JOURNAL OF CLINICAL MICROBIOLOGY, May 1999, p. 1265–1268 Vol. 37, No. 5
0095-1137/99/$04.00+0

Molecular Epidemiology of Tuberculosis in Malaysia

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Received 20 October 1998/Returned for modification 17 December 1998/Accepted 25 January 1999

Molecular typing with IS*6110* was applied to *Mycobacterium tuberculosis* isolates from all parts of Malaysia. The degree of clustering increased with patient age, suggesting that reactivation may contribute to clustering. Identical banding patterns were also obtained for isolates from widely separate regions. Therefore, the use of clustering as a measure of recent transmission must be treated with caution. Strains related to the Beijing family were common in Peninsular Malaysia but were less common in Sabah and Sarawak, while a distinct group of strains comprised nearly 40% of isolates from East Malaysia but such strains were rare in Peninsular Malaysia. Single-copy strains, common in South and Southeastern Asia, constituted nearly 20% of isolates from the peninsula but were virtually absent in East Malaysia. The marked geographical difference in the prevailing strains indicates not only a restricted dissemination of *M. tuberculosis* but also a considerable degree of stability in the banding patterns.

Probes based on the insertion sequence IS*6110* (IS*986*) (13, 18, 26) generate extensive restriction fragment length polymorphisms (RFLPs) with isolates of *Mycobacterium tuberculosis* (10, 17) and have therefore been used extensively for the identification of clusters of cases that are presumptively linked epidemiologically on the basis of identical fingerprint patterns. Population-based studies have been used to identify trends in transmission frequencies and risk factors associated with active transmission, using the assumption that clusters of identical fingerprints are a measure of recent, active transmission (1, 16, 20, 23). Most of these studies have been carried out in countries with a low incidence of tuberculosis. One purpose of the research reported in this paper was to test these assumptions by relating the similarities of fingerprint patterns to selected demographic data in Malaysia, where the incidence of the disease is higher (58 per 100,000 in 1995).

The use of IS*6110* fingerprinting relies on the pattern being sufficiently stable to give identical fingerprints for related isolates but, at the same time, sufficiently variable to show differences when the isolates are unlinked. The extent of the difference between two strains is therefore a function not only of the evolutionary or epidemiological distance between them but also of the rate of transposition of IS*6110*. Much of the discussion assumes, implicitly, that this rate is constant, despite evidence to the contrary (24). In particular, it is widely recognized that strains with a low number of copies (fewer than five) show little polymorphism, and identical patterns are commonly found for strains from apparently unconnected patients. Such low-copy-number strains, apart from those showing a single copy only, which appear to be much more frequent in patients from South and Southeast Asia, are, in general, widely distributed. This paper discusses the distribution of these single-copy strains of *M. tuberculosis* in Malaysia.

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MATERIALS AND METHODS

Bacterial isolates. Random samples of *M. tuberculosis* complex isolates from all parts of Malaysia, with associated demographic and other data, were provided on a monthly basis throughout 1993 and 1994 by the National Tuberculosis Centre (now the Institute for Respiratory Medicine), Kuala Lumpur, Malaysia. Isolates were not further differentiated, but spoligotyping of a sample of isolates with a single copy of IS*6110* showed patterns typical of those of *M. tuberculosis* rather than those of *Mycobacterium bovis* (see below). After eliminating repeat isolates from the same patient and isolates showing no bands or unreadable fingerprints, 439 fingerprints were available for analysis. This represents approximately 3% of the bacteriologically confirmed cases for this period.

DNA fingerprinting and spoligotyping. Southern blotting with an IS*6110*-derived probe was done by the standard fingerprinting method (21) with *M. tuberculosis* Mt14323 as an external standard for normalization. Spoligotyping (spacer oligonucleotide typing) of the first 28 single-copy isolates was performed as described by Kamerbeek et al. (12).

Computer analysis and statistics. IS*6110* fingerprints were analyzed with GelCompar, version 4 (Applied Maths, Kortrijk, Belgium), normalizing the gels with respect to the external marker tracks of Mt14323. Banding patterns were compared by unweighted pair group analysis of the Dice coefficients. Clusters were defined as collections of isolates with greater than 95% similarity, and groups were defined as collections of isolates with greater than 80% similarity; in each case these were subject to visual inspection. Spoligotyping results were also analyzed with GelCompar. Statistical analyses were carried out with SPSS for Windows.

RESULTS AND DISCUSSION

After eliminating repeat isolates from the same patient and isolates showing no bands or unreadable fingerprints, 439 patterns were available for further analysis (Table 1). Of these, 77 (17.5%) had a single copy; all of the single-copy strains fell into one of three clusters (Table 2). It has been shown in many other studies that strains with a single copy of IS*6110* can readily be subdivided by spoligotyping. We tested 28 single-copy strains (the complete set of such strains available at the time); Table 2 shows that they fell into 17 different spoligotypes, one of which contained seven isolates, while the others consisted of one to three strains. None of the spoligotypes was differentiated by IS*6110* typing. These results are similar to those described by Kamerbeek et al. (12).

