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Molecular clustering of patients with *Mycobacterium tuberculosis* strains cultured from the diabetic and non-diabetic newly diagnosed TB positive cases

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ABSTRACT

Background: Social determinants of health, biological, and individual variants have been associated with Pulmonary TB (PTB) case clustering. None of the studies have focused on diabetes mellitus (DM) despite it being one of the co-morbidity affecting TB patients. Minimal data is available and it is not clear whether patients with DM and TB are more likely than TB patients without DM to be grouped into similar molecular clusters thus indicating a bias in transmission among TB/DM co-morbidity patients.

Objective: To determine proportion of TB strains within TB and TB/DM cases that were clustered with their corresponding clinical outcomes and hence could be attributable to active TB transmission in the two urban counties of Nairobi, Kenya.

Methods: We carried out a prospective cohort study of non-pregnant patients aged 15 years and above that tested positive for TB in two peri-urban counties in Kenya between February 2014 and August 2015. Clinical and socio-demographic data were obtained from a questionnaire and medical records of the National TB program patient data base at two, three, five and six months. Spoligotyping data was then obtained and compared from previously identified strains in a data bank from the spolDB4.

Results: We identified 7 different TB strains out of which East Asia Beijing, Euro America and Indo oceanic being the most dominant strain within the two counties accounting for 92.4% of the infections. DM was not a significant factor in increasing the likelihood of PTB patients to cluster according to the genotype of the infecting *Mycobacterium tuberculosis* bacillus. TB lineages, DM and County of the patient were found to be independent of the clinical outcomes that were observed in the study

Conclusion: Diabetes mellitus is not a significant factor in increasing the molecular clustering among PTB patients.

Introduction

Mycobacterium tuberculosis and the other members of the *M. tuberculosis* complex (MTBC) remains one of the main causes of morbidity and mortality in low and medium-income countries, currently experiencing an upsurge in diabetes mellitus (DM) cases [1–3]. Studies indicates an increased risk of TB among patients with DM in addition to poor prognosis of patients suffering from DM/TB co-morbidity [4]. Some studies have further explored the relationship between diabetes mellitus (DM) and tuberculosis infecting *M. tuberculosis* bacillus. Socio-epidemiological data, molecular strain typing together with conventional epidemiologic methods have previously helped to further characterize *M. tuberculosis* strains and understand the dynamics and patterns of transmission of the pathogen in different regions and populations [5–8]. In molecular characterization, patients with identical strains of *M. tuberculosis* are considered to belong to one cluster resulting from recent transmission and rapid progression. Unique patterns results mainly from reactivation of latent infection or recent transmission from patients out of the period or area under study [9,10].

Variation in individual, biological and social determinants have also been associated with Pulmonary TB (PTB) case clustering. Other determinants include being male, young, country of birth, resident of an urban area, alcohol or drug consumption, homelessness, HIV infection or having acid-fast bacilli in sputum smear [11,12]. Similar determinants have been described in medium income countries. Despite numerous studies on the association of HIV and increased risk of *M.*

