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Comparison of Classic and Molecular Approaches for the Identification of Untypeable Enteroviruses†

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Members of the family *Picornaviridae* are the most common viruses infecting humans, and species in several genera also infect a wide variety of other mammals. Picornaviruses have traditionally been classified by antigenic type, based on a serum neutralization assay. However, this method is time-consuming and labor-intensive, is sensitive to virus aggregation and antigenic variation, and requires a large number of antisera to identify all serotypes, even when antiserum pools are used. We developed generic reverse transcription (RT)-PCR primers that will amplify all human enterovirus serotypes, as well as many rhinoviruses and other picornaviruses, and used RT-PCR amplification of the VP1 gene and amplicon sequencing to identify enteroviruses that were refractory to typing by neutralization with pooled antisera. Enterovirus serotypes determined by sequencing were confirmed by neutralization with monospecific antisera. Of 55 isolates tested, 49 were of known enterovirus serotypes, two were rhinoviruses, and four were clearly picornaviruses but did not match any known picornavirus sequence. All four untyped picornaviruses were closely related to one another in sequence, suggesting that they are of the same serotype. RT-PCR, coupled with amplicon sequencing, is a simple and rapid method for the typing and classification of picornaviruses and may lead to the identification of many new picornavirus serotypes.

Enteroviruses (EV) (family *Picornaviridae*) are among the most common of human viruses, infecting an estimated 50 million people annually in the United States and possibly a billion or more annually worldwide (10, 13). Most infections are inapparent, but EV may cause a wide spectrum of acute disease, including mild upper respiratory illness (common cold), febrile rash (hand, foot, and mouth disease and herpangina), aseptic meningitis, acute flaccid paralysis (poliomyelitis), and neonatal sepsis-like disease. Sixty-four human EV serotypes have been identified antigenically by the use of an antibody neutralization test (7, 9), and antigenic variants have been described within many serotypes (10).

The neutralization test, long the gold standard for EV typing, is generally reliable, but it is labor-intensive and time-consuming and may fail to identify an isolate because of aggregation of virus particles, antigenic drift, recombination within the capsid region (a rare event [1]), or the presence of multiple viruses in the specimen being tested. Isolates that are not of a known human EV serotype (new serotypes or serotypes that normally infect animals other than humans) would obviously also present difficulties in identification by antigenic means, as the method requires the use of serotype-specific reagents. While serotyping may have little influence on the clinical management of a given patient, identification of the serotype is important to firmly establish an epidemiological link among cases during an outbreak and to recognize serotype-specific clinical illness (e.g., poliomyelitis, acute hemorrhagic conjunctivitis, or encephalitis). From a public health standpoint, it is important to be able to distinguish sporadic cases from an outbreak so that intervention and prevention strategies may be targeted logically and effectively.

We recently showed that VP1 nucleotide and deduced amino acid sequences can be used to discriminate among the prototype strains of all human EV serotypes (15) and then successfully applied this information to the typing of 51 EV clinical isolates by PCR and sequencing of the 3′ end of VP1 (14). However, the isolates tested in that study had been previously typed by the neutralization method and thus represented the simplest possible test of the method. To evaluate these molecular techniques more rigorously, we have tested their ability to identify EV that could not be typed by the use of standard immunological reagents and techniques. We report here the results of that evaluation and compare the classical (ultrastructural, biophysical, and immunological) and molecular (PCR and sequencing) methods for typing human EV.

MATERIALS AND METHODS

Viruses. Fifty-five virus isolates that could not be typed with standard enterovirus antiserum pools were chosen from among those processed in our laboratory during the period 1983 to 1990 for routine EV reference testing. The virus strains were isolated from a wide range of clinical specimens, including cerebrospinal fluid, stool, rectal swab, throat swab, oral swab, and ear fluid, on several different cell lines, including primary monkey kidney, RD, MRC-5, WI-38, and HEp-2C. All samples except one were isolated from the original clinical specimens by the submitting laboratory, and all isolates were passaged in RD (ATCC CCL 136), HLF (ATCC CCL 199), or LLC-MK_2 (ATCC CCL 7) cells in our laboratory prior to undergoing typing. For all isolates, infection of susceptible cells resulted in the appearance of a characteristic EV cytopathic effect (data not shown).

