

Worldwide emergence of multiple clades of enterovirus 68

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Human enterovirus 68 (EV-D68) is a historically rarely reported virus linked with respiratory disease. In the past 3 years, a large increase in respiratory disease associated with EV-D68 has been reported, with documented outbreaks in North America, Europe and Asia. In several outbreaks, genetic differences were identified among the circulating strains, indicating the presence of multiple clades. In this report, we analyse archived and novel EV-D68 strains from Africa and the USA, obtained from patients with respiratory illness. Phylogenetic analysis of all EV-D68 sequences indicates that, over the past two decades, multiple clades of the virus have emerged and spread rapidly worldwide. All clades appear to be currently circulating and contributing to respiratory disease.

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INTRODUCTION

Enteroviruses (family *Picornaviridae*, genus *Enterovirus*) are non-enveloped, positive-sense ssRNA viruses. Enteroviruses can cause a wide range of clinical symptoms, ranging from mild febrile illness to fatal meningitis and encephalitis and are among the most common human pathogens. A typical enterovirus genome consists of an RNA strand of approximately 7500 nt and contains a single ORF encoding a polyprotein that is processed post-translationally to yield individual viral proteins. The polyprotein ORF is flanked by a UTR at each end, with the 5' UTR containing the highly conserved internal ribosome entry site (IRES) (Racaniello, 2001). The VP1 gene, which encodes one of four capsid proteins, has traditionally been used to distinguish between enterovirus serotypes and, based on molecular and biological characteristics, four human enterovirus (HEV) species are currently recognized, designated HEV-A, -B, -C and -D (Oberste *et al.*, 1999a, b).

Human enterovirus 68 (EV-D68) was first isolated from samples obtained in California in 1962 from four children with pneumonia and bronchiolitis (Schieble *et al.*, 1967). Along with EV-D70, EV-D94 and EV-D-111, EV-D68 is one of four serotypes assigned to HEV-D. Unlike other enteroviruses, EV-D68 is acid-labile and biologically more similar to human rhinoviruses in being mainly associated with respiratory disease; however, until recently, reports of respiratory disease due to EV-D68 were rare (Oberste *et al.*, 2004). Between 1970 and 2005 only 26 clinical isolates of EV-D68 were reported in the USA, representing 0.1% of all clinical EV isolates (Khetsuriani *et al.*, 2006). Over the past 3 years, however, outbreaks in Japan, the Philippines and the Netherlands, as well as several clusters in the USA, have implicated EV-D68 as an emerging respiratory pathogen (Hasegawa *et al.*, 2011; Imamura *et al.*, 2011; Kaida *et al.*, 2011; Meijer *et al.*, 2012; Rahamat-Langendoen *et al.*, 2011; Tokarz *et al.*, 2011; Ikeda *et al.*, 2012; Jacobson *et al.*, 2012). The clinical presentation of EV-D68 infections in these outbreaks has ranged from mild illness to complications requiring hospitalization and, in rare instances, death. In all reports, children represented the majority of symptomatic infections. In several clusters, novel genetic variants were described (Hasegawa *et al.*, 2011; Imamura

The GenBank/EMBL/DDBJ accession numbers for the sequences of EV-D68 strains reported in this study are JX101786–JX101846.

A supplementary figure is available with the online version of this paper.

et al., 2011; Kaida *et al.*, 2011; Meijer *et al.*, 2012; Rahamat-Langendoen *et al.*, 2011; Ikeda *et al.*, 2012; Jacobson *et al.*, 2012).

In the context of pursuing respiratory virus surveillance during the 2009 H1N1 pandemic, we detected a cluster of EV-D68 cases in New York City (NYC) in the autumn of 2009 (Tokarz *et al.*, 2011). Thereafter we detected EV-D68 in respiratory disease samples collected from several other countries within the last decade. Here, we present the first analysis of EV-D68 from Africa, and compare it to the virus responsible for the NYC cluster, as well as currently circulating strains. Our analysis indicates that several recently emerged distinct clades are circulating globally.

