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## Human Parechoviruses in Infants with Systemic Infection

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**Shortened title:** Parechovirus-3 in systemic infections

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**ABSTRACT**

Human parechoviruses (HPeVs) are RNA viruses related to neonatal sepsis, meningoencephalitis and other infections in young children. Little clinical and epidemiological information is available on these viruses. HPeVs were sought in cerebrospinal fluid from 397 infants aged less than 12 months from whom a sample was obtained to exclude meningitis or encephalitis from 2006 to 2009. HPeV infections were also tested in stool samples from 271 children aged less than 3 years old with gastroenteritis from November 2008 to March 2009. HPeV detection was by real-time polymerase chain reaction assay (region 5'UTR), followed by genotyping (region VP3/VP1). HPeVs were detected in the cerebrospinal fluid of nine infants (2.3%), one aged 6 months and eight aged 14-55 days old. All were admitted to hospital for febrile syndrome with abrupt clinical deterioration and suspected systemic infection without clear laboratory signs of meningeal inflammation. The same virus was detected in all the available nasopharyngeal aspirates, stool and/or serum samples from each patient. At least eight of the nine cases were caused by HPeV3. HPeVs were detected in stool samples from 17 children (6.3%), the most prevalent types being types 1 and 3. In conclusion, HPeV infection is common in the Basque Country (Spain) and HPeV3 is a significant cause of hospital admission due to systemic infection in the first few months of life. In these patients, HPeVs should be investigated as part of routine tests for enterovirus.

**KEY WORDS:** Human parechovirus, systemic infection, febrile syndrome, meningitis

## INTRODUCTION

Human parechoviruses (HPeVs) are RNA viruses belonging to the *Picornaviridae* family and have been associated with distinct types of acute infection, mainly systemic, gastrointestinal, respiratory, and central nervous system (CNS) infections in infants and young children [Harvala and Simmonds, 2009]. Initially identified as enteroviruses because of their clinical and biological characteristics, in 1999 HPeVs were reassigned to the new *Parechovirus* genus on the basis of phylogenetic studies. The two oldest members of the genus, HPeV types 1 and 2 (HPeV1 and HPeV2), previously known as echovirus 22 and 23 respectively, were isolated in 1956 and have mainly been detected in children with gastrointestinal and respiratory infections, as well as in asymptomatic children [Stanway et al., 2000]. Since 2004, eight new types have been discovered and at least a further four are under investigation. Consequently, this genus appears to be highly diverse [[www.picornaviridae.com/parechovirus/hpev/hpev.htm](http://www.picornaviridae.com/parechovirus/hpev/hpev.htm); Kim Pham et al., 2010]. HPeV3 was described in Japan in 2004 and has been related to severe disease, especially sepsis-like illness, meningitis and encephalitis in the first few months of life, although this virus has also been detected in other types of infections [Ito et al., 2004; Harvala et al., 2009]. Knowledge of the pathogenicity, geographical distribution and other epidemiological features of these viruses is limited, since the tests required for their detection are not routinely undertaken in most clinical microbiology laboratories. The aims of the present study were to analyze the presence of HPeVs in the Basque Country (Spain) and their possible association with systemic infection in infants aged less than 12 months.

## MATERIAL AND METHODS

### Patient Groups and Clinical Samples

This study was performed in two groups of samples. The first group consisted of 424 samples from 397 infants aged less than 12 months (median 1.1 months) in whom cerebrospinal fluid (CSF) samples were taken between January 2006 and September 2009 to exclude CNS infection. These samples represented 90% of the patients of this age in whom CSF was extracted during the study period. The CSF or cDNA aliquot were stored at  $-80^{\circ}\text{C}$  and were studied retrospectively. From January 2009 diagnosis was made prospectively. These samples were taken for routine investigation of enterovirus.

The second group comprised 271 stool samples from a distinct group of children aged less than 3 years old with acute gastroenteritis (median age 13.8 months), obtained between November 2008 and March 2009. These samples were suspended in B199 medium (Sigma-Aldrich, St Louis MO, USA) and were refrigerated at  $4^{\circ}\text{C}$  for a maximum of 14 days until processing. To do this, the samples were pooled in groups of nine samples each, and samples from groups with a positive result were studied individually. The aim of the study of this second group was to obtain preliminary information on the presence of HPeVs and their distinct types in the Basque Country (Spain), since the gastrointestinal tract is believed to be one of the main sites of replication [Harvala and Simmonds, 2009].

