EV and HPeV on blood and CSF and did not perform tests to discover viral infections other than EV, HPeV, and herpes simplex virus in our patients. So, we did not determine the influence of other viruses and did not uncover

dual infections of infants with an EV or HPeV infection. Our objective was to identify the impact of EV and HPeV on sepsis-like illness in our population of young infants and this lack of testing for other viruses did not influence our outcome. But it would be of interest for further research.

This study adds a large prospective cohort of young infants with sepsis-like illness to current knowledge. We describe similar findings in epidemiology, with a higher detection rate than previously reported, of HPeV in epidemic years. And although less common than in EV infection, HPeV can cause pleocytosis and aseptic meningitis. Testing for EV and HPeV in plasma and CSF should be considered in young infants with sepsis-like illness.

References

1. Thompson M, Van den Bruel A, Verbakel J, et al. Systematic review and validation of prediction rules for identifying children with serious infections in emergency departments and urgent-access primary care. *Health Technol Assess*. 2012;16(15):1-100.

2. Arora R, Mahajan P. Evaluation of child with fever without source: review of literature and update. *Pediatr Clin North Am.* 2013;60(5):1049-62.

3. 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(10):869-74.

4. 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(2):204-10.

5. Rittichier KR, Bryan PA, Bassett KE, et al. Diagnosis and Outcomes of Enterovirus Infections in Young Infants. *Pediatr Infect Dis J.* 2005;24(6):546-50.

6. Cabrerizo M, Trallero G, Pena MJ, et al. Comparison of epidemiology and clinical characteristics of infections by human parechovirus vs. those by enterovirus during the first month of life. *Eur J Pediatr.* 2015;174(11):1511-6.

7. Miller DG, Gabrielson MO, Bart KJ, et al. An epidemic of aseptic meningitis, primarily among infants, caused by echovirus 11-prime. *Pediatr.* 1968;41(1):77-90.

8. Lee BE, Davies HD. Aseptic meningitis. *Curr Opin Infect Dis.* 2007;20(3):272-77.

9. Harvala H, Robertson I, Chieochansin T, et al. Specific association of human parechovirus type 3 with sepsis and fever in young infants, as identified by direct typing of cerebrospinal fluid samples. *J Infect Dis.* 2009;199(12):1753-60.

10. Selvarangan R, Nzabi M, Selvaraju SB, et al. Human Parechovirus 3 Causing Sepsis-like Illness in Children From Midwestern United States. *Pediatr Infect Dis J.* 2011;30(3):238-42.

11. Verboon-Maciolek MA, Krediet TG, Gerards LJ, et al. Severe Neonatal Parechovirus Infection and Similarity With Enterovirus Infection. *Pediatr Infect Dis J.* 2008;27(3):241-45.

12. Piñeiro L, Vicente D, Montes M, et al. Human parechoviruses in infants with systemic infection. *J Med Vir.* 2010;82(10):1790-96.

13. Verboon-Maciolek MA. White matter damage in neonatal enterovirus meningoencephalitis. *Neurol.* 2006;66(8):1267-69.

14. Verboon-Maciolek MA, Groenendaal F, Hahn CD, et al. Human parechovirus causes encephalitis with white matter injury in neonates. *Ann Neurol.* 2008;64(3):266-73.

15. King RL, Lorch SA, Cohen DM, et al. Routine Cerebrospinal Fluid Enterovirus Polymerase Chain Reaction Testing Reduces Hospitalization and Antibiotic Use for Infants 90 Days of Age or Younger. *Pediatr.* 2007;120(3):489-96.

16. Verboon-Maciolek MA, Krediet TG, Gerards LJ, et al. Clinical and Epidemiologic Characteristics of Viral Infections in a Neonatal Intensive Care Unit During a 12-Year Period. *Pediatr Infect Dis J.* 2005;24(10):901-04.

17. Jeziorski E, Schuffenecker I, Bohrer S, et al. Relevance of human parechovirus detection in cerebrospinal fluid samples from young infants with sepsis-like illness. *J Clin Lab Anal.* 2015;29(2):112-5.

18. Harvala H, Griffiths M, Solomon T, et al. Distinct systemic and central nervous system disease patterns in enterovirus and parechovirus infected children. *J Infect.* 2014;69(1):69-74.

