Journal of Medical Virology 82:649–657 (2010)

A Comparison of the VP1, VP2, and VP4 Regions for Molecular Typing of Human Enteroviruses

David Perera,[1]* Hiroyuki Shimizu,[2] Hiromu Yoshida,[2] Phan Van Tu,[3] Hiroaki Ishiko,[4] Peter C. McMinn,[5] and Mary J. Cardosa[1]

[1]*Institute of Health & Community Medicine, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia*
[2]*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan*
[3]*Pasteur Institute of Ho Chi Minh City, Ho Chi Minh City, Vietnam*
[4]*Host Defense Laboratory, Mitsubishi Chemical Medicine Corporation, Tokyo, Japan*
[5]*Discipline of Infectious Diseases and Immunology, Central Clinical School, University of Sydney, Sydney, NSW, Australia*

The VP4, VP2, and VP1 gene regions were evaluated for their usefulness in typing human enteroviruses. Three published RT-PCR primers sets targeting separately these three gene regions were used. Initially, from a total of 86 field isolates (36 HEV-A, 40 HEV-B, and 10 HEV-C) tested, 100% concordance in HEV-A was identified from all three gene regions (VP4, VP2, and VP1). However, for HEV-B and HEV-C viruses, only the VP2 and VP1 regions, and not VP4, showed 100% concordance in typing these viruses. To evaluate further the usefulness of VP4 in typing HEV-A enteroviruses, 55 Japanese and 203 published paired VP4 and VP1 nucleotide sequences were also examined. In each case, typing by VP4 was 100% in concordance with typing using VP1. Given these results, it is proposed that for HEV-A enteroviruses, all three gene regions (VP4, VP2, and VP1), would be useful for typing these viruses. These options would enhance the capability of laboratories in identifying these viruses and would greatly help in outbreaks of hand, foot, and mouth disease. ***J. Med. Virol. 82:649–657, 2010.***

KEY WORDS: human enterovirus; RT-PCR detection; hand, foot, and mouth disease

INTRODUCTION

Human enteroviruses are small non-enveloped RNA viruses that belong to the family *Picornaviridae*. Classification of human enteroviruses using molecular methods has divided these viruses into dive separate species (poliovirus, HEV-A, HEV-B, HEV-C, and HEV-D) [Stanway et al., 2005]. To date 62 antigenically distinct serotypes and more than 30 additional genetically distinct types have been described (http://www.picornaviridae.com/enterovirus/enterovirus.htm). In the last decade, a succession of large outbreaks of hand, foot, and mouth disease (HFMD) has been reported in the Asia-Pacific region [McMinn, 2002; Cardosa et al., 2003; Miyazawa et al., 2008; Ang et al., 2009; Zhang et al., 2009]. Although, the disease is generally characterized as a mild childhood illness, recent outbreaks have shown that there can be severe symptoms that sometimes lead to fatal outcomes associated with human enterovirus 71 (HEV71), which belongs to the HEV-A species [McMinn, 2002]. HEV-A viruses are associated most commonly with HFMD outbreaks [Yamashita et al., 2005; Podin et al., 2006]. Of these, HEV71 and human coxsackievirus A16 (CVA16) are the viruses most often isolated from these outbreaks and co-circulation of both viruses during HFMD outbreaks is a frequent occurrence [Lin et al., 2003; Li et al., 2005; Podin et al., 2006; Hosoya et al., 2007]. Of the two, infection with CVA16 is associated with a milder form of the disease as compared with HEV71 which is associated with acute neurological symptoms in a small proportion of cases [Lin et al., 2003; Li et al., 2005; Podin et al., 2006]. In view of this, it is important to be able to identify the enterovirus serotype during HFMD outbreaks so that informed decisions on public health intervention and treatment can be made.

Several different RT-PCR methods targeting different regions of the enterovirus genome, particularly those encoding the structural proteins of the virus have been used to determine serotype identity [Casas et al., 2001;

Grant sponsor: The Wellcome Trust; Grant number: WT071588MA; Grant sponsor: Universiti Malaysia Sarawak (Operational Fund).

*Correspondence to: David Perera, Institute of Health & Community Medicine, Universiti Malaysia Sarawak, Kota Samarahan, 94300 Sarawak, Malaysia. E-mail: davidperera@yahoo.com

Accepted 11 August 2009

DOI 10.1002/jmv.21652

Published online in Wiley InterScience (www.interscience.wiley.com)

Ishiko et al., 2002; Oberste et al., 2003; Nasri et al., 2007]. These methods involve RT-PCR coupled with sequencing of the amplified product to identify the "molecular serotype" of the virus. Methods based on the VP1 gene region in particular have been shown to provide serotype identification that is comparable to standard neutralization tests [Oberste et al., 1999]. Although the majority of these methods have been designed to work on viral isolates, methods that utilize primary clinical specimens have also been described [Casas et al., 2001; Nix et al., 2006]. During large outbreaks of HFMD, the ability to determine the human enterovirus serotype quickly and efficiently in a cost effective manner would definitely contribute towards decisions on public health intervention and management. In this study, the accuracy of sequencing three separate gene regions of the enterovirus genome (VP4, VP2, and VP1) for typing enterovirus isolates of HEV-A, HEV-B, and HEV-C was compared. Three separate published RT-PCR protocols [Ishiko et al., 2002; Oberste et al., 2003; Nasri et al., 2007] that target all three gene regions of interest were used. Sequences generated from these gene regions were then compared to each other, using the VP1 gene region as a reference, in its ability to type HEV-A, HEV-B, and HEV-C viruses.

