Molecular and epidemiological characterization of HIV-1 infection networks involving transmitted drug resistance mutations in Northern Greece

Lemonia Skoura1*, Simeon Metallidis2, Andrew J. Buckton3, Jean L. Mbisa3, Dimitrios Pilalas2, Evagelia Papadimitriou1, Androniki Papoutsi1, Anna-Bettina Haidich4, Theofilos Chrysanthidis2, Olga Tsachouridou2, Zoe A. Antoniadou1, Panagiotis Kollaras2, Pavlos Nikolaidis2 and Nikolaos Malisiovas1

1National AIDS Reference Centre of Northern Greece, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece; 2Infectious Diseases Division, AHEPA University Hospital, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece; 3Virus Reference Department, Microbiology Services, Health Protection Agency, Colindale, London, UK; 4Department of Hygiene and Epidemiology, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece

*Corresponding author. Tel: +30-2310-999156; Fax: +30-2310-999082; E-mail: mollyskoura@gmail.com

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Objectives: To determine the contribution of transmission clusters to transmitted drug resistance (TDR) in newly diagnosed antiretroviral-naive HIV-1-infected patients in Northern Greece during 2000–07.

Methods: The prevalence of TDR was estimated in 369 individuals who were diagnosed with HIV-1 infection in the period 2000–07 at the National AIDS Reference Laboratory of Northern Greece. Phylogenetic analysis was performed using a maximum likelihood method on partial pol sequences. TDR was defined in accordance with the surveillance drug resistance mutation list (2009 update).

Results: The overall prevalence of TDR in our population was 12.5% [46/369, 95% confidence interval (CI) 9.1%–15.8%], comprising 7.6% (28/369) resistant to nucleoside reverse transcriptase inhibitors, 5.4% (20/369) resistant to non-nucleoside reverse transcriptase inhibitors and 3.3% (12/369) resistant to protease inhibitors. Dual class resistance was identified in 3.8% (14/369). Infection with subtype A was the sole predictor associated with TDR in multivariate analysis (odds ratio 2.15, 95% CI 1.10–4.19, \(P = 0.025\)). Phylogenetic analyses revealed three statistically robust transmission clusters involving drug-resistant strains, including one cluster of 12 patients, 10 of whom were infected with a strain carrying both T215 revertants and Y181C mutations.

Conclusions: Our findings underline the substantial impact of transmission networks on TDR in our population.

Keywords: HIV/AIDS, molecular epidemiology, resistance

Introduction

Transmitted drug resistance (TDR) in HIV-1 has been well documented and shown to be an impediment to the success of antiretroviral therapy and raises the possibility of the further spread of resistant strains.1,2 The limited therapeutic options for antiretroviral treatment-experienced patients in the past led to high rates of replication of drug-resistant viruses, which consequently increased the risk of transmission. The rate of new diagnoses showing drug resistance ranges from very low in countries with limited use of antiretroviral therapy to 20% in some populations.3 Understanding the pattern of HIV transmission chains will help to optimize the prevention of HIV infection and control the spread of TDR.

Drug resistance testing has been routinely implemented in clinical practice in Greece and in other European countries, providing a set of viral sequences for phylogenetic analysis to study the molecular epidemiology of HIV transmission.4

Our objectives were to determine the prevalence of TDR and identify phylogenetic relationships of HIV strains derived from newly diagnosed antiretroviral-naive patients in Northern Greece.

Methods

The study population comprised 369 individuals newly diagnosed with HIV-1 infection between 2000 and 2007 at the National AIDS Reference Laboratory of Northern Greece. The laboratory is affiliated to the Aristotle University of Thessaloniki and is the only centre commissioned by the Hellenic Centre for Disease Control and Prevention to provide serology and molecular testing for diagnosis and monitoring of HIV infection in Northern Greece. The laboratory operates in conjunction with the

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Infectious Diseases Division of the AHEPA University Hospital, which provides clinical care for HIV-infected patients. Informed consent to maintain medical and epidemiological data for therapeutic and research purposes was obtained routinely at each patient’s first visit to the clinic. Demographics and relevant clinical data were retrieved from the database and cross-referenced with the patients’ medical files. The HIV-1 serostatus of each subject was confirmed by western blotting. All patients were antiretroviral naive at the time genotypic resistance testing was performed.

