A novel enterovirus and parechovirus multiplex one-step real-time PCR-validation and clinical experience

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ABSTRACT

As the number of new enteroviruses and human parechoviruses seems ever growing, the necessity for updated diagnostics is relevant. We have updated an enterovirus assay and combined it with a previously published assay for human parechovirus resulting in a multiplex one-step RT-PCR assay. The multiplex assay was validated by analysing the sensitivity and specificity of the assay compared to the respective monoplex assays, and a good concordance was found. Furthermore, the enterovirus assay was able to detect 42 reference strains from all 4 species, and an additional 9 genotypes during panel testing and routine usage.

During 15 months of routine use, from October 2008 to December 2009, we received and analysed 2187 samples (stool samples, cerebrospinal fluids, blood samples, respiratory samples and autopsy samples) were tested, from 1546 patients and detected enteroviruses and parechoviruses in 171 (8%) and 66 (3%) of the samples, respectively.

180 of the positive samples could be genotyped by PCR and sequencing and the most common genotypes found were human parechovirus type 3, echovirus 9, enterovirus 71, Coxsackievirus A16, and echovirus 25. During 2009 in Denmark, both enterovirus and human parechovirus type 3 had a similar seasonal pattern with a peak during the summer and autumn. Human parechovirus type 3 was almost invariably found in children less than 4 months of age.

In conclusion, a multiplex assay was developed allowing simultaneous detection of 2 viruses, which can cause similar clinical symptoms.

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1. Introduction

Many new and clinically important picornaviruses have been discovered during the last few years due to advances in metagenomics and other molecular biological approaches. Both the enterovirus (EV) genus and the human parechovirus (HPeV) species of the parechovirus genus have been expanded with a number of new genotypes (Benschop et al., 2008; Oberste et al., 2007). The EV genus now contains more than 100 genotypes, and the HPeV species contains 16 genotypes (www.picornaviridae.com). As the number of genotypes increases, the diagnostic methods need to be validated and updated if necessary in order to ensure a high sensitivity and specificity. From a clinical perspective, both the EVs and the HPeVs are capable of causing meningitis and septicaemia, as well as more trivial illnesses like rhinitis and unspecific

fever without other symptoms, and diseases with these viruses have been described as indistinguishable in neonates (Verboon-Maciolek et al., 2008). Therefore, it seemed prudent to develop a diagnostic test that included both EV and HPeV. Consequently, the EV assay in use at Statens Serum Institut (SSI) was updated and combined with a previously published HPeV assay (Baumgarte et al., 2008), thereby creating a multiplex one-step real-time RT-PCR (reverse transcriptase polymerase chain reaction) assay for simultaneous detection of EV and HPeV.

In this article, the initial validation of this EV and HPeV multiplex assay, and the results of the first 15 months of routine diagnostic usage are presented.

2. Study design

2.1. Multiplex real-time RT-PCR

2.1.1. Multiplex assay design

The EV assay was designed by aligning available sequences from a highly conserved area of the 5′ untranslated region

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from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the ClustalW software (http://www.clustal.org/). Primers (EV08-1 GGTGCGAAGAGTCTATTGAGC; EV08-2 CACCCAAAG-TAGTCGGTTCC) were designed using the primer3 software (http://frodo.wi.mit.edu/primer3/), and the probe (Fam-CCGGCCCCTGAATG) was designed as a MGB (minor groove binder) probe using the Primer Express software (Applied Biosystems, Life Technologies, Naerum, Denmark). This new EV assay was combined with an already published HPeV assay (Baumgarte et al., 2008) resulting in a multiplex assay. The HPeV specific probe was labelled with a Hex-dye. The HPeV assay was not validated beyond the multiplex combination as the assay had already been published.

2.1.2. Reagents, amplification and detection

For both multi- and monoplex amplification, 5 μl of extracted nucleic acids were used as a template (total reaction volume 25 μl) using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). The reaction mixtures contained 1 μM of each primer and 0.2 μM of each probe. The Mx3005P real-time thermocycler (Agilent Technologies, Hoersholm, Denmark) was used for amplification and detection with the following settings: 50 °C for 20 min, 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s and 55 °C for 1 min.

