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Molecular characterization of drug-resistant *Mycobacterium tuberculosis* among Filipino patients derived from the national tuberculosis prevalence survey Philippines 2016

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ABSTRACT

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains a high burden disease and leading cause of mortality in the Philippines. Understanding the genetic diversity of *M. tuberculosis* strains in the population, including those that are multi-drug resistant (MDR), will aid in formulating strategies for effective TB control and prevention. By whole genome sequencing of *M. tuberculosis* isolates (n = 100) from patients of the Philippine 2016 National Tuberculosis Prevalence Survey, we sought to provide a baseline assessment of the genotypic and phylogenetic characteristics of the isolates. The majority (96/100) of the isolates were EAI2-Manila strain-type (lineage 1), with one Lineage 2 (Beijing), one Lineage 3 (CAS1), and two Lineage 4 (LAM9) strains. The EAI2-Manila clade was not significantly associated with patient's phenotypic and *in silico* drug resistance profile. Five (5/6) MDR-TB isolates predicted by *in silico* profiling were concordant with phenotypic drug resistance profile. Twenty-one mutations were identified in nine drug resistance-related genes, all of which have been reported in previous studies. Overall, the results from this study contribute to the growing data on the molecular characteristics of Philippine *M. tuberculosis* isolates, which can help in developing tools for rapid diagnosis of TB in the country, and thereby reducing the high burden of disease.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major cause of morbidity and mortality throughout the world, with estimated 1.5 million deaths and 10 million incident cases in 2020 alone. Much of the burden of TB is felt in developing countries where *M. tuberculosis* transmission has been associated with factors like poverty, crowding and weak public health systems. Furthermore, the overlap of TB with the HIV pandemic and the emergence of drug-resistant *M. tuberculosis* strains complicate current strategies for control, hence the need for evaluating the relative contribution of these factors in the current global epidemiology of the disease. Multidrug-resistant (MDR) TB, which is caused by *M. tuberculosis* resistant to first-line isoniazid and rifampicin drugs, is posing significant challenges to the prevention and control of disease [1].

The burden of TB in the Philippines remains substantial despite efforts to control the disease. The Philippines is one of 30 countries identified by the WHO to have high burden of TB, MDR-TB and TB/HIV co-infection [2]. It ranked fourth in terms of the number of incident cases in 2019, behind only India, China and Indonesia [1]. Results of the 2016 National Tuberculosis Prevalence Survey (NTPS) showed that around 760,000 Filipinos older than 14 years of age have pulmonary TB. The estimated prevalence of bacteriologically confirmed pulmonary TB (i.e., positive for TB culture and/or Xpert MTB/RIF) in this age group was 1159 per 100,000 population, and that of smear positive pulmonary TB was 434 per 100,000 population. Pulmonary TB prevalence

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estimated using the Xpert MTB/RIF diagnostic was 983 per 100,000 population, and that by TB culture was 587 per 100,000 population [3].

Recent advances in microbiology have allowed the development of genomic and molecular tools for the sequencing, genotyping and genetic analysis of *M. tuberculosis* strains, which subsequently is providing better insights on the epidemiology of TB. These insights include the identification of strain-types and lineages in the *M. tuberculosis* complex (MTBC), transmission clusters, and drug resistance. Several molecular genotyping tools, including IS6110 Restriction fragment length polymorphism (RFLP) typing, spoligotyping, and Mycobacterial interspersed repetitive units - variable number tandem repeat (MIRU-VNTR) typing, which use a small proportion of the *M. tuberculosis* genome (size 4.4 Mb) [4,5], are being replaced by higher resolution whole genome sequencing (WGS) approaches [6]. WGS data can be used to *in silico* profile sub-lineages [7] and spoligotypes [8], where some strain-types within the phylo-geographically distributed MTBC are thought to be more virulent and drug-resistant (*e.g.* Lineage 2 Beijing strains). *In silico* drug resistance profiles can be inferred through identifying known causal and potentially novel genetic mutations in *M. tuberculosis* genes coding for drug-targets or -converting enzymes [9,10]. Further, transmission clusters can be inferred through identifying *M. tuberculosis* isolates with (near-)identical genome variation, usually using a single nucleotide polymorphism (SNP) difference cut-off between samples, with relationships and events supported using epidemiological data.

In the Philippines, there is a need to understand the transmission dynamics of TB, including MDR-TB, as well as the prevalence and distribution of different strains across community settings. Differences in settings may reveal important information, such as the interaction of different *M. tuberculosis* strains in the community and the variable infectiousness of these different strains as they affect patient contacts and subsets of high-risk populations within the communities (*e.g.,* elderly, diabetics, PLHIV). There have been few studies covering the molecular epidemiology of circulating *M. tuberculosis* isolates in the Philippines. A study in 2003 found that *M. tuberculosis* isolates sourced from TB patients (without HIV co-infections) belonged to a unique EAI2-Manila Family spoligotype [11]. A molecular epidemiological study in 2013 in Santa Rosa City, Laguna, characterized 116 isolates covering 14 unique spoligotypes, where the majority of the source pulmonary TB patients were young (<30 years old) and male. No significant associations between the EAI2-Manila clade and patient characteristics (*e.g.* sex and age groups) were found, but there was some evidence to suggest recent transmission of TB in Laguna [12]. A recent study using WGS of *M. tuberculosis* isolates from the Second National TB Drug Resistance Survey in 2012 (DRS 2) confirmed the predominance of EAI2-Manila genotype among the isolates. Phylogenetic analysis revealed around 200 SNPs that are specific for the EAI2-Manila strain. Drug mutation profiling also showed novel, as well as previously published, mutations in the drug resistance-conferring genes of the isolates [6].