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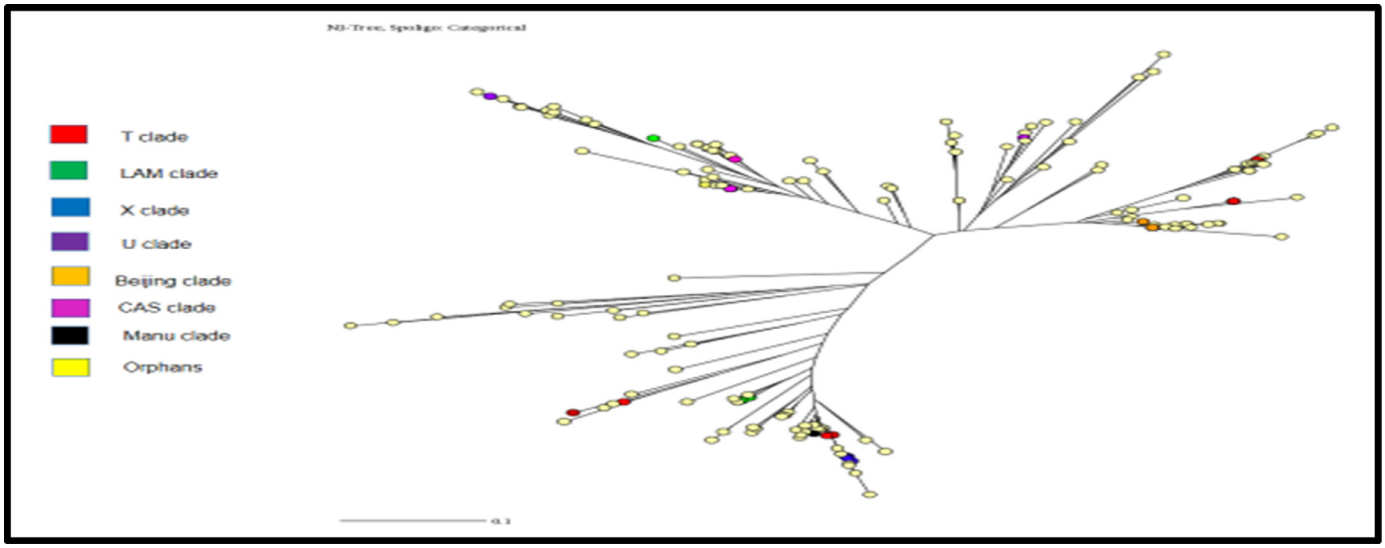

Fig. 1. Bio geographical structure of MTB lineage identified: 7 major spoligotype based families were identified: Beijing clade (n = 55 as East Asia Beijing), Latin American and Mediterranean (LAM) (n = 80 as Euro America), a European family X clade (n = 4 as Euro America), Central and Middle Eastern Asia (CAS) (n = 63 as Indo oceanic), U clade(n = 7 as M bovis), a default family T clade(n = 1 as M Africanus), Menu clade(n = 3 as West African 1 and n = 4 as West African 2), and Orphans (n = 157).

Table 1
Difference in various clinical outcomes among different TB lineages: C-Completed, D- Died, F – Failure, NC- Not Complete, OOC – Out of Control, TC– Treatment Complete, TO- Transfer Out. The results presented in tables using frequencies with corresponding percentages within parenthesis. Treatment failures were reported in East Asia Beijing and Indo oceanic.

	Outcome							
Lineage	C	D	F	NC	OOC	TC	TO	Total
East Asia Beijing	55 (90.2)	0 (0.0)	1 (1.6)	0 (0.0)	2 (3.3)	2 (3.3)	1 (1.6)	61 (24.5)
Euro America	84 (85.7)	0 (0.0)	0 (0.0)	4 (4.1)	2 (2.0)	6 (6.1)	2 (2.0)	98 (39.4)
Indo oceanic	63 (88.7)	1 (1.4)	1 (1.4)	0 (0.0)	1 (1.4)	3 (4.2)	2 (2.8)	71 (28.5)
M Africanum	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.4)
M bovis	7 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (10.0)
West Africa 1	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	4 (1.6)
West Africa 2	4 (57.1)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	1 (14.3)	7 (2.8)
Total	217	2	2	4	6	12	6	249

Table 2
Difference in TB lineage among diabetics and non-diabetics.

Lineage	Diabetes condition		Total
	Yes	No	
East Asia Beijing	46 (75.4)	15 (24.6)	61 (24.5)
Euro America	62 (63.3)	36 (36.7)	98 (39.4)
Indo oceanic	54 (76.1)	17 (23.9)	71 (28.5)
M Africanum	0 (0.0)	1 (100.0)	1 (0.4)
M bovis	4 (57.1)	3 (42.9)	7 (2.8)
West Africa 1	3 (75.0)	1 (25.0)	4 (1.6)
West Africa 2	4 (57.1)	3 (42.9)	7 (2.8)
Total	173	76	249

Table 3
Difference in TB lineages within Nairobi and Kiambu counties.

