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Molecular Epidemiology and Genetic Diversity of Echovirus Type 30 (E30): Genotypes Correlate with Temporal Dynamics of E30 Isolation

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Echovirus type 30 (E30) (genus, *Enterovirus*; family, *Picornaviridae*) has caused large outbreaks of aseptic meningitis in many regions of the world in the last 40 years. U.S. enterovirus surveillance data for the period 1961 to 1998 indicated that the annual proportion of E30 isolations relative to total enterovirus isolations has fluctuated widely, from a low of 0% in 1966 to a high of 42% in 1998. Peaks of E30 isolations occurred in the years 1968 to 1969, 1981 to 1984, 1990 to 1993, and 1997 to 1998, coincident with large nationwide outbreaks of E30-associated aseptic meningitis. Analysis of the complete VP1 sequence (876 nucleotides) of 136 E30 strains isolated in geographically dispersed regions of the United States and nine other countries between 1956 and 1998 indicated that the currently circulating E30 strains are genetically distinct from those isolated 30 to 40 years ago. Phylogenetic reconstruction demonstrated the existence of at least four distinct genetic groups, three of which have not been isolated in North America since 1981. Two of the three groups disappeared during periods when E30 was isolated infrequently. All North American E30 strains isolated after 1988 were closely related to one another, and all post-1993 isolates were of the same lineage within this group. Surveillance data indicate that E30 causes large national outbreaks of 2- to 4-year durations, separated by periods of relative quiescence. Our results show that shifts in the overall genetic diversity of E30 and the predominant genetic type correlate temporally with the dynamics of E30 isolation. The sequence data also provide a basis for the application of molecular techniques for future epidemiologic investigations of E30 disease.

Enteroviruses (EV) (family, *Picornaviridae*) are the major etiologic agents of aseptic meningitis, resulting in approximately 50,000 hospitalizations per year in the United States and Canada (reviewed in reference 20). Sporadic cases of aseptic meningitis are relatively common, and numerous epidemics of EV aseptic meningitis have been described (reviewed in reference 4). Echovirus type 30 (E30) is one of the most frequently isolated EV in the United States, comprising 6.8% of all reported EV isolations from 1970 to 1983 (27) and 9.5% of EV isolated from 1993 to 1996 (3). Large outbreaks of E30-associated meningitis occurred in the United States in the years 1959 to 1960, 1968 to 1969, 1981 to 1982, and 1991 to 1993 (2, 16–18, 26, 27) and in Canada in the years 1959 to 1960 and 1991 to 1992 (6, 12). In a seven-state study of the 1968 U.S. epidemic, E30 was isolated from 64% of the aseptic meningitis cases from which an EV was isolated (18). E30 was by far the most frequently isolated EV in the United States during 1991 and 1993, accounting for 21% of the total EV isolations reported in 1991 and 26% in 1993 (3).

As a group, E30 strains are antigenically heterogeneous, and three major antigenic groups have been defined, but the basis for the antigenic differences is unknown (7, 29). Previous studies on the molecular epidemiology of E30 have focused on strains isolated over a relatively short time period or in limited geographic regions (5, 6, 10). The overall genetic diversity and molecular evolution within the E30 complex and its correlation with the epidemiologic features of E30-associated disease have not yet been studied in detail. To address these questions, we determined the complete VP1 sequences for 136 geographically dispersed E30 strains isolated in 10 countries between 1956 and 1998. The sequences were analyzed in the context of 38 years of E30 virologic surveillance data. We describe here the implications of E30 temporal dynamics and genetic diversity for transmission, epidemiology, and laboratory diagnosis of EV-associated disease.

MATERIALS AND METHODS

Epidemiologic surveillance for nonpolio EV. National EV surveillance was initiated in the United States in 1961, through cooperation between the Centers for Disease Control and Prevention (CDC) and the state and territorial public health laboratories. From 1961 to 1968, the unit of reporting was the isolate, such that multiple isolations from a given case were reported separately (21). Since 1969, the unit of reporting has been the individual patient, so that the number of isolations reported and the number of cases are directly comparable (1). Because isolations of nonpolio EV are reported voluntarily, the number of states reporting may vary from year to year. U.S. EV surveillance data were summarized in aggregate most recently in 1997 (3). For each year from 1961 to 1998, the E30 isolation frequency in the United States was calculated as a fraction of the total EV isolations and plotted versus the year of isolation (Fig. 1). EV surveillance data are not routinely collected in Canada. Canadian E30 isolation frequencies were calculated as described above, using data obtained by the National Centre for Enteroviruses (NCEV) from the provincial public health laboratories in Manitoba (1987 to 1998), Ontario (1975, 1977, 1979 to 1980, and 1982 to 1988), and Saskatchewan (1998) and from the Laboratory Centres for Disease Control (1988 and 1990), as well as NCEV isolation data for the years 1991 to 1998. NCEV data include information about isolates from British Columbia, New Brunswick, Newfoundland, Nova Scotia, and Prince Edward Island. Australian

