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Mycobacterium tuberculosis Population Structures Differ Significantly on Two Indonesian Islands[∇]

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Comparison of *Mycobacterium tuberculosis* genotype distributions in different areas might help to find determinants of the emergence of certain genotypes, such as the Beijing family. In this study, *M. tuberculosis* isolates originating from patients from two Indonesian islands were genotyped, and possible associations with patients' characteristics and drug resistance were explored. A high degree of genetic diversity was observed among the *M. tuberculosis* strains, and a significant difference was found in the geographical distribution of genotype families. The predominant Beijing genotype family was isolated from 268 of 813 patients from West Java (33.0%) versus 12 of 84 patients from Timor (14.3%) ($P = 0.002$). Family F (East African-Indian) (33.3%) and family D (Latin American and Mediterranean) (20.0%) were more prevalent in Timor. No significant associations were found between genotype families and age, vaccination with *Mycobacterium bovis* BCG, previous treatment, disease localization, or drug resistance. Possible explanations for the differences in the geographical distribution of the *M. tuberculosis* genotypes are discussed.

The introduction of DNA fingerprint methods in the early 1990s has greatly improved the possibilities for examining the transmission of tuberculosis (TB) and the phylogeny of *Mycobacterium tuberculosis* (22). The available genetic fingerprinting methods have different characteristics and applicability. In particular, analysis of large chromosomal deletions (4) and single-nucleotide polymorphisms can facilitate meaningful exploration of the population structure of the *M. tuberculosis* complex. However, these methods require sophisticated and costly techniques. More widespread is the use of spoligotyping to examine the strain diversity of *M. tuberculosis* in given areas (5, 22). By this method, the presence of 43 spacers in the direct repeat (DR) region of *M. tuberculosis* complex can be detected. The loss of spacers in the DR region seems to be in line with the evolutionary development of the *M. tuberculosis* complex lineages, and therefore, this currently appears to be the simplest approach to studying the population structure of *M. tuberculosis* complex strains (18). Because a high number of spoligotype patterns have been added to a central database, many *M. tuberculosis* genotype families could be identified in the past 15 years, including the Beijing, East African-Indian (EAI), Haarlem (H), Latin American and Mediterranean (LAM), and Central Asian (CAS) genotype families (5).

One of the best studied and most widespread evolutionary lineages of *M. tuberculosis* is the Beijing genotype family, which was first found in China and has been reported worldwide (8, 11). The emergence of Beijing genotype strains suggests that

they may have a selective advantage over other *M. tuberculosis* strains. Indeed, studies with animal models have shown enhanced virulence and distinctive histopathology following infection with *M. tuberculosis* Beijing genotype strains (6, 16). Extrapolating from this, the emergence of Beijing and other genotype families may be the consequence of the application of two major measures against TB in the last century: *Mycobacterium bovis* BCG vaccination and anti-TB treatment (22). Possibly, BCG vaccination is less protective against (more-virulent) Beijing genotype strains than against other strains, but so far, this has not been proven (2). Similarly, anti-TB treatment might be less effective in eradicating Beijing strains than other strains. Indeed, in one study, failure of TB treatment and subsequent relapse were more common among patients infected with Beijing strains (15). Studies examining a relationship between the Beijing genotype and drug resistance have encountered major differences in widespread geographic areas, according to a systematic review (11).

After India and China, Indonesia has the third highest TB case load in the world, with an estimated 525,000 new cases and an estimated 90,000 deaths per year (23). To date, only one molecular epidemiological study of tuberculosis has been performed in Indonesia, in 1998 (20). Of 94 TB patients, all from the capital city, Jakarta, 32.4% were infected with Beijing genotype strains. The Indonesian archipelago consists of 17,504 islands, inhabited by many different ethnic groups. Comparison of the distribution of *M. tuberculosis* genotypes in different areas or among different human populations and study of the patients' characteristics might help to find determinants of the emergence of particular genotype families. Therefore, we have spoligotyped *M. tuberculosis* isolates from a large cohort of patients from two Indonesian islands: West

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Java and Timor. Furthermore, we have explored the distribution of *M. tuberculosis* genotype families and analyzed the possible association of these genotypes with age, sex, BCG vaccination status, previous TB treatment, disease localization, and drug resistance.

