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Genetic Characteristics of Human Enterovirus 71 and Coxsackievirus A16 Circulating from 1999 to 2004 in Shenzhen, People's Republic of China†

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The genetic and phylogenetic characteristics of human enterovirus 71 (EV71) and coxsackievirus A16 (CA16) sampled from children with hand, foot, and mouth disease in Shenzhen, People's Republic of China, over a 6-year period (1999 to 2004) were examined with reverse transcription-PCR and DNA sequencing. Out of 147 stool specimens, 60 showed positive signals when screened with EV71- and CA16-specific primers. EV71 was identified in 19 specimens, and CA16 was identified in 41 specimens; coinfection by EV71 and CA16 was not observed. Phylogenetic analysis of all EV71 strains isolated from the mainland Chinese samples established C4 as the predominant genotype. Only one other known strain (3254-TAI-98; AF286531), isolated in Taiwan in 1998, was identified as belonging to genotype C4. Phylogenetic analysis of CA16 strains allowed us to identify three new genetic lineages (A, B, and C), with lineage C recently predominating in Asian countries, such as the People's Republic of China, Malaysia, and Japan. These new observations indicate that CA16 circulating in the People's Republic of China is genetically diverse, and additional surveillance is warranted.

Hand, foot, and mouth disease (HFMD), a common illness in children, can be caused by many human enteroviruses, including coxsackieviruses A4, A5, A8, A10, A16, B3, and B7 and enterovirus 71. Of these, human enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are two major causative agents of HFMD. EV71 and CA16 infections manifesting as HFMD and herpangina are clinically indistinguishable, but EV71 infection is more frequently associated with serious neurological complications and fatalities (13, 20, 24, 27, 30). Since 1997, several large epidemics of HFMD have been reported in the Asia-Pacific region, especially in Southeast Asia. Outbreaks with multiple cases of severe neurological pathologies have occurred in Taiwan, Malaysia, and Singapore. Historically, these HFMD epidemics involve major proportions of EV71 and CA16, with differing ratios. For example, the 1998, 1999, and 2000 enterovirus infections in Taiwan included 44.4, 2.0, and 20.5% EV71, respectively, and 18.2, 1.7, and 18.0% CA16, respectively (31).

Here we examine the frequencies of EV71 and CA16 in HFMD cases in the city of Shenzhen, People's Republic of China. Shenzhen is located on the southern coast of China, has a prosperous mutual exchange with other regions of Southeast Asia, and has a relatively high incidence of HFMD. For this study, 147 stool specimens from pediatric patients diagnosed with HFMD or suspected enterovirus infection in Shenzhen from 1999 to 2004 were collected for epidemiological surveillance. The frequencies of EV71 and CA16 infections were determined, and partial viral genome sequences were used to analyze the genetic characteristics and genotypes of the viral strains.

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MATERIALS AND METHODS

Stool specimens and viruses. Stool specimens ($n = 147$) were obtained from the Department of Microbiology at Shenzhen Center for Disease Control and Prevention (Shenzhen, People's Republic of China). All specimens were collected from pediatric patients in the city of Shenzhen with clinical diagnoses of HFMD or suspected enterovirus infection between 1999 and 2004.

Extraction of RNA. Stool specimens were mixed thoroughly with 5 to 10 volumes of phosphate-buffered saline (pH 7.4) to yield homogeneous suspensions. The mixtures were clarified by centrifugation at 13,000 × *g* for 5 min. Viral RNA was extracted from supernatants of stool suspensions using either the QIAamp viral RNA Mini kit (QIAGEN, Germany) or the High Pure viral RNA kit (Roche, Germany). Extracted RNA was stored at −80°C for later use.

Reverse transcription-PCR and nucleotide sequencing. cDNA was generated in a 20-μl reaction volume for 1.5 h at 42°C using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the provided instructions. PCR was performed in 50-μl reaction volumes containing 2 μl of cDNA, 2 U of recombinant *Taq* DNA polymerase (Takara, Japan), 50 pmol specific forward and reverse primers, 0.4 mM concentrations of deoxynucleoside triphosphates, 20 mM Tris-HCl (pH 8.4), 20 mM KCl, and 1.5 mM $MgCl_2$. The cycling conditions consisted of 2 min at 94°C, followed by 34 cycles of 94°C for 30 s to 1 min, 55°C for 30 s to 1 min, and 72°C for 1 to 3 min, and then 72°C for 10 min. The PCR products were examined by agarose gel electrophoresis and purified with a QIAquick PCR purification kit (QIAGEN, Germany).

