

# Genetic heterogeneity of HIV-1 in Greece

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**ABSTRACT** – The aim of this study was to detect and determine the genetic variation of HIV-1 in Greece and to analyze the phylogenetic relationships and transmission dynamics of identified variants. Eighty-six blood samples from HIV-1 seroconverted patients of different risk groups were collected from the AIDS clinic, AHEPA Hospital, Thessaloniki, Greece. Retroviral DNA was extracted from uncultured peripheral blood mononuclear cells. HIV-1 DNA sequences encoding a 500-bp fragment of the gp120 C2-C3 region were amplified from each study subject, and they were genetically subtyped by heteroduplex mobility assay and DNA sequencing. Genetic distances and phylogenetic relationships of DNA sequences were estimated using PHYLIP software. Our results revealed that 82 out of 86 (95.3%) subjects carried subtype B sequences, while four (4.7%) carried subtype A sequences. Subtype A in Greek individuals not having traveled abroad was documented. An average of intrasubtype B genetic divergence of 15% was noted. Our findings demonstrate the presence of at least two genetic subtypes of HIV-1 in northern Greece – subtype B and subtype A. The predominant subtype is subtype B, which was transmitted into Greece by multiple sources. Our observations lend support to the argument that the distribution of HIV-1 subtypes is determined by founder effects or other processes rather than any tropism for particular cell types or mode of transmission. © 2000 Éditions scientifiques et médicales Elsevier SAS

HIV-1 / genetic heterogeneity / subtypes / Greece

## 1. Introduction

HIV-1 is characterized by an unusually high degree of genetic variation. A majority of HIV-1 sequences cluster into one large group called M (major), whereas few are clearly distinct, and they constitute the group O (outlier) [1]. Group M sequences have been further subdivided into 10 genetic subtypes or clades, A through J [1–3]. Subtype B predominates in western Europe, North America, Latin America, and Australia, whereas one half of the strains from eastern Europe have been other than clade B, including A, D, C, F, and G. Clades A, C, and D are prevalent in Africa and clade C in India. Clades B and E predominate in East Asia and Thailand, where other subtypes seem to be less prevalent [1, 4]. The envelope glycoprotein, a major target of antiviral immunity and a pivotal element of current vaccine strategies, differs by up to 30% between subtypes, while up to 15% or more within subtypes [5].

The existence of these ten subtypes has many important implications for the global evolution of HIV, future vaccine development, diagnostic problems, pathogenicity, and transmissibility [6–10].

In Greece, which is a country with a population of 10.5 million, a total of 1 882 AIDS cases have been reported up to 31 December 1998; 1 103 (58.6%) of them were fatal; 1 852 of the total cases were adults and adolescents (87.5% males, 12.5% females) and 30 were children (60% boys, 40% girls). Among the 1 852 adult cases, 75.4% were infected sexually, 4.4% were intravenous drug users, 8.6% were infected through multiple transfusion, and for 11.6% the route of transmission was unknown [11].

## 2. Materials and methods

Blood samples were obtained from 86 HIV-1-seropositive patients (68 males, 18 females) from different risk groups, who were visiting the regional AIDS clinic of the AHEPA Hospital in Thessaloniki, northern Greece

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**Table I.** Patients from whom *env* region of proviral DNA was amplified and sequenced.

ID	Accession number	Year of birth	Sex	Risk group	Infection date
GR20	AJ224953	1943	Male	Unknown	1996
GR23	AJ224954	1969	Male	Homosexual	1995
GR34	AJ224955	1944	Male	Bisexual	1997
GR48	AJ224956	1965	Male	Bisexual	1997
GR50	AJ224947	1961	Male	IDU	1993
GR51	AJ224948	1976	Male	Homosexual	1997
GR63	AJ224949	1955	Male	Homosexual	1994
GR71	AF094523	1964	Female	IDU	1994
GR74	AJ224951	1960	Male	Heterosexual	1997
GR76	AJ224952	1967	Female	Heterosexual	1996
GR94	AF094524	1967	Female	IDU	1990
GR107	AF201735	1967	Male	Bisexual	1997
GR138	AF094525	1964	Male	IDU	1992
GR167	AF094526	1960	Male	Homosexual	1991
GR175	AF094527	1963	Male	IDU	1994
GR187	AF094528	1966	Female	IDU	1997
GR190	AF094529	1963	Male	Homosexual	1997
GR191	AF094530	1957	Male	Heterosexual	1996