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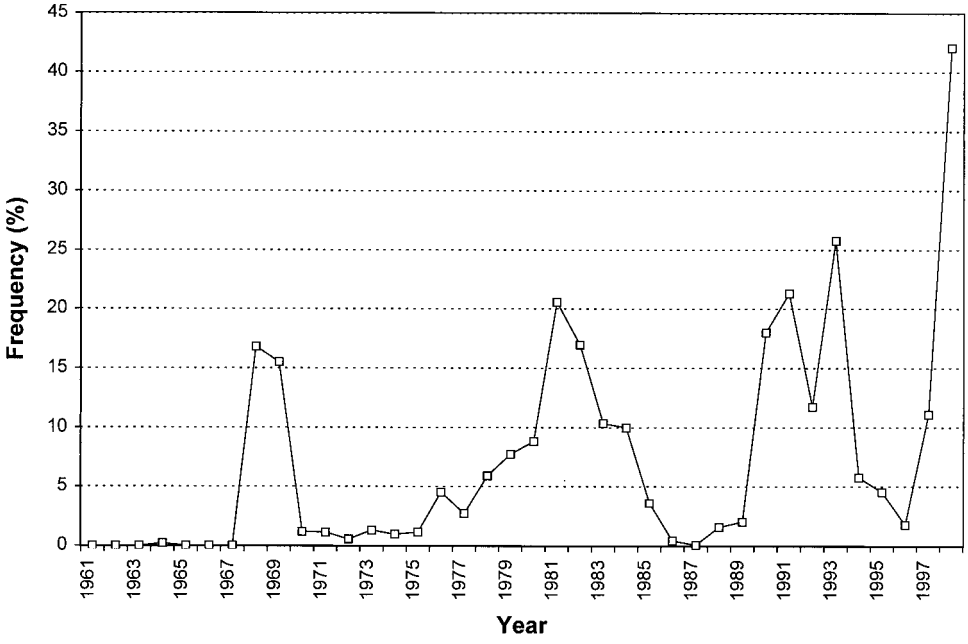

FIG. 1. Frequency of E30 isolation in the United States between 1961 and 1998. E30 isolations are expressed as a proportion of total EV isolations for a given year, as reported to the CDC by state and territorial public health laboratories through the U.S. EV surveillance program.

EV surveillance data are collected independently by each state. E30 data for the state of Victoria are included here, based on EV isolation and typing by the Entero-Respiratory Laboratory, Fairfield Hospital and Victorian Infectious Diseases Reference Laboratory (VIDRL), Melbourne, Victoria, Australia.

Viruses. The E30 strains studied were chosen from the EV collections of the CDC, Atlanta, Ga.; Fairfield Hospital and VIDRL; the NCEV, Halifax, Nova Scotia, Canada; and the California Department of Health Services, Berkeley, Calif. The selected strains represent a wide range, both temporally and geographically, of all E30 isolates in our collections. Recent strains were also solicited from other laboratories, including the Instituto Nacional de Salud, Bogotá, Colombia, and the Robert Koch-Institut, Berlin, Germany. Where possible, at least three isolates were chosen for each year, with isolates from different states or provinces that were geographically dispersed within their respective countries. For years in which a virologically confirmed E30 outbreak had occurred, up to 10 isolates were chosen to determine the dynamics of E30 evolution within an epidemic period. Viruses were isolated from original clinical specimens and propagated in cell culture by standard methods (11). Isolates were typed by neutralization assay, using standard antiserum pools (19), and most typings were confirmed with monospecific antisera. Virus isolates were stored as unpurified cell culture supernatants at −20°C. Some of the isolates had been stored for as long as 30 years without additional passage prior to RNA extraction for reverse-transcription PCR (RT-PCR).