There were 31 further isolates with a low number (two to four) of copies of IS*6110* (Table 1) and 331 isolates with more than four copies. In contrast to the widely held belief that IS*6110* fingerprinting shows poor discrimination with low-copy-number isolates, the degree of clustering was not signif-

TABLE 1. Clustering and IS*6110* copy number of *M. tuberculosis* isolates from Malaysia

IS*6110* copy no.	No. of isolates[a]	Percentage of total isolates	No. of clustered isolates	% Clustering[b]	No. of clusters	Maximum cluster size (no. of isolates)
1	77	17.5	77	100.0	3	63
2–4	31	7.1	4	12.9	2	2
>4	331	75.4	36	10.9	17	3
Total	439	100	117	26.7	22	

[a] Since repeat isolates from individual patients have been eliminated, the number of isolates corresponds to the number of patients.

[b] Percent clustering is the number of isolates forming part of a cluster, for each copy number, as a percentage of the number of isolates with that copy number.

icantly higher for those patterns with two to four copies (13% clustering) than for those with more than four copies (11% clustering). For subsequent analyses the data were therefore used to divide the isolates into two groups: single-copy strains and isolates with more than one copy. The relatively low degree of clustering and the small sizes of the clusters (other than those containing the single-copy strains) are probably due to the low percentage of patients represented in the sample and should not be taken as a measure of the absolute level of clustering in Malaysia. Nevertheless, assuming that there is no bias in the sampling, it is valid to examine the factors that are associated with clustering.

First, it is a common working hypothesis that the degree of clustering can be used as a measure of the level of recent transmission and that reactivation of previous infections will be more likely to lead to a diverse set of RFLPs. Second, it is to be expected that older patients will exhibit a higher ratio of reactivation to recent infection than younger subjects, simply due to the fact that the cumulative effects of the longer period of possible exposure make it more likely that older patients have been infected at some time in the past. Furthermore, if the strains in circulation change over time, then the older age groups will have been exposed to a wider variety of strains. Combining these hypotheses leads to the prediction that the degree of clustering would tend to diminish with age. However, we found that the converse was true: the median age for the patients from whom the clustered strains were isolated was 49 years, whereas for the patients from whom the nonclustered strains were isolated the median age was 42 years ($P = 0.04$). The significance in the difference of the median values remained when single-copy isolates were excluded and also when only isolates with more than four copies were analyzed. It is unlikely that the picture is distorted by specific outbreaks, since (apart from the single-copy isolates) no cluster was represented by more than three strains in the sample studied.

Of the other possible confounding factors, one likely candidate was the ethnic group; the median age for the Chinese patients (53 years) was significantly higher than that for all other ethnic groups combined (40 years) ($P < 0.0001$), possibly due to social differences between the ethnic groups. However, the degree of clustering among strains from Chinese patients (10.1%, excluding single-copy strains) was not significantly different from that for the whole sample (11.0%), suggesting that the different age distributions of patients between the ethnic groups does not account for the apparent increase in clustering with age. A definitive answer to this question would require a much larger sample. Nevertheless, the data presented here suggest that the hypotheses outlined above need to be reexamined, and in particular, the possibility that strains with stable fingerprint patterns may be in circulation for some time, resulting in apparent clusters of cases due to reactivation rather than recent transmission, must be considered.

The limited sampling of isolates and the nature of the epidemiological data available made it impossible to attempt to trace routes of infection at a microepidemiological scale. However, analysis of the regional distribution of the identified clusters showed, surprisingly, that in only 7 clusters (of 19 clusters, excluding the clusters of single-copy isolates) were the isolates from the same state, with isolates in 2 other clusters originating from adjacent states. The isolates in the remaining 10 clusters were from nonadjacent states, including in three clusters strains obtained from both Peninsular Malaysia and East Malaysia (Fig. 1), suggesting that it is unlikely that there is a direct epidemiological connection between the patients concerned; it would be surprising for migration to account for such a high proportion of the observed clusters. In some cases, the similarity of the fingerprints may be apparent rather than real, but it seems unlikely that this could explain all of these apparently unconnected clusters. However, it is known that IS*6110* does have preferred sites of integration, and studies of banding patterns have shown that the distribution of band positions is not uniform (5, 9, 14). Convergence of banding patterns is therefore a possibility; i.e., identical patterns could arise independently for isolates with different origins. However, it should be noted that isolates in 6 of these 10 clusters exhibited more than 10 bands. Consideration of such data also must take account of the possibility of conservation of strains; i.e., a widespread fingerprint type, will, if it is sufficiently stable, give rise to "clusters" of fingerprints that are epidemiologically unrelated. Whatever the explanation, if we accept the fact that the geographical separation of these clusters makes an epidemiological connection unlikely, then this also leads to the conclusion that the use of clustering as a measure of recent transmission becomes unreliable. Further evidence to this effect comes from a study, performed in the United States (23), which detected identical fingerprint patterns for isolates from different states with no evident epidemiological connection.