The primers used for EV71 and CA16 detection, partial genome amplification, and sequencing are listed in Table S1 in the supplemental material. The primers were numbered according to EV71 strain SHZH03 (AY465356) and CA16 strain Tainan-5079-98 (AF177911).

All amplicons were bidirectionally sequenced, first with the amplification primers and then with the sequencing primers. Cycle sequencing was performed with the Big Dye Terminator cycle sequencing kit (Applied Biosystems) and an ABI3730 automated DNA sequencer (Applied Biosystems).

TABLE 1. Sequence comparisons of genome regions among EV71 Shenzhen strains

Genome region of EV71	Nucleotide identity (%)[a]	Amino acid identity (%)[a]
Partial 5′ UTR	93.3–100	
VP4	92.7–100	100
VP2	91.0–99.7	97.2–100
VP3	89.9–100	98.3–100
VP1	92.7–100	98.3–100

[a] The data indicate the range of sequence identities (percent) among EV71 strains of Shenzhen, China.

Data analysis. The nucleotide sequence data were inspected and prepared with the Bioedit (version 5.0) sequence analysis program (9). Further sequence analysis employed programs of the LaserGene package (DNAstar). Multiple sequence alignments were performed with the ClustalW program (29). Phylogenetic analysis was performed using the neighbor-joining method with PHYLIP (version 3.6), and the reliability was evaluated by bootstrap analysis with 1,000 data sets (8). Cladograms were drawn with the TREEVIEW program (18).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this study have been deposited in the GenBank database under accession numbers AY895091 to AY895145, AY821794 to AY821797, and AY790926. The EV71 and CA16 nucleotide sequences retrieved from GenBank and primary sequences obtained in this study are listed in Table S2 in the supplemental material.

RESULTS

From 1999 to 2004, there were no epidemics of HFMD reported in Shenzhen, but each year there were small, local outbreaks associated with only a few cases of neurological disease and no reported fatalities. For this study, 147 stool specimens were collected, and the case number each year ranged from 22 to 29. The stool specimens were reverse transcription-PCR screened with EV71- and CA16-specific primers. We identified 60 positive specimens; EV71 was detected in 19 specimens and CA16 in 41 specimens. Coinfection by EV71 and CA16 was not identified in these samples. Of the patients with molecularly confirmed EV71 or CA16 infection, the age ranged from 6 months to 8 years, with 52 of the patients (87%) being less than 5 years old. There were 39 boys and 21 girls, for a male-to-female ratio of 1.9 to 1. Interestingly, no EV71 infection was detected in the samples from 1999 and 2000, whereas the samples from 2001, 2002, 2003, and 2004 showed 3, 2, 3, and 11 cases positive for EV71, respectively. CA16 was detected from samples taken in each year from 1999 to 2004, with a distribution of 7, 2, 8, 13, 2, and 9 cases, respectively. In 2004, out of eight positive specimens collected from a single kindergarten in Shenzhen, five were positive for EV71 infection.

We next sequenced and analyzed the EV71 strain viral genomes from the 5′ untranslated region (5′ UTR) and the VP4 and VP2 to VP1 regions (about 3,200 bp). Pairwise nucleotide and amino acid comparisons of the five individual regions showed that the variability was minor among the EV71 strains detected in Shenzhen over the 4-year period (Table 1). The nucleotide identities of the partial 5′ UTR (616 bp) sequences were higher than 93.3%. Nucleotide insertions and deletions were observed in this region compared to comparable sequences from the SHZH98 strain and the prototype, BrCr. The VP4 region showed greater than 92.7% nucleotide identity and 100% amino acid identity across the detected EV71 strains.