Neutralization and VDN tests. Pools of serotype-specific equine antisera (8, 11), in-house pools of serotype-specific rabbit antisera (specific for coxsackie B viruses, polioviruses, and most echovirus serotypes), in-house pools of serotype-specific mouse antisera (specific for coxsackie A virus serotypes), and individual equine or rabbit antisera raised against serotypes not represented in the pools have been used in our laboratory for routine EV typing since the early 1960s (P. Feorino, personal communication) (Table 1). The individual antisera also included those against well-characterized antigenic variants of E4 (DuToit and Shropshire), E6 (E6′-Cox and E6″-Burgess), E11 (E11′-Silva), and E30 (Frater, Giles, and PR-17), as well as a few duplicates of pool antiserum specificities

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† This paper is dedicated to the memory of our friend and colleague George Marchetti.

‡ Deceased.

TABLE 1. Antiserum pools and individual antisera used in standard neutralization tests in the Centers for Disease Control and Prevention (CDC) Enterovirus Reference Laboratory[a]

Pool	Component antiserum specificities
LBM (horse)	
A	CA7, CB1, CB4, E1, E4, E5, E7, E15, E29, E33
B	PV2, CA7, CA9, CB2, E2, E3, E9, E19, E21, E26
C	PV1, CB1, CB5, E2, E6, E12, E24, E29, E30
D	PV3, CB2, E6, E13, E14, E16, E25, E26, E32, E33
E	PV2, CB4, CB5, E5, E11, E13, E17, E18, E22, E30, E32
F	PV1, CB6, E7, E14, E18, E19, E20, E26, E27, E29
G	CA9, E4, E5, E16, E17, E20, E23, E30, E31
H	PV3, CA16, CB6, E1, E3, E9, E12, E22, E23, E32
CDC (rabbit)	
H′	E1, E2, E8, E15, E20
I	E5, E6, E14, E22
J	E9, E16, E17, E18
K	E23, E24, E25, E26, E29
L	CA9, CB3, CB4, CB5, CB6
M	CB1, E7, E11, E12
N	CB2, E27, E30, E31, E32
O	E3, E13, E19, E21
P	PV1, PV2, PV3
Coxsackie A virus (mouse)	
i	CA1, CA2, CA3, CA4, CA5, CA6, CA7
ii	CA8, CA9, CA10, CA11, CA12, CA13, CA14, CA15
iii	CA16, CA17, CA18, CA19
iv	CA20, CA21, CA22, CA23, CA24
Individual antisera	
E4 (Dutoit, Pesacek)	
E6″	
E11′	
E30 (Bastianni, Frater, Giles, PR-17)	
E34	
EV68	
EV69	
EV70	
EV71	

[a] LBM pools A to H (11) are distributed by the World Health Organization (Statens Seruminstitut, Copenhagen, Denmark). Other antisera were produced in limited quantities by the CDC for in-house use (P. Feorino, personal communication, 1998). CA, coxsackie A virus; CB, coxsackie B virus; E, echovirus; PV, poliovirus.

(E4-Pesacek and E30-Bastianni, the prototype strains of their respective serotypes). Typing of each virus isolate by the neutralization test in tubes or microtiter plates was attempted, using standard methods (4). For the virus dilution neutralization (VDN) test, eight twofold serial dilutions, containing from 100 to 0.78125 50% tissue culture infectious dose, were prepared and each dilution was tested in a standard microneutralization test (4) with each of the Lim and Benyesh-Melnick (LBM) antiserum pools, A to H (8, 11). Isolates were considered typed if the pattern of neutralization made sense according to the LBM scheme, and all positive pools neutralized within a fourfold virus dilution range.

Stability to acid. The stability of each virus isolate to acid was tested by incubation of the virus for 1 h at pH 3.0 and 4°C, adjustment to pH 7.0, and inoculation of cell cultures, using the same cell line as that employed for passage of the isolate. A decrease in virus titer of at least 100-fold indicated acid lability (4).

