EPIDEMIOLOGICAL TYPING

Genetic differentiation of Australian isolates of *Mycobacterium tuberculosis* by pulsed-field gel electrophoresis

M. M. FEIZABADI, I. D. ROBERTSON, R. EDWARDS*, D. V. COUSINS† and D. J. HAMPSON

School of Veterinary Studies, Murdoch University, Murdoch, Western Australia 6150, *Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital, Melbourne 3078, Australia and †Australian Reference Laboratory for Bovine Tuberculosis, Department of Agriculture, South Perth, Western Australia 6151, Australia

As part of an epidemiological study of tuberculosis in Australia, 84 isolates of *Mycobacterium tuberculosis* from patients were analysed by pulsed-field gel electrophoresis (PFGE). The isolates were genetically heterogeneous, with 66 different DNA banding patterns obtained following digestion of genomic DNA with *Dra*I and 53 patterns with *Xba*I. When the results were compared with those previously obtained in restriction fragment length polymorphism analysis (RFLP), in 87% of cases the results with *Dra*I were consistent with those obtained with insertion sequence IS6110 as a probe in RFLP. However, PFGE was able to differentiate four of eight isolates which were identical with IS6110 typing. The high polymorphism amongst strains and the high average age of the patients (51 years) suggested that most organisms were cultured from patients who had reactivation of existing infections. Isolates with identical DNA patterns were found in different states of Australia, but no one strain predominated in any area. This suggests that tuberculosis has been introduced into Australia from various sources.

Introduction

Tuberculosis is re-emerging as a major global problem [1]. In Australia the rate of new cases is 5.62 per 100 000 population per year [2], with 70% of patients being born outside the country [3, 4]. The median age of Australian patients is 50 years, suggesting that most cases represent reactivation of infection [5]. Risk factors in Australia include migration from countries with a high prevalence of the disease, and homelessness [5]. Surprisingly, unlike the situation in other countries, concurrent HIV infection does not appear to be an important risk factor in Australia [6, 7].

When studying the epidemiology of tuberculosis it is important to be able to differentiate between different strains of *Mycobacterium tuberculosis* [8]. Bacteriophage typing has been a useful strain marker in several investigations [9–11], but it requires a high level of technical expertise, and is time-consuming and labour-intensive [11, 12]. The use of DNA typing techniques has revolutionised the investigation of outbreaks [13]. Restriction endonuclease analysis of genomic DNA has been used in several epidemiological studies [14, 15], but it produces large numbers

of sometimes overlapping DNA fragments and this causes difficulty in analysis [8]. Distinct DNA bands are produced by restriction fragment length polymorphism analysis of DNA (RFLP), and these are much easier to read and interpret. The RFLP method, with a DNA probe directed against the insertion sequence IS6110 has been used in several epidemiological studies in various countries, including Australia [5, 16]. However, some isolates lack IS6110 and, therefore, cannot be differentiated with this probe [17]. Pulsed-field gel electrophoresis (PFGE) can be used to type a broader array of bacterial species, including mycobacteria, and comparative studies have indicated that isolates that are indistinguishable by PFGE are unlikely to demonstrate substantial differences by other typing techniques [18–20].

The current study is the first to use PFGE to investigate diversity among Australian isolates of *M. tuberculosis*. The technique has been used previously to study the population genetics of the *M. tuberculosis* complex, and for differentiation of isolates from the USA and the UK [21, 22]. The Australian isolates of *M. tuberculosis* examined in this study were characterised previously by RFLP with pTBN12 and IS6110 as probes [17, 23, and Edwards R, unpublished data], and in the current study these results were compared with those obtained by PFGE.

Materials and methods

Bacterial collection and culture

Eighty-four isolates of *M. tuberculosis* were examined; 60 isolates were obtained from the Mycobacterium Reference Laboratory, Fairfield Hospital, Victoria and 24 were received from the State Health Laboratory in Western Australia. The isolates were from patients who were born in Australia ($n = 15$), born in other countries ($n = 38$), or information on the place of birth was not available ($n = 31$). The patients' ages ranged from 17 to 87 years, with an average of 51 years. For comparison, two isolates of *M. bovis* BCG and one isolate of *M. africanum*, which have been analysed previously [24], were examined.

All isolates were identified as *M. tuberculosis* by standard procedures including positive nitrate reduction, niacin production and pyrazinamidase production, or resistance to thiophen-2-carboxylic acid hydrazide 5 µg/ml, or both. All isolates had been analysed previously by RFLP with the pTBN12 probe, and isolates received from Victoria had been analysed with the IS6110 probe [17, 23, and Edwards R, unpublished data].

Isolates were subcultured on slopes of Stonebrinks medium for 2–3 weeks and single fresh colonies were cultured on five slopes of E8 medium [25]. Cultures were incubated for 2 weeks at 37°C, and cells were harvested by centrifugation at 2500 *g* at room temperature for 20 min, washed twice in phosphate-buffered saline (PBS) and then stored overnight at –20°C.

