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Mycobacterium Genotypes in Pulmonary Tuberculosis Infections and Their Detection by Trained African Giant Pouched Rats

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Abstract Tuberculosis (TB) diagnosis in low-income countries is mainly done by microscopy. Hence, little is known about the diversity of *Mycobacterium* spp. in TB infections. Different genotypes or lineages of *Mycobacterium tuberculosis* vary in virulence and induce different inflammatory and immune responses. Trained *Cricetomys* rats show a potential for rapid diagnosis of TB. They detect over 28 % of smear-negative, culture-positive TB. However, it is unknown whether these rats can equally detect sputa from patients infected with different genotypes of *M. tuberculosis*. A 4-month prospective study on diversity of *Mycobacterium* spp. was conducted in Dar es Salaam, Tanzania. 252 sputa from 161 subjects were cultured on Lowenstein-Jensen medium and thereafter tested by rats. Mycobacterial isolates were subjected to molecular identification and multispacer sequence typing (MST) to determine species and genotypes. A total of 34 *Mycobacterium* spp. isolates consisting of 32 *M. tuberculosis,* 1 *M. avium* subsp. *hominissuis* and 1 *M. intracellulare* were obtained. MST analyses of 26 *M. tuberculosis* isolates yielded 10 distinct MST genotypes, including 3 new genotypes with two clusters of related patterns not grouped by geographic areas. Genotype MST-67, shared by one-third of *M. tuberculosis* isolates, was associated with the Mwananyamala clinic. This study shows that diverse *M. tuberculosis* genotypes ($n = 10$) occur in Dar es Salaam and trained rats detect 80 % of the genotypes. Sputa with two *M. tuberculosis* genotypes (20 %), *M. avium hominissuis* and *M. intracellulare* were not detected. Therefore, rats detect sputa with different *M. tuberculosis* genotypes and can be used to detect TB in resource-poor countries.

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Introduction

Few laboratories in low-income countries with high-burden tuberculosis (TB) routinely culture sputum specimens for the isolation of mycobacteria and drug-resistance testing [15]. This situation limits insight into the distribution of mycobacteria and the *Mycobacterium tuberculosis* genotypes responsible for pulmonary TB in low-income countries, such as Tanzania. The most commonly used diagnostic method in these countries is direct sputum smear microscopy with low sensitivity ranging from 20 to 62 % [20]. This method only detects 55 % of TB culture-positive HIV-infected patients [10] and fails to distinguish

