Genetic diversity and drug resistance profiles of *Mycobacterium tuberculosis* complex isolates from patients with extrapulmonary tuberculosis in Ghana and their associated host immune responses

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ABSTRACT

Objectives: This study sought to determine the genetic diversity and drug resistance profiles of *Mycobacterium tuberculosis* complex (MTBC) isolates from extrapulmonary tuberculosis (EPTB) patients in Ghana, and their associated immune responses.

Methods: Spoligotyping was performed on 102 MTBC isolates from EPTB patients. Lineages/sub-lineages were assigned by comparing spoligotyping patterns primarily with the SITVIT2 database and subsequently with the TB-Lineage online tool for unknown isolates in SITVIT2. Drug susceptibility testing was performed using MGIT (BD BACTEC 960), Lowenstein-Jensen media (indirect proportion method), and GenoType MTBDR*plus*/MTBDR*sl* assays. Differential cytokine levels in the serum of 20 EPTB patients infected with MTBC lineage 4 were determined using the Luminex multiplex immunoassay.

Results: Around 95% (97/102) of isolates were *Mycobacterium tuberculosis*, predominantly lineage 4 (95%; 92/97). Of the lineage 4 isolates, the majority were sub-lineage Cameroon (37%, 34/92). Prevalence was significantly higher in the 15–34 years age group among EPTB patients infected with lineage 4 strains ($p = 0.024$). Fifteen isolates were resistant to at least one anti-TB drug tested. Decreased levels of IL-1β, IL-17A, and IFN-α were observed in individuals infected with Cameroon sub-lineages compared with other lineage 4 sub-lineages.

Conclusions: Our study confirms Cameroon (SIT61) as the most common spoligotype causing human EPTB in Ghana, and that it is associated with decreased serum IL-1β, IL-17A, and IFN-α.

Introduction

The World Health Organization (WHO) declared tuberculosis (TB) a global health emergency in 1993. Despite several interventions, TB remains a major threat to public health (WHO, 1993). Primarily, TB affects the lungs – pulmonary TB (PTB) – but can manifest in almost every part of the body as extrapulmonary TB (EPTB). EPTB constitutes around 20–30% of TB cases globally (Addo et al., 2021). However, many TB control activities and the majority of studies undertaken are focused mostly on PTB. This may be attributed to the misguided belief that EPTB constitutes a minimal threat to public health, because it is often not contagious. However, recent studies have shown that EPTB can have a huge impact on morbidity and mortality (Arega et al., 2020; Ohene et al., 2019).

Tuberculosis is caused by the Mycobacterium tuberculosis complex (MTBC), comprising human-adapted species [*Mycobacterium tuberculosis* (*M. tuberculosis*), *Mycobacterium africanum* (*M. africanum*)] and animal-adapted species [*Mycobacterium bovis* (*M. bovis*), *Mycobacterium canettii* (*M. canettii*), *Mycobacterium caprae* (*M. caprae*), *Mycobacterium microti* (*M. microti*), *Mycobacterium pinnipedii* (*M. pinnipedii*)]. *M. bovis* is a zoonotic species, which primarily infects cattle but can infect humans. The human-adapted species can further be classified into nine phylogenetic lineages (Kanabalan et al., 2021). These lineages differ in some genotypic and phenotypic characteristics, such as host specificity, growth kinetics, metabolism, virulence, and geographical niche (Kanabalan et al., 2021; Osei-Wusu et al., 2021). Studies have shown that the genotyping of MTBC species and determining their drug-
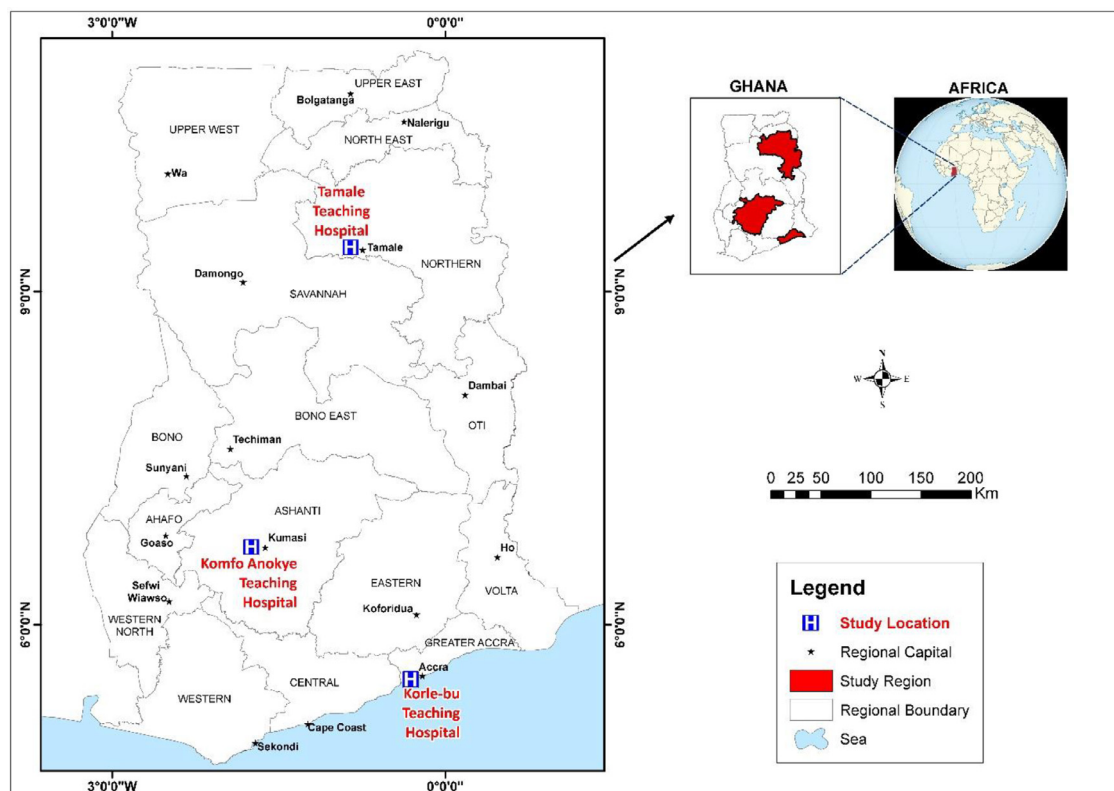
Figure 1. Map of Ghana showing the locations of the study sites

