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Analysis of Echovirus 30 Isolates from Russia and New Independent States Revealing Frequent Recombination and Reemergence of Ancient Lineages▽

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We studied two genome regions, VP1 and 3D, of 48 echovirus 30 (E30) isolates from Russia and the new independent states. In VP1, most isolates were similar to European strains reported earlier, and frequent change of circulating subgroups was noticed. We also observed, in 2003–2006, the reemergence of a group of E30 strains with a VP1 region very distant from most modern E30 strains and remotely similar to E30 isolates from the 1960s and the 1970s. A study of the 3D genome region detected multiple recombination events among the studied strains. Recombination presumably occurred every few years, and therefore, the study of a single VP1 genome region cannot accurately describe the phylogenetic history of the virus or predict pathogenetic properties of an isolate. In general, a comparison of the VP1 and 3D genome region phylogenies revealed, in some instances, virtually independent circulation of enterovirus genome fragments on a scale of years.

Echovirus 30 (E30) is a member of the human enterovirus B (HEV-B) species, which also includes 28 serotypes of echoviruses, 6 coxsackie B viruses, coxsackie A9 virus and several new enterovirus serotypes. Enteroviruses cause a wide spectrum of clinical manifestations. Most frequently, the infection is asymptomatic or subclinical; however, severe and life-threatening forms are not rare (18). E30 has recently been a common cause of meningitis in many countries (4, 5, 7, 9, 12, 14, 19) and was a subject of several epidemiological studies (2, 15, 21); therefore, many sequences for the VP1 genome region are available in GenBank for comparison. Previously, however, most studies of E30 epidemiology were conducted by using only the VP1 genome region. A number of recent publications revealed that intertypic recombination is a very frequent event in circulating enteroviruses, thus allowing virtually independent evolution of different genome regions (8, 10, 16). In this work, we studied the epidemiology of E30 in Russia and the new independent states (NIS) in 1998–2006 by using two genome regions, VP1 and 3D, to estimate the role of recombination in the short-term epidemiology of enteroviruses.

MATERIALS AND METHODS

We used 48 strains of E30 isolated in the course of the WHO polio surveillance program and enterovirus surveillance (Table 1). Virus isolation and identification were carried out according to a standard WHO protocol (24) by using RD and Hep-2 cell cultures. Strain serotypes were identified by neutralization test with antisera produced by RIVM (Bilthoven, The Netherlands). Virus RNA was isolated from cell culture supernatant by guanidine thiocyanate lysis and adsorption to silica (3). Reverse transcription was carried out using Moloney murine leukemia virus reverse transcriptase (Promega) and random hexanucleotide primers. PCR was performed with previously published oligonucleotides for VP1 (15) and 3D (11). All nucleotide positions are given according to the prototype E30 strain Bastianni (GenBank accession no. AF311938). After amplification, bands were excised from agarose gels, purified using QIAquick kits (Qiagen), and sequenced directly with the PCR primers. Nucleotide sequences were aligned using ClustalX software (23). Phylogenetic trees were constructed with ClustalX (neighbor-joining algorithm), with correction for multiple substitutions and excluding positions with gaps, and with 1,000 bootstrap pseudoreplicates. RNA and protein sequence distances were calculated with the PHYLIP software package (6). GenBank accession numbers for sequences are provided in Table 1.