Molecular characterization of viruses. Viral RNA extraction, reverse transcription (RT)-PCR, nucleotide sequencing, and sequence analysis were performed as described previously (14). All isolates were screened with PCR primers that anneal at conserved sites in the 5′ nontranslated region (5′ NTR) of all enteroviruses and some rhinoviruses (19; M.A.P., unpublished data, 1999) (Table 2) to confirm that they were picornaviruses. For typing of each isolate, VP1 PCR with primer pairs 012-011 and 040-011 was initially attempted (Table 2), as described previously (14). For isolates that were not amplified with primer pair 012-011 or 040-011, amplification with primer pairs 187-222, 188-222, and 189-222 was attempted (Table 2). The serotype was determined by comparing the sequence of the VP1 amplicon to a database containing the complete sequences of all human EV (15), as well as to other picornavirus sequences that were available in the GenBank database, as described previously (14). In this scheme, a VP1 sequence identity of at least 75% to any EV prototype strain indicates that the isolate is of the homologous serotype, provided that the second-highest identity score (next closest serotype) is less than 70%. A high score of between 70 and 75% or a second-highest score of more than 70% indicates a tentative identification that must be confirmed by other means, whereas a high score of less than 70% indicates that the sequence of the isolate does not match any sequence in the database (14). Molecular typing results for all isolates were confirmed by neutralization with monospecific polyclonal antisera (described above) specific for each of the viruses with the four highest sequence identity scores. That is, antisera specific for the highest-scoring EV prototype strain specifically neutralized the unknown and the other antisera did not.

Nucleotide sequence accession numbers. The sequences described here have been deposited in the GenBank database under accession no. AF152248 to AF152302.

RESULTS

Fifty-five virus isolates obtained during the period 1983 to 1990 that repeatedly produced indeterminate typing results in the neutralization test with standard EV typing antisera and protocols were chosen for further investigation. All isolates had been identified as putative EV on the basis of a characteristic EV cytopathic effect in an EV-susceptible cell line (data not shown), and all were successfully amplified with pan-EV RT-PCR primers (Table 3). Fifty-one of fifty-five isolates were acid stable, a hallmark of viruses in the genus *Enterovirus*, retaining infectivity following a 1-h incubation at 4°C in pH 3.0 buffer (Table 3). The remaining four isolates—MD84-5914, OK88-8162, TX88-9121, and CA90-0150—were acid labile, suggesting that they may not be enteroviruses (Table 3).

To determine whether the initial neutralization tests had failed because of antigenic variation within one of the serotypes included in the LBM pools or the presence of virus mixtures, we attempted to type each of the acid-stable virus isolates by a VDN test. In theory, this test should be capable of detecting antigenic relationships among strains by assessing neutralization at higher-than-normal antibody-antigen ratios. Only 19 of 50 isolates tested could be typed unambiguously by this method (Table 3). PER89-9426 was not tested by VDN. In addition, the method was highly subjective, as there were often multiple individual wells in which cytopathology was incomplete and clear endpoints were difficult to determine.

For molecular typing, RT-PCR was attempted with each of the 55 isolates, using five different VP1-specific primer pairs, 012-011, 040-011, 187-222, 188-222, and 189-222 (Table 2). All isolates except IL85-6642 were amplified by at least one of the five VP1 primer pairs (Table 3). VP1 amplicons from each isolate were sequenced, and the nucleotide sequences were

TABLE 2. Primers used for PCR amplification or sequencing

Primer[a]	Sequence[b]	Gene	Nucleotide positions[c]
EV2	TCCGGCCCCTGAATGCGGCTAATCC	5′ NTR	446–470
EV1	ACACGGACACCCAAAGTAGTCGGTCC	5′ NTR	559–533
006	GGCAACTTCCACCACCACC	VP2	1197–1179
011	GCICCIGAYTGITGICCRAA	2A	3408–3389
012	ATGTAYGTICCICCIGGIGG	VP1	2951–2970
040	ATGTAYRTICCIMCIGGIGC	VP1	2951–2970
187	ACIGCIGYIGARACIGGNCA	VP1	2612–2631
188	ACIGCIGTIGARACIGGNG	VP1	2612–2630
189	CARGCIGCIGARACIGGNGC	VP1	2612–2631
222	CICCIGGIGGIAYRWACAT	VP1	2969–2951

[a] EV1 and EV2 are from reference 19, 006 is from reference 5, and 011, 012, and 040 are from reference 15.