resistance profiles are key surveillance tools in supporting TB control. These help in identifying the sources of infection and recent TB transmission or reactivation patterns, as well as the phylogenetic and evolutionary relationships among strains (Biadglegne et al., 2015).

Researchers have shown that certain lineages elicit increased expression of certain cytokines (Domingo-Gonzalez et al., 2016; Ranaivomanana et al., 2021; Reiling et al., 2018). Previous studies have reported the types of MTBC genotype circulating in Ghana, their drug resistance profiles, and the types of immune response they trigger in the hosts by measuring certain cytokine levels (Acquah et al., 2021; Basingnaa et al., 2018). However, all these studies involved patients with PTB, and there has been no such study conducted involving EPTB patients. Therefore, our study aimed to identify the circulating MTBC strains causing EPTB, and establish their drug resistance patterns and cytokine levels in the serum of EPTB patients.

Methods

Study design

This was a hospital-based study in which both prospective and retrospective samples were used.

Study settings

The study was conducted at three tertiary hospitals in Ghana: Korle Bu Teaching Hospital (KBTH), Komfo Anokye Teaching Hospital (KATH), and Tamale Teaching Hospital (TTH), located in Accra (southern Ghana), Kumasi (central Ghana), and Tamale (northern Ghana), respectively (Figure 1). As major referral hospitals, KBTH, KATH, and TTH receive patients from all over Ghana and neighbouring countries, such as Burkina Faso, Cote d'Ivoire, and Togo.

Patient recruitment and sample collection

Patients with clinically suggestive EPTB ($n = 286$) receiving routine laboratory services at the included hospitals were prospectively recruited into the study after obtaining informed consent and subject to the availability of enough samples (aspirates, biopsies, fluids, and pus from affected body sites) after routine diagnostic testing. Demographic and clinical data were obtained by administering study-specific questionnaires and retrieving information from laboratory request forms and registers. Samples were stored briefly at –20°C and transported to Noguchi Memorial Institute for Medical Research (NMIMR) within 48 hours for laboratory testing. Due to the low culture positivity rate among the prospective samples (65/286; 23%), 37 MTBC isolates obtained from routine laboratory testing of extrapulmonary samples at the same hospitals between 2015 and 2018 were added retrospectively, increasing the total sample size to 323 and available MTBC isolates to 102.

Culture and identification

All specimens except cerebrospinal fluid (CSF) were processed using the NALC-NaOH digestion/decontamination technique, followed by refrigerated centrifugation at 3000 × g for 20 minutes. The supernatant was decanted, and the sediment was resuspended in 2 ml of sterile phosphate-buffered saline (PBS). Next, 0.5 ml was added to an MGIT (Mycobacteria Growth Indicator Tube) and 0.1 ml inoculated onto LJ (Lowenstein-Jensen) media. The tubes were incubated at 37°C for up to 42 days for MGIT and 8 weeks for LJ media. Isolates were identified as MTBC using an MGIT TBID Kit (Becton Dickenson, USA), an immunochromatographic assay based on the MPT64 protein.

DNA extraction

Genomic DNA was extracted from MTBC isolates using the heat-killing method. Briefly, three to five colonies from the LJ media were

harvested into 500 ml of sterile distilled water, or three to five drops from the MGIT were placed into a sterile Eppendorf tube. The tubes were centrifuged at 10 000 × *g* for 15 minutes. The supernatant was discarded, and the pellet was resuspended in 100 ml of sterile distilled water and heat-killed at 95˚C for 1 hour, using a heat block. This was followed by sonication for 15 minutes and centrifugation at 13 000 × *g* for 8 minutes. The supernatant containing DNA was used for line probe assays (MTBC speciation and drug susceptibility testing) and spoligotyping.

Speciation of MTBC

The MTBC isolates were speciated using GenoType MTBC (Hain Lifescience, Nehren, Germany) according to manufacturer's instructions. Briefly, extracted DNA was amplified by multiplex PCR with biotinylated primers and amplicons reverse hybridized onto complementary oligonucleotide test strips.