RESULTS AND DISCUSSION

In the VP1 genome region (nucleotides [nt] 2460 to 3335), all E30 strains differed by up to 31% of nucleotide sequence (DNADIST, Kimura model) and by up to 20.5% of protein sequence (PROTDIST, Jones-Taylor-Thornton distance matrix). All strains grouped with the prototype E30 Bastianni relative to enteroviruses of other serotypes, thus confirming the serotype. We used 75 of over 150 GenBank sequences available for E30 VP1 that were reported previously (1, 15, 21), excluding very similar (less than 2% nucleotide sequence difference) strains from the same geographic region. The nucleotide sequence of the prototype E21 Farina, the closest HEV-B serotype to E30, was used to root the phylogenetic tree, but was omitted from Fig. 1. Most of the strains studied fell into a major phylogenetic group that included most E30 strains isolated worldwide since 1978 and sequenced so far. This group was reported previously in several studies (15, 21); therefore, the majority of the strains from Russia and the NIS studied here were similar to other recent E30 isolates reported elsewhere. One explanation for the observed global group of modern strains that differ from each other far less (below 13% nucleotide sequence, PHYLIP Dnadist, Kimura model) than do the E30 isolates from the 1960s and the 1970s (up to 32% of nucleotide sequence) is probably the higher fitness of this genotype, as was hypothesized previously (21). As shown by Fig. 1, most of the modern strains had the same putative ancestor relative to the strains from the 1960s and 1970s. We also suggest that an increase in economical ties and the spread

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TABLE 1. E30 strains used for the study[a]

Isolate no.	Place of isolation	Sampling date[b]	Clinical data	VP1 GB no.	3D GB no.
8477	Kola Peninsula, Russia	1998	Meningitis	AY371478	AY896767*
10331	Azerbaijan	05-07-1999	Healthy child	AY371461	EU280275
10334	Azerbaijan	05-07-1999	Healthy child	AY371456	EU280261
11202	Ukraine	02-10-1999	Encephalitis	AY371469	EU280264
11203	Ukraine	28-09-1999	Meningitis	AY371471	EU280270
11272	Georgia	19-11-1999	Healthy child	AY371464	EU280259
11273	Georgia	19-11-1999	Healthy child	AY371465	EU280267
11388	Central Russia	18-08-1999	Meningitis	AY371472	EU280268
12202	Georgia	13-04-2000	AFP	AY371457	EU280266
13022	Southern Russia	05-07-2000	Healthy child	AY371458	EU280260
13162	Georgia	13-07-2000	Gastroenteritis	AY371462	EU280263
13168	Georgia	11-07-2000	Neurological disorder	AY371463	EU280271
13484	Southern Russia	26-09-2000	Meningitis	AY371459	EU280274
13726	Southern Russia	11-2000	Meningitis	AY371460	EU280279
14121	Ukraine	22-11-2000	AFP	AY371470	EU280265
14125	Ukraine	30-11-2000	Healthy child, AFP contact	AY371467	AY896766*
17891	Kalmykia, Russia	02-06-2002	Meningitis	AY371481	EU280272
17909	Kalmykia, Russia	09-06-2002	Meningitis	AY371479	EU280269
18102	Kalmykia, Russia	27-06-2002	Healthy, meningitis contact	AY371480	EU280262
18113	Kalmykia, Russia	26-06-2002	Healthy, meningitis contact	AY371482	EU280258
18121	Kalmykia, Russia	27-06-2002	Healthy, meningitis contact	AY371483	EU280276
18733	Moldova	16-07-2002	Meningitis	AY371476	EU280290
19167	Far East Russia	14-09-2002	Meningitis	AY371474	EU280277
20798	Siberia, Russia	13-09-2003	Meningitis	EU280304	EU280249
20829	Far East Russia	31-07-2003	AFP	EU280305	EU280255
20885	Moldova	05-08-2003	Meningitis	EU280301	EU280284
20965	Central Russia	12-09-2003	Sewage	EU280295	EU280254
21003	St. Petersburg, Russia	23-09-2003	AFP	EU280303	EU280257
21093	Southern Russia	03-10-2003	AFP	EU280297	EU280285
21460	Georgia	20-10-2003	Healthy	EU280292	EU280256
21740	Ukraine	04-11-2003	Sewage	EU280302	EU280253
22127	Kyrgyzia	29-04-2004	Suspected EV infection	EU280298	EU280252
22541	Moscow, Russia	15-08-2004	AFP	EU280312	EU280250
22663	Moscow, Russia	01-08-2004	Meningitis	EU280300	EU280283
22696	Central Russia	20-07-2004	Suspected EV infection	EU280296	EU280281
22763	Georgia	05-08-2004	Sewage	EU280291	EU280248
22885	Siberia, Russia	24-07-2004	Meningitis	EU280299	EU280251
23184	Central Russia	14-07-2003	Meningitis	EU280293	EU280280
23199	Siberia, Russia	08-2004	Meningitis	EU280310	EU280273
23202	Siberia, Russia	08-2004	Meningitis	EU280309	EU280289
23324	Southern Russia	02-11-2004	Meningitis	EU280294	EU280282
24881	Ukraine	23-08-2005	AFP	EU280308	EU280278
25039	Ukraine	15-09-2005	AFP	EU280311	EU280288
25093	Kirghizia	03-10-2005	Polyradiculoneuritis	EU280307	EU280286
25105	Uzbekistan	31-08-2005	AFP	EU280306	EU280287
25729	Uzbekistan	28-04-2006	AFP	EF397645	EF397664
26337	Far East Russia	09-08-2006	Meningitis	EF397655	EF397660
26346	Far East Russia	29-07-2006	Meningitis	EF397656	EF397661

