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Identification of 20 Common Human Enterovirus Serotypes by Use of a Reverse Transcription-PCR-Based Reverse Line Blot Hybridization Assay▽

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The more than 100 human enterovirus (HEV) serotypes can also be classified into four species, HEV-A to -D, based on phylogenetic analysis of multiple gene regions. Current molecular typing methods depend largely on reverse transcription-PCR (RT-PCR) amplification and nucleotide sequencing of the entire or 3′ half of the VP1 gene. An RT-PCR-based reverse line blot (RLB) hybridization assay was developed as a rapid and efficient approach to characterize common HEVs. Twenty HEV serotypes accounted for 87.1% of all HEVs isolated at an Australian reference virology laboratory from 1979 to 2007. VP1 sequences of all known HEV prototype strains were aligned to design one sense primer and three antisense primers for RT-PCR. After sequencing of the complete VP1 genes of 37 previously serotyped examples of the commonest 20 serotypes and alignment of these VP1 sequences with GenBank sequences, four serotype-specific probes for each serotype were designed for RLB. The RT-PCR–RLB assay was then applied to 132 HEV isolates, made up of the previously sequenced 37 isolates and another 95 serotyped clinical isolates. The RT-PCR–RLB genotypes corresponded with the serotypes for 131/132 isolates; the one exception was confirmed by VP1 sequencing, and the genotype was confirmed by repeat conventional serotyping. Genotyping by RT-PCR–RLB complements traditional serotyping methods and VP1 sequencing and has the advantages of convenience, speed, and accuracy. RT-PCR–RLB allows detection of specific enteroviral serotypes or genotypes associated with HEV outbreaks and significant disease.

Human enteroviruses (HEVs), members of the *Enterovirus* genus of the family *Picornaviridae*, are common human pathogens associated with a wide spectrum of diseases. These range from asymptomatic infection to serious illness, especially in children (20) and the immunocompromised. Clinical syndromes include aseptic meningitis (1, 25), encephalitis (8, 26), fever and rash, acute flaccid paralysis (29), and gastrointestinal and respiratory illness. Some HEVs (e.g., EV71) have caused large outbreaks in Southeast Asia (21). Polioviruses (PVs) (including vaccine strains) have reemerged recently and have been associated with clinical disease (30). Although Australia is "polio-free," there have been recent outbreaks of polio in nearby Indonesia (5) and recent importation of a case from Pakistan (31).

The original classification of HEVs consisted of PVs, coxsackieviruses A (CVA) and B (CVB), and echoviruses (E), based on biological activity and disease. Conventional approaches for detecting and characterizing HEVs are based on the time-consuming and labor-intensive procedures of viral isolation in cell culture and neutralization using reference antisera (7). Current classification of the over 100 HEV serotypes (http://www.picornaviridae.com) divides the members of HEV genera into four species (HEV-A, HEV-B, HEV-C, and HEV-D), based on genome organization, sequence similarity, and biological properties (23). At present, molecular typing methods largely depend on reverse transcription-PCR (RT-PCR) amplification and nucleotide sequencing of the entire or 3′ half of the VP1 gene (23). Comparison of the VP1 sequence with databases of VP1 sequences of HEV prototype and variant strains (18) allows genotype assignation. Other molecular methods that assist in serotype-specific identification include multiplex real-time hybridization probe RT-PCR for specific detection and differentiation of enterovirus type 71 (EV71) and CVA16 (28), combined multiplex RT-PCR and microarray assay to detect and differentiate EV71 and CVA16 (4), molecular characterization of PV strains by RT-PCR–restriction fragment length polymorphisms (2), and an integrated micro-RT-PCR system for automatic detection of microorganisms, including EV71 (11). Experience with microwell oligonucleotide arrays (27) and other non-sequencing-based molecular methods to genotype other HEV serotypes is limited.

The 20 most frequent HEV serotypes identified at the reference virology laboratory of the Centre for Infectious Diseases and Microbiology (CIDM) in New South Wales, Australia, from 1979 to 2007 included HEV-A (CVA16 and EV71), HEV-B (E3, -6, -7, -9, -11, -14, -17, -18, and -30, CVB1, -2, -3, -4, and -5, and CVA9), and HEV-C (PV1, -2, and -3) serotypes. These 20 serotypes accounted for 87.1% of all HEVs referred for typing during this period.

In order to establish a quick, accurate, and cost-effective

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TABLE 1. Oligonucleotide primers and probes for RT-PCR–RLB assay

