Direct detection of heteroresistance in *Mycobacterium tuberculosis* using molecular techniques

The risk of resistance emerging during the treatment of tuberculosis (TB) is ever present. The simultaneous detection of both ethambutol-sensitive and -resistant genotypes in sputum specimens predicted or known to have drug-resistant TB has been reported, a phenomenon described as heteroresistance (Rinder *et al.*, 2001a). We extend this observation by detecting pre-existing mutants in the sputum of patients from a population in Tanzania in which resistance is unlikely.

Two samples of sputum, either freshly collected (six samples) or frozen (16 samples), from each of 11 newly diagnosed TB patients were examined. DNA extraction was performed at Kilimanjaro Christian Medical College (KCMC), Moshi, Tanzania, using the chelating resin Chelex 100 (Bio-Rad) (Giraffa *et al.*, 2000). The presence of *Mycobacterium tuberculosis* DNA was confirmed by strand

displacement amplification (SDA; BD Probe-Tec) at the Centre for Medical Microbiology, University College London, UK, in nine samples (six fresh and three frozen). Seven frozen samples were equivocal by repeated SDA testing, indicating that the sample DNA had deteriorated on storage (six samples were not tested due to insufficient volume of extract). To detect mutations in codon 306 of the ethambutol-resistance-determining region of *embB*, which is responsible for most ethambutol resistance (Sreevatsan *et al.*, 1997), restriction digestion of nested PCR products was carried out as previously described (Rinder *et al.*, 2001b).

Nested *embB* PCR was performed on all 22 samples at KCMC. Twelve extracts gave PCR products of the expected size (103 bp). To identify pre-existent ethambutol mutants, six PCR-positive DNA extracts from three patients were selected, on which

nested *embB* PCR was performed 10 times under identical conditions. The PCR product was digested with *Nla* III (New England Biolabs), which cut the PCR products at three sites, including codon 306 of the wild-type gene, producing four fragments of 11, 18, 30 and 44 bp in length. Mutation at codon 306, which indicated the presence of a resistant gene, would be detected by the presence of a 74 bp product (30 + 44 bp due to loss of a restriction site). The restriction products were visualized by electrophoresis in a 3 % agarose gel containing ethidium bromide (Fig. 1). All six samples demonstrated the presence of the wild-type sequence in each of the 10 identical tests. However, two samples from one patient and one sample from a second patient demonstrated an additional 74 bp band in at least one of the 10 tests, indicating the presence of a mutation at codon 306 of the *embB* gene.

Identification of mutations encoding ethambutol resistance in samples from two of three patients indicates that pre-existent mutants are common, even when the risk of drug resistance is low, and that the molecular heterogeneity described previously also occurs in patients at low risk of resistance. Our data demonstrate the ever-present risk of the emergence of resistance if therapy is less than optimal. Due to the possibility of preferential amplification of low levels of a mutant sequence, our data indicate that there is a risk of false-positive results in rapid ethambutol-resistance testing by a nucleic acid amplification method. This may have clinical implications for the initial choice of anti-TB therapy if used to predict phenotypic susceptibility.

Acknowledgements

The authors would like to acknowledge financial support received for M. M. C. from the Association of Medical Microbiologists.

Fig. 1. Demonstration of both wild-type and mutant restriction products in one out of 10 identical *embB* PCR reactions (sample: patient 10/day 5).

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