2.1.3. Specificity testing

In order to test the specificity of the EV assay, known non-EV viruses were tested as well as the 2007 Quality Control for Molecular Diagnostics (QCMD) rhinovirus proficiency panel (containing rhinovirus types 16, 72, and 90) and samples positive for HPeV types 2 and 3. Furthermore, the following viruses were tested in the multiplex assay: Herpes simplex types 1 and 2, varicella zoster virus, adenovirus, respiratory syncytial virus A and B, parainfluenza virus 1–3, influenza virus A and B, human metapneumovirus, rhinovirus, coronavirus OC43, 229E, NL63, norovirus, rotavirus, sapovirus, and astrovirus. These virus strains were used as positive controls in other accredited diagnostic tests in the SSI laboratory.

In order to ensure that the EV assay could detect the desired viruses the assay was evaluated in three different ways. First, 42 EV reference strains, supplied by the WHO collaborative centre for virus reference and research were tested (supplementary table); second, the primer and probe sequences were evaluated in silico against the nucleotide sequences of all known EV genotypes (supplementary table). Finally, two QCMD enterovirus panels from 2007 and 2008 were tested. In addition, the 2009 and 2010 QCMD enterovirus/parechovirus panels, and the 2009 and 2010 WHO poliovirus panels have also been evaluated.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2013.06.038.

2.1.4. Sensitivity testing of the multiplex assay

The sensitivity of the assay was measured in three different ways: The sensitivity of the multiplex assay and the monoplex assays were compared by analysing 10-fold serial dilutions of three EV positive patient samples (echovirus 11, echovirus 7 and enterovirus 71) and two HPeV positive patient samples (HPeV 1 and 3). The assay was tested against stock dilutions from the following quality control panels – 2007 and 2008 QCMD enterovirus panels, 2009 and 2010 QCMD enterovirus/parechovirus panels, and 2009 and 2010 WHO poliovirus panels.

The limit of detection was determined for the enterovirus PCR by testing 10 replicates of a 10-fold serial dilution of a Sabin type 2 stock (WHO reference virus) with a viral titre of 5.6.

2.2. Clinical specimens

2.2.1. Sample material

In the 15-month period from October 2008 until December 2009, a total of 2187 samples from 1546 patients were submitted to SSI for EV diagnostics and analysed in the multiplex assay for EV and HPeV. The types of samples analysed were: stool samples ($n = 495$), spinal fluids ($n = 597$), serum/plasma ($n = 383$), throat swabs ($n = 152$), broncheoalveolar lavage ($n = 128$), autopsy biopsies ($n = 135$), and samples from the upper respiratory tract ($n = 40$). For 35 patients with positive virus findings more than one sample was submitted during the same infection episode (within 21 days). Two patients had more than one infection episode, with different viruses detected in each episode.

2.2.2. Sample preparation

Only stool samples and biopsy samples required special preparation prior to nucleic acid extraction. Stool samples were prepared as a 10% (weight/volume) suspension in minimal essential medium and centrifuged at $3500 \times g$ for 30 min. When this preparation method was introduced 10 years ago, inhibition was seen in <0.5% of the samples. Therefore no internal control was used. Biopsy samples were suspended in Lysis/Binding Buffer from the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany), followed by homogenisation.

2.2.3. Nucleic acid isolation

Nucleic acids were extracted from 200 μl sample material. All sample types, except spinal fluid samples, were processed using the MagNa Pure LC Total Nucleic Acid Isolation Kit on the MagNa Pure LC or MagNa Pure 96 (Roche Diagnostics) instruments according to the manufacturer's specifications. Nucleic acids from spinal fluid samples were isolated using the QIAamp DNA Blood Mini Kit on the QIAcube instrument (QIAGEN) following the manufacturer's specifications.

2.3. Genotyping

2.3.1. Enterovirus genotyping PCRs

Extracted RNA was used as a template for two separate PCR's amplifying part of the VP1 and VP2 genes, respectively.

For the VP1 amplification, cDNA was synthesised with primers AN32, AN33, AN34, AN35 (Nix et al., 2006), followed by a nested PCR using primers 224 and 292 (Nix et al., 2006) for the first round, and primers AN88 and AN89 (Nix et al., 2006) for the second round in a model PTC-225 thermo cycler (MJ Research/Bio-Rad, Copenhagen, Denmark). cDNA was synthesised by denaturing the samples at 97 °C for 5 min, cooling on ice before adding RT-mix, incubating at 37 °C for 60 min, 95 °C for 5 min, and cooling at 4 °C for 30 min prior to PCR set-up. The VP1 first and second rounds of amplifications were carried out according to the published protocol (Nix et al., 2006).