Spoligotyping

Spoligotyping was performed using a spoligotyping kit (Mapmygenome, Hyderabad India) as per the manufacturer's instructions. Briefly, a membrane precoated with 43 spacer oligos was hybridized with amplicons from PCR of the direct-repeat (DR) region of MTBC isolates, using oligonucleotide primers. The membrane was incubated with streptavidin-peroxide and enhanced chemiluminescence (ECL) used to visualize the presence of spacers on X-ray film as small black squares. The patterns obtained were compared with existing patterns in SITVIT2. SITVIT2 (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/) is an updated version of SpolD4 and MIRU-VNTR*plus* databases, comprising 111 635 clinical isolates from 131 countries of isolation (Couvin et al., 2019). The TB-Lineage (http://tbinsight.cs.rpi.edu/run_tb_line age.html) online tool was used to predict major lineages and sub-lineages of isolates whose lineages could not be defined by SITVIT2.

Drug susceptibility testing

Drug susceptibility testing (DST) with isoniazid, INH (0.2 mg/mL), rifampicin, RIF (40 mg/mL), streptomycin, STR (4 mg/mL), and ethambutol, EMB (2 mg/mL) was determined phenotypically by MGIT AST algorithm on the BACTEC MGIT 960 instrument, as previously described (Siddiqi and Rüsch-Gerdes, 2006) and the indirect proportion method on LJ slants. The GenoType MTBDR*plus* version 2 assay (Hain Lifescience, Nehren, Germany) was performed to determine INH- and RIF-resistance patterns and mutations associated with them, according to the manufacturer's instructions. In addition, all first-line drug-resistant isolates were further tested for resistance to fluoroquinolones (ofloxacin/moxifloxacin) and second- line injectable drugs (kanamycin/amikacin, capreomycin/viomycin) using GenoType MTBDR*sl* version 2 (Hain Lifescience, Nehren, Germany). Briefly, this involved DNA extraction, multiplex PCR with biotinylated primers, reverse hybridization, stringent, conjugate and substrate reactions, and evaluation of bands using an evaluation sheet developed by the manufacturer.

Luminex Multiplex Immunoassay

To determine whether there was any significant association between host immune response and the genotype of the infecting *M. tuberculosis*, the levels of different cytokines, chemokines, and growth factors in the serum of EPTB patients infected with lineage 4 strains (Cameroon, T1, or 'other' comprising H3, T2, T3, X3) were measured. Prior to commencement of anti-TB treatment, 20 bacteriologically confirmed (Xpert MTB/RIF assay) EPTB patients with HIV-negative status from study population were selected for collection of blood samples. About 5 ml of

venous whole blood was drawn into serum separator tubes (SST) (Becton Dickenson, USA). The SST were centrifuged to obtain serum, and the levels of cytokines (IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ), chemokines (MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP-1) and growth factors (GM-CSF) were measured using a Human Cytokine Magnetic 25-Plex Panel (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions. The multiplex immunoassay involved three main steps: (i) analytes (cytokine/chemokine/growth factor) capture by antibody beads; (ii) analytes detection by biotinylated detector antibody; and (iii) reading and analysis of results on the Luminex™ 200™ equipment using xPONENT® software (Luminex Corporation, TX, USA). All test samples and seven standard controls (included in the kit) were diluted and run in duplicates, and the average of the two concentrations for each test sample recorded.

Data analysis

Descriptive statistics, such as frequencies, percentages, medians, and interquartile ranges (Microsoft Excel 365; Microsoft Corporation, WA, USA), were used to describe patients' sociodemographic and clinical characteristics. Sociodemographic and clinical characteristics associated with MTBC lineages were then evaluated using chi-square analysis. The rate of diversity among isolates was calculated by dividing the number of spoligotype patterns by the total number of isolates. Two or more isolates with identical spoligo patterns defined a cluster. Recent transmission was estimated by calculating clustering rate, using a formula by Glynn et al., (1999): $CR = (nc − c)/n$, where CR is the clustering rate, nc is the total number of clustered isolates, c is the total number of clusters, and n is the total number of isolates. An online tool was used to calculate the discriminatory power (Hunter and Gaston, 1988) (http://insilico.ehu.es/mini_tools/discriminatory_power). Data from the Luminex immunoassays were analyzed with Kruskal-Wallis and Mann-Whitney U tests, using GraphPad Prism version 9.2.0 (GraphPad Software Inc., CA, USA). A *p*-value < 0.05 was considered statistically significant.

Results

Sociodemographic characteristics

A summary of the demographic and clinical characteristics of study participants is presented in Table 1. Around 57% (184/323) were males and the median (interquartile range) age was 39 (24–55). Patients aged 25–34 and 35–44 years had the same and highest number of isolates (16.7%, 54/102). Around 87% (281/323) were urban dwellers and 53% (172/323) were employed. The majority (95%, 307/323) of patients had no history of TB and 8% (27/323) were HIV positive. Out of 323 samples, 102 were culture positive, comprising 65 from prospective samples and 37 from archived samples.

Site of infection

The samples were classified into eight categories based on site of infection. The pleura accounted for the majority of infected sites (116, 35.9%). The rest comprised lymph nodes (50, 15.5%), gastrointestinal (48, 14.9%), multiple sites (42, 13.0%), central nervous systems (CNS) (28, 8.7%), and bones and joints (15, 4.6%). Sites with fewer than 10 occurrences were further grouped as 'other' (24, 7.4%) (Figure 2).

