A unique strain of *Mycobacterium tuberculosis* and a cautionary tale for users of molecular techniques

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Molecular techniques are now widely applied in Australia and elsewhere for the direct detection of *Mycobacterium tuberculosis* DNA in clinical specimens and culturally enhanced material. All nucleic acid testing methods have the potential to give false negative results due to mutations that may arise at primer or probe binding sites. We describe one such strain of *M. tuberculosis* that was encountered in 1995 and that has not been encountered in Australia since.

Introduction

Mycobacterium tuberculosis (MTB) remains an important pathogen for Australia and many laboratories within the Australian Mycobacterium Reference Laboratory Network (AMRLN) use sophisticated molecular techniques to detect this pathogen. These techniques are widely applied for the direct detection of *M. tuberculosis* DNA in clinical specimens as well as to culturally enhanced material.

The most common polymerase chain reaction (PCR) technique that is applied to cultures in Australia today is the multiplex PCR developed by Steve Wilton and Debby Cousins in 1992 [1]. The TB primers described in this technique target the MPB70 gene of the *M. tuberculosis* complex (MTBC) and have also been applied to the direct detection of these organisms in specimens [2].

Case study

This report describes a strain of *M. tuberculosis* that was encountered in 1995 but not again since then. Although it failed to react with these primers, it was in all other respects characteristic of *M. tuberculosis.* The isolate was recovered from a sputum sample from a 69 year old female with a right upper lobe cavity in her lung who presented with a cough. The strain was isolated from BACTEC 12B medium after 12 days incubation and microscopy revealed AFB showing good serpentine cording. It was confirmed as MTBC by MTBC-specific probe [Accuprobe; Gen Probe, San Diego, Calif.] and 16SrRNA gene-sequence analysis. Species-specific tests identified the organism as *M. tuberculosis.* It accumulated niacin, produced pyrazinamidase, reduced nitrate and was susceptible to 10µg/ml thiophene-2-carbooxylic acid hydrazide [3, 4]. Susceptibility testing was performed using the BACTEC 460 radiometric assay proportion method [5] and the strain was susceptible to streptomycin, isoniazid, rifampicin and ethambutol (SIRE).

Patient details

The female patient, aged 69, had migrated to Australia from Burma 28 years previously in 1967. Two years before presentation, she had travelled around Australia but there was no other history of travel abroad. She was a non-smoker and did not consume alcohol.

She presented in May 1995 with a dry chronic cough, lethargy and anorexia. A chest x-ray showed a right upper lobe cavity. Mantoux testing showed a 21x 10mm response to human purified protein derivative (PPD) but no response to avian PPD. She had osteoporosis but no other significant past or present medical problems. A bronchoscopy showed swollen erythematous airways and purulent secretions. These were negative for acid-fast bacilli (AFB) and she had the fine needle aspirate of her lung lesion, which was diagnostic.

She received isoniazid, rifampicin, ethambutol and pyrazinamide (HREZ) from June 95 until August 95, and then continued on HR until Dec 95, i.e. 6 months of treatment. She tolerated the treatment well and responded with near complete resolution of the chest x-ray abnormality, and no relapse.

Strain investigations

No amplifiable PCR product could be obtained with this patient strain using the published TB1F and TB1R primers [1]. An additional primer was designed using Primer Express Version 1.5 [Applied Biosystems, Foster City, Calif.] that was outside the 372bp target region for TB1F & TB1R of the MBP70 gene. This new primer (MPB70fwd) has the sequence: 5'-ATCGGCTGGCGTCCGAAA-3'. PCR was performed using the combination of primers MBP70fwd and TB1R. When MPB70fwd combined with TB1R in the reference strain of *M. tuberculosis* (H37Rv) the large product expected (736bp) was detected. When MPB70fwd combined with TB1R in the patient strain, a smaller product (368bp) was detected

since the target was now smaller, due to the deletion (Figure 1).

This double stranded 368bp product from the patient strain was purified by QIAquick PCR purification kit [Qiagen, Valencia Calif.] and two sequencing reactions performed using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit [Applied Biosystems, Foster City, Calif.]. 3.2 pmol of sequencing primers MPB70Fwd and TB1R were used. The sequencing products was purified using cold ethanol precipitation and the reaction loaded onto the ABI prism 310 Genetic Analyser in accordance with the manufacturers' instructions [Applied Biosystems]. The results of the sequence reaction were analysed using Sequencher Version 3.0 [Gene Codes Corporation, Ann Arbor, MI] and a comparison made with the sequences obtained with Gen Bank Accession X17086 (H37Rv, a reference strain of *M. tuberculosis).* A 368bp deletion in the MPB70 gene, corresponding to bases 38 to 405, was detected. Figure 1 indicates the location of the primers used, the region of deletion detected and the primer binding sites in the MBP70 gene of these strains.

Typing

MTB strain typing was performed using the method of Frothingham & Meeker-O'Connell [6] targeting five exact tandem repeat loci (ETR), ETR A, B, C, D and E to obtain a numerical profile for the strain based on variable numbers of tandem DNA repeats (VNTR) at each loci. A VNTR profile of 46463 was obtained. We examined our database of VNTR profiles (Queensland data for the period 1999-2003) to determine if there were other MTB strains with the same VNTR profile, which could harbour the same deletion. We found no strains with this VNTR profile. MIRU analysis [7] gave a profile of 164 326 223 332, again unique in Australia, albeit against our limited database. Restriction Fragment Length Polymorphism (RFLP) analysis of the insertion sequence IS*6110* [8] showed the strain to have an 11-band pattern, again unique in Australia (Figure 2). International databases were not consulted to determine the worldwide distribution for this strain.