The VP2 region was amplified in a semi-nested PCR. cDNA synthesis and the first round of PCR were carried out using a OneStep RT-PCR kit (QIAGEN) and primers AM11, AM12, AM31, and AM32 (Nasri et al., 2007), and the second round of amplification was carried out using primers AM21, AM22, AM31, and AM32 (Nasri et al., 2007). OneStep thermocycler conditions were 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 48 °C for 45 s, 72 °C for 45 s, and a final elongation step at 72 °C for 5 min. The second round of amplification was carried out using the published thermocycling programme (Nasri et al., 2007).

2.3.2. HPeV genotyping PCR

Extracted RNA was used as a template for a nested PCR, which amplified part of the VP3/VP1 gene area. The primers used for

genotyping (Harv1-F, Harv1-R, Harv2-F, and Harv2-R) have been previously published by Harvala et al. (2008). For the first round of amplification, the Harv1-F and Harv1-R primers were used together with the QIAGEN Onestep RT-PCR kit and RNAse inhibitor using the same thermocycler and the same conditions as the corresponding step of the EV VP2 genotyping. For the second round of amplification, the Harv2-F and Harv2-R primers were used together with the AmpliTAQ polymerase (Invitrogen, Naerum, Denmark) on the same cycler at the following conditions: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s, and a final elongation step at 72 °C for 5 min.

2.3.3. Genotyping by sequencing

Prior to sequencing, PCR products were purified using the High Pure PCR purification kit (Roche Diagnostics). Purified PCR products were sequenced in both directions by using primers AN88 and AN89 (EV VP1), AM21, AM22, AM31, and AM32 (EV VP2), and Harv2-F and Harv2-R (HPeV VP3/VP1), using BigDye v1.1 chemistry, and an automated ABI-377 DNA sequencer (Applied Biosystems, Darmstadt, Germany). The lengths of the EV sequences were 314 bp (VP1) and 368 bp (VP2), respectively. The length of the HPeV sequence was 255 bp (VP3/VP1). Sequences were assembled in BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium), and genotypes were assigned either by BLAST analyses against reference sequences in the internal database, or against all published sequences in GenBank (http://blast.ncbi.nlm.nih.gov/).

3. Results

3.1. Multiplex real-time RT-PCR

3.1.1. Limit of detection

The sensitivity of the enterovirus assay, both in single and multiplex format, was determined using a 10-fold serial dilution of Sabin type 2 strain to be 30 virus particles per ml specimen (detected in 95% of tests) corresponding to a dilution of 10^{-4}. Since the primary evaluation of the PCR, the 2009 and 2010 QCMD enterovirus/parechovirus panels and the 2009 and 2010 WHO poliovirus panels have also been tested in the multiplex assay. The multiplex assay could detect all EV positive samples in the 2010 QCMD panel (10^{-7} dilution of CA9, E11, E30 and EV71; 10^{-6} dilution of CB3, 10^{-5} dilution of CA9, E11, E30, and EV71). In the 2009 panel, the new assay detected all (10^{-7} dilution of CB3; 10^{-6} dilution of CA16, E16, and P3; 10^{-5} dilution of CB3, E11, and EV71) but the most diluted preparations of CB3 (10^{-8}) and EV71 (10^{-7}) tested positive by 27% and 41% of all other participants, respectively. In the parechovirus part of the 2010 QCMD panel, HPeV type 3 was detected in the 10^{-4}, but not the 10^{-6} dilution. The latter dilution, categorised by QCMD as infrequently detected, had been detected by 63% of all other participants. In the 2009 QCMD panel, none of the 2 HPeV type 3 positive samples (10^{-5} and 10^{-7}) were found positive.

3.1.2. Comparison of sensitivity between the multiplex assay and the two monoplex tests

As shown in Fig. 1 the sensitivity of the multiplex was equal to the monoplex analyses, as the 10 fold serial dilutions of the patient samples tested had the same CT-values (cycle threshold) in the multiplex assay as in the monoplex assay. Furthermore, no difference was observed in the CT-value of the endpoint dilutions of the EV monoplex assay and the HPeV monoplex assay in comparison to the multiplex assay.

