Phylogenetic analysis of complete VP1 sequences of echoviruses 11 and 6: high genetic diversity and circulation of genotypes with a wide geographical and temporal range

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Echovirus 6 (E6) and echovirus 11 (E11) are common causes of meningitis and other human diseases; they are among the most frequently isolated enteroviruses worldwide. In the present work we have studied genetic variability over the entire VP1 gene of selected isolates representing a wide geographical and temporal range. Fifty new sequences from North Africa were included, together with previously published sequences from different countries. The sequence diversity between strains of the same type was high: 22 and 30 % for E6 and E11, respectively. Phylogenetic analysis revealed five genogroups within each type, the genetic diversity within a genogroup generally being <20 %. Some genogroups were further subdivided into genotypes, most containing isolates that had circulated over a wide geographical (several countries from different continents) and temporal (up to two decades) range. Several genotypes were also shown to co-circulate in a region during the same period of time. These features differ from other enteroviruses that divide into temporal or geographical clusters. This study reports new sequences from North Africa, updates the molecular epidemiology of E6 and E11, and proposes a new genogroup in each type.

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INTRODUCTION

Human enteroviruses (HEVs) are small non-enveloped viruses with a worldwide distribution. Laboratory diagnosis of related infections currently is based on virus detection by isolation in cell culture or by direct PCR amplification from clinical samples. HEV types are identified by seroneutralization or partial sequencing of the VP1 region (Caro *et al.*, 2001; Norder *et al.*, 2001; Oberste *et al.*, 1999a, b, 2000; Palacios *et al.*, 2002). Similar to other RNA viruses, HEVs have a high capacity to evolve genetically. Genotypes are identified based on the genetic variability and phylogenetic relationships among isolates. The circulation of these genotypes throughout the world and over time has been studied to increase our understanding of the dynamics of their transmission, to evaluate their endemicity and to explain the extent of epidemics

when they occur. Beyond type identification, sequencing of the VP1 region has also proved to be a reliable method for such molecular epidemiological studies.

Echovirus 6 (E6) and echovirus 11 (E11) are among the most commonly isolated HEVs worldwide, and are frequently associated with outbreaks and sporadic cases of aseptic meningitis, as well as with several diseases ranging from mild non-specific illness to encephalitis, paralysis, myocarditis and severe systemic infections in neonates (Abe *et al.*, 2000; Ashwell *et al.*, 1996; Atkinson *et al.*, 1998; Bahri *et al.*, 2005; Belguith *et al.*, 2007a; Boyd *et al.*, 1987; Cabrerizo *et al.*, 2008; Chomel *et al.*, 2003; Druyts-Voets, 1997; El-Sageyer *et al.*, 1998; Joo *et al.*, 2005; Khetsuriani *et al.*, 2006; Mao *et al.*, 2010; Mirand *et al.*, 2008; Miwa & Sawatari, 1994; Somekh *et al.*, 2001; Ventura *et al.*, 2001). However, despite their important impact on human health, studies of the molecular epidemiology of E6 and E11 remain limited. Other studies have attempted to classify the isolates into genogroups and genotypes (Bouslama *et al.*, 2007; Belguith *et al.*, 2007b; Mao *et al.*, 2010; McWilliam Leitch *et al.*, 2010; Oberste *et al.*, 2003). In the present work, E6 and E11 isolates detected in four countries from

Abbreviations: E6, echovirus 6; E11, echovirus 11; HEV, human enterovirus.

The GenBank/EMBL/DDBJ accession numbers for the VP1 sequences of the E6 and E11 isolates reported in this study are HQ674700–HQ674749.

North Africa were sequenced over the entire VP1 gene. The sequences obtained were added to those previously published from other countries to study their genetic diversity and to update the genogroups and genotypes within each type.

