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Heterogeneity of *Mycobacterium tuberculosis* strains in Makassar, Indonesia

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SUMMARY

SETTING: Patients with suspected pulmonary tuberculosis (TB) visiting government TB diagnostic and treatment centres in Makassar City, South Sulawesi Province, Indonesia, from February to October 2008 were included in the study.

OBJECTIVE: To determine the distribution of *Mycobacterium tuberculosis* genotypes in Makassar.

DESIGN: Cross-sectional study. Spoligotyping, mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) and principal genetic grouping (PGG) were used to genotype the *M. tuberculosis* clinical isolates.

RESULTS: Among 179 isolates derived from pulmonary TB patients, distribution of spoligotypes comprised the East Africa-Indian (30.2%), T (17.9%), H (12.3%) and

Beijing (9.5%) lineages. Other lineages found in smaller proportions were the Latin American-Mediterranean, MANU, S and X lineages. Nineteen isolates (10.6%) could not be grouped into any of the reported lineages or shared types. Single nucleotide polymorphism analysis of *katG*⁴⁶³ and *gyrA*⁹⁵ grouped these isolates primarily into PGG1 (9/19, 47%).

CONCLUSION: Only a few genetically identical clustered isolates were identified within the 9-month study period, and most isolates were genetically diverse. Furthermore, 15 spoligopatterns identified in our study have not been reported previously. To our knowledge, this is the first comprehensive study describing genotypes of *M. tuberculosis* clinical isolates in Sulawesi.

KEY WORDS: genotyping; spoligotyping; MIRU-VNTR

INDONESIA has a population of ~240 million inhabitants who speak more than 750 languages and belong to over 300 different ethnic groups. It ranks fourth in the list of the 22 high TB burden countries in the world.¹ The Health Office of South Sulawesi Province reported that a total of 1396 smear-positive TB patients were enrolled in DOTS in 2008 in Makassar. The notification rate for smear-positive cases in Makassar was estimated to be ~110 per 100 000 population, which was higher than the national average rate (74/100 000).¹ Although the TB burden of the country is high, only two TB genotyping studies have been reported from Indonesia to date.^{2,3} Parwati et al.'s study shows that diverse *M. tuberculosis* genotypes are observed in both the eastern and western parts of Indonesia.³

Several molecular typing methods are available

to characterize *M. tuberculosis* genotypes. Spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) are commonly used polymerase chain reaction (PCR) based genotyping methods.⁴ Sreevatsan et al.² demonstrated that *M. tuberculosis* could be divided into three principal genetic groups (PGGs), 1, 2 and 3, based on single nucleotide polymorphisms (SNP) of *katG*⁴⁶⁶ encoding the catalase-peroxidase enzyme, and of *gyrA*⁹⁵, encoding the A subunit of DNA gyrase.⁵

This study aimed to determine the distribution of *M. tuberculosis* genotypes in Makassar using spoligotyping, MIRU-VNTR and PGG-SNP typing methods. Our work is the first study to describe the distribution of genetically defined *M. tuberculosis* clinical isolates in the island of Sulawesi.

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MATERIALS AND METHODS

Patients and *M. tuberculosis* isolates

The study protocol was approved by the institutional review boards of the Faculty of Medicine, Hasanuddin University and Eijkman Institute. A total of 657 TB suspects visiting the central lung clinic, six community health centres and three hospitals in Makassar during the study period, February–October 2008, were included in the study. Sputum samples from patients were cultured in Löwenstein-Jensen medium and BACTEC™ MGIT™ (BD, Sparks, MD, USA), as previously described.⁶ Of the 657 study patients, 234 (35%) were culture-positive. Isolates from 179/234 patients (76%) were confirmed as *M. tuberculosis* complex using the AccuProbe test (Gen-Probe Inc, San Diego, CA, USA). Demographic data were collected from all 179 patients, and *M. tuberculosis* isolates were subjected to drug susceptibility testing (DST) and genotyping. A total of 179 *M. tuberculosis* complex isolates from 179 patients were included in the study. Genomic DNA extraction was performed using the CTAB/NaCl method, as described previously.⁷