### Molecular Methods

HPeV detection was by a nucleic acid amplification technique, with reverse transcription of viral RNA followed by polymerase chain reaction (RT-PCR). RNA

1 extraction was performed in the NucliSENS® Easy-Mag system (bio-Mérieux SA,  
2 Marcy l'Etoile, France) with an input volume of 200 µL of CSF and 400 µL of the other  
3 samples. Reverse transcription of RNA to cDNA was performed with M-MuLV reverse  
4 transcriptase (USB Corp., Cleveland, OH, USA), using random primers. PCR was  
5 performed in real time (Ligthcycler, Roche) by amplifying a fragment of the 5'UTR  
6 gene [van der Sanden et al., 2008]. Each PCR run included a positive control (a  
7 previously positive clinical sample, GenBank accession number GU477688) and a  
8 negative control (water control), which were treated identically to the clinical samples  
9 throughout. The presence of amplification inhibitors in the clinical samples was  
10 excluded through amplification of a fragment of the human  $\beta$ -globin gene, or the  
11 GAPDH gene from 2009 onwards. All procedures were carried out using routine  
12 precautions to avoid contamination. In patients with positive CSF, HPeVs were  
13 investigated in other samples related to the episode (nasopharyngeal aspirates, faeces  
14 and/or serum). For parechovirus genotyping, in the HPeV-positive samples, a nested  
15 RT-PCR was performed, which amplified a 300 bp fragment of the VP3/VP1 region  
16 [Harvala et al., 2008]. The amplicons obtained from both regions (5'UTR and  
17 VP3/VP1) were sequenced in an ABIPRISM 3100 Genetic Analyzer (Applied  
18 Biosystems, Foster City, CA, USA) and the sequences were analyzed in the Basic Local  
19 Alignment and Search Tool (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). A  
20 phylogenetic analysis was performed using the ClustalW2 and Mega4.1 programs.  
21 Detection of enterovirus RNA in CSF was performed with the cDNA sample by a real-  
22 time PCR method, based on amplification of the 5'UTR gene [Rotbart et al., 1994].

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## Nucleotide Sequence Accession Numbers

The gene sequences described in the present study were deposited in the GenBank database under accession numbers GU477688-GU477711 and GU574202-GU574203.

## RESULTS

### Human Parechoviruses in CSF

HPeVs were detected in CSF from nine infants aged less than 12 months old (2.3% [9/397]; range 14-185 days), this percentage being 3.0% in infants aged less than 2 months old (8/265) (Table I). In these age groups, the percentages of enterovirus detection were 16.1% (64/397) and 18.9% (50/265). HPeV strains were detected in all 4 years of the study, mainly (7/9) between June and November (Table II). The nine infants with HPeV in CSF had been discharged from hospital after birth and were readmitted due to HPeV infection. Eight of these infants were born at term ( $\geq 37$  weeks) and one was premature (week 34 of pregnancy). At admission to the pediatric emergency department, the infants showed febrile syndrome (temperature  $>38^{\circ}\text{C}$ ) accompanied by abrupt clinical deterioration and suspected infection (sepsis-like illness), with no clear laboratory signs of meningeal inflammation in CSF (low cellularity [1-12 leukocytes/ $\text{mm}^3$ ] and slightly elevated proteinorrachia in three patients with normal glucorrachia). In three patients no focus was found, five showed mild respiratory symptoms and three showed gastrointestinal symptoms. In all patients, concentrations of C reactive protein and procalcitonin in the acute phase were  $<10$  mg/dl and  $<0.5$  ng/ml, respectively, and the results of blood and urine cultures were



1 negative. Bacterial cultures and investigation of enterovirus in CSF were also negative.  
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3 Eight patients were treated with antibiotics for 4-14 days. In the nine infants, HPeVs  
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5 were also detected in all the samples available from other sites: in nasopharyngeal  
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7 aspirates in four (in one of these, the serum sample was also positive), in stool samples  
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9 and nasopharyngeal aspirates in three, and in stool samples only in two. Eight cases  
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11 were attributed to HPeV3 infection based on the amplicon sequence of the VP3/VP1  
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13 region in the CSF or other samples (Table II). The nucleotide sequence identity among  
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15 samples from the same patient was  $\geq 99\%$ , while that among the eight cases and the  
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17 prototype HPeV3 strain A308/99 (GenBank accession number AB084913) was 94-96%  
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19 (Fig. 1). The amplicon sequence of the 5'UTR region of the patient without  
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21 amplification of the VP3/VP1 region had 94% nucleotide identity with the prototype  
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23 HPeV3 strain A308/99.  
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### Human Parechoviruses in Stool Samples

