When is an outbreak not an outbreak? Fit, divergent strains of *Mycobacterium tuberculosis* display independent evolution of drug resistance in a large London outbreak

R. J. Shorten1,2, A. C. McGregor3, S. Platt4, C. Jenkins2,5, M. C. I. Lipman6, S. H. Gillespie7, B. M. Charalambous2 and T. D. McHugh2*

1Department of Medical Microbiology, Royal Free Hospital, London, UK; 2Centre for Clinical Microbiology, University College London, UK; 3Department of Infection, St Thomas’ Hospital, London, UK; 4Applied Laboratory and Bio Informatics, Centre for Infections, Health Protection Agency, London, UK; 5Microbiology Services Colindale, Centre for Infections, Health Protection Agency, London, UK; 6Centre for Respiratory Medicine, Royal Free Hospital, University College London, UK; 7School of Medicine, University of St Andrews, Scotland, UK

*Corresponding author. Tel: +44-20-7472-6402; Fax: +44-20-7317-7710; E-mail: t.mchugh@ucl.ac.uk

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**Objectives:** To study the evolutionary relationship of *Mycobacterium tuberculosis* isolates from 13 patients in a large outbreak of isoniazid-resistant tuberculosis in London.

**Methods:** Genotypic and phenotypic susceptibility tests were performed. Molecular genotyping using restriction fragment length polymorphisms and mycobacterial interspersed repetitive units was carried out. Additionally, the generation times of 13 strains of *M. tuberculosis* from the outbreak were measured to determine relative fitness.

**Results:** Genotypic and phenotypic susceptibility testing demonstrated variations between isolates. Polymorphisms causing isoniazid resistance varied within clusters of isolates that were indistinguishable by standard genotyping. The measurement of *in vitro* generation times demonstrated that the fitness of the resistant strains was not significantly different from either wild-type or susceptible isolates in the outbreak, indicating that apparently no fitness cost was associated with the acquisition of drug resistance.

**Conclusions:** It appears that this outbreak comprised a heterogeneous collection of closely related strains, which appear to exhibit more variation than would usually be associated with a point source outbreak. These strains appear to have evolved by acquisition of additional antimicrobial resistance mutations while remaining competitive. The acquired resistance and retained competitiveness may be partly responsible for the difficulty in controlling the outbreak.

**Keywords:** transmission, fitness, MIRU, RFLP

**Introduction**

The largest outbreak of isoniazid-resistant *Mycobacterium tuberculosis* in Western Europe is centred in London. The outbreak was first identified in 2000, but cases were identified retrospectively 5 years earlier than this. The demographics of patients in this outbreak were different from those normally seen in this population. Cases were more likely to be born in the UK (OR 2.4; 95% CI 1.7–3.4) and of white (OR 2.9; 95% CI 1.8–4.8) or black Caribbean (OR 12.5; 95% CI 7.7–20.4) ethnicity, a prisoner at the time of diagnosis (OR 20.2; 95% CI 6.7–60.6), unemployed (OR 4.1; 95% CI 3.0–5.6), or a drug dealer or sex worker (OR 187.1; 95% CI 28.4–1232.3). A total of 113 (39%) cases used drugs and 54 (18%) were homeless. The first 100 cases were investigated to ascertain the number of secondary cases of active and latent disease. Analysis revealed that a large number of contacts (11%) were diagnosed with active disease, while 13% were reported to have latent tuberculosis (TB) infection. These rates were considerably higher than generally described. The UK National Institute for Health and Clinical Excellence guidance suggests that approximately 1% of contacts are likely to progress to active disease. The apparent increased rate of transmission in this outbreak was associated with a specific drug-resistant phenotype, raising the possibility that increased transmission may be directly related to acquired resistance.

Decreased virulence of isoniazid-resistant *M. tuberculosis* in guinea pigs was reported in the 1950s, with organisms displaying...
the highest levels of resistance exhibiting the greatest loss in virulence.\textsuperscript{5,6} It is assumed that the acquisition of antimicrobial resistance comes at a biological price.\textsuperscript{7} However, the correlation between acquired resistance mutations and fitness cost is uncertain.\textsuperscript{8}

There are several documented reports of fitness compensation for drug resistance mutations in bacteria other than \textit{M. tuberculosis}.\textsuperscript{9–11} The only compensatory mechanism reported in \textit{M. tuberculosis} is the hyperexpression of the alkyl hyperperoxidase reductase gene, \textit{ahpC}, in isoniazid-resistant isolates with \textit{katG} mutations.\textsuperscript{12} However, even this is not certain, as the mutation has been seen in isolates that do not possess \textit{katG} mutations.\textsuperscript{13}