Here, to provide additional genomic characterization of Philippine drug-susceptible and drug-resistant *M. tuberculosis* isolates for surveillance purposes, we present a study of 100 isolates from the NTPS in 2016. Our study aimed to determine the genotypic and phylogenetic characteristics of drug-susceptible and MDR-TB isolates from the NTPS 2016 patients, and link them to demographic features and clinical outcomes of the host. The resulting data adds significantly to a proposed country-level database of *M. tuberculosis* strains. Such data could be used by the National TB Program, scientists and healthcare providers in refining current strategies and interventions to curtail the burden of TB in the Philippines. This can be achieved through applications of more effective treatment and prevention strategies for the management and control of TB.

2. Materials and methods

2.1. Sampling and data collection

Based on the NTPS 2016 study [3], out of the total 61,466 individuals declared eligible to participate in the survey from 106 clusters, based on age and residency criteria, only 46,689 (76%) contributed through providing an interview and chest x-ray. A total of 18,597 individuals (39.8%) were declared eligible for sputum collection, out of whom 16,242 (87.3%) provided at least one sample for examination. Samples were tested with direct sputum smear microscopy (DSSM), TB culture and Xpert MTB/Rif. There were 466 bacteriologically confirmed pulmonary TB cases, 173 (37.1%) of which were smear positive, 159 (34.1%) were positive by both TB culture and Xpert MTB/Rif, 69 (14.8%) by TB culture alone, and 238 (51.1%) by only Xpert MTB/RIF. A total of 232 (49.8%) patients gave positive culture results, 190 (81.9%) of which were drug-susceptible to first-line *anti*-TB drugs and Levofloxacin, and 42 (18.1%) were resistant to any drug (Fig. 1).

From the 232 patients, only 120 were included in the final analysis. At least five representative patients from each geographic region of the Philippines were randomly selected. Information on patient's age, sex, marital status, address, occupation, history of previous TB infection or treatment, smear grade based on DSSM, chest x-ray findings, Gene Xpert results and phenotypic DST results were collected from the NTPS 2016 database.

2.2. M. tuberculosis isolate revival and DNA extraction

One hundred twenty *M. tuberculosis* isolates stored at the National Tuberculosis Reference Laboratory (NTRL) biobank of the Research Institute for Tropical Medicine (RITM) were revived and sub-cultured into Middlebrook 7H9 broth and Ogawa media, and subsequently confirmed as *M. tuberculosis* through TB Ag MPT64 Rapid test (SD Diagnostics). Mycobacterial DNA extraction was performed using the QIAGEN Gentra® Puregene® Extraction kit, following the manufacturer's instructions. Mycobacterial DNA extracts were stored at −80 °C until used for WGS. All laboratory procedures involving *M. tuberculosis* isolates were done in a BSL 2-enhanced facility.

2.3. Whole genome sequencing and bioinformatics analysis

The extracted genomic DNA samples were submitted to the UP - Philippine Genome Center (UP-PGC) for WGS. Quality control of the submitted DNA extracts was done by UP-PGC using Qubit Fluorometer. Library preparation was done through Qiaseq FX DNA, and sequencing was performed using the Illumina NextSeq platform (Mid-output, 150 cycles). Only 100 (of 120, 83.3%) isolates had sufficient sequencing data and genomic coverage for further analysis. Sequencing reads were mapped against the H37Rv reference genome (GCA_000195955.2) using *bwa-mem* software (v 0.7.17). Variants were called using *bcftools* software (v1.10). SNPs were called using the BCF/VCF tool suite (v1.8) in regions where at least 10 reads were present. SNPs were removed from non-unique regions of the genome (*e.g., ppe* genes). TB-Profiler (v2.8.12) was used to predict drug resistance and strain-type based on genomic sequences [7,13]. *IQ-Tree* (v1.6.12) was used to create a phylogenetic tree based on genomic SNPs (n = 9893). Trees were annotated in *iTOL*.

2.4. Data availability

The generated raw sequence data from this study can be found in the European Nucleotide Archive (accession number PRJEB46482).

2.5. Data analysis

The socio-demographic and clinical characteristics of the patients included in the study were summarized and described. Proportions and