MATERIALS AND METHODS

Virus Isolation

Human enterovirus isolates from Sarawak used in this study were cultured during the course of several surveillance programs run by this laboratory in collaboration with the Sarawak Health Department over a 10-year period (1998–2008). South Vietnamese human enetrovirus isolates were obtained through collaboration within the Asia-Pacific Enterovirus Surveillance Network (APNET, http://www.apnet.org.au/) formed in 1999. All human enteroviruses were propagated in human rhabdomyosarcoma (RD) or human embryonic kidney (293) cell lines.

RNA Extraction

Viral nucleic acids were extracted from all culture harvests using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Manheim, Germany) according to instructions provided by the manufacturer. Typically, extraction was done from 200 μl of culture harvest, eluted in 50 μl of sterile ultra high quality RNase-free water and stored at −80°C until use.

RT-PCR for HEV Identification

The presence of human enterovirus RNA was determined using a previously published pan-HEV RT-PCR method [Romero and Rotbart, 1993] that targets a region of the 5′UTR. Electrophoresis gel analysis was used to determine the presence of amplified human enterovirus RNA.

RT-PCR and Sequencing for HEV Typing

Three different published RT-PCR methods targeting the VP4 [Ishiko et al., 2002], VP2 [Nasri et al., 2007], and VP1 [Oberste et al., 2003] gene regions were used for human enterovirus typing. Each RT-PCR method was performed according to conditions published by the respective authors. All amplicons were analyzed by agarose gel electrophoresis. Amplicons were purified from the gel using the GENECLEAN III kit (BIO101, Vista, San Diego, CA). Purified products were sequenced in both directions using both the sense and antisense PCR primers of that method. Sequencing was done using the BigDye v3.2 (Applied Biosystems, Foster City, CA) and performed in the 3130 Genetic Analyzer (Applied Biosystems). Sequences were prepared and primer sequences removed using SeqMan software (DNASTAR, Madison, WI). The final consensus sequence for the VP4, VP2, and VP1 gene regions were 207, 368, and 357 bp, respectively.

Sequence Data Set

Sequence information for the VP4, VP2, and VP1 regions using methods described above, were generated from 72 Sarawak and 14 South Vietnam human enterovirus isolates examined in this study (Table I). Additionally, a total of 55 paired VP4 and VP1 sequences of Japanese HEV-A isolates mainly from cases with HFMD or herpangina were used [Hosoya et al., 2007]. A further 203 published paired VP4 and VP1 HEV-A sequences from Sarawak, Australia, South Korea, South Vietnam, Peninsular Malaysia, Saudi Arabia, Thailand, China, and United Kingdom [McMinn et al., 2001; Cardosa et al., 2003; Li et al., 2005; Simmonds and Welch, 2006; Perera et al., 2007; Tu et al., 2007] were obtained from GenBank and also evaluated.

Sequence Analysis and Phylogenetic Relationships

To determine the human enterovirus type, sequences of the three different gene regions were aligned separately. Each alignment included the corresponding sequence of each human enterovirus prototype strain obtained from GenBank. Alignments were performed using the MegAlign software (DNASTAR) which was also used to generate the nucleotide identity scores (percent of nucleotide homology) of each query sequence compared to the human enterovirus prototype sequences. As proposed by Oberste et al. [2003], if the highest score was >75% and the next highest score was <70%, then the sequence would be assigned the same serotype identity as the prototype human enterovirus paired to the highest score. However, if the highest score was between 70% and 75%, then the sequence was assigned a preliminary serotype identity of the matching prototype human enterovirus pending more sensitive typing methods.

Separate phylogenetic trees for the VP4, VP2, and VP1 gene regions were prepared using the *MEGA*

TABLE I. A Comparison of Three Different Gene Regions to Type Human Enterovirus (HEV) Isolates by Using the Nucleotide Identity Score