Primer or probe[a]	Target	T_m (°C)	GenBank accession no.	Sequence (5′–3′)[c]	No. of aligned sequences[d] (prototype strain GenBank no.)	% Covered strains (no. of covered strains/total no. of strains)
VP1-ALL-Sn	HEVs	59.09	AF029859 (E1)	2540NYT NMM NGC NGY NGA RAY NGG2560	86 prototype strains	
VP1-A-Ab	HEV-A	61.42	AY421760 (CVA2)	3066AAN GWN GGR TAN CCR TCR TAR AAC CA3041	16 prototype strains	
VP1-B-Ab	HEV-B	60.38	AF029859 (E1)	2953GGR TTN GTN GAN GWY TGC C2935	54 prototype strains	
VP1-C-Ab	HEV-C	59.34	AF499635 (CVA1)	3069RWA NCC RTC RTA RAA RTG NGM RTA NGC3043	13 prototype strains	
VP1-CVA16-Sp1	Serotype CVA16	64.47	FJ868280[b]	224CAC AGG AGA CAG CCA TTG G242	104 (U05876)	95.2 (99/104)
VP1-CVA16-Sp2	Serotype CVA16	64.62	AB292123	63GCA GGA GAC AGC CAT TGG80		
VP1-CVA16-Sp3	Serotype CVA16	61.94	AB119644	60CAC AAG AGA CAG CCA TTG G78		
VP1-CVA16-Sp4	Serotype CVA16	60.85	U05876	2669CTC AAG AGA CCA CGA TTG G2687		
VP1-EV71-Sp1	Serotype EV71	63.04	EF063152	3017CAT TCA TGT CAC CTG CGA G3035	622 (U22521)	70.4 (438/622)
VP1-EV71-Sp2	Serotype EV71	63.03	FJ868281[b]	577CCA TTT ATG TCA CCT GCG AG596		
VP1-EV71-Sp3	Serotype EV71	62.14	U22521	3013TGC CAT TTA TGT CAC CTG C3031		
VP1-EV71-Sp4	Serotype EV71	63.16	DQ841993	578CGT TCA TGT CAC CCG CGA G596		
VP1-CVA9-Sp1	Serotype CVA9	63.45	FJ868282[b]	226ATG GAG GAA TAC AAG ACC ACA GA248	20 (D00627)	95 (19/20)
VP1-CVA9-Sp2	Serotype CVA9	60.26	AY466022	2673ATG GAG GAG TAT AAA ACC ACA GA2695		
VP1-CVA9-Sp3	Serotype CVA9	61.51	D00627	2673ATG GAA GAG TAC AAG ACC ACT GA2695		
VP1-CVA9-Sp4	Serotype CVA9	63.22	AB167978	91ATG GAG GAT TAC AAA ACC ACA GA113		
VP1-CVB1-Sp1	Serotype CVB1	63.62	FJ868283,[b] FJ868284[b]	358ATA ACG AGT GCC CAA CAA CC377	18 (M16560)	88.9 (16/18)
VP1-CVB1-Sp2	Serotype CVB1	63.99	AB167989	234ATA ACG AGC GCT CAA CAA CC253		
VP1-CVB1-Sp3	Serotype CVB1	65.10	M16560	2809ATA ACA AGT GCC CAG GAG CC2828		
VP1-CVB1-Sp4	Serotype CVB1	62.67	AY186745	2790ATA ACG AGT GCC CAA CAG C2808		
VP1-CVB2-Sp1	Serotype CVB2	62.14	AB234339	132TGG AAA GTG AGT GTT AGA CAA GC154	11 (AF085363)	100 (11/11)
VP1-CVB2-Sp2	Serotype CVB2	64.27	FJ868285[b]	280TGG AAG GTG AGT GTT AGA CAA GC302		
VP1-CVB2-Sp3	Serotype CVB2	63.03	AF225468	280TGG AAA GTG AGT GTT AGG CAA G301		
VP1-CVB2-Sp4	Serotype CVB2	61.82	AM711056	280TGG AAA GTC AGT GTT AGG CAG300		
VP1-CVB3-Sp1	Serotype CVB3	62.61	AM711026	256CGG TAT GCT GAA TGG GTG273	63 (M16572)	88.9 (56/63)
VP1-CVB3-Sp2	Serotype CVB3	63.40	M16572	2705GCG GTA TGC TGA ATG GGT A2723		
VP1-CVB3-Sp3	Serotype CVB3	67.29	EU144042	2708CGG TAT GCC GAA TGG GTG2725		
VP1-CVB3-Sp4	Serotype CVB3	65.24	FJ868286,[b] FJ868287[b]	256CGG TAC GCT GAA TGG GTG273		
VP1-CVB4-Sp1	Serotype CVB4	58.24	FJ868288,[b] FJ868289[b]	222GAT TTA CAT CAA ATA CTC AAG TGC245	93 (X05690)	71 (66/93)
VP1-CVB4-Sp2	Serotype CVB4	58.98	X05690	2666CGT AAT TTA TAT AAA ATA CTC CAG TGC2692		
VP1-CVB4-Sp3	Serotype CVB4	60.10	AF160047	254ATC TAC ATC AAA TAC TCA AGT GCT G278		
VP1-CVB4-Sp4	Serotype CVB4	58.89	AF160069	244TGC ATG TGT GAT TTA TAT AAA ATA CTC270		
VP1-CVB5-Sp1	Serotype CVB5	60.56	AY695109	310CGC AAA CTT GAG ATG TTT ACA TA332	36 (AF114383)	77.8 (28/36)
VP1-CVB5-Sp2	Serotype CVB5	64.76	FJ868290,[b] FJ868291[b]	316TTG GAA ATG TTC ACA TAT GCC A337		
VP1-CVB5-Sp3	Serotype CVB5	61.99	AF114383	2756CGA AAG CTT GAG ATG TTT ACG TA2778		
VP1-CVB5-Sp4	Serotype CVB5	60.56	AM711080	315CGC AAA CTA GAA ATG TTC ACA TA337		
VP1-E3-Sp1	Serotype E3	60.22	FJ868292,[b] FJ868293[b]	157GTC AAG AAT TAT CAT TCC AGG AC179	24 (AY302553)	83.3 (20/24)
VP1-E3-Sp2	Serotype E3	59.75	AJ241446	156TGT TAA GAA TTA TCA TTC CAG GAC179		
VP1-E3-Sp3	Serotype E3	60.07	AY302553	2603TGT TAA GAA CTA TCA CTC CAG GAC2626		
VP1-E3-Sp4	Serotype E3	62.55	AY919580	157GTC AAG AAC TAC CAT TCC AGG AC179		
VP1-E6-Sp1	Serotype E6	60.57	FJ868294[b]	367ATA ACC AGT GTG CAG GAT GA386	63 (AY302558)	82.5 (52/63)
VP1-E6-Sp2	Serotype E6	60.87	FJ868295[b]	363TGT CAT TAC AAG TGT ACA GGA TGA386		
VP1-E6-Sp3	Serotype E6	63.18	AY302558	2813ATA ACC AGC GTG CAA GAT GA2832		
VP1-E6-Sp4	Serotype E6	59.57	AB198867	252ATA ACC AGC GTA CAG GAT GA271		
VP1-E7-Sp1	Serotype E7	62.17	FJ868296,[b] FJ868297[b]	271ATG TCG TGG ACC ATA AAT GC290	17 (AY302559)	100 (17/17)
VP1-E7-Sp2	Serotype E7	63.38	AY302559	2717ATG TCG TGG ACA ATA AAT GCC2737		
VP1-E7-Sp3	Serotype E7	63.14	DQ227458	1975ATG TCG TGG GCC ATA AAT G1993		
VP1-E7-Sp4	Serotype E7	62.55	AJ241447	270CAT GTC ATG GAC CAT AAA TGC290		
VP1-E9-Sp1	Serotype E9	62.53	FJ868298,[b] FJ868299[b]	271TTT GAT GCG TGG GAG ATA AG290	23 (X84981)	91.3 (21/23)
VP1-E9-Sp2	Serotype E9	63.79	X84981	2718ACG CAT GGG AGA TAA GCG2735		
VP1-E9-Sp3	Serotype E9	61.01	X92886	2717CTT TGA TGC ATG GGA GAT AAG2737		
VP1-E9-Sp4	Serotype E9	64.92	AB167995	110TTT GAC GCG TGG GAG ATA AG129		
VP1-E11-Sp1	Serotype E11	59.53	FJ868300,[b] FJ868301[b]	307ATG AGA AGG AAA CTT GAA ATG TT329	92 (X80059)	81.5 (75/92)
VP1-E11-Sp2	Serotype E11	62.27	X80059	2765ATG AGA CGC AAG CTA GAG ATC TT2787		
VP1-E11-Sp3	Serotype E11	66.75	AY121418	307ATG AGG CGC AAG CTG GAG324		
VP1-E11-Sp4	Serotype E11	66.47	AY121423	297CAT GGT GCA AAT GAG GCG314		
VP1-E14-Sp1	Serotype E14	62.63	AB268217	169GAC AGC TGG GAC ATC AAC AT188	25 (AY302540)	80 (20/25)
VP1-E14-Sp2	Serotype E14	58.94	FJ868302[b]	274GAC AGC TGG GAT ATC AAC AT293		
VP1-E14-Sp3	Serotype E14	58.99	FJ868303[b]	274GAC AGC TGG GAC ATA AAC AT293		
VP1-E14-Sp4	Serotype E14	61.20	AB268225	151CGA TAG TTG GGA CAT CAA CAT171		