The use of a lower degree of matching of fingerprint patterns, in which the similarity is set at 80% to define groups rather than clusters, enables the examination of potential longer-range relationships. In this way, 41 groups were identified, and these comprised some 75% of the isolates. Most of these groups contained only a few isolates; Table 3 lists all groups with 10 or more members and the distribution of these groups between East Malaysia (Sabah and Sarawak) and the states of Peninsular Malaysia. Apart from the single-copy isolates,

TABLE 2. Spoligotyping of *M. tuberculosis* isolates from Malaysia with a single copy of IS*6110*

IS*6110* typing		Spoligotyping[a]		
Cluster name[b]	No. of isolates in cluster	No. of isolates spoligotyped[c]	No. of spoligotypes	Maximum no. of isolates in one type
A	3	0		
C	11	5	4	2
T	63	23	13	7
Total	77	28	17	7

[a] All the spoligotypes were typical of *M. tuberculosis* rather than *M. bovis*.

[b] The single-copy strains formed three clusters, depending on the position of the single band on the Southern blots.

[c] Spoligotyping was performed at an intermediate stage in the project. The sample tested represents the total number of single-copy isolates at the time.

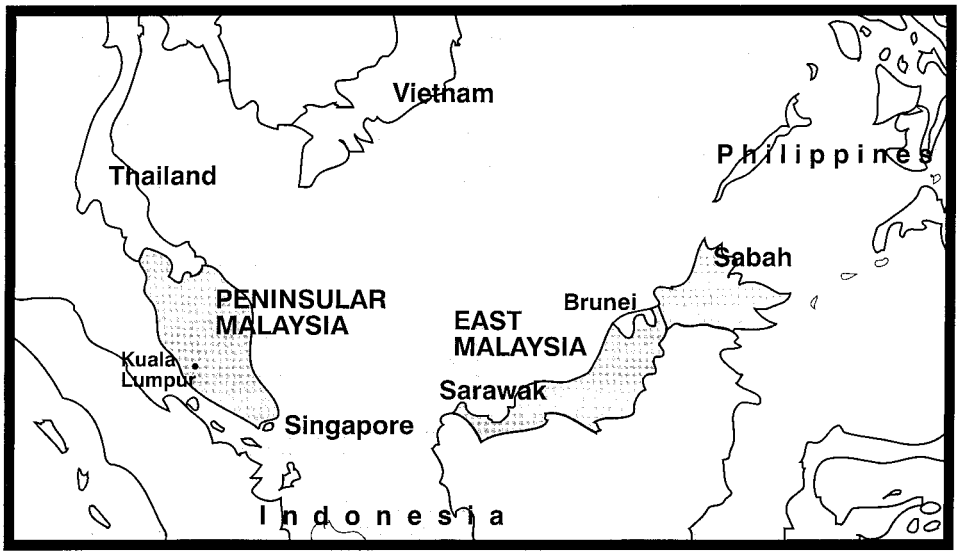

FIG. 1. Map of Malaysia and neighboring countries.

which are considered separately below, two of these groups were of particular interest. Group 1 is similar to the Beijing family of strains that have been shown to be predominant in some countries of East Asia including China, Mongolia, Korea, and Thailand (15, 22); these strains were significantly less frequent in Sabah and Sarawak (10.2% of all isolates) than in Peninsular Malaysia (19.2% of isolates). After excluding single-copy isolates, these figures become 11 and 24%, respectively (by chi-square analysis, $P = 0.04$). A converse distribution is exhibited by the group 4 strains, which were almost entirely confined to East Malaysia, forming 39% of isolates from Sabah and Sarawak, as opposed to 1.6% in Peninsular Malaysia (if single-copy isolates are eliminated, these figures become nearly 40% and less than 3%, respectively). These results not only indicate an epidemiological distinction between these geographically separated regions of Malaysia (further supported by the analysis of single-copy isolates; see below) but also indicate the potential benefit of analysis of lower degrees of similarity between isolates in developing an understanding of the evolution of the organism.