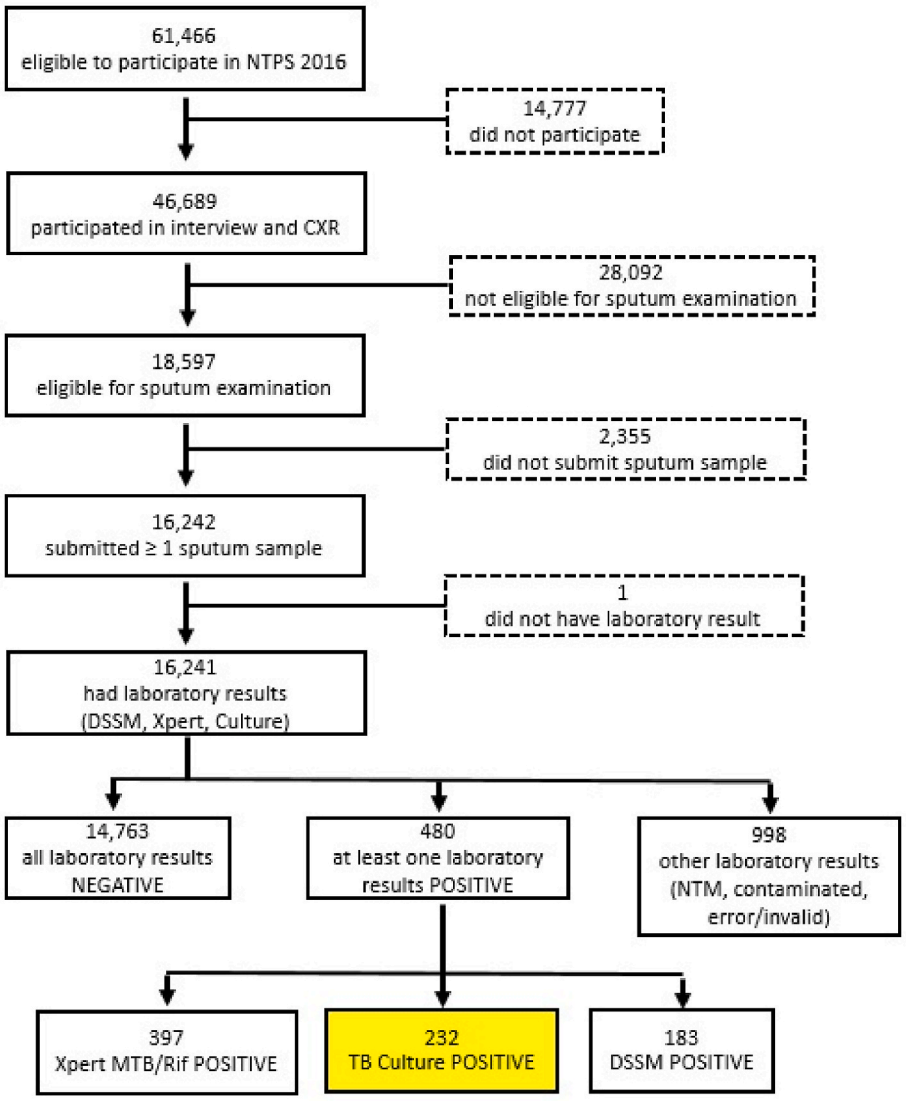

Fig. 1. Patient recruitment and flowchart, from NTPS 2016 [3].

associations between the patient characteristics among different *M. tuberculosis* genotypes or strains were determined using Fisher's exact test. Univariate and logistic regression analyses were performed for possible predictors of genotype. All statistical tests were two-sided and statistical significance was set at a p-value < 0.05.

2.6. Ethical considerations

This study underwent review by the RITM – Institutional Review Board. Stored *M. tuberculosis* isolates were anonymized. Patient records were given alphanumeric codes to ensure privacy and confidentiality of data in compliance to the Philippine Data Privacy Act (2012).

3. Results

Demographic and clinical characteristics for the 100 patients with high quality sequenced isolates are summarized (Table 1). The majority of the patients were male (75, 75%) and belong to the economically active age groups (25–64 years old, 56%), with mean age of 45.3 years (±15.7). Fifty-two (52%) of the TB culture-positive patients had negative acid-fast bacilli smear.

In silico genotyping revealed four lineages of the *M. tuberculosis* isolates sequenced. Most isolates (96%) were identified as the EAI2-Manila strain (Lineage 1.2.1.1). One isolate (1%) was identified as a Beijing strain (lineage 2.2.1.1), which was isolated from a male TB patient from the Bicol Region (Region 5). Another isolate (1%) was identified as a CAS strain (lineage 3) isolated from an elderly female from Bicol region also. Two isolates (2%) were identified as a LAM strain (lineage 4.3.4), one of which was from a male TB patient from Eastern Visayas (Region 8), and the other from a female patient from the National Capital Region (NCR). Patient's age, sex, TB severity, bacillary load and phenotypic DST results were not significantly associated with the genotype of *M. tuberculosis* ($p > 0.05$) (Table 2).

The phylogenetic-geospatial distribution of the 100 *M. tuberculosis* isolates was assessed using a phylogenetic tree constructed from 9893 high quality genome-wide SNPs (Fig. 2). Isolates were linked to the administrative district (barangay) where the patients were sampled, and no clear geospatial/phylogenetic clustering was observed. A reconstructed phylogeny of the EAI2-Manila isolates and those from other publicly available datasets showed that the EAI2-Manila isolates did not form a monophyletic group, but were scattered throughout the phylogenetic tree. Interestingly, one isolate was interleaved with strains from Thailand, while other isolates were interleaved with strains from North America, Europe and Vietnam, among others.

Table 1
Socio-demographic and clinical characteristics of patients derived from the NTPS 2016 (n = 100) [35].

Variable	Frequency (n = 100)	%	Proportion in the Population (%)[a]
Age [Mean ± SD (Range)]	45.3 ± 15.7 (16 – 83)		
15 – 24	14	14.0	
25 – 34	11	11.0	
35 – 44	15	15.0	63.4
45 – 54	30	30.0	
55 – 64	20	20.0	
≥ 65	10	10.0	4.7
Sex			
Male	75	75.0	50.6
Female	25	25.0	49.4
Region of Residence			
Region I	4	4.0	5.0
Region II	3	3.0	3.4
Region III	12	12.0	11.1
Region IV-A	14	14.0	14.3
Region IV-B	4	4.0	2.9
Region V	6	6.0	5.7
Region VI	5	5.0	4.4
Region VII	3	3.0	6.0
Region VIII	5	5.0	4.4
Region IX	4	4.0	3.6
Region X	4	4.0	4.6
Region XI	4	4.0	4.8
Region XII	5	5.0	4.5
ARMM	3	3.0	3.7
CAR	0	0	1.7
CARAGA	6	6.0	2.6
NCR/Metro Manila	12	12.0	12.8
NIR	6	6.0	4.4
Occupation			
Office Worker	3	3.0	
Farmer/Forester	8	8.0	
Laborer / unskilled worker	10	10.0	
Trader / businessperson	9	9.0	
Service worker / sales worker	28	28.0	
Technician / associate professional	1	1.0	
No employment	41	41.0	
History of previous TB treatment			
Yes	17	17.0	
No	83	83.0	
Severity of TB[b]			
Non-severe	59	59.0	
Severe	41	41.0	
Bacillary Load[c]			
Low	93	93.0	
High	7	7.0	
Gene Xpert Results			
MTB detected, RR not detected	60	60.0	
MTB detected, RR detected	11	11.0	
MTB not detected	27	27.0	
Error / Invalid	2	2.0	
Phenotypic DST Results			
Drug-susceptible	70	70.0	
Resistant to any drug	30	30.0	
Rifampicin - monoresistance	4	4.0	
Isoniazid (INH) - monoresistance	13	13.0	
Streptomycin (STR) - monoresistance	4	4.0	
MDR (Rifampicin + Isoniazid)	5	5.0	
INH + STR resistance	3	3.0	
MDR + STR resistance	1	1.0	