If a resistant strain is prevalent in the community, it implies that it has suffered minimal fitness cost, and therefore must be transmitting successfully between individuals. For this reason, not only is the prevalence of a particular mutation important, but also the size and frequency of clusters of cases associated with the mutated strains. In a Dutch cohort, strains with the most frequently occurring isoniazid resistance-conferring mutation (amino acid 315 in \textit{katG}) were found in clusters as often as isoniazid-susceptible strains.\textsuperscript{14} This was not the case for isoniazid-resistant strains with other mutations.\textsuperscript{15} A study in San Francisco reported that isoniazid-resistant strains of \textit{M. tuberculosis} with the \textit{katG} mutation Ser-315-Thr or a mutation in the \textit{inhA} promoter region were more likely to be found in clusters than strains with other mutations.\textsuperscript{13}

The phylogeographic background of the bacterium may also play a role in the fitness and drug resistance of \textit{M. tuberculosis}. Differences in immunogenicity and virulence have previously been reported and reviewed.\textsuperscript{16} An association between drug resistance and the Beijing family of strains has been noted,\textsuperscript{17} although in a comparison of 12 studies in Southeast Asia and countries of the former Soviet Union showing a high proportion of multidrug-resistant (MDR) strains, poor tuberculosis control strategies are undoubtedly the major cause of MDR-TB rather than the strain type.\textsuperscript{18}

As part of a larger project, \textit{M. tuberculosis} isolates from the Royal Free Hampstead NHS Trust were investigated between 2002 and 2007. A lineage, defined as strains related at $\geq$70% similarity by two genotyping methods,\textsuperscript{19} was identified as having a high proportion of drug-resistant strains (87.5%; 14 of 16), and it was subsequently confirmed that these strains were part of the North London isoniazid-resistant outbreak.\textsuperscript{2,14} This provided an opportunity to investigate the lineage’s evolution and explore the relationship between the isoniazid resistance-causing mutation and fitness.

**Methods**

**Genotyping**

The first available isolate from each TB patient at our centre was genotyped prospectively using IS6110 restriction fragment length polymorphism (RFLP) analysis\textsuperscript{20} and 24-loci mycobacterial interspersed repetitive units – variable number tandem repeats (MIRU-VNTR) analysis.\textsuperscript{21}

**Antimicrobial susceptibility testing**

Phenotypic antimicrobial susceptibilities were determined by the Health Protection Agency National Mycobacterium Reference Laboratory, Whitechapel, London. First-line drugs were tested using the proportional method on Lowenstein Jensen media. Second-line drugs were tested with the proportional method in an automated liquid culture system.

**DNA extraction, PCR and sequencing**

Genomic DNA was extracted from liquid culture by heat-killing at $\geq$95°C for 30 min followed by physical disruption using a Ribolyser with glass beads (Becton Dickinson, Oxford, UK) in a FastPrep 24 (MP Biomedicals, Illkirch, France). The resistance associated regions of the \textit{inhA} and \textit{katG} genes of all strains were amplified using primers previously described (\textit{katG}\textsuperscript{72}, \textit{inhA}\textsuperscript{73}) and the following PCR conditions: 1 x KCl buffer (Bioline, London, UK), 0.04 $\mu$M of each primer, 0.15 mM dNTPs and 2 U Taq polymerase (Bioline, London, UK). Water was added to make a final volume of 90 $\mu$L, and 10 $\mu$L of extracted DNA was then added. Thermocycling conditions consisted of 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, either 56°C (\textit{katG}) or 65°C (\textit{inhA}) for 2 min and 72°C for 3 min. A final elongation step of 72°C for 7 min was used. Sequencing was performed as described previously.\textsuperscript{24} Sequences were analysed using BioNumerics, version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). IS6110 RFLP fingerprints were analysed using the Dice similarity coefficient with a tolerance setting of 1.2% and tree generation using the UPGMA algorithm. Minimum spanning tree analysis of MIRU-VNTR profiles was performed using a categorical coefficient with locus 3690 excluded.

**In vitro fitness measurement**

The generation time of each isolate was measured in automated liquid culture (MGIT; Becton Dickinson, Oxford, UK) as previously described.\textsuperscript{8} Fresh cultures were diluted 1:10 (termed dilution A) and 1:10000 (termed dilution B). A 0.5 mL aliquot of each dilution was inoculated in triplicate into fresh MGIT tubes containing growth supplement. These tubes were incubated in the MGIT 960. This was performed in triplicate for all isolates. A modified Youmans and Youmans method\textsuperscript{25} was used to calculate the growth rate constant, $k = (\log A - \log B)/t$, where $A$ is the longest inoculum (dilution A), $B$ is the smallest inoculum (dilution B) and $t$ is equal to the difference of the three replicates of the time to positivity (TTP) between dilution A and B. To determine the generation time $G$, the equation $G = \log 2/k$ was used.