Genetic diversity and assignment of lineages/sub-lineages

Out of 102 isolates, 97 (95.10%) were *M. tuberculosis*, four (3.92%) *M. africanum* and one (0.98%) *M. bovis*. Spoligotyping showed 36 different spoligotype patterns, representing an overall diversity rate of 35%

Table 1

Sociodemographic characteristics of extrapulmonary tuberculosis patients (*n* = 323) at three teaching hospitals in Ghana, 2015–2019.

Characteristics	All patients, *n* = 323	%	Patients with culture-positive samples, *n* = 102	%
Sex (male:female)	1.3:1		1.5:1	
Male	184	56.97	61	59.8
Female	139	43.03	41	40.12
Age, median (IQR)	39 (55–24)		39 (49.75–26)	
< 15	44	13.62	7	6.86
15–24	40	12.38	14	13.72
25–34	54	16.72	19	18.63
35–44	54	16.72	26	25.49
45–54	47	14.56	13	12.75
55–64	40	12.38	9	8.82
65+	44	13.62	14	13.73
Residence				
Urban	281	87	85	83.33
Rural	42	13	17	16.67
Ethnicity				
Akan	132	40.87	32	31.38
Ewe	22	6.81	6	5.88
Ga-Dangme	31	9.59	6	5.88
Mole-Dagbani	93	28.79	19	18.63
[a]Other	8	2.48	2	1.96
[b]Unknown	37	11.46	37	36.27
Employment				
Employed	172	53.25	47	46.08
Unemployed	22	6.81	7	6.86
[c]Other	112	34.68	31	30.39
Unknown	17	5.26	17	16.67
Educational status				
Pre-school/primary	71	21.98	11	10.79
Middle/JHS	90	27.86	21	20.59
SHS	85	26.32	21	20.59
Tertiary	24	7.43	6	5.88
Non-formal	16	4.95	6	5.88
[b]Unknown	37	11.46	37	36.27
Smoking habit				
Yes	7	2.17	4	3.93
No	279	86.37	61	59.8
[b]Unknown	37	11.46	37	36.27
Alcohol drinking habit				
Yes	26	8.05	9	8.83
No	260	80.49	56	54.9
[b]Unknown	37	11.46	37	36.27
TB history				
Yes	16	4.95	9	8.83
No	307	95.05	93	91.17
Contact with TB patient				
Yes	26	8.05	8	7.84
No	297	91.95	94	92.16
HIV status				
Positive	27	8.36	12	11.76
Negative	166	51.39	74	72.55
Unknown	130	40.25	16	15.69

IQR – interquartile range

[a]Patients belonging to ethnic groups other than Akan, Ewe, Ga-Dangme, and Mole-Dagbani.

[b]Thirty-seven patients whose isolates were retrospectively added to the prospective study samples, and hence their sociodemographic and clinical information were not available.

[c]Includes children below 18 years old, non-working students, pensioners, and elderly persons (above 75 years old)

(36/102). Of these, 16 patterns comprising 82 isolates matched pre-existing Shared International Types (SIT) in SITVIT2, whereas 18 different patterns (18 isolates) were orphans without pre-existing SITs. Two patterns (two isolates) matched an orphan in SITVIT2 and were considered 'new' (Table 2). Seventy-six isolates were grouped into 10 clusters ranging from two to 23 isolates per cluster, with a clustering rate of 65%. The remaining 26 isolates were unique/singletons. The discriminatory power of spoligotyping was 0.82. All the isolates were distributed among the six major lineages of human-adapted MTBC species and *M. bovis*. Lineage 4 was predominant (91.0%, 92/102) among the human-adapted

MTBC species. Other lineages included lineage 3 (3.0%, 3/102), lineage 5 (2.0%, 2/102), lineage 6 (2.0%, 2/102), lineage 1 (1.0%, 1/102), and lineage 2 (1.0%, 1/102). Sub-lineages of around 81% (83/102) of isolates were identified in SITVIT2, with Cameroon (37%, 31/83) and T1 (28%, 23/83) being in the majority. Among the Cameroon and T1 sub-lineages, SIT61 (23/31) and SIT53 (21/23) were the most common (Table 2). Isolates designated as atypic, not defined, and unknown in SITVIT2 were identified using the TB-Lineage online tool as Indo-Oceanic (1), East African Indian (3), Euro-American (Cameroon, H1, H2, H4_Ural_2, Manu2, T2, T3, X2) (10), *Mycobacterium africanum*_AFRI_1
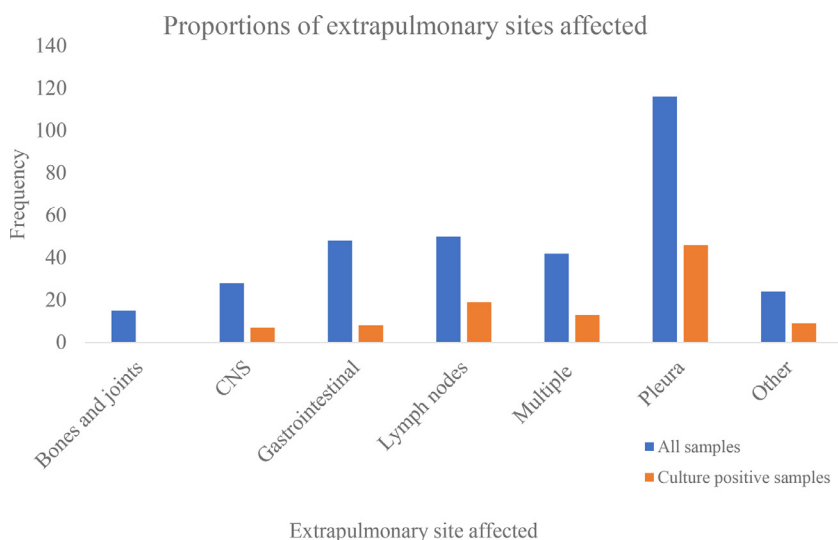
Figure 2. Proportions of sites affected among extrapulmonary tuberculosis patients. CNS: central nervous system. Multiple: multiple sites, including disseminated EPTB. Other: sites with proportions less than 10.