	VP4[a]		VP2[b]		VP1		
Isolate	Type	Highest % nucleotide identity	Type	Highest % nucleotide identity	Type	Highest % nucleotide identity	HEV species[c]
SB1806	HEV71	82.1	HEV71	80.4	HEV71	82.3	A
SB1988	HEV71	82.6	HEV71	79.7	HEV71	80.7	A
SB2278	HEV71	81.6	HEV71	80.8	HEV71	82.7	A
SB10280	HEV71	80.7	HEV71	80.8	HEV71	81.7	A
SB12682	HEV71	83.1	HEV71	82.9	HEV71	83.7	A
MY104-9	HEV71	82.1	HEV71	80.1	HEV71	84.0	A
SB64543	HEV71	82.6	HEV71	81.1	HEV71	84.0	A
VN51192	HEV71	80.2	HEV71	80.8	HEV71	83.7	A
VN5540	HEV71	83.1	HEV71	81.1	HEV71	86.0	A
VN5707	HEV71	82.1	HEV71	82.5	HEV71	83.3	A
VN5718	HEV71	82.1	HEV71	82.5	HEV71	82.3	A
VN5730	HEV71	82.6	HEV71	79.4	HEV71	82.3	A
VN5776	HEV71	80.7	HEV71	80.4	HEV71	83.7	A
VN5777	HEV71	81.6	HEV71	79.4	HEV71	82.3	A
VN5925	HEV71	80.2	HEV71	79.7	HEV71	85.3	A
VN5933	HEV71	80.7	HEV71	80.8	HEV71	82.7	A
SB2001	CVA16	81.2	CVA16	74.8	CVA16	78.3	A
S1283	CVA16	80.7	CVA16	75.5	CVA16	78.7	A
VN5607	CVA16	81.2	CVA16	76.2	CVA16	78.7	A
S114351	CVA14	84.1	CVA14	85.3	CVA14	84.7	A
SB65972	CVA12	76.8	CVA12	78.9	CVA12	81.8	A
SB4000	CVA10	80.7	CVA10	74.7	CVA10	79.1	A
SB12012	CVA10	78.3	CVA10	73.7	CVA10	76.8	A
SB12326	CVA10	76.3	CVA10	73.7	CVA10	76.8	A
SB13031	CVA10	76.3	CVA10	73.7	CVA10	76.8	A
CNS31323	CVA10	76.3	CVA10	74.0	CVA10	77.4	A
SB67851	CVA8	85.0	CVA8	82.4	CVA8	85.0	A
SB12030	CVA6	79.2	CVA6	81.5	CVA6	84.2	A
VN5710	CVA6	77.8	CVA6	81.5	CVA6	84.5	A
SB10052	CVA5	79.7	NS	—	CVA5	83.5	A
SB64742	CVA5	84.1	CVA5	82.7	CVA5	84.2	A
SB11605	CVA4	88.9	CVA4	88.4	CVA4	85.9	A
SB13128	CVA4	86.0	CVA4	84.2	CVA4	86.9	A
SB610474	CVA3	75.8	CVA3	76.1	CVA3	75.9	A
SB7470	CVA2	78.3	CVA2	80.6	CVA2	80.1	A
S17271	CVA2	78.3	CVA2	81.0	CVA2	80.1	A
CNS32573	E1,HEV75	84.5	E1	80.1	E1	79.4	B
CNS613151	CVB4	83.1	NA	—	E1	79.4	B
SB11993	E20	82.1	E3	77.2	E3	81.9	B
SB13332	E20	81.2	E3	77.2	E3	81.9	B
CNS34292	E20	82.6	E3	77.2	E3	81.6	B
CNS34293	E20	82.6	E3	77.9	E3	81.6	B
SB2598	E5	82.6	E5	81.0	E5	81.5	B
SB2640	E5	83.1	E5	80.3	E5	81.8	B
SB12804	E6	82.1	E6	78.8	E6	74.7	B
D94S1	E7	82.1	E6	78.2	E6	76.2	B
SB6067	E6,7,17;HEV75	83.1	E7	78.5	E7	78.8	B
SB15056	HEV75	83.6	E7	76.9	E7	79.4	B
VN51140	CVB3	83.1	E7	78.8	E7	81.6	B
SB12978	E29	81.2	E9	82.6	E9	84.7	B
VN5773	E32	82.6	NA	—	E9	86.9	B
SB611217	CVB1	86.5	E12	80.8	E12	81.9	B
SB9352	E4	78.7	E14	77.7	E14	75.3	B
B132S2	E15	79.7	E15	78.1	E15	78.7	B
SB7629	E16	83.6	E16	76.1	E16	80.6	B
CNS996392	E19	86.5	E19	80.1	E19	79.6	B
B11S2	E12,20	85.5	E19	78.5	E19	80.2	B
SB600724	E12,20	85.5	E19	79.2	E19	80.6	B
CNS21263	CVB4,E20	83.1	E20	80.1	E20	80.4	B
SB13652	E27	81.6	E27	84.0	E27	85.5	B
CNS21593	E19	82.6	E29	77.9	E29	78.8	B
SB14374	CVB3,4;E24	79.2	E30	79.8	E30	80.9	B
SB14486	CVB4	82.6	E30	81.1	E30	80.9	B

(*Continued*)

TABLE I. (*Continued*)

Isolate	VP4[a] Type	VP4[a] Highest % nucleotide identity	VP2[b] Type	VP2[b] Highest % nucleotide identity	VP1 Type	VP1 Highest % nucleotide identity	HEV species[c]
SB11373	E19	84.1	E33	77.9	E33	78.5	B
CNS3583	E19	84.1	E33	77.9	E33	78.8	B
SB10405	E19	83.6	CVB2	81.6	CVB2	82.1	B
SB10406	E19	83.6	CVB2	81.6	CVB2	82.1	B
SB12296	E19	84.1	CVB2	82.0	CVB2	81.8	B
SB13078	E19	84.1	CVB2	81.6	CVB2	81.2	B
CNS22473	E32	83.6	CVB2	81.6	CVB2	81.8	B
VN5555	HEV75	82.6	NS	—	CVB2	84.9	B
SB12597	E7	81.2	CVB3	78.9	CVB3	74.7	B
D29S1	CVB4	84.1	CVB4	82.4	CVB4	81.0	B
SB3951	CVB1	85.5	CVB5	81.8	CVB5	81.8	B
SB18674	CVB2	83.1	CVA9	80.1	CVA9	81.0	B
SB6376	E20	82.6	NS	—	HEV73	76.9	B
B105S1	CVA13	81.6	NS	—	CVA13	72.4	C
SB1220	CVA11	81.6	CVA17	77.7	CVA17	78.0	C
SB1543	CVA11	81.6	NA	—	CVA17	78.0	C
SB7508	CVA21	90.8	CVA21	87.6	CVA21	89.4	C
CNS20514	CVA21	90.3	CVA21	90.3	CVA21	90.0	C
SB7293	CVA24	83.6	CVA24	84.8	CVA24	85.0	C
SB8403	CVA24	84.1	CVA24	84.8	CVA24	85.0	C
CNS30014	CVA11	83.1	CVA24	72.6	CVA24	71.3	C
CNS51093	CVA24	84.1	CVA24	85.1	CVA24	84.7	C
CNS51214	CVA24	84.1	CVA24	84.4	CVA24	85.3	C

