

## Population genetics of *Mycobacterium tuberculosis* complex in Scotland analysed by pulsed-field gel electrophoresis

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### SUMMARY

The results of typing of 121 strains in the *Mycobacterium tuberculosis* complex by PFGE are presented. Every isolate from patients in Scotland over a 3-month period for *M. tuberculosis* and for 1 year for *M. bovis* were included along with several laboratory strains including those of BCG. The PFGE results suggest that the population structure of all the strains in this complex is distinctly simple with limited genetic diversity and also suggest that *M. bovis* is not a distinct species.

### INTRODUCTION

The incidence of tuberculosis in Scotland has declined over the last two centuries [1]. By 1987 notifications had fallen to 561. Since then they have remained at around this level. In 1992 there were 529 notifications, 280 of these cases were confirmed bacteriologically. Of these 93·5% were in natives of the United Kingdom. 5·7% of isolates were drug resistant with two-thirds of these only resistant to isoniazid [2].

Typing methods for *Mycobacterium tuberculosis* complex isolates have suffered from a lack of discriminatory power. Phenotyping has allowed the division of the complex into four species *M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum* [3] but is of limited use in epidemiological studies [4–9]. Phage typing of *M. tuberculosis* produces only four major groups [10]. Pyrolysis mass spectrometry produced similar divisions to conventional phenotyping [11]. High performance liquid chromatography has only been successful at discriminating between the vaccine strain BCG and wild type organisms [12]. SDS-PAGE is useful for speciation of strains but of limited value in subdividing species [7, 13–17]. Conventional genomic DNA digests produce patterns with almost complete similarity although sufficient differences have been noted to be of some use in epidemiological work [14, 16, 17–22]. The most successful typing methods use repetitive DNA sequences such as the transposable element *IS6110* as markers [23–25]. These markers although successful for epidemiological purposes, particularly over short time periods, are not ideal for population genetic studies as they can vary rapidly in number and position in the chromosome.

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Infrequent-cutting restriction endonucleases digest chromosomal DNA into a small number of large fragments which can be resolved by pulsed-field gel electrophoresis (PFGE) into simple banding patterns which are ideal for use as a genotyping method. PFGE has in practice been found to be one of the most discriminatory typing methods for bacteria and a useful tool for chromosome mapping [26]. PFGE has been successfully used to type many bacterial species including *Mycobacterium paratuberculosis* [27]. In 1991 it was decided to develop PFGE for *M. tuberculosis* to investigate the population structure of the organism in Scotland and to estimate the genetic diversity of this organism by analysing strains from every patient in Scotland over a limited period.

Initial work concentrated on the use of the infrequent cutting enzymes *Xba* I and *Dra* I. When these enzymes started to give conflicting results it was decided to also include the more frequent cutting enzyme *Hind* III. As our results with PFGE differed dramatically from the experiences of others with *IS6110* Southern blotting it was decided to apply this method also to our collection of strains.

#### METHODS

All clinical isolates had been referred to the Scottish Mycobacteria Reference Laboratory, Edinburgh. The collection of 121 unique strains analysed comprised 99 strains of *M. tuberculosis* collected between 1 August 1992 and 1 December 1992 (one isolate from every culture-positive patient in Scotland between these dates) and 12 strains of *M. bovis* of human origin collected between 1 August 1992 and 1 August 1993 all from different patients (again every culture positive patient in Scotland between these dates), also a type strain of *M. tuberculosis*-H37Ra, three clinical isolates of *M. bovis* BCG from the collection of the Scottish Mycobacteria Reference Laboratory and six BCG vaccine strains (BCG's Brazil, Copenhagen, Dakar, Glaxo, Pasteur and USSR) which were kindly donated by Dr J. Dale [28]. Repeat isolates from several patients were studied in a pilot study but as no variation was found between isolates separated in time or from different body sites in the same patient it was decided to analyse only one isolate per patient in the main study.

