Association of specific mutations in \textit{katG}, \textit{rpoB}, \textit{rpsL} and \textit{rrs} genes with spoligotypes of multidrug-resistant \textit{Mycobacterium tuberculosis} isolates in Russia

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ABSTRACT

Most multidrug-resistant (MDR) \textit{Mycobacterium tuberculosis} isolates in Russia belong to the Beijing or Latino-American and Mediterranean (LAM) spoligotype families. The objective of this study was to investigate possible associations between genotype and the frequencies of mutations that confer drug resistance in a population that has two large families of circulating strains. Spoligotyping, IS6110 restriction fragment length polymorphism typing, and sequencing of the \textit{katG} and \textit{rpoB} genes, were performed for 217 consecutive MDR \textit{M. tuberculosis} isolates from patients. The \textit{rpsL} and \textit{rrs} genes were also sequenced for selected streptomycin-resistant isolates. Of the 217 MDR isolates, 99 (46\%) belonged to the LAM family, 92 (42\%) to the Beijing family, 21 (10\%) to the Haarlem family and four (2\%) to the T family. There was one unique spoligotype. Mutations in the \textit{katG} gene were identified in 207 (95\%) isolates, all of which had mutations in codon 315. Mutations in the \textit{rpoB} gene were identified in 200 (92\%) isolates; 75\% of LAM isolates carried a mutation in codon 516, whereas 71\% of Beijing isolates carried a mutation in codon 531. In the 33 isolates resistant to streptomycin 50 mg/L, the 43AGG \textit{rpsL} mutation was found in 27\% of Haarlem, 75\% of Beijing and 0\% of LAM isolates, and \textit{rrs} mutations were found in 17\% (516C $\rightarrow$ T) of Beijing and 100\% (513A $\rightarrow$ C) of LAM isolates. Overall, there appeared to be a correlation between the genotype and specific mutations conferring resistance to rifampicin or streptomycin in the Beijing and LAM families. The biological implications of this correlation remain to be explored.

Keywords Genotype, multidrug resistance, mutations, \textit{Mycobacterium tuberculosis}, resistance, spoligotype

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INTRODUCTION

Tuberculosis (TB) is an urgent public health problem in both industrialised and developing countries. Efforts to control TB have recently been complicated by the worldwide spread of multi-drug-resistant (MDR; resistant to at least isoniazid and rifampicin) strains of \textit{Mycobacterium tuberculosis} [1]. In several regions of the world, the spread of MDR TB has been associated with the expansion of strains belonging to the Beijing family [2]. Drug resistance results from the accumulation of chromosomal mutations, in part because tubercle bacilli have no known efficient mechanism for horizontal gene transfer [3]. Despite the need for several independent mutations, resistance to multiple drugs is not uncommon, as successive rounds of inadequate or inappropriate therapy, or incomplete compliance, can lead to the stepwise accumulation of drug resistance mutations [1]. In the mycobacteria, rifampicin resistance is associated with changes in the \textit{rpoB} gene, and isoniazid resistance is associated primarily with changes in the \textit{katG}, \textit{inhA} or \textit{aphC} genes [4–7].

Genotyping of \textit{M. tuberculosis} isolates has proven to be a powerful approach for investigating transmission dynamics, circulating strains, and the natural history of TB. The commonly used
genotyping methods are spoligotyping, IS6110 restriction fragment length polymorphism (RFLP) typing, and mycobacterial interspersed repetitive unit typing [8–10]. Families of *M. tuberculosis* isolates are defined on the basis of shared spoligotypes or closely related IS6110 RFLP patterns. The distribution of *M. tuberculosis* genotype families varies greatly by region [11,12]. In Russia, the majority of *M. tuberculosis* isolates belong to either the Beijing or Latino-American and Mediterranean (LAM) families [13–17].

Because genotyping techniques measure variations in the *M. tuberculosis* genome unrelated to the acquisition of drug resistance, it was somewhat surprising to find that certain mutations appear to predominate in drug-resistant members of some families. For example, the *katG* 315 mutation appears to predominate in isoniazid-resistant isolates of the Beijing family [7,17,18]. The objective of the present study was to take advantage of the presence of two large families of strains in Russia to investigate correlations between specific drug resistance mutations and the genotype of MDR strains of *M. tuberculosis*.