RT-PCR and sequencing. RNA isolation and RT-PCR were carried out as described previously (23). The complete VP1 region of each isolate was amplified as a single fragment by using flanking primers in VP3 (008, GCRTGCAATGA YTTCTCWGT; nucleotides [nt] 2411 to 2430, poliovirus type 1 [PV1] numbering) and 2A (011, GCICCIGAYTGITGICCRAA; nt 3408 to 3389, PV1 numbering) or as two overlapping fragments by using primer 008 with 013 (GGIGC RTTICCYTCIGTCCA; nt 3051 to 3032, PV1 numbering) and primer 012 (AT GTAYGTICCICCIGGIGG; nt 2951 to 2970, PV1 numbering) with 011 (23). The PCR products were gel isolated and purified for sequencing by using a QIAquick Gel Extraction kit (Qiagen, Inc., Santa Clarita, Calif.). Both strands were sequenced by automated methods, using fluorescent dideoxy-chain terminators (PE Biosystems, Foster City, Calif.). These sequences have been deposited in the GenBank database.

Sequence analysis. Nucleotide and deduced amino acid sequences were aligned by using the Pileup program (9). The maximum likelihood method of Kishino and Hasegawa (15) with an empirical transition/transversion ratio, calculated as 4.87 by Puzzle (28), was used to construct a distance matrix for phylogenetic analysis. Phylogenetic relationships were inferred by using the programs DNAdist/Neighbor (PHYLIP version 3.57 [8]) and Puzzle (version 4.0 [28]). Support for specific tree topologies was estimated by bootstrap analysis with 100 pseudoreplicate data sets (DNADist/Neighbor) or by using 1,000 puzzling steps (Puzzle). Branch lengths of the neighbor-joining trees were calculated by the maximum likelihood quartet-puzzling method, using Puzzle.

Nucleotide sequence accession numbers. The sequences described above have been deposited in the GenBank database under accession no. AF127983 to AF128090 and AF152866 to AF152891.

RESULTS

Temporal and geographic patterns of E30 isolation in the United States and Canada. Very few E30 isolates were reported between 1961 and 1967, but E30 accounted for almost 17% of all EV isolations in the United States in 1968 and over 15% in 1969 (Fig. 1), coinciding with a widespread epidemic of E30-associated aseptic meningitis (18). From 1970 to 1975, E30 once again comprised less than 2% of all EV isolations in the United States. The frequency of E30 isolations began to increase in 1976, reaching 20% of all EV isolates in 1981, and then declined to less than 1% in 1986. E30 remained a minor proportion of EV isolates until 1990, when another nationwide epidemic of E30 meningitis began, and the E30 isolation frequency increased from 2% in 1989 to 18% in 1990. The frequency remained high throughout the epidemic period, reaching 25% in 1993, and then declined to about 6% in 1994 and remained low through 1996. The frequency again increased in 1997, to 11%, and swelled to 42% in 1998, coincident with another widespread epidemic of E30 meningitis.

During 1998, E30 was reported by 10 of the 12 states that provided EV isolation data to the CDC (Arizona, California, Connecticut, Florida, New Mexico, Oklahoma, South Carolina, Tennessee, Texas, and West Virginia), as well as 4 other states that sent isolates to the CDC for reference testing (Hawaii, Idaho, Maine, and South Dakota), indicating that the epidemic was widespread across the United States. Texas and Oklahoma accounted for 83% of the reported U.S. E30 isolates in 1998, reporting 182 and 35 isolates, respectively. On the basis of limited available data, periods of increased E30 activity in Canada have coincided with those observed in the United States, with E30 accounting for 35% of EV isolates in 1991 and 49% in 1998 (data not shown). As in the United