METHODS

New sequences from North Africa. A total of 50 virus isolates belonging to E6 ($n=25$) and E11 ($n=25$) types, isolated in Tunisia, Algeria, Libya and Morocco during the period 1991–2010, were sequenced (Table 1). The isolates were obtained mainly from clinical specimens (stools and cerebrospinal fluid) from healthy individuals and patients with aseptic meningitis or acute flaccid paralysis; a few isolates were obtained from environmental (sewage) samples. The viruses were obtained by inoculation of the treated samples onto two cell culture lines, human rhabdomyosarcoma (RD) cells and transgenic murine cells expressing the human poliovirus receptor (L20B cells), following World Health Organization standard protocols (WHO, 2004). The type was determined primarily by seroneutralization of the cytopathic effect using the anti-enterovirus serum pools A–G from RIVM (National Institute of Public Health and the Environment, Bilthoven, The Netherlands). RNA extraction was performed from 140 μ l infected cell supernatant using a QIAamp viral RNA mini kit (Qiagen), and the extracted RNA was then processed for RT-PCR amplification. The reverse transcription reaction mixture comprised 5 μ l viral RNA, 1 μ l RNasin (Promega), 10 pmol PIC reverse primer (5'-AGCTGATCGATGGGCTACCATGCGTACCCT-TTTTTTTTTTTTTTTTTTTC-3'), 4 μ l 5 $\times$ transcriptase buffer, 2 μ l 0.1M DTT, 1 μ l dNTP mix (20 mM each), 100 U SuperScript II reverse transcriptase (Invitrogen) and 5 μ l nuclease-free water, in a final volume of 20 μ l. The mixture was incubated at 42 °C for 45 min, heat inactivated at 95 °C for 5 min and immediately chilled on ice. Two reported primer pairs (490/492 and 491/493) were used to amplify two overlapping fragments of the VP1 region (Oberste *et al.*, 2006). PCR amplification was performed with 5 μ l cDNA in a 50 μ l reaction mixture containing 3 μ l 10 $\times$ polymerase buffer, 3 μ l MgCl₂, 2.5 U recombinant *Taq* polymerase, and 50 pmol each forward and reverse primer. The mixture was subjected to 35 amplification cycles of 95 °C for 1 min, 42 °C for 1 min and 72 °C for 1 min, with a final extension cycle of 10 min at 72 °C. The PCR products were separated and visualized on 1% agarose gels containing ethidium bromide (0.2 μ g ml⁻¹). Depending on the presence of one or multiple bands in the gel, PCR products were either directly purified using enzymic methods (exonuclease I and shrimp alkaline phosphatase; USB) or excised from the gel and purified with a QIAquick gel extraction kit (Qiagen). The purified template DNA was sequenced in both directions using a BigDye terminator ready reaction cycle sequencing kit (Applied Biosystems) and the same PCR primers on an ABI Prism 3130 automated sequencer (Applied Biosystems). The sequence of each isolate was deduced by aligning the respective forward and reverse sequences using CLUSTAL_X.

GenBank sequences. A total of 128 E6 and E11 sequences published in GenBank were selected for the phylogenetic tree construction. The sequences were selected from those containing the complete VP1 gene and represented a wide temporal and geographical range, having been isolated in 24 different countries between 1955 and 2009.

Phylogenetic analyses. Phylogenetic analyses were performed using the programs included in the MEGA package (version 4.0.2). Genetic distances were calculated using the neighbour-joining and Kimura two-parameter methods. The reliability of the phylogenetic

constructions was estimated by bootstrap analysis with 1000 pseudoreplicate datasets.

RESULTS AND DISCUSSION

Fifty isolates, all obtained with RD cells and identified previously by seroneutralization as E6 ($n=25$) or E11 ($n=25$), were successfully amplified by PCR and sequenced. The sequences of 876 and 867 nt for E11 and E6, respectively, were first aligned with HEV prototype strains to confirm that they belonged to the E6 or E11 type (data not shown). All E6 isolates clustered with the E6 Burgess, Charles and D'Amori reference strains, whilst the E11 isolates clustered with the E11 Gregory and E11 Silva reference strains. No discordance was found with the serotype determined previously using seroneutralization.