[a]Nucleotide identity scores >75% to multiple serotypes was observed.
[b]NA, no amplification by RT-PCR; NS, no sequence obtained after multiple attempts to sequence from either strand.
[c]Classification done according to VP1 data.

version 4, available at http://www.megasoftware.net [Tamura et al., 2007]. Neighbor-joining (NJ) phylogenetic trees were generated using a maximum composite likelihood model of nucleotide substitution. The statistical significance of the phylogenies constructed was estimated by bootstrap analysis using 1,000 pseudo-replicate data sets.

RESULTS

Performance of the Different RT-PCR Methods

A total of 86 different human enterovirus isolates comprising those from Sarawak and South Vietnam were included in this study. The presence of human enterovirus RNA in all nucleic acid extracts from these isolates was confirmed by a pan-human enterovirus RT-PCR method that targets the 5′UTR region [Romero and Rotbart, 1993]. Extracts from all 86 isolates were positive for human enterovirus using this method. To type these isolates, three different RT-PCR methods that target the VP4, VP2, and VP1 gene regions, respectively, were used. Both the VP4 and VP1 methods successfully amplified all 86 isolates tested and sequence data were obtained from all amplicons. However, the VP2 method failed to amplify three isolates (CNS613151, SB1543, and VN5773) (Table I). Additionally, sequence could not be obtained from amplicons generated by the VP2 method for four other isolates (SB10052, SB6376, B105S1, and VN5555) even after multiple attempts to generate sequence data from either strand (Table I).

HEV Typing

The partial VP1 sequence data (this study and all human enterovirus prototype sequences from GenBank) was used to initially prepare a bootstrapped phylogenetic tree (data not shown) to determine the species group (HEV-A to D) of all the 86 human enterovirus isolates in this study. From this analysis, a total of 36 HEV-A, 40 HEV-B, and 10 HEV-C were identified (Table I). All further analysis was done separately for each human enterovirus species group. Results were compared to typing by VP1 which was used as the reference standard.

HEV-A

Using the criteria proposed by Oberste et al. [2003] as described in the methods section, the highest nucleotide identity (HNI) score for each gene region (VP4, VP2, and VP1) was used to generate preliminary serotype identification for each of the 86 human enterovirus isolates in this study (Table I). For the 36 HEV-A isolates investigated, a 100% concordance of serotype identification by all three gene regions was observed. This was observed despite the fact that some of the HNI scores for the VP2 region were below 75% (indicating a tentative identification by that method) and all the HNI scores generated from VP4 sequences had multiple nucleotide identity scores greater than 75% to other serotypes (indicating potential inconclusive serotype identification). However, separate phylogenetic analysis of each gene region showed that in every case, HEV-A isolates

clustered (bootstrap support of >70%) with the prototype strain of the serotype determined using the HNI score (Fig. 1). Taken together, HNI scores coupled with phylogenetic analysis provide a useful way of typing HEV-A isolates using any of the three gene regions (VP4, VP2, and VP1) investigated in this study.

HEV-B

Preliminary serotype identification of HEV-B isolates using the HNI scores showed that there was a 100% concordance between the VP2 and VP1 gene regions. However, only seven isolates (17.5%) typed by VP4 matched the preliminary serotype identification obtained using VP2 and VP1 (Table I). As with the HEV-A isolates, VP4 sequences showed multiple HNI scores >75% to other serotypes. In addition, identical HNI scores to more than one serotype (for isolates CNS32573, SB6067, B11S2, SB600724, CNS21263, and SB14374) were observed with VP4. It was also observed that for VP4, serotype identification with HNI scores generally did not match clustering to the same prototype strain in the phylogenetic tree (Fig. 2). For example, isolates SB11993, SB13332, CNS34292, and CNS34293 had HNI scores >75% to E20 but clustered together with E3 in the phylogenetic tree (Fig. 2). On the contrary, for both VP2 and VP1, there was a 100% match between HNI scores to, and phylogenetic clustering with, the same prototype strain for all HEV-B isolates tested. The results suggest that only the VP2 and VP1 gene regions would be useful for serotype identification of HEV-B viruses.

HEV-C

HEV-C isolates investigated in this study also showed a 100% concordance between the VP2 and VP1 data for serotype identification using the HNI scores (Table I). With the exception of two isolates (B105S1 and CNS30014), there was a correlation between serotype identification with HNI scores and phylogenetic clustering (Fig. 3) to the same prototype strain using either of these gene regions. However, for isolates B105S1 (for VP1 only; no sequence was obtained from VP2) and CNS30014 (for both VP1 and VP2), with HNI scores between 70% and 75% to CVA13 and CVA24,