IDU, intravenous drug user.

between 1997 and 1998. All of the patients were living in northern Greece and seroconverted between 1990 and 1998. Eighty-one (94.2%) of these subjects were Greek citizens, whereas five (5.8%) were foreigners from different nationalities of Africa. The age range of the patients was between 21–67 years (average age 32 years). Twenty-five of them (29.1%) were male homosexuals, 12 (14%) were male bisexuals, 24 (27.9%) (ten males and 14 females) were heterosexuals, nine (10.5%) (six males and three females) were intravenous drug users, two (2.3%) were male hemophiliacs, whereas for 14 of them (16.2%) (13 males and one female) the route of transmission was unknown.

Proviral DNA was extracted from uncultured peripheral blood mononuclear cells using the 'whole blood specimen preparation kit' (Amplicor, Roche Diagnostic System, USA) as described by the manufacturers. A two-step nested PCR was performed. All primers and PCR conditions were described by Delwart et al. [12]. Five  $\mu$ L of second PCR product were mixed with 2  $\mu$ L of loading buffer and were electrophoresed at 120 V for 30 min in TBE buffer. DNA was detected by UV transillumination. Heteroduplexes were formed according to the method described elsewhere [12]. Heteroduplex products were mixed with 1.5  $\mu$ L loading buffer and loaded on 3% Meta-Phor XR agarose gel (FMC BioProducts) [13]. The PCR products from 18 samples taken from patients listed in table I were sequenced with amplification primers ED31 and ED33, using fluorescent dye termination method (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli Taq DNA polymerase, FS, Perkin Elmer) and an Applied Biosystems (Foster City, CA) 373A DNA sequencer.

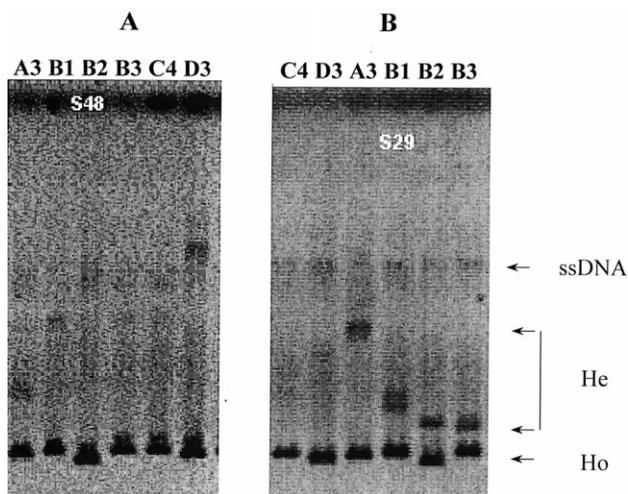
The DNA sequences of this study were aligned using the Clustal version W multiple alignment program [14]. Sites at which there was a gap in any of the aligned sequences were excluded from all comparisons. The

sequences were aligned with other envelope reference sequences of all ten known group M subtypes obtained from the Los Alamos database [15]: subtype A (U455 and 92UG037.1); B (MN and B1); C (D747 and UG268); D (NDK and ELI); A/E (90CF402 and 93TH253); F (BZ163 and BZ126); G (GA.LBV217 and 94-47621); H (90CF056 and RU1.MLY10A); A/G/I (94CY.HO32 and GR11); J (SE9173 and SE9280). Group O (CM.MVP5180) envelope sequences were used as outgroup. After alignment, the sequences were transferred to the Phylip program for creation of distance matrix files and phylogenetic analysis and subsequent construction of the phylogenetic tree [16]. Briefly, the sequence alignment was opened in Seqboot, which read in the data set and produced multiple data sets from it by bootstrap resampling. The 'multiple data sets' option was 100. Then pair-wise distances were generated using the Kimura two-parameter algorithm of DNADIST. The distances were then used in the Fitch distance matrix program which estimated phylogenies. A consensus phylogenetic tree was constructed by Consense.