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35 HPeVs were detected in stool samples from 17 children with gastroenteritis  
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37 (6.3% [17/271]; median age 210 days). Of these, the nested RT-PCR for genotyping  
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39 was positive in 14, corresponding to type 1 (n=5), type 3 (n=6) and type 4 (n=2) strains.  
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41 One strain (two isolates, Spa250229/08 and Spa254577/08) could not be allocated to  
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43 any HPeV type (nucleotide sequence similarity  $< 75\%$  with known types) (Fig. 1).  
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47 Another enteropathogenic agent, norovirus, was found only in a stool sample of one of  
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49 these 17 children.  
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### DISCUSSION

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57 Few studies have investigated the presence of HPeVs in CSF samples requested  
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59 for viral diagnosis. In the present study, HPeVs were found in 3.0% (8/264) of CSF  
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1 samples in infants aged less than 2 months under investigation for possible CNS  
2 infection, representing a 16% increase in diagnostic confirmation with respect to the 50  
3 samples positive for enterovirus. These data are similar to those reported in a large  
4 study performed in Edinburgh between 2006 and 2008, in which HPeV3 was detected  
5 in 2.9% of CSF samples sent for virological analysis in infants aged less than 2 months  
6 old (n=278) [Harvala et al., 2009]. Likewise, in Amsterdam, HPeVs were found in 4.6%  
7 of CSF samples from children investigated in 2004-2006, who were mainly aged less  
8 than 2 years old [Wolthers et al., 2008]. HPeVs also accounted for most of the isolates  
9 that could not be typed in a Dutch enterovirus surveillance program between 2000 and  
10 2007, representing 12.2% of the total number of viruses obtained, mostly from young  
11 children with systemic infections [van der Sanden et al., 2008]. Therefore, HPeVs can  
12 be a significant cause of severe illness in young children, especially in the first few  
13 months of life.

14         Eight of the nine HPeV strains detected in the present study in CSF samples  
15 were confirmed as type 3, and the ninth was probably also HPeV3, providing evidence  
16 that, among HPeVs, this type is the main agent of CNS infections, in line with recent  
17 observations [Boivin et al., 2005; Benschop et al., 2006; van der Sanden et al., 2008;  
18 Harvala et al., 2009]. In contrast, HPeV1, the most prevalent type in respiratory and  
19 stool samples, is rarely detected in CSF and is normally found in slightly older children,  
20 aged 3 to 24 months [Abed and Boivin, 2006; Benschop et al., 2006; van der Sanden et  
21 al., 2008; Harvala et al., 2009]. In the present study, HPeV1 was also detected in stool  
22 samples, but not in CSF. The reason for the differences observed between these types is  
23 unknown, although HPeV3 lacks the RGD motif involved in cell entrance of HPeV1  
24 and other viruses, which could translate into differences in tissue tropism and  
25 pathogenicity [van der Sanden et al., 2008]. Another hypothesis is that the differences

1 may be related to the lower prevalence of HPeV3 antibodies observed in adults, making  
2 passive transfer of antibodies to the newborn less frequent [Benschop et al., 2006;  
3 Harvala and Simmonds, 2009].

4       The nine patients with HPeV detection in CSF were admitted to hospital due to  
5 febrile syndrome and suspected severe acute infection (one patient in the pediatric  
6 intensive care unit), with a mean stay of 6.8 days, and eight were treated with  
7 antibiotics. Febrile syndrome with no apparent source of infection in the first few  
8 months of life is a common reason for consulting hospital emergency services, as well  
9 as for hospital admission for microbiological analysis and antibiotic therapy, since this  
10 syndrome may be caused by potentially life-threatening bacterial infections [Goldman et  
11 al., 2009]. However, the most frequent cause of these episodes are viral infections,  
12 mainly due to enterovirus, which are sometimes serious [Verboon-Maciolek et al.,  
13 2003; Jordán et al, 2009]. The results of this study and other recent observations support  
14 the causal role of HPeV3 in this type of episodes (febrile syndrome, sepsis-like illness)  
15 in the first few months of life [Boivin et al 2005; Benschop et al, 2006; Verboon-  
16 Maciolek et al, 2008a; Harvala et al., 2009; Levorson et al., 2009].

