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Human Parechovirus Type 1, 3, 4, 5, and 6 Detection in Picornavirus Cultures▽

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Picornavirus cultures that could not be typed in neutralization assays were analyzed by VP1 reverse transcription-PCR (RT-PCR) and a virus discovery tool (VIDISCA). Human parechoviruses (HPeVs) were frequently identified, among which were the uncommon isolates HPeV-4, HPeV-5, and HPeV-6. The HPeV-5 isolate could be amplified only by VIDISCA and not by VP1 RT-PCR.

Enteroviruses and human parechoviruses (HPeVs) belong to the family *Picornaviridae*. Infections can cause relatively mild symptoms such as diarrhea but also life-threatening diseases such as encephalitis (14). HPeVs were formerly known as echovirus 22 (HPeV-1) and echovirus 23 (HPeV-2; two strains) but were reclassified by phylogenetic analysis as a new genus (6, 8, 10, 13, 15). Additional genotypes have been identified recently (1–3, 7, 17), and one of the two strains of HPeV-2 (strain CT86-6760) was reclassified as HPeV-5 (2). The VP1 protein of enteroviruses and HPeV is the most variable and immunogenic protein. Serotyping is performed with specific antisera directed to the different enteroviruses and HPeV types. Nowadays, genotyping is used as an alternative method for typing. In genotyping, a clinical isolate with a VP1 nucleotide identity to known genotypes of higher then 75% or with an amino acid identity to known genotypes of 85% or higher is considered the same enterovirus type (11, 12).

In the study presented here, we analyzed 25 virus isolates that showed an enterovirus-like cytopathic effect (CPE) in cell culture. We used a universal enterovirus VP1 reverse transcription-PCR (RT-PCR) and a universal HPeV VP1 RT-PCR that should theoretically amplify all enteroviruses and all HPeVs (3, 9). Furthermore, we employed the VIDISCA method. VIDISCA is a virus discovery tool that can identify any RNA or DNA virus regardless of the virus family. The method uses restriction enzyme recognition sites and subsequent PCR amplification and was successfully used to identify a novel human coronavirus (16).

At the Municipal Health Service of Amsterdam, clinical samples that are sent in for virus diagnostics are routinely incubated on various cell cultures. If CPE is observed, a chloroform sensitivity test is performed to check whether the virus contains an envelope. For a nonenveloped virus, an acid stability test is performed to distinguish between rhinoviruses and other picornaviruses such as enteroviruses and HPeV. When viruses are acid stable, the isolates are tested with neutralization assays using antisera directed against poliovirus types 1 to 3; echoviruses 1 to 7, 9, 11 to 14, 20, 21, 25, 27, 29, 30, and 33; coxsackievirus B types 1 to 6; and HPeV-1 (RIVM, Bilthoven, The Netherlands). For some virus cultures these neutralization assays remain negative, although the CPE indicates the presence of a virus.

The 25 virus isolates that we analyzed could not be typed by the antiserum panel (Table 1). We first analyzed the virus isolates with the universal enterovirus and parechovirus VP1 RT-PCRs (3, 9). Twenty-three isolates were amplified with these PCRs, whereas two samples remained negative. Subsequent sequence analysis identified one echovirus 9, one echovirus 15, one echovirus 16, four echovirus 18, one echovirus 21, and five coxsackievirus type A2, A6, A7, A10, and A16 isolates. The parechoviruses included four HPeV-1 isolates, four HPeV-3 isolates, one HPeV-4 isolate, and one HPeV-6 isolate (Table 1).

To determine the efficacy of VIDISCA in detecting and identifying picornaviruses, we also tested all of the isolated viruses with this virus discovery method. The VIDISCA method was performed exactly as described previously (16). Twenty-four of 25 samples yielded virus-specific DNA fragments. Cloning and sequencing confirmed the presence of viral sequences. The VIDISCA confirmed the typing by VP1 sequencing for all 23 cases (Table 1). Interestingly, there was one sample that remained negative in the VP1 RT-PCR but for which viral fragments were obtained by VIDISCA. The VIDISCA sequences displayed a high similarity with HPeV-5. Via genome walking the complete VP1 sequence was obtained, which confirmed that isolate 2000-1108 is indeed an HPeV-5 isolate (88% nucleotide identity) (GenBank accession numbers EU077510 to EU077513). We then compared the VP1 sequences of isolate 2000-1108 with those of the primers used for the VP1 RT-PCR. A mismatch close to the 3′ end of one

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TABLE 1. Virus isolates typed by VP1 RT-PCR or VIDISCA