MATERIALS AND METHODS

Study population. In Indonesia, *M. tuberculosis* culture is not routinely performed, and there is no national archiving of isolates. Therefore, we prospectively collected isolates from well-characterized patients in two different clinical studies. In West Java, from January 2001 through December 2006, consecutive patients over the age of 16 years with microscopically proven pulmonary TB (PTB) at two outpatient clinics and two hospitals in Jakarta and Bandung (West Java) were included as part of a large case-control study examining host susceptibility to TB (1). The diagnosis of TB was based on clinical presentation, chest X-ray examination, microscopic detection of acid-fast bacilli by Ziehl-Neelsen staining of sputum smears, and culture of *M. tuberculosis* on 3% Ogawa medium. As part of the same study, a smaller cohort of microbiologically proven extrapulmonary TB (EPTB) cases was included. All cultured *M. tuberculosis* isolates from patients with PTB ($n = 740$) and EPTB ($n = 73$) were used for the current study. In addition, in West Timor, as part of a study of micronutrient supplementation in TB, 84 cultured isolates from PTB patients were included (T. Pakasi et al., unpublished data). Of note, these were the first isolates ever cultured in this less-developed part of Indonesia. For all patients, age, history of previous TB treatment, the presence or absence of a BCG scar on the left deltoid muscle (as an approximation of BCG vaccination status), and disease localization were recorded.

Spoligotyping. DNA was extracted by taking two loopfuls of bacterial mass from a *M. tuberculosis* culture in saline and subsequently heating them at 95°C for 5 min. Spoligotyping was performed using a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands). The presence or absence of 43 spacers in the DR region of *M. tuberculosis* isolates was determined as follows: the DR region was amplified by primers, one of which was biotinylated; the amplified products were reverse hybridized to spacer sequence oligonucleotide probes immobilized on a Biodyne C membrane; and spacer sequences were detected with peroxidase-labeled streptavidin and enhanced chemiluminescence (9, 13). Spoligotyping was done at the Hasan Sadikin Hospital, Bandung, Indonesia. For quality control, spoligotyping of 10% of the isolates, and of all isolates lacking hybridization, was repeated at Gelre Hospital, Apeldoorn, The Netherlands.

Phylogenetic reconstruction. The spoligotyping results were recorded in octal and binary formats on an Excel spreadsheet and compared to the international SpolDB4.0 database (5). Phylogenetic analysis was done using Bionumerics software (Applied Maths, Sint-Maartens-Latem, Belgium). Spoligotype patterns were imported into Bionumerics as a character type; similarities between the patterns were calculated by using the categorical coefficient; and dendrograms were prepared by using the unweighted-pair group method using arithmetic linkages.

DST. Drug susceptibility testing (DST) was performed on cultured isolates using an absolute concentration method on 7H10 Middlebrook agar in 25-well plates with supranational control by the National Mycobacteria Reference Laboratory at the RIVM in Bilthoven, The Netherlands. In The Netherlands, this method has shown 96 to 100% accuracy in 10 rounds of WHO/IUATLD proficiency testing (21). Approximately 10% of the isolates from the current study were retested at the supranational reference laboratory in The Netherlands.

Data analysis and statistics. Spoligotype patterns were correlated with patients' age, sex, and BCG vaccination status (indicated by the presence or absence of a BCG scar), previous treatment, and resistance to isoniazid (INH), rifampin, or both drugs (multidrug-resistant [MDR] TB). Differences between groups were statistically examined using nonparametric tests for continuous variables and Fisher's χ^2 test. To avoid possible confounding by geographical area, the correlations were examined separately for Java and Timor.

RESULTS

Distribution of *M. tuberculosis* genotype families. Eighty-four *M. tuberculosis* isolates from Timor and 813 isolates from West Java were collected. Among the isolates from West Java, 740 were isolated from PTB patients and 73 from patients with EPTB. The latter category consisted of isolates associated with

meningitis ($n = 42$), lymphadenitis ($n = 9$), pleuritis ($n = 13$), and other diseases ($n = 9$).

A high degree of genetic diversity among the *M. tuberculosis* isolates was observed (Table 1). On the basis of the similarities of their spoligotype patterns, 739 of the 897 isolates were grouped into nine major spoligotype groups of more than 20 isolates. These nine major spoligotype groups were designated family A to family I. Spoligotype patterns outside of those nine major groups were designated "other." The largest spoligotype group comprised the Beijing genotype (family I) and contained 280 strains (31.2%). The second largest group was family B (T1), which consisted of 100 isolates (11.1%). Families E and A, both representing the Haarlem genotype, contained 82 (9.1%) isolates. In this study, 198 patterns were designated "orphan" patterns, because they had no shared type pattern in the international spoligotype database SpolDB4 (Fig. 1). No unique Indonesian strains were identified.