19. Harvala H, Robertson I, McWilliam Leitch EC, et al. Epidemiology and clinical associations of human parechovirus respiratory infections. *J Clin Microbiol.* 2008;46(10):3446-53.

20. Chiu CH, Lin TY, Bullard MJ. Application of criteria identifying febrile outpatient neonates at low risk for bacterial infections. *Pediatr Infect Dis J.* 1994;13(11):946-9.

21. McCarthy PL, Sharpe MR, Spiesel SZ, et al. Observation scales to identify serious illness in febrile children. *Pediatr.* 1982;70(5):802-9.

22. Greenberg RG, Smith PB, Cotten CM, et al. Traumatic lumbar punctures in neonates: test performance of the cerebrospinal fluid white blood cell count. *Pediatr Infect Dis J.* 2008;27(12):1047-51.

23. Kliegman RM SB, St. Geme J, Schor NF. *Nelson Textbook of Pediatrics,* 20th edition Philadelphia, PA: Elsevier Inc 2016.

24. Kestenbaum LA, Ebberson J, Zorc JJ, et al. Defining Cerebrospinal Fluid White Blood Cell Count Reference Values in Neonates and Young Infants. *Pediatr.* 2010;125(2):257-64.

25. 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(4):555-62.

26. Jokela P, Joki-Korpela P, Maaronen M, et al. Detection of human picornaviruses by multiplex reverse transcription-PCR and liquid hybridization. *J Clin Microbiol.* 2005;43(3):1239-45.

27. Noordhoek GT, Weel JF, Poelstra E, et al. Clinical validation of a new real-time PCR assay for detection of enteroviruses and parechoviruses, and implications for diagnostic procedures. *J Clin Virol.* 2008;41(2):75-80.

28. Leitch EC, Harvala H, Robertson I, et al. Direct identification of human enterovirus serotypes in cerebrospinal fluid by amplification and sequencing of the VP1 region. *J Clin Virol.* 2009;44(2):119-24. Erratum in J Clin Virol. 2011 Aug;51(4):286.

29. Leggiadro RJ, Darras BT. Viral and bacterial pathogens of suspected sepsis in young infants. *Pediatr Infect Dis.* 1983;2(4):287-9.

30. Wolthers Katja C, Benschop Kimberley SM, Schinkel J, et al. Human Parechoviruses as an Important Viral Cause of Sepsislike Illness and Meningitis in Young Children. *Clin Infect Dis.* 2008;47(3):358-63.

31. Harvala H, McLeish N, Kondracka J, et al. Comparison of human parechovirus and enterovirus detection frequencies in cerebrospinal fluid samples collected over a 5-year period in edinburgh: HPeV type 3 identified as the most common picornavirus type. *J Med Vir.* 2011;83(5):889-96.

32. van der Sanden SM, Koopmans MP, van der Avoort HG. Detection of human enteroviruses and parechoviruses as part of the national enterovirus surveillance in the Netherlands, 1996-2011. *Eur J Clin Microbiol Infect Dis.* 2013;32(12):1525-31.

33. Krajden S, Middleton PJ. Enterovirus infections in the neonate. *Clin Pediatr (Phila).* 1983;22(2):87-92.

34. Lee ST, Ki CS, Lee NY. Molecular characterization of enteroviruses isolated from patients with aseptic meningitis in Korea, 2005. *Arch Virol.* 2007;152(5):963-70.

35. Chang LY, Lin TY, Huang YC, et al. Comparison of enterovirus 71 and coxsackie-virus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. *Pediatr Infect Dis J.* 1999;18(12):1092-6.

36. Lim CTK, Jiang L, Ma S, et al. Basic reproduction number of coxsackievirus type A6 and A16 and enterovirus 71: estimates from outbreaks of hand, foot and mouth disease in Singapore, a tropical city-state. *Epidemiol Infect.* 2016;144(5):1028-34.

37. Ho M, Chen ER, Hsu KH, et al. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. *N Engl J Med.* 1999;341(13):929-35.

38. Wright HT, Landing BH, Mcallister RM, et al. Fatal Infection in an Infant Associated with Coxsackie Virus Group a, Type 16. *N Engl J Med.* 1963;268(19):1041-&.

