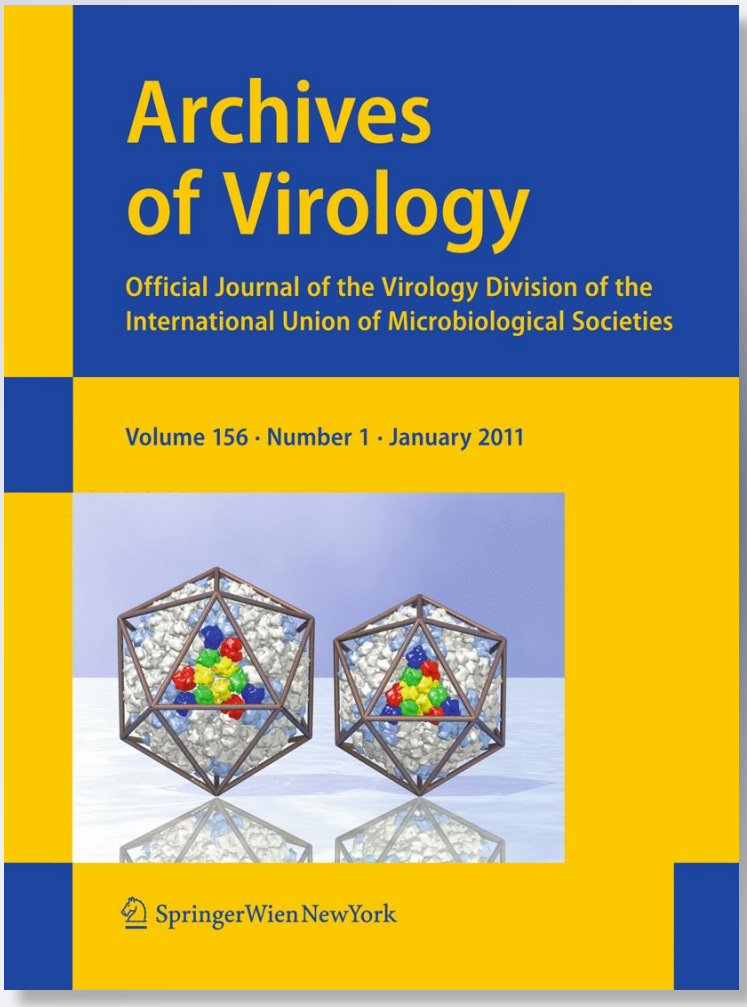
Molecular identification of human enteroviruses in children with neurological infections from the central region of Argentina

Archives of Virology
Official Journal of the Virology Division of the International Union of Microbiological Societies

ISSN 0304-8608
Volume 156
Number 1

Arch Virol (2010) 156:129-133
DOI 10.1007/
s00705-010-0828-4



Springer

Arch Virol (2011) 156:129–133
DOI 10.1007/s00705-010-0828-4

BRIEF REPORT

Molecular identification of human enteroviruses in children with neurological infections from the central region of Argentina

Adrián Farías · María Cabrerizo · Viviana Ré · Nora Glatstein · Belén Pisano · Lorena Spinsanti · Marta Silvia Contigiani

Received: 12 August 2010 / Accepted: 27 September 2010 / Published online: 8 October 2010

Abstract In the central area of Argentina, epidemiological and molecular characteristics of human enterovirus infections are still unknown. RT-nested PCR of the highly conserved 5′NCR was used to detect enteroviruses in 168 samples of cerebrospinal fluid from hospitalized patients with suspected infection of the central nervous system (2007–2008), and 13 (7.7%) were positive. Molecular typing was performed by sequencing of the 3′-half VP1 region. Echovirus 30 was the predominant type detected, followed by coxsackie viruses A9 and B4. All echovirus 30 strains of 2007 clustered in lineage H, whereas the echovirus 30 isolate obtained in 2008 was more distantly related, possibly representing a new lineage.