The distribution of genotypes showed little variation over time. From 2000 to 2006, 28.9% to 32.7% of the strains belonged to the Beijing genotype family, with no obvious trend in the number of cases over time. Because the distributions of genotypes in outpatient clinics and hospitals in Jakarta and Bandung were highly similar, those data were combined ("West Java"). When the spoligotype patterns originating from West Java were compared to those from Timor, there appeared to be a significant difference in the distribution of genotype families. In West Java, *M. tuberculosis* Beijing genotype strains were predominant (33.0%; 95% confidence interval [95% CI], 29.7 to 36.2%), but in Timor, Beijing strains were much less common (14.3%; 95% CI, 6.8 to 21.8%) ($P = 0.002$). On the other hand, family F (EAI) and family D (LAM) were highly prevalent in Timor (33.3% and 20.0%, respectively) but uncommon in West Java (6.2% and 8.7%, respectively) (Fig. 2). All these differences were statistically significant.

Association of genotype families with patient characteristics. We compared the characteristics of patients infected with different *M. tuberculosis* genotype families, including sex, age, BCG vaccination, previous treatment, and disease localization (PTB versus EPTB). Because of the strong geographic differences in the distribution of genotypes, the data from West Java and Timor were analyzed separately. For the 813 patients included in West Java, the results are shown in Table 2. The various genotype families were associated with slight differences in patient characteristics, none of which were statistically significant. A BCG scar as an indication of BCG vaccination was found in only 28.7% of patients. The smallest percentage of patients with BCG scars was found for those infected with strains from family C (T in SpolDB4) (16.3%), and the highest was found for those infected with strains from family A (H in SpolDB4) (46.3%), but these differences did not reach statistical significance. Similarly, different genotype families showed slightly different age distributions and percentages of extrapulmonary localization and previous treatment, but none of these were statistically significant (Table 2).

To relate the *M. tuberculosis* genotype to patient characteristics in Timor (not included in Table 2), we compared the two largest families in Timor, family F (EAI) (33.3%) and family D (LAM) (22.0%), with the remaining group including all strains of other genotypes (46.7%). The median age of patients infected with strains of family D/LAM (23 years) was signifi-

TABLE 1. Spoligotype diversity and spoligotype family designation of 897 *M. tuberculosis* isolates from West Java and Timor

Spoligotype family designation from SpoIDB4	No. of isolates with the following spoligotype family designation from this study:										Total no. of isolates	No. of patterns
	A	B	C	D	E	F	G	H	I	Other		
BEIJING										275	275	6
BEIJING-LIKE										5	5	2
CAS											2	1
CAS1_DELHI											3	1
EAI2_MANILLA						13					13	3
EAI2_NTB											7	1
EAI3_IND						3					3	1
EAI5						16					16	8
EAI6_BGD1						1					1	1
EAI1_SOM						16					16	4
H1					20						20	6
H3	40				9						50	9
H3-T3		2									2	1
LAM1				8							9	2
LAM3				1							1	1
LAM3 and S/convergent											2	1
LAM6				3							3	2
LAM8				28							28	1
LAM9				15		1					16	5
MANU2											3	1
Orphan	1	17	15	33	8	24	7				108	213
S		7									1	8
T1		55	4	2	3						10	74
T1(T4-CE1?)		1									1	1
T2		9									1	10
T2-T3		4										4
T3		3	24									27
T5_RUS1											1	1
U		1			1		7	3			15	27
U (LIKELYH3)							27					27
U (likelyS)								26				26
X1		1									3	4
Total	41	100	43	90	41	74	41	29	280	158	897	307

cantly different from the median age of those infected with strains of family F/EAI (29 years) or infected with other genotypes (32 years) ($P = 0.010$). No significant differences between genotype families were found for other patient characteristics. When the 12 (14.3%) patients infected with the Beijing genotype were analyzed separately, they appeared to be older than patients infected with other strains (median age, 46 years; interquartile range, 24.3 to 57.5 years), but this difference was not statistically significant.