Spoligotyping

Spoligotyping was performed according to the manufacturer's instructions (Isogen Lifescience, De Meern, The Netherlands, or Ocimum Biosolutions, Hyderabad, India). *M. tuberculosis* H37Rv and *M. bovis* bacille Calmette-Guérin strains were used as positive controls. Isolates were tested independently in duplicate. Spoligopatterns of isolates were compared with the SpolDB4 database⁸ and/or analysed according to Filliol et al.⁹ Isolates with spoligopatterns not matching any shared-type (ST) in the SpolDB4 database, but which could still be assigned to a lineage, were defined as 'established-lineage-orphans'. In contrast, when no lineage designation was possible, the isolates were designated 'non-established-lineage-orphans'. The spoligopatterns from the current study were also compared with those included in an unpublished version of an updated Institut Pasteur Paris SITVIT2 database (C. Sola et al., 55 266 clinical isolates from 134 countries, unpublished results).

MIRU-VNTR analysis and principal genetic grouping

All isolates were subjected to 24 loci MIRU-VNTR typing in duplicate and analysed as described by Supply et al.¹⁰ *M. tuberculosis* H37Rv was used as a positive control. The MIRU copy number per locus was calculated as described previously.¹⁰ Isolates were also classified as one of the three PGGs based on the *gyrA*⁹⁵ and *katG*⁴⁶³ SNPs, according to Sreevatsan et al.⁵

Data mining, statistical and computational analyses

Cluster analyses of MIRU-VNTR profiles were performed using BioNumerics 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms

were generated using the categorical characterisation and neighbour-joining clustering method.¹¹ A cluster was defined as isolates from different patients (two or more) with identical MIRU-VNTR patterns. Sipina[®] software (Université de Lyon 2, Lyon, France, http://eric.univ-lyon2.fr/~ricco/sipina_download.html) was used to assign any 24 VNTR patterns of an individual isolate to a predefined phylogenetic lineage. The Sipina software is based on an induction algorithm to generate decision trees under Ole (Windows environment). Sipina was first used on VNTR results in a study conducted on 3454 clinical isolates from the Netherlands.¹² For statistical analysis, the mean age of patients among different groups was compared using one-way analysis of variance. Categorical variables among patient groups were compared using Fisher's exact test. Results were considered statistically significant if $P < 0.05$.

RESULTS

Patient characteristics

Of 179 patients, 59% were male and the median age was 37 years (range 17–85); 45% were aged >45 years and 34% had previously been treated with anti-tuberculosis drugs.

Spoligotyping

Spoligotyping of 179 isolates generated 88 distinct spoligopatterns (Figure 1). Of the 88 spoligopatterns, 47 (119 isolates) were exact matches with the existing spoligopatterns in the SpolDB4 database⁸ and Filliol et al.⁹ Although they were not exact matches, a further 26 spoligopatterns (41 isolates) could be grouped within established spoligotype lineages and were designated 'established lineage orphans'. Consequently, 73 (47 + 26) spoligopatterns were grouped into nine distinct spoligotype lineages according to SpolDB4: notably East African (EAI), T, Haarlem (H), Beijing, Latin American-Mediterranean (LAM), MANU, S, X and the unknown/undesigned (U) lineages (Figure 1). The most commonly found lineages were EAI (30.2%), T (17.9%), H (12.3%), Beijing (23.5%), U (8.6%) and LAM (5.6%; Figure 1). Of the isolates belonging to U lineages 23 were 'U', two were 'U likely H3', two were 'U likely H', and five were 'U likely S'. The most frequent STs were as follows: ST1, Beijing lineage ($n = 16$); ST19, EAI2-Manila ($n = 13$); ST53, T ($n = 7$); and ST655, H ($n = 7$; Figure 1). The remaining 15 spoligopatterns (19 isolates) could not be assigned to any single spoligotype lineage and were designated 'non-established lineage orphans' strains, accounting for 10.6% of the isolates.