[a]The population of the Philippines in 2015 is 100,979,303.[33]
[b]Severe pulmonary TB is defined as pulmonary involvement with cavitary lesions, miliary TB, extensive involvement of one or both lungs, or presence of pleural effusion, in chest x-ray
[c]Bacillary load, determined by acid-fast bacilli (AFB) smear examination using Ziehl-Nielsen method, is classified as "Low" if AFB smear grade ≤ +2 or positive only for TB culture; or "High" if AFB smear grade ≥ +3; RR - Rifampicin resistance

Table 2
Association of *M. tuberculosis* genotype with demographic and clinical characteristics of patients derived from the NTPS 2016 (n = 100).

Patient Characteristics	Genotypes/Sublineages		Total	p-value[a]
	EAI2-Manila	Others		
Age				0.587
15-24	14	0	14	
25-34	11	0	11	
35-44	14	1	15	
45-54	28	2	30	
55-64	20	0	20	
≥65	9	1	10	
Sex				0.260
Male	73	2	75	
Female	23	2	25	
Severity of TB				0.142
Non-severe	55	4	59	
Severe	41	0	41	
Bacillary Load				1.000
Low	89	4	93	
High	7	0	7	
Drug-resistance (phenotypic)				1.000
Susceptible	67	3	70	
Monoresistant	20	1	21	
INH + STR resistance	3	0	3	
MDR	6	0	6	

[a] Fisher's exact test, two-sided; INH - Isoniazid; STR - Streptomycin.

Inferred *in silico* drug resistance profiling revealed that 66 isolates (66%; 63 EAI2-Manila strains) were pan drug-susceptible (Table 3). Twenty-five EAI2-Manila isolates and one Beijing strain were classified as pre-MDR, that is, resistant to either isoniazid or rifampicin. Five isolates were MDR-TB, all of which were EAI2-Manila strain. Three isolates (all EAI2-Manila) were resistant to other drugs streptomycin, pyrazinamide or ethambutol only. *M. tuberculosis* genotype was not significantly associated with *in silico* drug resistance.

A total of 21 point mutations in nine genes related to drug resistance were identified by bioinformatic analysis of the genome sequences (Table 4). However, only eight mutations have frequencies greater than one. All mutations were found in genes related to resistance to first-line drugs (*i.e.,* isoniazid, rifampicin, streptomycin, pyrazinamide and ethambutol), and none were found in genes related to resistance to fluoroquinolones and second-line injectable drugs (*e.g.,* kanamycin, capreomycin, amikacin). The C-15 T mutation in the *inhA* promoter and the S315T mutation in the *katG* gene are the most common mutations in isoniazid-monoresistant and MDR isolates, but they were also found in phenotypically drug-susceptible isolates. Notably, the S450L mutation in the *rpoB* gene was identified in the MDR and rifampicin-monoresistant isolates, but not in drug-susceptible isolates. The H445Y mutation in the *rpoB* gene was identified in two rifampicin-monoresistant and one MDR isolates. Surprisingly, three isolates with rifampicin-resistance detected by Xpert MTB/Rif were not confirmed by WGS. No gene mutations were found in three isoniazid-monoresistant, one rifampicin-monoresistant and two streptomycin-monoresistant isolates. Ten of the phenotypically drug-susceptible isolates were found to harbor mutations in the *inhA* promoter, *rpoB*, *embB*, *rrs* or *rpsL* genes.

4. Discussion

Our study reports the predominance of the EAI2-Manila strain among *M. tuberculosis* isolates collected from patients derived from the NTPS 2016. This finding is consistent with previously published molecular epidemiologic studies on *M. tuberculosis* in the Philippines – both in prison and community settings [12,13]. The EAI2-Manila strain also comprised 80% of the isolates derived from the Philippines' Second TB Drug Resistance Survey (DRS 2) in 2012 [6]. More generally, the EAI lineage is the dominant clade of *M. tuberculosis* in Southeast Asian countries, such as Malaysia, Philippines, Singapore, Myanmar, and Thailand, and some South Asian countries like Bangladesh and Sri Lanka. Sub-lineages of EAI have been found to be endemic in specific counties, such as the EAI1-MYS strain in Malaysia [14], EAI4-VNM strain in Vietnam [15], and EAI2-Manila strain in the Philippines [11]. The EAI2-Manila genotype has been found among TB patients in countries where there are large Filipino communities or migrant workers, like Saudi Arabia [16], Tuscany, Italy [17], Acapulco, Mexico [18] and Hawaii [19].