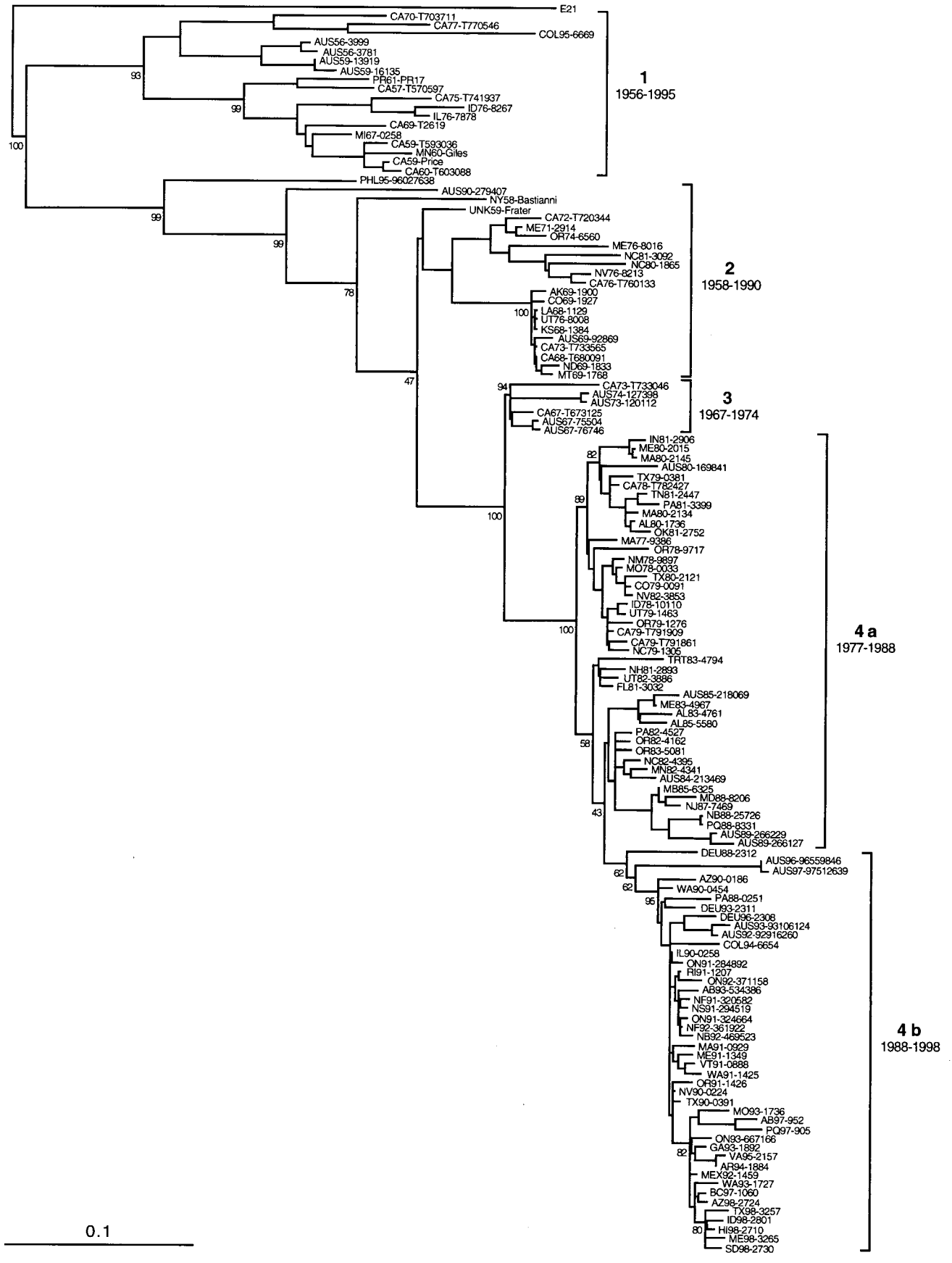
E21
CA70-T703711
CA77-T770546
COL95-6669
AUS56-3999
AUS56-3781
AUS59-13919
AUS59-16135
PR61-PR17
CA57-T570597
CA75-T741937
ID76-8267
IL76-7878
CA69-T2619
MI67-0258
CA59-T593036
MN60-Giles
CA59-Price
CA60-T603088
1
1956-1995
PHL95-96027638
AUS90-279407
NY58-Bastianni
UNK59-Frater
CA72-T720344
ME71-2914
OR74-6560
ME76-8016
NC81-3092
NC80-1865
NV76-8213
CA76-T760133
AK69-1900
CO69-1927
LA68-1129
UT76-8008
KS68-1384
AUS69-92869
CA73-T733565
CA68-T680091
ND69-1833
MT69-1768
2
1958-1990
CA73-T733046
AUS74-127396
AUS73-120112
CA67-T673125
AUS67-75504
AUS67-76746
3
1967-1974
IN81-2906
ME80-2015
MA80-2145
AUS80-169841
TX79-0381
CA78-T782427
TN81-2447
PA81-3399
MA80-2134
AL80-1736
OK81-2752
MA77-9386
OR78-9717
NM78-9897
MO78-0033
TX80-2121
CO79-0091
NV82-3853
ID78-10110
UT79-1463
OR79-1276
CA79-T791909
CA79-T791861
NC79-1305
TRT83-4794
NH81-2893
UT82-3886
FL81-3032
AUS85-218069
ME83-4967
AL83-4761
AL85-5580
PA82-4527
OR82-4162
OR83-5081
NC82-4395
MN82-4341
AUS84-213469
MB85-6325
MD88-8206
NJ87-7469
NB88-25726
PQ88-8331
AUS89-266229
AUS89-266127
4 a
1977-1988
DEU88-2312
AUS96-96559846
AUS97-97512639
AZ90-0186
WA90-0454
PA88-0251
DEU93-2311
DEU96-2308
AUS93-93106124
AUS92-92916260
COL94-6654
IL90-0258
ON91-284892
RI91-1207
ON92-371158
AB93-534386
NF91-320582
NS91-294519
ON91-324664
NF92-361922
NB92-469523
MA91-0929
ME91-1349
VT91-0888
WA91-1425
OR91-1426
NV90-0224
TX90-0391
MO93-1736
AB97-952
PQ97-905
ON93-667166
GA93-1892
VA95-2157
AR94-1884
MEX92-1459
WA93-1727
BC97-1060
AZ98-2724
TX98-3257
ID98-2801
HI98-2710
ME98-3265
SD98-2730
4 b
1988-1998
0.1