[b] Sequences are shown 5′ to 3′, using standard IUB nucleotide ambiguity codes. I, deoxyinosine.

[c] Nucleotide sequence coordinates are given relative to the sequence of PV1-Mahoney (GenBank accession no. J02281).

TABLE 3. Summary of results for acid stability, VDN test, pan-EV PCR, sequencing, and confirmatory neutralization test using monospecific antisera[a]

Isolate[b]	Acid stability[c]	Type by VDN[d]	PCR/sequencing primer pair	Type by sequence	% Identity	Confirmed by neutralization[e]	Category[f]
NC83-5515	S	E24	012-011	E24	76.0	Y	1
NC84-5530	S	E24	012-011	E24	76.5	Y	1
NC84-5531	S	E25	012-011	E25	77.6	Y	1
TAI84-5839	S	CA16	040-011	CA16	77.9	TI	1
MD84-5914	L	NT	012-011	CB2	86.2	Y	2
TX84-5915	S	E15	187-222	CB4	81.5	Y	2
HON84-6016	S	UNT	040-011	CB2	85.2	Y	2
MOR83-6264	S	UNT	189-222	CA18	77.7	Y	3
MOR83-6266	S	UNT	188-222	CA20	81.4	Y	3
MOR83-6282	S	UNT	040-011	CA20	81.0	Y	3
MOR83-6286	S	E15	187-222	CB5	84.9	Y	2
OR85-6323	S	UNT	012-011	E18	78.8	Y	2
OR85-6329	S	UNT	187-222	CB2	84.1	Y	2
OK85-6388	S	UNT	012-011	UNT EV	E1: 68.0	N	4
MD86-6393	S	UNT	187-222	E3	84.4	Y	2
GA84-6536	S	UNT	189-222	CA24	74.5	TI	3
IL85-6642	S	UNT	EV2-006	E4	81.3	Y	2
VA86-6765	S	UNT	012-011	UNT EV	E1: 67.5	N	4
VA86-6776	S	UNT	012-011	E13	72.3	Y	2
HON86-6843	S	E25	040-011	E25	79.4	Y	1
CT87-7122	S	UNT	012-011	UNT EV	E1: 66.5	N	4
CT87-7123	S	E15	012-011	UNT EV	E4: 66.8	N	2
MD86-7277	S	UNT	040-011	CA21	93.0	Y	3
MD86-7286	S	E33	187-222	CB5	97.2	Y	2
MT87-7421	S	UNT	012-011	E3	76.6	Y	2
SC87-7477	S	UNT	012-011	E18	80.9	Y	2
GUT88-8020	S	UNT	040-011	CA21	78.0	Y	3
AL88-8149	S	UNT	040-011	EV71	81.6	Y	3
MD88-8157	S	UNT	012-011	E3	75.6	Y	2
OK88-8162	L	NT	187-222	UNT HRV	HRV2: 73.4	NT	3
MD88-8208	S	UNT	012-011	E18	78.1	Y	2
ELS88-8236	S	UNT	012-011	E12	80.8	Y	2
TN88-8321	S	UNT	040-011	CA17	79.1	TI	3
PA88-8412	S	UNT	012-011	E4	79.5	Y	2
GUT88-8438	S	UNT	040-011	CA21	78.0	Y	3
PER88-8830	S	E11	187-222	E11	80.0	Y	1
PER88-8831	S	E29	187-222	E29	78.4	Y	1
PA88-8885	S	UNT	012-011	CB5	83.5	Y	2
MEX88-8931	S	CB5	012-011	CB5	86.0	Y	1
TX88-9121	L	NT	187-222	CB3	76.6	Y	3
WA89-9165	S	UNT	040-011	CA21	93.3	Y	3
TX89-9166	S	UNT	040-011	EV71	80.8	Y	3
BRA88-9169	S	E4	040-011	CB3	83.7	TI	3
BRA88-9171	S	E29	012-011	CB3	81.5	Y	3
BRA88-9172	S	E4	012-011	CB3	83.5	Y	3
BRA88-9173	S	E4	040-011	CB3	83.9	Y	3
OK89-9243	S	UNT	040-011	EV71	81.1	Y	3
MD87-9256	S	UNT	040-011	EV71	81.3	Y	3
PA89-9262	S	CA16	040-011	CA16	77.7	TI	1
PER89-9426	S	NT	012-011	CB3	80.3	Y	3
OK89-9448	S	UNT	012-011	E18	77.6	Y	2
OK89-9452	S	E5	040-011	EV71	80.7	Y	3
NM90-9873	S	UNT	040-011	EV71	80.3	Y	3
HON88-8429	S	E11	187-222	CB4	80.9	Y	2
CA90-0150	L	NT	187-222	HRV2	92.2	NT	3