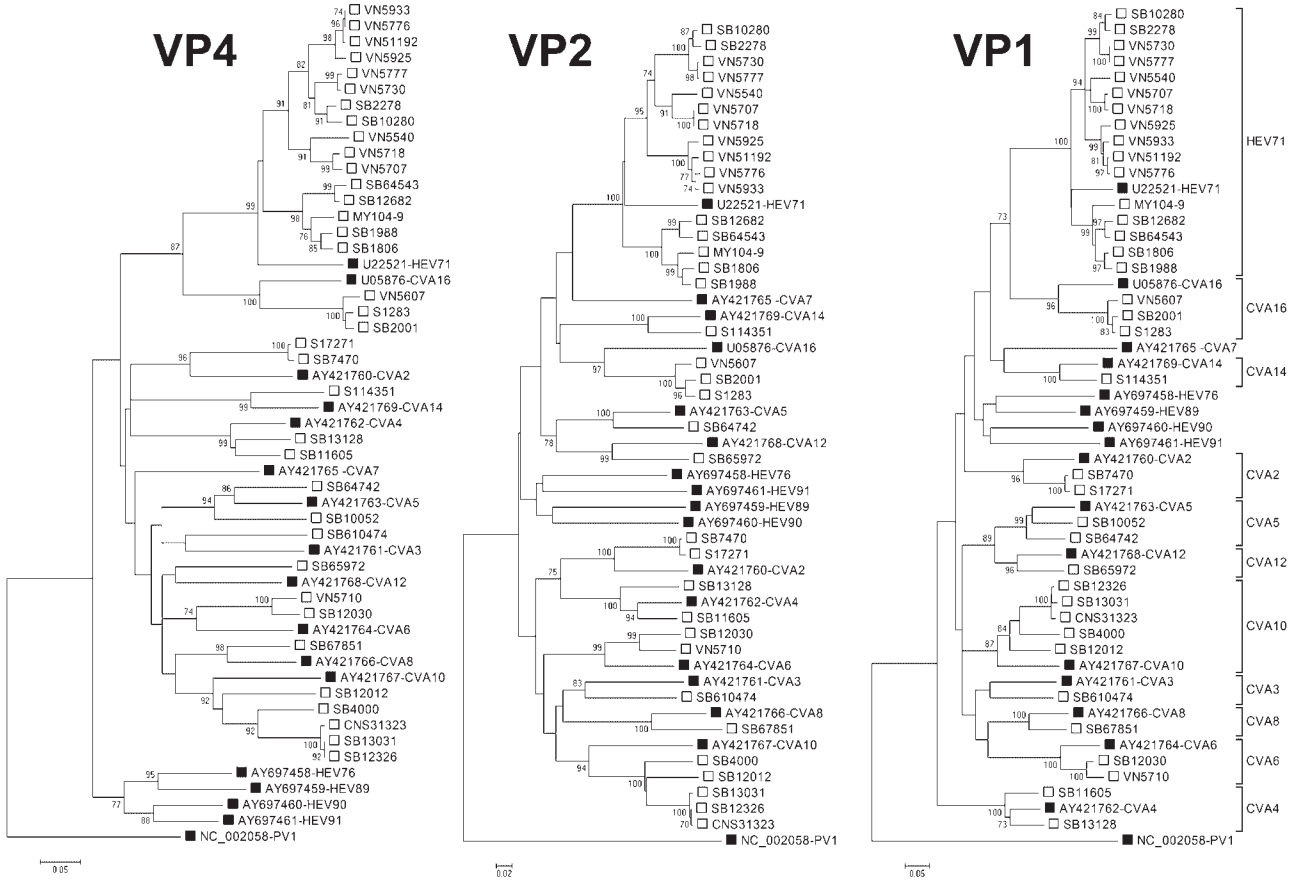

Fig. 1. Phylogenetic trees showing the relationships of HEV-A isolates based on the VP4, VP2, and VP1 gene regions. All trees include the HEV-A isolates listed in Table I (open boxes) and prototype sequences obtained from GenBank (closed boxes). Trees are rooted using PV1 (GenBank accession no. NC_002058), all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site, and bootstrap value >70% are indicated at key nodes. All the different serotypes identified from the study set using VP1 are indicated to right of the VP1 tree.