(2), *Mycobacterium africanum*_AFRI_2 (2), and *Mycobacterium bovis*_BOV (1) (Table 2).

Association of sociodemographic and clinical characteristics with MTBC lineages

As shown in Table 3, there was no significant association between sex, residence, TB history, contact with TB patient, major site of infection, and HIV status of EPTB patients and MTBC lineages. However, age group was a significant characteristic, with those aged 15–34 years, followed by those in the 35–54 years age group, being significantly more prevalent among EPTB patients with lineage 4 strains ($p = 0.024$).

Drug susceptibility testing (DST) and mutations associated with resistance

Out of the 102 isolates tested, 87 (85.3%) were pan-susceptible to all first-line anti-TB drugs (STR, INH, RIF, EMB). Fifteen (14.7%) isolates were resistant to at least one of the four drugs tested, including nine with INH monoresistance, three with RIF monoresistance, two with INH/EMB polyresistance, and one with INH/RIF multidrug resistance (MDR) (Table 4). Isoniazid mono-resistance was associated with *katG* (S315T1) (7/9), *katG* (S315T2) (1/9), and *inhA* (C-15T) (1/9). For INH/EMB polyresistance, *katG* (S315T1)/*embB* (M306V) was associated with resistance in both isolates. All three RIF monoresistant isolates had an *rpoB* (S531L) mutation. The MDR was due to mutations in *katG* (S315T1) and *rpoB* (H526D) (Table 4). The patients with INH monoresistant TB and MDR TB had a previous history of TB disease. All the first-line-drug-resistant isolates ($n = 15$) were pan-susceptible to fluoroquinolones (ofloxacin/moxifloxacin) and second-line injectable drugs (kanamycin/amikacin, capreomycin/viocin) (Table 4).

Drug resistance pattern and MTBC lineages

The majority of pan-susceptible isolates (78/87, 89.9%) belonged to lineage 4. Of the rest, three (3.4%) were lineage 3 and two (2.3%) were lineage 6, with one (1.1%) each for lineage 1, lineage 2, lineage 5, and the *M. bovis* lineage. Among the nine INH monoresistant isolates, eight belonged to lineage 4 (T1 = 5, H =1, T3 = 1, X3 = 1) and one belonged to lineage 5. All the RIF monoresistant (Cameroon = 3), INH/EMB polyresistant (T1 = 1, X3 = 1), and MDR (T1 = 1) isolates belonged to lineage 4. Of the 15 resistant isolates, 10 isolates, including MDR (66.7%), were found in clusters (Table 4).

Differential immune responses among patients infected with lineage 4 (L4) strains

According to our analysis, none of the chemokines or growth factors measured showed significant differences when compared across all three categories of patients and pairwise. Conversely, among the cytokines, the serum levels of IL-1β ($p = 0.049$), IL-17A ($p = 0.041$), and IFN-α ($p = 0.012$) were significantly different across all three groups and in some pairwise comparisons. The levels of IL-1β, IL-17A, and IFN-α were significantly lower in individuals infected with Cameroon when compared pairwise with T1 and 'other' (Figure 3). However, differences in levels of IL-1β, IL-17A, and IFN-α observed between T1 and 'other' were not statistically significant.

Discussion

For this study, the lineages of MTBC strains circulating among individuals diagnosed with EPTB in Ghana, as well as their associated drug-resistance patterns and host immune responses, were determined. Spoligotyping, DST, and immunological assays were performed using extrapulmonary samples collected from clinically diagnosed EPTB patients at three teaching hospitals in Ghana.

The diversity of EPTB cases and MTBC strains observed in this study was unsurprising because tertiary-level/teaching hospitals provide diagnostic and treatment services for patients with complex and rare cases of EPTB from all over the country. Pleural TB and TB lymphadenitis (cervical lymph nodes most affected) were the most common EPTB forms. This observation correlated strongly with previous findings from Ghana and elsewhere (Addo et al., 2021; Arega et al., 2020; Ohene et al., 2019). The low culture positivity (65/286, 23.0%) was consistent with the 3–57% reported elsewhere (Cantres-Fonseca et al., 2019; Mbuh et al., 2019; Orvankundil et al., 2019). This could be due to the paucibacillary nature of EPTB samples, and suppression of growth due to the possibly harsh decontamination process.