The EAI2-Manila strain belongs to the *M. tuberculosis* lineage 1, which is considered as an ancient lineage because of the presence of the 52-bp TbD1 region in the mycobacterial genome, which was deleted in the modern lineages (*i.e.,* lineages 2, 3, and 4) [5]. Lineage 1 strains are endemic in 11 of the 30 high TB-burden countries, including the Philippines. A recent genomic analysis of *M. tuberculosis* isolates from patients in northern Thailand revealed a total of 18 EAI sublineages or strains distributed into three major clades, namely sub-lineages 1.1, 1.2.1 (EAI2 clade) and 1.2.2. In this Thai study, 12 isolates were identified as EAI2-Manila strain [20]. In a more recent whole genome analysis of Philippine *M. tuberculosis* isolates derived from DRS 2, a large number (n = 114, 64%) of EAI2-Manila (lineage 1.2.1) isolates formed a large monophyletic clade comprising mainly of Thai strains, suggesting that lineage 1.2.1 strains in Thailand originated from the Philippines and later diversified [6]. In our study, however, only one (1%) EAI2-Manila isolate was found to be interleaved with Thai strains. The rest of the isolates were scattered in the phylogeny with isolates collected from other parts of the world, suggesting the spread of this clade in other regions.

While the exact reason for the predominance of *M. tuberculosis* lineages in specific geographical regions is not known, several studies attribute this to human migration, climate conditions, trade activities, among others [21]. The spread, expansion and predominance of EAI lineages in some Asian countries are hypothesized to have been caused by ancient human migration and trade routes between Eastern Africa, Southern Asia and Southeast Asia [22]. The origin of the EAI lineage is thought to be Africa, where this lineage was first described, after which it was transported through human migration to East Asia where it continued to spread and flourish [23,24]. Southern Taiwan, the origin of the Austronesian language-speaking people who further migrated to the Pacific islands, is believed to be the source of the EAI strains that later diversified to sub-lineages in specific geographical areas, such as the EAI2-Manila strain in the Philippines [20,21]. This observation is supported by a report that a significant portion of the *M. tuberculosis* isolates in Southern Taiwan are EAI lineage, the majority of which are the EAI2-Manila strain [23].

Moreover, there have been studies pointing to host-pathogen co-adaptation or co-evolution as a key to the predominance of specific *M. tuberculosis* lineages in certain geographical regions. The ancient lineages 1, 5 and 6 are observed to be more restricted in terms of geographic distribution, compared to the modern lineages 2, 3 and 4. For example, the EAI clade under lineage 1 is prevalent in South Asia, Southeast Asia and Pacific regions, while the Beijing clade under lineage 2 is globally distributed [25]. These findings also reflect the differences in transmissibility and virulence between the lineages. Gagneux [4] hypothesized that less virulent strains, like EAI sublineages, can persist longer in the host population compared to more virulent ones, like the Beijing or lineage 2 strains, because the latter cause higher mortality. However, more virulent strains can transmit TB infection better by causing more severe (*e.g.*, cavitary disease) or higher bacillary load in TB patients [25,26]. The increased virulence of the modern *M. tuberculosis* lineages has also been demonstrated in *vitro*, animal and human studies [25]. Similarly, one study performed in Montreal, Canada showed that EAI lineage was found to have reduced transmissibility and less associated with severe forms of TB [27]. In our study, *M. tuberculosis* lineage, particularly EAI2-Manila strain, was not significantly associated with TB severity and bacillary load, as well as with patient's age and sex. This finding is consistent with a molecular epidemiologic analysis of 116