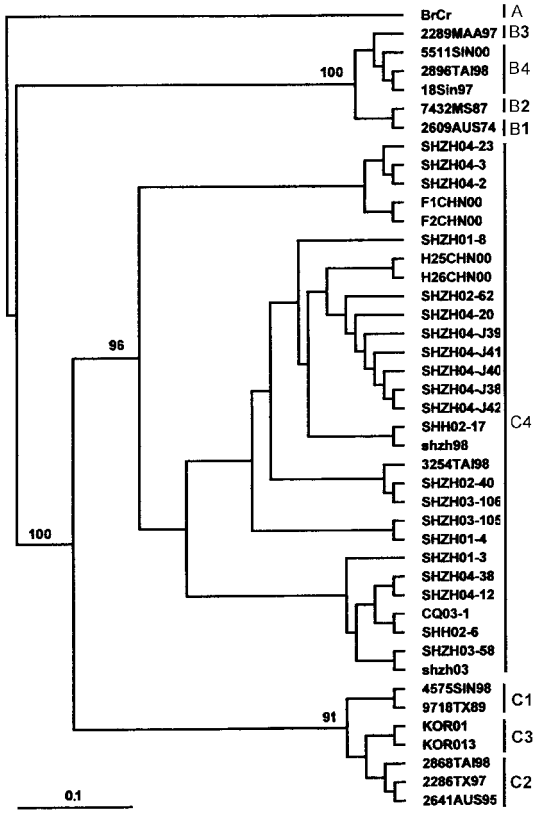

FIG. 1. Phylogenetic analysis based on EV71 VP1 nucleotide sequences (891 bp). Details of the EV71 strains included in the dendrogram are provided in Table S1 in the supplemental material. The marker denotes the percentage of bootstrap frequency of the main branch. The VP1 nucleotide sequence of EV71 prototype BrCr was used as an outgroup in the analysis.

The VP2 region showed the largest variation in amino acid sequence, with identities ranging from 97.2% to 100%. The VP3 region had the highest nucleotide variation (>89.9% identity) but showed amino acid identities higher than 98.3%, with 10 strains detected from four different years yielding identical amino acid sequences. The nucleotide and amino acid identities of the VP1 region were higher than 92.7% and 98.3%, respectively.

Phylogenetic analysis of these strains was based on the alignment of complete VP1 or VP4 gene sequences. A total of 43 EV71 strains were used for phylogenetic analysis of the VP1 gene (Fig. 1), including the 19 EV71 strains identified in this study, 9 EV71 strains previously detected from the Chinese mainland (GenBank), and 15 other EV71 strains (GenBank) included as genotype references. Consistent with the results of previous studies, all 43 strains clustered into three distinct genotypes on the phylogenetic tree. The EV71 strains detected from the Chinese mainland were closely related to each other and grouped into genotype C to form a new genetic lineage (C4) distinct from the previously described C1, C2, and C3 lineages. The VP4 gene was similarly analyzed in 30 EV71 strains, including 13 reference strains (Fig. 2). As in the VP1 analysis, the VP4 sequences of 17 EV71 strains detected from Shenzhen also fell into genotype C, clustered in the C4 lineage.

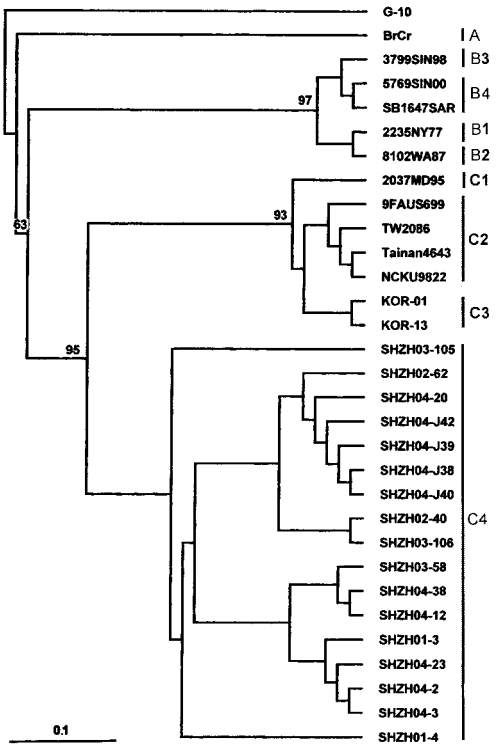

FIG. 2. Phylogenetic analysis based on EV71 VP4 nucleotide sequences (207 bp). Details of the EV71 strains included in the dendrogram are provided in Table S1 in the supplemental material. The marker denotes the percentage of bootstrap frequency of the main branch. The VP4 nucleotide sequence of coxsackievirus A16 prototype G10 was used as an outgroup in the analysis.

