GENOTYPING OF *MYCOBACTERIUM TUBERCULOSIS* IN NEW SOUTH WALES: RESULTS FROM 18 MONTHS OF A STATEWIDE TRIAL

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Several molecular typing methods are available to assist public health practitioners in identifying clusters of recently acquired tuberculosis cases.[1,2] Molecular typing or fingerprinting investigates variations in microbial populations, defines specific clones and identifies outbreaks by matching molecular fingerprints of epidemiologically linked isolates. The combination of two or more methods, with different preselected genomic loci in the *Mycobacterium tuberculosis* genome, have been used to identify and track outbreaks, define high-risk groups and target prevention strategies.[2,3,4] Table 1 compares three current typing methods.

In contrast to epidemiological methods, the use of genotypic methods to define clusters is controversial. Genotype-defined clusters are used to calculate the transmission index or average number of secondary cases from a single source case. These clusters appear to result from recently transmitted infection with rapid progression to clinical disease.[5] Routine genotyping has shown that transmission of tuberculosis occurs more readily than previously thought,[6] with substantial proportions (28–72 per cent) of urban cases occurring in clusters.[7,8,9] By contrast, conventional contact tracing may identify only 10 per cent of clustered cases.[7] DNA fingerprinting has demonstrated the existence and worldwide transmission of families of genetically related strains and local dissemination of successful clones.

The Centre for Infectious Diseases and Microbiology (CIDM) at Westmead has been genotyping all *M. tuberculosis* complex (including *M. tuberculosis, M. africanum, M. bovis, M. bovis* bacillus Calmette-Guerin (BCG), the rarely isolated species *M. microti*, *M. canettii,* and the newly described seal pathogen, *M. pinnipedii)* isolates from NSW since 2003. This report is a review of the results for the period December 2003 to May 2005.

METHODS

Isolates

All 420 *M. tuberculosis* complex isolates referred to the NSW Mycobacterium Reference Laboratory, CIDM, between December 2003 and May 2005, are included.

Molecular typing methods

All isolates were tested by mycobacterial interspersed repetitive units (MIRU) typing and spoligotyping, according to published methods.[10,11] Clinical isolates with matching MIRU and spoligotype numerical codes were then subjected to IS*6110* restriction fragment length polymorphism (RFLP) analysis.[12] Quality control strains of *M.tuberculosis* and *M.bovis* BCG were used to monitor the performance of the genotyping techniques.

Cluster analysis

Comparison of IS*6110* RFLP gel profiles was performed using the Bionumerics Edition 3.0 package (Applied Maths, Koutrai, Belgium) using standard methods. A cluster was

TABLE 1

COMPARISON OF CURRENT GENOTYPING METHODS USED FOR *M.TUBERCULOSIS*

Name of method	Genomic target	Method	Result format	Turnaround-time	Discriminatory power	Cost
Spoligotyping	Direct repeat region	Single PCR; dot-blot hybridisation to detect presence/ absence of 43 spacer sequences	15 digit code	Days; can be done directly on specimens	High sensitivity; low specificity	Relatively low
Mycobacterial interspersed repetitive units (MIRU) typing	12 loci (can be more or less)	Multiple PCR; amplicons size indicates number of repeat sequences at each locus	12 digit code	Days; can be done directly on specimens	Depends on number of loci targeted; 12 loci high sensitivity; moderate specificity	Medium; depends on number of loci targeted
IS6110 restriction fragment length polymorphism (RFLP) analysis	IS6110 (0-20 copies)	DNA cut with restriction enzyme; fragments separated on gel; probed for presence of IS6110	Image – number/size of fragments containing IS	Weeks (requires lots of high quality DNA)	Gold standard – high specificity. Not suitable for strains with <5 copies of IS6110	High

IS = insertion sequence; PCR = polymerase chain reaction.

Adapted from Malik and Godfrey-Faussett.[4]

defined as a group of isolates that were indistinguishable by all three methods. Laboratory cross-contamination, as a possible source of clustering, was investigated by checking the time of the processing in the laboratory and the clinical history of patients. The rate of recent transmission (RRT)[13] was calculated as:

RRT (per cent) = (No. of isolates clustered – No. of clusters)/Total isolates typed x100

RESULTS

Spoligotyping identified seven of 420 (1.7 per cent) isolates as members of *M. tuberculosis* complex other than *M. tuberculosis,* namely *Mycobacterium bovis* (2 isolates), *M.bovis* BCG (3 isolates), *M. canettii* (1 isolate), and *M.caprae* (1 isolate).

Molecular diversity

Of 413 sequential isolates of *M.tuberculosis*, 273 (66 per cent) and 176 isolates (43 per cent) were individually grouped by spoligotyping and MIRU typing, respectively, and 105 isolates (25 per cent) were clustered by both methods. Of 273 isolates grouped by spoligotyping, 71 (26 per cent) belonged to the Beijing family of *M. tuberculosis* strains. The numbers of isolates belonging to other recognised groups are shown in Figure 1.