3.1.3. Specificity testing

No cross-reactivity of the EV assay was seen against the 2007 rhinovirus QCMD panel or against parechovirus type 2 and 3.
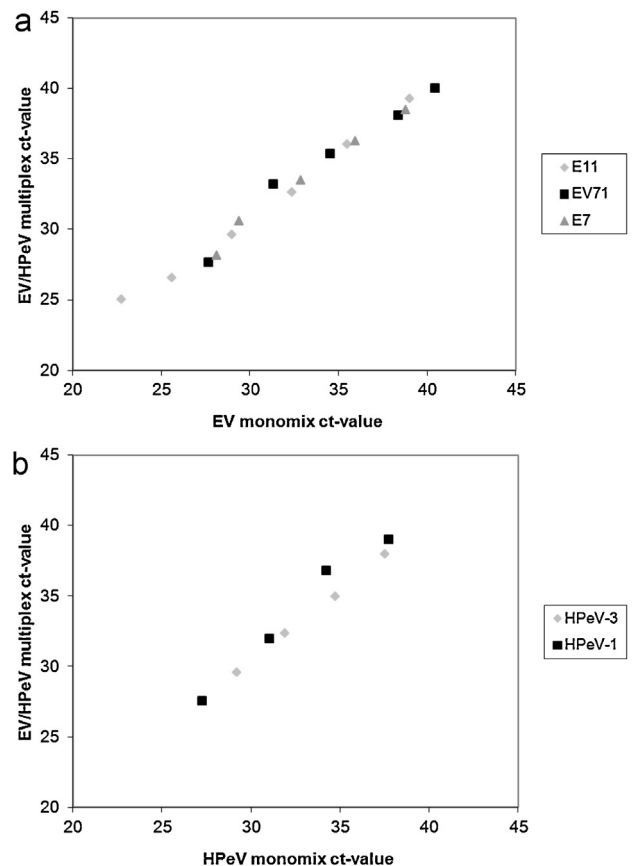

Fig. 1. Comparison of the sensibility of the HPeV and EV monomix assays against the multiplex assay by determining the CT-values of serial dilutions of two stool samples positive for HPeV (a): H1238 (echovirus 11, dilutions 10^0–10^{-5}), F6531 (enterovirus 71, dilutions 10^0–10^{-4}) and W16504 (echovirus 7, dilutions 10^0–10^{-4}) (b): T809 (HPeV type 3, dilutions 10^0–10^{-3}) and M33290 (type 1, dilutions 10^0–10^{-3}), and three stool samples positive for EV.

Furthermore, no cross-reactivity of the EV/HPeV multiplex assay was seen against any of the other 20 viruses tested.

The EV assay could detect all 42 reference strains from all 4 species as well as another 5 strains in the 2007, 2008 QCMD enterovirus panels (see supplementary table). Furthermore, the nucleotide sequences of the primers and probe were evaluated against all known prototype strains showing acceptable alignment results (see supplementary table), meaning that only four (4%) genotypes (E-12, PV-1, EV-104, and EV-108) had more than 2 substitutions against the forward primer. There were substitutions against the probe and the reverse primer in only another four (4%) genotypes (CV-A5, CV-B4, E-18, and EV-104). During the 15-month study period another 4 EV genotypes were detected among the EV positive samples, resulting in a total of 51 different EV genotypes identified. During the same period, HPeV genotypes 1, 3, and 6 were detected among the diagnostic samples.

3.2. Analysis of clinical specimens

3.2.1. Sample distribution

Of a total of 2187 samples received, during the 15-month study period, 171 (8%) and 66 (3%) were positive for EV and HPeV, respectively. The highest proportion of positive virus findings was seen in stool samples and spinal fluids, where EV and HPeV were detected in 14% and 8% of stool samples, and in 5% and 2% of spinal fluid specimens, respectively (Fig. 2). EV could also be detected in blood samples, throat swabs, autopsy material, BALs and upper respiratory samples (URS), whereas the only additional sample material
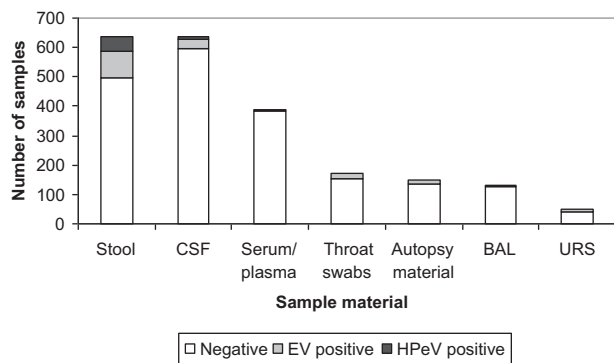
Fig. 2. Number of samples tested and number of EV and HPeV positive findings in relation to sample material in all 2187 samples received during the 15-month study period. CSF = cerebrospinal fluid; BAL = broncheoalveolar lavage; and URS = upper respiratory sample.