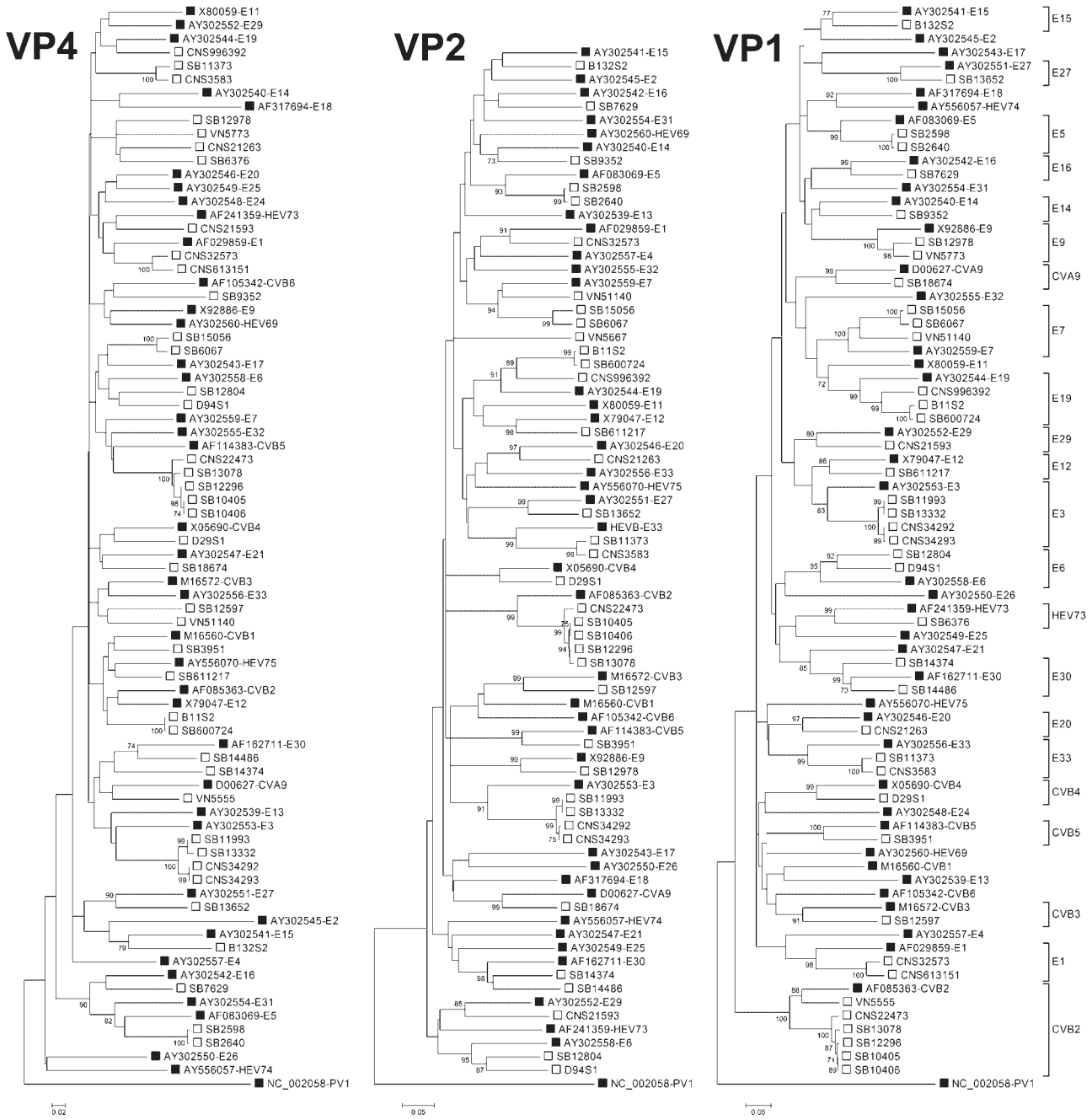

Fig. 2. Phylogenetic trees showing the relationships of HEV-B isolates based on the VP4, VP2, and VP1 gene regions. All trees include the HEV-B isolates listed in Table I (open boxes) and prototype sequences obtained from GenBank (closed boxes). Trees are rooted using PV1 (GenBank accession no. NC_002058), all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site, and bootstrap value >70% are indicated at key nodes. All the different serotypes identified from the study set using VP1 are indicated to right of the VP1 tree.

respectively, clustering to the same prototype strain in the phylogenetic tree did not occur (Fig. 3). As such, using the definition proposed by Oberste et al. [2003], serotype identification of both these isolates remains tentative pending more sensitive identification methods. When using VP4, there was a 70% concordance of serotype identification by HNI scores when compared to VP1. However, as observed with HEV-B isolates, VP4 HNI scores to a particular serotype did necessarily mean clustering to the same prototype strain in the phylogenetic tree. For example, isolate CNS30014 had a VP4 HNI score of 83.1% to CVA11 (Table I) but clustered with CVA20 in the phylogenetic tree (Fig. 3). Even when there was a match between VP4 HNI identification and clustering to the same prototype strain in the phylogenetic tree, a potential misassignment of the

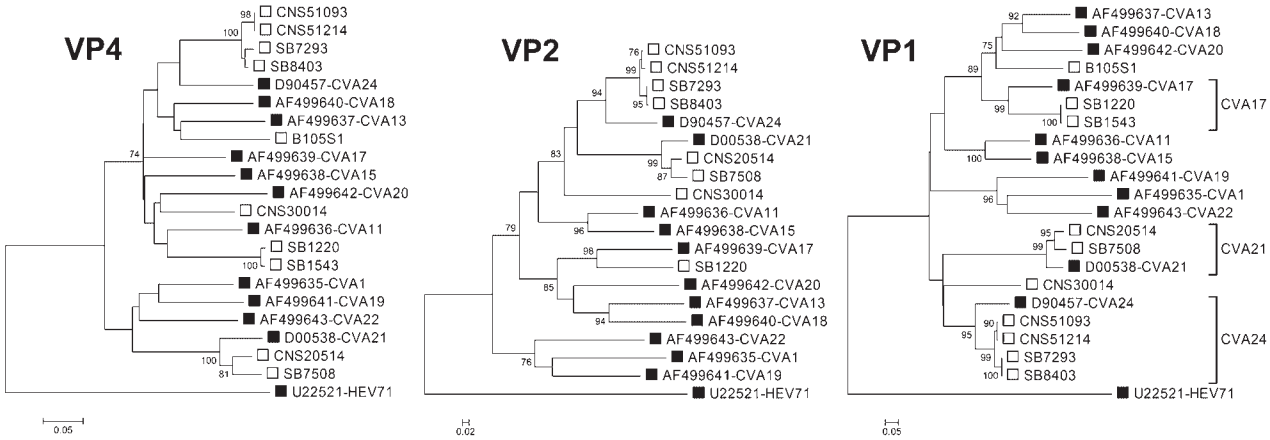

Fig. 3. Phylogenetic trees showing the relationships of HEV-C isolates based on the VP4, VP2, and VP1 gene regions. All trees include the HEV-C isolates listed in Table I (open boxes) and prototype sequences obtained from GenBank (closed boxes). Trees are rooted using HEV71 (GenBank accession no. U22521), all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site, and bootstrap value >70% are indicated at key nodes. All the different serotypes identified from the study set using VP1 are indicated to right of the VP1 tree.

serotype identity may occur. For example, isolates SB1220 and SB1543 typed as CVA11 by VP4 were identified as CVA17 by VP1 (Table I). Taken together, only the VP2 and VP1 gene regions are useful for serotype identification of HEV-C viruses.