DNA was extracted and digested using a modification of the method of Zhang and colleagues [29]. Briefly, strains were cultured in Middelbrook 7H9 broth in a 50 ml centrifuge tube at 37 °C for 4 weeks without shaking or aeration. The cultures were checked for bacterial contamination, heat-killed in a waterbath at 80 °C for 30 min and left to cool. The cultures were centrifuged at 3000 g for 25 min and the pellet stored at -20 °C. To extract the DNA the pellets were suspended in 1 ml of 10 mM Tris-EDTA (pH 8.0) containing 2 mg of lysozyme; incubated at 37 °C for 4 h; then mixed with 1 ml of 1.5% low-melting point agarose and dispensed into 200 µl insert formers. The inserts were incubated at 55 °C in 2 ml of 0.5 M-DTA (pH 8.0) with 1% sodium dodecyl sulphate and 1 mg/ml proteinase K for 24 h then stored at 4 °C. Proteinase K in the inserts was removed by washing six times in 20 ml of 10 mM Tris-EDTA (pH 8.0) at room temperature. The samples were stored at 4 °C until digestion was performed.

For restriction endonuclease analysis a suitably sized portion of each insert was incubated at 37 °C for 2 h in a predigestion solution of 1 × restriction enzyme

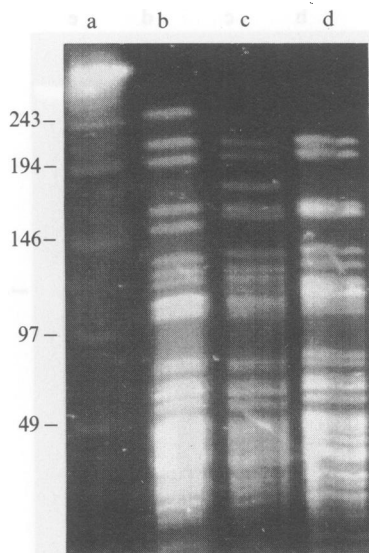


Fig. 1. *Xba* I digest patterns. Patterns I (lane b), II (lane c) and III (lane d). Size standard (lane a) is lambda ladder with fragments of 49, 97, 146, 194 and 243 kb from the bottom of the gel.

buffer, 1 mM dithiothreitol, 50  $\mu\text{g/ml}$  skim milk powder to a final volume of 200  $\mu\text{l}$  (including insert portion). Digestion was in fresh solution containing 50U of restriction enzyme (*Dra* I, *Xba* I or *Hind* III), incubated for 20 h at 37 °C and terminated by incubation in 0.5 M-EDTA (pH 8.0) containing 1% sodium lauroyl sarkosinate at 50 °C for 2 h.

Samples were electrophoresed in 1% agarose in 0.5  $\times$  Tris-borate -EDTA buffer and run in a CHEF DR-II system at 14 °C at 200 V. The pulse times were ramped from 3–12 sec for *Xba* I for 20 h, for *Hind* III 2–5 seconds for 20 h and for *Dra* I 5–15 sec for 12 h then 60–70 sec for 6 h. Gels were stained with ethidium bromide and photographed using UV transillumination. Size standards were polymerized bacteriophage lambda DNA or *Saccharomyces cerevisiae* chromosomal DNA. Results were compared visually.

For Southern blotting a modification of the method of van Embden and colleagues (24) was used. A portion of insert was digested with *Pvu* II by the method above and then loaded into a conventional gel and run until smallest size band of the digoxigenin labelled lambda/*Hind* III marker had migrated 6.6 cm. The gel was then transferred onto a nylon membrane and probed using a digoxigenin labelled probe. Detection was with the chemiluminescent digoxigenin detection system and photographic film.