VP1-E17-Sp1	Serotype E17	63.77	FJ868304,[b] FJ868305[b]	223GTG GTC AAA ACA TAC AAA ATG GG245	11 (AY302543)	90.9 (10/11)	
VP1-E17-Sp2	Serotype E17	60.70	AY302543	2667GTG GTC AAA ACA TAT AAA ATG GG2689			
VP1-E17-Sp3	Serotype E17	60.87	AY919430	223GTG GTT AAG ACA TAC AAA ATG GG245			
VP1-E17-Sp4	Serotype E17	58.82	AF295482	223GTA GTC AAA ACA TAC AAA ATG GG245			
VP1-E18-Sp1	Serotype E18	61.79	FJ868306,[b] FJ868307[b]	329TTG ACA TTG AGA TGA CAA TGG T350	50 (AF317694)	96 (48/50)	
VP1-E18-Sp2	Serotype E18	60.76	AF317694	2773TTG ATA TCG AGA TGA CAA TGG T2794			
VP1-E18-Sp3	Serotype E18	61.60	AB167997	201TTG ACA TTG AAA TGA CAA TGG T222			
VP1-E18-Sp4	Serotype E18	58.48	AM711103	329TTG ACA TAG AGA TGA CAA TGG T350			
VP1-E30-Sp1	Serotype E30	64.63	AM711074	328ATG TTC ACA TAC ATG AGG TTT GAC C352	614 (AF162711)	75.1 (461/614)	
VP1-E30-Sp2	Serotype E30	61.59	FJ868308,[b] FJ868309[b]	328ATG TTT ACA TAC ATG AGG TTT GAC C352			
VP1-E30-Sp3	Serotype E30	59.64	AF162711	2782ATG TTT ACG TAT ATG AGA TTT GAC CT2807			
VP1-E30-Sp4	Serotype E30	64.32	AM711084	328ATG TTC ACA TAC ATG AGA TTT GAC CTT354			
VP1-PV1-Sp1	Serotype PV1	62.94	AY923837	578GCA TTT CAG TAC CAT ACG TTG G599	652 (AY560657 and PV Sabin 1 strain V01150)	73 (476/652)	
VP1-PV1-Sp2	Serotype PV1	63.01	AY560657	3061GGA TCT CGG TAC CGT ATG TTG3081			
VP1-PV1-Sp3	Serotype PV1	63.16	AY255678	580ATC TCC GTG CCG TAC GTT597			
VP1-PV1-Sp4	Serotype PV1	62.90	AJ237885	578GAA TTT CGG TTC CAT ACG TTG598			
VP1-PV2-Sp1	Serotype PV2	64.54	FJ868310[b]	204GAG ACG AAC GCG ATC AGA GT223	78 (AY082680 and PV Sabin 2 strain X00595)	78.2 (61/78)	
VP1-PV2-Sp2	Serotype PV2	62.74	M12197	2696GGT CGG AGT CTA CGG TTG A2714			
VP1-PV2-Sp3	Serotype PV2	65.03	AY082680	205AAG CGA ACG CGA TCA GAG T223			
VP1-PV2-Sp4	Serotype PV2	66.31	AM158276	2492GGC GAA CGC GAT CAG AAT2509			
VP1-PV3-Sp1	Serotype PV3	63.51	FJ868311,[b] FJ868312[b]	430AAT AAT GGG CAT GCA CTC AAC450	67 (K01392 and PV Sabin 3 strain X00596)	79.1 (53/67)	
VP1-PV3-Sp2	Serotype PV3	64.95	AY221227	430AAT AAT GGG CAC GCT CTC AAC450			
VP1-PV3-Sp3	Serotype PV3	66.39	AY278864	430AAC AAT GGG CAT GCA CTC AA449			
VP1-PV3-Sp4	Serotype PV3	65.17	POLVP4D	2164AAT AAC GGG CAT GCA CTG AAT2184			