**MATERIALS AND METHODS**

**Patients**

In total, 312 consecutive *M. tuberculosis* isolates resistant to at least one anti-TB drug were obtained from 312 pulmonary TB patients in two neighbouring regions, Tula and Serpukhov District of Moscow, between 1998 and 2001. Of these patients, 241 (77.2%) were chronic cases, 285 (91.3%) were male, and none was infected with human immunodeficiency virus; the mean age was 43 years. Resistance to both isoniazid and rifampicin was detected in isolates from 217 patients (of whom 93.1% were chronic cases and 92.2% were male). Only the 217 MDR isolates were included in this study.

**Drug susceptibility testing**

Isolates were tested for susceptibility to isoniazid, streptomycin, rifampicin, kanamycin and ethambutol, using the absolute concentration method on Loewenstein–Jensen medium [19]. The concentrations of the drugs used in the medium were: for isoniazid, 1, 5 and 25 mg/L; for streptomycin, 5, 10 and 50 mg/L; for rifampicin, 20 and 50 mg/L; for kanamycin, 30 and 50 mg/L; and for ethambutol, 2 and 5 mg/L. Isolates that grew on the lowest concentration of the drug tested were considered to be resistant to that drug.

**Genotyping**

Spoligotyping was performed as described previously [8], using membranes made at the CDC. IS6110 RFLP typing of *M. tuberculosis* isolates was carried out as described previously [9]. IS6110 RFLP images on films were scanned (200 dpi, 256 grayscales) and processed with GelCompar II v.2.5 (Applied Maths, Sint-Martens-Latem, Belgium) software. Sets of restriction fragments were considered as vectors in multidimensional Euclidian space [15] and processed with the unweighted pair-group method using arithmetic averages (Jaccard’s similarity coefficient, tolerance 1.0%, optimisation 0.7%).

**DNA sequencing**

DNA samples were prepared as described previously [9]. Primers for PCR amplification and sequencing are listed in Table 1. The primers were selected to amplify and sequence the rifampicin resistance-determining region (RRDR) of the *rpoB* gene (bases 1366–1772; *Escherichia coli* numbering system), 697 bp of the *katG* gene, including the region around codon 315 (bases 872–1568), the entire *katG* gene (bases –23 to +2232), 248 bp and 283 bp of the *inhA* (bases –168 to +79) and *ahpC* (bases –182 to +100) regulatory regions, 373 bp of the *rpsL* gene, including the region around codon 43 (bases 12–384), and 553 bp of the *rrs* gene, including the region around nucleotide 530 (bases 427–980).

The amplification mixture consisted of PCR buffer containing 2.5 mM MgCl₂ (Fermentas, Vilnius, Lithuania), DNA 0.2 ng/μL, 0.15 mM each dNTP (Fermentas), and 0.4 μM forward and reverse primers (Syntol, Moscow, Russia), and 0.05 U/μL *Taq* DNA polymerase (Fermentas). Thermocycling conditions comprised 95°C for 4 min, 25 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by 3 min at 72°C. Amplification products were electrophoresed in 1% agarose gel, stained with ethidium bromide and visualised in UV light.

**Sequence analysis**

DNA sequences were aligned with Escherichia coli numbering system, using membranes made at the CDC. IS6110 RFLP typing of *M. tuberculosis* isolates was carried out as described previously [8]. DNA sequencing was performed using membranes made at the CDC. The *rpoB* sequence was converted into a binary sequence of ‘1’s and ‘0’s using the order of codons as described recently [8]. A similarity matrix was calculated for each binary sequence, with the similarity coefficient optimised at 0.7%.