Sample[a]	Cell culture with CPE[b]	VP1 PCR result			VP1 nucleotide identity (%)[c]	VIDISCA result
		Enterovirus	HPeV	Virus type		
06-5030513	tMK	+[d]	−	Echovirus 9	92	Echovirus 9
2000-870	tMK	+[e]	−	Echovirus 15	82	Enterovirus B
2005-721	Rd	+[d]	−	Echovirus 16	79	Enterovirus B
2000-634	tMK	+[e]	−	Echovirus 18	98	Echovirus 18
2006-649	tMK/CaCo2	+[d]	−	Echovirus 18	97	Enterovirus B
06-5030740	tMK	+[d]	−	Echovirus 18	97	Echovirus 18
06-5031010	tMK	+[d]	−	Echovirus 18	97	Enterovirus B
2004-1573	tMK	+[d]	−	Echovirus 21	84	Enterovirus B
2005-421	Rd	+[f]	−	Coxsackievirus A2	93	Coxsackievirus A2
2000-684	CaCo2	+[f]	−	Coxsackievirus A6	96	Enterovirus A
2006-652	tMK/CaCo2/LLC-MK2	+[f]	−	Coxsackievirus A7	94	Enterovirus A
2004-1052	Rd	+[f]	−	Coxsackievirus A10	99	Enterovirus A
2005-415	tMK	+[f]	−	Coxsackievirus A16	98	Enterovirus A
2004-1608	tMK	−	+	HPeV-1	95	HPeV-1
2005-938	tMK	−	+	HPeV-1	87	HPeV-1
2005-939	tMK	−	+	HPeV-1	91	HPeV-1
1999-1096	tMK	−	+	HPeV-1	96	HPeV-1
2000-632	tMK	−	+	HPeV-3	97	HPeV-3
2000-752	tMK	−	+	HPeV-3	98	HPeV-3
2000-759	tMK	−	+	HPeV-3	98	HPeV-3
2000-976	tMK	−	+	HPeV-3	98	HPeV-3
2006-99[g]	tMK/HT29	−	+	HPeV-4	88	HPeV-4
2000-1108[h]	tMK	−	−	HPeV-5	88	HPeV-5
2005-823[i]	HT-29	−	+	HPeV-6	96	HPeV-6
2005-830	HT-29	−	−	NA[j]	NA	Negative

[a] All cultures were obtained from stool samples except for 2005-721, which was obtained from a nose swab.
[b] tMK, tertiary cynomolgus monkey kidney cells; Rd, rhabdomyosarcoma cells; CaCo2 and HT29, colorectal adenocarcinoma epithelial cell lines; LLC-MK2, epithelial monkey kidney cell line.
[c] Nucleotide identity to the most homologous strain of each genotype.
[d] Enterovirus primer set: 011-012 (primer sets are described in reference 9).
[e] Enterovirus primer set 187-222.
[f] Enterovirus primer set 011-040.
[g] Clinical symptoms: vomiting and acute, life-threatening episode of loss of consciousness. Age 4 months.
[h] Clinical symptoms: fever, dull affect, refusal to drink, and late-onset sepsis neonatorum. Age, 10 days.
[i] Clinical symptoms: fever, dull affect, refusal to drink (dehydration), bilateral otitis media, and anemia. Age, 2.5 months.
[j] NA, not applicable.

of the primers may explain the negative result in the VP1 RT-PCR (target sequence in strain 2000-1108, 5′-**T**CA**G**AAT TC**T**TGGGG**A**TC-3′; primer sequence, 5′-CCAAAATTCRT GGGGTTC-3′).

For one sample (sample 2005-830), neither the VIDISCA method nor the VP1 RT-PCR supplied viral amplification products. We therefore tested this sample in a universal 5′ untranslated region (5′UTR) RT-PCR for enterovirus and HPeV, but this assay also remained negative (data not shown).

Another sample (2005-823) was identified as HPeV-6. Since only a single full genome sequence of an HPeV-6 isolate from Japan (17) is available, full genome sequencing was performed with VIDISCA, combined with degenerate primers designed to anneal at conserved regions, and genome walking. The complete genome sequence except for the first 70 nucleotides of the 5′UTR was obtained (GenBank accession number EU077518). The HPeV-6 strain NII561-2000 and our HPeV-6 variant have identities in the coding region of 96.4% and 99.1% for nucleotides and amino acids, respectively.