Keywords Human enterovirus · Echovirus 30 · VP1 · Aseptic meningitis · Argentina

A. Farías (✉) · V. Ré · B. Pisano · L. Spinsanti · M. S. Contigiani
Instituto de Virología "Dr. J. M. Vanella", Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Cordoba, Argentina
e-mail: adrianalefarias@hotmail.com

A. Farías · N. Glatstein
Departamento de Epidemiología, Ministerio de Salud de la Provincia de Córdoba, Cordoba, Argentina

M. Cabrerizo
National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain

A. Farías
Enfermera Gordillo Gómez s/n Ciudad Universitaria, CP 5016 Cordoba, Argentina

Human enteroviruses (HEVs) are the most common agents responsible for aseptic meningitis and constitute the majority of the infections of the central nervous system worldwide. HEVs are small, single-stranded RNA viruses that belong to the family *Picornaviridae*, within the genus *Enterovirus*, together with the recently included human rhinoviruses (http://www.ictvonline.org/virusTaxonomy.asp). HEVs are currently classified in four species: HEV-A (17 serotypes), HEV-B (56 serotypes), HEV-C (16 serotypes, including the three poliovirus serotypes), and HEV-D (3 serotypes) [22]. HEVs infect a large number of people every year. Although HEVs are distributed worldwide, some serotypes are endemic, and others can be introduced periodically, producing epidemic outbreaks. The great majority of the infections are asymptomatic or cause only mild respiratory diseases, especially in children. Severity depends on the age and constitution of the host, as well as the type and virulence of the circulating strain [20]. HEV outbreaks are not usually associated with sequelae, except in immunocompromised patients, but affect large numbers of people, producing significant economic damage [6]. To perform effective surveillance for variants, identify sources of infection, detect viruses in the environment, observe changes in circulating patterns and establish their association with symptomatic human infections, detailed information about sequence variation of the HEV types is necessary. In this sense, molecular methods have proved to be useful for identifying serotypes in clinical samples, improving the epidemiological study of these viruses [2, 4, 16–18].

In Argentina, meningitis notification is mandatory since 1960 (Law 15.465) [10]; however, only a few reports of diagnosis and typing have been published [7, 9–11]. Two of them describe outbreaks of meningitis caused by echovirus 4 in two different northern regions, Tucuman and

Misiones, and the other two reports are retrospective studies about enterovirus epidemiology in Buenos Aires province. Since Argentina is a large country, epidemiological and molecular characteristics of HEV infections may be different depending on the region and climate. Since data from the central area of Argentina have not been described yet, this report is a useful contribution to epidemiology and molecular identification of HEV, which was responsible for neurological infections in Cordoba (central Argentina) between January 2007 and March 2008.

One hundred sixty-eight samples of cerebrospinal fluid (CSF) from hospitalized patients (89 males and 79 females; mean age, 21 years, $r = 0$–84 years) in Cordoba city (second most populated inland province of Argentina) were analyzed between January 2007 and March 2008. The specimens were screened for HEV using molecular methods described previously [3]. Viral nucleic acids were extracted from 200 µl of CSF using a Purelink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RT-PCR uses primers from the highly conserved 5-non-coding region (NCR) of the genome and amplifies a fragment of 310 nt. The samples that tested positive for HEV were sent to the Enterovirus Laboratory of the National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain, for typing, sequencing, and phylogenetic analysis.

For enterovirus typing, a species-specific RT-nested PCR using 5 µl of each RNA extract was used to amplify the 3′-half of the VP1-coding region of the genome. The PCR reaction was carried out as described previously by Cabrerizo et al. [2]. The sizes of the fragments were about 454, 758 and 458 bp, for HEV-A, B and C, respectively. The sequences obtained were compared pairwise with the enterovirus sequences available in GenBank to determine the identity score (http://www.ncbi.nlm.nih.gov/blast).

HEV serotype identification was confirmed by phylogenetic analysis. The 3′-half VP1 fragment (420 nt) sequences obtained were compared with all HEV-B species prototype strains and other sequences available in GenBank from different countries. Multiple sequence alignments were performed with the ClustalW program. Genetic distances were calculated using the Kimura 2-parameter model of nucleotide substitution, and statistical significance of phylogenies was estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. A phylogenetic tree was constructed using the neighbor-joining method in the MEGA version 3.1 program.