**Table 1. Primers used for PCR amplification and sequencing**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer, 5′–3′</th>
<th>Reverse primer, 5′–3′</th>
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<tbody>
<tr>
<td><em>katG</em> fragment (217 isolates)</td>
<td>Amplification and sequencing CTCGGCTGACAGCATGGGC</td>
<td>Amplification and sequencing TCTCCAGGTTCCGGAATGGCT</td>
</tr>
<tr>
<td><em>katG</em> full (four isolates)</td>
<td>Amplification and sequencing CACCAACTCTGGAAAGGAATGC</td>
<td>Amplification and sequencing GATCTGCTCCAGCCGAGCA</td>
</tr>
<tr>
<td><em>inhA</em> (regulatory region)</td>
<td>Amplification and sequencing CGGTACACTTTCCGTAAGACC</td>
<td>Amplification and sequencing CACCGAGACCCGCAATGG</td>
</tr>
<tr>
<td><em>ahpC</em> (regulatory region)</td>
<td>Amplification and sequencing CATTGCACCTCTGGAAAGAGATC</td>
<td>Amplification and sequencing CAGGGGTTCCAGCCGAG</td>
</tr>
<tr>
<td><em>rpsL</em></td>
<td>Amplification and sequencing CCTGGCTCAGCCAGAAAGCGA</td>
<td>Amplification and sequencing ATCGCCGGCTTCTCCTGG</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>Amplification and sequencing CCGGACACCGTGTCGCTTCA</td>
<td>Amplification and sequencing CGATGGACCCGCGCGACCT</td>
</tr>
<tr>
<td><em>rpoB</em></td>
<td>Amplification and sequencing CTACCAAGCTCCGCGC</td>
<td>Amplification and sequencing TCAGTTGACCCGCGCGACCT</td>
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57°C and 60 s at 72°C, followed by 30 s at 57°C, 5 min at 72°C, and cooling to 10°C. Amplicons were purified by electrophoresis on agarose 1% w/v gels, followed by recovery with a DNA extraction kit (Fermentas). Sequencing was performed using the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing kit (Fermentas) according to Sanger et al. [20], with a thermocycling profile of 94°C for 3 min, 25 cycles of 30 s at 94°C, 30 s at 57°C and 60 s at 72°C, followed by 30 s at 57°C, 5 min at 72°C, and cooling to 10°C. Sequences were compared to those of the wild-type (WT) genes of strain H37Rv (http://www.ncbi.nlm.nih.gov).

Statistical methods

Clin (95%) and the probability, p, that a difference between mean values is casual, were estimated using binomial distribution.

RESULTS

The spoligotyping analysis identified four (1.8%) T family, 21 (9.7%) Haarlem family, 92 (42.4%) Beijing family and 99 (45.6%) LAM family isolates. The spoligotype of one isolate did not belong to a known family. IS6110 RFLP typing revealed 94 (43.3%) different strains with unique profiles, comprising 33 LAM, 37 Beijing, 19 Haarlem and four T family strains. The results of IS6110 RFLP typing were used to select isolates for sequencing (rpsL, rrs, inhA and ahpC genes) in order to avoid sequencing multiple isolates of the same strain.

In addition to being resistant to isoniazid and rifampicin, all 217 MDR isolates were resistant to streptomycin, 150 (69%) were resistant to kanamycin, and 178 (82%) were resistant to ethambutol. The frequency of kanamycin-resistant isolates was greater in the LAM family than in the Beijing and Haarlem families (83%, 60% and 38%, respectively; p <0.0003). There was no significant difference in the frequency of ethambutol-resistant isolates among the LAM, Beijing, Haarlem and T families.

Mutations in the katG gene were identified in 207 (95.4%) of the 217 MDR isolates, including 99% of the MDR isolates in the LAM family (codon 315ACC, n = 97; 315AGA, n = 1), 57% of the MDR isolates in the Haarlem family (315ACC, n = 12), and all of the MDR isolates in the Beijing (315ACC, n = 91; 315AGA and 335GTC, n = 1) and T families (315ACC, n = 4). All Beijing family isolates also carried the codon 463CTG polymorphism. However, this polymorphism is not associated with isoniazid resistance [21], but is one of the genetic polymorphisms used to identify the principal genotypic groups of M. tuberculosis isolates [3]. The frequency of isoniazid-resistant isolates with a WT katG gene in the region sequenced (697 bp) was greater in the Haarlem family than in the LAM and Beijing families (43%, 1%, and 0%, respectively; p <0.001). The isoniazid-resistant isolate with the unclassified spoligotype had mutations in codons 315ACC and 463CTG of the katG gene. The frequency of isoniazid-resistant isolates that carried a mutation in katG codon 315 and were resistant to the high (25 mg/L) concentration of isoniazid was greater in the LAM family (n = 47, 48%) than in the Beijing (n = 8, 8.7%; p <0.001) and Haarlem (n = 2, 17%; p <0.04) families (Fig. 1).