positive for parechovirus was biopsies (autopsy material from the gastrointestinal tract), and URS. Neither EV nor HPeV was found in urine, pleural fluid, amniotic fluid, or ascites fluid.

The age distribution of the patients sampled, and the proportion of positive virus findings can be seen in Fig. 3. Primarily children below 11 years of age were sampled (42% and 24% were <11 years and <1 year of age, respectively), and the majority of enterovirus positive samples, 85/118 (72%), was found in this group. Among HPeV positive samples an even higher proportion, 48/55 (87%), was found among children <1 year of age. 41 of the children were infected with HPeV type 3, of these 39 (95%) were less than 4 months of age. EV infections were detected in all age groups except among patients >81 years of age (n = 24).

The seasonal distribution of positive virus findings of EV and HPeV can be seen in Fig. 4. The occurrence of both viruses peaked during the summer and autumn of the study period.

3.2.2. Genotype distribution

The genotype could be established in 134 of the 171 EV positive samples (95 of 120 patients), and in 46 of the 66 HPeV positive samples (46 of 55 patients). The genotype distribution is presented in Table 1, showing that HPeV type 3 was the most common single genotype, followed by a range of different EV genotypes, with echovirus 9, enterovirus 71, Coxsackievirus A16, and echovirus 25 being the most prevalent. A total of 21 different EV genotypes were identified – 4 of these – CB6, E9, E21, and EV68 – were not among
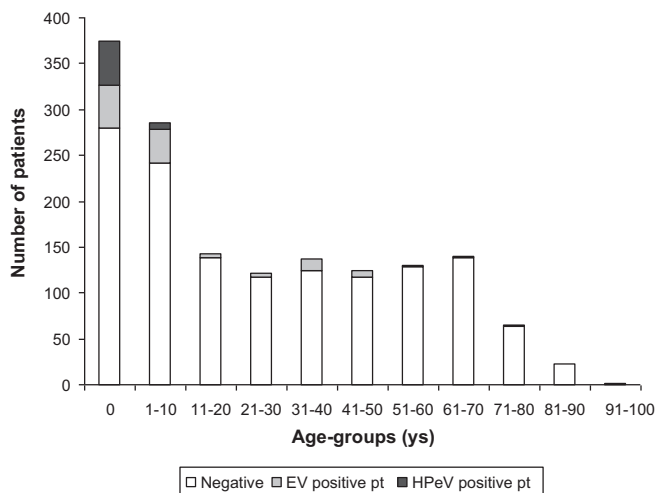

Fig. 3. Number of patients and number of EV and HPeV positive findings in relation to the age of all the 1546 patients sampled. Only one sample per patient is included.
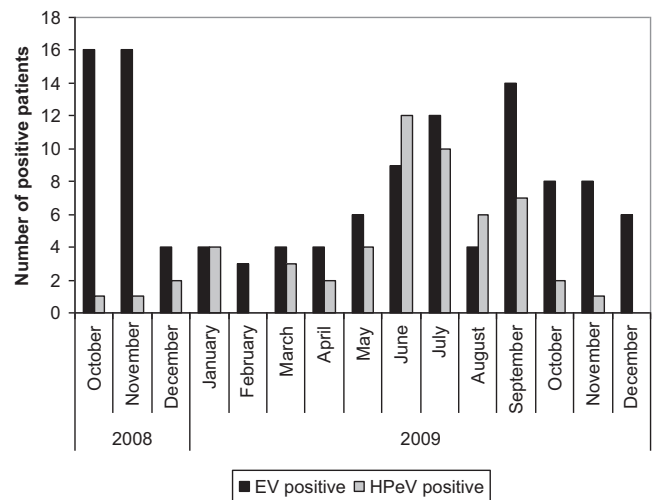

Fig. 4. Seasonal distribution of EV and HPeV positive findings of all 171 EV positive patients and all 66 HPeV positive patients during the 15 months period.