The majority of isolates (95%; 97/102) were *M. tuberculosis*, which was in line with results from previous studies (Addo et al., 2017; Damena et al., 2019). Moreover, our study identified 3.9% (4/102) of isolates as *M. africanum*, consistent with the 2–20% previously reported in Ghana (Acquah et al., 2021; Addo et al., 2017; Asante-Poku et al, 2016). Similar to previous findings from Ghana, *M. bovis* constituted less than 2% of isolates from human TB patients (Addo et al., 2007; Asante-Poku et al., 2016; Otchere et al., 2019). In contrast, researchers from other countries have reported relatively higher outcomes (Kazwala et al., 2001; Kidane et al., 2002; Portillo-Gómez & Sosa-

Table 2

Description of lineages/sub-lineages of *Mycobacterium tuberculosis* complex isolates identified in clinical samples collected from extrapulmonary tuberculosis patients at three teaching hospitals in Ghana, 2015–2019.

Species	SIT	Major lineage	[a]SITVIT2 lineage	[b]CBN/KBBN lineage	Clustering	Number of isolates
M. tuberculosis	1	L2	Beijing	Not applicable	Unique	1
M. tuberculosis	37	L4	T3	Not applicable	Cluster	2
M. tuberculosis	42	L4	LAM9	Not applicable	Unique	1
M. tuberculosis	53	L4	T1	Not applicable	Cluster	21
M. tuberculosis	57	L4	Cameroon	Not applicable	Cluster	3
M. tuberculosis	61	L4	Cameroon	Not applicable	Cluster	23
M. tuberculosis	86	L4	T1	Not applicable	Unique	1
M. tuberculosis	200	L4	X3	Not applicable	Cluster	5
M. tuberculosis	316	L4	H3	Not applicable	Cluster	6
M. tuberculosis	384	L4	H1	Not applicable	Cluster	3
M. tuberculosis	472	L4	H3	Not applicable	Cluster	3
M. tuberculosis	742	L4	H	Not applicable	Unique	1
M. tuberculosis	772	L4	Cameroon	Not applicable	Unique	1
M. tuberculosis	838	L4	Cameroon	Not applicable	Cluster	4
M. tuberculosis	848	L4	T2	Not applicable	Cluster	6
M. tuberculosis	New	L4	T1	Not applicable	Unique	1
M. tuberculosis	New	L4	X3	Not applicable	Unique	1
M. tuberculosis	2810	L1	ATYPIC	Indo-Oceanic_Manu1	Unique	1
M. tuberculosis	Orphan	L4	Unknown	Euro-American_Manu2	Unique	1
M. tuberculosis	Orphan	L3	Not defined	East African Indian	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_H2	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_T3	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_Cameroon	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_X2	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_Cameroon	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_Cameroon	Unique	1
M. tuberculosis	Orphan	L3	Not defined	East African Indian	Unique	1
M. tuberculosis	Orphan	L3	Not defined	East African Indian	Unique	1
M. tuberculosis	Orphan	L4	Not defined	H4_Ural_2	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_T2	Unique	1
M. tuberculosis	Orphan	L4	Not defined	Euro-American_H1	Unique	1
M. africanum	Orphan	L5	Not defined	*M. africanum_AFRI*	Unique	1
M. africanum	Orphan	L5	Not defined	*M. africanum_AFRI_2*	Unique	1
M. africanum	Orphan	L6	Not defined	*M. africanum_AFRI_1*	Unique	1
M. africanum	Orphan	L6	Not defined	*M. africanum_AFRI_1*	Unique	1
M. bovis	Orphan	Bovis	Not defined	*M. bovis_BOV*	Unique	1

CBN – conformational Bayesian network; KBBN – knowledge-based Bayesian network; SIT – shared international type; *M. africanum – Mycobacterium africanum*; *M. bovis – Mycobacterium bovis*; *M. tuberculosis – Mycobacterium tuberculosis*
[a]Assignment of lineage/sub-lineage according to SITVIT2 database (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/)
[b]Assignment of lineage/sub-lineage according to TB-Lineage online tool (http://tbinsight.cs.rpi.edu/run_tb_line age.html)
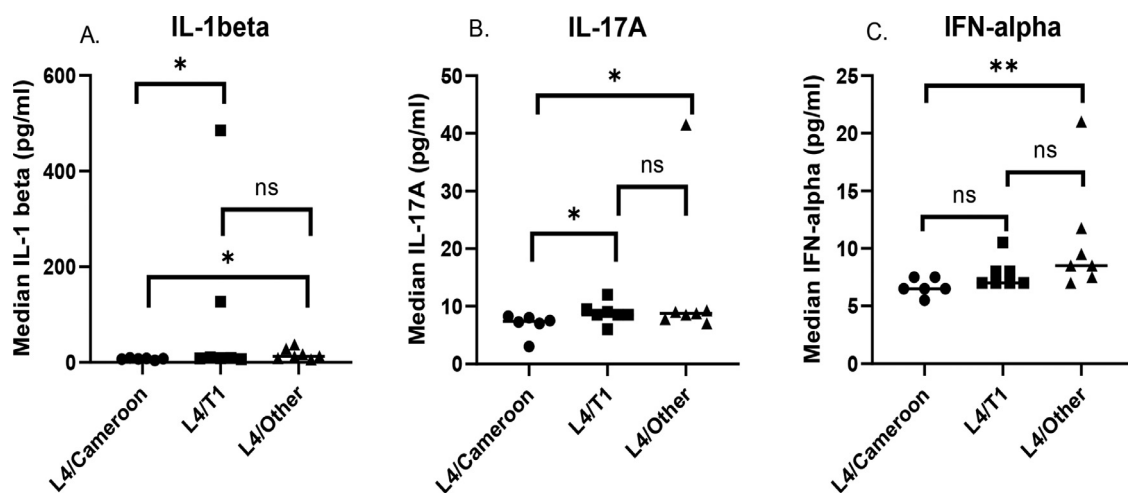