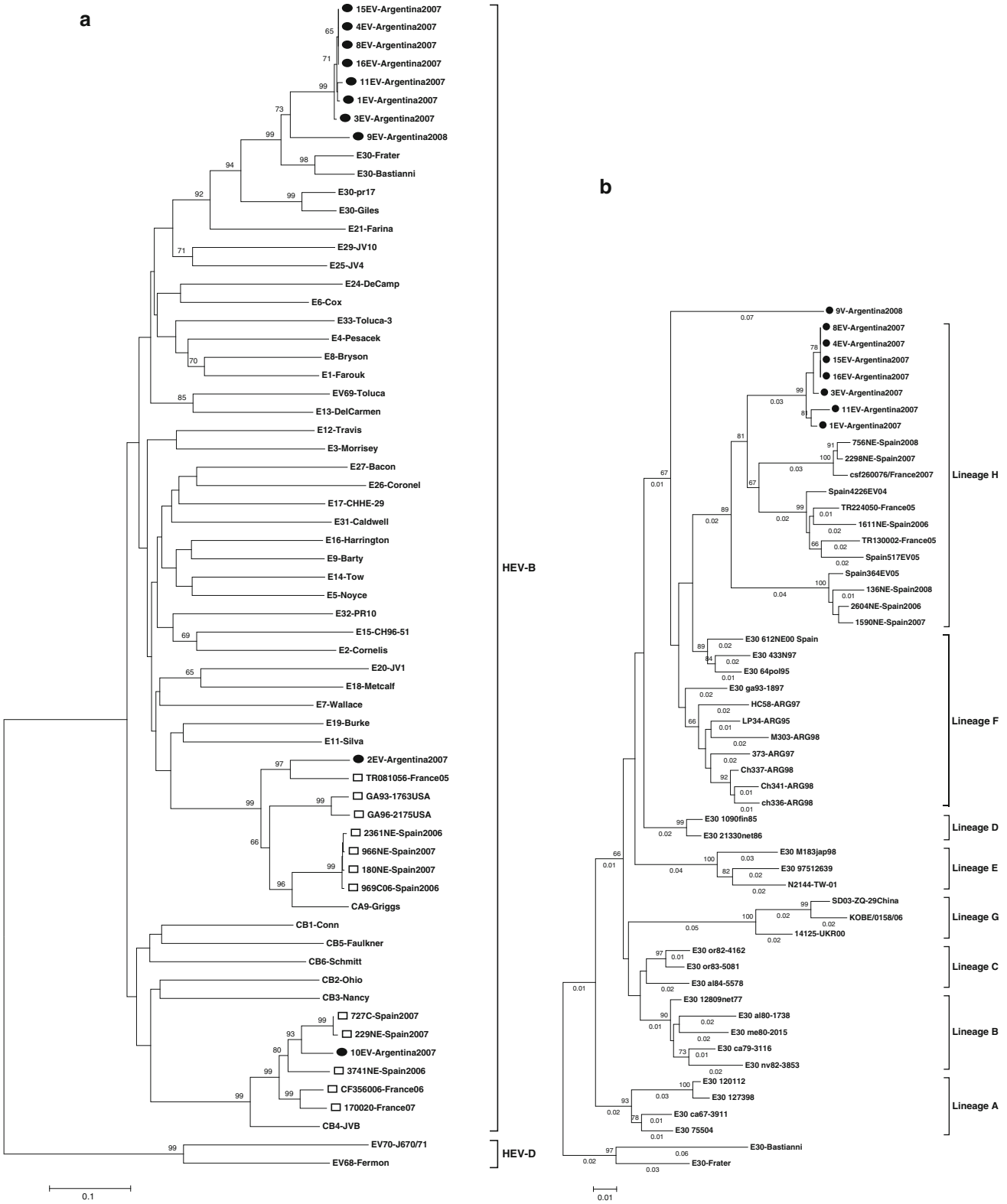
Among the 168 patients studied, 13 (7.7%) were HEV RNA positive by PCR. The mean age of the infected patients was 4.3 years ($r = 21$ days–13 years). The male/female ratio was 1. Fifty-four percent (7/13) of the patients were schoolchildren (age: 5–14 years). Regarding the distribution of positive cases per month, the analysis showed prominent summer seasonality; in Argentina, this means from November 2007 to March 2008. No differences were noted between the summer months. Aseptic meningitis was the most frequent diagnosis (46%, 6/13), followed by encephalitis (31%, 4/13) and meningoencephalitis (23%, 3/13).