Association of genotype families with drug resistance. Drug resistance patterns were available for 694 isolates: 620 from West Java, and 74 from Timor. Among the isolates of previously treated patients ($n = 77$), 25.4% were INH resistant, 31.2% were rifampin resistant, and 17.9% were MDR. Among newly treated patients ($n = 617$), these rates were 7.5%, 6%, and 2.6%, respectively. The various genotype families showed slightly different rates of drug resistance. Resistance to INH ranged from 2.7% to 10%, and resistance to rifampin ranged from 2.7% to 11.2%. Because the number of drug-resistant isolates per genotype family was small, no meaningful statistical analysis could be done on those differences between the nine genotype families.

Several previous studies have reported an association between the Beijing genotype and drug resistance. However, the drug resistance patterns of 209 Beijing isolates (30.1%) and

485 non-Beijing isolates (69.9%) were not significantly different (Table 2). Also, when strains isolated from Java were analyzed separately, no association was found between the Beijing genotype and drug resistance.

DISCUSSION

This is the second study of the molecular epidemiology of TB in Indonesia, involving the two most densely populated cities in West Java and a more rural area on Timor at the eastern end of the archipelago. Consecutive patients with microscopically proven TB from a large cohort study in various clinics in 2001 to 2006 were included, and patients were carefully characterized and followed prospectively. Because the isolates came from only three areas, the study is not representative of Indonesia as a whole. However, the four clinics in West Java showed very similar *M. tuberculosis* genotype family distributions, suggesting that these data are indeed representative for this area.

A high genetic diversity among *M. tuberculosis* isolates was found in Indonesia: nine major *M. tuberculosis* genotypes were identified. This high genetic *M. tuberculosis* strain diversity is in contrast with the findings of studies from other high-prevalence areas, including, e.g., Vietnam, where only two major genotype families were found: Beijing and EAI (15). The *M.*