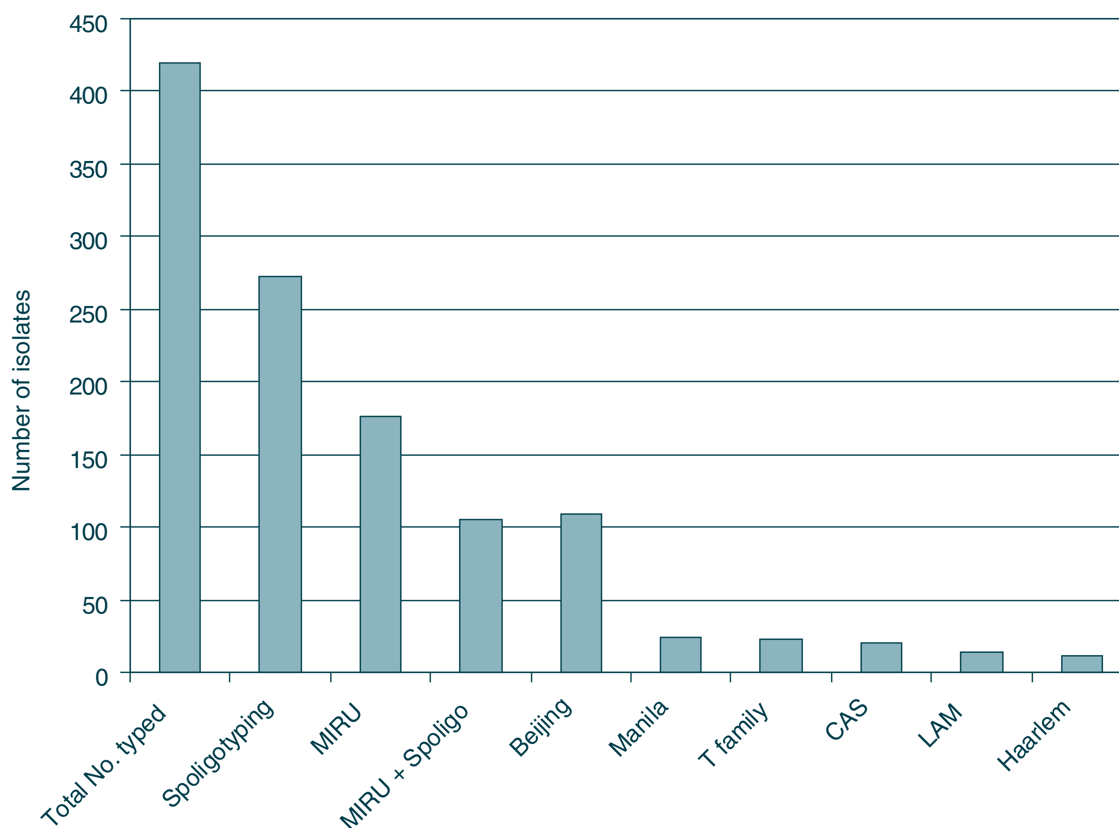
Clusters

Eight clusters, involving a total of 20 isolates (4.8 per cent), were identified, based on all three typing methods. Five clusters contained only two isolates, two contained three isolates and the other contained four isolates. Three stored isolates were later identified as belonging to cluster 1.

Cluster 1 comprised six isolates: three collected during the study period and a further three isolates collected outside the study period. They were linked, but not initially recognised as being epidemiologically related, by geographic proximity and risk factors. The index case was diagnosed and treated in 2000. A sixth case was identified by routine genotyping later in the study period. The RFLP pattern for this cluster consisted of 12 bands.

FIGURE 1

CLUSTERED ISOLATES BASED ON TWO PCR-BASED TYPING METHODS AND MAJOR *M. TUBERCULOSIS* FAMILIES IDENTIFIED BY SPOLIGOTYPING



PCR = polymerase chain reaction
MIRU = mycobacterial interspersed repetitive units
CAS = Central and Middle Eastern strain
LAM = Latin American

Clusters two, three and four consisted of isolates from patients who had recently migrated from the Philippines (four cases), the Sudan (two cases) and the Indian subcontinent (two cases), respectively. There were no identifiable links between patients within clusters; the patients' infections were probably independently acquired in their countries of origin.

Cluster five consisted of two isolates from patients who resided in different Australian states and had no obvious epidemiological links (but warrant further investigation).

Clusters six, seven and eight represented probable cross-contamination. In all three clusters there was one isolate from a patient with typical smear-positive tuberculosis; the others were from patients in whom the diagnosis of tuberculosis was considered unlikely. Clusters six and seven comprised two and three isolates respectively, referred for confirmatory identification. Isolates in both clusters were recovered from specimens from different patients, processed in the same laboratories at the same time. The two isolates in cluster eight were recovered from patients who had attended the same clinic for bronchoscopy two weeks apart; the same bronchoscope was used for both procedures.