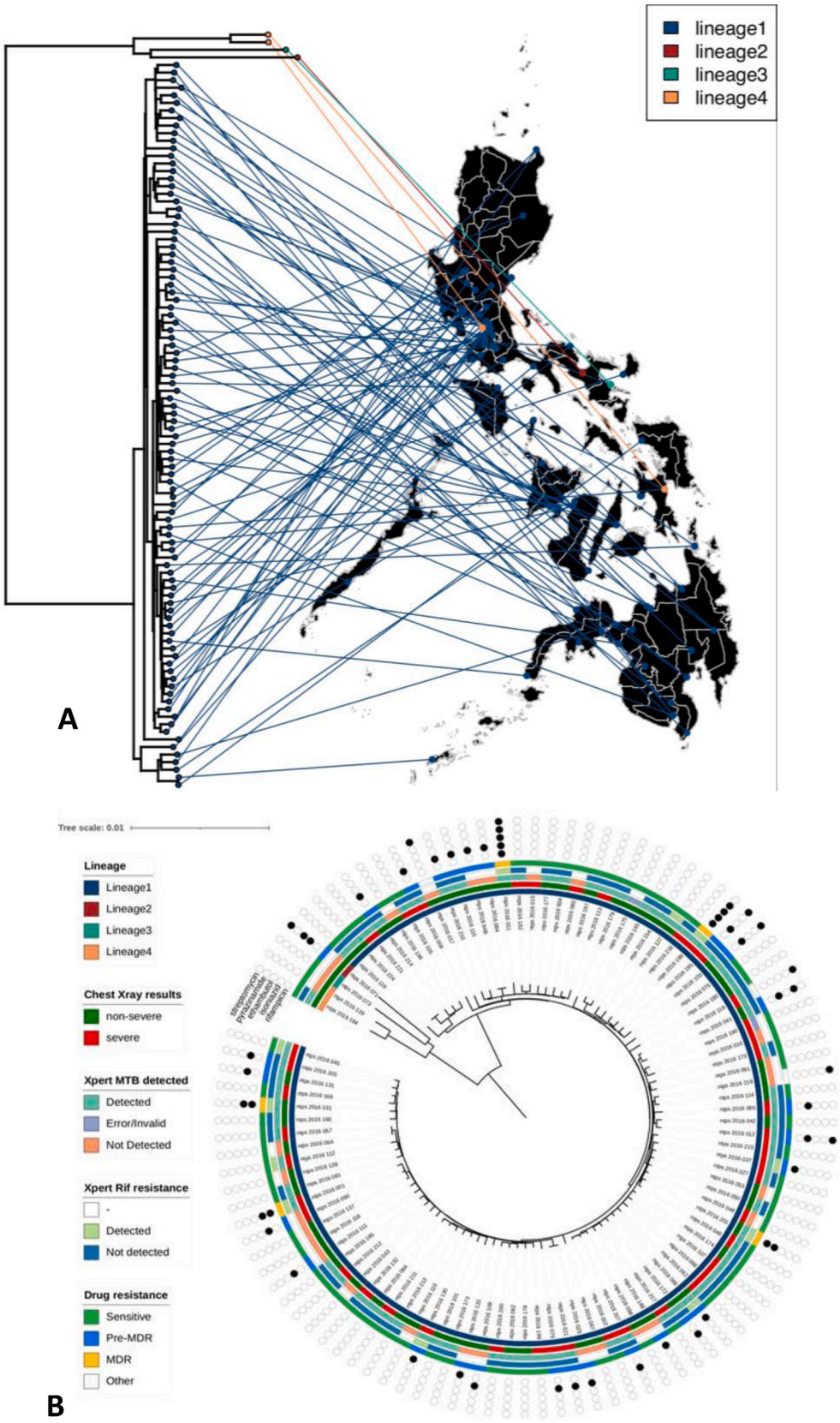

Fig. 2. The 100 *M. tuberculosis* isolates derived from NTPS 2016: **(A)** The phylogenetic-geospatial distribution of isolates from this study. Isolates have been linked to the barangay where they have been sampled; **(B)** Whole genome phylogeny of the isolates annotated with lineage, chest X-ray results, Xpert MTB/Rif results, and *in silico* drug resistance prediction by TB-Profiler.

Table 3
The *in silico* inferred drug resistance and lineage profiles of *M. tuberculosis* isolates sequenced.

Sub-lineage	Sensitive	Pre-MDR	MDR	Others	Total	p-value[a]
EAI2-Manila	63	25	5	3	96	1.000
Others	3	1	0	0	4	
Total	66	26	5	3	100	

[a] Fisher's exact test, two-sided; MDR multi-drug resistant.

Table 4
Drug resistance-related nucleotide mutations, amino acid substitution and their frequencies in the isolates sequenced.

Drug	Gene	Nucleotide Mutation	Amino Acid Substitution	Frequency
Isoniazid	*katG*		S315T	13
			Q195K	1
Isoniazid/ Ethionamide	*inhA promoter*	−15 C → T −8 T → C		12 1
Isoniazid	*inhA*		I21V	2
Rifampicin	*rpoB*		Q432E	1
			D435G	1
			D435Y	1
			H445Y	2
			H445 N	1
			S450L	4
Ethambutol	*embB*		M306V	1
			M306I	1
Ethambutol	*embA*	−12 C → T		1
Pyrazinamide	*pncA*	−11 A → C	D12A	1
				1
			W119*	1
			A146T	1
Streptomycin	*rrs*	514 A → C		4
Streptomycin	*rpsL*		K88R	2
			K43R	2

M. tuberculosis isolates from patients in a suburban community in the Philippines [23]. The prevalence of the EAI lineage in regions like the Philippines is also attributed to the strain's ability to adapt to tropical climate or high-temperature environments. This insight was based from the observation that EAI strains are predominant in southern Taiwan, which has more tropical climate compared to northern Taiwan where the Beijing strain is predominant [21]. A systematic review also reported that lineage 1 strains, such as the EAI clade, were associated with increased transmission in East Asia only, suggesting that geographic location may have some effect on the behavior of specific *M. tuberculosis* strains [28].

No significant association between the EAI2-Manila strain and drug resistance was found in our work, consistent with a study of prison inmates in the Philippines [13]. The EAI clade of *M. tuberculosis* has not been reported by studies to be associated with drug resistance, in contrast to the Beijing strains that are commonly associated with drug-resistant TB [25,28,29]. Even in settings where EAI clade is predominant and MDR-TB burden is high, EAI strains have not been significantly associated with resistance to *anti*-TB drugs [30]. Moreover, drug-susceptible TB cases caused by lineage 1 strains have been shown to respond better to treatment, as demonstrated by faster sputum culture conversion, compared to TB cases caused by modern *M. tuberculosis* clades [26]. While our study reports the predominance of drug-susceptible TB in cases caused by EAI2-Manila strain, we do not have data on the treatment outcomes of the patients studied, which can be explored in future research.

The Philippine NTPS 2016 reported a prevalence rate of 18% for phenotypic resistance to any drug, 5.6% for rifampicin resistance, and 2.6% for MDR among 232 patients with positive TB culture [3]. We performed WGS analysis of 100 of these isolates and report 21 mutations in nine genes. Only eight mutations have frequencies greater than one, all of which are found in genes related to resistance to first-line drugs. All eight mutations were also reported by the WGS analysis on *M. tuberculosis* isolates derived from the Philippine DRS 2 [6]. Consistent with their findings, we also report the most common mutations in the *katG* gene and *inhA* promoter region associated with isoniazid resistance to be S315T and C-15 T, respectively, and S450L mutation in *rpoB* gene associated with rifampicin resistance. These mutations were classified as high-frequency SNPs in a genome-wide analysis study of MDR and XDR-TB isolates from more than 30 countries [31]. No pre-XDR and XDR isolates were noted from the *in silico* analysis, which is consistent with findings of low incidence of quinolone resistance among *M. tuberculosis* isolates in the Philippines [32]. The sensitivity and specificity of drug resistance prediction using mutations from WGS, with phenotypic DST as gold standard, has been reported to be high (>90%) for rifampicin and isoniazid [7]. Due to the relatively small sample size of this study, the sensitivity and specificity of the identified mutations to predict phenotypic drug resistance were not calculated.