We performed a similar analysis of the CA16 strains detected from Shenzhen during the 6-year period of this study. We sequenced and analyzed genome regions from the 5′ UTR, VP4, VP2, and VP3 to VP1 (about 3,200 bp) and used pairwise nucleotide and amino acid comparisons to assess the heterogeneity among the strains (Table 2). The nucleotide identities of the partial 5′ UTR (617 bp) were higher than 90.2%, with nucleotide insertions and deletions noted in comparison to the sequence of the CA16 prototype, G10. In the VP1 region, the nucleotide identities ranged from 88.3 to 100%, and amino acid identities were higher than 95.6%. In 1999 and 2000, the nucleotide differences seldom resulted in amino acid variations. From 2001 to 2004, the accumulated nucleotide changes resulted in new amino acid variations as nonsynonymous substitutions increased. The nucleotide and amino acid identities between two particular strains isolated in 2003 were 99.5 and 99.3%, respectively, with three out of four nucleotide variations forming nonsynonymous substitutions. This tendency was more obvious in VP1 than in VP2 or VP3. The nucleotide identities in the VP4 region ranged from 85.9 to 100%, and the amino acid sequences of 34 strains isolated from six different years were identical. In contrast, the VP4 regions of four strains (SHZH99-11, 99-48, 99-79, and 00-2) showed higher variation, with identities of >97.1% among the four strains and identities with the other strains ranging from 85.9 to 91.7%.

TABLE 2. Sequence comparisons of genome regions among CA16 Shenzhen strains

Year	Sequence identity (%)[a]				
	5′ UTR	VP1	VP2	VP3	VP4
1999	92.1–99.6	90.1–99.4	89.5–99.6	88.9–99.4	89.3–99.5
		99.6–100	*98.8–100*	*99.1–100*	*94.2–100*
2000	92.6	89.3	89.5	88.4	91.3
		99.6	*99.2*	*99.1*	*100*
2001	95.6–100	96.1–99.6	97.1–100	96.1–99.4	95.6–100
		97.6–100	*96.8–100*	*97.9–100*	*94.2–100*
2002	96.9–99.8	92.0–100	94.4–98.9	93.8–99.1	94.2–100
		95.6–100	*96.0–100*	*98.3–100*	*97.1–100*
2003	99.8	99.5	98.9	98.6	98.5
		99.3	*98.8*	*99.5*	*95.6*
2004	96.0–100	93.0–100	92.5–99.8	92.9–100	93.7–100
		98.6–100	*98.4–100*	*99.5–100*	*98.5–100*
1999-2004	90.2–100	88.3–100	87.1–100	87.0–100	85.9–100
		95.6–100	*96.0–100*	*97.9–100*	*88.4–100*

[a] The data indicate the range of sequence identities (percent) among CA16 strains of each year and from 1999 to 2004. Amino acid identities are shown in italics.

Phylogenetic analysis of the CA16 strains was based on the alignment of complete VP4 gene sequences among the detected strains and 53 additional previously characterized CA16 strains, allowing us to newly clarify the genetic lineages of CA16 strains detected from 1951 to 2004 in the United States, the United Kingdom, Japan, Malaysia, People's Republic of China, and Taiwan. While there were a few outliers, the CA16 strains clustered in three distinct genetic lineages (A, B, and C) supported by the indicated bootstrap values (Fig. 3). Lineage A contains the CA16 prototype, G10, and a single isolate from Japan (G10JPN). The nucleotide identity of the two strains was 99.5%, and the nucleotide sequences differed from all other strains by 20.4 to 27.4%. Lineage B (>93% identity) comprised four strains detected in this work during 1999 and 2000, some early Japanese strains, and some Malaysian strains detected in 2000. Most of the Asian strains detected during the 1990s and the United Kingdom strain Epsom/15290/99 belonged to lineage C and shared nucleotide identities higher than 90.8%. The nucleotide divergence between lineages B and C was 7.0 to 18.6%.