Typing of Additional HEV-A Viruses

Since the data showed that VP4 could be used to accurately assign serotype to HEV-A viruses, it was decided to investigate if this observation holds true using data obtained by other laboratories. VP2 was not included in this part of the analysis because there is very little published data of paired VP4 and VP2 sequences. A total of 55 Japanese and 203 published paired VP4 and VP1 sequences comprising eight different serotypes of HEV-A (HEV71, CVA2, CVA4, CVA5, CVA6, CVA10, CVA12, and CVA16) were analyzed in the same way as described above (data not shown). There was a 100% concordance of serotype identification by using the HNI scores of either VP4 or VP1. Additionally, all serotypes identified by HNI scores of either VP4 or VP1 correctly clustered with their prototype strain in the phylogenetic analysis (data not shown), thus confirming that indeed VP4 sequence can assign reliably serotype to HEV-A strains.

DISCUSSION

In recent years, molecular typing methods have been reported as useful and rapid tools for typing human enteroviruses [Casas et al., 2001; Ishiko et al., 2002; Oberste et al., 2003; Nasri et al., 2007]. Most of these methods have targeted the virus capsid protein coding regions. Of these methods, the sequencing of a portion of the VP1 capsid protein coding region has been widely used by several laboratories as a means for human enterovirus identification [Oberste et al., 2003; Blomqvist et al., 2008]. The VP1 nucleotide sequence has been shown to provide human enterovirus typing identification that correlates to neutralization tests [Oberste et al., 1999]. However, little has been done to compare directly other protein coding regions to VP1 in identification of human enteroviruses although methods using other regions have separately been published. In this study the usefulness of the complete VP4 and partial VP2 gene regions in human enterovirus typing using a partial VP1 nucleotide region as a reference standard was investigated. This results have shown that the percentage of nucleotide homology to prototype sequences (HNI scores) coupled with phylogenetic analysis provide a useful way to type human enteroviruses. Altogether, this study has shown that typing by VP1 or VP2 would be useful for HEV-A, HEV-B, and HEV-C viruses, confirming results obtained by others [Nasri et al., 2007] whereas typing using VP4 would be useful only for assigning serotype to HEV-A viruses.

The high rate of mutation [Takeda et al., 1994; Brown et al., 1999; Martin et al., 2000] observed for human enteroviruses could potentially lead to newer strains with sufficient mutations that could affect the performance of primers used in RT-PCR-based typing methods. Thus, the ability to use more than one method or gene region to type HEV would certainly enhance the capability of a laboratory to type these viruses. As an example, in this study, it has been observed that the partial VP2 primer set failed to amplify or sequence some viruses of HEV-A, HEV-B, and HEV-C which was successfully amplified by both the VP4 and VP1 methods. Similar observations have also been reported by others in which recent human enterovirus strains have been typed successfully using the VP2 method but failed to be amplified using the VP1 method [Nasri et al., 2007]. Interestingly, in the study by Nasri et al. [2007], four of the viruses that failed to be amplified by the VP1 method were typed as HEV71 (HEV-A) using the VP2

method. Based on the results of this study, these HEV71 viruses could potentially have been typed by the VP4 method to confirm the typing results obtained with the VP2 method.

The sequences obtained from these typing methods could also be useful for epidemiological studies that track evolution, migration, and emergence of HEV. For example, in reported studies of HFMD, for two of the major causative agents (HEV71 and CVA16), genotyping using the VP4 sequence has been shown to correlate with the genotype profile obtained using complete VP1 sequence [Cardosa et al., 2003; Perera et al., 2007]. Generating a shorter PCR amplicon (207 bp for VP4 vs. 891 bp for VP1 for both HEV71 and CVA16), the VP4 gene region is useful as a rapid screening tool to determine genotype profiles of theses viruses during large outbreaks of HFMD [Hosoya et al., 2007]. This information can then be used for informed decisions on which isolates to sequence for the complete VP1 as a way to improve efficiency and cost when dealing with very large numbers of isolates. In addition, sequencing for the VP4 gene region reduces cost as only a single sequencing reaction of one strand of the amplicon is sufficient to generate complete VP4 sequence as the primer binding sites are located outside of the target region (in 5′UTR and VP2). In contrast, the published method for molecular typing using the partial VP1 gene region requires two sequencing reactions targeting both strands of the amplicon to generate the partial VP1 sequence as the primer binding sites are immediately flanking the target region.

In conclusion, it is proposed that typing for HEV-A, HEV-B, and HEV-C can be achieved using either the partial VP1 or VP2 method or a combination of both if one fails to amplify or generate sequence of a particular isolate. In addition, any of the three methods evaluated would be useful for typing HEV-A viruses. Therefore, in large HFMD outbreaks where the predominant causative agents are HEV-A viruses, the VP4 method offers not only cost-saving measures to the laboratory but additionally the VP4 sequence itself offers a rapid way to provide information about the genotype of the virus in the case of HEV71 and CVA16. These options for typing human enteroviruses would certainly help in enhancing the capability of any laboratory working with these viruses.