TABLE 1. Summary of pairwise nucleotide and amino acid sequence comparisons among E30 isolates[a]

Group	Sequence differences within and among the indicated groups							
	Nucleotide				Amino acid			
	1	2	3	4	1	2	3	4
1	0.1–20.6	21.6–25.1	21.6–24.8	21.5–26.4	0.3–6.9	6.9–12.7	7.2–11.3	7.2–13.0
2		0.2–12.3	7.1–13.5	10.1–16.0		0.0–6.2	3.1–6.5	3.8–7.9
3			0.6–7.9	6.2–12.0			1.0–2.8	1.4–5.1
4				0.1–10.8				0.0–6.9

[a] The data indicate the range of sequence differences (percent) within and among the four E30 genetic groups.

States, E30 was widespread in Canada in 1998, as it was, by far, the most commonly isolated EV in seven provinces stretching across the country (British Columbia, Manitoba, Saskatchewan, New Brunswick, Newfoundland, Nova Scotia, and Prince Edward Island).

Phylogenetic clustering of E30 isolates. To analyze the genetic diversity and molecular aspects of E30 epidemiology, 136 strains isolated from patients in 10 countries during the period 1956 to 1998 were chosen as described in Materials and Methods. The complete VP1 sequences were determined, and all were 876 nt in length (292 amino acids). Phylogenetic trees were constructed from the aligned nucleotide sequences by using the maximum likelihood and neighbor-joining methods (Fig. 2). E30 isolates were monophyletic with respect to all other EV serotypes and E21 was the nearest outgroup taxon, in accordance with previously published EV VP1 phylogenies (23). E30 sequences segregated into four distinct major groups based largely on year of isolation, with some temporal overlap. Reference strains MN60-Giles, PR61-PR17, and CA59-Price, as well as 17 other isolates from the period 1956 to 1995, constituted genetic group 1. COL95-6669 was the only post-1977 isolate in group 1. Group 2 included the UNK59-Frater reference strain, a 1969 Victoria isolate, seven 1968 to 1969 U.S. epidemic isolates (from seven states), and ten viruses isolated from six states (California, Maine, Oregon, Nevada, North Carolina, and Utah) during 1971 to 1981. Phylogenetic analysis placed NY58-Bastianni and AUS90-279407 in a position intermediate between groups 1 and 2, but amino acid sequence comparisons suggested that they are part of group 2 (see below). Two California isolates, from 1967 and 1973, and four Australian isolates, from 1967, 1973, and 1974, were the only members of genetic group 3. Group 4 was composed of two subgroups of distinct, but closely related viruses. Subgroup 4a contained viruses isolated between 1977 and 1989 in Victoria, Australia, three Canadian provinces, and 24 U.S. states, including most of the states represented in group 2. All E30 strains isolated after 1989, except COL95-6669, AUS90-279407, and PHL95-96027638, were members of subgroup 4b. All strains isolated in North America after 1993 were members of a single lineage, suggesting that the current North American epidemic strains are all derived from a common ancestor. Isolates from Australia in 1996 and 1997 formed a cluster distinct from the other subgroup 4b strains. Canadian isolates, available only for the period 1988 to 1997, were closely related to isolates that circulated in the United States during the same period, as were the small number of German isolates. PHL95-96027638 was phylogenetically distinct from all other isolates and occupied a position between groups 1 and 2.