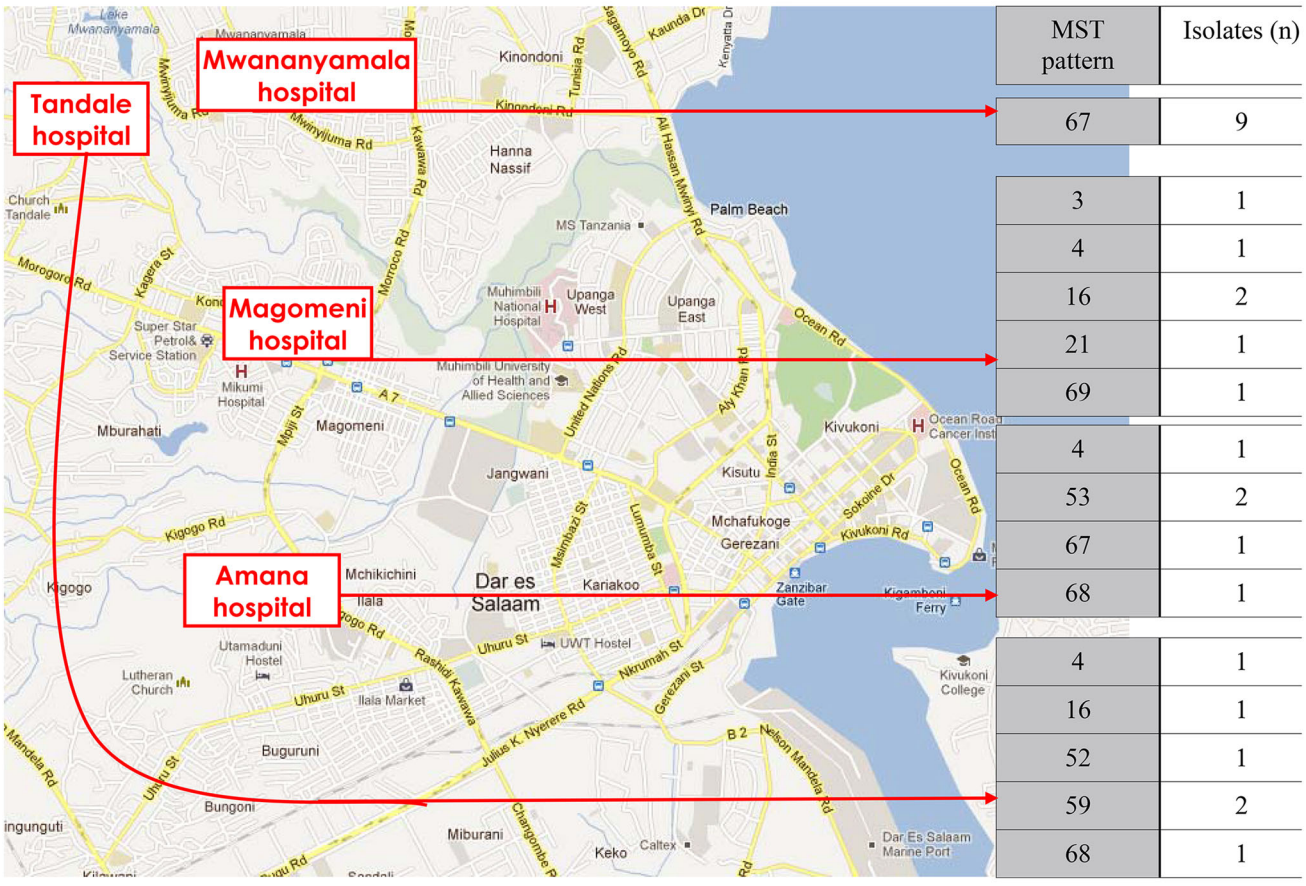
M. tuberculosis from nontuberculous mycobacteria (NTM) [16]. It is, therefore, imperative to increase our knowledge of prevalent mycobacteria in TB patients to improve management of TB and to develop novel rapid diagnostic tools, such as TB detection by African giant pouched rats (*Cricetomys gambianus*) [11, 21]. Previous reports have shown that different genotypes/lineages of *M. tuberculosis* vary in virulence and in the levels of immunity, inflammation and pathology that they induce in TB patients [3, 13]. The ability of trained rats to detect sputum samples from TB patients infected with different genotypes of *M. tuberculosis* remains to be established.

In this study, sequence-based methods for identification and typing were used to investigate the diversity of *Mycobacterium* spp. in individuals attending TB clinics in Tanzania's largest city, Dar es Salaam, which also provided the sputum samples used for the routine training of TB detection rats [11, 21]. Better knowledge about the prevalence of mycobacterial genotypes forms the basis for assessing the diagnostic performance of rats on clinical samples containing different *Mycobacterium* genotypes and species.

Materials and Methods

Study Setting, Population and Design

Sputum specimens for isolation of mycobacteria and training TB detection rats at SUA-APOPO laboratory, Sokoine University of Agriculture, Morogoro, Tanzania, were prospectively investigated from March to June 2009. These included 252 sputa from 161 patients attending four selected Directly Observed Treatment—Short course (DOTS) centres in different geographic areas of Dar es Salaam (Fig. 1). The DOTS centres and the numbers (and percentages) of patients recruited per DOTS centre were as follows: Magomeni 18 (11.25 %), Tandale 24 (15 %), Amana 29 (18.12 %) and Mwananyamala 90 (55.62 %). The age of patients ranged from <1 to 85 years (mean ± SD = 31 ± 3 years). Patients comprised 90 males and 71 females (sex ratio 1.27M:1F). Informed consent for participation in the study was obtained from participants and parents or guardians of child participants. Only samples with volumes adequate to provide aliquots for culture (≥1 ml) and for TB detection (≥ 3 ml) by rats



MST pattern	Isolates (n)
67	9
3	1
4	1
16	2
21	1
69	1
4	1
53	2
67	1
68	1
4	1
16	1
52	1
59	2
68	1

Fig. 1 Map of Dar es Salaam showing the location of tuberculosis clinics where sputum samples were collected, with respective *Mycobacterium tuberculosis* genotypes based on multispacer sequence typing (MST). Number (*n*) of isolates exhibiting an MST pattern

were included in this study, to enable subsequent determination of the rats' performance on sputa with different *M. tuberculosis* genotypes and NTM. The aliquots for cultures were aseptically transferred using sterile disposable transfer pipettes into sterile screw-capped microtubes and the remaining volumes were processed for routine training of rats [21]. This study is part of the wider SUA-APOPO training of African giant pouched rats for diagnosis of pulmonary TB, and was done in compliance with the "Animal Research: Reporting In Vivo Experiments" (ARRIVE) guidelines and the Helsinki Declaration. Ethics Committee of the National Institute for Medical Research (NIMR, Tanzania) approved the study.