The reference strain \textit{M. tuberculosis} H37Rv, two unrelated isoniazid-resistant isolates and one unrelated susceptible isolate were additionally investigated as controls.

**Results**

Analysis of the lineage showed that the IS6110 RFLP fingerprints for these isolates were related at $\geq$80% similarity (Figure 1). We showed that there were a total of eight insertions and seven deletions of the IS6110 element. MIRU-VNTR genotyping confirmed that the isolates were not identical, but closely related (Figure 2), but only 23 of 24 MIRU-VNTR loci were detected for all isolates: PCR products were not obtained for locus 3690.

When compared with the other lineages in the complete North London dataset, the proportion of drug-resistant strains was statistically significantly higher ($P=0.0001$; Fisher’s exact test with two-tailed analysis). Of the six lineages in the dataset ($n=294$) containing 10 or more strains, five possessed $<10$% resistant strains. The lineage in this study contained 87.5% resistant strains.

The majority of the isolates (11/13; the other 3 isolates were not viable and were therefore not included in any analysis) showed resistance to one or more antimicrobial agent. Isoniazid resistance (with or without other resistances) was
Figure 1. Dendrogram of IS6110 fingerprints of strains within the lineage. Analysed using the Dice similarity coefficient with a tolerance setting of 1.2%.

Figure 2. Minimum spanning tree of MIRU-VNTR profiles presented in Table 1. Node population is also indicated by relative node diameter. Branch values indicate the number of loci that differ between nodes. Each node (containing one or more strains) is named A–I. These node names are also used in Figure 4. The analysis was performed using the categorical coefficient where locus 3690 was excluded from all profiles.
seen in 10 isolates. Streptomycin monoresistance was present in two isolates. Amplification and sequencing of katG and the promoter region of inhA showed heterogeneous polymorphisms, which are summarized in Table 1. Cytosine was substituted for thymine at a locus 15 bp upstream from the start codon of the mabA gene (which is co-transcribed with inhA) in 8 of the 10 isolates that were isoniazid resistant. The two other isoniazid-resistant isolates possessed mutations that substituted serine for threonine at codon 463, was detected in one of the isoniazid-susceptible controls (04:011).

The generation time of all isolates and controls was measured. The median generation time of each isolate was compared with that of the laboratory strain M. tuberculosis H37Rv as well as to susceptible controls. The mean generation time of each isolate was compared using a one-way ANOVA with Kruskal–Wallis post-test (Figure 3). Antimicrobial susceptibilities, isoniazid resistance-causing mutations and relative fitness are summarized in Table 1.

Using the methods described previously we analysed the IS6110 RFLP fingerprints, MIRU-VNTR types and antimicrobial susceptibility profiles. There were two fully susceptible isolates (05:046 and 02:113) that we identified as the earliest strains in the network and which had not yet acquired antimicrobial resistance. From this it was possible to develop a map of evolution through the alteration of IS6110 patterns and acquired resistance-conferring mutations from that point on. The hypothetical relationship between the strains, the insertion/deletion of IS6110 and the acquisition of antimicrobial resistance are shown in Figure 4.

**Discussion**

The strains in this lineage are closely related but divergent, and although they cluster together with the North London isoniazid-resistant outbreak strains, they are no longer homogeneous, as minimum spanning tree (MST) analysis (Figure 2) indicates that evolution has occurred during the course of transmission between individuals. The isolates were cultured from patients between 2002 and 2007, so there was ample opportunity for the acquisition of drug resistance as well as insertions or deletions of IS6110, assuming a half-life of 2–5 years. Evidence of evolution is indicated by the antibiograms of the strains, which vary widely. This includes drug-susceptible, isoniazid- and streptomycin-monoresistant as well as MDR strains. However, resistance is not acquired in a sequential manner. This is in agreement with the reports of MDR cases in the North London outbreak. It may be reasoned that the strain was already successfully circulating in the North London area and the acquisition of antimicrobial resistance was a secondary event. We have previously demonstrated that strains in this outbreak have become multidrug resistant by the

<table>
<thead>
<tr>
<th>Type/isolate</th>
<th>Resistance</th>
<th>Polymorphism</th>
<th>Median generation time (h)</th>
<th>Relative fitness to H37Rv</th>
<th>to 02:113</th>
<th>to 05:046</th>
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<tr>
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<td></td>
<td></td>
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<td>fully susceptible</td>
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<td>0.90</td>
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<tr>
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<td>0.88</td>
<td>0.91</td>
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<td>21.2</td>
<td>0.81</td>
<td>0.84</td>
<td>0.79</td>
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*H, isoniazid; R, rifampicin; S, streptomycin; Eth, ethionamide; C, clarithromycin. Polymorphisms: katG S315T, serine to threonine substitution at codon 315; inhA C→T, cytosine to threonine substitution at 15 bp upstream of the start codon of mabA.*
independent acquisition of unusual \(rpoB\) mutations.\(^{24}\) We have not analysed the individual patients’ adherence to therapy, but this is likely to be a strong driver in this outbreak. Indeed, treatment completion rates in individuals in this outbreak were low.\(^{2}\) If treatment completion is not maximized, there is likely to be ongoing transmission, new drug resistance and continued lack of control of this outbreak.