Lineage	Country		Total
	Kiambu	Nairobi	
East Asia Beijing	13 (21.3)	48 (78.7)	61 (24.5)
Euro America	10 (10.2)	88 (89.8)	98 (39.4)
Indo oceanic	12 (16.9)	59 (83.1)	71 (28.5)
M Africanum	0 (0.0)	1 (100.0)	1 (0.4)
M bovis	1 (14.3)	6 (85.7)	7 (2.8)
West Africa 1	2 (50.0)	2 (50.0)	4 (1.6)
West Africa 2	2 (28.6)	5 (71.4)	7 (2.8)
Total	40	209	249

tuberculosis transmission within the sub-Saharan region, fewer studies have investigated if DM increases the likelihood of PTB patients to cluster according to the genotype of the infecting *M. tuberculosis* bacillus. Furthermore, there is dearth of data addressing DM-TB co-infection and it remains unclear whether patients with DM and TB are more likely than TB patients without DM to be grouped into molecular clusters defined according to the genotype of the infecting *M. tuberculosis* bacillus. Specifically, the question as to whether there is convincing molecular epidemiological evidence for TB transmission among DM patients or the impact of lineage in the clinical outcome of a patient on treatment is yet to be addressed.

Information derived from DM-TB co-infection will contribute to developing effective preventive strategies of TB transmission. Alternatively, those not clustered might represent patients latently infected in the remote past presenting with reactivated active TB disease or might be recently infected but with unique strains to that population [7,8,13,14]. In any case, this important information can be used for outbreak investigation or contact tracing, active case-finding or instituting other control measures to mitigate further TB transmission in at risk population (i.e., those identified as clustered or un-clustered). Here, we sought to identify the most frequently isolated TB strains among patients with TB/DM and also to determine proportion of TB/

Table 4
Strain Characterization and the various clinical outcomes: The pr value of 0.328 is > 0.05. This indicate no relationship between the lineage and the clinical outcome.

DM = < 7 (Normal)

	Outcome				
TB Lineage 2	Cured	Treatment	Failed/Died	Unknown	Total
East Asian (Beijing)	44(89.79)	2(4.08)	1(2.04)	2(4.08)	49(100)
Euro -American	58(84.05)	5(7.24)	0(0)	6(8.69)	69(100)
Indo- Oceanic	54(93.1)	1(1.72)	1(1.72)	2(3.44)	58(100)
M. african	5(71.42)	0(0)	1(14.28)	1(14.28)	7(100)
M.bovis	5(100)	0(0)	0(0)	0(0)	5(100)
Orphan strain (unknown)	26(78.78)	3(9.09)	2(6.06)	2(6.06)	33(100)
Spoligotyping not done	31(73.8)	6(14.28)	2(4.76)	3(7.14)	42(100)
Total	223(84.79)	17(6.46)	7(2.66)	16(6.08)	263(100)
	Pearson chi2(18) = 20.0866 Pr = 0.328				

DM cases that were clustered with their corresponding clinical outcomes and hence could be attributable to active TB transmission in the two urban counties of Nairobi, Kenya.

Materials and methods

Study design

A prospective cohort study was carried out in Kiambu and Nairobi counties of, Kenya between February 2014 and August 2015. Patients aged above 15 years who tested positive for *M. tuberculosis* complex on sputum smear microscopy were registered at the TB clinic once they were confirmed as smear positive. The diagnosis was based on the KNTP guideline criteria. The exclusion criteria included participants who were pregnant, within the gestational period, with chronic renal failure or on TB therapy. Ethical approval for the study was obtained from the Kenyatta National Hospital Ethical Research Committee (KNH/UoN-ERC) and the study was undertaken in accordance with the principles of the Helsinki Declaration.

Written consent was obtained from patients who agreed to participate. Venous blood was collected at baseline in two separate tubes (one for fasting or random blood glucose levels and the other for HbA1c levels. This was followed by physical examination and questionnaire administration by trained healthcare personnel where detailed history, including signs and symptoms of diabetes mellitus, cigarette smoking and other life-style information were ascertained. Patients were then followed at two, three, five, six months and, at end of therapy to assess adherence and clinical evaluation with sputum microscopy examination at each time when possible. The initial sputum examination was submitted for culture and pathogen identification. Spoligotyping was then performed on all the positive cultures to characterize the strains. Patients were examined at each visit for both TB and DM. Loss to follow up were then compared between the TB-DM and TB without DM. MTB strains characterized and classified to their specific strains and MTB lineage compared with the demographic data from the questionnaire.