To investigate the genetic relationships among isolates, phylogenetic trees were generated for E6 and E11 (Figs 1 and 2, respectively), including the new sequences from North Africa reported here and representative sequences from different countries of the world. Table 2 shows the nucleotide divergence rates within and between the different clusters identified by the phylogenetic constructions. The results showed that the viruses from both types shared common properties in their genetic diversity and evolution. High sequence diversity within each type was found, reaching 22 and 30% for E6 and E11, respectively. Such high genetic diversity, exceeding 20%, has also been reported for other enteroviruses such as E30 (Bailly *et al.*, 2000; Lukashev *et al.*, 2008; Oberste *et al.*, 1999c; Wang *et al.*, 2002) and coxsackievirus A9 (Santti *et al.*, 2000). In contrast, other types appear to be more conserved, such as enterovirus 71 (Brown *et al.*, 1999) and coxsackievirus B4 (Mulders *et al.*, 2000). Other studies have also reported high sequence diversity within E6 and E11. The mean genetic distance between E6 strains isolated in Greece between 2006 and 2007 was 22% (Papa *et al.*, 2009), based on analysis of a 358 nt fragment in the 5' end of the VP1. For E11, the genetic diversity over the entire VP1 gene was up to 27.6% (Oberste *et al.*, 2003).

Our phylogenetic analyses also showed that the sequences from both types could be divided into different genetic groups, designated genogroups I–V; most were supported by high bootstrap values (Figs 1 and 2). The genogroups contained isolates generally with <20% nucleotide differences (Table 2); they were subdivided into clusters containing isolates sharing >85% nucleotide similarity. These clusters may correspond to genotypes.

E6

The tree in Fig. 1 compares the sequences of 89 E6 isolates, 25 from our region and 64 from 11 other countries, published in GenBank. The majority of the field sequences grouped within genogroups III and IV. The three reference strains, Charles, Burgess and D'Amori, grouped together in genogroup I, and were genetically very close to each other

Table 1. Clinical and epidemiological data for the studied E6 and E11 isolates

GenBank no.	Isolate name	Serotype	Country of isolation	Year of isolation	Specimen	Clinical symptoms
HQ674725	S127-09	E6	Algeria	2002	CSF	Meningitis
HQ674726	S129-09	E6	Algeria	2002	CSF	Meningitis
HQ674727	S130-09	E6	Algeria	2002	CSF	Meningitis
HQ674728	S136-09	E6	Algeria	2002	CSF	Meningitis
HQ674729	S94-09	E6	Algeria	2006	Stool	AFP
HQ674730	S79-09	E6	Algeria	2006	Stool	AFP
HQ674731	S132-09	E6	Algeria	2002	CSF	Meningitis
HQ674732	S128-09	E6	Algeria	2002	CSF	Meningitis
HQ674733	S125-09	E6	Algeria	2002	CSF	Meningitis
HQ674734	C49-01	E6	Tunisia	2001	CSF	Meningitis
HQ674735	C60-01	E6	Tunisia	2001	CSF	Meningitis
HQ674736	S48-94	E6	Tunisia	1994	Stool	AFP
HQ674737	E120-01	E6	Tunisia	2001	Stool	Healthy
HQ674738	S10-93	E6	Tunisia	1993	Stool	AFP
HQ674739	ESs72-93	E6	Tunisia	1993	Stool	Healthy
HQ674740	EGf143-92	E6	Tunisia	1992	Stool	Healthy
HQ674741	S63-97	E6	Tunisia	1997	Stool	AFP
HQ674742	S83-01	E6	Morocco	2001	Stool	AFP
HQ674743	S157-97	E6	Tunisia	1997	Stool	AFP
HQ674744	S65-97	E6	Tunisia	1997	Stool	AFP
HQ674745	S206-06	E6	Libya	2006	Stool	AFP
HQ674746	E91-00	E6	Tunisia	2000	Stool	AFP
HQ674747	ETt111-97	E6	Tunisia	1997	Stool	Healthy
HQ674748	S319-05	E6	Morocco	2005	Stool	AFP
HQ674749	S25-01	E6	Morocco	2001	Stool	AFP
HQ674705	S93-09	E11	Algeria	2008	Stool	AFP
HQ674701	S89-09	E11	Algeria	2008	Stool	AFP
HQ674704	S87-09	E11	Algeria	2008	Stool	AFP
HQ674723	S267-09	E11	Libya	2009	Stool	AFP
HQ674722	E155-03	E11	Tunisia	2003	Stool	Healthy
HQ674716	ETz175-92	E11	Tunisia	1992	Stool	Healthy
HQ674707	EJn113-97	E11	Tunisia	1997	Stool	Healthy
HQ674720	S40-98	E11	Tunisia	1998	Stool	AFP
HQ674717	LCR8-98	E11	Tunisia	1998	CSF	Meningitis
HQ674721	S53-98	E11	Tunisia	1998	Stool	AFP
HQ674706	EBj16-99	E11	Tunisia	1999	Stool	Healthy
HQ674700	S58-09	E11	Algeria	2005	Stool	AFP
HQ674702	S59-09	E11	Algeria	2005	Stool	AFP
HQ674703	S61-09	E11	Algeria	2005	Stool	AFP
HQ674719	S39-10	E11	Libya	2010	Stool	AFP
HQ674718	S3-94	E11	Tunisia	1993	Stool	AFP
HQ674715	Es39-01	E11	Tunisia	1995	Environment	NA
HQ674714	Es37-01	E11	Tunisia	1995	Stool	Healthy
HQ674710	Es27-01	E11	Tunisia	1995	Stool	Healthy
HQ674708	Es14-01	E11	Tunisia	1995	Environment	NA
HQ674711	Es3-01	E11	Tunisia	1991	Environment	NA
HQ674724	Es9-01	E11	Tunisia	1994	Environment	NA
HQ674712	Es31-01	E11	Tunisia	1995	Environment	NA
HQ674713	Es36-01	E11	Tunisia	1995	Stool	Healthy
HQ674709	Es18-01	E11	Tunisia	1995	Environment	NA