In order to investigate the high isoniazid resistance of some isolates carrying the codon 315 katG mutation, the regulatory regions of two other genes (inhA and ahpC) known to influence isoniazid resistance were sequenced in isolates that carried a codon 315 mutation and did not belong to the same IS6110 RFLP cluster. None of the isolates of any family had more than one mutation in the regulatory sequences of the inhA and ahpC genes. A mutation in the regulatory region of the ahpC gene (−30C → T) was detected in one of the eight Beijing family isolates resistant to isoniazid 25 mg/L. Mutations in the regulatory region of the inhA gene (−15C → T) were detected in three of the eight Beijing family isolates resistant to isoniazid 25 mg/L, in two of the nine Beijing family isolates resistant to isoniazid 1 mg/L, in all 14 LAM family isolates resistant to isoniazid 25 mg/L, in nine of the ten LAM family isolates resistant to isoniazid 1 mg/L, in neither of two Haarlem family isolates resistant to isoniazid 25 mg/L, and in two of four Haarlem family isolates resistant to isoniazid 1 mg/L. The difference between the frequencies of this

![Fig. 1. Level of resistance to isoniazid in isoniazid-resistant Mycobacterium tuberculosis isolates belonging to different families.](http://www.ncbi.nlm.nih.gov)
mutation in isolates resistant to isoniazid 25 mg/L and those resistant to isoniazid 1 mg/L in any family was not significant (p ≥0.4).

In order to determine the molecular basis for resistance to the high concentration of streptomycin, the rpsL and rrs (16S rRNA) genes were sequenced in selected isolates that did not belong to the same IS6110 RFLP cluster and had resistance to streptomycin 50 mg/L. The 513A → C mutation in the rrs gene was detected in all ten LAM family isolates tested. The codon 43AAG → AGG mutation was detected in the rpsL gene of three (27%) of 11 Haarlem family isolates tested, and in nine (75%) of 12 Beijing family isolates tested, while the 516C → T mutation in the rrs gene was detected in two (17%) Beijing family isolates (Fig. 2). The frequency (73%) of streptomycin-resistant (50 mg/L) isolates with mutations in neither the rpsL nor the rrs genes in the Haarlem family was greater than that in the LAM (0%, p <0.001) or Beijing (8%, p <0.001) families.

The rpsL and rrs genes were also sequenced in five Beijing family isolates showing resistance to streptomycin 10 mg/L and susceptibility to streptomycin 50 mg/L; the 43AAG → AGG mutation in the rpsL gene was detected in one (20%) isolate, and the 516C → T mutation in the rrs gene was detected in four (80%) isolates. The frequency of the 43AAG → AGG mutation in the Beijing family isolates with resistance to streptomycin 50 mg/L was greater than that in isolates with resistance only to streptomycin 10 mg/L (75% vs. 20%; p 0.06).

Mutations in the RRDR of the rpoB gene were identified in 200 (92%) of the 217 MDR isolates. The most frequent mutations were substitutions in codons 516, 526 or 531 of the rpoB gene, which were found in 194 (89%) of the 217 isolates. The mutations in the rpoB gene of the 92 MDR isolates of the Beijing family were 511CCG (n = 2), 513CCA (n = 1), 514–515 deletion CAT (n = 1), 516GTC (n = 3), 516TAC (n = 2), 522TTG (n = 1), 525ACG + 526TAC + 527CAG (n = 1), 526CGC (n = 1), 526CTC (n = 1), 526GAC (n = 6), 526TAC (n = 5), 531TTG (n = 1), 531TTG (n = 64) and 533CCG (n = 1); two isolates had no mutations in the RRDR of the rpoB gene. Mutations in codon 531 of the rpoB gene were detected in 65 (71%) of 92 MDR isolates of the Beijing family.