RESULTS

Sample origin

The EV-D68-positive samples originated from both children and adults (Table 1). All South African samples were collected from hospitalized children, while all US samples were originally collected in outpatient clinics. The US sample from Arizona came from a child with fever, cough and rhinitis, while 14 NYC samples came from patients between 14 and 47 years of age that were seen in outpatient clinics throughout the city from individuals presenting with respiratory illness. The initial survey of the samples by MassTag PCR identified EV-D68 as the lone viral pathogen present, with the exception of two South African samples that were co-infected with human respiratory syncytial virus A and one Gambian sample co-infected with human parainfluenza virus 1.

Prevalence of EV-D68 varied between sample sets. The Gambian and South African EV-D68-positive samples originated from large studies of paediatric respiratory disease and accounted for <1% of all screened samples from each region. The eight South African cases occurred over a 2 year period between late April and July, which corresponds to the autumn–winter season in the southern hemisphere. All five samples from the Gambia were collected within a 1 month period, indicating a focal outbreak at the beginning of the rainy season. In contrast, the three EV-D68 samples from Senegal accounted for 7%

of the total number of respiratory disease samples collected during a 2 month interval during the dry season. In NYC, the EV-D68-positive samples accounted for approximately 2% of all respiratory samples analysed over 13 months. However, EV-D68 was the second most frequently detected respiratory virus during a 4 week period in September and October 2009, when detection peaked (Tokarz *et al.*, 2011).

Phylogenetic analysis

The Bayesian phylogenetic tree inferred using the VP1 gene of all available EV-D68 sequences revealed the presence of three primary clades (A, B and C) with strong nodal support [Bayesian posterior probability (BPP)=1 in all cases, Fig. 1]. Notably, all three clades were distributed globally, although clade A contained a larger proportion of sequences from all four sampled continents (Asia, Europe, Africa and North America). All newly sequenced strains from NYC (2009), the Gambia (2008) and Senegal (2010), along with one South African sequence (2001), were found within clade A, while the remaining South African samples (2000–2001) formed a cluster with sequences from Europe and the USA ancestral to clades B and C. The sequence from Arizona (2009) belonged to clade C. We did not detect any EV-D68 that clustered within clade B in our sample set. Based on the data currently available, the USA appears to have been a source for much of the EV-D68 diversity that we have sampled. Sequences from the USA are present at the root of the tree, as well as positioned at the base of all major lineages. However, the EV-D68 phylogeny is characterized by the frequent presence of long branches throughout the tree, indicative of a general lack of global surveillance and detection of EV-D68, as well as the presence of *in situ* evolution in individual locations. It is therefore possible that retrospective or broader sampling may reveal additional (and deeper) diversity.

The high correlation between root-to-tip genetic distance and sampling time ($R^2=0.84$; Fig. S1, available in JGV Online) indicates that EV-D68 evolution has been characterized by rapid, clock-like dynamics over the timescale of sampling. The combination of a relaxed molecular clock and the SRD06 model of nucleotide substitution resulted in the best fit of the model to the data (data not shown). Despite the strong clock-like dynamics

Table 1. Origin of EV-D68-positive samples

Location	No. of samples	Collection date	Age
The Gambia	5	June 2008	<2 years
Senegal	3	February–March 2010	2, 23 and 32 years
South Africa	8	May 2000–May 2001	5–23 months
USA (NYC)	16	August–October 2009	14–47 years*
USA (Arizona)	1	October 2009	23 months

*Age unknown for two cases.