**HIV-1 genotyping and sequence analysis**

Samples with HIV-1 RNA above 1000 copies/mL before initiation of anti-retroviral therapy underwent population-based sequencing of HIV-1 reverse transcriptase (RT, codon 40–247) and protease genes (codons 1–99) (TRUGEN™ HIV-1 genotyping kit; Siemens), according to the manufacturer’s instructions.

Mutations conferring resistance to nucleoside RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs) and protease inhibitors (PIs) were analysed using the Stanford University HIV drug resistance database (http://hivdb.stanford.edu). Prevalence of mutations associated with TDR was analysed using the surveillance drug resistance mutation (SDRM) list. The updated 2009 SDRM list has 93 mutations comprising 34 NRTI-associated resistance mutations at 15 RT positions, 19 NNRTI-associated resistance mutations at 10 RT positions and 40 PI-associated resistance mutations at 18 protease positions. Notably absent were V90I, A98G, V106I, V108I and E138A (RT) and L33F, M36I, H69K and L89V (protease).

The subtype was assigned using the Stanford University HIV drug resistance database subtyping tool (http://hivdb.stanford.edu). Inconsistent results were resolved using the REGA HIV-1 subtyping tool, version 2.0 (http://www.bioafrica.net/subtypetool). For purposes of analysis, HIV isolates were categorized as subtype B, subtype A and non-A/non-B subtypes.

**Phylogenetic analysis**

Nucleotide sequences derived from 167 patient samples, including the majority of patients with TDR and a representative proportion of subtypes observed that were randomly selected, were imported into MEGA4 software and aligned using the ClustalW algorithm. This was followed by manual editing and the removal of positions containing gaps. The alignment matrices were imported into the tree-building software PAUP® and an initial neighbour-joining (NJ) tree was built under a Jukes–Cantor model of evolution. The best fitting nucleotide substitution model was then estimated on the basis of the initial NJ tree by comparing up to 56 different models as implemented by the software Modeltest within PAUP®. The derived parameters of the selected model, together with the initial NJ tree were used to perform a heuristic search for a maximum likelihood tree under the GTR+1 model of substitution and the tree bisection and reconnection (TBR) branch-swapping algorithm. The assumed proportion of invariable sites (I) was 0.5664. An HIV-1 subtype K sequence (GenBank accession number AJ249239) retrieved from the Los Alamos HIV database was used to root the tree. Robustness and statistical support of the internal branches of the maximum likelihood tree were evaluated by non-parametric bootstrap analysis with 500 rounds of replication. Transmission clusters were defined as sequences sharing a most recent common ancestor with >85% bootstrap support and a mean genetic distance of <0.015 nucleotide substitutions per site.

To assess the potential bias in the clustering of TDR sequences caused by drug resistance mutations, 6 and 12 drug resistance-associated codons in protease and RT respectively, were removed from the alignment. The codons removed were as follows: D30, M46, F53, V82, N88 and L90 from protease; and M41, T69, F77, K101, K103, V118, E138, Y181, M184, L210, T215 and K219 from RT. The new alignment was then used to infer a maximum-likelihood tree in PAUP® as outlined above.

**Statistical analysis**

For statistical analysis we grouped the patients by the calendar year of diagnosis into three time periods: January 2000 to December 2003, January 2004 to December 2005 and January 2006 to December 2007. This allocation led to three patient groups of comparable size. Time trends were evaluated using the Cochran–Armitage trend test. Epidemiological characteristics and parameters such as the viral load and CD4 T cell count at baseline were assessed as predictors of TDR in univariate logistic regression models. Those significantly associated with TDR (P<0.05) were tested in a multivariable logistic regression model by forced entry.