17       A notable finding in this series was the poor or absent cellular reaction and the  
18 frequent normal values of CSF protein concentrations despite HPeV detection in the  
19 CNS. This finding, recently reported in another study [Verboon-Maciolek et al., 2008a],  
20 is also observed in enterovirus infections [Ritichier et al., 2005], and could be due to the  
21 systemic nature of some HPeV3 infections rather than to localization of the infection in  
22 the CNS. HPeV3 detection in the samples investigated in each patient in this series  
23 (nasopharyngeal aspirate, stools, serum) supports the disseminated nature of these  
24 infections [Harvala et al., 2009]. Although most of the HPeV3 isolates in CSF reported  
25 to date correspond to children with sepsis-like illness [Wolthers et al., 2008; Harvala et

1 al., 2009], as in our series, cases of neonatal meningoencephalitis with white matter  
2 injury have also been reported, sometimes with neurological sequelae and almost  
3 always without pleocytosis or biochemical alterations in CSF [Verboon-Maciolek et al.,  
4 2008b].

5 In contrast with previous findings, the present series showed no predominance of  
6 cases among males [Benschop et al., 2006] or a biannual circulation pattern for HPeV3  
7 (paired years), as reported in Holland and Scotland [van der Sanden et al., 2008;  
8 Benschop et al., 2008a; Harvala et al., 2009]. The present data suggest that viral  
9 circulation was higher in autumn 2006 and summer 2009, an observation that is  
10 coherent with variations in the annual incidence and indicates the possibility of  
11 geographical differences in the circulation of this virus. However, a preferential  
12 distribution in the warmest months (between late spring and autumn) was found, as  
13 reported by other authors [van der Sanden et al., 2008; Wolthers et al., 2008] and as  
14 usually occurs with enterovirus [Khetsuriani et al., 2006].

15 The non-coding 5' UTR region of HPeV shows preserved areas in the distinct  
16 types of HPeVs and consequently this region is useful for *Parechovirus* genus detection.  
17 However, because antigenicity lies in the VP region, this region is chosen for  
18 genotyping, even though VP is sometimes difficult to amplify because of its greater  
19 variability, especially in clinical samples such as CSF, which normally have a low viral  
20 load [Benschop et al., 2008a]. As with other genera of the *Picornaviridae* family,  
21 recombination events resulting in the exchange of non-coding regions among HPeV are  
22 frequent [Benschop et al., 2008b]. In the present series, among the nine HPeV3 strains  
23 causing systemic infection, eight (88.9%) could be genotyped based on direct  
24 sequencing of the VP3/VP1 region, since other samples obtained simultaneously with  
25 the CSF sample were available. In the remaining strain, the similarity observed in the

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3 1 5'UTR region with the A308/99 reference strain and the fact that HPeV3 is less variable  
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5 2 and less frequently recombines than other HPeV types [Benschop et al., 2008b]  
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8 3 suggests that it probably also belongs to type 3.  
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10 4 Clinical microbiology laboratories do not test routinely for HPeVs in biological  
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12 5 samples due to lack of awareness of the role of these viruses in human disease and  
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15 6 technical difficulties. However, current real-time PCR methods allow rapid etiological  
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17 7 diagnosis of HPeV infections, which could aid the management of children with febrile  
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19 8 syndromes, thus avoiding unnecessary treatments and shortening length of hospital stay.  
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21 9 The results of the present study, the first to demonstrate the presence of HPeV3 in  
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23 10 southern Europe, indicate that investigation of HPeVs in CSF should be undertaken as  
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25 11 part of routine tests for enterovirus in young infants admitted to hospital for sepsis-like  
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27 12 syndrome and/or meningoencephalitis.  
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34 14 With regard to the issue of Ethical Committee approval and informed consent,  
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36 15 none of the human samples were obtained for research purposes. The study involves  
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38 16 only samples taken for routine diagnosis of meningeal, acute respiratory, or acute  
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40 17 enteric infections. For this reason, informed consent from the parents or guardians was  
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42 18 not obtained (most of the study was retrospective). Nevertheless, a statement by the  
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44 19 Ethical Committee for the study of new potentially pathogenic viruses in samples taken  
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46 20 for routine diagnosis was obtained in December 2007.  
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#### 55 23 **DECLARATION OF INTEREST**