Figure 3. Differential serum levels (pg/ml) of IL-1β, IL-17A, and IFN-α among extrapulmonary TB patients infected with lineage 4 (L4), the most dominant *Mycobacterium tuberculosis* complex lineage identified in the study. The serum levels of [A] IL-1β, [B] IL-17A, and [C] IFN-α were measured by Luminex multiplex immunoassay among L4/Cameroon ($n = 6$), L4/T1 ($n = 7$), and L4/other ($n = 7$) strain-infected patients. The data are shown as scatter plots in which each circle/square/triangle represents a single individual, and the bar represents the geometric mean. Data were analyzed using Kruskal-Wallis tests followed by Mann-Whitney U tests on GraphPad Prism v. 9.2.0. *P*-values are represented as *(< 0.01), **(< 0.001), and ns (not significant).

Table 3

Distribution of sociodemographic and clinical characteristics of EPTB patients and the occurrence of the most predominant *Mycobacterium tuberculosis* complex lineage (L4-Euro-American) and other lineages (non-lineage 4).

Characteristics	Major lineage, *n* (%)		*p*-value*
	Lineage 4 (*n* = 92)	[a]Non-lineage 4 (*n* = 10)	
Sex			0.537
Male	54 (58.7)	7 (70.0)	
Female	38 (41.3)	3 (30.0)	
Age group (years)			0.024
< 15	6 (6.5)	1 (10)	
15–34	32 (34.8)	1 (10)	
35–54	31 (33.7)	8 (80)	
55+	23 (25.0)	0 (0)	
Residence			0.237
Urban	77 (83.7)	8 (80)	
Rural	15 (16.3)	2 (20)	
TB history			0.983
Yes	9 (9.8)	0 (0)	
No	83 (90.2)	10 (100)	
Contact with TB patient			0.770
Yes	7 (7.6)	1 (10)	
No	85 (92.4)	9 (90)	
Major site of infection			0.177
Pleura	40 (43.5)	6 (60)	
Central nervous system	6 (6.5)	1 (10)	
Gastrointestinal	7 (7.6)	1 (10)	
Lymph node	18 (19.6)	1 (10)	
[b]Multiple	13 (14.1)	0 (0)	
[c]Other	8 (8.7)	1 (10)	
HIV status			0.480
Positive	10 (10.9)	2 (20)	
Negative	67 (72.8)	7 (70)	
Unknown	15 (16.3)	1 (10)	

∗Tested by Pearson's chi-squared test, $p < 0.05$.

[a]Lineages other than lineage 4 (L1-Indo-Oceanic, L2-East-Asian, L3-East-African Indian, L5-West African 1, L6-West African 2, animal source-bovis).

[b]More than one site affected in an individual patient.

[c]Group of affected sites whose frequency of occurrence was less than 5.

Iglesias, 2011). It could therefore be inferred that *M. bovis* contributes minimally to human TB in Ghana, but the incidence may be higher in specific populations and geographical areas. For instance, the only *M. bovis* isolate in our study was obtained from a patient living in the northern region of Ghana, where livestock farming as well as consumption of meat and dairy products are very common. Direct and constant contact with infected cattle and the consumption of infected meat and dairy products are potential risk factors for *M. bovis* infection (Silva et al., 2018).

The spoligotypes observed in our study were mostly similar to those found in PTB isolates from previous studies in Ghana (Acquah et al., 2021; Ameke et al., 2021). All six major lineages of human-adapted MTBC were observed, with a predominance of lineage 4 (Cameroon and T1/Ghana) as well as *M. bovis*. The high prevalence of lineage 4 was unsurprising, since it is the most geographically widespread and successful MTBC lineage (Stucki et al., 2016). The near absence of the Beijing genotype (1/102, 0.98%) in our study is noteworthy since it is known to be hypervirulent and has a propensity for causing drug-resistant TB (Yeboah-Manu et al., 2011). Nevertheless, it is recommended that genotyping of isolates – particularly from high-risk areas – should be carried out routinely as a surveillance tool for genotypes of concern.

Age group was the only characteristic in our study to be significantly associated with major MTBC lineages. This finding was in contrast with that of a study in South Africa (Sibandze et al., 2020), where sex was the significant variable, with females significantly overrepresented among patients with lineage 3, and a study in Bangladesh (Uddin et al., 2021), which reported no significant associations. The variation in associations between sociodemographic and clinical characteristics and MTBC lineages across various studies could be attributed to factors such as ge-

ographical variation, predominant major MTBC lineages, sample sizes, and study methodology (Singh et al., 2021).

The overall clustering rate of 65% may suggest recent transmission, but for EPTB, which is less transmissible from person to person compared with PTB, this observation may have been due to the discriminatory power of the genotyping tool used. The discriminatory power of spoligotyping was 0.82, classified as medium high, but it can be extremely high when combined with other genotyping methods, such as MIRU-VNTR.