Only patients from clusters one and five were included in the calculation of the rate of recent transmission (RRT), which was calculated as 1.4 per cent (Table 2).

DISCUSSION

Our results highlight the diversity of *M. tuberculosis* strains involved in tuberculosis infections in this country, most of these infections being acquired elsewhere. The most prominent strains identified during the study by spoligotyping belong to the W-Beijing family (more than one quarter of all isolates examined), which was first described in China and neighbouring countries in 1995[14] and has since spread to many parts of the world, especially Asia and Russia.[2,14,15,16] They are highly transmissible and often found predominantly in younger patients and they have an increased tendency to develop multidrug-resistance.[15,16] There is some evidence that BCG vaccination is less effective against Beijing genotype strains than others.[17] The high proportion of Beijing genotype strains reflects the migration patterns into NSW.

The low level of clustering of *M.tuberculosis* isolates in this study confirms that recent transmission of tuberculosis in NSW is uncommon. Several clusters may reflect reactivation of latent tuberculosis infections in migrants from high incidence countries where *M. tuberculosis* strains are more homogenous.[3,18] However, the possibility of recent transmission from direct contact, for example in a refugee camp or detention centre before arrival in Australia, cannot always be excluded. The rate of recent transmission (1.4 per cent) in this study is lower than that reported from other low-incidence countries (Table 2). However, these findings should be interpreted with caution. Studies of short duration (i.e. less than two years) may significantly underestimate the level of clustering because of the long incubation period of tuberculosis.[2,20,21] Cluster 1, in this study, was identified because of genotyping of more recent isolates several years after the first three cases had presented. Cluster size can be significantly underestimated unless a high proportion of the total isolates from a population over a significant period (usually at least 3 years) are genotyped.[22]

There is a growing body of evidence to support the role of *M. tuberculosis* genotyping in the detection and tracking of outbreaks of infection.[4,6,18] Increased migration from high-prevalence areas increases the risk of spread of multidrug-resistant *M. tuberculosis* and the need for earlier detection of outbreaks.[23] Clustering reflects the efficiency of therapy, the interval between disease onset and the start of treatment and the regional dominance of more successful strains of *M. tuberculosis*.[22] A better knowledge of expanding clones, such as the Beijing strain, is urgently needed in order to define better control measures.[4,23,24]

TABLE 2

COMPARISON OF FINDINGS FROM NSW WITH THREE RECENT INTERNATIONAL STUDIES THAT HAVE USED MOLECULAR TYPING OF *M.TUBERCULOSIS* TO DESCRIBE THE EPIDEMIOLOGY OF THE DISEASE

	London, UK[13]	Denmark[18]	Italy[19]	NSW (this study)
Number of isolates genotyped	57	1549	248	420
Methods used	IS6110 RFLP, spoligotyping	IS6110 RFLP	IS6110 RFLP, spoligotyping	IS6110 RFLP, spoligotyping, MIRUs
Duration of study	3 years	5 years	1 year	1.5 years
Rate of clustering %	15.8	49	33	4.9
Recent transmission rate %	8.8	57*	15	1.4
Percentage of tuberculosis due to Beijing strain	Not reported	Not reported	2.8	25.9

RFLP = restriction fragment length polymorphism; MIRU = mycobacterial interspersed repetitive units.

*Active transmission among native Danes reported only (two strains were responsible for 40% of all clustered cases among native Danes; the sample included two large clusters among HIV positive drug users).

No single typing method is ideal. PCR-based methods are rapid and relatively inexpensive; when combined, they can quickly exclude clustering in three quarters of cases, significantly reducing the need for IS*6110* RFLP typing. Patients who need additional follow-up can be identified more rapidly, secondary cases treated more quickly and new cases prevented. Although IS*6110* is regarded as the 'gold standard' it often requires several weeks' culture of *M. tuberculosis* to obtain adequate DNA and inter-laboratory comparison of results can be difficult. Spoligotyping alone is relatively non-discriminatory but provides valuable data about the prevalence of various *M. tuberculosis* families and can rapidly differentiate sub-species within the *M. tuberculosis* complex (for example *M.bovis, M.bovis* BCG, and *M.canetii*), which can otherwise only be identified by time-consuming biochemical tests. As far as we know, this is the first time that *M. canetti* and *M. caprae* have been identified in Australia.[25] The combination of three methods, as used in NSW, is probably the most cost-effective approach in the long term if clustered cases are rapidly identified and investigated, but more detailed analysis of data, over a longer period, is required.

These data will be used in future as a baseline for real-time monitoring of transmission dynamics of tuberculosis cases in NSW. They will contribute to a national genotyping project (based on MIRU typing only, initially), which may identify links between patients travelling interstate (such as those in cluster five). A project is currently in progress in NSW to link the genotyping database with tuberculosis case notification data. A comprehensive national tuberculosis genotyping network linked to the National Notifiable Diseases Surveillance System would provide continuous monitoring of transmission trends and allow identification of widespread outbreaks.

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