[a] AFP, acute flaccid paralysis; GB, GenBank; *, complete genome sequence is available for this strain.
[b] Dates are presented as year, day-month-year, or month-year.

of air travel have resulted in the emergence of a common epidemiological space in Europe and North America, where a more fit capsid could efficiently prevail in a matter of several years.

Within the "modern" group, phylogenetic subgrouping did not always correlate with isolation location, but rather correlated with the time of isolation, which indicates a very dynamic epidemiological pattern. In general, we observed limited temporal overlap between subgroups that circulated in Russia and the NIS. Sublineages of E30 demonstrate quick and wide spreading within months over vast distances and then vanish to give space to newer lineages. It seems unlikely to us that the epidemiology of E30 is significantly driven by herd immunity pressure, as the sequence difference between most subgroups implies only minor serological differences.

Some strains in the major modern group, such as those in subgroups 1, 2, and 3 (Fig. 1), presumably arrived from Western Europe not very long before isolation in Russia. These strains originated mainly from Russia and the western NIS; however, strains of subgroup 2 could be found in Middle Asia (in Kirghizia). This observation is not unexpected and shows that the epidemiological space of the former USSR is significantly integrated into the European epidemiological space. Strains that comprise subgroup 4 seemingly diverged from the main E30 lineage over two decades ago, as they are almost an

FIG. 1. Phylogenetic tree (ClustalX, neighbor joining) for complete VP1 genome region (nt 2460 to 3335). Numbers at tree nodes are a percentage of bootstrap pseudoreplicates that supported a group below; bootstrap values below 70% were omitted. The tree was rooted with E21 Farina strain (omitted). E30 sequences from GenBank have accession numbers. ■, group 1; +, group 2; *, group 3; ●, group 4; ▲, group 5.