AFP, Acute flaccid paralysis; CSF, cerebrospinal fluid; NA, not applicable.

(1.4–1.5 % divergence) and clearly different from the other genogroups (23.3–29.5 % divergence) (Table 2). An old isolate from the USA from 1955 also remained alone in one

branch of the tree, with 23.3–29.7 % divergence from all E6 isolates (genogroup II). Three sequences from China from 1998 and 2006 grouped together (genogroup V) with a

Fig. 1. Phylogenetic trees of E6 strains, based on analysis of the complete VP1 coding region (nt 2447–3313 according to the E6 D'Amori prototype strain). The tree includes the sequences of the North African isolates reported in this study and 61 previously published sequences from other countries, in addition to the sequences of the 3 E6 reference strains (Charles, Burgess and D'Amori). The E29.JV-10 reference strain was used as the outgroup. The segregation of all E6 sequences into five genogroups (I–V) is shown in (a), whilst (b) is a representation of the same phylogenetic tree showing in more detail the members of the different genotypes within each genogroup. Bootstrap values (%) are indicated at the tree nodes of the different genogroups (I–V) and genotypes (1–6 for genogroup III and 1–8 for genogroup IV). The isolates from North Africa reported in this study are shown in bold, and indicated by the type followed by the laboratory code, country code and year of isolation. The reference sequences are shown in italic. The sequences of field isolates from GenBank are indicated by their accession number followed by the country code and the year of isolation. AUS, Australia; AZE, Azerbaijan; BLR, Belarus; CHN, China; DZA, Algeria; FRA, France; GEO, Georgia; KAZ, Kazakhstan; KOR, Republic of Korea; LBY, Libyan Arab Jamahiriya; MAR, Morocco; RUS, Russian Federation; TUN, Tunisia; UKR, Ukraine; USA, United States of America.