## RESULTS

*Xba* I digests showed very little variation. One hundred strains had an indistinguishable banding pattern including strain H37Ra and the 12 *M. bovis* (pattern II in Fig. 1) The BCG Glaxo and two clinical isolates of BCG produced a similar but distinct pattern (Pattern I in Fig. 1). Four isolates of *M. tuberculosis*

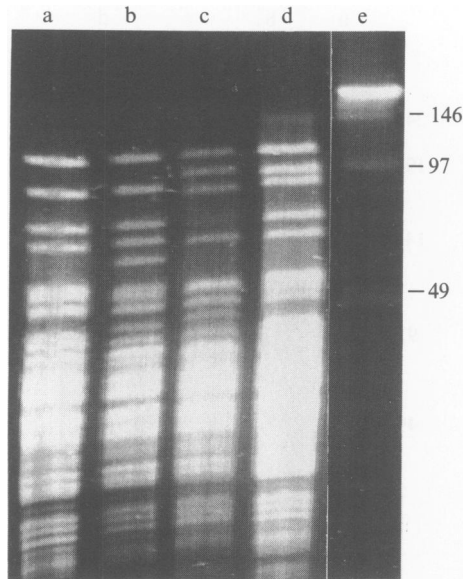


Fig. 2. *Hind* III digest patterns. Patterns a (lane a), b (lane b), c (lane c) and d (lane d). Size standard (lane e) lambda ladder-sizes of fragments 49, 97 and 146 kb from the bottom of the gel.

Table 1. *Combinations of Hind III and Xba I digest patterns of isolates*

|                        | <i>Hind</i> III pattern |    |   |   |
|------------------------|-------------------------|----|---|---|
|                        | a                       | b  | c | d |
| <i>Xba</i> I pattern I | 3                       | 0  | 0 | 0 |
| II                     | 80                      | 17 | 3 | 0 |
| III                    | 8                       | 0  | 1 | 1 |

Patterns I-III and a-d are illustrated in Figures 1 and 2 respectively. The numbers are the number of isolates with that particular combination of digest patterns.

Seven *Hind* III pattern a isolates failed to digest with *Xba* I and one isolate failed to digest with either enzyme.

and six BCG strains- Brazil, Copenhagen, Dakar, Pasteur, USSR and a clinical isolate of BCG gave another similar pattern (Pattern III in Fig. 1). Eight strains failed to produce a banding pattern on electrophoresis.

*Hind* III digestion gave four very similar patterns. 98 strains produced pattern a. This group contained strain H37Ra, the 12 *M. bovis* and all nine isolates of BCG, 15 isolates pattern b, 3 pattern c and one pattern d (see Fig. 2). One strain failed to produce a banding pattern.

The *Xba* I and *Hind* III results are combined in Table 1. With *Dra* I 75 strains (including the six BCG vaccine strains) on electrophoresis gave smears of DNA or no digestion at all. Thirty-one strains of *M. tuberculosis* gave patterns of poor quality but with every strain apparently different (data not shown). Eleven strains of *M. bovis* produced banding patterns which were indistinguishable. The twelfth isolate of *M. bovis* differed by one band from this pattern. The three clinical isolates of the BCG were indistinguishable but the pattern was different from those of *M. bovis*.

By *IS6110* Southern blotting 26 of the strains of *M. tuberculosis* produced a visible banding pattern. Each of these strains had a unique *IS6110* fingerprint pattern.

#### DISCUSSION

Since our work began PFGE methods have also been developed for *Mycobacterium fortuitum* [30], *M. tuberculosis* [29–32], *M. avium* [33–35], *M. chelonae* and *M. abscessus* [36].

Digestion of genomic DNA by *Hind* III and *Xba* I gave indistinguishable patterns for most strains. Calculation of the similarity indices by Dice analysis [37] for the most diverse strains gives indices of > 97% when compared with the main group of strains. A useful comparison can be made with *Mycobacterium avium*, a slow-growing environmental mycobacterium which appears to be as genetically diverse as *Escherichia coli* [35]. *M. avium* isolates with differences of this order of magnitude when analysed by PFGE were not be considered to be genotypically distinct by Von Reyn and colleagues [38].