The high proportion of single-copy isolates in this study provided an opportunity to examine the distribution of these strains. Analysis by age group showed that the proportion of isolates with a single copy increased from 12% in patients under 20 years old to 25% in individuals aged 70 years or older. This could suggest either that these strains were more common in the past than they are now or that for other reasons there is a higher ratio of reactivation to recent transmission with single-copy strains. However, although the median age for patients from whom single-copy isolates were isolated (49 years) was greater than that for patients from whom isolates with two or more copies were isolated (43 years), the statistical significance of the difference was low ($P = 0.43$).

The proportion of single-copy isolates varied quite markedly between states of the Malaysian Federation, although the numbers were too small to test statistical significance at the level of individual states. However, the two East Malaysian states (Sabah and Sarawak) combined had a lower percentage (4%) of single-copy isolates (Table 4) than the states of Peninsular Malaysia when the results for the Peninsular Malaysia states are pooled (19.5%; by chi-square analysis, $P = 0.009$). Thus, Peninsular Malaysia is similar in this respect to neighboring

TABLE 3. Geographical distribution of major groups of *M. tuberculosis* isolates from Malaysia

Group[a]	East Malaysia		Peninsular Malaysia		Missing data[b] (no. of isolates)	Total	
	No. of isolates	% of isolates[c]	No. of isolates	% of isolates[c]		No. of isolates	% of isolates[c]
1	5	10.2	73	19.2	5	83	18.9
3	4	8.2	20	5.3		24	5.5
4	19	38.8	6	1.6	2	27	6.2
6[d]	2	4.1	60	15.8	1	63	14.4
8[d]			11	2.9		11	2.5
14	1	2.0	9	2.4		10	2.3
17			10	2.6		10	2.3
25			11	2.9		11	2.5
Other groups[e]	11	22.4	81	21.3		92	21.0
Not grouped	7	14.3	99	26.1	2	108	24.6
Total	49	100.0	380	100.0	10	439	100.0

[a] Groups of strains show 80% similarity in their IS*6110* RFLP patterns. Groups with 10 or more members are identified by number.
[b] Isolates for which the geographical origin was not available.
[c] Percentage of the total isolates from that region belonging to that group.
[d] Isolates containing a single copy of IS*6110*.
[e] Thirty-three other groups with fewer than 10 members in each group.

TABLE 4. Geographical distribution of *M. tuberculosis* isolates with a single copy of IS*6110*

Geographical area	No. of isolates		% Isolates within geographical area[a]	
	Single copy	Two or more copies	Single copy	Two or more copies
East Malaysia	2	47	4.1	95.9
Peninsular Malaysia	74	306	19.5	80.5
Total	76	353	17.7	82.3

[a] Isolates with a single copy or with two or more copies of IS*6110* as a percentage of the total number of isolates from that region.

countries of Southeast Asia, in which studies in Thailand and of patients of Vietnamese origin have shown a high proportion of single-copy strains (20 and 12%, respectively) (15, 25); a similar study in Madras, South India, showed that as many as 40% of isolates had a single copy of IS*6110* (4). On the other hand, East Malaysia exhibits a low percentage of such strains (4%); low frequencies (less than 5%) of single-copy strains have been reported from many other countries including not only Europe and North America (6, 8, 23) but also China and Mongolia (22), Korea (11), Hong Kong (3), and French Polynesia (19). These results therefore bring into sharper focus the geographically restricted distribution of these distinctive strains (7).

We considered the possibility that the occurrence of these strains might be ethnically restricted, reflecting a hypothetical genetic predisposition to infection with a specific strain showing this pattern. When the analysis was restricted to Peninsular Malaysia, the occurrence of single-copy isolates among Indians and Malays was virtually identical (21.4 and 22.9%, respectively) but was lower among Chinese patients (13.4%). The difference between the Chinese patients and the combined data for Indians and Malays was statistically significant (by chi-square analysis, $P = 0.05$), but not at a level to lend much credence to a genetic predisposition hypothesis. The reasons for the restricted distribution of single-copy strains therefore remain obscure.

The high frequency of these single-copy strains, at least in some countries, indicates a high degree of stability of this pattern, presumably due to the virtual absence of transposition of the element in such strains. This is supported by a study of sequential isolates in San Francisco, Calif. (24), in which changes in banding patterns were seen only in strains with intermediate numbers (8 to 14) of bands. Since the insertion sequence itself is identical in low- and high-copy-number strains (2), the lack of transposition must be imposed by the context of the insertion sites involved. The implication that rates of variation not only may differ between isolates but also may change following rare transposition into a more active site means that it will be difficult or impossible to devise quantitative rules that relate the degree of similarity between isolates to their evolutionary or epidemiological distances.

ACKNOWLEDGMENTS

This work was supported by the Commission of the European Communities under the International Scientific Co-operation Programme (contract CI1-CT91-0905) and the BIOMED Programme (provision of GelCompar software).

We are grateful to J. D. A. van Embden for providing facilities and assistance with the spoligotyping.

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