Laboratory analysis

Laboratory sample processing was done at the National Reference Tuberculosis laboratory (NTRL) Kenya. Staining was done with ZN and FM, while sputa were decontaminated with Sodium hydroxide (NaOH). Ten microliters of the processed sputa was inoculated on 2 slopes of LJ and incubated at 37 °C for 8 weeks and read weekly. Positive LJ was confirmed by use of ZN, conventional biochemical tests to discriminate MTB complex mycobacteria other than Tuberculosis (MOTT).

Table 5
Significance of the county, diabetes status and TB lineage on the clinical outcomes: There is no relationship between the county, Tb lineage and the diabetes status on the clinical outcomes of the patients. Similar information is corroborated in Fig. 2 below.

		Total		DNA fingerprints		
Variable	Level	N = 340 (%)	Not done n = 58 (%)	Done, fully Characterized n = 241 (%)	Orphan strains n = 41 (%)	*P*-value
Outcomes	Favorable	308 (90)	50 (86)	221 (92)	37 (90)	0.761
	Unfavorable	13 (4)	3 (5)	8 (3)	2 (5)	
	Unknown (D/LD/TO)	19 (6)	5 (9)	12 (5)	2 (5)	
Time-to-negative smears*	Baseline (positive)	335/336 (99)	58 (100)	236/237 (100)	41 (100)	0.811
	2/3-months (positive)	32/307 (10)	5/49 (10)	23/220 (10)	4/38 (11)	0.998
	5-months	4/290 (1)	1/44 (2)	2/212 (1)	1/34 (3)	0.559
	6-months	3/290 (1)	1/44 (2)	1/212 (0)	1/34 (3)	0.283
Diabetes Mellitus (DM)	Non-DM	258 (76)	42 (72)	183 (76)	33 (80)	0.798
	Pre-DM	60 (18)	13 (22)	41 (17)	6 (15)	
	DM	22 (6)	3 (5)	17 (7)	2 (5)	
HIV test result	Positive	77 (23)	12 (21)	56 (23)	9 (22)	0.941
	Negative	238 (70)	41 (70)	169 (70)	28 (68)	
	Not done	25 (7)	5 (9)	16 (7)	4 (10)	
TB regimen	2HRZE/4HR	308 (91)	52 (90)	217 (90)	39 (95)	0.568
	2SHRZE/1HRZE/5HRE	32 (9)	6 (10)	24 (10)	2 (5)	
County	K	56 (17)	7 (12	39 (16)	10 (24)	*0.037*
	N	283 (83)	51 (88)	202 (84)	30 (73)	
	Missing	1 (0)	0	0	1 (2)	
Gender	Female	96 (28)	8 (14)	79 (33)	9 (22)	*0.010*
	Male	244 (72)	50 (86)	162 (67)	32 (78)	
Ever smoked	Yes	97 (29)	20 (35)	66 (27)	11 (27)	0.838
Age (years)	(mean [SD])	32.11 (8.80)	32.15 (9.13)	32.18 (9.04)	31.60 (6.81)	0.979
Weight (KG)	(mean [SD])	54.61 (10.32)	54.61 (11.47)	54.86 (10.34)	53.14 (8.39)	0.615
BMI (KG/m^2)	(mean [SD])	19.55 (3.91)	19.37 (3.56)	19.71 (4.11)	18.93 (3.23)	0.456
Blood glucose	(mean [SD])	3.61 (1.19)	3.81 (1.23)	3.59 (1.18)	3.39 (1.20)	0.220
HbA1c (%)	(mean [SD])	5.75 (2.22)	5.64 (2.42)	5.82 (2.18)	5.54 (2.21)	0.699
BUN	(mean [SD])	3.76 (1.17)	4.1 (1.36)	3.68 (1.08)	3.73 (0.32)	0.172
Creatinine	(mean [SD])	89.39 (20.33)	96.56 (20.37)	87.99 (19.92)	87.46 (21)	*0.012*