[a] All isolates were successfully amplified with pan-EV RT-PCR primers.

[b] Isolates were named according to the state or country of origin and the year of original specimen collection and the laboratory identifier. Two-letter codes are standard state abbreviations. Three-letter codes are standard World Health Organization country codes (BRA, Brazil; ELS, El Salvador; GUT, Guatemala; HON, Honduras; MEX, Mexico; MOR, Morocco; PER, Peru; and TAI, Taiwan).

[c] S, acid stable; L, acid labile.

[d] UNT, untypeable by VDN; NT, not tested.

[e] Typed with monospecific antisera as described in Materials and Methods. Y, antigenic type agreed with molecular type; N, not neutralized by monospecific antisera against any of the four highest-scoring serotypes; TI, virus titer insufficient for antigenic typing; NT, not tested.

[f] As defined in Table 4.

compared with those of the prototype EV strains, available rhinovirus VP1 sequences, and VP1 sequences of other picornaviruses, using the program Gap (3). Forty-six isolate sequences were at least 75% identical to one of the EV prototype strain sequences and were assigned that serotype (Table 3). The VA86-6776 VP1 sequence was 72.3% identical to that of the E13 prototype, Del Carmen (Table 3), and 80.7% identical to that of a recently recovered E13 strain, TX95-2089 (14). The VP1 sequence of GA84-6536 was 74.5% identical to that of the CA24 prototype strain, Joseph, and less than 70% identical to those of all other prototype strains (Table 3). IL85-6642 consistently failed to amplify with any of the VP1 primer pairs, but amplification with primer pair EV2-006 (5′ NTR-VP2) yielded a product that was 81.3% identical in sequence to the E4 prototype strain, Pesacek (Table 3). The VP1 sequences of the remaining six strains were less than 70% identical to those of all prototype enterovirus strains. CA90-0150 was identified as human rhinovirus 2 (HRV2), based on its 92.2% identity to the HRV2 prototype sequence. The VP1 sequence of OK88-8162 was 73.4% identical to that of HRV2, suggesting that it is probably of an HRV2-related serotype whose sequence is not available. For four isolates, the highest identity score was less than 70%. The VP1 sequences of CT87-7122 and CT87-7123 were 99.5% identical to one another, 84.5% identical to that of OK85-6388, and 84.8% identical to that of VA86-6765 (Table 3), indicating that all four were of the same serotype. Phylogenetic analysis indicated that OK85-6388, VA86-6765, CT87-7122, and CT87-7123 were monophyletic with respect to all known EV serotypes, supporting the conclusion that the four strains represent a single new serotype (data not shown).

Five isolates (BRA88-9169, GA84-6536, PA89-9262, TAI84-5839, and TN88-8321) grew poorly in all cell lines tested (RD, HLF, BGM, and LLC-MK_2) and could not be typed antigenically because of the low titers. For all molecularly typed EV isolates with sufficient titers (44 strains), neutralization with monospecific antisera specific for the four serotypes whose VP1 sequences most closely matched that of the unknown confirmed the result obtained by sequencing.