DISCUSSION

EV71 and CA16 belong to the family *Picornaviridae*, which is characterized by a single positive-strand genomic RNA known to have a high mutation rate caused by low-fidelity replication and frequent recombination. Different genotypes and subgenotypes of the two viruses have alternated and cocirculated in the Asia-Pacific region, leading to repeated outbreaks of HFMD. Although a few previous studies have attempted to clarify the genetic characteristics and phylogenetic relationships of the EV71 and CA16 strains detected from the Chinese mainland (7, 26), we have herein broadened these analyses to include more viral strains collected over a longer time span in order to provide a more comprehensive overview of the molecular epidemiology of the EV71 and CA16 viruses circulating on the Chinese mainland.

Ever since the 1980s, large and small EV71 epidemics caused by distinct genotypes have occurred in Asian countries and regions sharing trade with China. In Hong Kong, sporadic EV71 infections with incidences of monoplegia were reported in 1987, and a fatal case was documented in 2001 (16, 22). A

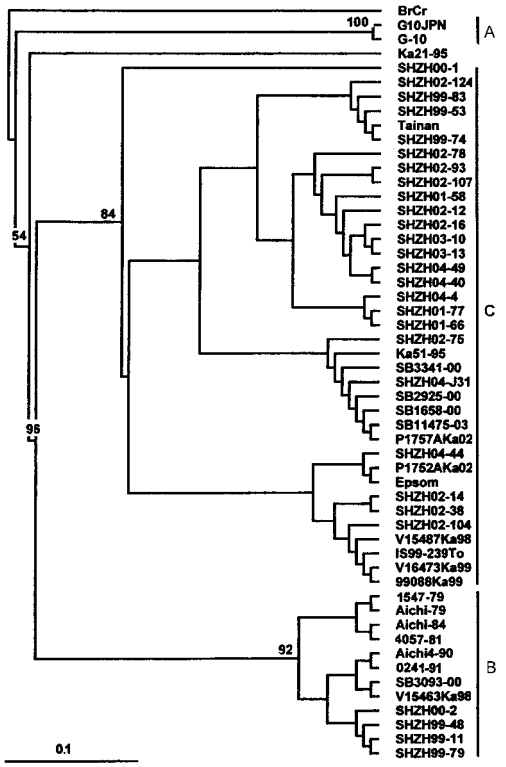

FIG. 3. Phylogenetic analysis based on CA16 VP4 nucleotide sequences (207 bp). Details of the CA16 strains included in the dendrogram are provided in Table S1 in the supplemental material. The marker denotes the percentage of bootstrap frequency of the main branch. The dendrogram shows only one isolate number to represent isolates with identical sequences. The VP4 nucleotide sequence of EV71 prototype BrCr was used as an outgroup in the analysis.

large outbreak of HFMD due to EV71 infection occurred in Taiwan in 1998, including 129,106 case reports, 405 children with severe complications, and more than 80 deaths (6, 12, 25). After 1998, smaller EV71 epidemics occurred almost annually in Taiwan, primarily associated with EV71 viruses of genotypes C2 and B4. In Malaysia, EV71 outbreaks occurred in 1997 and 2000, mainly associated with genotypes B3 and B4 (1, 14). Since 1997, EV71 epidemics (genotypes B and C) have been reported annually in Singapore, with genotype B4 forming the predominant causative agent of a large outbreak in 2000 (5). In Korea, an EV71 epidemic in 2000 was caused by a relatively new genotype, C3, which has only rarely been identified outside of Korea in recent years (10). Thus, the EV71 genotypes of these HFMD epidemics seem to be regionally and temporally unrestricted.

We compared the available published data for 9 EV71 strains detected from the Chinese mainland with the 19 sequences determined in this study. These 28 EV71 strains were detected from Shenzhen, Shanghai, and Chongqi during the years 1998 to 2004, excluding 1999. The nucleotide identities of the 28 strains were higher than 92.7%, and the amino acid identities were at least 96.6%. All of the strains clustered close together in our phylogenetic analyses, with phylogenies based on the VP1 and VP4 gene sequences providing similar results. The VP1-based analysis indicated that the strains detected from the Chinese mainland formed a distinct cluster in genotype C, having 90.2 to 92.2% nucleotide sequence identities with the selected reference strains of the C1, C2, and C3 subgroups but only 81.3 to 83.8% identities with genotype A and B strains. These results were consistent with the genetic lineage definition of Brown et al. (3), allowing us to establish subgroup C4 for the 28 strains isolated from the Chinese mainland. A phylogenetic analysis based on the VP4 sequences confirmed this result. Furthermore, analysis of the EV71 VP1 sequence data available in GenBank revealed only one other C4 genotype strain, 3254-TAI-98 (AF286531), which was isolated from Taiwan in 1998.