39. Goldberg MF, McAdams AJ. Myocarditis possibly due to Coxsackie Group A, type 16, virus. *J Ped.* 1963;62(5):762-65.

40. Wang CY, Li Lu F, Wu MH, et al. Fatal coxsackievirus A16 infection. *Pediatr Infect Dis J.* 2004;23(3):275-6.

41. Astrup BS, Johnsen IBG, Engsbro AL. The role of Coxsackievirus A16 in a case of sudden unexplained death in an infant – A SUDI case. *Forensic Sci Int.* 2016;259:e9-e13.

42. Pajkrt D, Benschop KSM, Westerhuis B, et al. Clinical Characteristics of Human Parechoviruses 4–6 Infections in Young Children. *Pediatr Infect Dis J.* 2009;28(11):1008-10.

43. Harvala H, Simmonds P. Human parechoviruses: Biology, epidemiology and clinical significance. *J Clin Vir.* 2009;45(1):1-9.

44. Yun KW, Choi EH, Cheon DS, et al. Enteroviral meningitis without pleocytosis in children. *Arch Dis Child.* 2012;97(10):874-8.

Figure 1: Flowchart of study population and details of diagnoses in the 'severe infection' subgroup.

Figure 2: Seasonal distribution per diagnosis

Legend: black = EV; dotted = HPeV; dark gray = severe infection; light gray = FUO.

SDC Legend:

SDC 1. Criteria for sepsis-like illness according to age.doc

SDC 2. Enterovirus serotyping details.doc

Table1: Clinical characteristics and laboratory indices of total study population

	Severe infections^ (n=8, 2%)	**EV or HPeV*** (EV, n=132, 37%) (HPeV, n=52, 15%)	**FUO*** (n=161, 46%)	**p-value**
Sex (males)	6/8 (75%)	116/184 (62%)	85/161 (52%)	0.063
Positive Medical History	1/8 (13%)	9/184 (5%)	10/161 (6%)	0.641
Prematurity	1/6 (17%)	6/165 (5%)	7/148 (5%)	1.000
Age <28 days	4/8 (50%)	101/184 (55%)*	62/161 (39%)*	0.003
Rash	2/8 (25%)	29/178 (16%)	23/154 (15%)	0.764
Body temperature (^{0}C)	38.7 (38.0-39.1)	38.7 (38.3-39.1)*	38.5 (38.1-38.9)*	0.001
Heart Frequency (/min)	164 (147-187)	172 (158-188)*	167 (150-180)*	0.007
Breathing frequency (/min)	37 (32-56)	50 (40-60)*	44 (35-52)*	0.001
Oxygen Saturation (%)	99 (97-100)	100 (98-100)	99 (98-100)	0.134
Capillary refill >2 sec (%)	3/7 (43%)	46/177 (26%)	32/155 (21%)	0.299
Abnormal behaviour	5/8 (63%)	140/182 (77%)*	102-161 (63%)*	0.006
Duration of illness before presentation	0.5 (0.1-2.5)	0.5 (0.5-1.0)	0.5 (0.5-1.5)	0.450
White blood cell count (x10^9/L)	14.0 (3.9-18.5)	7.7 (5.6-10.1)*	10.1 (8.0-14.3)*	0.000
Blood neutrophil count (x10^9/L)	8.6 (1.6-12.0)	3.6 (2.2-4.8)*	4.8 (2.8-7.0)*	0.000

C-Reactive Protein (mg/L)	22 (7-41)	6 (3-20)	6 (3-19)	0.964
Pleocytosis (%)	3/8 (38%)	51/152 (34%)	17/91 (19%)	0.013
CSF white blood cell count (x/3µL)	13 (5-359)	8 (2-58)*	3 (2-11)*	0.003
CSF glucose (mmol/L)	2.4 (1.0-3.1)	2.9 (2.6-3.3)	3.1 (2.8-3.3)	0.044
CSF protein (mg/L)	0.79 (0.55-1.58)	0.54 (0.41-0.72)	0.49 (0.34-0.68)	0.087

p-values <0.01 were considered to indicate statistical significance (subgroup analysis) Mann-Whitney-U tests were used for numerical data and Fisher's Exact tests for categorical data.