[a] S, sense; A, antisense; b, biotin-labeled primer (primers were biotin labeled at the 5′ end); p, probe (probes were 5′ end labeled with a C-6 amine); n, non-biotin-labeled primer.
[b] One of 33 GenBank accession numbers for new complete VP1 sequences for 37 isolates.
[c] M, A or C; R, A or G; W, A or T; S, C or G; Y, C or T; K, G or T; N, A, C, G, or T. Numbers represent the base positions at which primer/probe sequences start and finish (starting at position 1 of the corresponding GenBank sequence).
[d] The database of prototype strains is based on previously published sequences (23), except for EV94 (GenBank accession no. EF107097). The sequence lengths for alignment were at least 600 bases.

method to characterize different isolates belonging to these common serotypes, we developed an HEV RT-PCR–reverse line blot (RLB) assay, using modifications from previously published RLB assays (9, 10, 32, 33).

MATERIALS AND METHODS

Viruses. Our laboratory database contained 6,383 HEVs isolated between 1979 and 2007. Isolates were obtained by inoculation of clinical specimens, including specimens where HEV had been detected by real-time fluorescence PCR (24), into cultures of primary monkey kidney cells and a variety of continuous cell lines, including A549 (human lung carcinoma), MRC-5 (human embryonic lung fibroblast), and RD (human rhabdomyosarcoma) cells. The serotype of each isolate was identified by neutralization with serotype-specific horse antisera, originally purchased from the National Institute of Allergy and Infectious Diseases (Bethesda, MD). Virus isolates were stored as unpurified cell culture supernatants at −70°C.

In this study, 132 HEV isolates collected from 1990 to 2007 and belonging to the commonest 20 serotypes were randomly selected from 6,383 HEV isolates for testing by RT-PCR–RLB. Of these 132 isolates, 37 (one or more examples of the 20 commonest serotypes) were also used for sequencing of the VP1 gene.

Primer design for VP1. To amplify the VP1 region, the VP1 sequences of all 85 known prototype HEV strains (23) plus EV94 (GenBank accession no. EF107097) were aligned using the ClustalW tool provided in Biomanager (Sydney Bioinformatics; http://www.angis.org.au/). This allowed the design, in highly conserved regions of VP1, of one sense primer (VP1-All-Sn) and three antisense primers (VP1-A-Ab, VP1-B-Ab, and VP1-C-Ab) to cover three different species (HEV-A, -B, and -C) (Table 1). Primer sequences were evaluated using the Sigma DNA calculator (Sigma; http://www.sigma-genosys.com/calc/dnacalc.asp) and synthesized by Sigma-Aldrich (Sydney, Australia).