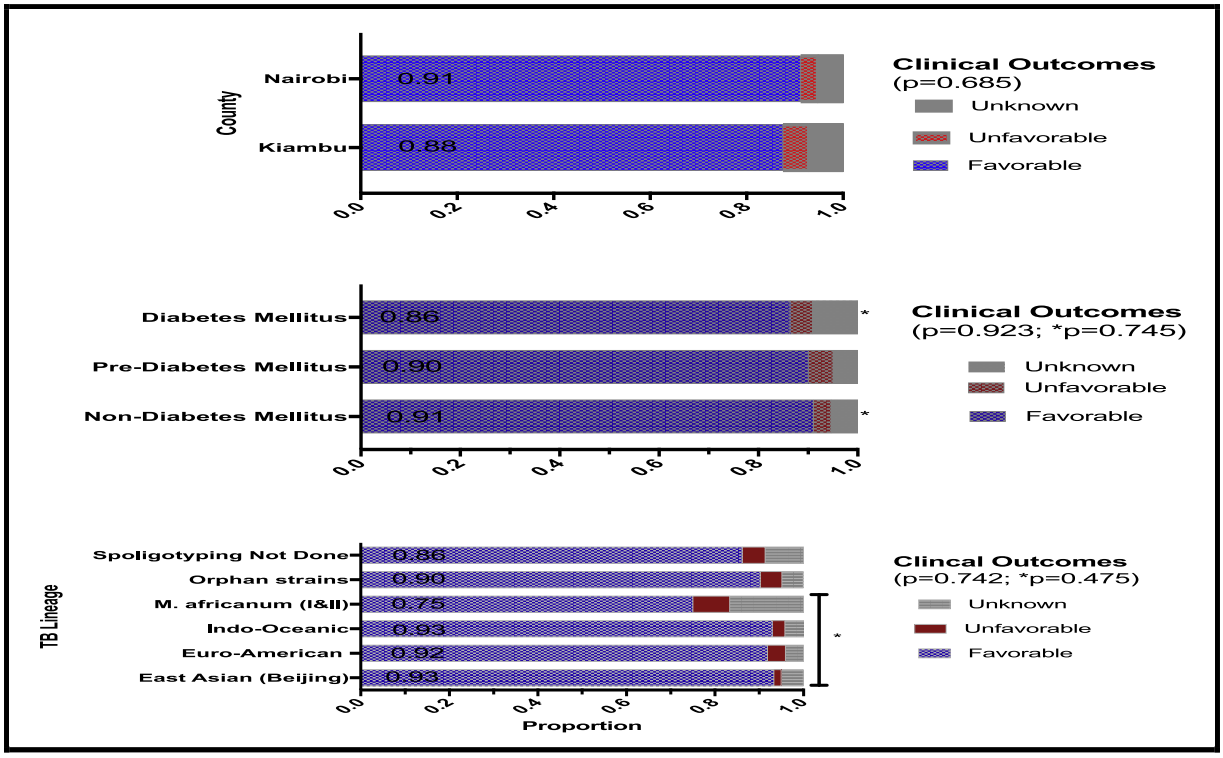

Fig. 2. Summary of the results obtained using SpolDB derived prototypes for model initiation. There was no significance difference in the clinical outcomes within the two counties or TB strain.

DNA extraction and spoligotyping analyses

Extraction of genomic DNA from TB positive culture slopes and spoligotyping were done at the South Africa Medical Research Council (SAMRC, Pretoria) as described by Kamerbeek et, al. [9]. Spoligotyping was performed using the spoligotype kit (Ocimum bio solutions company, Netherlands) according to the manufacturer's instructions. The direct repeats (DR) were amplified by ABI 9700 thermocycler using oligonucleotides primers (DRa: 5′ GGT TTT GGG TCT GAC GAC 3′ and DRb: 5′ CCG AGA GGG GAC GGA AAC 3′) derived from the DR sequence. The DRa primer is biotinylated at the 5′-end. Biotinylation exploitss polymorphisms in the spacer sequences found in the direct repeats (DR) locus in the chromosomes of MTBC Strains. Currently the 94 different spacers sequences were identified of which 43 are used for MTBC strain differentiation which determine the absence or presence of the 43 defined spacer sequences.