Fig. 1 **a** Phylogenetic tree with Argentine sequences (*circle*) and reference strains of species HEV-B based on 420 nucleotides within the 3′-VP1 region. Enterovirus (EV) 68 and EV70 (species HEV-D) were used to root the dendrogram. The analysis included several CA9 and CB4 sequences (*square*) isolated in other countries and available from the GenBank database. **b** Phylogenetic tree showing the relationship between the Argentine E30 strains (*circle*) and other E30 sequences available from GeneBank database. The tree is rooted with Bastianni and Frater prototype strains. The dendrograms were constructed by the neighbor-joining method, with 1,000 bootstrap pseudoreplicates. Only bootstrap values >65% are shown at nodes. Genetic distances were calculated with the Kimura 2-parameter model of evolution, and horizontal branch lengths are drawn to scale (displayed below branch in Fig. 1b). The sequences described in this study have been deposited in the GenBank database, under accession numbers: GU199338-GU199347

Ten out of 13 HEV-positive samples (77%) were amplified using a VP1 PCR protocol and classified by phylogenetic analysis, demonstrating the presence of members of HEV species B. Almost 80% of the viruses detected corresponded to E30 ($n = 8$) followed by coxsackie virus A9 (CA9) ($n = 1$) and coxsackie virus B4 (CB4) ($n = 1$). Members of HEV species A or C were not detected. Three HEV-positive samples could not be typed, probably because the sensitivity of the RT-PCR in VP1 region is from 10- to 100-fold lower than the 5′-NCR-RT-PCR [2]. Another reason for failure to type could be mutations in the primer-binding site(s) of the partial VP1 gene. This is a problem frequently encountered in viral diagnosis and HEV typing [8, 21].

To investigate the genetic relationships between HEV strains, in the phylogenetic analysis, we included sequences from different geographical regions and periods of isolation available from the GenBank database (Fig. 1a). Argentinean CB4 strain (10EV-Argentine2007) clustered with Spanish and French isolates from 2006 to 2007 in a separate branch compared to the reference strain. Argentinean CA9 sequence (2EV-Argentine2007) was closely related to a strain isolated in France in 2005 (TR081056) and formed a cluster distinct from the North American and Spanish CA9 strains included in the phylogenetic analysis. The 10EV-Argentine 2007 (CB4) sequence was obtained from a 21-day-old girl with meningitis, and sequence 2EV-Argentine2007 (CA9) was from a 9-year-old boy with meningoencephalitis.

E30 was the most frequently detected serotype. A phylogenetic analysis with Argentinean sequences and other E30 strains available in GenBank was carried out (Fig. 1b). The tree was constructed based on the phylogenetic



analysis previously performed by Palacios et al. [18]. In that study, E30 viruses were divided into two genotypes and further subdivided into subgroups, and these subgroups could be divided into lineages based on their nucleotide distances and levels of bootstrapping. All but one of the Argentinean sequences were closely related to each other and clustered to the recently described lineage H, together with Spanish and French strains from 2004 to 2008 [2].

The nucleotide distance within the clustered sequences ranged from 0 to 0.04. These Argentinean sequences were detected in samples collected between November and December 2007. However, the phylogenetic tree showed that the only strain detected in 2008 (February, 9EV) was placed in a separate branch from all E30 sequences included in the analysis (nucleotide distance, 0.07). The phylogenetic analysis also included previously reported Argentinean strains from 1995, 1997 and 1998, which clustered with the previously described lineage F [18].

In this study, we demonstrated that HEVs are responsible for 7.7% of the infections of the central nervous system analyzed by molecular methods. Different from other methods developed in temperate areas, which report the occurrence of enteroviruses mainly during the summer–autumn period [1], we observed a higher incidence during spring–summer, as Mistchenko et al. [11] have reported previously.

In our study, HEV detection was performed by PCR of the highly conserved 5′NCR of the genome, and later, the isolated strains were typed by amplification of the fragment of the 3′-half of the VP1-coding region of the genome [2]. The advent of molecular methods has greatly improved the diagnosis and management of HEV diseases. Several approaches for molecular "serotyping" that involve the amplification of the genomic fragment encompassing the VP1, VP2 or VP4 coding regions have been developed [4]. Although these methods require virus cultures to obtain enough viral RNA, we used nucleic acid amplification directly from clinical samples for HEV detection. Direct genotyping of clinical samples along with phylogenetic analysis of the 3′-end VP1 gene allows results to be obtained quickly during epidemics and molecular epidemiology studies, as previous reports have demonstrated [2, 4, 18]. In addition, this assay can be of great help for deepening the surveillance and molecular epidemiology of possible vaccine-derived cases of poliovirus that could circulate in our region [14].