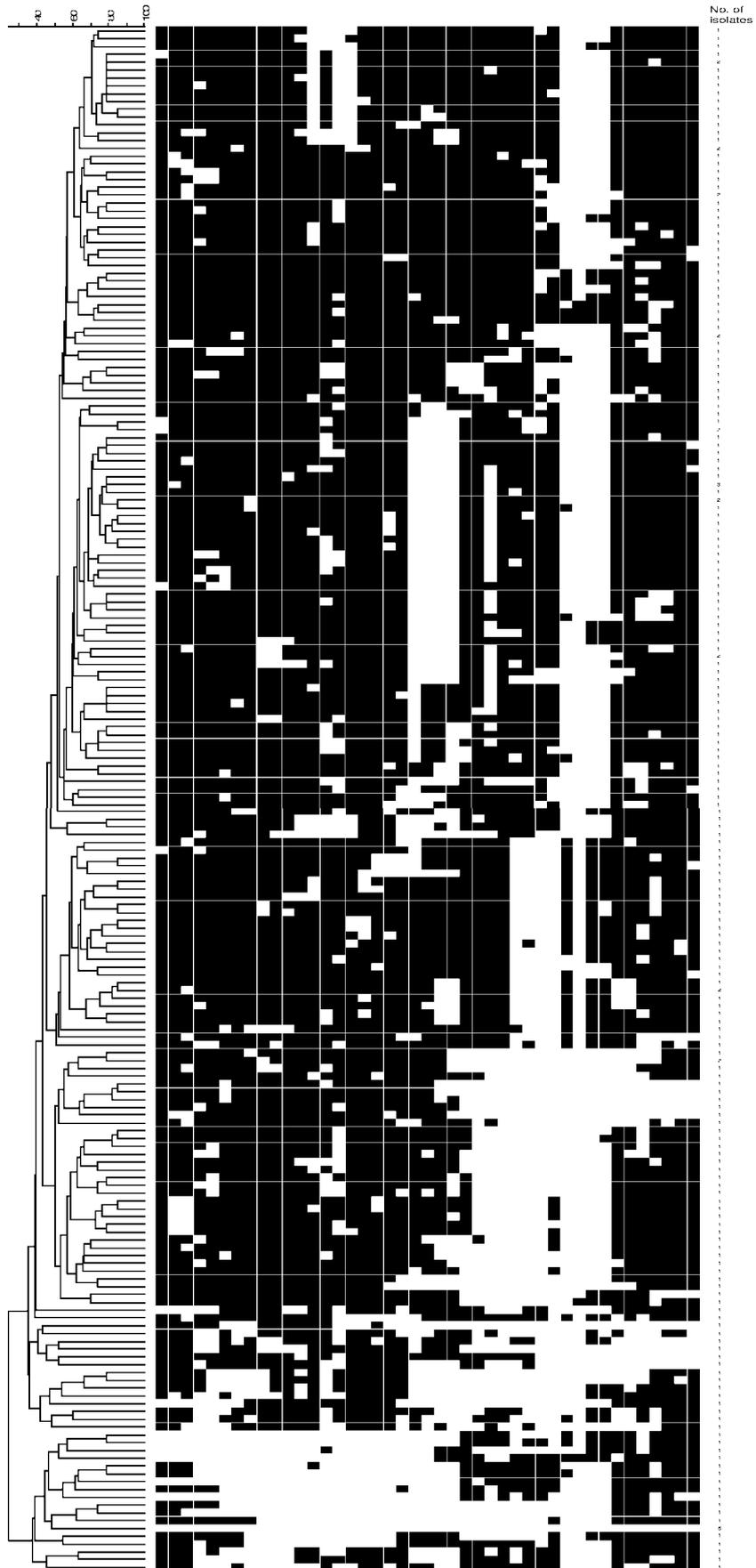


FIG. 1. Diagram of the 198 orphan spoliotype patterns found in this study, with a dendrogram on the left showing the similarities among the patterns. The number of isolates of each spoliotype is given on the right.

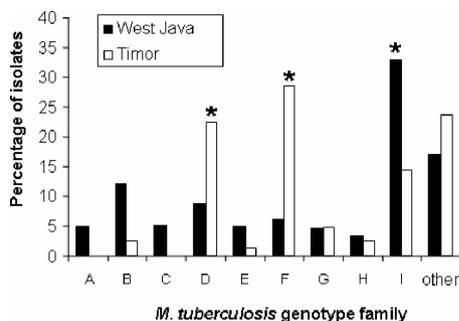


FIG. 2. Distribution of *M. tuberculosis* genotype families in West Java and Timor. *, $P < 0.05$.

tuberculosis Beijing genotype family was the most prevalent genotype family in Indonesia (31.2%), and the proportion of Beijing strains was stable during the study period and similar to the prevalence recorded in a previous, smaller study (32.4%) (20). In contrast to what has been reported in other studies (2, 7, 15), no association was found between the Beijing genotype and age, previous treatment, or BCG vaccination. As demonstrated previously in other studies from Asia, no clear association was found between drug resistance and Beijing strains, in contrast to reports from, e.g., the republics of the former Soviet Union, Cuba, South Africa, and Vietnam (8).

Interestingly, a strong difference was found between the *M. tuberculosis* population structures in West Java and Timor. The Beijing genotype family was found in 33.0% of patients in Java versus 14.3% of patients in Timor. This is in line with a recent observation in Vietnam, where a frequency of 50% in Ho Chi Minh City, but only 30% in the Mekong Delta, was found for Beijing genotype strains (unpublished data). These differences suggest that transmission of Beijing genotype strains may ben-

efit from highly dense populations. Inversely, in Timor, the EAI and LAM genotype families were predominant, while these genotypes were uncommon in Java. One can only hypothesize about the explanation for this difference. First, this difference may be due to a “founder effect,” with a higher chance of finding a particular genotype family closer to where it originated. Second, it may also indicate that particular mycobacterial lineages have adapted to particular human populations. This concept of genetic host-pathogen compatibility is supported by data showing a preferential spread of particular lineages among patient populations from the same area rather than from other areas (10, 12). So far, no studies showing direct associations between the genetic characteristics of TB patients and their (own) mycobacterial isolates have been reported.

Finally, the predominance of certain genotype families, particularly the Beijing genotype family, might also be explained by other mechanisms, such as higher transmission rates or “escape” from BCG vaccination. Our results do not support these hypotheses, since no significant associations were found between particular genotype families and patient characteristics, especially age and BCG vaccination status. However, the coverage of BCG vaccination is very high in Indonesia. Hence, transmission from vaccinated to nonvaccinated individuals is so frequent that differences between the populations of strains in the two groups will be diluted easily.

