Genome sequencing reveals novel deletions associated with secondary resistance to pyrazinamide in MDR *Mycobacterium tuberculosis*

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**Objectives:** Detection of pyrazinamide resistance in *Mycobacterium tuberculosis* isolates presents significant challenges in settings with no dominant clonal lineages, such as Australia. We assessed the utility of WGS versus standard PCR amplification assays for the characterization of pyrazinamide resistance in MDR-TB isolates identified in New South Wales, Australia, over an 8 year period.

**Methods:** PCR amplicon sequencing was used to identify molecular markers associated with antibiotic resistance in pyrazinamide-resistant MDR-TB isolates recovered by the New South Wales Mycobacterium Reference Laboratory between 2007 and 2014. WGS was subsequently performed on two isolates for which *pncA* amplification failed.

**Results:** WGS identified two novel genomic deletions associated with *in vitro* resistance to pyrazinamide in MDR-TB. One isolate also carried a second deletion involving the genes *dfrA* and *thyA* associated with resistance to para-aminosalicylic acid.

**Conclusions:** Steadily decreasing sequencing costs are increasing the appeal of WGS as an alternative approach for detecting complex patterns of pyrazinamide resistance in MDR-TB.

**Keywords:** multidrug-resistant tuberculosis, whole-genome sequencing, pyrazinamide resistance

**Introduction**

Every year, MDR-TB causes almost half a million new cases of disease worldwide. A significant proportion of MDR-TB is associated with secondary resistance due to inappropriate selection of antimicrobial agents, poor adherence to therapy, malabsorption or low quality of drugs. Control of MDR-TB depends on the timely recognition and understanding of mechanisms of drug resistance. While resistance to isoniazid and rifampicin can be reliably identified by phenotypic testing and through the detection of molecular markers, assessing the activity of another first-line antituberculous drug, pyrazinamide, presents significant challenges. Firstly, routine drug susceptibility testing of pyrazinamide is complicated as the growth of bacilli is impeded by the acidic conditions required for optimal drug activity. Secondly, our understanding of the mechanisms of resistance to pyrazinamide remains limited. This drug is converted into its active form, pyrazinoic acid, by the bacterial enzyme pyrazinamidase, which is encoded by the 561 nt *pncA* gene of *Mycobacterium tuberculosis*. Non-synonymous mutations in *pncA* result in lost or reduced activity of the enzyme and have been considered the primary mechanism of pyrazinamide resistance. However, the presence of *pncA* mutations does not correlate well with phenotypic susceptibility testing and recent reports of mutations in the *rpsA* gene and *pncA* gene deletions suggest that pyrazinamide resistance might be more complex than previously thought. In this report, we present two novel large deletions in *M. tuberculosis* associated with pyrazinamide resistance and argue that WGS could be the most informative method of estimating *in vitro* resistance to pyrazinamide in MDR-TB.

**Methods**

To determine the molecular markers of resistance, we examined all MDR-TB isolates identified by the New South Wales (NSW) Mycobacterium Reference Laboratory between 2007 and 2014, which were *in vitro* phenotypically resistant to pyrazinamide by reference BACTEC 460 or Mycobacterial Growth Indicator Tube 960 (Becton Dickinson, MD, USA) methods. Fourteen isolates obtained from patients diagnosed in NSW but born in high-incidence countries and successfully
treated for culture-confirmed TB in Australia were included in the study. There were no known epidemiological links between the patients. Table 1 presents the main characteristics of the isolates including demographics of the patients as well as susceptibility profiles determined by phenotypic reference methods, GenoTypeMDRTBplus (Hain LifeScience) and in-house PCR for isoniazid, rifampicin, ethambutol and ofloxacin resistance (see Table S1, available as Supplementary data at JAC Online). Pyrazinamide resistance was initially investigated by PCR and sequencing of the pncA gene. Primers used to amplify and sequence the pncA gene included the 5\'-untranslated region (105 bp upstream of the start codon), as drug resistance has also been associated with mutations in this regulatory region. Twelve isolates (86%) had different mutations scattered across the pncA gene or its promoter region. However, PCR amplification failed for two isolates (Mtb97 and Mtb24) that were resistant to pyrazinamide. Both isolates had mycobacterial interspersed repetitive unit (MIRU) profiles (Mtb97, 223325163533; Mtb24, 2233225173532) indicative of the Beijing lineage (Table 1). WGS was performed on these two isolates to investigate the genetic basis of drug resistance.

Genomic DNA was extracted from pure cultures on Middlebrook medium using the Wizard genomic DNA kit (Promega, Australia) and sequenced on Ion Torrent PGM (Life Technologies) with 400 bp sequencing chemistry. Reads were trimmed by quality (>20) and length (<50 bp) and aligned to the reference genome of M. tuberculosis H37Rv (GenBank accession number AL123456). De novo assembly was also performed and contigs aligned against the reference. Bioinformatic analyses were conducted using CLC Genomics Workbench (CLC Bio, version 7.0).