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57 24 None  
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**Table I.** Distribution by age and detection of parechoviruses and enteroviruses in infants aged less than 12 months in whom a cerebrospinal fluid sample was taken to exclude central nervous system infection

Age	N° investigated	Parechovirus		Enterovirus	
		N°	(%)	N°	(%)
<14 days	121	0	-	13	(10.7)
14-30 days	69	4	(5.8)	21	(30.4)
1 month	75	4	(5.3)	16	(21.3)
2 months	25	0	-	6	(24.0)
3 months	18	0	-	1	(5.6)
4 months	17	0	-	1	(5.9)
5 months	15	0	-	0	-
6-11 months	57	1	(1.8)	6	(10.5)
Total	397	9	(2.3)	64	(16.1)

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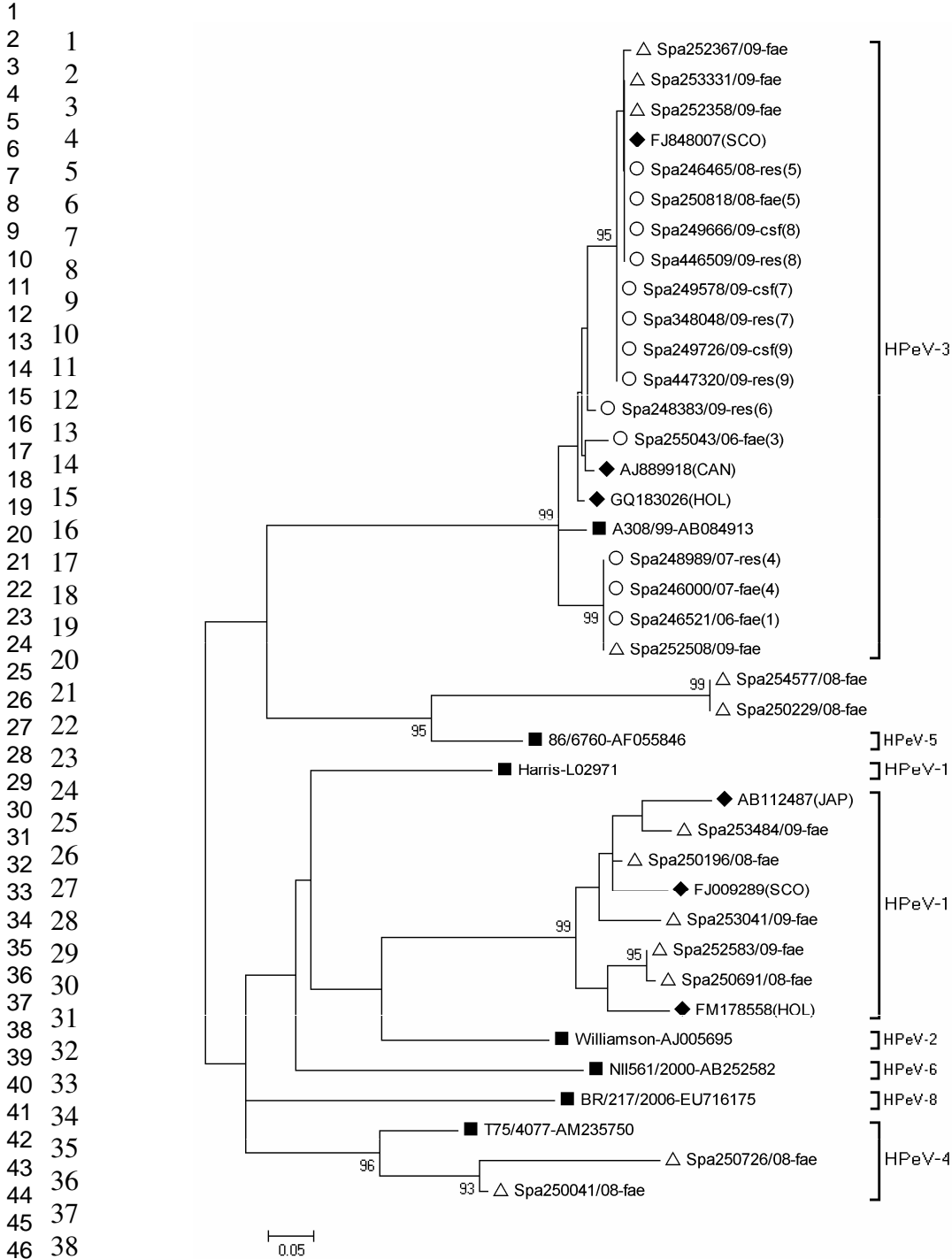
1 **Table II.** Main clinical characteristics and laboratory data of nine infants with systemic infection by human parechovirus type 3