Fisher’s exact test and Mann–Whitney U-test were used to evaluate differences between subgroups of patients.

All reported P values are two-tailed at a 0.05 significance level. Analyses were conducted using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) and STATA 10 (StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP).

**Seroconversion date estimation**

Due to the lack of systematic documentation of seroconversion dates during the study period, we resorted to results from a Markov chain model of HIV progression to infer time since seroconversion based on CD4 cell count at diagnosis. The model was derived from the San Francisco Men’s Health Study and implemented as described in a recent UK study of HIV epidemiology in adults over 50 years old. The generated data were used to undertake a sensitivity analysis of temporal trends in TDR.

**Results**

**Study population**

The study population characteristics are depicted in Table 1.

**HIV-1 subtypes**

In total, 196/369 (53.1%) patients were infected with subtype B HIV-1 isolates, 120 (32.5%) with subtype A and 53 (14.4%) with other subtypes. Time trends suggest an increase in the incidence of subtype A HIV-1 infection during the study period (P=0.023).

**TDR**

The overall prevalence of TDR to any drug class among treatment-naive patients over the 8 years surveyed was 12.5% [46/369, 95% confidence interval (CI) 9.1%–15.8%]. The per drug class prevalence was as follows: 7.6% (28/369) for NRTIs; 5.4% (20/369) for NNRTIs; and 3.3% (12/369) for PIs. Dual class resistance was identified in 3.8% (14/369) of patients (12 to NNRTIs plus NNRTIs and 2 to NNRTIs plus PIs).

Overall, time trends indicated an increase in the prevalence of TDR (P=0.038) and in particular in the prevalence of TDR associated with NNRTIs (P=0.035). No significant time trend was detected with regard to NRTI (P=0.092) and PI (P=0.437) resistance (Figure 1).
A sensitivity analysis based on estimated time since seroconversion resulted in similar rates of TDR during the three estimated seroconversion periods of approximately equal sample size (before 1997, 1998–2000 and 2001–05) ($P=0.097$).

In multivariate logistic analysis, subtype A was the sole predictor associated with TDR to any drug class [odds ratio (OR) 2.15, 95% CI 1.10–4.19, $P=0.025$] (Table 2). In univariate logistic models, the OR of TDR to NNRTIs was significantly higher with subtype A than other subtypes (9.90, 95% CI 2.82–34.75, $P<0.001$). However, the association between TDR related to NRTIs and subtype A was borderline significant (OR 2.19, 95% CI 0.99–4.86, $P=0.054$). No such association was observed for resistance to PIs ($P=0.778$). Dual drug resistance was linked to subtype A ($P=0.001$) and men having sex with men as a risk group ($P=0.037$). Moreover, logistic regression analysis was performed to assess risk factors for TDR within the subset of patients infected with subtype A but no significant associations became evident (data not shown).

Among the NRTI mutations, thymidine analogue mutations (TAMs) were found in 25/369 patients (6.8%, 21 had T215C/D/E/S/V, 6 had M41L, 3 had L210W, 3 had K219Q/E and 1 had K70R). For NNRTI mutations, Y181C was the most common (13/369, 3.5%) and occurred in 12 HIV-1 subtype A patients. With regard to PIs, the most common mutation was M46L and was present in five individuals (5/369, 1.4%).