The genome of HPeV-6 strain 2005-823 has the characteristic picornavirus organization, putatively encoding a single polyprotein of 2,182 amino acids that is predicted to be cleaved into 10 mature proteins by viral proteases. HPeV-6 strain 2005-823 contains a *cis*-acting replication element that is located in VP0, as described recently (2). Furthermore, HPeV-6 strain 2005-823 has a 28-amino-acid extension in the N terminus of VP3 and conserved motifs in the 2A protein that share homology with the H-rev-107 family of cellular proteins (5, 15). An imperfect repeat in the 3′UTR region is present, as described previously (2), which contains some nucleotide changes. The first repeat has the AUUAGACACUAAUCUG sequence, and the second has AUUGGAACACUAAUUCG.

HPeV-6 strain 2005-823 contains an RGD motif at the end of VP1 (Fig. 1). This RGD motif is critical for infectivity of HPeV-1 (2, 4, 15), and it has been suggested that this motif facilitates entry into the cell by binding to integrins. Such an RGD motif is present in all HPeVs except HPeV-3 isolates. Interestingly, an insertion of 3 amino acids is present 7 amino acids upstream of the RGD motif. This insertion is not observed in HPeV types 1, 2, 3, 4, and 5 but is present in our isolate and in the HPeV-6 isolate from Japan, which makes it unique for type 6 (Fig. 1).

HPeV-6 was described recently by Watanabe et al. (17). The virus was isolated from a cerebrospinal fluid specimen from a 1-year old child with Reye syndrome with a fatal outcome. HPeV-6 was only observed in Japan in the years 2000 and 2001

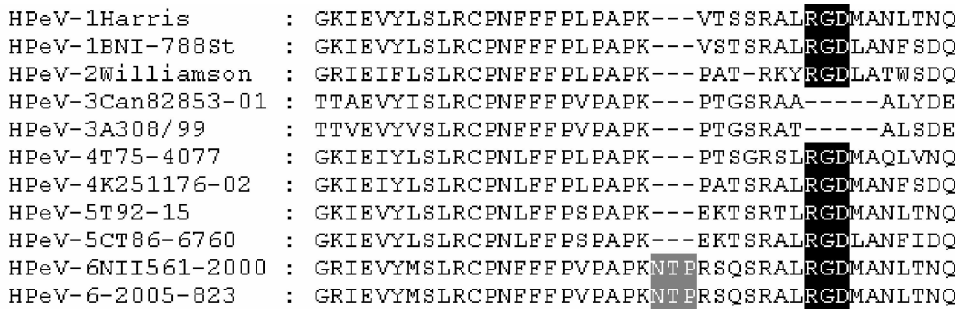

FIG. 1. Alignment of predicted amino acid sequence in the C terminus of the VP1 protein. The RGD motif is indicated by a black background. The insertion of 3 amino acids, located 7 amino acids upstream of the RGD motif, is marked with gray.

and not in the years 1991 to 1999 and 2002 to 2005. Our sample was isolated in 2005 from a stool sample from a 2.5-month-old girl with a history of 9 days of fever, dehydration, bilateral otitis media, and anemia.

For all samples a neutralization assay was performed with a panel of antibodies before molecular analysis. This panel contains antibodies against the most prevalent enteroviruses and HPeV-1. For six of the viruses that we identified, antisera were present in this panel but neutralization assays remained negative. The antisera used in this assay were generated several years ago and possibly, due to evolutionary changes, do not recognize the currently circulating strains.

Since the assignment of parechoviruses as a separate genus, additional genotypes have been identified with the use of molecular biological techniques. Furthermore, phylogenetic analysis with these novel genotypes allowed the reclassification of HPeV-2 (strain CT86-6760) as HPeV-5 (2). Most of the novel genotypes have been identified in a short time period by independent groups in different continents, as with HPeV-6, described by Watanabe et al., and our variant (1–3, 7, 17). It is tempting to speculate that in addition to HPeV types 1 to 6, more HPeV types will be discovered in the near future. Within 2 years two new genotypes (HPeV-4 and HPeV-6) have been discovered (2, 3, 17). These viruses probably are not emerging viruses but were previously unrecognized. Universal RT-PCRs facilitated identification of these previously unknown genotypes. However, the sequence variation that we noticed in the primer-binding site of HPeV-5 isolate 2000-1108 illustrates that the degenerative primers that are currently in use have their limitations. With the discovery of the additional genotypes and monitoring of the sequence variation, the universal HPeV VP1 primers can be improved and redesigned, which might lead to detection of additional previously unrecognized HPeVs.

In conclusion, we successfully typed picornaviruses in 24 out of 25 cell cultures, which were previously untypeable using serology. Most interesting is the identification of HPeV-4, HPeV-5, and HPeV-6 in clinical samples. Infection with HPeV-5 and HPeV-6 has not been described previously in The Netherlands, whereas infection with HPeV-4 has been described only once (3).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers EF155423, EF155422, EU077500 to EU077509, and EU077514 to EU077524.

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