Isolation of *Mycobacterium* spp. and Genotyping

Sputum samples were cultured on Lowenstein–Jensen (LJ) medium with pyruvate and LJ with glycerine following standard decontamination with 4 % sodium hydroxide (1:1) for 45 min, centrifugation at 3000×*g* for 20 min and neutralisation of the sediment with 14 % potassium dihydrogen phosphate. Cultures were incubated at 37 °C for a minimum of 8 weeks with intermittent observation for growth [22]. The isolates were Ziehl Neelsen-stained, and DNA was extracted from all acid-fast bacilli by the bead-beating method and subjected to multiplex real-time PCR for preliminary affiliation to the genus *Mycobacterium*, the *M. tuberculosis* complex (MTC) or the *M. avium* complex (MAC) [18, 19]. MTC isolates were further analysed by multispacer sequence typing (MST) of the MST 4, MST 11, MST 12 and MST 13 spacers [6]. MAC isolates were analysed by *rpoB* gene sequencing to determine their specific identification [1, 2]. The negative control for all PCR-based analyses consisted of distilled water at a ratio of 1:3 (negative control:specimen). The performance of rats on all mycobacteria culture-positive sputum specimens was determined following procedures described by Weetjens et al. [21]. Numerical data were compared using the Chi-squared test, and a P value <0.05 was used as cut-off for statistical significance of comparisons. Correlation analysis was used to determine whether rats' detection of sputum was correlated with smear positivity.

Results

Mycobacterial Isolation

Isolates of the *Mycobacterium* genus were cultured from sputum of 34 of the 161 patients (21.1 %) with a prevalence of 23.3 % in males (21 out of 90) and 18.6 % in females (13 out of 71). This difference is not statistically significant. Of the 28 mycobacterial isolates with DNA quality suitable for further molecular studies, 26 isolates (93 %) were identified as *M. tuberculosis* and 1 each as *M. avium* subsp. *hominissuis* and *M. intracellulare* (Table 1).

Genotyping

MST analysis of the 26 *M. tuberculosis* isolates yielded 10 distinct genotypic patterns (mean molecular diversity, 0.38): (pattern 1) MST 67 comprised 10 of the 26 isolates (38.5 %); (patterns 2 and 3) MST 4 and MST 16 were found in 3 out of 24 isolates each (11.5 %); (patterns 4–6) MST 53, MST 59 and MST 68 were found in 2 isolates each; and (patterns 7–10) MST 3, MST 21, MST 52 and MST 69 were found in 1 isolate each. Sequence clustering analysis revealed two clusters of related patterns for the four groups (52, 67, 59 and 4, respectively), which were differentiated by a single-sequence repeat or a single-nucleotide polymorphism. The five remaining genotypes exhibited higher degree of variability with genotypes 16 and 69 being the most distant patterns. New genotypes were found in the course of this study: MST 67 and MST 68 were new genotypes due to a new combination of previously known spacer sequences, whereas MST 69 was a new genotype due to a novel sequence within spacer 12, combining six modules of a 77-bp repeat unit. With one exception, we found only one MST genotype per patient; in one patient, an MST 69 genotype isolate and a further MST 21 genotype isolate were found. No other patient had MST 21 or MST 69 genotype isolates. Data gathered herein are interpreted as authentic, as all negative controls remained negative.

The map displaying the distribution of genotypes according to the sample location (Fig. 1) revealed that related patterns were not significantly grouped together in geographical areas. However, the genotype MST 67 was significantly associated with the Mwananyamala clinic ($P < 0.05$).