RT-PCR, sequencing, and analysis of complete VP1 genes. Viral RNA was extracted from 200 μl of virus-infected cell culture supernatant by use of a High Pure viral RNA kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's procedures and eluted with 50 μl elution buffer. RT was performed under the following conditions: 22°C for 5 min, 50°C for 40 min, 72°C for 10 min, and 4°C for 5 min. Each RT mix contained 10 μl RNA, 1 μl deoxynucleoside triphosphates (dNTPs) (10 mM concentration of each dNTP), 4 μl 5× first-strand buffer, 1 μl dithiothreitol (0.1 M), 0.5 μl SuperScript III reverse transcriptase (Invitrogen, CA), 0.5 μl RNasin Plus RNase inhibitor (Promega, Madison, WI), 1 μl random primers (hexamers) (200 ng μl^{-1}) (Roche Applied Science, Mannheim, Germany), and water to 20 μl. The cDNA was stored at −70°C.

The complete VP1 genes of 37 isolates, providing one or two examples of each of our commonest 20 serotypes, were sequenced in two fragments by use of published primers (17). These were primer pairs 486-488 and 487-489 for HEV-A, primer pairs 490-492 and 491-493 for HEV-B, and primer pairs 494-496 and 495-497 for HEV-C. Primer 492 was modified to GGR TTI G*T*N *GA*I GWY TGC CA (R is A or G, I is deoxyinosine, N is A, C, G, or T, W is A or T, and Y is C or T) (modifications are shown in italics). PCR was performed using an AmpliTaq DNA polymerase kit (Appled Biosystems, Foster City, CA) and the following cycling conditions: 95°C for 10 min; 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 90 s; 72°C for 10 min; and a hold at 22°C. The PCR mixture contained the following: 2 μl template cDNA, 0.4 μl each of forward (100 pmol μl^{-1}) and reverse (100 pmol μl^{-1}) primers, 5 μl dNTPs (2 mM concentration of each dNTP), 5 μl 10× PCR buffer, 4 μl $MgCl_2$ (25 mM), 0.3 μl AmpliTaq DNA polymerase (5 units μl^{-1}), and water to 50 μl.

PCR products (10 μl) were analyzed by electrophoresis on 2% ethidium bromide-stained agarose gels. For sequencing, PCR products of the appropriate size that produced visible bands upon UV illumination were further purified using a PCR product presequencing kit (USB Corporation, Cleveland, OH) according to the manufacturer's procedures. These products were sequenced using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences of the two fragments of each isolate were assembled, and the previously tested serotype was confirmed by pairwise comparison of the complete VP1 sequence with a database containing VP1 sequences for the prototype and variant strains of all known HEV serotypes (18).

Serotype-specific probe design for VP1. After sequencing of the complete VP1 regions of 37 isolates and alignment of these VP1 sequences with at least 600 bases of VP1 sequence from GenBank for each of the 20 serotypes, using the ClustalW tool in Biomanager (Sydney Bioinformatics), four serotype-specific probes per serotype were designed in relatively conservative regions of VP1