maximum of 11.2 % divergence between each other and up to 29.7 % divergence with the other genogroups. Genogroup IV included the largest number of sequences ($n=61$), followed by genogroup III with 21 sequences. Several genotypes could be distinguished within genogroups III and IV, designated 1–6 and 1–8, respectively (Fig. 1b). Based on similar phylogenetic criteria, other studies have tried to classify E6 isolates into genetic clusters and subclusters. Belguith *et al.* (2007b) sequenced a 300 nt fragment in VP1 and suggested nine clusters within the E6 type named I–IX. A more recent study (Mao *et al.*, 2010) divided E6 isolates into three clusters, named A–C, with four subclusters within cluster C. The authors sequenced the entire VP1 gene, allowing us to include in our phylogenetic tree representative isolates from the clusters and subclusters identified by these authors (Fig. 1). Clusters B and C corresponded to genogroups III and IV in the present work. Most of the field isolates published previously in GenBank clustered in genogroup IV, whereas only a few clustered in genogroup III. This study added 11 new sequences from our region to the latter genogroup. Genogroups III and IV were characterized by a high level of genetic diversity; our results suggest the existence of at least six and eight different genotypes within genogroups III and IV, respectively. The three E6 reference sequences and an old isolate from the USA from 1955 were classified within cluster A by Mao *et al.* (2010); however, the isolate from the USA had >23 % divergence with the three reference sequences. We suggest its classification into a separate genogroup (genogroup II), assigning the three reference sequences to genogroup I. Analysis of the temporal and geographical characteristics of the different E6 genotypes showed that some included isolates from distant countries in different continents, detected over a long period of time (i.e. IV-1 and IV-8), whilst others comprised isolates with limited geographical and temporal ranges (i.e. III-5 and III-6). There were also genotypes comprising isolates from countries of same geographical region but detected over a long period of time (9–15 years), for example genotypes III-1, III-4 and IV-4. Genotype IV-3 could also be added to this group as it comprised isolates from Tunisia, Morocco and France detected between 1999 and 2005; although from two continents, these countries can be considered as being from the same geographical region (the Mediterranean

region). Finally, genotype IV-7 included isolates from distant countries (Australia, France and Ukraine) detected in 2006 and 2007 only. We also found that viruses from different genotypes had circulated during the same period in our region: isolates from genotypes IV-8 and IV-3 in 2001 and others from genotypes III-4 and III-2 in 2006.

E11

The trees in Fig. 2 compared our E11 sequences with those of 64 isolates from 16 other countries. Similar to E6, five genogroups and several genotypes were identified. Genogroup IV comprised the majority of the field isolates included in the tree (61/93) and could be divided into five genotypes. Genogroups I and III included far fewer sequences; however, the high genetic diversity between the different isolates and subclusters suggested the existence of at least five genotypes in genogroup I and eight genotypes in genogroup III. The Gregory reference strain was alone in a separate branch of the tree, forming genogroup II; it had 21.7–30.1 % divergence from all E11 isolates. Two isolates, one from Tunisia in 1992 and another from Iraq in 2000, grouped together in genogroup V; they had 13.3 % divergence between each other and differed from the other E11 isolates by 20.2–31.6 % divergence rates. A first study by Oberste *et al.* (2003) classified E11 isolates into four genogroups (A–D), comprising isolates with <20 % nucleotide divergence among each other; genogroup D was subdivided into five lineages, designated D1–D5. The study included isolates from different regions of the world, sequenced over the entire VP1 region. It showed that most field isolates clustered within genogroup D, whilst genogroup B contained the Gregory prototype strain alone, and genogroups A and C contained only a few isolates but from different and distant countries. A subsequent study added isolates from Tunisia and other countries sequenced at the 3' end of VP1 and found the same clustering, and all the isolates from Tunisia also clustered within genogroup D (Bouslama *et al.*, 2007). A recent study by McWilliam Leitch *et al.* (2010) included E11 isolates from Europe, sequenced over the 5' half of VP1. The authors found that most of the European sequences clustered within genogroups D and A, and they suggested the existence of three new genogroups, as well as