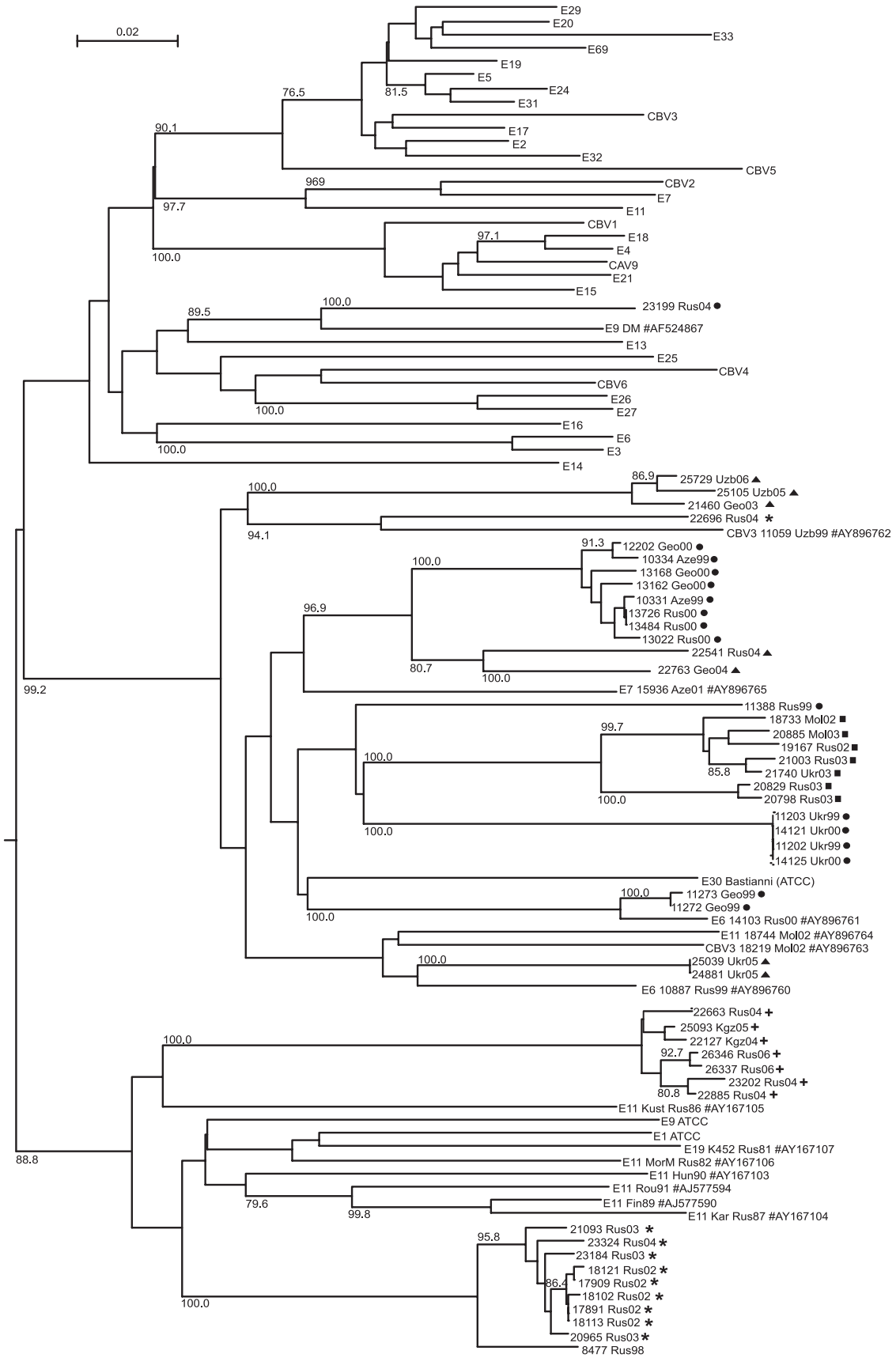

FIG. 2. Phylogenetic tree (ClustalX, neighbor-joining) for partial 3D genome region (nt 6468 to 6931). Numbers at tree nodes are a percentage of bootstrap pseudoreplicates that supported a group below; bootstrap values below 70% were omitted. The tree was rooted with poliovirus 1 (omitted). Prototype HEV-B strains do not have additional indications, studied E30 strains do not have serotypes indicated, and all modern HEV-B strains of different serotypes are given with isolation data and GenBank accession number. ■, group 1; +, group 2; *, group 3; ●, group 4; ▲, group 5.

out-group within the major modern group. Viruses of subgroup 4, especially strain 23199, were similar to recent Chinese isolates and probably represent an Asian sublineage. It should be noted that we observed nonuniform rates of E30 isolation within Russia and the NIS. Most strains studied here came from Russia and Caucasia and Western countries, and only several E30 strains were among many HEV-B isolates from Middle Asia.

Rather unexpectedly, we observed a group of six strains (subgroup 5) (Fig. 1) that was strikingly different from most other modern E30 strains. These isolates were only somewhat similar to a Columbian strain isolated in 1995 and probably originated from countries where enterovirus surveillance was not performed. A close look at subgroup 5 does not allow inferring the spreading pattern of these strains. Older Georgian strains make a subset within subgroup 5; therefore, multiple introductions of different sublineages of this group likely took place (see below). Reemergence of subgroup 5 strains that fell outside the modern E30 major group demonstrates that surveillance carried out in developed countries for E30, arguably the best-studied nonpolio enterovirus, does not explicitly reflect world epidemiology of E30. We speculate that strains of this group originated from less-developed regions with less dynamic and less "global" epidemiologies, where they could have been maintained for decades. This observation not only is important for an understanding of E30 or HEV-B epidemiology but also implies that "eradicated" polio may reemerge decades later from secluded reservoirs.