All the mutations (S315T1, S315T2, C-15T, S531L, H526D, M306V) associated with drug resistance among isolates in our study had been reported previously for PTB samples from Ghana (Addo et al., 2017; Osei-Wusu et al., 2018; Otchere et al., 2016). Among the INH-resistant isolates, 10/12 (83.3%) had *katG* (S315T1), which should be a matter of concern because mutations in *katG* are associated high levels of INH resistance (Charan et al., 2020). Although almost 3% of the isolates (3/102) were resistant to RIF only, this is of serious concern because RIF-resistant TB (RR-TB) serves as a proxy marker for MDR-TB. Two of the DR-TB patients had a history of TB disease, which highlights the need for strict adherence to anti-TB drug regimens.

The serum levels of 25 analytes, including pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines, and growth factors in EPTB patients infected with different *M. tuberculosis* strains of lineage 4, were compared. The levels of three pro-inflammatory cytokines – (interleukin 1-beta (IL-1β), interleukin 17A (IL-17A), and interferon-alpha (IFN-α) – were significantly different among individuals infected with Cameroon, T1, and 'other' sub-lineages. IL-1β, IL-17A, and IFN-α were present in low concentrations in the serum of patients infected with Cameroon strains compared with T1 and 'other'. IFN-α is associated with ongoing active clinical TB (Taneja et al., 2020). Decreased IFN-α levels in EPTB may suggest less severe infection or disease with Cameroon strains. In contrast, IL-1β and IL-17A are important for host resistance to *M. tuberculosis* infection, through enhanced clearance of intracellular *M. tuberculosis* (Ling et al., 2013). Thus, an increase in IL-1β and IL-17A levels during *M. tuberculosis* infection impacts positively on the host. The significant differences observed in the levels of some cytokines among individuals infected with *M. tuberculosis* lineage 4 strains in our study population suggest that lineage of infecting *M. tuberculosis* may play a role in the type of immune response elicited by the host.

Our study had some limitations, including a relatively small sample size due to the low culture yield. Also, spoligotyping – a method known to have lower discriminatory power compared with other molecular typing tools, such as IS6110 restriction fragment length polymorphism (RFLP), MIRU-VNTR, and whole genome sequencing – was used in isolation. While spoligotyping tends to overestimate clustering rates, very high discriminatory power might limit the detection of clusters. Hence, spoligotyping with above-average discriminatory power could be helpful in determining clustering tendencies in a relatively less transmissible disease like EPTB.

Conclusion

This study provides insight into the genetic diversity, drug resistance patterns, and host immune responses of MTBC strains from EPTB patients mostly in urban areas of Ghana, where population density is high. The findings show Cameroon (SIT61) as the most predominant spoligotype among EPTB patients in Ghana, and to be associated with decreased serum IL-1β, IL-17A, and IFN-α. Overall, our study provides useful molecular epidemiological information on EPTB in Ghana. The results could serve as reference data for further studies in other aspects of EPTB and TB control activities.

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Table 4

Distribution of drug-resistance patterns and lineages of *Mycobacterium tuberculosis* complex isolates from extrapulmonary tuberculosis patients

| Lineage | DST patterns for first-line drugs (N = 102) | | | | | | | DST patterns for second-line drugs (N = 15) OFX/MOX, KAN/AMK, CAP/VIO Pan-susceptible n = 15 |
	Pan-susceptible, n = 87	[a]INH^mono n = 9 katG (S315T1)	katG (S315T2)	inhA (C-15T)	[b]RIF^mono n = 3 rpoB (S531L)	[c]INH/EMB^poly, n = 2 katG (S315T1)/embB (M306V)	INH/RIF (MDR), n = 1 katG (S315T1)/rpoB (H526D)	gyrA, gyrB, rrs, eis
L1	1	0	0	0	0	0	0	0
L2	1	0	0	0	0	0	0	0
L3	3	0	0	0	0	0	0	0
L4	78	6	1	1	3	2	1	14
L5	1	1	0	0	0	0	0	1
L6	2	0	0	0	0	0	0	0
M. bovis	1	0	0	0	0	0	0	0

M. bovis – *Mycobacterium bovis*; INH – isoniazid; RIF – rifampicin; EMB – ethambutol; MDR – multidrug-resistant; OFX – ofloxacin; MOX – moxifloxacin; KAN – kanamycin; AMK – amikacin; CAP – capreomycin; VIO – viomycin.

[a]Isoniazid monoresistance.

[b]Rifampicin monoresistance.

[c]Isoniazid/ethambutol polyresistance.

Ethical approval

Ethical approval was sought from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 093/17-18), Korle Bu Teaching Hospital Institutional Review Board (KBTH-IRB 000137/2019), and the Kwame Nkrumah University of Science and Technology/Komfo Anokye Teaching Hospital Committee on Human Research and Publication Ethics (CHRPE/AP /048/19).

Author contributions

Conceptualization: Samuel Ofori Addo, Kennedy Kwasi Addo, Gloria Ivy Mensah. Data curation: Samuel Ofori Addo. Formal analysis: Samuel Ofori Addo, Gloria Ivy Mensah. Investigation: Samuel Ofori Addo. Resources: Kennedy Kwasi Addo, Gloria Ivy Mensah. Supervision: Kennedy Kwasi Addo, Gloria Ivy Mensah, Lydia Mosi, Afua Owusua Darkwah Abrahams. Validation: Kennedy Kwasi Addo, Gloria Ivy Mensah. Writing – original draft: Samuel Ofori Addo. Writing – review and editing: Samuel Ofori Addo, Gloria Ivy Mensah, Lydia Mosi, Afua Owusua Darkwah Abrahams, Kennedy Kwasi Addo.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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