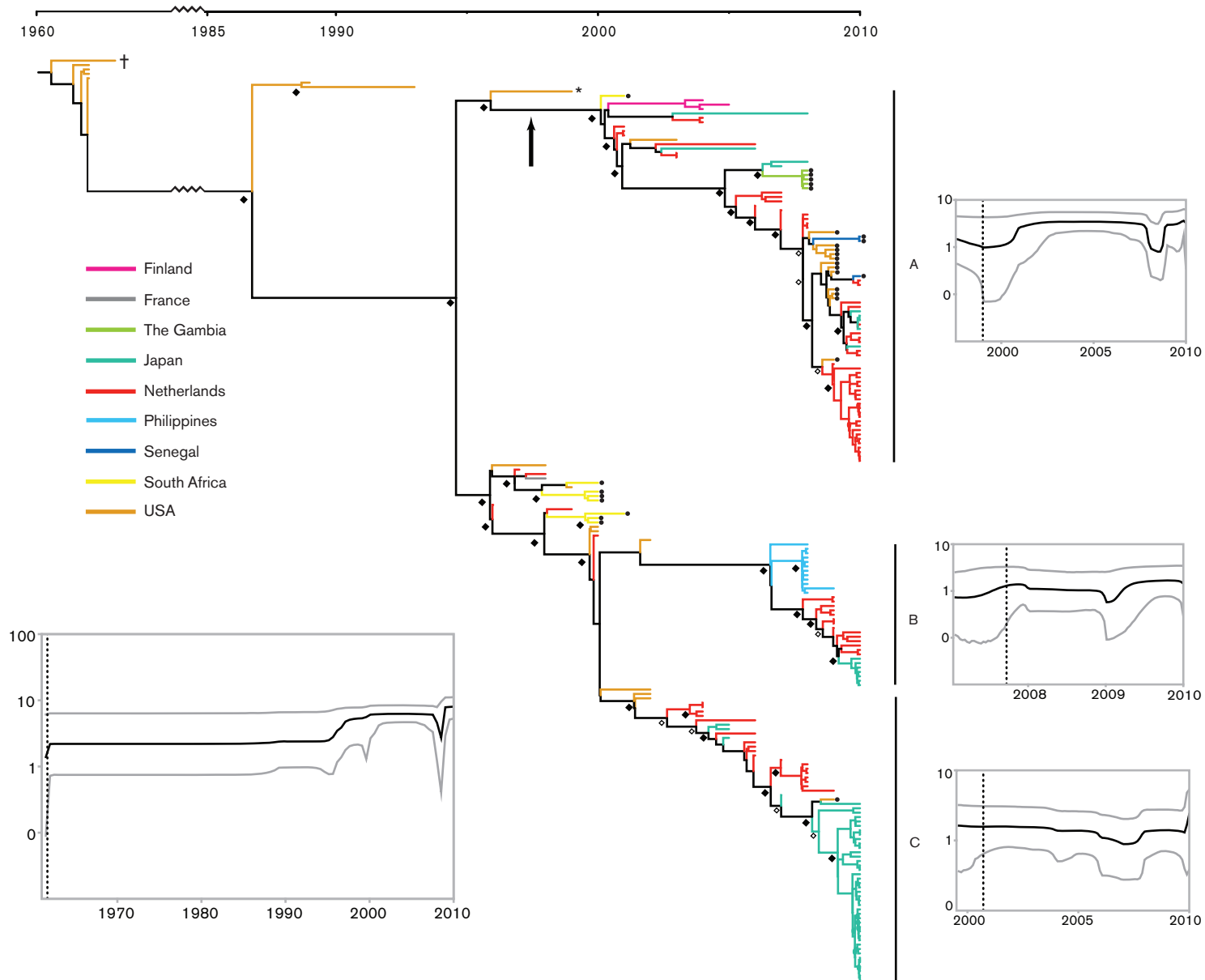


Fig. 1. Evolutionary history of EV-D68 based on complete VP1 sequences. The maximum clade credibility tree is shown with BPP values of 0.7–0.8 and 0.9–1.0 indicated by \blacklozenge and \diamond at the nodes, respectively. Clades A, B and C are indicated and supported by BPP=1.0. The Bayesian skyline plots (BSPs) are shown for the overall dataset (bottom left) and for clades A, B and C. The black line indicates the median relative genetic diversity (\log_{10} scale) over time and the grey curves represent the upper and lower 95% HPD intervals. \bullet at the tips of the branches indicates novel sequences generated in this study. The vertical arrow indicates the branch along which the VP1 asparagine deletion occurred; \dagger indicates the Fermon strain (1962), and $*$ indicates the initial clade A strain from Maryland, USA (1999).