Definition of terms

A clustered case was defined as any TB case from the study populations whose strain type, based on the standard spoligotyping assay, was not indistinguishable from that of at least one other case, while a non-clustered cases shared a unique strain not found in the study population. Since the TB cases were from adjacent and proximate counties and all cases were notified within the same calendar year, the specific geographic and temporal timing of cases were not used in the definition of a cluster. This means that recent transmission was assumed to have occurred between TB cases who shared the same strain, either directly or indirectly via another identified or missed case, within the same population.

Data analysis

We defined diabetes using a cut-off of the variable HB1AC such that a person is considered to be diabetic if HB1AC > 6.5, else non-diabetic. TB treatment outcome was grouped into seven categories (C, D, F, NC, OOC, TC, and TO), diabetic conditions grouped into two groups (diabetic and non-diabetic), and two counties considered (Nairobi and Kiambu). Cross tabulation of TB lineage was done with Outcome, diabetic conditions, and counties to compare the proportion of each lineage across each of the categories of the other variables. The results presented in tables using frequencies with corresponding percentages within parenthesis.

All genotyping data were entered into a Microsoft Excel sheet. Spoligotyping molecular analysis patterns was converted into binary and octal codes designations for easier analysis and interpretation [15]. This data was compared from previously identified strains in a data bank from the spolDB4 and for binary spoligotype was entered in the Share international types (SITVIT2 web) database from which contains more than 75,000 MTB isolates from different countries as opposed to orphans which designates patterns reported as a single isolates while the lineage of the Mycobacteria was obtained by SPOTCLUST online software (http://www.tbinsight.cs.rpi.edu). MLVA Compare V1.03 software (Genoscreen; Lille, France) was used to draw the minimum spanning trees (MSTs). The SIT or orphan spoligotype number appeared inside each node, and the distance (number of spacers of difference) between two nodes was shown on the edge linking these nodes. These phylogenetic trees were colored in function of various characteristics such as the MTBC lineages described in SITVIT and county of isolation. The MST is a graph which is undirected and connected. The MST links all isolates together with the fewest possible linkages between nearest neighbors. Furthermore, a spoligoforest was drawn as a “hierarchical layout” using the SpolTools software (available through http://www.emi.unsw.edu.au/spolTools. As opposed to the MST, the spoligoforest is a directed and not necessarily connected phylogenetic tree illustrating the parent to descendant relationships between spoligotype (considering the fact that spoligotype rather evolve by loss of spacers). TBVis tool (available at http://tbinsight.cs.rpi.edu/; [29, 30]) was used to visualize and map the spoligotype shared between different lineages and split by county of isolation. Maps were reproduced and designed

Results

Newly diagnosed TB patients (374) were enrolled to participate in the study. We collected 347 isolates from the 5 health facilities in both Kiambu and Nairobi counties between August 2014 and August 2015 and analyzed in terms of sample size and within the Health facilities or tuberculosis lineages by genetic analysis using Spacer oligonucleotide typing direct repeat (spoligotype) originating from the population based on the cross sectional study. This was done through strains detected by spoligotyping and matching it the with the Spoligotype43 spacer. The spoligotype analysis builds upon previous research that has classified MTBC strain isolates into 9 major spoligotype based families: *Mycobacterium africanum, M. bovis*, East African Indian (EAI), Beijing, Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asia (CAS), a European family X and a default family T. These 9 families are further subdivided into 36 more subfamilies in the global data base SpolDB. Groups of the related spoligotype are interchangeably called (sub) families, (sub) clades and (sub) classes. Spoligotype that have no match in the SpolDB database are defined as orphans or unclustered. Distinguishing both the clustered and the orphans are unique cases are more likely to be directly or indirectly involved in the same chain of Transmission and the unique genotype is difficult to get plausible origin.