MIRU-VNTR analysis

Overall, the 179 isolates generated 165 MIRU-VNTR patterns. The MIRU-VNTR patterns of 54 isolates belonging to the EAI lineage are shown in Figure 2A.

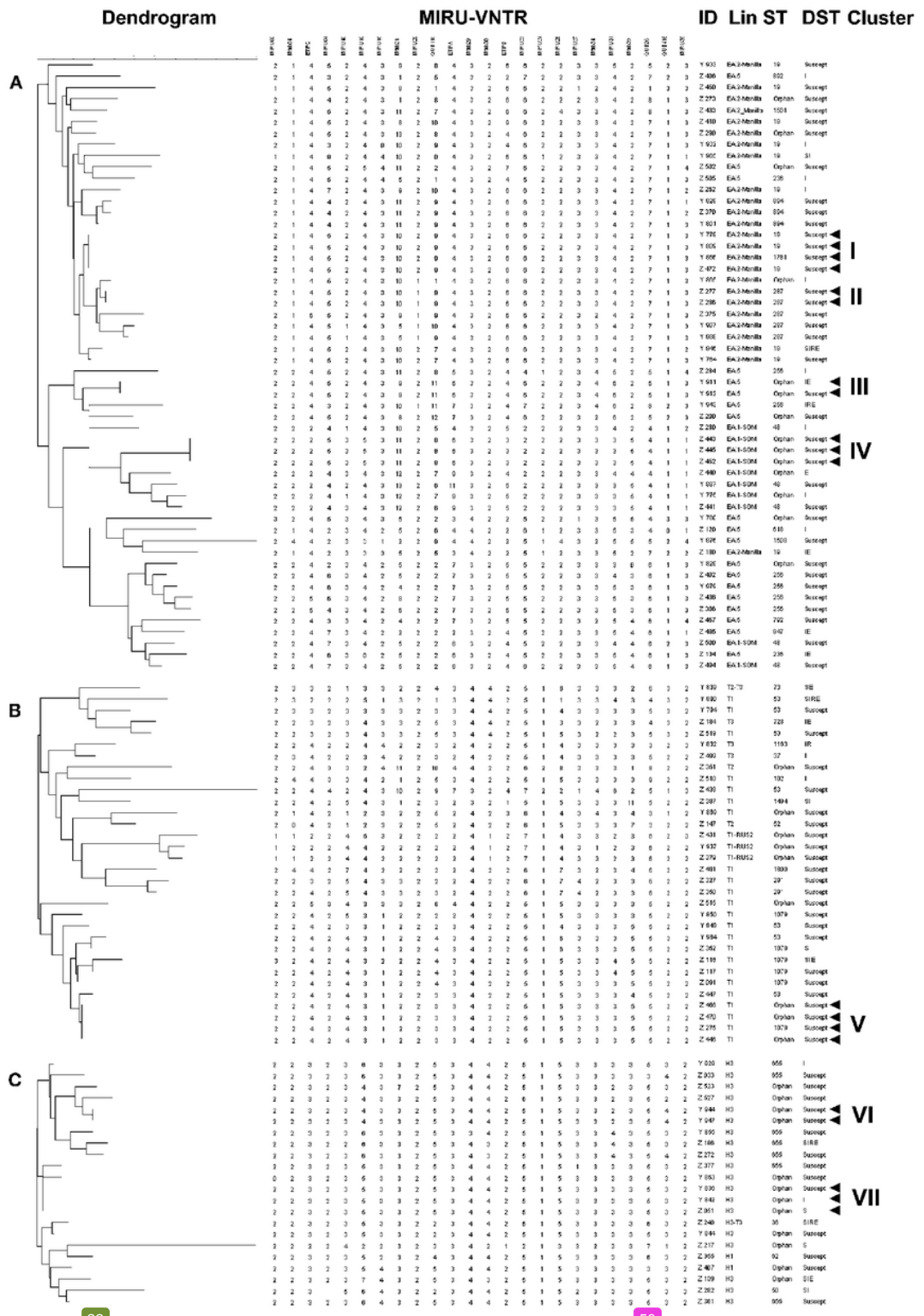


Figure 2 24-locus MIRU-VNTR typing of EAI lineage (A), T lineage (B), and H lineage (C). *M. tuberculosis* isolates are subjected to MIRU-VNTR analysis as described in Materials and Methods. Arrowheads denote clustered isolates. MIRU = mycobacterial interspersed repetitive units; VNTR = variable number of tandem repeats; 149 = sample identification number; Lin = lineage; ST = shared type; DST = drug susceptibility testing; EAI = East African Indian; S = streptomycin; I = isoniazid; R = rifampicin; E = ethambutol.

Table Characteristics of patients infected with different lineages

	EAI lineage (n = 54) n (%)	T lineage (n = 32) n (%)	H lineage (n = 22) n (%)	Beijing lineage (52/17) n (%)	P value
Age, years, mean \pm SD	41 \pm 13.8	39.1 \pm 15.1	38.7 \pm 16.9	34.8 \pm 16.3	0.53
Male sex	34 (63)	20 (63)	8 (36)	9 (53)	0.16
History of previous treatment for TB	15 (28)	9 (28)	8 (36)	5 (30)	0.9
Infected with MDR-TB isolates	2 (4)	2 (6)	2 (9)	2 (12)	0.92
Infected with any drug resistant isolates	15 (28)	7 (22)	6 (27)	6 (35)	0.79

EAI = East Africa-Indian; SD = standard deviation; TB = tuberculosis; MDR-TB = multidrug-resistant TB.

DISCUSSION

In this study, we examined the population structure of *M. tuberculosis* in Makassar City (population: 1.3 million), the capital of South Sulawesi Province in Sulawesi Island and the largest city in the eastern part of the archipelago. The predominant lineage observed in this study was EAI (30%). The other main lineages were T (18%), H (12%) and Beijing (10%; Figure 1). Parwati et al., who examined a collection of isolates from West Timor (eastern part of Indonesia), showed that the EAI lineage accounted for ~33% and Beijing ~14%.³ Thus, our findings in Makassar are similar to the West Timor findings reported by Parwati et al.³