Partial sequences for 3D genome fragments (nt 6468 to 6931 of E30 Bastianni) of the strains studied were aligned with available sequences of HEV-B strains of different serotypes, as E30 could be expected to recombine extensively with other HEV-B strains in the nonstructural protein (NSP) region (8, 10). Enterovirus B strains differed by up to 31.5% of nucleotide sequence and by up to 9.7% of protein sequence; thus, a majority of substitutions in this genome region were synonymous. A phylogenetic tree was created with poliovirus 1 sequence added to provide a correct root (Fig. 2). E30 strains studied here followed a grouping pattern reported previously for most modern HEV-B strains that cluster either with prototype E30 or with E1/E9, yet again supporting an observation of ubiquitous recombination in enteroviruses (10, 17, 20). In this work, however, we were mostly interested in a short-term incidence of recombination among strains isolated within only 7 years. A comparison of phylogenetic grouping in VP1 and 3D genome regions indicates many occasions of recombination over this short time frame. Only two phylogenetic groups observed in VP1, subgroups 1 and 2, were fully maintained in the 3D genome region. Within subgroup 3, strain 22696, indistinguishable from the group in VP1 and isolated close in time and location with strain 23184, was clearly recombinant in 3D. Viruses of subgroups 4 and 5, which were very similar and grouped very reliably in VP1, bore multiple marks of recombination in 3D. Strains of subgroup 4 possessed five different 3D polymerase regions (Fig. 2). These strains were hardly distinguishable in the VP1 region, which is traditionally used for typing, yet very diverse in the 3D region and, presumably, in most of the nonstructural genome region. Interestingly, strain 23199 had a 3D region very distant from those of most modern strains, again showing that rare enterovirus genome parts can persist and then reemerge in a virus that would not seem unusual from the conventional VP1 region analysis. Strains of subgroup 5 have also undergone multiple recombination events and fell into three distinct groups in the 3D genome region. Again, as was observed for subgroup 3, strains isolated at the same location in Georgia, within a 10-month interval and very similar in VP1, bore notably different nonstructural genome regions. We can thus conclude that subgroup 5 in VP1 indicates the emergence not of a new virus lineage, but of a new capsid lineage.

Phylogenetic trees for structural and nonstructural genome regions indicate that different genome regions in enteroviruses circulate independently even on a scale of a few years. While it would not be correct to estimate the exact frequency of recombination in circulating E30 from our data, it is perfectly obvious that it is about once every few years, much as was recently shown for HEV-B (22). Indeed, 16 of 47 strains studied probably recombined within the last five years before being isolated and all E30 strains of the major modern group in the VP1 genome region were recombinant in 3D compared not only to the prototype strains of the 1950s but also to the HEV-B isolated in the 1980s and 1990s. Considering our results, especially those for subgroups 4 and 5, it would be incorrect to say that one can trace the circulation of individual strains; rather, one can trace the circulation of discrete capsid or nonstructural genome regions that only temporarily coexist as a distinct virus. Importantly, in the NSP region, E30 can recombine with any HEV-B strain; therefore, study of a single serotype would always produce a rather limited result that would not clearly reflect the epidemiology of NSP genome regions of a species. Flexible and highly dynamic genetics and epidemiology of circulating E30 and presumably of other enteroviruses oblige us to expect the emergence and rapid spread of strains with unusual properties. Our results also indicate that as long as enterovirus surveillance is carried out using only the VP1 genome region, either molecularly or in a neutralization test, we have a significantly reduced chance of linking the clinical manifestations and epidemiology of enterovirus infection.

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ADDENDUM

After the manuscript was submitted, a study of E30 epidemiology in France also reported frequent recombination between VP1 and 3D regions (13).

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