The dendrogram in Fig. 1 indicated high clustering at 33% among the dominant strains with a divergent evolutionary trend among the orphan clusters. The results below in Tables 1–5 and Fig. 2 shows there was no difference in the clinical outcomes depending on TB lineage, county or diabetes status.

Discussion

In Countries like Kenya with high burden of TB, studies determining the population structure of strains in different geographical areas are important to monitor transmission. Information regarding MTB strains circulating in Nairobi and Kiambu counties, situated at the center of Kenya with huge influx of people across the country, is lacking. The only data available are from a previous study including few isolates thus not representative of MTB strains in Nairobi and Kiambu counties. The study documents 7 different lineages with East Asia Beijing, Euro America and Indo oceanic being the most dominant within the two counties. DM and county were not significant factors in increasing the likelihood of PTB patients to cluster according to the genotype of the infecting *M. tuberculosis* bacillus. Difference in TB lineages, DM and County of the patient were also found to be independent of the various clinical outcomes that were observed in the study.

Within the two counties, the most dominant strains were Euro America (39.4%), Indo oceanic (28.5%) and East Asia Beijing (24.5%). This indicates that the modern strains are the most circulating in the two counties and not ancestral lineages. The three clades were also the most prevalent genotypes in East Africa [16–18]. The East Asia Beijing, Euro American and Indo oceanic lineages occurred in all the countries within the region, but the highest frequencies of the Indo oceanic lineage were in Kenya, depicting it as the dominant strain within the two counties. Our clustering rate of 33% was similar to that observed in the Ethiopian study [19–21]. However, 125/374 (33.42%) isolates were not described before and regarded as orphans. This is higher compared to the Ghanaian study that had 16% [22–24]. The divergent evolutionary trend of the orphan cluster suggest that the cluster may be involved in the evolution of adaptive traits, and thus are not particularly candidate clusters for identifying lineage-specific adaptations.

We documented that DM and county were not significant factors in increasing the likelihood of PTB patients to cluster according to the genotype of the infecting *M. tuberculosis* bacillus. This may indicate non-clonal transmission with diverse strains contributing to TB dynamics [25–27]. In addition there remains a need to type current isolates to get a clear understanding on the genotypic population structure of TB strains and its transmission dynamics. The heavy clustering among the three strains might also be due to recent transmission and rapid progression [21,26,27]. The few Unique genetic patterns observed are likely due to low reactivation of latent infection or almost non-existence recent transmission from patients out of the period or area under study [26–28]. Being that there was no relationship between DM and clustering of the cases also points to being no convincing molecular epidemiological evidence of higher TB transmission among DM patients similarly as observed in other settings [15,18,21]. These settings have observed no association between DM and molecular clustering of PTB in the community at a pooled estimate of 0.84 (CI 95% 0.40 ± 1.72) [19,21].

Our study did not indicate any relationship between the TB lineages, DM and County of the patient to the various clinical outcomes observed. Other studies show poorer outcomes among patients with TB and DM [29,30]. Public health impact of the comorbidity is greater in regions of low and middle income, where 84% of patients with DM live, many of whom are unaware of their condition [28,30]. Lack of association between the DM and poor clinical outcomes in our study could be attributed to high economic status of the two counties. It also indicates that the two counties almost have similar health systems and economic status.

Limitations

The study is limited by the fact that it is a single center study, the results of which cannot be generalized. However this provide a window of opportunity for clinicians and researchers to carry out further study and follow the patients on early screening among Newly diagnosed *M. tuberculosis* for DM as we may have underestimated the prevalence of DM and also cross transmission through contacts.

Conclusion

East Asia Beijing, Euro America and Indo oceanic are the most dominant strain within the two counties of Kiambu and Nairobi Counties. DM and county were not significant factors in increasing the likelihood of PTB patients to cluster according to the genotype of the infecting *M. tuberculosis* bacillus indicating no convincing molecular epidemiological evidence of higher TB transmission among DM patients similarly as observed in other settings.

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