The cells were suspended in 1 ml of PBS, killed by heating at 80°C for 30 min and washed twice in 50 mM EDTA. DNA was extracted as described previously [24]. Briefly, 50 µl of the cells were suspended in 250 µl of prelysing solution – 6 ml of 50 mM EDTA, 6 ml of 10 mM Tris, 0.1 M sodium citrate, 150 µl of β-mercaptoethanol and 760 units of crude lyticase (Sigma). The cell suspension was mixed with an equal volume of low melting temperature agarose (BioRad Laboratories, California) 1.4% prepared in 125 mM EDTA (pH 8) and cooled at 50°C. This mixture was poured into plug moulds. The agarose plugs were kept at 4°C for 30 min and then submerged in 2 ml of a solution containing 0.5 M EDTA plus β-mercaptoethanol 7.5%, and incubated for 24 h at 37°C in a water bath. Agarose plugs were washed four times with TE buffer (10 mM Tris, 1 mM EDTA, pH 8) for 10 min each and incubated for 5 h in 3 ml of TE buffer containing lysozyme 1 mg/ml. The solution was changed to 0.5 M EDTA containing proteinase K (Boehringer GmbH) 2 mg/ml, sodium lauryl sarcosine (Sigma) 1%, and incubated at 55°C for 48 h. The plugs then were washed with TE, three times for 30 min each, at room temperature, and incubated at 55°C in TE plus phenylmethylsulphonyl fluoride (Sigma)

0.04 mg/ml, to inactivate the proteinase K. They were washed three times with TE, stored in 0.5 M EDTA at 4°C and then washed extensively with TE before being subjected to restriction endonuclease digestion.

Restriction endonuclease digestion

The agarose plugs were cut by scalpel to fit the size of the combs of the gel casting (4 × 3 mm). They were washed in restriction buffer at 4°C for 30 min and digested for 24 h with 25 units of *Dra*I, *Xba*I or *Spe*I (Boehringer GmbH) in the appropriate restriction buffer recommended by the supplier.

PFGE

Plugs containing digested DNA were loaded into an agarose 1% gel, prepared and run in 0.5 × TBE buffer (1 × TBE contains 0.025 M Tris, 0.5 mM EDTA and 0.025 M boric acid). PFGE was carried out with a contour-clamped homogenous electric field-DR II system (BioRad) at 14°C for 24 h at 180 V and 16 A. Following *Dra*I digestion, pulse time was ramped from 5 to 15 s for 16 h and then from 60 to 70 s for 8 h. Following *Xba*I and *Spe*I digestion it was ramped from 5 to 15 s for 16 h, and then from 1 to 20 s for 8 h. Gels were stained with ethidium bromide 0.5 µg/ml for 30 min and photographed with polaroid film. Bacteriophage lambda DNA (BioRad) was used as a marker. The patterns obtained in PFGE analysis were labelled D1-D66 and X1-X53, following digestion with *Dra*I and *Xba*I respectively.

Results

The PFGE patterns obtained, and comparison with results of RFLP with pTBN12 and IS6110 probes are summarised in Table 1. The 84 isolates were divided into 66 patterns with *Dra*I, with 14–22 DNA bands present. One pattern was possessed by nine isolates (all from Vietnamese immigrants), two patterns were each possessed by three isolates, seven patterns were each possessed by two isolates, and all other isolates had distinct patterns. Some isolates with the same pattern were isolated from patients in different Australian states, but no one strain predominated. Most isolates cultured from patients born in Australia and Northern Europe produced more DNA bands (19–22) than isolates cultured from patients born in Asian countries such as the Philippines and Vietnam (14–16). However, a high degree of polymorphism was found amongst isolates of both groups.

Fewer patterns were obtained by *Dra*I (66) than were obtained by RFLP with IS6110 (71). Despite the higher discriminatory power of RFLP, PFGE with *Dra*I differentiated four isolates which were identical with the IS6110 probe. Eleven isolates were not differentiated by *Dra*I but were differentiated by the

Table 1. Origin and number of DNA patterns amongst isolates of *M. tuberculosis* obtained by PFGE with *Dra*I and *Xba*I, and RFLP analysis with IS6110 and pTBN12 probes

Country of birth	Number of isolates	Number of patterns obtained with			
		<i>Dra</i> I PFGE	<i>Xba</i> I PFGE	IS6110 RFLP	pTBN12 RFLP
Australia	15	12	11	13	9
Burma	3	2	2	2	2
China	2	2	2	2	2
India	2	2	2	2	2
Indonesia	4	3	2	4	2
Philippines	6	5	4	6	4
Vietnam*	12	4	4	5	4
Others†	9	7	6	7	7
Unknown	31	30	25	31	25
All countries	84	66	51	71	40

*A total of six patterns with PFGE.

†Including isolates cultured from patients from nine countries.

IS6110 probe. All remaining isolates were differentiated by *Dra*I in a similar order to that obtained with IS6110 (Fig. 1).

Differences between the number of patterns obtained by *Dra*I in the PFGE analysis and RFLP with pTBN12 were apparent (66 *versus* 42 patterns respectively). Two isolates with the same pattern by pTBN12 had differences in 12 bands when analysed by *Dra*I.