The rpoB mutations in the 99 MDR isolates of the LAM family were 516GTC (n = 73), 516TAC (n = 1), 522CAG (n = 1), 526CTC (n = 1), 526GAC (n = 11), 526TAC (n = 1) and 531TTG (n = 7); four isolates had no mutations in the RRDR of the rpoB gene. Mutations in codon 516 of the rpoB gene were detected in 74 of the 99 MDR isolates of the LAM family.

The rpoB mutations in the 21 MDR isolates of the Haarlem family were 516GTC (n = 1), 516TAC (n = 1), 526GAC (n = 2), 526TAC (n = 2), 531TTG (n = 4) and 533CCG (n = 1); ten isolates had no mutations in the RRDR of the rpoB gene. The frequency (48%) of MDR isolates in the Haarlem family with a WT rpoB gene was greater than that in the LAM (4%, p <0.001) or Beijing (2%, p <0.001) families (Fig. 3).

The rpoB mutations in the four MDR isolates of the T family were 516GTC (n = 2), 526GAC (n = 1) and 526TAC (n = 1). The MDR isolate with the unclassified spoligotype had a WT rpoB gene.

Isolates in the same IS6110 RFLP cluster often had different mutations in the rpoB gene. For example, the correlation coefficient between the

Fig. 2. Mutations in the rpsL and 16S rRNA genes of streptomycin-resistant (50 mg/L) Mycobacterium tuberculosis isolates belonging to different families (WT, wild-type).

Fig. 3. Mutations in the rpoB gene in rifampicin-resistant Mycobacterium tuberculosis isolates belonging to different families (WT, wild-type).
presence of the mutation 531TTG and the affiliation of an isolate with the largest IS6110 RFLP cluster (n = 35) of the Beijing family was 0.032. A correlation between the position of a mutation in the rpoB gene and the affiliation of an isolate with a particular IS6110 RFLP cluster was detected for only one cluster containing 43 isolates of the LAM family (p 0.51), but affiliation of an isolate with this cluster and the level of rifampicin resistance were not correlated (p 0.02).

Of the 217 MDR isolates, ten carried no mutations in the sequenced region of the katG gene, 17 carried no mutations in the RRDR of the rpoB gene, and eight carried no mutations in the sequenced regions of both the katG and rpoB genes. One of these eight isolates belonged to the LAM family and produced 20 colonies on Loewenstein–Jensen medium containing rifampicin 20 mg/L, which was the minimum number required to consider an isolate as rifampicin-resistant; the other seven isolates belonged to the Haarlem family. Regulatory regions of the inhA and ahpC genes were sequenced for these eight MDR isolates, and all eight had a WT regulatory sequence for the inhA gene. Three isolates of the Haarlem family had either the −6G → A, −10C → T or −32G → A mutations in the regulatory sequence of the ahpC gene. As mutations in this regulatory region often coincide with deletions in the katG gene, the entire katG sequence of these isolates was determined, revealing that each isolate had a WT katG sequence.

**DISCUSSION**

Beijing family strains of *M. tuberculosis* are prevalent in many regions of the world and are often associated with drug resistance [2,12]. According to published data [14–16], LAM family strains are also prevalent in some regions of Russia, and are also associated with drug resistance. It is interesting that members of two of the principal genetic groups (Beijing family, group 1; LAM family, group 2 [3]) of *M. tuberculosis* are highly prevalent in TB patients in the same regions of Russia. It has been suggested that the prevalence of Beijing family strains is a result of the ability of these strains to overcome the protective effects of vaccination of the population with bacille Calmette-Guérin [22]. A similar explanation may apply to the LAM family strains, which occur at a high frequency in the Russian population, which has a high rate of bacille Calmette-Guérin vaccination.

As has been observed previously for isolates in Russia, mutations in codon 315 of the katG gene are found in the vast majority of isoniazid-resistant isolates [16,18]. However, the frequency of codon 315 mutations in isoniazid-resistant isolates in other populations has been reported to range from 35% to 97% [7]. Mutations in codon 315 do not significantly decrease the peroxidase activity of the katG gene product, but do decrease its ability to activate isoniazid [23]. These features allow the mutants to maintain the peroxidase activity required for virulence, and to resist killing by isoniazid. Such isolates often display resistance to only lower levels of isoniazid, and resistance to higher levels appears to correlate with loss of catalase activity or acquisition of mutations in multiple genes implicated in isoniazid resistance, e.g., inhA or ahpC [4,5]. However, in the present study, resistance of the isolates carrying the codon 315 mutations to a high concentration of isoniazid (25 mg/L) did not correlate with the presence of mutations in either the inhA or ahpC genes.