The GenBank database accession numbers for the Greek sequences obtained in this study are: AJ224947 to AJ224949, AJ224951 to AJ224956, AF094523 to AF094530 and AF201735.

### 3. Results

Eighty-two of the 86 subjects tested by heteroduplex mobility assay (HMA) were identified as subtype B (95.3%), while four other isolates (4.7%) (two obtained from heterosexuals and two from bisexuals) were identified as subtype A (figure 1). Phylogenetic analysis of the 18 sequenced Greek subjects showed that 16 carried subtype B sequences, while two carried subtype A sequences (figure 2). The results of sequencing were in concordance

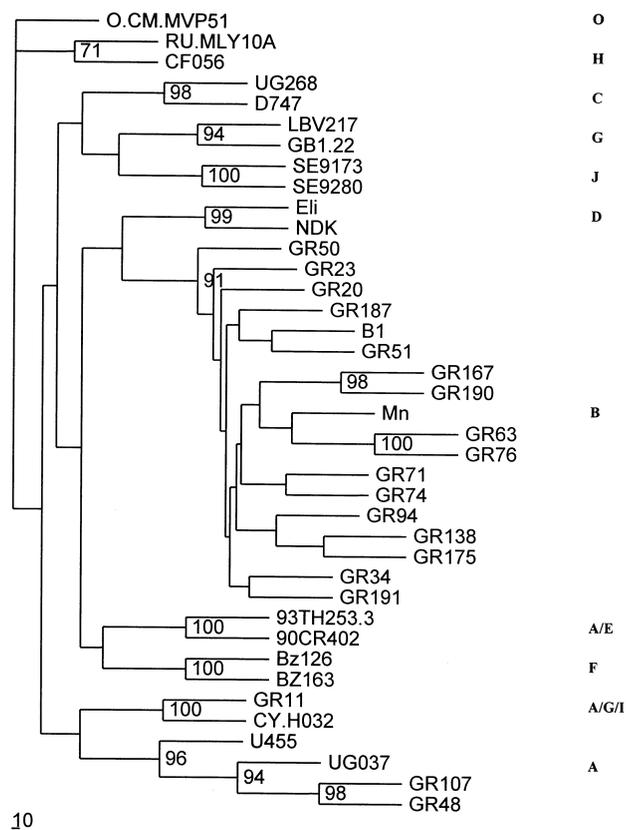


**Figure 1.** Heteroduplexes formed between pairs of PCR-amplified DNA fragments from subtype references. ED31 and ED33 *env* gene fragments were separated in a 3% Metaphor XR agarose gel. ssDNA: single-stranded DNA; He: position of heteroduplex migration; Ho: position of homoduplex double-stranded DNA. A: HMA of sample GR48 (subtype A) with different reference strains listed below with the clone name and GenBank accession number: lane A3: SF170, M66533; lane B1: BR20, UO8691; lane B2: TH14, UO8801; lane B3: SF162, M65024; lane C4: BR25, UO9133; lane D3: UG46, UO8809. B: HMA of sample GR29 (subtype B) with reference strains: lane C4: BR25; lane D3: UG46; lane A3: SF170; lane B1: BR20; lane B2: TH14; lane B3: SF162.

with the HMA results. After measuring the genetic distances among the Greek subtype B sequences, a genetic divergence of 15% (range 1.4 to 20%) was observed. The diversity between the Greek subtype A sequences was 7.5%, whereas the diversity among the Greek subtype A sequences and the Greek subtype B sequences was 23.5% (range 18.3 to 29.5%).

## 4. Discussion

HIV-1 subtypes are unevenly distributed geographically in different population subsets, and may be present at widely different frequencies, making adequate sampling difficult. Epidemiological surveillance of HIV-1 subtypes is an important and ongoing element of preparation of global antiviral interventions. Both vaccine design and vaccine evaluation are complicated by the diversity within and among subtypes and by the geographic intermixing of them [4, 10, 17]. For any HIV-1 vaccine to be successful globally, it should be able to counter viruses from all subtypes, which may require multiple variants to be included in a complex vaccine formulation [18]. For HIV vaccine efficacy trials, it is of critical importance to consider a comprehensive scope of activities, including detailed virological information, prevalence, distribution, and incidence of the genetic subtypes and antigenic variants of HIV-1.