Transmission clusters

The maximum likelihood tree revealed three transmission clusters highly supported by bootstrapping (>85%) and a mean genetic distance of <0.015 nucleotide substitutions per site (Figure 2). Among the newly diagnosed patients with TDR, 17 out of 46 (37%, 95% CI 23%–50.9%) were part of transmission clusters (Table 3).
The first cluster included two individuals infected with a subtype B strain carrying the RT T215E and M41L mutations and had a bootstrap support value of 100%. The second cluster included 12 individuals who were all infected with a subtype A strain; most infections were associated with sex between men, nevertheless, the route of transmission was unclear for some of the individuals in that cluster. Dual class resistance mutations T215 variants and Y181C were detected in 10 of 12 individuals. This cluster had a bootstrap support value of 88%. The third cluster, which had a bootstrap support value of 100%, was composed of three individuals infected with subtype A strain; in two of them K219Q was detected. To assess the sensitivity of the bootstrapping analysis and the potential bias in the clustering of TDR sequences caused by drug resistance mutations, we performed phylogenetic reconstruction using the same sequences with drug resistance codons removed. This showed that the transmission clusters were maintained with high bootstrap support values of 100%, 78% and 99% for clusters i, ii and iii, respectively, and a mean genetic distance of 0.015 nucleotide substitutions per site. Overall, infection with HIV subtype A was linked to being part of a TDR transmission cluster (P = 0.001), whereas the association with risk factors for HIV infection was not statistically significant (P = 0.467). To further illustrate the impact of transmission clusters on TDR rates, we found that 14 out of 25 cases of TDR in subtype A isolates were cluster related compared with 2 out of 19 TDR cases in subtype B isolates.

**Table 2. Predictors of TDR related to any drug class: univariate and multivariate analysis**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Subtype (reference subtype B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>subtype A</td>
<td>2.45 (1.28–4.68)</td>
<td>0.007</td>
</tr>
<tr>
<td>other</td>
<td>0.37 (0.08–1.62)</td>
<td>0.185</td>
</tr>
<tr>
<td>Risk factor (reference MSM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterosexual contact</td>
<td>0.32 (0.11–0.94)</td>
<td>0.039</td>
</tr>
<tr>
<td>IDU</td>
<td>1.01 (0.33–3.10)</td>
<td>0.993</td>
</tr>
<tr>
<td>other/unknown</td>
<td>1.14 (0.47–2.79)</td>
<td>0.772</td>
</tr>
<tr>
<td>Age at diagnosis (per 10 years increase)</td>
<td>1.16 (0.91–1.48)</td>
<td>0.229</td>
</tr>
<tr>
<td>Gender (reference female)</td>
<td>0.32 (0.10–1.06)</td>
<td>0.062</td>
</tr>
<tr>
<td>Baseline CD4 (per 100 cells/mm³ increase)</td>
<td>1.11 (1.00–1.22)</td>
<td>0.156</td>
</tr>
<tr>
<td>Log₁₀ HIV RNA (per 1 log increase)</td>
<td>0.82 (0.56–1.22)</td>
<td>0.336</td>
</tr>
<tr>
<td>Country of origin (reference Greece)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td>0.17 (0.02–1.28)</td>
<td>0.086</td>
</tr>
<tr>
<td>Residence (reference urban)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td>0.27 (0.08–0.95)</td>
<td>0.042</td>
</tr>
<tr>
<td>unknown</td>
<td>0.6 (0.27–1.36)</td>
<td>0.22</td>
</tr>
<tr>
<td>Time period (reference 2000–03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2004–05</td>
<td>2.37 (0.97–5.78)</td>
<td>0.059</td>
</tr>
<tr>
<td>2006–07</td>
<td>2.52 (1.08–5.90)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

**Discussion**

We performed a retrospective cohort study of newly diagnosed individuals over an 8 year period in the geographical area of Macedonia, Northern Greece.

A marked increase in the prevalence of subtype A in Greece was previously reported in a multicentre study conducted in Athens. More specifically, the prevalence of subtype A exceeded that of subtype B in 2004 and it also affected predominantly the native population. No specific risk groups for subtype A infection were identified. Previous reports from Northern Greece are consistent with these findings, suggesting a high prevalence of subtype B before year 2000 (>90%), which was followed by a gradual increase in the prevalence of subtype A, which until 2007 had not surpassed that of subtype B, as documented by our study.