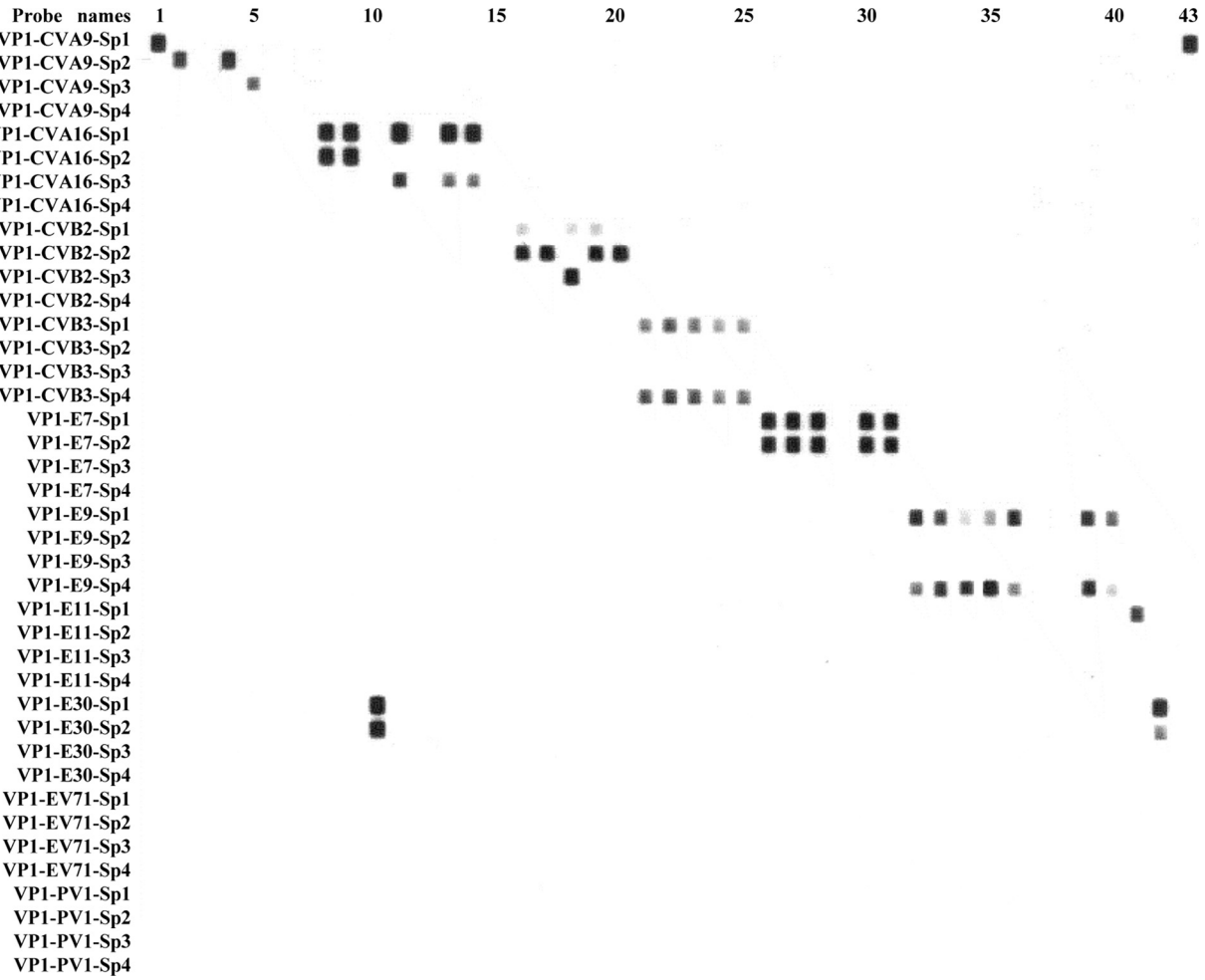

FIG. 1. Representative RT-PCR–RLB results for 43 clinical isolates (see Table 1 for description and targets of probes listed on the left). The first membrane (shown here) was bound with 40 probes (4 probes for each of 10 common serotypes), while the second (not shown) was bound with 40 probes for the other 10 common serotypes. Conventional serotype–RT-PCR–RLB results for the isolates shown, from left to right, were CVA9 (positive control), CVA9, E6*, CVA9, CVA9, E6*, E3*, CVA16, CVA16, E30, CVA16, CVB1*, CVA16, CVA16, CVB1*, CVB2, CVB2, CVB2, CVB2, CVB2, CVB3, CVB3, CVB3, CVB3, CVB3, E7, E7, E7, CVB4*, E7, E7, E9, E9, E9, E9, E9, E17*, E3*, E9, E9, E11, E30, and CVA9 (positive control). Isolates marked with asterisks belong to one of the 10 common serotypes and were identified by the second membrane (data not shown).

(Table 1) for use in RLB. Probe sequences were evaluated using the Sigma DNA calculator (Sigma), and the probe specificity was confirmed by a BLASTn search in Biomanager. All probes were synthesized by Sigma-Aldrich (Sydney, Australia).

RT-PCR for RLB. PCR (using VP1-All-Sn, VP1-A-Ab, VP1-B-Ab, and VP1-C-Ab) was performed for all 132 isolates (the 95 isolates not sequenced in VP1 were tested blinded to prior neutralization results), using a Qiagen Hotstar *Taq* polymerase kit (Qiagen, Clifton Hill, Victoria, Australia) and the following cycling conditions: 95°C for 15 min; 35 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min; 72°C for 10 min; and a hold at 22°C. The PCR mixture contained the following: 2 μl template cDNA, 1 μl VP1-All-Sn (100 pmol μl^{-1}), 0.5 μl (each) of VP1-A-Ab, VP1-B-Ab, and VP1-C-Ab (100 pmol μl^{-1}), 2.4 μl dNTPs (2.5 mM concentration of each dNTP), 3 μl 10× PCR buffer, 4.2 μl $MgCl_2$ (25 mM), 0.2 μl Qiagen Hotstar *Taq* polymerase (5 units μl^{-1}), and water to 30 μl.

RLB hybridization assay. The RLB assay was performed as previously described, with minor modifications (10). The PCR product (30 μl) was halved for hybridization over the two membranes, each of which was bound to 40 probes (4 probes for each of 10 serotypes) (Fig. 1). Briefly, the PCR product was hybridized with membrane-bound probes at 58°C for 1 h, and the two membranes were washed separately twice (10 min each time) at 58°C with prewarmed (58°C) 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.5% sodium dodecyl sulfate (SDS) and incubated in 15 ml of streptavidin-peroxidase conjugate (Roche Diagnostics, Mannheim, Germany) diluted 1:4,000 in 2× SSPE–0.5% SDS for 60 min at 42°C. These two membranes were further washed with 2× SSPE–0.5% SDS at 42°C and then with 2× SSPE at 25°C. If present, bound PCR products were detected by chemiluminescence, using ECL detection liquid (GE Healthcare Limited, Buckinghamshire, United Kingdom), and visualized by exposure for 20 min to an X-ray film (Hyperfilm; GE Healthcare Limited, Buckinghamshire, United Kingdom).