No large EV71 epidemic has been reported on the Chinese mainland, but sporadic infections are common in the southeast coastal area as well as inland regions, such as Beijing, Chongqing, and Jinan. Our results indicate that the EV71 viruses circulating throughout the Chinese mainland do not have as great a genetic diversity as those from Taiwan, Singapore, or Malaysia (11, 15, 22, 28). Indeed, from 1998 to 2004, only viruses belonging to genotype C4 were identified on mainland China. Interestingly, a C4 genotype was also identified in Taiwan, which has otherwise yielded strictly C2 and B4 epidemics (with one historical report of genotype C3). There are two possible explanations for this finding: either the Taiwanese strain circulated to the Chinese mainland through one or more of the mutual exchanges between two regions or viruses were transmitted from the Chinese mainland to Taiwan and then underwent mutational variations. The observation site we selected was located on the Asia-Pacific coast and had many affiliations with adjacent regions where EV71 is epidemic. Thus, it is quite interesting that the observed viral genotypes were not affected by epidemic situations in adjacent regions. This may indicate that the Chinese mainland forms a relatively independent area with specific geographic and climatic features allowing EV71 to be sustained with little outside effect. Observations such as this and a similar report by Cardosa et al. (4) in regard to SHZH98 (People's Republic of China, 1998) tend to support the hypothesis that EV71 viruses isolated on the Chinese mainland may be the ancestors of the EV71 strains currently circulating in Taiwan.

The original data of the study were obtained from enterovirus 71 strains collected from Shenzhen, People's Republic of China, and the data of enterovirus 71 strains collected from Chongqing and Shanghai were also included for phylogenetic analysis. To provide a more comprehensive epidemiologic picture of enterovirus 71 in mainland China, more data from different surveillance sites in a broad geographic area will be necessary.

We similarly examined the prevalence and genotype distribution of CA16, another main pathogen associated with HFMD that is less well studied, likely due to its relatively benign clinical symptoms. In this study, we subjected all available CA16 sequence data to phylogenetic analysis as a novel attempt to provide a primary outline of its molecular typing. Though a phylogenetic analysis based on the longer VP1 gene would have provided greater confidence, we were limited to using VP4, as there were relatively few VP1 sequences available in GenBank. Our results indicate that there are three genetic lineages circulating in Asia, namely, A, B, and C. These

are called lineages because the data were insufficient for us to concretely identify the groupings as genotypes (2, 23).

Our results showed that in Shenzhen, lineages B and C cocirculated in 1999 and 2000, but only lineage C was found from 2001 to 2004. A single CA16 strain isolated from Taiwan in 1998 belonged to lineage C. Japan has monitored CA16 ever since the 1970s; a few of the Japanese strains isolated prior to the 1990s belonged to lineage B, while a single strain (G10JPN) isolated in the early 1990s shared lineage A with the CA16 prototype, G10. Viruses of lineages B and C cocirculated in Japan beginning in 1995, with lineage C gradually becoming predominant. In Malaysian strains detected in 2000 and 2003, viruses of lineages B and C coexisted (with lineage C predominating) in 2000, while lineage C but not lineage B was identified in 2003 (19). These results indicate that three genetic lineages of CA16 (A, B, and C) have existed in Asia. Lineage A and B viruses cocirculated before the early 1990s, with B predominating. Since the late 1990s, nucleotide variations have led to the emergence of lineage C viruses, which have gradually replaced lineage B as the predominant CA16 genetic lineage in Asia (17, 21). Unfortunately, CA16 sequences from Western countries are scarce. We were able to identify only the prototype G10 (United States, 1951) and strain Epsom/15290/99 (United Kingdom, 1999), which belong to lineages A and C, respectively. Further data will be required to determine whether the CA16 genetic lineages show the same tendency in Western countries as in Asia.

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