^ No statistically significant difference existed between the severe infection and other subgroups.

* p-values indicate a difference between the 'EV or HPeV' and 'FUO' groups.

Table 2: Comparison of EV and HPeV positive infants

	EV (n=132)	HPeV (n=52)	p-value
Sex (males)	84/132 (64%)	32/52 (62%)	0.866
Positive Medical History	7/132 (5%)	2/52 (4%)	1.000
Prematurity	5/126 (4%)	4/48 (8%)	0.263
Age <28 days	77/132 (58%)	24/52 (46%)	0.143
Rash	19/127 (15%)	10/51 (20%)	0.502
Body temperature (^{0}C)	38.6 (38.3-38.9)	38.8 (38.3-39.1)	0.223
Heart frequency (/min)	170 (156-185)	182 (161-195)	0.012
Breathing frequency (/min)	50 (40-60)	48 (40-59)	0.447
Oxygen Saturation (%)	100 (98-100)	100 (98-100)	0.469
Capillary refill >2 sec (%)	28/128 (22%)	18/49 (37%)	0.055
Abnormal behaviour	96/131 (73%)	44/51 (86%)	0.078
Duration of illness before presentation	0.5 (0.5-2.0)	0.5 (0.25-1.0)	0.013
White blood cell count (x10^9/L)	8.2 (6.6-10.8)	5.2 (4.0-8.1)	0.000
Blood neutrophil count (x10^9/L)	4.0 (2.7-5.1)	2.3 (1.6-3.6)	0.000
C-Reactive Protein (mg/L)	8 (3-24)	5 (2-9)	0.027
Pleocytosis (%)	48/113 (43%)	3/39 (8%)	0.000
CSF white blood cell count (x/3µL)	13 (3-151)	4 (2-9)	0.001
CSF glucose (mmol/L)	2.8 (2.6-3.2)	3.2 (2.9-3.4)	0.000
CSF protein (mg/L)	0.54 (0.43-0.74)	0.47 (0.38-0.64)	0.149

P-values <0.01 were considered to indicate statistical significance (subgroup analysis). Mann-Whitney-U tests tests were used for numerical data and Fisher's Exact tests for categorical data.