The results of *Dra* I digestion for *M. tuberculosis* differed considerably from the results with other enzymes. A *Dra* I restriction site (TTTAAA) occurs within the insertion sequence *IS6110* at 328 nt. from the 5' end [39], and since this insertion sequence is transposable with a varied number of copies and sites in the genome of different strains it is likely that the differences in the banding patterns with *Dra* I are due to variations in the number and position of copies of *IS6110*. The lower level of diversity found among *M. bovis* and the BCG strains probably reflects the lower copy number and less positional variation of the insertion sequence found in these species [28, 40]. There was no correlation between success with *Dra* I PFGE and the results of the Southern blotting. The high failure rate with Southern blotting in our hands probably reflects the fact that PFGE DNA preparations contain less DNA than those made by using the conventional protocol for *IS6110* typing.

We are unable to distinguish *M. bovis* isolates from those of *M. tuberculosis*. These results provide further evidence in support of the view that *M. bovis* is not a distinct species: DNA hybridization studies have shown 100% homology between *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* [41, 42]. Conventional DNA restriction endonuclease analysis showed almost complete similarity between the species [18, 19, 21, 22]. A study looking for variation in the 16S-to-23S rDNA internal transcribed spacer demonstrated none between the species of the complex [43]. Isolates that showed phenotypic characteristics intermediate between *M. tuberculosis* and *M. bovis* have been described [5] and analysis of a large series of phenotypic characteristics showed 92% similarity between *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* [44]. Under the taxonomic rules *M. bovis* should be reduced to a biovar of *M. tuberculosis* rather than be classified as a discrete species [3].

Our results differ from those achieved by Zhang and colleagues using similar PFGE techniques [29] on a smaller collection of American isolates. Our success rate with *Dra* I PFGE was much lower than theirs and the greater diversity found by Zhang and colleagues with *Xba* I digestion- every unrelated strain had a unique pattern, was not found by us. Examination of their published results suggests that

the genetic diversity of their strains was limited. The slightly greater genetic diversity in American isolates probably reflects differences in the human population. The other two studies using PFGE in the *M. tuberculosis* complex were largely concerned with characterising *M. bovis* BCG [31, 32]. Varnerot and colleagues [31] were able to differentiate the virulent and avirulent strains of *M. tuberculosis* H37R and to distinguish between the Pasteur, Glaxo, Russian and Japanese BCG strains using *Dra* I digestion. Kristjansson and colleagues [32] were able to discriminate *M. bovis* from *M. bovis* BCG by *Asn* I digestion. We were unable to repeat the work of Varnerot and colleagues but it was possible to distinguish the Glaxo vaccine strain which is used in the UK from the others by its unique *Xba* I pattern. Two of three Scottish clinical isolates from vaccine ulcers shared this pattern. Whether the third isolate represents strain divergence within the patient or vaccination with an alternative strain is not discernible from the records.

Several hypotheses have been put forward by Frothingham and colleagues [43] for the lack of diversity found in the *M. tuberculosis* complex. First it is suggested that the complex may be of recent origin; second that the latent state of the disease may slow the rate of evolution; third the organisms are obligate parasites which may restrict population numbers; fourth their intracellular existence limits their exposure to other organisms and thus chances for recombination; and fifth that the constant host environment provides little selection pressure for divergence.

The poor discrimination found with other typing methods is most readily explained by the intrinsic lack of genetic diversity in genomic sequences of the *M. tuberculosis* complex. This was the case even when the sensitive technique of PFGE was used to characterize isolates from widely separated geographical regions. We have presented evidence that the genomic diversity that is observed with *Dra* I is a consequence of a *Dra* I restriction site in *IS6110*. Others have demonstrated that this element transposes in the genome [45] and therefore the techniques of *Dra* I PFGE fingerprinting and *IS6110* Southern hybridisation of genomic restriction fragments are both identifying the same genetic polymorphism-*IS6110* transposition. This transposition occurs in a genetically very stable genomic background.

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