Table 2. Estimated mean TMRCA (years) and nucleotide substitution rates (nucleotide substitutions per site year⁻¹) for EV-D68 based on all available complete VP1 gene sequences, as well as for clades A, B and C

	Mean TMRCA (95 % HPD)	Mean substitution rate (95 % HPD)
Complete dataset: relaxed clock	1961 (1960–1962)	6.2×10^{-3} ($5.4\text{--}7.1 \times 10^{-3}$)
Complete dataset: strict clock	1960 (1959–1961)	5.8×10^{-3} ($5.2\text{--}6.4 \times 10^{-3}$)
Clade A	1997 (1995–1999)	7.1×10^{-3} ($5.9\text{--}8.3 \times 10^{-3}$)
Clade B	2007 (2006–2008)	6.8×10^{-3} ($5.6\text{--}8.1 \times 10^{-3}$)
Clade C	1999 (1998–2001)	6.2×10^{-3} ($5.1\text{--}7.2 \times 10^{-3}$)

suggested by our regression analysis, the values for the coefficient of variation of the relaxed molecular clock were always >0, indicating a significant deviation from strict clock-like evolution. The mean rate of nucleotide substitution for the VP1 gene for EV-D68 was 6.2×10^{-3} substitutions per site year⁻¹ [95% highest probability density (HPD) value, $5.4\text{--}7.1 \times 10^{-3}$ substitutions per site year⁻¹] with a corresponding time to most recent common ancestor (TMRCA) for the entire EV-D68 sample set of 1961 (1960–1962). This value corresponds closely to the TMRCA estimated under a strict clock, as well as by the regression analysis (1960).

The diversity within each of the three primary clades appears to have arisen only recently, with mean TMRCA of 1997 and 1999 for clades A and C, respectively, and of 2007 for clade B (Table 2). However, the long branch leading to clade B indicates that the ancestral diversity of this group has probably not been sampled, and the age of this group may correspond more closely to that of the other clades. Analysing the population dynamics of EV-D68 revealed an overall trend towards increasing genetic diversity in the present, as well as within each of the three main clades (Fig. 1). However, based on the 95% HPDs, no significant departure from a constant level of genetic diversity could be detected. Again, the long branches observed in the tree between the initial characterization of EV-D68 in 1962 and the recent burst of sampling that began around the year 2000 suggest strongly that a significant fraction of the evolutionary history of EV-D68 has not been sampled, making conclusions about the origin and spread of this virus difficult.

Full genome comparison

In addition to the Fermon strain, there are only three other full-length EV-D68 sequences currently available, all from clade C. These consist of strain 37-99 isolated from France in 1999, and strains JPOC10-290 and JPOC10-378 from a 2009 Japanese outbreak. We sequenced the full genome of one of the clade A strains from NYC and compared it with the published genomes (Table 3). The overall length of the NYC strain was 7340 nt. The nucleotide identity compared with clade C strains ranged from 91.4 to 93.8%, and was 88.5% compared with the Fermon strain. The predicted polyprotein identity was 97.4–98% compared with clade C and 95.3% compared with the Fermon strain.

Among the major differences observed in the structure of the genome, all but one of the clade A sequences contained a 3 nt deletion in the VP1 gene at positions 2806–2808, resulting in a missing asparagine residue that is present in all sequences of clades B and C (Fig. 2). The lone clade A sequence without the deletion was a 1999 isolate from Maryland, USA, which is the earliest known representative of the clade (Fig. 1). In addition, we observed variation in the 5' UTR spacer region between the end of the IRES and the beginning of the polyprotein ORF (Fig. 2). The UTR region between positions 162 and 623 was highly conserved and was >95% identical across all EV-D68 strains. Within nt 624–732, both clades A and C had a 24 nt deletion at positions 681–704 relative to the Fermon strain, and clade C had an additional 11 nt deletion at positions 721–731 that was absent in clade A. 5' UTR sequences from representatives of clade B were not available for analysis. To determine whether the pattern of deletions was conserved throughout clades A and C, we amplified and sequenced a fragment containing the 5' UTR spacer region in all our remaining samples. The UTR pattern was observed in all clade A and C sequences, irrespective of year of collection and geographical area.