Notably, only 60 of the phenotypically drug-sensitive isolates do not harbor mutations in any gene, and nine isolates have a single mutation in one of the genes related to first-line drug resistance. Interestingly, the Beijing isolate, which was phenotypically pan-susceptible, was found to contain single mutations in the *katG*, *embB* and *rpsL* genes. This observation is not uncommon because Beijing strains have been associated with drug resistance [28,29]. Discrepancies between the phenotypic drug resistance results and WGS analysis can be attributed to many factors, as drug resistance in *M. tuberculosis* is said to be an interplay of molecular mechanisms or mutations in several resistance-conferring genes. Some mutations are associated with low-level drug resistance, which cannot be detected by routine phenotypic DST because of standard concentrations of drugs used for testing [33]. Other mechanisms of drug resistance have also been postulated, such as the compensatory evolution, efflux-mediated resistance and deficient DNA repair mechanisms [31,33]. The coexistence of wildtype and resistant isolates in a culture can also explain the discrepancy between the phenotypic DST and gene sequencing results [34].

Our study is one of the largest WGS studies of the EAI2-Manila clade, showing that this strain is prevalent all over the Philippines. SNP variability is high among EAI2-Manila isolates, consistent with previous work [6], and in keeping with the genetically diverse nature of lineage 1, as shown by the WGS analysis of isolates from Thailand [20] and other countries [25]. The SNPs identified as specific to the EAI2-Manila clade can be used as basis for strain barcoding for surveillance of circulating *M. tuberculosis* strains in the country and other regions.

5. Conclusions

Whole genome analysis of *M. tuberculosis* isolates from patients derived from NTPS 2016 revealed the predominance of EAI2-Manila clade in the Philippines, consistent with previous results from molecular epidemiologic studies on TB in the country. EAI2-Manila clade was not significantly associated with patient's age, sex, TB severity, bacillary load and phenotypic drug resistance profile. Analysis also revealed high SNP variability among EAI2-Manila isolates, confirming the large genetic diversity among lineage 1 clades. There was no clear geospatial-phylogenetic clustering observed, and EAI2-Manila isolates were scattered throughout the phylogenetic tree that included EAI2-Manila isolates from other countries, suggesting that this clade has spread to other parts of the world, probably through the influx of Filipino migrant workers. Mutations identified in the genes related to drug resistance were similar to those reported by previous studies that sequenced Philippine *M. tuberculosis* isolates. Whole genome sequencing of additional drug-susceptible and drug-resistant isolates from national surveys would provide more information on TB transmission and development of drug resistance among Filipino TB patients and assist the development of databases to inform clinical and infection control decision making.

Author contributions statement

JCM: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Roles/Writing – original draft; Writing – review & editing; JCMM: Data curation; Investigation; Methodology; Resources; Roles/Writing – original draft; Writing – review & editing; CFA: Data curation; Investigation; Methodology; Project administration; Resources; Roles/Writing – original draft; Writing – review & editing; LTR: Data curation; Investigation; Methodology; Resources; RPB: Data curation; Investigation; Methodology; Resources; Supervision; Writing – review & editing; DRL: Data curation; Methodology; Project administration; Resources; Writing – review & editing; MLEA: Data curation; Methodology; Formal Analysis; Writing – review & editing; MCGA: Methodology; Project administration; Resources; Supervision; Writing – review & editing; JEP: Data curation; Methodology; Formal Analysis; Writing – review & editing; MLH: Data curation; Methodology; Formal Analysis; Writing – review & editing; TGC: Data curation; Methodology; Formal Analysis; Writing – review & editing.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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References