Genetic divergence among E30 isolates. Pairwise sequence comparisons showed that all E30 isolates differed from the E21 prototype strain, Farina, by 27.9 to 33.1% (data not shown). Pairwise comparisons among E30 isolates are summarized in Table 1. Isolates of group 1 were the most divergent from strains of other groups, differing in nucleotide sequence from other E30 isolates by 21.5 to 26.4% (up to 13.0% amino acid sequence difference). Within group 1, strains differed from one another by up to 18.2% in nucleotide sequence (up to 6.9% amino acid sequence difference). Within group 2, nucleotide sequences varied by up to 12.3% (up to 5.5% amino acid sequence difference). In amino acid sequence, the E30 prototype strain (NY58-Bastianni) was at least 91% identical to all other E30 strains, except those of group 1. Group 4 was genetically more homogeneous than groups 1 and 2, with a maximum of 10.8% nucleotide sequence divergence within the group.

DISCUSSION

EV surveillance data for the United States and Canada are incomplete, due to the voluntary nature of reporting and the fact that only a subset of state and provincial public health laboratories type their EV isolates. Nevertheless, the data show the basic pattern of activity for a given virus serotype and define temporal peaks and major shifts in serotype predominance (3, 27). The many EV serotypes differ in their isolation frequency and in the shape of the isolation frequency curve. For example, coxsackievirus B3 is isolated at a relatively constant rate, whereas coxsackievirus B5 is most often associated with epidemics and is otherwise relatively rare (24). The factors influencing EV prevalence are poorly understood but may include duration of serotype-specific immunity, efficiency of transmission, severity of symptoms, and ease of isolation, all of which could influence identification and reporting. The annual frequency of E30 isolation in the United States and Canada has varied widely during the past 38 years, with several major peaks corresponding to large regional or nationwide E30 asep-

FIG. 2. Phylogram depicting the phylogenetic relationships among 136 E30 strains isolated between 1956 and 1998. U.S. and Canadian isolates are listed by state or province (by their postal abbreviations), year of isolation (last two numbers of year), and laboratory identifier; all others are listed by country, year (last two numbers of year), and laboratory identifier, using World Health Organization-standard three-letter country codes: AUS, Australia; COL, Colombia; DEU, Germany; MEX, Mexico; PHL, Philippines; TRT, Trinidad and Tobago; and UNK, United Kingdom. All Australian isolates were from Victoria. Phylogenetic reconstruction was performed by using the neighbor-joining method with a maximum likelihood distance matrix (8). Branch lengths were calculated by the maximum likelihood method by using the Puzzle program (28). E21 was included as the outgroup, but the tree is unrooted. Numbers adjacent to nodes represent percent bootstrap support for the clusters to the right of the node for major genetic groups and for clusters of at least five members with greater than 80% bootstrap support. Major genetic groups and the years in which they have been observed are shown.

tic meningitis epidemics (Fig. 1 and data not shown). The pattern of E30 isolation was similar in Victoria, Australia, but the peaks were offset by 2 to 3 years from those in North America (13). Together, the surveillance data indicate that E30 follows an epidemic mode of transmission, causing large outbreaks and then becoming quiescent for a period of several years, rather than an endemic mode, with a relatively constant isolation frequency. The quiescence is most probably due to the development of population immunity that occurs in a high-infection-rate epidemic. The virus may cause only sporadic cases until a large cohort of nonimmune individuals has developed, often over a period of several years, setting the stage for another large epidemic.