Table 3. Percentage nucleotide identity between clade A representative and EV-D68 strain Fermon as well as clade C strains 37-99, JPOC10-378 and JPOC10-290

Gene	Clade A NYC403	Fermon	Clade C		
			37-99	JPOC10-378	JPOC10-290
VP4	100	93.2	95.7	94.2	93.7
VP2	100	86.4	93.3	90.7	90.7
VP3	100	85.8	93.5	89.9	90.1
VP1	100	87.6	92.5	90.7	90.3
2A	100	87.3	93.0	90.2	90.9
2B	100	86.2	92.9	90.6	90.2
2C	100	89.4	95.3	93.5	93.4
3A	100	90.3	93.3	90.6	90.3
3B	100	93.9	90.9	92.4	90.9
3C	100	88.4	94.7	90.5	90.6
3D	100	88.9	93.8	91.8	92.2
ORF	100	88.1	93.6	91.5	91.5

DISCUSSION

Reports identifying EV-D68 as the aetiological agent of respiratory disease have increased steadily over the last decade, most notably over the last 3 years. EV-D68 was identified in San Diego, CA, USA, in seven military recruits in 2004–2005 and in respiratory disease surveillance screens in France in 2008 and Italy in 2008–2009 (Petitjean-Lecherbonnier *et al.*, 2011; Piralla *et al.*, 2011; Wang *et al.*, 2010). In the Philippines in late 2008 and early 2009, EV-D68 was detected in 21 patients, two of whom died (Imamura *et al.*, 2011). In Japan, the virus has been detected each year since 2003, culminating in a report describing 14 cases in the autumn of 2010 (Kaida *et al.*, 2011). In the USA, we documented the EV-D68 cluster in NYC in 2009, and the Centers for Disease Control and Prevention (CDC) reported EV-D68 clusters in several states in 2010 (CDC, 2011; Tokarz *et al.*, 2011). In the Netherlands, 24 EV-D68 cases were recorded between August and November of 2010 (Rahamat-Langendoen *et al.*, 2011). A more recent retrospective study of EV-D68 from the Netherlands and Finland described the presence of three distinct clades within the past 10 years (Meijer *et al.*, 2012). Another recent report documented the circulation of these clades within the past decade in Japan (Ikeda *et al.*, 2012). Our analysis of EV-D68 sequences from these reports, in addition to new sequences reported here, indicates that there are at least three major clades of EV-D68 currently in circulation worldwide, consistent with the data in these recent reports.

Between the early 1960s and mid-1990s, the EV-D68 genome underwent a rearrangement in the spacer region of the 5' UTR between the end of the IRES and the polyprotein ORF. This resulted in a 24 nt deletion in all EV-D68 strains that we examined. The virus then underwent a large diversification in the mid-1990s that culminated in a division into clades A and C. Not long after

this division, the genomes of clade C-type viruses underwent further rearrangements, resulting in an additional 11 nt deletion in the spacer region. This deletion, first reported by Kaida *et al.* (2011), may have a significant effect on the initiation of translation. Although it is established that variations within the IRES can have major effects on virulence (Gromeier *et al.*, 1996; Li *et al.*, 2011), little is known about the spacer region and its role in viral fitness. The length of the EV 5' UTR spacer region is approximately 150 nt, and deletions such as those that have occurred in EV-D68 are not common. In rhinoviruses, the spacer is >50 nt and it may be noteworthy that EV-D68, which shares respiratory tropism with rhinoviruses, appears to be undergoing a reduction in the UTR length. Additionally, these deletions may affect the virulence of the virus by enhancing translational efficiency and may be correlated with the recent increase in EV-D68 cases worldwide.

Clade A lacks the secondary deletion observed in the spacer region of clade C viruses, but has a 3 nt deletion in the VP1 gene. According to our calculations, the deletion arose early after the split from clades C and B, sometime between 1997 and 2000. Meijer *et al.* (2012) showed that this deletion, as well as additional VP1 amino acid substitutions in clades A–C relative to the Fermon strain, were in the exposed portions of the VP1. Such changes were proposed to have a role in modulating the immune response to the virus and thus may aid in viral persistence. Following the divergence of the three clades, clades A and C appear to have expanded rapidly as both clades were present in North America, Europe and Africa by 2001. Clade B appears to have diverged from clade C around 2007, although the lack of sampling prior to the early 2000s makes this assumption difficult to corroborate.