Isolates from the EAI lineage/sub-lineages are highly prevalent in South-East Asia, South India, and, to a lesser extent, in East Africa,^{8,14,15} while isolates of the Beijing lineage are highly prevalent in China, South Korea, Japan and South-East Asian countries; its prevalence is lower in other continents.¹⁶ In our study, the most predominant EAI sub-lineage was EAI2-Manila (Figure 1). EAI2-Manila isolates were first described in Manila, the Philippines,¹⁷ and subsequently identified in other Asian countries, including Thailand, Viet Nam, Singapore and Taiwan.^{8,18} In the International SpolDB4 database, only a single EAI2-Manila strain has been reported from Java⁸ and two from West Timor-Indonesia.³ Other EAI sub-lineages present in Makassar were EAI1-SOM and EAI5 (Figure 1). Among the EAI1-SOM and EAI5 sub-lineage isolates, ST48 and ST256 were most commonly observed. ST48 isolates are prevalent in East African countries⁸ and a few ST 256 isolates have been reported from Myanmar, India, Thailand, Viet Nam and Malaysia.^{8,14} According to the SpolDB4 database, T lineage strains are prevalent in the Middle East and Europe, and the H lineage strains in Europe.⁸ Some of the *M. tuberculosis* strains commonly found in Madagascar,¹⁹ such as ST 52, ST 50, ST 32 and ST 34, were also found in Makassar (Figure 1). Among non-established lineage orphan strains (19 isolates), the majority were categorised as PGG 1 (47%), while the remainder belonged to PGG 2 (26%), and PGG 3 (26%). The PGG 1 comprised both 'ancestral' and 'modern' strains of *M. tuberculosis*, including the EAI, Beijing and CAS lineages, while PGG 2 and 3 comprised only modern strains, such as the H, LAM and X lineages, as described previously.²⁰