At least four distinct North American E30 genetic groups have existed since the serotype was discovered in the late 1950s. With the exception of AUS90-279407, recent isolates from Australia and Germany were closely related genetically to North American strains from the same time period, suggesting either a global E30 reservoir or the rapid transmission of viruses from one region to another. Until the mid-1970s, at least three different E30 genotypes circulated simultaneously in the United States, with group 1 in California, Idaho, and Illinois, group 2 in California, Maine, Nevada, Oregon, and Utah, and group 3 in California. Group 1 apparently disappeared from North America shortly thereafter, during a period of relatively low E30 activity (Fig. 1 and 2), but it apparently continues to circulate in South America, as demonstrated by the isolation of COL95-6669 in 1995. In the United States, group 2 was apparently replaced by group 4 in about 1981, near the peak of a large U.S. epidemic. Group 2 accounted for only 2 of 42 isolates analyzed from the period 1977 to 1986, suggesting that it was a very minor contributor to the 1980s epidemic. The breadth of the 1980 to 1984 epidemic peak (Fig. 1) suggests that it may actually represent two overlapping epidemics, one caused by group 2 viruses and the other due to group 4 viruses. Group 4, which arose in about 1977 and became dominant after 1981, could be subdivided into two subgroups. Subgroup 4a circulated only from 1977 to 1989 and was the predominant E30 genotype during the 1980s epidemic. Viruses isolated during epidemics in the 1990s were all of subgroup 4b, which had apparently arisen in about 1988. In 1991 and 1998, closely related viruses were isolated from throughout North America (Fig. 2), confirming the potential of E30 to transmit rapidly over a large geographic region.

It is possible that groups 1 to 3 continue to circulate in North America but were absent from our analysis because we had sampled an insufficient number of isolates or because these virus groups were associated with only minor disease and thus would not have been isolated. We consider these explanations unlikely, as we have sequenced many recent isolates and none have been of groups 1 to 3. Although isolation studies are usually performed only on specimens from ill patients who seek medical care, there is no evidence that disease caused by group 1 to 3 viruses is any different from that caused by viruses of group 4. Other than the decline of group 2, all shifts in E30 group prevalence have occurred during a period of relatively low E30 activity, suggesting that the occurrence of a large epidemic somehow may contribute to the change in predominant genotype. One result of a large epidemic is to reduce the population of nonimmune (susceptible) individuals available for additional cycles of virus transmission. In the case of poliovirus, reduction in the population of susceptibles by immunization results in virus population bottlenecks that ultimately lead to genotypic extinction and reduced genetic diversity (14). It is possible that large-scale natural E30 infection may have the same effect as large-scale polio immunization in reducing the population of susceptibles, thus interrupting chains of transmission.

Early studies demonstrated broad antigenic heterogeneity among E30 strains and the existence of three major antigenic groups, with up to a 100-fold difference between homologous and heterologous reciprocal neutralization titers (7, 29). Genetic characterization of five strains included in those studies indicated that their nucleotide sequences differed from one another by 10 to 25% (2 to 12% amino acid difference) in both the 5′ end of VP2 (22) and in the complete VP1 gene (23). The VP1 sequence relationships among strains NY58-Bastianni, UNK59-Frater, MN60-Giles, CA59-Price, and PR61-PR17 mirror their antigenic relationships (Fig. 2) (23). Antigenic group I, represented by the Bastianni and Frater strains, appears to correspond to genetic group 2. Giles and Price, genetic group 1 strains, comprise antigenic group II. PR17, also a member of genetic group 1, is the sole isolate in antigenic group III, based on cross-neutralization data. Complement fixation and hemagglutination inhibition tests suggested that PR17 may be antigenically related to Giles and Price (29), in agreement with the genetic comparisons; therefore, antigenic groups II and III may actually represent variation within a single genetic group. It will be interesting to see whether sequence differences in other parts of the capsid correlate with the observed antigenic differences. For example, phylogenetic trees constructed with partial VP2 sequences of NY58-Bastianni, UNK59-Frater, MN60-Giles, and PR61-PR17 were not congruent to those constructed with VP1 sequences (22, 23). Because of its broader antigenicity, Bastianni (genetic group 1) was designated the E30 prototype for production of standard diagnostic reagents (19, 25). The antigenic relationship of genetic group 3 and 4 viruses to genetic group 1 and 2 strains has not yet been addressed. The nature of EV serotype-specific epitopes is unknown, as is the nature of the epitopes responsible for E30 antigenic heterogeneity; however, the genetic divergence of currently circulating strains from Bastianni and other early E30 isolates suggests that the typing reagents produced almost 40 years ago, using Bastianni as the immunogen, may be inadequate for the identification of recent and future E30 isolates. The monophyly of E30 VP1 sequences with respect to other EV serotypes and the low genetic diversity among recent isolates suggest that molecular typing based on VP1 sequence may provide a more reliable means for the typing of current and future E30 isolates.

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