ACKNOWLEDGMENTS

We thank collaborators in Vietnam and Japan for sharing their sequences used in this study.

REFERENCES

Ang LW, Koh BK, Chan KP, Chua LT, James L, Goh KT. 2009. Epidemiology and control of hand, foot and mouth disease in Singapore, 2001–2007. Ann Acad Med Singapore 38:106–112.

Blomqvist S, Paananen A, Savolainen-Kopra C, Hovi T, Roivainen M. 2008. Eight years of experience with molecular identification of human enteroviruses. J Clin Microbiol 46:2410–2413.

Brown BA, Oberste MS, Alexander JP, Kennett MLJ, Pallansch MA. 1999. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol 73:9969–9975.

Cardosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, Cho H, McMinn PC. 2003. Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: Comparative analysis of the VP1 and VP4 genes. Emerg Infect Dis 9:461–468.

Casas I, Palacios GF, Trallero G, Cisterna D, Freire MC, Tenorio A. 2001. Molecular characterization of human enteroviruses in clinical samples: Comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. J Med Virol 65:138–148.

Hosoya M, Kawasaki Y, Sato M, Honzumi K, Hayashi A, Hiroshima T, Ishiko H, Kato K, Suzuki H. 2007. Genetic diversity of coxsackievirus A16 associated with hand, foot, and mouth disease epidemics in Japan from 1983 to 2003. J Clin Microbiol 45:112–120.

Ishiko H, Shimada Y, Yonaha M, Hashimoto O, Hayashi A, Sakae K, Takeda N. 2002. Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. J Infect Dis 185:744–754.

Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y, Jin Q. 2005. Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. J Clin Microbiol 43:3835–3839.

Lin TY, Twu SJ, Ho MS, Chang LY, Lee CY. 2003. Enterovirus 71 outbreaks, Taiwan: Occurrence and recognition. Emerg Infect Dis 9:291–293.

Martin J, Dunn G, Hull R, Patel V, Minor PD. 2000. Evolution of the Sabin strain of type 3 poliovirus in an immunodeficient patient during the entire 637-day period of virus excretion. J Virol 74:3001–3010.

McMinn PC. 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev 26:91–107.

McMinn PC, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ. 2001. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. J Virol 75:7732–7738.

Miyazawa I, Azegami Y, Kasuo S, Yoshida T, Kobayashi M, Shiraishi T. 2008. Prevalence of enterovirus from patients with herpangina and hand, foot and mouth disease in Nagano Prefecture, Japan, 2007. Jpn J Infect Dis 61:247–248.

Nasri D, Bouslama L, Omar S, Saoudin H, Bourlet T, Aouni M, Pozzetto B, Pillet S. 2007. Typing of human enterovirus by partial sequencing of VP2. J Clin Microbiol 45:2370–2379.

Nix WA, Oberste MS, Pallansch MA. 2006. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol 44:2698–2704.

Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. 1999. Molecular evolution of the human enteroviruses: Correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 73:1941–1948.

Oberste MS, William AN, Maher K, Pallansch MA. 2003. Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing. J Clin Virol 26:375–377.

Perera D, Yusof MA, Podin Y, Ooi MH, Thao NT, Wong KK, Zaki A, Chua KB, Malik YA, Tu PV, Tien NT, Puthavathana P, McMinn PC, Cardosa MJ. 2007. Molecular phylogeny of modern coxsackievirus A16. Arch Virol 152:1201–1208.

Podin Y, Gias ELM, Ong F, Leong Y-W, Yee S-F, Yusof MA, Perera D, Teo B, Wee T-Y, Yao S-C, Yao S-K, Kiyu A, Arif MT, Cardosa MJ. 2006. Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: Lessons from the first 7 years. BMC Public Health 6:180.

Romero JR, Rotbart HA. 1993. PCR detection of the human enteroviruses. In: Persing DH, Smith TF, Tenover FC, White TJ, editors. Diagnostic molecular microbiology: Principles and applications. Washington DC: American Society for Microbiology. pp 401–406.

Simmonds P, Welch J. 2006. Frequency and dynamics of recombination within different species of human enteroviruses. J Virol 80:483–493.

Stanway G, Brown F, Christian P, Hovi T, Hypia T, King AMQ, Knowles NJ, Lemon SM, Minor PD, Pallansch MA, Palmenberg AC, Skern T. 2005. Family *Picornaviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: Eight report of the International Committee on Taxonomy of Viruses. United Kingdom: Elsevier/Academic Press. pp 757–778.

Takeda N, Tanimura M, Miyamura K. 1994. Molecular evolution of the major capsid protein VP1 of enterovirus 70. J Virol 68:854–862.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol and Evol 24:1596–1599.

Tu PV, Thao NT, Perera D, Huu TK, Tien NT, Thuong TC, How OM, Cardosa MJ, McMinn PC. 2007. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerg Infect Dis 13:1733–1741.

Yamashita T, Ito M, Taniguchi A, Sakae K. 2005. Prevalence of coxsackievirus A5, A6, and A10 in patients with herpangina in Aichi Prefecture, 2005. Jpn J Infect Dis 58:390–391.

Zhang Y, Tan XJ, Wang HY, Yan DM, Zhu SL, Wang DY, Ji F, Wang XJ, Gao YJ, Chen L, An HQ, Li DX, Wang SW, Xu AQ, Wang ZJ, Xu WB. 2009. An outbreak of hand, foot, and mouth disease associated with subgenotype C4 of human enterovirus 71 in Shandong, China. J Clin Virol 44:262–267.