[1] World Health Organization. Global tuberculosis report 2021. 2021.
[2] World Health Organization. https://www.who.int/news/item/17-06-2021-who-releases-new-global-lists-of-high-burden-countries-for-tb-hiv-associated-tb-and-drug-resistant-tb; 2021.
[3] Lansang, et al. High TB burden and low notification rates in the Philippines: the 2016 National TB Prevalence Survey. PLoS One 2021;16(6):e0252240. https://doi.org/10.1371/journal.pone.0252240.
[4] Gagneux S. Genetic diversity in *Mycobacterium tuberculosis*. Curr Top Microbiol Immunol 2013. https://doi.org/10.1007/82_2013_329.
[5] Galagan JE. Genomic insights into tuberculosis. Nat Rev Genet 2014. https://doi.org/10.1038/nrg3664.
[6] Phelan JE, et al. *Mycobacterium tuberculosis* whole genome sequencing provides insights into the Manila strain and drug-resistance mutations in the Philippines. Nat. Sci. Rep. 2019;9:9305.
[7] Napier G, et al. Robust barcoding and identification of *Mycobacterium tuberculosis* lineages for epidemiological and clinical studies. Genome Med 2020;12:114. https://doi.org/10.1186/s13073-020-00817-3. 2020.
[8] Coll F, et al. SpolPred: rapid and accurate prediction of *Mycobacterium tuberculosis* spoligotypes from short genomic sequences. Bioinformatics 2012;28:2991–3. http://www.ncbi.nlm.nih.gov/pubmed/23014632.
[9] Phelan J, et al. Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculous drugs. Genome Med 2019;11:41. https://doi.org/10.1186/s13073-019-0650-x.
[10] Coll, et al. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Med 2015;7(1):51. 2015.
[11] Douglas JT, Qian L, Montoya JC, Musser JM, Van Embden JD, Van Soolingen D, Kremer K. Characterization of the manila family of *Mycobacterium tuberculosis* in Filipino patients with tuberculosis. J. Clin. Microbiol. 2003;41(6):2723–272615.
[12] Montoya JC, Murase Y, Ang CF, Solon J, Ohkado A. Molecular epidemiologic analysis of *Mycobacterium tuberculosis* among Filipino patients in a suburban community in the Philippines. Kekkaku 2013;88(6):543–52.
[13] Montoya JC, et al. Molecular epidemiologic analysis of Mycobacterium tuberculosis among prison inmates in selected prisons in the Philippines. Philipp J Sci 2021;150(2):417–27.
[14] Ismail F, et al. Study of *Mycobacterium tuberculosis* complex genotypic diversity in Malaysia reveals a predominance of ancestral east-african-Indian lineage with a Malaysia-specific signature. PLoS One 2014;9(12):e114832. https://doi.org/10.1371/journal.pone.0114832.
[15] Nguyen V, Choisy M, Nguyen D, et al. High prevalence of beijing and EAI4-VNM genotypes among *Mycobacterium tuberculosis* isolates in northern Vietnam: sampling effect, rural and urban disparities. PLoS One 2012;7(9):e45553.
[16] Varghese B, Supply P, Shoukri M, et al. Tuberculosis transmission among immigrants and autochthonous populations of the eastern province of Saudi Arabia. PLoS One 2013;8(10):e77635. https://doi.org/10.1371/journal.pone.0077635.
[17] Lari N, Rindi L, Bonnani D, et al. Three-year longitudinal study of genotypes of *Mycobacterium tuberculosis* isolates in Tuscany, Italy. J Clin Microbiol 2007;45(6):1851–7.
[18] Nava-Aguilera E, Lopez-Vidal Y, Harris E, et al. Clustering of *Mycobacterium tuberculosis* cases in Acapulco: spoligotyping and risk factors. Clin Dev Immunol 2011;2011. Article ID 408375.
[19] Koster KJ, Largen A, Foster JT, et al. Genomic sequencing is required for identification of tuberculosis transmission in Hawaii. BMC Infect Dis 2018;18:608–21.
[20] Palittapongarnpim P, et al. Evidence for host-bacterial coevolution via genome sequence analysis of 480 Thai *Mycobacterium tuberculosis* lineage 1 isolates. Nat. Sci. Rep. 2018;8:11597. https://doi.org/10.1038/s41598-018-29986-3.
[21] Conceicao E, Refregier G, Gomes H, et al. *Mycobacterium tuberculosis* lineage 1 genetic diversity in Pará, Brazil, suggests common ancestry with east-African isolates potentially linked to historical slave trade. Infect Genet Evol 2019;73:337–41. https://doi.org/10.1016/j.meegid.2019.06.001.
[22] O'Neill M, Shockey A, Zarley A, et al. Lineage specific histories of *Mycobacterium tuberculosis* dispersal in Africa and Eurasia. Mol Ecol; 2019 (2019), 10.1111/mec.15120.
[23] Chang J, Chen Y, Huang T, et al. Clonal expansion of both modern and ancient genotypes of *Mycobacterium tuberculosis* in southern taiwan. PLoS One 2012;7(8):e43018.
[24] Reed M et al. Major *Mycobacterium tuberculosis* lineages associate with patient country of origin. J Clin Microbiol 47(4): 1119-1128. DOI: 10.1128/JCM.02142-08.
[25] Coscolla M, Gagneux S. Consequences of genomic diversity in *Mycobacterium tuberculosis*. Semin Immunol 2014;26(6):431–44. https://doi.org/10.1016/j.smim.2014.09.012.
[26] Click ES, et al. Association between *Mycobacterium tuberculosis* lineage and time to sputum culture conversion. Int J Tubercul Lung Dis 2013;17(7):878–84.
[27] Albanna A, Reed M, Kotar K, et al. Reduced transmissibility of east african Indian strains of *Mycobacterium tuberculosis*. PLoS One 2011;6(9):e25075.
[28] Wiens K, Woyczynski L, Ledesma J, et al. Global variation in bacterial strains that cause tuberculosis disease: a systematic review and meta-analysis. BMC Med 2018;16(1):196. https://doi.org/10.1186/s12916-018-1180-x.
[29] Ramazanzadeh R, Sayhemiri K. Prevalence of beijing family in *Mycobacterium tuberculosis* in world population: systematic review and meta-analysis. Int J Mycobacteriol 2014;1:41–5. https://doi.org/10.1016/j.ijmyco.2014.01.001.
[30] Shanmugama S, Selvakumar N, Narayanan S. Drug resistance among different genotypes of *Mycobacterium tuberculosis* isolated from patients from Tiruvallur, South India. Infect Genet Evol 2011;11:980–6. https://doi.org/10.1016/j.meegid.2011.03.011.
[31] Coll F, et al. Genome-wide analysis of multi- and extensively drug-resistant *Mycobacterium tuberculosis*. Nat Genet 2018. https://doi.org/10.1038/s41588-017-0029-0.
[32] Lim DR, et al. Low prevalence of fluoroquinolone resistance among patients with tuberculosis in the Philippines: results of a national survey. Eur Respir J 2018;51:1702571. https://doi.org/10.1183/13993003.02571-2017.
[33] Dookie N, et al. Evolution of drug resistance in *Mycobacterium tuberculosis*: a review on the molecular determinants of resistance and implications for personalized care. J Antimicrob Chemother 2018;73:1138–51.
[34] Aye KS, et al. Genotypic characterization of multidrug-resistant *Mycobacterium tuberculosis* isolates in Myanmar. J Infect Chemother 2016;22(3):174–9.
[35] Philippine Statistics Authority. Philippines in figures 2019. Available at: https://psa.gov.ph/sites/default/files/PIF2019_revised.pdf; 2019.