In the present study, TDR was defined as the presence of at least one of the mutations present in the 2009 SDRM list, which is optimized for surveillance of TDR and enables comparisons across different populations.

The prevalence of antiretroviral resistance among treatment-naïve patients was 12.5% (95% CI 9.1%–15.8%). The point estimate for the prevalence of TDR to any drug class in our population is higher than that reported by the European TDR surveillance programme, but the difference was not statistically significant. Moreover, in a previous study of 101 newly diagnosed HIV patients conducted in Greece during the period 2002–03, the reported prevalence of TDR according to the IAS-USA 2004 mutation list was 9% (95% CI 4.2%–11.2%).
The rates of TDR increased in our population during the study period. Taking into consideration the limitations inherent in the categorization of patients according to the year of diagnosis, we performed a sensitivity analysis based on estimates of the seroconversion date, which confirmed the trend for increase in TDR. This trend could be attributed to a reduced awareness among HIV-infected individuals with regard to their infectiousness, due to either high-risk sexual behaviours or high viral replication as a result of low adherence and limited therapeutic options.

The finding that subtype A is strongly associated with TDR in our population is not consistent with previous studies in which infection with non-B subtypes was associated with a lower prevalence of TDR. In an analysis restricted to subtype A HIV-infected individuals, TDR was not associated with any of the predictors tested in the comprehensive analysis. TAMs and in particular revertants at codon 215 (T215C/D/E/S) were the most common type of TDR mutation, a finding consistent with the results of the European TDR surveillance programme. This fact could be attributed to the extensive use of

Figure 2. Phylogenetic analysis of TDR lineages. (a) Maximum likelihood inferred genealogy showing TDR clusters circulating among treatment-naive individuals in Northern Greece. Branch lengths are drawn to scale with the bar at the bottom representing 0.02 nucleotide substitutions per site. The tips of sequences with drug resistance mutations are shown in red. Boxes labelled (i), (ii) and (iii) indicate drug resistance-associated transmission clusters with significant statistical support (>85% bootstrap support) for the branch subtending the cluster and a mean genetic distance of <0.015 nucleotide substitutions per site. The subtype of the sequences (A, B or non-AB) is shown on the right. The tree was rooted using a subtype K sequence (GenBank accession number AJ249239). (b) The three TDR-associated clusters are shown in detail. The drug resistance mutations associated with each sequence are indicated at the tips of the branches.
zidovudine or stavudine in the era before highly active antiretroviral therapy (HAART) and the early HAART period. T215C/D/E/S emerge from T215Y/F to increase virus fitness in the absence of selective drug pressure.\textsuperscript{16,17} They occur more frequently than reversion to wild-type T because most of the T215 revertants require only a single nucleotide mutation rather than the double nucleotide mutation required for Y or F to revert to T. The presence of T215 revertants is associated with increased risk of virological failure of zidovudine or stavudine in antiretroviral-naive patients.\textsuperscript{18}

The next most frequent mutation was Y181C, which confers high-level resistance to nevirapine, is associated with low-level resistance to efavirenz (\(\approx\)2-fold susceptibility reduction) and reduces etravirine susceptibility.\textsuperscript{19,20} Both T215 revertants and Y181C mutations are non-polymorphic and do not occur at highly polymorphic positions. According to Bennett et al.,\textsuperscript{5} T215 revertant mutations occur at a frequency of <0.5\% in subtypes B and A and Y181C mutations occur in 0.1\% and 0\% in subtypes A and B, respectively, in treatment-naive patients.

Interestingly, 12/13 viral isolates carrying Y181C were of subtype A, in contrast to a previous report in which Y181C arose more commonly in subtype B.\textsuperscript{21} The most frequent PI resistance mutation was M46L, which was excluded from the former SDRM list.\textsuperscript{5} M46L confers intermediate-level resistance to nelfinavir and potential low-level resistance to PIs such as lopinavir and atazanavir.