It has been suggested that EV-D68 may have a shifted seasonality in comparison to other enteroviruses, which in

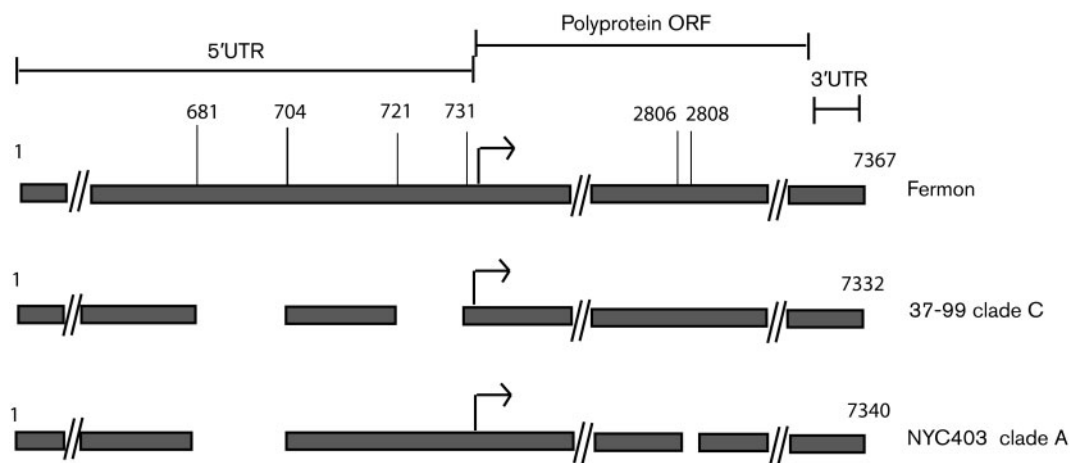


Fig. 2. Major variations in the genomes of clades A and C in comparison with the EV-D68 Fermon strain. Dashed lines (//) indicate extended genomic regions without variation. Arrows represent the start of the polyprotein ORF.

temperate climates traditionally circulate in the summer and early autumn (Khetsuriani *et al.*, 2006). The recently reported EV-D68 outbreaks appeared later than this time frame, with most cases reported throughout the autumn and into winter. EV-D68-positive samples in this report originating from the USA and South Africa were obtained between late summer and winter. Outside of an outbreak in the Philippines, there is little information on the seasonality of EV-D68 in tropical regions. We analysed EV-D68-positive samples from Senegal and the Gambia, where the climate consists of primarily dry and rainy seasons. Within the small sample set that we analysed, the circulation pattern was unclear, as EV-D68 cases in the Gambia occurred during the rainy season, while the cases from Senegal occurred during the dry season. A much larger sample set would be necessary to assess the seasonal circulation pattern of EV-D68 in non-temperate regions.

In previously documented clusters, approximately 80 % of reported EV-D68 cases occurred in children. One explanation for this finding is that most reports highlighted only paediatric cases. Our EV-D68 sequences from the Gambia, South Africa and Arizona were all obtained from children. However, a recent report from the Netherlands, which focused on screening for EV-D68 in archived respiratory samples without age bias, noted a preponderance of adult cases (Meijer *et al.*, 2012). Additionally, NYC cases presented in our report were from individuals aged 14 years or older, which is the largest EV-D68 cluster of non-paediatric origin to date. We anticipate that, with increased screening, EV-D68 may ultimately be recognized as a respiratory pathogen in all age groups.

Although there appears to be a global upsurge of EV-D68-associated respiratory disease, we cannot ignore the possibility that this increase merely reflects improved case ascertainment. In previous studies of respiratory disease, EV-D68 may have been misidentified as a rhinovirus, which would lead to underestimation of its prevalence and importance (CDC, 2011). Continued efforts towards the improvement of diagnostic techniques and surveillance are necessary to further assess the epidemiology of EV-D68 and to assess its role in respiratory disease.