Figure 1

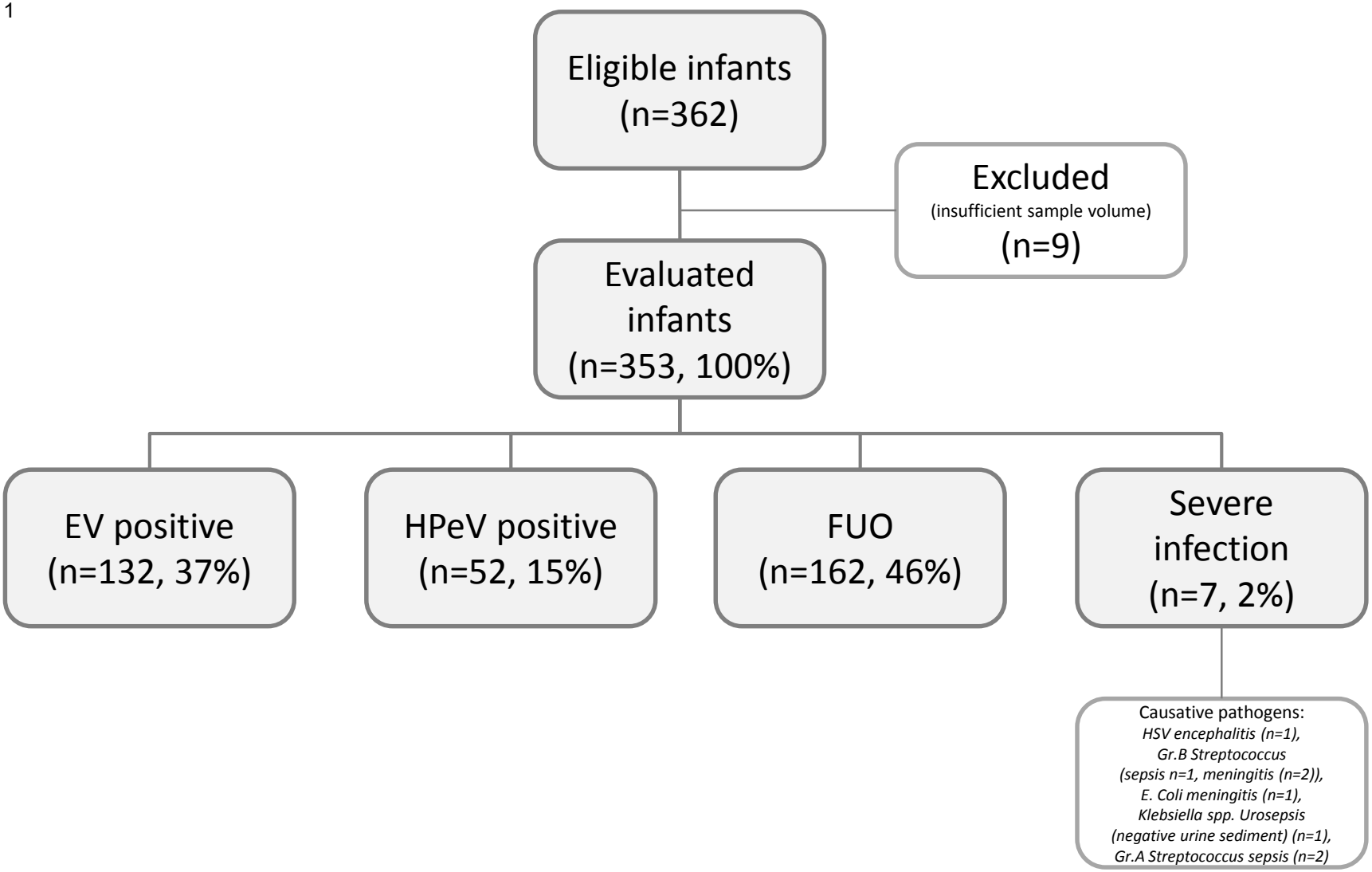
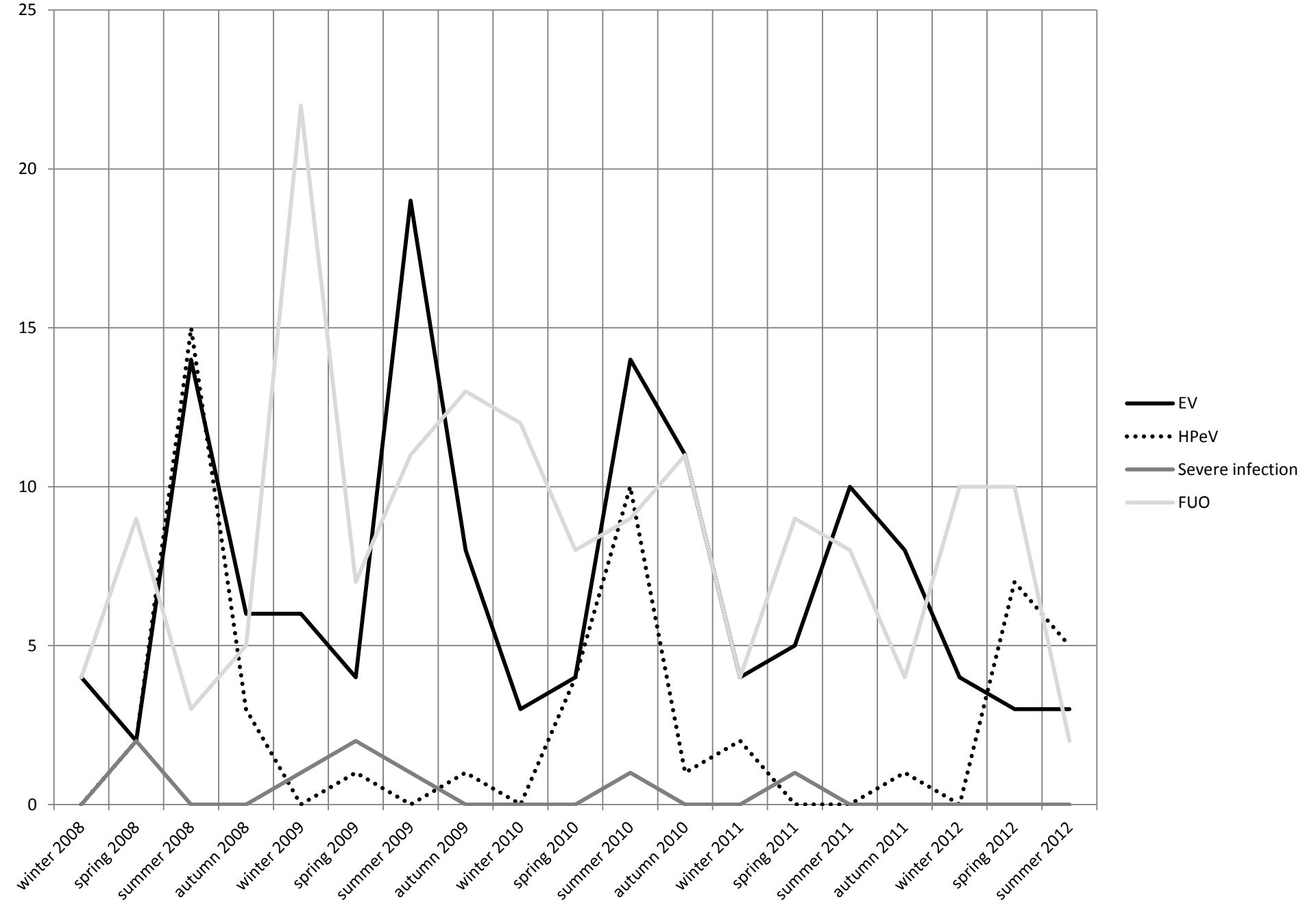