In this sense, the molecular approach was adequate for determining the serotype in most of the cases that were positive for HEV (7.7%). Echovirus 30 was the most prevalent serotype in our study and also the most commonly reported serotype in several studies of enterovirus surveillance from European, Asian and other South American countries, such as Brazil [1, 2, 5, 12, 13, 19, 24]. In a previous Argentine study [7], E30 was the main type detected only during 1998, and CB4 was not found; in other study [11], E30 was shown to be the predominant serotype, and CB4 was barely detected. CA9 was not isolated in any of the studies. These previous reports show analyses of larger samples and provide valuable information about the epidemiology of enteroviruses in Argentina. Both of them, however, are retrospective studies of samples obtained from 1991 to 1998 and 1998 to 2003, respectively, and most of them were collected in two hospitals of the same city, Buenos Aires. Our results, although limited due to the small size of the sample, help to determine which serotypes of enteroviruses are currently circulating in the rest of the country.

In addition, our study reports the first molecular characterization of E30 in specimens collected from patients with neurological infections in central Argentina. The E30 pattern of evolution has been extensively described [15, 16, 18, 23], and all of the studies suggest that this serotype is not geographically restricted, but that a particular genotype circulates in different regions of the world simultaneously. In our study, phylogenetic analysis showed that all Argentine E30 strains from 2007 clustered with strains isolated during the same time period from several European countries formed the named lineage H [2]. However, the only Argentine sequence detected in 2008 appeared as a unique sequence on a separate branch from the rest of E30 strains included in the analysis. This strain was detected in a town located 600 km away from the region in which the rest of the Argentine E30 strains were isolated; this result suggests that E30 from 2008 and later might represent a new lineage, but further studies with more sequences are necessary to confirm this hypothesis.

In conclusion, this study demonstrates the importance of HEV as etiological agents of aseptic meningitis and acute encephalitis in the central area of Argentina.

The use of modern molecular techniques has increased the ability to diagnose and characterize these viral diseases. The phylogenetic analysis shown here was capable of discriminating between lineages within a serotype. Further studies involving specimens from different time periods and regions throughout the country will be necessary to identify emergent new variants or serotypes of E30 in Argentina.

Acknowledgments This study was supported in part by grants from SECyT (Secretaría de Ciencias, Tecnología e Innovación Productiva, Argentina). M. Cabrerizo is supported by the Spanish Ministry of Health (DGEG-1304/08). V. Ré is a scientific member of CONICET, Argentina. M.B. Pisano is recipient of the CONICET scholarships of Argentina.

References

1. Antona D, Leveque N, Chomel JJ, Dubrou S, Levy-Bruhl D, Lina B (2007) Surveillance of enteroviruses in France, 2000–2004. Eur J Clin Microbiol Infect Dis 26:403–412
2. Cabrerizo M, Echevarria JE, González I, de Miguel T, Trallero G (2008) Molecular epidemiological study of HEV-B enteroviruses involved in the increase of meningitis cases occurred in Spain during 2006. J Med Virol 80:1018–1024
3. Casas I, Tenorio A, Echevarria JM, Klapper PE, Cleator GM (1997) Detection of enteroviral RNA and specific DNA of herpes