The contribution of transmission clusters to the epidemiology of TDR has been evaluated in previous studies.\textsuperscript{22–25} In our study, phylogenetic analysis revealed three clusters that involved 17 individuals, all diagnosed after 2005. Of note, 16 out of 17 harbour drug resistance mutations conferring resistance to NRTIs, NNRTIs or both. The resistant lineages involved mostly subtype A strains, although subtype B remained more prevalent throughout the study period.

The strong correlation between subtype A and TDR could be attributed to two complementary factors: the dynamics of the subtype A HIV epidemic in Greece and the presence of transmission chains within our study population. Patients infected with subtype A HIV predominantly belong to the native population and receive treatment through a public health programme.\textsuperscript{9} Transmission chains provide evidence of high-risk behaviours that could potentially lead to the establishment of a pool of drug-resistant HIV circulating among men having sex with men.

Importantly, 10 out of 12 individuals who were infected with subtype A and grouped in cluster ii carried both T215 revertants and Y181C. The sequences in this cluster were closely related and descend from a common ancestor, indicating that spread of the virus occurred within that population. Nine out of 12 individuals in this cluster had reported having sex with men as a risk factor for HIV infection, but this association was not statistically significant (\(P=0.091\)). Although the presence of TDR clusters is well documented,\textsuperscript{22–25} to our knowledge this is the first time a T215 revertant and Y181C subtype A cluster of that size has been described. The emergence of a cluster of dual drug-resistant HIV-1 strains, first documented after 2005 and involving 12 persons, raises therapeutic implications for the individual patients as well as public health concerns and highlights the importance of surveillance of HIV TDR on a regional basis.

There are limitations associated with our study. As has been discussed, the use of year of diagnosis instead of year of seroconversion raises concerns about the reliability of estimates. Despite this fact, our approach allows direct comparisons with the rates reported by the European TDR surveillance programme.

Table 3. Clusters of TDR: patient characteristics

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Patient ID</th>
<th>Gender</th>
<th>Age at diagnosis (years)</th>
<th>Route</th>
<th>Subtype</th>
<th>Year of diagnosis</th>
<th>NRTI</th>
<th>NNRTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>P1</td>
<td>female</td>
<td>23</td>
<td>Het</td>
<td>B</td>
<td>2005</td>
<td>M41L, T215E</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>P2</td>
<td>female</td>
<td>27</td>
<td>Het</td>
<td>B</td>
<td>2005</td>
<td>M41L, T215E</td>
<td></td>
</tr>
<tr>
<td>ii</td>
<td>P3</td>
<td>male</td>
<td>31</td>
<td>MSM</td>
<td>A</td>
<td>2007</td>
<td>T215CTS, Y181C</td>
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<td>ii</td>
<td>P4</td>
<td>male</td>
<td>30</td>
<td>MSM</td>
<td>A</td>
<td>2005</td>
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<td>P5</td>
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<td>MSM</td>
<td>A</td>
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<tr>
<td>ii</td>
<td>P6</td>
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<td>44</td>
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<td>2007</td>
<td>T215D, Y181C</td>
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<td>ii</td>
<td>P7</td>
<td>male</td>
<td>69</td>
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<td>2006</td>
<td>T215D, Y181C</td>
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<td>male</td>
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<td>MSM</td>
<td>A</td>
<td>2005</td>
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<td>A</td>
<td>2006</td>
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<tr>
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<td>MSM</td>
<td>A</td>
<td>2005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Het, heterosexual contact; MSM, men having sex with men.

All patients (P1–P17) were of Greek origin.

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Further limitations include the use of population sequencing, which underestimates the presence of drug resistance mutations in viral subpopulations below the detection limit.²⁶

In conclusion, our data indicate that transmission networks have a major impact in our study population, necessitate enhanced surveillance and prompt preventive measures to increase awareness regarding HIV infection. Further studies should evaluate the clinical relevance of the HIV-1 subtype A infection carrying the pattern of dual resistance mutations observed in our area.

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Transparency declarations
None to declare.

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