Fig. 2. Phylogenetic trees of E11 strains, based on analysis of the complete VP1 coding region (nt 2459–3334 according to the Gregory prototype strain). The tree includes the sequences of the North African isolates reported in this study and 64 previously published sequences from other countries, in addition to the sequences of the 2 E11 reference strains (Gregory and Silva). The E19 Burke reference strain was used as the outgroup. The segregation of all E11 sequences into five genogroups (I–V) is shown in (a), whilst (b) is a representation of the same phylogenetic tree showing in more detail the members of the different genotypes within each genogroup. Bootstrap values (%) are indicated at the tree nodes for the different genogroups (I–V) and genotypes (1–5 for genogroup I, 1–8 for genogroup III and 1–5 for genogroup IV). The isolates from North Africa reported in this study are shown in bold, and indicated by the type followed by the laboratory code, country code and year of isolation. The reference sequences are in italic. The sequences of field isolates from GenBank are indicated by their accession number followed by the country code and year of isolation. Four of these sequences were from Tunisia and are shown in bold and marked with an asterisk. ARG, Argentina; AUS, Australia; BGD, Bangladesh; CHI, China; COL, Columbia; COD, Democratic Republic of the Congo; DOM, Dominican Republic; DZA, Algeria; FRA, France; IRQ, Iraq; KWT, Kuwait; LBY, Libyan Arab Jamahiriya; NLD, The Netherlands; OMA, Oman; PAR, Paraguay; PER, Peru; ZAF, South Africa; TUN, Tunisia; USA, United States of America.

another genotype within genogroup D. In the present work, we identified five genogroups that we designated I–V; genogroups I, II, III and IV correspond to the previously described genogroups A, B, C and D, respectively. Within genogroup IV (formerly D), we also found five genotypes, as reported previously. Genogroup V is a newly proposed genogroup, comprising two isolates: one from Tunisia (E11.Etz175.92.TUN1992), reported here for what is believed to be the first time, and the second from Iraq (E11.AY121423.IRQ2000). The Iraqi isolate has been reported previously but was classified within genogroup I (formerly A) by Oberste *et al.* (2003) and within genogroup II (formerly B) by Bouslama *et al.* (2007). We found that the two isolates grouped together in a separate branch of the phylogenetic tree and had >20 % nucleotide divergence (20.2–31.6 %) from the isolates from the other genogroups, suggesting their classification in a separate genogroup. To assess whether this new genogroup corresponded to one of the three recently suggested by McWilliam Leitch *et al.* (2010), we compared our sequences with those of the newly suggested ones, in the 5' half of VP1 (data not

shown). The high divergence rates that we found (24.9–30.4 %) suggested that it probably is another genogroup.

Similar to E6, analysis of the geographical and temporal characteristic of E11 genotypes showed no systematic association of the phylogenetically determined genotypes with a region or a period of time. In genogroup IV (formerly D), genotype IV-5 contained isolates from eight countries in four different continents and our data showed that it has circulated at least from 1998 to 2010. Genotype IV-4 included isolates from the USA and Tunisia only. It has been circulating in the USA for more than 20 years (1981–2001), which indicates that it may be specific to this region. In contrast, the sequences from Tunisia were detected over only a short period of time (1994–1995), suggesting occasional introduction of genotype IV-4 to Tunisia at that time followed by its rapid extinction in favour of other locally circulating or newly introduced genotypes. McWilliam Leitch *et al.* (2010) found that few E11 isolates from Europe clustered within this genotype (D4). The sequences could not be included in our phylogenetic tree, given that they covered only part of

Table 2. Nucleotide divergence rates between E6 and E11 isolates within each genogroup and between isolates from different genogroups

Results are shown as a mean value, with the range in parentheses.