DISCUSSION

All of the isolates tested in this study were resistant to typing by the widely employed standard EV neutralization test using antiserum pools. The standard pools contain antibodies against only 40 of the 64 known human EV serotypes, plus E22 and E23, so their failure to neutralize a given isolate could have been due simply to the absence of homologous antibodies in the pools used (11, 12). Virus aggregation (16), antigenic variation (2, 17, 18), or the presence of a virus mixture could explain the failure of the neutralization test to identify viruses whose specificities are represented in the standard pooled antisera. In many cases, aggregation can be overcome by filtration or by treatment of the virus preparation with chloroform, reducing agents, or a nonionic detergent prior to neutralization (6, 16), but pretreatment significantly adds to an already labor-intensive procedure. The presence of a virus mixture can be overcome by plaque purification or limiting dilution, but again, these steps add significantly to the labor and cost of typing EV isolates. The detection of antigenic variants, well known among many enterovirus serotypes (2, 4, 17, 18), requires additional antisera not generally available to most clinical laboratories. Other viruses that would not be identified with the LBM pools include new EV serotypes and non-EV that replicate in EV-susceptible cells (rhinoviruses, for example). Stability to acid, traditionally used to differentiate EV from rhinoviruses, may not always be a reliable indicator, as MD84-5914 (CB2) and TX88-9121 (CB3) were acid labile and the molecular typing for both was confirmed by neutralization with monospecific antisera.

TABLE 4. Categories of typing results

Category	Description of results	No. of isolates
1	Type represented in LBM pools; VDN and sequencing agree	9
2	Type represented in LBM pools; VDN result indeterminant, VDN and sequencing disagree, or VDN not attempted[a]	19
3	Type not represented in LBM pools; serotype determined by sequencing[b]	23
4	Both VDN and sequencing indeterminant	

[a] Includes one CB2 isolate (MD84-5914) for which VDN was not attempted.
[b] Includes two CB3 isolates (TX88-9121 and PER89-9426) and two HRV isolates (OK88-8162 and CA90-0150).

Typing by antigenic and molecular methods resulted in four categories of results, summarized in Table 4. Category 1 isolates were of a serotype represented in the LBM pools whose VDN and sequencing results agreed (nine isolates). Isolates in category 2 were those determined by sequencing to be of a serotype represented in the LBM pools, but the VDN result was indeterminate or was discordant with the sequencing result (19 isolates). Sequence divergence from the prototype strains did not correlate with success or failure of the VDN test, as the partial VP1 sequences of category 1 isolates were 76 to 80% identical to the VP1 sequences of their respective prototype strains and those of category 2 isolates were 72 to 97% identical to the VP1 sequences of their respective prototypes. The wide range of sequence identities in category 2 suggests that some isolates may be antigenic variants, while others may fail in the VDN test because of aggregation or other factors. Viruses of serotypes not represented in the LBM pools could be typed only by sequencing (23 isolates) and were classified as category 3. With the exception of five low-titer isolates, the identity (determined by sequencing) of each of the EV in categories 1, 2, and 3 was confirmed by neutralization with monospecific antisera. Category 4 included isolates that could not be typed by VDN or that yielded a VP1 sequence that did not match that of any known picornavirus prototype strain by at least 70% nucleotide sequence identity (four isolates). Further characterization is needed to determine whether category 4 isolates represent new serotypes or are simply highly divergent isolates of known serotypes. In previous studies, the lowest VP1 sequence identity score for two viruses of the same serotype was 72.4% (88.7% amino acid identity) (14, 15). In the absence of a known epidemiological link, the presence of closely related viruses (OK85-6388, VA86-676 CT87-7122, and CT87-7123) in different parts of the United States in three different years suggests that these strains may represent a circulating new EV serotype.

Partial sequencing of VP1, coupled with confirmation by neutralization with monospecific antisera, successfully identified 49 of 55 virus isolates (89%), while VDN correctly identified only 9 isolates (16%). Neutralization was not attempted for the two HRV isolates. The remaining four isolates could not be identified by sequencing and appeared to represent EV other than one of the recognized 64 serotypes. VDN is not recommended as a typing method as it was much less reliable than sequencing. These results expand on our previous study, which demonstrated the use of the VP1 sequence as an EV typing tool (14), by inclusion of additional primers of broader specificity that extend to other genera of the family *Picorna-*

viridae. In addition, we have identified several strains that appear to represent new picornavirus serotypes. This method should find broad application for EV typing in the clinical virology laboratory and in reference laboratories, as well as for the identification and classification of new picornaviruses.