Results and discussion

WGS confirmed that the pncA gene was missing in both genomes of MDR-TB, but also revealed that they contained different genomic deletions. Specifically, in isolate Mtb24, 13 genes upstream of pncA and 4 genes downstream were missing (Rv2030–Rv2047), a deletion of 20021 bp compared with the H37Rv genome (Figure 1a). The missing region included the gene coding for the stress protein HspX (Rv2031), a recently suggested vaccine candidate, the repressor protein ArsR (Rv2034) and an ABC transport protein complex (Rv2038c–Rv2040c). Mapping de novo-assembled contigs to H37Rv indicated that a 15115 bp contig spanned this deletion (2993 supporting reads, 54% average coverage). MDR-TB Mtb97 carried a smaller but still significant pncA genomic deletion (2539 bp), extending from gene Rv2042 to Rv2045 (Figure 1a). As above, the alignment of de novo-assembled contigs to H37Rv showed that a single 7663 bp contig spanned the deletion (1671 supporting reads, 63% average coverage). WGS was also performed on another three isolates from the same cohort to exclude possible bias in coverage in the regions where the deletions were observed. Sequence reads were deposited at the Sequence Read Archive with the following accession numbers: SRR1952721 (Mtb97), SRR1948177 (Mtb24), SRR1952684 (Mtb59), SRR1952705 (Mtb45) and SRR1952706 (Mtb15).

Importantly, WGS simultaneously provided additional information about markers associated with resistance to second-line antimycobacterial agents. Mtb24 contained a synonymous mutation (A1401G) in the rs gene associated with amikacin resistance. In contrast, MDR-TB Mtb97 carried a second 3001 bp, four-gene deletion (Rv2762–Rv2765) involving the genes dfrA and thyA (Figure 1b), the latter of which is known to be associated with resistance to para-aminosalicylic acid. A contig of 2447 bp extended across the deletion, confirming it. Furthermore, MDR-TB Mtb97 also contained two non-synonymous mutations in the katG (R463L and R705L) that were not initially detected by in-house PCR as they fell outside the primer-amplified region, which could explain the observed low-level phenotypic resistance to isoniazid in this strain (MIC 0.125 mg/L).

Sequencing reads that did not map to H37Rv were assembled de novo, resulting in six additional contigs (>1000 bp) for each isolate. Comparison of these contigs using the ProgressiveMauve alignment tool (Mauve) demonstrated no major differences in the genetic content between the contigs from the two isolates. Blastn searches found that all of the contigs aligned to genomic regions from Beijing lineage genomes stored in the NCBI GenBank database. SNP comparisons indicated that isolates had >300 differences between them, suggesting that they did not share a close evolutionary sublineage.
Both MDR-TB isolates were recovered from patients with pulmonary TB who had a history of treatment in their country of origin prior to arriving in Australia (Table 1). In particular, isolate Mtbb7 was associated with a recurrent disease lasting >30 years. The first episode of the TB was treated with 12 months of isoniazid, ethambutol and para-aminosalicylic acid. The latter could have selected for the deletion of the thyA gene region. Following recurrence, the patient received several rounds of isoniazid, rifampicin and ethambutol for a period of 3 years. After 5 years of smear-negative cultures, another relapse was treated with 14 months of isoniazid, rifampicin and pirazinamide followed by 18 months of isoniazid, pirazinamide and ofloxacin with the addition of kanamycin for 4 weeks. This extended selection pressure of treatment with pirazinamide may have driven the deletion of the pncA gene. The disease was eventually cured by 24 months of directly observed therapy that included isoniazid, moxifloxacin, capreomycin, prothionamide and cycloserine. The patient harbouring the MDR-TB Mtbb24 also had a history of TB treatment for 12 months, 5 years prior to the recovery of this isolate and curative treatment with second-line drugs in Australia.

Accurate detection of pirazinamide resistance is critical as its rates in MDR-TB have been increasing in Northern America and Asia. Here, we report two novel large deletions of the pncA gene associated with secondary resistance to pirazinamide in MDR-TB. While this study focused on pirazinamide resistance in phenotypically confirmed isolates of MDR-TB, further examination of genomic changes in pirazinamide-monoresistant and phenotypically susceptible isolates is warranted. Our findings reconfirm recent reports of pirazinamide resistance resulting from the complete loss of the pncA gene in four clinical isolates of MDR-TB from Germany and Japan. These isolates were reported to have different deletions ranging from 1565 to 6258 bp. These results further strengthen the case for using WGS for rapid resistance testing of M. tuberculosis. The complexity of pirazinamide resistance mechanisms, which can involve several highly diverse mutations or deletions scattered over the pncA gene cluster and beyond as well as significant genetic diversity within M. tuberculosis complex, make it difficult to rely on PCR-based methods targeting a single gene. Decreasing costs and turnaround times for performing WGS experiments and data analyses mean that WGS has the potential to improve both the accuracy and timelines of detection of complex patterns of mutations associated with pirazinamide resistance in MDR-TB.

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### Transparency declarations

None to declare.
**Supplementary data**

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**