Identification of isolates negative by RLB. For isolates that were negative using the RLB assay, a modified species-specific RT-PCR amplification method (17) was applied. First, to determine the species (HEV-A, HEV-B, HEV-C, or HEV-D) of each isolate, four individual PCRs for each isolate were performed using an AmpliTaq DNA polymerase kit (Appled Biosystems, Foster City, CA) and the following cycling conditions: 95°C for 10 min; 35 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 30 s; 72°C for 10 min; and a hold at 22°C. Four separate PCR mixtures for each isolate, using four different primer pairs (480-423 for HEV-A, 481-424 for HEV-B, 482-425 for HEV-C, and 483-426 for HEV-D), were prepared to contain the following: 2 μl template cDNA, 0.4 μl each of forward (100 pmol μl^{-1}) and reverse (100 pmol μl^{-1}) primers, 5 μl dNTPs (2 mM concentration of each dNTP), 5 μl 10× PCR buffer, 4 μl $MgCl_2$ (25 mM), 0.3 μl AmpliTaq DNA polymerase (5 units μl^{-1}), and water to 50 μl. Ten microliters of each of the four PCR products for each isolate was analyzed by electrophoresis on 2% ethidium bromide-stained agarose gels. After deter-

TABLE 2. RT-PCR–RLB and serotype identification results for 132 clinical isolates

Serotype(s)	No. of isolates at CIDM (% of total no. of isolates)	No. of isolates tested by RLB	RT-PCR–RLB result
CVA16	177 (2.8)	7	CVA16
EV71	91 (1.4)	6	EV71
CVA9	323 (5.1)	6	CVA9
CVB1	97 (1.5)	5	CVB1
CVB2	200 (3.1)	6	CVB2
CVB3	521 (8.2)	5	CVB3
CVB4	226 (3.5)	5	CVB4
CVB5	307 (4.8)	5	CVB5
E3	92 (1.4)	5	E3
E6	177 (2.8)	4	E6
E7	247 (3.9)	7	E7
E9	418 (6.6)	9	E9
E11	751 (11.8)	7	E11
E14	129 (2.0)	7	E14
E17	109 (1.7)	5	E17
E18	104 (1.6)	11	E18
E30	312 (4.9)	20	E30
PV1	546 (8.6)	3	PV1
PV2	463 (7.3)	4	PV2
PV3	268 (4.2)	4	PV3
E25[a]		1	Negative

[a] This isolate, previously identified as E14 by serotyping but negative by RLB, was confirmed as E25 by a modified species-specific RT-PCR amplification method (17) showing it as HEV-B and by VP1 sequencing (GenBank accession no. FJ868313). Repeat conventional serotyping confirmed the E25 serotype, consistent with the genotype.

mining the species of each isolate, the complete VP1 gene was amplified, sequenced, and analyzed using the above methods.

Nucleotide sequence accession numbers. Thirty-three new complete VP1 sequences for 37 isolates have been deposited in GenBank (Table 1).

RESULTS AND DISCUSSION

Analysis of HEV database. A total of 6,383 HEV isolates were obtained from 1979 to 2007 from a variety of clinical samples, including cerebrospinal fluid, stools, vesicular skin lesions, throat swabs, and nasopharyngeal aspirates. These included isolates of PV (1,360 isolates, all vaccine strains), CVA (508 isolates), CVB (1,357 isolates), E (2,837 isolates), EV68 (2 isolates), EV69 (1 isolates), EV71 (91 isolates), and untypeable HEVs (227 isolates). Of these isolates, the commonest 10 serotypes were E11, PV1, CVB3, PV2, E9, CVA9, E30, CVB5, PV3, and E7, which accounted for 65.1% of isolates. The next 10 common serotypes were CVB4, CVB2, E6, CVA16, E14, E17, E18, CVB1, E3, and EV71 (Table 2). The commonest 20 serotypes accounted for 87.1% (5,558/6,383 isolates) of all isolates over approximately 30 years.

Analysis of complete VP1 sequences. The original classification of HEVs (PV, CVA, CVB, and E) was based on biological activity and disease, even though HEVs are sometimes difficult to culture in vitro and serological typing using reference antisera is time-consuming and labor-intensive. The current molecular classification scheme divides HEVs into four species (HEV-A to -D): in this newer system, members of an EV species share >70% amino acid (aa) identity in P1, share >70% aa identity in the nonstructural proteins 2C and 3CD, share a limited range of host cell receptors, share a limited natural host range, have a genome base composition (C+G) which varies by no more than 2.5%, and share a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination (6). Recombination has been observed to occur in nature only among members of the same species, except in the 5′-nontranslated region (3, 12–14, 16, 19, 22).