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REFERENCES

1. **Duggal, R., and E. Wimmer.** 1999. Genetic recombination of poliovirus *in vitro* and *in vivo*: temperature-dependent alteration of crossover sites. Virology **258:**30–41.
2. **Duncan, I. B.** 1968. A comparative study of 63 strains of ECHO virus type 30. Arch. Gesamte Virusforsch. **25:**93–104.
3. **Genetics Computer Group.** 1997. Wisconsin sequence analysis package, version 9.1. Genetics Computer Group, Madison, Wis.
4. **Grandien, M., M. Forsgren, and A. Ehrnst.** 1989. Enteroviruses and reoviruses, p. 513–569. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral, rickettsial, and chlamydial infections, 6th ed. American Public Health Association, Washington, D.C.
5. **Huttunen, P., J. Santti, T. Pulli, and T. Hyypiä.** 1996. The major echovirus group is genetically coherent and related to coxsackie B viruses. J. Gen. Virol. **77:**715–725.
6. **Kapsenberg, J. B., A. Ras, and J. Korte.** 1979. Improvement of enterovirus neutralization by treatment with sodium deoxycholate or chloroform. Intervirology **12:**329–334.
7. **King, A. M. Q., F. Brown, P. Christian, T. Hovi, T. Hyypiä, N. J. Knowles, S. M. Lemon, P. D. Minor, A. C. Palmenberg, T. Skern, and G. Stanway.** 2000. Picornaviridae. *In* M. H. V. Van Regenmortel, C. M. Fauquet, and D. H. L. Bishop (ed.), Virus taxonomy. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, New York, N.Y.
8. **Lim, K. A., and M. Benyesh-Melnick.** 1960. Typing of viruses by combinations of antiserum pools. Application to typing of enteroviruses (coxsackie and ECHO). J. Immunol. **84:**309–317.
9. **Mayo, M. A., and C. R. Pringle.** 1998. Virus taxonomy—1997. J. Gen. Virol. **79:**649–657.
10. **Melnick, J. L.** 1996. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses, p. 655–712. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Channock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
11. **Melnick, J. L., V. Rennick, B. Hampil, N. J. Schmidt, and H. H. Ho.** 1973. Lyophilized combination pools of enterovirus equine antisera: preparation and test procedures for the identification of field strains of 42 enteroviruses. Bull. W. H. O. **48:**263–268.
12. **Melnick, J. L., I. Tagaya, and H. von Magnus.** 1974. Enteroviruses 69, 70, and 71. Intervirology **4:**369–370.
13. **Morens, D. M., and M. A. Pallansch.** 1995. Epidemiology, p. 3–23. *In* H. A. Rotbart (ed.), Human enterovirus infections. ASM Press, Washington, D.C.
14. **Oberste, M. S., K. Maher, D. R. Kilpatrick, M. R. Flemister, B. A. Brown, and M. A. Pallansch.** 1999. Typing of human enteroviruses by partial sequencing of VP1. J. Clin. Microbiol. **37:**1288–1293.
15. **Oberste, M. S., K. Maher, D. R. Kilpatrick, and M. A. Pallansch.** 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J. Virol. **73:**1941–1948.
16. **Wallis, C., and J. L. Melnick.** 1967. Virus aggregation as the cause of the nonneutralizable persistent fraction. J. Virol. **1:**478–488.
17. **Wenner, H. A., P. Harmon, A. M. Behbehani, H. Rouhandeh, and P. S. Kamitsuka.** 1967. The antigenic heterogeneity of type 30 echoviruses. Am. J. Epidemiol. **85:**240–249.
18. **Wigand, R., and A. B. Sabin.** 1962. Intratypic antigenic heterogeneity of coxsackie B viruses. Arch. Gesamte Virusforsch. **12:**29–41.
19. **Yang, C.-F., L. De, S.-J. Yang, J. Ruiz Gómez, J. Ramiro Cruz, B. P. Holloway, M. A. Pallansch, and O. M. Kew.** 1992. Genotype-specific in vitro amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala. Virus Res. **24:**277–296.