Our study therefore shows that *M. tuberculosis* isolates from patients in Makassar displayed highly diverse spoligo patterns. During the thirteenth and fourteenth centuries, prior to the arrival of the first Portuguese settlers in 1511,²¹ Makassar, then the port and capital of the Gowa and Tallo Sultanates, flourished as a trading centre for regional societies extending from South-East Asia to foreign traders from Europe, the Middle East, India and China.^{22,23} During the sixteenth and seventeenth centuries, Dutch trading routes were established, including Makassar and Madagascar, other parts of Africa, Indian subcontinent and several islands in the Eastern part of Indonesia, including Timor.²⁴ Makassar's early trading connections with different parts of the world and neighbouring islands may account for the diverse *M. tuberculosis* lineages found in this study.

Beijing lineage isolates are more prevalent than EAI lineage isolates among TB patients in the western part of Indonesia (West Java), contrary to reports from eastern parts of the country,^{2,3} including our study. This diversity may reflect the adaptation of a given lineage to a particular human population.^{25,26} It is possible that the EAI lineage is well established among east Indonesian populations, including those from Makassar and West Timor.³ However, this remains speculative, as human population genetic data and analysis are currently absent.

It should be noted that our study also shows that small clusters of TB cases were present in Makassar (Figures 1 and 2). We did not observe obvious possible geographic links among patients infected with clustered isolates (data not shown). One could thus assume that ongoing community TB transmission is not a major issue in this area. Alternatively, small clusters of TB cases and possible non-geographically linked patients may reflect undetected sampling bias. Although the exact numbers of TB patients in Makassar during the sampling period were not available, according to the South Sulawesi Health Department office, a total of 1396 smear-positive TB patients were identified in 2008 in Makassar. Based on these data, it was estimated that the total TB population during the data collection period (9 months) in Makassar was ~1050. We thus estimated that the sample size included in our study accounted for about 17% of the total culture-positive *M. tuberculosis* isolates

in Makassar. Sampling was done continuously for 9 months among TB patients attending the lung clinic, six government out-patient health care centres and three hospitals in Makassar. We therefore believe that, despite the small sample size, continuous collection of samples from multiple sites represents the bacillary populations in Makassar. A further contact investigation study is needed to determine whether genetically clustered isolates have an epidemiological link. It would also be interesting to explore [44](#) TB transmission in Makassar occurs mainly via [casual contacts in public places such as public institutions, places of worship, markets, schools, offices etc, as reported by Classen et al.](#)²⁷

We also observed that there was no correlation between the *M. tuberculosis* lineages and patient characteristics, including drug resistance. This lack of correlation has also been observed in other regions in Indonesia such as Jakarta, Bandung and West Timor.³

Sipina software was experimentally used to assign individual isolates to a predefined phylogenetic lineage based on 24 VNTR patterns (Figure 1). This experimental approach (knowledge discovery using data) has previously been used successfully for spoligotyping classification.²⁸ However, data generated using Sipina software showed some mislabelling in the current study. This may be due, in part, to the limited amount of reference data of samples originating from South-East Asia included in the present database. Furthermore, some lineages remain poorly differentiated by either VNTR or spoligotyping, making phylogenetic grouping difficult.^{11,29,30}

In summary, our findings indicate that TB patients in Makassar have been infected with genotypically diverse isolates. Among these, the EAI lineage was predominately present. Furthermore, 15 spoligopatterns identified in our study had not been reported previously. Findings from this study could form the basis for continued monitoring of *M. tuberculosis* clusters and drug resistance patterns in Makassar. Such monitoring may be used to elucidate the effectiveness of the TB control programme.