Several studies, such as one in Vietnam, have reported a lower age of patients infected with Beijing strains (2), suggesting more-recent transmission of Beijing strains. A second study from a different area in Vietnam has found a similar relationship between age and genotype (T. N. Buu, submitted for publication), but we, like others (7, 19), did not find this association. In fact, among patients from Timor, those infected

TABLE 2. *M. tuberculosis* genotype families and associated patient characteristics in West Java

Patient characteristic ^a	Result for the following spoligotype family ^b :										
	A (H)	B (T)	C (T)	D (LAM)	E (H)	F (EAI)	G (U likely H3)	H (U likely S)	I (Beijing)	Other	Total
No. (%)	41 (5)	98 (12.1)	43 (5.3)	71 (8.7)	40 (4.9)	50 (6.2)	37 (4.6)	27 (3.3)	268 (33.0)	138 (17.0)	813 (100)
Age (yr)											
Median	26.0	28.5	30.0	28.0	27.0	34.5	27.0	27.0	30.0	35.0	30
IQR	20.5–36.0	23.0–42.0	24.0–46.0	23.0–39.0	21.3–41.8	24.0–46.3	22.5–40.0	20.0–41.0	23.0–41.0	25.0–47.0	23.0–43.0
Male	43.9	58.2	48.8	56.3	52.5	52.0	54.1	48.1	49.3	50.7	51.4
BCG scar ^c	46.3	24.5	16.3	29.6	30.0	22.0	24.3	22.2	28.0	35.5	28.7
Previous TB treatment	12.2	16.3	4.7	8.5	10.0	20.0	10.8	18.5	9.7	12.3	11.7
Disease localization											
Extrapulmonary	2.4	9.2	7.0	7.0	0.0	4.0	2.7	25.9	11.2	10.9	9.0
Pulmonary	97.6	90.8	93.0	93.0	100.0	96.0	97.3	74.1	88.8	89.1	91.0
Drug resistance ^d											
Isoniazid (R + I)	17.1	14.3	11.6	7.0	17.5	14.0	10.8	11.1	13.1	9.4	12.3
Isoniazid (R only)	9.8	7.1	7.0	4.2	7.5	10.0	2.7	3.7	8.2	5.1	6.9
Rifampin	7.3	11.2	4.7	5.6	10.0	8.0	2.7	7.4	5.6	7.2	6.9
MDR	7.3	4.1	2.3	2.8	5.0	6.0	0.0	3.7	3.7	4.3	3.9

^a Except where otherwise indicated, each data point is the percentage of patients with the indicated characteristic. IQR, interquartile range; R, resistant; I, intermediate.

^b Given as the spoligotype family designation from this study (spoligotype family designation from SpolDB4).

^c Evaluated for 565 patients.

^d DST was successful for 694 isolates.

with a Beijing strain were older, although the number was small. The difference between Indonesia and Vietnam may be explained by the existence of different evolutionary lineages of the Beijing genotype family circulating in different geographic areas, as has been demonstrated previously (17).

In Indonesia, DST of *M. tuberculosis* is a matter of concern. Indonesia is not included in international surveillance of drug resistance (3), and there is no national surveillance or quality control system. We have previously compared the quality of conventional (proportional) DST with that of the 25-well DST method (21) in Indonesia, and the 25-well DST method used in this study showed better performance on WHO reference strains (B. Alisjahbana et al., submitted for publication). However, the high rates of drug resistance found in this study are in contrast with the only recent peer-reviewed data on drug resistance in Indonesia and with rates in neighboring countries (14). Because most of the isolates in this study came from clinics, care should be taken in the interpretation of the high drug resistance rate observed. Drug resistance rates in a more nationally representative DST survey may not be as high. It is clear that there is an urgent need for widespread implementation of quality-assured DST in Indonesia. Because of the limited number of strains belonging to certain genotype families, a possible relationship between particular genotypes and drug resistance cannot be excluded.

In conclusion, this molecular epidemiological study in Indonesia shows a considerable degree of heterogeneity among *M. tuberculosis* isolates and a significant difference in *M. tuberculosis* population structures at the different geographical study sites. A nationwide survey can provide more detail on the distribution of different genotype families in Indonesia, and a study of possible associations between host and mycobacterial genetics may help to establish if differences in *M. tuberculosis* population structures are caused by evolutionary adaptation of particular mycobacterial lineages to certain human populations.

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