METHODS

Sample collection and sequencing. A total of 33 EV-D68-positive nasal-pharyngeal swab samples were identified from five different respiratory disease sample sets (Table 1). Samples were initially analysed by MassTag PCR for respiratory pathogens (Briese *et al.*, 2005; Lamson *et al.*, 2006) and EV-positive samples were typed based on amplification of the VP4/VP2 gene region (Coiras *et al.*, 2004; Lamson *et al.*, 2006). Sixteen EV-D68-positive samples from NYC were identified from August to October 2009. One sample collected in October 2009 came from Arizona in south-west USA. Sixteen samples originated from Africa: eight samples from South Africa were obtained between May 2000 and May 2001: two were collected in May, four in July of 2000 and one each in April and May of 2001. Five samples from the Gambia were collected in June 2008 and three

EV-D68-positive samples were obtained from an outbreak of respiratory disease in Senegal in February and March 2010.

To acquire the sequence of the full-length VP1 gene from these samples, two sets of primer pairs were used. The first (5'fwd1, CCTTAATAGGGTTCATAGCAGC and 5'rev1, CTGGGCCGG-TGGTYACTA) generated a 1009 bp fragment, while the second pair (5'fwd2, ATGAGAGAYAGYCCTGACATTG and 5'rev2, CATT-GAGBGCATTTGGTGCT) amplified a 900 bp fragment. In instances where the first primer failed to generate the desired PCR product, the second primer pair was used. An additional primer pair (5'fwd3, GGTC AAGCACTTCTGTTTCCC and 5'rev3, TGGCAATGTT-GGCATYTC) was used to amplify a 606 bp fragment spanning most of the 5' UTR and a portion of VP4. The full genome sequence of a single NYC representative of clade A was generated by overlapping consensus PCR using existing EV-D68 genomes as reference. All sequences were deposited in GenBank under accession numbers JX101786–JX101846. All base pair coordinates are provided relative to the original 1962 EV-D68 isolate, the Fermon strain (GenBank accession no. AY426531).

Phylogenetic analysis. A nucleotide alignment incorporating all EV-D68 sequences available in GenBank was constructed manually (928 nt, $n=210$) using Se-AL (v2.0a11 Carbon, <http://tree.bio.ed.ac.uk/software/seal>). To assess the molecular clock-like behaviour of the data, a maximum-likelihood (ML) phylogenetic tree was first constructed using PhyML (v3.0, Guindon & Gascuel, 2003) incorporating the Hasegawa, Kishino and Yano 1985 (HKY85) model of nucleotide substitution with an among-site rate heterogeneity parameter (γ , G) and a heuristic SPR (subtree pruning and regrafting) branch-swapping search. The root-to-tip genetic distance inferred from the ML tree was then regressed against the time of sampling (in years) using the program Path-O-Gen (v1.2, <http://tree.bio.ed.ac.uk/software/pathogen/>). In this analysis, the correlation coefficient provides an estimate of the amount of variation in genetic distance that is explained by sampling time and the x -intercept serves as an estimator of the time to most recent common ancestor (TMRCA).

The evolutionary relationships of the viral sequences, along with the rate of nucleotide substitution per site year⁻¹ and the TMRCA, were inferred using a Bayesian Markov chain Monte Carlo (MCMC) method implemented in the BEAST package (v1.7, Drummond & Rambaut, 2007). Both strict and relaxed (uncorrelated lognormal) molecular clocks were employed with a flexible Bayesian skyline plot (BSP) coalescent prior (10, 15 and 20 piece-wise constant groups) and both the HKY85+G and the SRD06 models of nucleotide substitution. The MCMC chains were run for 200 million iterations, with subsampling every 20 000 iterations. A 10 % burnin was removed and maximum clade credibility trees were summarized using TreeAnnotator (v1.5.4), with Bayesian posterior probability (BPP) values providing a measure of statistical support at each node. Statistical confidence in the parameter estimates was represented by values for the 95 % highest probability density (HPD) intervals around the marginal posterior parameter means. The output of the BSP model was also used to infer the population dynamics of EV-D68 through time, both for the complete dataset and for each of three monophyletic clades we identified with BPP=1.0.

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