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RÉSUMÉ

CONTEXTE : On a inclus entre février et octobre 2008 les patients suspects de tuberculose (TB) pulmonaire consultant dans des centres de diagnostic et de traitement de la TB dans la ville de Makassar, Province du Sud Sulawesi, Indonésie.

OBJECTIF : Déterminer la distribution des génotypes de *Mycobacterium tuberculosis* à Makassar.

SCHEMA : Etude transversale. Pour réaliser le génotype des isolats cliniques de *M. tuberculosis*, on a utilisé le spoligotypage, les unités mycobactériennes intercalées répétées-nombre variable de répétitions en tandem (MIRU-VNTR) ainsi que le groupement génétique principal (PGG).

RÉSULTATS : Sur 179 isolats provenant de patients atteints d'une TB pulmonaire la distribution des spoligotypes était la suivante : lignées East Africa-Indian (30,2%), lignées T (17,9%), lignées H (12,3%) et lignées Beijing

(9,5%). D'autres lignées ont été observées en proportions plus faibles, en l'occurrence le Latin American-Mediterranean, MANU, S et X. Dix-neuf isolats (10,6%) n'ont pu être regroupés dans aucun des lignées rapportés ou des types partagés. L'analyse des polymorphismes des nucléotides simples de *katG*⁴⁶³ et de *gyrA*⁹⁵ a permis de regrouper ces isolats principalement dans PGG1 (9 sur 19, soit 47%).

CONCLUSION : Au cours d'une période d'étude de 9 mois, on a pu identifier seulement un petit nombre de grappes génétiquement identiques alors que la plupart des isolats étaient génétiquement différents. De plus, 15 des types de spoligotypage identifiés dans notre étude n'avaient pas été signalés antérieurement. A notre connaissance, il s'agit ici de la première étude complète décrivant les génotypes des isolats cliniques de *M. tuberculosis* à Sulawesi.

RESUMEN

MARCO DE REFERENCIA: Se estudiaron los pacientes con presunción clínica de tuberculosis (TB) pulmonar que acudieron los centros de diagnóstico y tratamiento de la TB del sector público en la ciudad de Makassar de la provincia Sulawesi en el sur de Indonesia entre febrero y octubre del 2008.

OBJETIVO: Determinar la distribución de los genotipos de *Mycobacterium tuberculosis* en Makassar.

MÉTODO: Fue este un estudio transversal encaminado a establecer el genotipo de los aislados clínicos de *M. tuberculosis* mediante el espiligotipado, el genotipado con marcadores para locus múltiples de las secuencias repetitivas en tándem (MIRU-VNTR) y la agrupación en tres grupos genéticos principales (PGG) según los polimorfismos de nucleótido único del gen *katG*.

RESULTADOS: Los espiligotipos de las 179 cepas aisladas de pacientes con TB pulmonar se distribuyeron entre los linajes Este Africano e India (30,2%), T (17,9%),

H (12,3%) y Beijing (9,5%). Otros linajes encontrados en menor proporción fueron el Latino Americano Mediterráneo, el MANU, el linaje S y el linaje X. Diecinueve aislados (10,6%) no correspondieron a ninguno de los linajes o tipos compartidos de la base de datos. Mediante el análisis de polimorfismos de nucleótido único de los genes *katG*⁴⁶³ y *gyrA*⁹⁵, estos aislados se asignaron principalmente al PGG 1 (9 de los 19, 47%).

CONCLUSIÓN: Durante el período de 9 meses del estudio se detectaron solo unos pocos aislados idénticos agrupados en conglomerados y la mayoría de los aislados fueron diversos desde el punto de vista genético. Además, 15 de los espiligotipos detectados en el presente estudio no habían sido notificados previamente. Este es el primer estudio descriptivo exhaustivo de los genotipos de los aislados clínicos de *M. tuberculosis* obtenidos en Sulawesi.

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