viruses by multiplex genome amplification. J Virol Methods 66:39–50
4. Casas I, Palacios GF, Trallero G, Cisterna D, Freire MC, Tenorio A (2001) Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. J Med Virol 65:138–148
5. Castro CM, Oliveira DS, Macedo O, Lima MJ, Santana MB, Wanzeller AL, Silveira E, Gomes M de L (2009) Echovirus 30 associated with cases of aseptic meningitis in state of Pará, Northern Brazil. Mem Inst Oswaldo Cruz 104:444–450
6. Center of Diseases Control (2003) Outbreaks of aseptic meningitis associated with echoviruses 9 and 30 and preliminary surveillance reports on enterovirus activity-United States. MMWR Morb Mortal Wkly Rep 52:761–764
7. Cisterna DM, Palacios G, Rivero K, Girard D, Lema C, Freire MC (2007) Epidemiology of enterovirus associated with neurologic diseases. Medicina 67:113–119
8. Choi YJ, Park KS, Baek KA, Jung EH, Nam HS, Kim YB, Park JS (2010) Molecular characterization of echovirus 30-associated outbreak of aseptic meningitis in Korea in 2008. J Microbiol Biotechnol 20:643–649
9. Freire MC, Cisterna DM, Rivero K, Palacios GF, Casas I, Tenório A, Gomez J (2003) Analysis of an outbreak of viral meningitis in the province of Tucuman, Argentina. Rev Panam Salud Publica 13:246–251
10. Grenón SL, Robledo ML, von Specht MH, Cisterna DM, Lema CL, Freire MC (2008) Outbreak of viral meningitis caused by echovirus type 4 in Misiones province. Rev Argent Microbiol 40:41–46
11. Mistchenko AS, Viegas M, Latta MP, Barrero PR (2006) Molecular and epidemiologic analysis of enterovirus B neurological infection in Argentine children. J Clin Virol 37:293–299
12. Mirand A, Archimbaud C, Henquell C, Michel Y, Chambon M, Peigue-Lafeuille H, Bailly JL (2006) Prospective identification of HEV-B enteroviruses during the 2005 outbreak. J Med Virol 78:1624–1634
13. Mirand A, Henquell C, Archimbaud C, Peigue-Lafeuille H, Bailly JL (2007) Emergence of recent echovirus 30 lineages is marked by serial genetic recombination events. J Gen Virol 88:166–176
14. Mueller JE, Bessaud M, Huang QS, Martinez LC, Barril PA, Morel V, BalanantJ BocacaoJ, Hewit J, Gessner BD, Delpeyroux F, Nates SV (2009) Environmental poliovirus surveillance during oral poliovirus vaccine and inactivated poliovirus vaccine use in Cordoba province, Argentina. Appl Environ Microbiol 75: 1395–1401
15. Lukashev AN, Ivanova OE, Eremeeva TP, Gmyl LV (2008) Analysis of echovirus 30 isolates from Russia and new independent states revealing frequent recombination and reemergence of ancient lineages. J Clin Microbiol 46:665–670
16. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA (1999) Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol 37:1288–1293
17. Oberste MS, Maher K, Williams AJ, Dybdahl-Sissoko N, Brown BA, Gookin MS, Peñaranda S, Mishrik N, Uddin M, Pallansch MA (2006) Species-specific RT-PCR amplification of human enteroviruses: a tool for rapid species identification of uncharacterized enteroviruses. J Gen Virol 87:119–128
18. Palacios G, Casas I, Cisterna D, Trallero G, Tenorio A, Freire C (2002) Molecular epidemiology of echovirus 30: temporal circulation and prevalence of single lineages. J Virol 76:4940–4949
19. Roth B, Enders M, Arents A, Pfitzner A, Terletskaia-Ladwig E (2007) Epidemiologic aspects and laboratory features of enterovirus infections in Western Germany, 2000–2005. J Med Virol 79:956–962
20. Saeed M, Zaidi SZ, Naeem A, Masroor M, Sharif S, Shaukat S, Angez M, Khan A (2007) Epidemiology and clinical findings associated with enteroviral acute flaccid paralysis in Pakistan. BMC Infect Dis 7:6
21. She RC, Hymas WC, Taggart EW, Petti CA, Hillyard DR (2010) Performance of enterovirus genotyping targeting the VP1 and VP2 regions on non-typeable isolates and patient specimens. J Virol Methods 165:46–50
22. Stanway G, Brown F, Christian P, Hovi T, Hyypiä T, King AMQ, Knowles NJ, Lemon SM, Minor PD, Pallansch MA, Palmenberg AC, Skern T (2005). Family *Picornaviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus Taxonomy. 8th Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp. 757–778
23. Thoelen I, Moes E, Lemey P, Mostmans S, Wollants E, Lindberg AM, Vandamme AM, Van Ranst M (2004) Analysis of the serotype and genotype correlation of VP1 and the 50 noncoding region in an epidemiological survey of the human enterovirus B species. J Clin Microbiol 42:963–971
24. Trallero G, Casas I, Tenorio A, Echevarria JE, Castellanos A, Lozano A, Breña P (2000) Enteroviruses in Spain: virological and epidemiological studies over ten years (1988–1997). Epidemiol Infect 124:497–506