Serotype	Genogroup	Divergence rates within genogroups (%)	Divergence rates between isolates from different genogroups (%)			
			I	II	III	IV
E6	I	1.5 (1.4–1.5)				
	II	–	23.5 (23.3–23.8)			
	III	16.0 (0.0–21.5)	26.5 (24.5–28.6)	25.3 (23.3–27.9)		
	IV	14.0 (0.1–20.9)	26.7 (24.1–29.0)	26.0 (24.0–28.0)	19.6 (6.4–22.7)	
	V	9.6 (7.1–11.2)	28.1 (26.5–29.5)	27.9 (25.3–29.7)	23.9 (23.0–25.9)	24.3 (21.5–26.2)
E11	I	14.4 (0.0–21.7)				
	II	–	24.3 (22.3–26.4)			
	III	18.5 (0.6–20.7)	25.8 (23.2–28.6)	22.9 (21.7–24.8)		
	IV	12.2 (0.0–22.7)	30.3 (26.9–33.1)	27.6 (25.6–30.1)	28.9 (26.4–32.5)	
	V	13.2	21.6 (20.2–25.1)	25.2 (24.2–26.1)	25.3 (21.8–27.3)	28.9 (26.1–31.6)

the VP1; however, these results suggest that this genotype may have a wide geographical extent of circulation and that it is not restricted to the USA. Genotypes IV-1, IV-2 and IV-3 included very few and relatively old sequences; they may have become extinct, but it is also possible that they are still circulating but are not detected in some regions of the world. Finally, whilst genogroup II (formerly B) is still represented by the unique Gregory reference strain, the present study added further sequences to genogroups I and III (formerly A and C). The two genogroups included sequences with high genetic diversity between each other; most of the clusters comprised only one or two sequences. Thus, the geographical range for these genotypes cannot be estimated, but many included isolates with a wide temporal range of 6–8 years (genotypes III-8, III-2, III-1 and I-1). The recent isolates from Algeria in 2008 and Libya in 2009 reported here, together with the recently reported isolates from China in 2006, indicate that at least some genotypes from these two genogroups are still active but may be underdetected in some regions of the world.

Thus, in addition to the high genetic diversity within each type, this study highlights common genetic characteristics for E6 and E11, especially the lack of geographical or temporal specificity of the phylogenetically determined genotypes. Different genotypes were identified previously for several other enteroviruses, also on the basis of <15 % nucleotide divergence. In some cases, like that of poliovirus, the different genotypes cluster geographically and, even if long-range importations do occur, a genotype remains specific to a given geographical region (Kew *et al.*, 1995). In contrast, other enteroviruses, such as coxsackieviruses A24 and E30, have temporal genotypes, each having circulated during a given period of time, and seem to have become extinct in favour of newly emerging genotypes (Lin *et al.*, 2001; Oberste *et al.*, 2003; Triki *et al.*, 2007). The present work shows that, for both E6 and E11, genotypes containing isolates with wide geographical and temporal ranges can be found, meaning that some genotypes can persist over a long period of time and have a wide geographical range of circulation. In addition, several genotypes can also co-circulate during the same period of time in a given country or region. Although we also found genotypes with limited geographical and temporal ranges, it cannot be excluded that these may have circulated but were not detected in other regions or periods of time. Surveillance and molecular characterization of E6 and E11, as well as most of the non-polio enteroviruses, remains unperformed on a regular basis in many regions of the world.

It is also evident that, with the identification of new isolates from new geographical regions, the classification into genogroups and genotypes becomes more accurate. A consensus on the genomic region that should be considered to determine genogroups and genotypes should also be established internationally, as well as a standard designation of these genogroups and genotypes. In this work, we considered the sequence of the entire VP1 region,

which may give more accuracy to the classification and identification of new groups.

In conclusion, in this study we have reported new sequences from the North African region and have given an update on the global molecular epidemiology of E6 and E11, two of the most commonly isolated non-polio enteroviruses in the world. Based on the sequence data currently available, these two types are characterized by a high genetic diversity and the existence of at least five genogroups each and several genotypes that do not have a strict temporal or geographical specificity. However, substantial gaps remain, given the absence of enterovirus surveillance and the only occasional testing of patients with meningitis and other enterovirus-related diseases in many countries. With the increasing availability of E6 and E11 sequence data from various parts of the world, such updates are needed to increase our understanding of the molecular epidemiology of these pathogens.

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