Eligible infants
(n=362)
Excluded
(insufficient sample volume)
(n=9)
Evaluated
infants
(n=353, 100%)
EV positive
(n=132, 37%)
HPeV positive
(n=52, 15%)
FUO
(n=162, 46%)
Severe
infection
(n=7, 2%)
Causative pathogens:
HSV encephalitis (n=1),
Gr.B Streptococcus
(sepsis n=1, meningitis (n=2)),
E. Coli meningitis (n=1),
Klebsiella spp. Urosepsis
(negative urine sediment) (n=1),
Gr.A Streptococcus sepsis (n=2)

Figure 2


25
20
15
10
5
0
winter 2008
spring 2008
summer 2008
autumn 2008
winter 2009
spring 2009
summer 2009
autumn 2009
winter 2010
spring 2010
summer 2010
autumn 2010
winter 2011
spring 2011
summer 2011
autumn 2011
winter 2012
spring 2012
summer 2012
EV
HPeV
Severe infection
FUO

SDC 1: Criteria for sepsis-like illness according to age*

Age at presentation	0-28 days	1-3 months
Clinical signs and symptoms	One or more: - toxic appearance - temp <36.0 °C or >38.0 °C - feeding problems - lethargy or agitation - tachypnea - tachycardia - capillary refill > 2 sec	One or more: - toxic appearance - temp <36.0°C or >39.0°C - fever >48 hours - lethargy or agitation - capillary refill >2 sec - bulging fontanel
Criteria for toxic appearance	Rochester Criteria[20]	Yale observation scale[21] > 10

* Local adaptation of national guidelines for management of children with fever without source (Dutch Association of Paediatrics, NvK).

SDC 2: Enterovirus serotyping details

Species	**Serotype**	**Number of Patients**	**Season/year of diagnosis**
Entero B	E-25	3	Winter, Spring, and Summer 2008
Entero B	E-7	1	Summer 2008
Entero B	CV-B4	1	Summer 2008
Entero B	CV-B1	2	Summer and Autumn 2008
Entero B	E-9	3	Summer and Autumn 2009
Entero B	CV-B5	3	Winter, Spring and Summer 2009
Entero B	E-6	2	Summer 2009
Entero B	E-11	2	Winter 2009 and Summer 2010
Entero B	CV-B2	2	Summer and Autumn 2010
Entero B	E-30	2	Summer 2010 and Winter 2011
Entero A	CV-A16	1	Autumn 2010
Entero B	E-18	1	Summer 2012