The striking difference between the frequencies of various mutations in the rpsL and rrs genes of streptomycin-resistant isolates (Fig. 2) was unexpected. As only isolates with different IS6110 RFLP profiles were selected for PCR sequencing, this dissimilarity cannot be explained by the existence of multiple isolates of the same strain. High-level streptomycin resistance has been correlated previously [4] with acquisition of the rpsL codon 43AAG → AGG mutation, while low-level streptomycin resistance was correlated with changes in the rrs gene, as was observed in the present study for the Beijing family isolates. The correlation of the rrs 513A → C mutation with high-level streptomycin resistance in the LAM family isolates is unusual. The molecular basis for this difference is unknown.

The frequencies of mutations in rpoB codons 516 (5.4%), 526 (15%) and 531 (71%) of the Beijing family isolates are similar to those reported for isolates in the north-west region of Russia [13] and the Archangel region [17]. The mutation 531TTG in rpoB is the most commonly observed mutation in rifampicin-resistant isolates in many parts of the world, e.g., in Brazil (54%) [24], the USA (35%) [25], India (38.7%) [26], Germany (65%) [27] and Australia (52%) [28]. The
predominance of mutations in rpoB codon 531 of the rifampicin-resistant isolates belonging to the Beijing family is in good agreement with frequencies of mutations in codons of the rpoB gene and rates of growth of spontaneous rifampicin-resistant mutants of M. tuberculosis strain H37Rv [29], and Mycobacterium leprae, Mycobacterium avium and Mycobacterium africanum strains [30]. Thus, the predominance of mutations in rpoB codon 516 of the rifampicin-resistant isolates of the LAM family is unusual. Interestingly, mutations in codon 516 of the rpoB gene confer a low level of cross-resistance to rifabutin and KRM-1648 [30,31].

As different mutations in the rpoB gene were detected in the same IS6110 RFLP cluster, the rpoB gene mutations appear to have been acquired independently in most isolates during the course of chemotherapy. As such, differences in the frequencies of mutations in the 516, 526 and 531 codons of the rpoB gene among isolates of the Beijing, Haarlem and LAM families may primarily reflect differences in mutational frequencies or the relative fitness of the mutations in the strain families, as opposed to a possible sampling bias caused by extensive transmission of individual MDR strains.

The finding that eight (3.7%) of the 217 MDR isolates did not contain mutations in the sequenced regions of the katG and rpoB genes was initially surprising. Given the observed frequencies of mutations in the sequenced regions of the katG gene in isoniazid-resistant isolates (95%) and in the sequenced regions of the rpoB gene of rifampicin-resistant isolates (92%), fewer than two such isolates would be expected in a sample of 217 isolates if acquisition of isoniazid and rifampicin resistances was independent. However, seven of these eight isolates were members of the Haarlem family and, in this family, only 57% (12 of 21) of isoniazid-resistant isolates had mutations in the sequenced regions of the katG gene, and 52% (11 of 21) of rifampicin-resistant isolates had mutations in the RRDR of the rpoB gene. Thus, the number of observed isolates without mutations is consistent with the independent frequencies of mutations in the individual genes in isolates of the Haarlem family. The low frequency of mutations revealed in the katG and rpoB genes of MDR Haarlem isolates does not correspond with data concerning 21 MDR Haarlem isolates in Tunisia [32]. However, that study involved a clonal outbreak of an MDR Haarlem strain, confirmed by IS6110 RFLP typing [32], whereas the present study analysed the data for 19 isolates with different IS6110 RFLP profiles.

Overall, the data indicate that the frequencies of individual mutations in the genes associated with rifampicin and streptomycin resistance vary dramatically among isolates belonging to different genotype families. Such variation may influence the performance of molecular diagnostic tests designed to detect mutations associated with drug resistance in M. tuberculosis isolates. This emphasises the importance of validating the performance of a diagnostic test in the population being tested. The biological significance of the predominance of certain mutations in particular genotype families remains to be determined.

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