Table 1
EV and HPeV genotype distribution of positive patients among the 134 EV patients and 46 HPeV patients with successful genotyping. CA = coxsackievirus A, CB = coxsackievirus B, E = echovirus, and EV = enterovirus.

Species	Genotype	Number of positive patients
EV-A	CA2	3
	CA4	3
	CA5	1
	CA6	2
	CA16	11
	EV71	13
EV-B	CA9	5
	CB1	1
	CB2	2
	CB3	5
	CB4	4
	CB5	4
	CB6	1
	E2	1
	E3	1
	E5	1
	E6	3
	E9	14
	E11	2
	E25	9
	E30	7
EV-C	CA19	1
EV-D	EV68	1
HPeV	Type 1	1
	Type 3	41
	Type 6	4

the 47 EV genotypes tested in the initial validation of the test. Only 12 different EV genotypes were found in the cerebrospinal fluids.

4. Discussion

In this article, a multiplex assay capable of detecting enteroviruses from all four human species (A–D), as well as parechoviruses, is presented. The use of multiplex assays to detect more than one pathogen in each reaction is an improvement in the diagnostic laboratory, as it decreases both work load and costs. However, it is important that the sensitivity is as high in the multiplex format as in the monoplex tests and that the sensitivity of the multiplex assay presented is as high as for the corresponding monoplex assays.

Creating a multiplex assay without losing sensitivity is not always easy, and sometimes a drop in sensitivity is acceptable depending on the expected usage of the assay – for example, when analysing sample materials with an expected high concentration of virus such as stool samples (Pham et al., 2010). However, when analysing specimens with a suspected low concentration of virus, such as cerebrospinal fluid samples, a drop in sensitivity should not be tolerated (Bennett et al., 2011). A contemporary study by Bennett et al. (2011) has also described an enterovirus parechovirus multiplex PCR assay. Their study used two published EV and HPeV assays in multiplex format comparing two commercially available enzyme kits. In this study a new enterovirus assay has been developed and combined with an established parechovirus assay, and the functionality of this multiplex assay has been demonstrated. A continuous revalidation and update of existing EV assays is essential as the number of viruses has been increasing considerably over the last few years. Both the multiplex assay by Bennett et al. (2011) and the assay presented in this paper have been evaluated with the 2010 EV/HPeV QCMD panel. The same high sensitivity for EV was found with both tests, whereas the method presented by Bennett et al. seems to be slightly more sensitive for HPeV-3 as their method alone could detect the highest dilution (10^{-6}) of HPeV-3.

Furthermore, the results from 15 months of routine usage of the EV-HPeV multiplex assay have been presented. During this period a seasonal peak was observed during the summer and autumn months for both EV and HPeV infections. However, the biannual cycle (Harvala et al., 2011) of HPeV-3 described in Scotland has either changed or follows a different pattern in Denmark, as 2009 was a year with a high HPeV-3 incidence in Denmark but with no cases detected in Scotland. From a clinical perspective, a study (Verboon-Maciolek et al., 2008) has shown that HPeV-3 is primarily a neonatal infection capable of causing severe sepsis-like illness resembling infections with EV, which corresponds very well to the findings in this study.

The single most prevalent genotype found was HPeV-3, which is in line with the recent findings of Harvala et al. in Scotland. The most common EV genotypes in Denmark during the study period were echovirus 9 followed by EV 71, Coxsackievirus A16 and echovirus 25. In the study by Harvala et al. (2011) only one of these genotypes, echovirus 9, was among the four most common genotypes in Scotland in the same time period. They reported 10 different EV genotypes, in contrast to the 26 different EV genotypes detected in this study. However, the study by Harvala et al. (2011) included

only cerebrospinal fluid samples. Looking solely at spinal fluid samples in the present study, 12 different genotypes were found, which is in line with the results in the study by Harvala et al. (2011). Thus, a likely reason for the higher number of EV genotypes detected in the present study is that many different types of sample materials, mainly stool samples, were tested.

In conclusion, an EV and HPeV multiplex assay has been created that has performed well both during validation and during the first 15 months of routine diagnostic usage. The multiplex assay for EV and HPeV has been especially useful for diagnostics in children.

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