The hypothesized evolution of the organisms in the North London isoniazid-resistant TB outbreak is shown in Figure 4. The earliest identified strain (02:113) was isolated in 2002. This organism is fully susceptible to first-line anti-TB agents. A second strain (02:302), also isolated in 2002, is indistinguishable using both IS\(6110\) and MIRU-VNTR genotyping, but has developed streptomycin monoresistance. The figure shows hypothesized routes of evolution from these early strains (Figure 4).

It is noteworthy that the MIRU-VNTR locus 3690 could not be amplified in any of the isolates. Strains from this outbreak typically have a large number of copies at this locus and are therefore often difficult to enumerate (Dr Tim Brown, HPA National Mycobacterium Reference Laboratory, personal communication).

The outbreak strains were collected over a 6 year period, and there are a total of eight insertions and seven deletions of IS\(6110\) during this time, a higher rate than described elsewhere; an insertion or deletion every 2–5 years.\(^{26,28}\) This raises the possibility that this group of strains is hypermutable.

The fitness of the resistant strains is not significantly different from either the laboratory-adapted strain \(M.\) \(tuberculosis\) \(H_{37}Rv\), the susceptible strains in the chain of transmission or unrelated isoniazid-susceptible and -resistant strains \((P=0.2956;\) one-way ANOVA with Kruskal–Wallis post-test). This indicates that if there were any fitness cost initially associated with the acquisition of resistance-conferring mutations, this is either very small, or the organisms have compensated for it rapidly. Indeed, comparing the generation time of these strains with the work of others shows that these organisms are extremely fit.\(^{8,29}\) Again, it seems reasonable to conclude that, in the absence of interventions, the outbreak will expand.

In line with this, data indicating a high rate of infection in close contacts (11%)\(^{1}\) suggest that these are highly competitive strains. However, epidemiological factors may play their part; many patients in this outbreak are prisoners and drug users who reside in confined dwellings with limited ventilation—encouraging increased person-to-person transmission.

We have highlighted that this ‘outbreak’ has been difficult to control and it does not follow the usual definition of a ‘point source’ outbreak. In fact, our evidence indicates that this event, rather than being a classical point source outbreak caused by genetically identical strains, has a high incidence of disease caused by genetically related (but non-identical) strains. Although evidence of person-to-person transmission was noted, the term ‘outbreak’ in this context may not be helpful in describing the course of events.

It is worth acknowledging that this study only represents a small sample of the ongoing outbreak and thus associations may be speculative. Likewise, further genetic analysis, such as whole genome sequencing, may provide some crucial insight into the mechanisms driving this outbreak.
information. The true extent of genetic diversity of these strains across the entire outbreak would provide fascinating insights into this large outbreak. In conclusion, it appears that this lineage, which is associated with the North London isoniazid-resistant outbreak, comprises a heterogeneous collection of closely related strains. These strains appear to have thrived in the community, generating large numbers of clinical cases while evolving independently via the acquisition of antimicrobial resistance and the alteration of genotypes.

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Figure 4. Split tree analysis. Relationship of the strains in lineage 15, including insertion (large arrows) and deletion (small arrows) of IS6110 from the earliest identified strains (02:302 and 02:113). All strains are isoniazid resistant, except 05:046 and 02:113, which are fully susceptible (Fully S), and 02:302 and 03:313, which are streptomycin monoresistant (S mono-®). The isoniazid resistance-conferring mutations are noted and additional resistances (®) are included (R, rifampicin; clari, clarithromycin; ethi, ethionamide). The first two digits in the isolate number indicate the year of isolation, e.g. 02:113 was isolated in 2002. Dotted arrows indicate that a strain may have evolved from more than one possible precursor. The underlined letters A–I relate to the MIRU-VNTR genotypes. Isolates with the same letter are indistinguishable by MIRU-VNTR genotyping. The relative fitness to M. tuberculosis H37Rv is in brackets.

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Transparency declarations
None to declare.

References
Evolution of resistance in an outbreak of tuberculosis