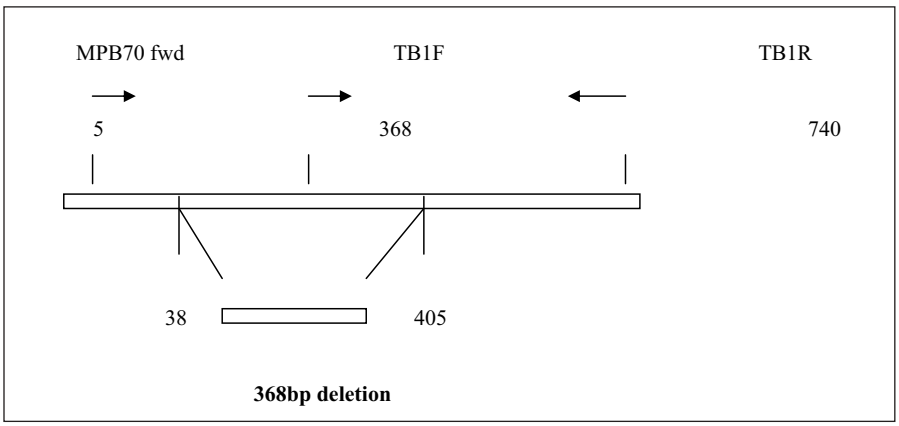

Figure1. Schematic diagram showing the region of deletion and the primer binding sites in the MPB70gene. Position numbers for bases are as given in Gen Bank for Accession Number X17086 (H37Rv).
When MPB70fwd combined with TB1R in the standard strain of *M. tuberculosis* (H37Rv), the large product expected (736bp) was detected. When MPB70fwd combined with TB1R in the patient strain, a smaller product (368bp) was detected since the target is now smaller, due to the deletion.

Figure 2. IS*6110* RFLP of the test strain showing its unique 11-band pattern. The test strain is in lane 1 followed by molecular weight markers in lane 2.

Discussion

The MPB 70 gene has been targeted in our respective laboratories and others for many years and has proved to be a highly reliable test in confirming identification of MTBC cultures. All nucleic acid testing methods have the potential to give false negative results due to mutations that may arise at primer or probe binding sites. In 2002, Gilpin *et al.* reported a deletion in a commercial direct nucleic acid amplification test that resulted in a false negative result in an AFB smear positive sputum sample [9]. Similarly IS*6110* has been widely used as a target region for direct nucleic acid testing and molecular strain discrimination; however, there are reports of strains containing no copies of this insertion sequence [10].

It is important to investigate the frequency of deletions or changes in target regions in circulating strains of *M. tuberculosis* in a population to appreciate what are the limitations of the molecular detection and identification tools we routinely apply in tuberculosis diagnosis. As examination of our VNTR and MIRU databases have shown that this isolate is not part of a circulating clade, our findings in this investigation should not preclude continued use of the MPB70 gene as an identification target region for MTBC.

All nucleic acid amplification results need to be interpreted in relation to other patient or clinical information. Our patient in this case was unaffected by the delayed laboratory diagnosis of tuberculosis as treatment for tuberculosis proceeded despite a negative MPB70 PCR.

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MIRU: the national tuberculosis genotyping strategy in Australia

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Introduction

Tuberculosis remains a low prevalence disease in Australia, with approximately 800 new bacteriologically confirmed cases detected each year. In Australia, this low incidence rate corresponds to less than five cases per 100,000 population.

The highest incidence occurs in migrants from high prevalence countries followed by indigenous Australian-born people. Among non-indigenous Australians, tuberculosis most often occurs among elderly males, largely due to re-activation of latent tuberculosis.

The general impression has been that most cases in Australia are due to re-activation of latent infection acquired many years earlier, when TB was more prevalent, in Australian-born population or in the country of origin in overseas born people. Transmission within Australia usually occurs in micro-epidemics as small clusters, particularly in indigenous communities but also in other social clusters.

Genotyping strategies

The control of tuberculosis requires powerful methods for detecting cases and tracing sources of infection, so that effective treatment can be correctly targeted and suitable public health measures implemented. The Australian Mycobacterium Reference Laboratory Network (AMRLN) is comprised of the five State reference laboratories and aims to coordinate genotyping of all strains of MTB in Australia, in addition to performing identification and susceptibility testing.

For several years, the individual reference laboratories have been genotyping their own MTB isolates using a variety of different methods, and the data have not been integrated into a national database. IS*6110*-restriction fragment length polymorphism (RFLP) is the standard reference method and has been widely applied for the discrimination of strains of MTB; however, the method is both technically demanding and time consuming. The comparison of large numbers of RFLP fingerprints remains difficult, and the method is not highly discriminatory among strains with low copies of IS*6110*.

Due to the limitations of using IS*6110* RFLP as a real-time tool for monitoring tuberculosis transmission, alternate strategies have been evaluated. PCR-based typing methods targeting loci containing variable numbers of tandem repeats (VNTR) and mycobacterial interspersed repetitive units (MIRU) have been described [1, 2]. MIRUs are typically 51-77 bp repetitive sequences and have been identified in 41 locations throughout the chromosome of MTB strain H37Rv [2].