Molecular methods with analysis of the VP1 gene can function as an excellent surrogate for serotyping by neutralization. The principles include the following: (i) a partial or complete VP1 nucleotide sequence identity of ≥75% (>85% aa sequence identity) between a clinical EV isolate and a prototype serotype strain may be used to confirm the serotype of the clinical isolate, with the proviso that the second highest score is <70%; (ii) a best-match nucleotide sequence identity of <70% may indicate that the isolate represents an unknown (that is, new) serotype; and (iii) a sequence identity of 70 to 75% indicates that further characterization is required before the isolate can be identified firmly (18). Using these criteria, strains of homologous serotypes can easily be distinguished from heterologous serotypes and new serotypes can be identified. Recently, 17 isolates were classified by this approach as members of 13 new HEV types, namely, EVs 79 to 88, 97, 100, and 101 (15). Following sequencing of VP1, we applied these guidelines to identify 37 isolates before then designing serotype-specific probes; these showed that the genotypes/serotypes of these isolates obtained by sequencing corresponded with serotypes determined by neutralization.

VP1 primer and probe design. The VP1 sequences of all 86 known prototype strains, including EV94, were aligned to allow design of one sense primer (VP1-All-Sn). Three antisense primers (VP1-A-Ab, VP1-B-Ab, and VP1-C-Ab) were also designed to differentiate the three different HEV species (HEV-A, -B, and -C) (Table 1). To design each of the species-specific VP1-A-Ab, VP1-B-Ab, and VP1-C-Ab primers, VP1 sequences of 16 HEV-A, 54 HEV-B, and 13 HEV-C prototype strains were aligned (Table 1). The primer lengths were 19 to 27 bases, and melting temperatures (T_m) were 59 to 62°C.

To construct probes for different common serotypes, the complete VP1 genes of 37 isolates were sequenced and analyzed (see above). After alignment of at least 600 bases of GenBank VP1 sequence for each of these 20 serotypes, together with the 37 complete VP1 genes sequenced in this project, four serotype-specific probes for each serotype were designed (Table 1). The main principles for probe design were as follows: (i) a conservative region of one specific serotype was selected after verifying that the probes would cover as many strains within that serotype as possible; (ii) within the conservative region, the four probes should be homologous with the sequenced isolates, the prototype strain, and other convergent strains; (iii) the lengths of probes should be 18 to 27 bases, and the T_m should be 58 to 67°C; and (iv) the specificity of all probes was confirmed by BLASTn search in Biomanager. The numbers of VP1 sequences aligned for probe design for each serotype are shown in Table 1. For the four probes per serotype, the proportions of covered strains among all strains within the serotype ranged from 70.4 to 100% (Table 1). These data demonstrate that four probes per serotype are capable of capturing the majority of serotype strains.

Validation of primers and serotype-specific probes by RT-PCR–RLB. PCR was performed using primers VP1-All-Sn,

VP1-A-Ab, VP1-B-Ab, and VP1-C-Ab to amplify the VP1 gene. For the first membrane, bound with 40 probes for 10 common serotypes, one sequenced CVA9 isolate (GenBank accession no. FJ868282) was used as the positive control to determine the location of the first probe (VP1-CVA9-Sp1) and the locations of the first and last samples of the RLB readout on the film (Fig. 1). Similarly, one sequenced E6 isolate (GenBank accession no. FJ868294) was chosen as the positive control for the second membrane, bound with 40 probes of the other 10 common serotypes (not shown).

After hybridization with serotype-specific probes for each of the commonest 20 serotypes, the RLB assay results were concordant with the serological identification for 131 isolates, which meant that at least one probe among the four serotype-specific probes detected each isolate (Table 2; Fig. 1). One isolate, previously identified as E14 by serotyping but negative by RLB, was confirmed as E25 by a modified species-specific RT-PCR amplification method (17) showing it as HEV-B and by VP1 sequencing (GenBank accession no. FJ868313). Repeat conventional serotyping showed that the isolate was E25, confirming the genotype.

Since the RLB assay cannot distinguish between sequences with a one-base difference, the probes for some serotypes that contain sequences with only one base difference from each other showed at least one positive result with PCR products (Fig. 1). However, given the specificity of the probes, any one among four probes per serotype can confirm the specific serotype/genotype of each isolate.

Most recently, Susi et al. (27) developed a straightforward assay for the rapid typing of HEVs by use of oligonucleotide arrays in microtiter wells. In their studies, 10 probes for 10 different HEV reference strains were designed and employed. Furthermore, they used serotype consensus oligonucleotide probes for CVA9 to detect 25 CVA9 isolates. Both this and our studies show that such methods could potentially be extended to cover all known HEV serotypes. Theoretically, this strategy can extend to other RNA virus detection and identification procedures.

Conclusion. In this study, we successfully used RT-PCR–RLB to identify 131/132 HEV isolates belonging to the commonest 20 serotypes detected by neutralization at our institution; the exception was an E25 isolate, a relatively uncommon serotype. Genotyping by RT-PCR–RLB complements traditional serotyping methods and VP1 sequencing and has the advantages of convenience, speed, and accuracy. This will allow examination of specific HEV serotypes or genotypes associated with outbreaks and significant disease. The ease and low cost of this RT-PCR–RLB strategy will improve the diagnosis and epidemiological investigation of enteroviral infections and outbreaks where other typing methods are unavailable.

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