**Figure 2.** Phylogenetic tree based on 400 nucleotides of the *env* region of 18 Greek samples, as well as representatives of each subtype from the Los Alamos HIV Sequence Database. The tree is drawn to scale, whereas vertical branches are for clarity only. The numbers at the nodes indicate the percentage of bootstrap values with which the cluster to the right is supported (only values above 70% are shown).

Our results from HMA revealed that at least two subtypes are found in northern Greece. Among the 86 patients, 82 (95.3%) carried subtype B sequences, which is considered the major subtype in Europe and North America, while four patients (4.7%) carried subtype A sequences. A recent study from southern Greece revealed that subtype B is predominant (86%), while subtypes A, C, D, and I were detected as well [19]. Population movements, such as migration and traveling, and population heterogeneity in this area, may explain the broader HIV-1 diversity in this area compared with northern Greece.

Four of our patients carried subtype A sequences. Two of them were bisexual males, the third was heterosexual male who had had many sexual partners of different nationalities, while the fourth was a man from Africa, which is considered an endemic area for subtype A, and he had probably acquired the virus heterosexually in Africa. From these results, a heterosexual or bisexual transmission of subtype A in Greek individuals not traveling abroad was documented, and this may be due to direct or indirect contact with persons from endemic areas, such as Africa. Two other individuals (GR74 and GR76) who

had resided in Africa acquired subtype B variants heterosexually, and they had probably been infected in Africa. Thus, even though subtype B is rare in Africa, it appears to be present in several parts of this continent [20]. These results agree with those of Alaeus et al. [21], who found that four individuals originating from four different countries carried subtype B variants, and the epidemiological investigations indicate that they all had been infected in Africa.

Generally, the predominant subtype in Greece is subtype B. However, during the last few years, multiple introduction and/or a growing range of non-B subtypes has been described in many parts of the world, especially in North America and several European countries, mainly in persons who have traveled to other continents or immigrated from them [21–24]. These data showed that in the future, the predominance of subtype B might change. The occurrence of non-B subtypes of HIV-1 in Greece, such as the newly described subtype I, will probably create inconsistencies in HIV-1 diagnosis and viral-load monitoring assays [6, 8, 25]. Concerning subtype I, the molecular analysis of the full-length genome gave evidence of A/G/I recombination [26, 27]. Concomitantly, the acquisition of HIV-1 subtype B infections from other countries could broaden subtype B diversity in the Greek population.

Phylogenetic analysis revealed that Greek sequences of subtype B have a high average of intrasubtype genetic divergence of 15%, suggesting that subtype B was transmitted to Greece by multiple sources. The boarder interpatient diversity among subtype B envelope sequences from foreign countries is noteworthy.

A relationship between intravenous drug users and the high prevalence of subtype B, and between heterosexual transmission and subtype E was reported in Thailand [28]. Subtype B viruses were associated with male homosexual transmission and subtype C viruses with heterosexual transmission were reported in republics of the former Soviet Union [29] and South Africa [24]; subtype B viruses were associated with male homosexual transmission in Baltic countries and Russia [30]. Our results do not show a genetic association between subtype B and the route of transmission, as most subjects from every risk group carried subtype B sequences. In addition, they do not support any association between genetic subtype and cell tropism like that found in Thai heterosexuals who carried subtype E viruses which grew more efficiently in the Langerhans' cells than any of the viruses from the USA homosexuals, which carried subtype B viruses [9]. However, recent studies disagree with cellular tropism association, as subtype B and E strains replicate in cutaneous dendritic cell-T-cell mixture or Langerhans' cells without displaying subtype-specific tropism [31, 32].

Although the number of the subjects tested in this study was not very large, it represents one-fourth of the HIV-patients in northern Greece, giving a picture of the general situation in this part of the country.

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