Case N° - year (month)	Sex and age (days)	Main diagnosis	Other diagnoses	Coinfections	Antibiotic therapy (days)	Hospital stay (days)	CSF (leukocytes/mm <sup>3</sup> and proteins mg/dL)	CRP (mg/dL) and PCT (ng/mL)	Positive samples (amplified region )	
									(5'UTR)	(VP3/VP1)
1 – 2006 (October)	M (45)	Febrile syndrome	Irritability, skin rash (face and trunk ), URTS	Rhinovirus in NPA	CTX+AM (5) and CFX (5)	5	1 and 43	0.3 and <0.5	CSF, NPA, FAE	FAE
2 – 2006 (October)	F (14)	Febrile syndrome	Irritability	-	CTX+AM (9)	8	8 and 53	2.6 and 0.5	CSF, FAE	-
3 – 2006 (November)	M (55)	Febrile syndrome	Irritability	-	CTX+AM (4)	5	2 and 22	7 and <0.5	CSF, FAE	FAE
4 – 2007 (June)	M (43)	Febrile syndrome	URTS	-	CTX+AM (5)	5	5 and 35	1.8 and <0.5	CSF, NPA, FAE	NPA, FAE
5 – 2008 (December)	F (16)	Febrile syndrome	Irritability, URTS, vomiting	-	CTX+AM (4)	4	10 and 42	9.6 and 0.12	CSF, NPA, FAE	NPA, FAE

6 – 2009 (February)	M (185)	Febrile syndrome	Skin rash, URTS, vomiting	Bocavirus in NPA	-	1 <sup>a</sup>	6 and 14	1.1 and 0.2	CSF, NPA	NPA
7 – 2009 (June)	F (14)	Febrile syndrome	-	-	GM+AM (7)	7	2 and 42	4.3 and 0.2	CSF, NPA	CSF, NPA
8 – 2009 (July)	F (33)	Febrile syndrome	Irritability, sepsis, skin rash (disseminated), ARI, microcephaly	-	VA+PTZ (14)	20 (9 in PICU)	12 and 122	6.23 and 0.1	CSF, NPA, serum	CSF, NPA
9 – 2009 (August)	M (19)	Febrile syndrome	Irritability, skin rash (sole and dorsum of foot), diarrhea	-	CTX+AM (4)	5	1 and 53	0.6 and 0.4	CSF, NPA	CSF, NPA

<sup>a</sup> After three medical consultations, this patient remained under observation in the emergency department for 24 hours.

M, male; F, female; URTS, upper respiratory tract symptoms; ARI, acute respiratory insufficiency; NPA, nasopharyngeal aspirate; CTX, cefotaxime; AM, ampicillin; CFX, cefixime; GM, gentamicin; VA, vancomycin; PTZ, piperacillin-tazobactam; PICU, pediatric intensive care unit; CRP, C reactive protein; PCT, procalcitonin; FAE, faeces.



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**Figure 1.** Phylogenetic analysis of 26 nucleotide sequences of human parechovirus (HPeV) (region VP3/VP1, positions 2159-2458) of isolates of cerebrospinal fluid (csf), faeces (fae) and nasopharyngeal aspirate (res), compared with reference strains (black squared: GenBank name and number) and other contemporaneous strains (black rhombus: GenBank number and country). Samples from infants with febrile syndrome are represented by a white circle (case number) and those from children with gastroenteritis by a

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2 1 white triangle. The tree was constructed through the neighbor-joining method with 1000 bootstrap  
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4 2 replications and shows bootstrap values higher than 70 in the branches. One strain (two isolates) could not be  
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6 3 attributed to any known HPeV type.  
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