Digestion of all isolates with *Xba*I resulted in 51 patterns, and the number of fragments obtained did not differ greatly amongst the isolates. More patterns were obtained with *Xba*I than in RFLP with pTBN12. All but nine of the isolates with the same *Xba*I pattern also were identical with the pTBN12 probe. Two isolates with different *Dra*I patterns were identical by *Xba*I. In 79% of cases, each *Xba*I pattern contained isolates which were identical by RFLP with pTBN12 (Fig. 2).

Fig. 1. DNA polymorphism amongst *M. tuberculosis* isolates demonstrated by *Dra*I in PFGE. Numbers show the cluster of isolates obtained by pTBN12 in RFLP analysis. **Fig. 2.** DNA polymorphism of *M. tuberculosis* isolates demonstrated with *Xba*I in PFGE. Numbers show the cluster of isolates obtained by pTBN12 in RFLP analysis.

Ten isolates with eight patterns obtained with pTBN12 were differentiated by *Xba*I.

A single isolate of *M. africanum* was distinguished from the isolates of *M. tuberculosis* by each of the methods employed. Isolates that were cultured on more than one occasion from three patients were shown to be identical. Nine isolates cultured from Vietnamese patients were identical by *Dra*I but one was differentiated by *Xba*I and *Spe*I. These were shown previously to contain either only a few copies of IS6110 or to lack this repetitive element [17], and were identical to both strains of *M. bovis* BCG by *Dra*I. However, *M. bovis* BCG could be differentiated from the Vietnamese isolates with *Spe*I and *Xba*I (Figs. 2 and 3).

Fifteen isolates cultured from patients who were born in Australia were diverse, belonging to 12 patterns after digestion with *Dra*I, and 11 after digestion with *Xba*I. In contrast, isolates cultured from certain ethnic groups born overseas, (e.g., in Vietnam), were more closely related.

Discussion

All isolates produced clear DNA bands with high resolution and the patterns were easy to read. In a previous study, Olson *et al.* [22] reported that 75% of the isolates they examined could not be digested with *Dra*I or gave smears of DNA on electrophoresis. The different results obtained in this study may be attributed to the use of different methods of DNA extraction.

Fig. 3. DNA polymorphism amongst *M. tuberculosis* isolates cultured from Vietnamese patients and an isolate of *M. bovis* BCG observed with *Spe*I in PFGE. Lanes 1 and 5, isolates that lack IS6110; 2 and 4, isolates which contain a single copy of IS6110; 3, *M. bovis* BCG.

PFGE with *Dra*I was more discriminatory for strain typing than was RFLP with the pTBN12 probe. Digestion with *Xba*I identified less polymorphism than with *Dra*I. This enzyme has been used previously to differentiate isolates of the *M. tuberculosis* complex from Scotland [22], but little polymorphism was reported. In contrast, Zhang *et al.* [21], using *Xba*I in PFGE analysis of isolates from the USA, found greater strain diversity. This was interpreted as arising from the more diverse origin of the human population, possibly due to migration, compared to Scotland [22]. In the current study, *Xba*I was shown to distinguish between unrelated strains cultured from ethnically different populations.

The polymorphism previously observed in strains of *M. tuberculosis* with *Dra*I in PFGE was attributed to the presence of and high copy numbers of IS6110 in the genome of different strains [22]. While the number of patterns obtained by *Xba*I digestion was close to that obtained by RFLP with the pTBN12 probe (49 versus 40), no clear relationship was found between the DNA patterns obtained with this endonuclease and RFLP with the IS6110 probe. This suggests that the combination of *Dra*I and *Xba*I can be useful for strain differentiation of *M. tuberculosis* isolates by PFGE, and that this method may be used as a substitute for RFLP analysis.

This study demonstrated considerable strain diversity amongst *M. tuberculosis* isolates from Australian patients. Although a few strains were present in patients in different states, no one strain predominated. This strain diversity was greater than that found amongst Australian isolates of the primary bovine pathogen *M. bovis* [24]. Interestingly, nine of the isolates from Vietnamese immigrants appeared to be closely related to *M. bovis* BCG.

Most cases of tuberculosis in Australia occur in patients > 50 years of age who are born overseas, suggesting that the condition arises from reactivation of a prior infection acquired outside Australia [5]. This belief is supported by the current findings. Where infections are mainly newly acquired as a result of active disease transmission, limited strain polymorphism would be anticipated whereas the reverse is true if disease results from reactivated latent infection [8]. As the isolates in this study were very diverse, most infections were likely to be reactivated infections in carriers who have migrated to Australia. Australian and European-born patients > 50 years of age yielded isolates which produced a large number of DNA bands with *Dra*I digestion which is a feature of European isolates [25]. Isolates with fewer *Dra*I bands came from patients of all age groups born in countries such as the Philippines and Vietnam, suggesting that the source of infection was the country of origin. Some preliminary evidence for transmission of infection between both groups of

patients was found, as a few patients from both groups yielded isolates which were common in another group.

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