Detection of Sputa with Different Mycobacterial Genotypes by Rats

The results of TB detection rats revealed that sputum samples with different genotypes of *M. tuberculosis* are detected by rats (Table 1). The rats detected 8 out of 10 MST genotypes (80 %). Two MST genotypes (MST 59 and MST 3) with 2 isolates and 1 isolate, respectively, were from sputum samples not detected by the rats (also referred to as rat-negative). However, the two isolates formed a cluster together with isolates from detected sputum (Fig. 2) indicating that they are genetically related and can be detected by rats given a larger sample size. Similarly, the nontuberculous *M. intracellulare* and *M. avium* subspecies

Table 1 Results of the multiplex real-time PCR, multispacer sequence typing of mycobacteria and detection of respective sputum by trained *Cricetomys gambianus* rats

S/N	Specimen ID	DOTS centre	Age	Gender	RT-PCR	MST type	Identification	Smear microscopy	TB detection rats
1	257837	Mwananyamala	24	M	MTC	67	*M. tuberculosis*	−	−
2	258362	Mwananyamala	17	M	MTC	67	*M. tuberculosis*	−	−
3	258429	Mwananyamala	25	F	MTC	67	*M. tuberculosis*	+	+
4	266786	Mwananyamala	40	F	MTC	67	*M. tuberculosis*	−	−
5	267265	Mwananyamala	35	F	MTC	67	*M. tuberculosis*	+	+
6	257729	Mwananyamala	22	M	MTC	67	*M. tuberculosis*	+	+
7	257763	Mwananyamala	32	F	MTC	67	*M. tuberculosis*	+	+
8	258471	Mwananyamala	28	M	MTC	67	*M. tuberculosis*	+	+
9	258473	Mwananyamala	27	F	MTC	67	*M. tuberculosis*	+	+
10	265826	Amana	33	F	MTC	4	*M. tuberculosis*	+	+
11	265903	Amana	<1	M	MTC	53	*M. tuberculosis*	−	+
12	265916	Amana	24	F	MTC	53	*M. tuberculosis*	−	−
13	266889	Tandale	<1	M	MTC	59	*M. tuberculosis*	−	−
14	266022	Amana	24	M	MTC	68	*M. tuberculosis*	+	+
15	265962	Amana	45	M	MTC	67	*M. tuberculosis*	+	+
16	266934	Tandale	29	M	MTC	52	*M. tuberculosis*	+	+
17	266865	Tandale	<1	F	MTC	59	*M. tuberculosis*	−	−
18	267017	Tandale	14	M	MTC	68	*M. tuberculosis*	+	+
19	267021	Tandale	50	M	MTC	16	*M. tuberculosis*	+	+
20	267005	Tandale	27	M	MTC	4	*M. tuberculosis*	−	+
21	267905	Magomeni	85	M	MTC	16	*M. tuberculosis*	−	−
22	267922	Magomeni	18	M	MTC	69	*M. tuberculosis*	+	+
23	267893	Magomeni	50	M	MTC	3	*M. tuberculosis*	−	−
24	263702	Magomeni	30	M	MTC	16	*M. tuberculosis*	+	+
25	267871	Magomeni	21	F	MTC	4	*M. tuberculosis*	+	+
26	263679	Magomeni	35	M	NTM	–	*M. intracellulare*	−	−
27	267841	Magomeni	31	M	MAC	–	*M. avium hominissuis*	−	−
28	267922	Magomeni	18	M	MTC	21	*M. tuberculosis*	+	+

MAC M. avium complex, *MST* multispacer sequence typing, *MTC Mycobacterium tuberculosis* (*M. tuberculosis*) complex, *NTM* nontuberculous mycobacteria, *RT* real-time PCR, *TB* tuberculosis

hominissuis were from rat-negative sputum samples. The performance of the rats on sputa containing the MST 67 genotype, which had a high proportion of isolates ($n = 10$), shows that 7 (70 %) of the 10 sputa with this genotype were detected by the rats (also known as rat-positive). Six of the seven samples were TB smear-positive. The smear-negative sputum detected by the rats was from a different locality. The remaining three specimens containing *M. tuberculosis* MST 67 genotypes, which were not detected by the rats, were smear-negative sputa. Sputa with nontuberculous *M. avium* subsp. *hominissuis* and *M. intracellulare* were rat-negative. The inclusion and exclusion criteria of study samples, based on quantity and quality, had no influence on the distribution patterns of the mycobacteria because they were applied randomly prior to mycobacteria isolation. The correlation between rats' detection of a sputum sample and smear positiveness was strong ($r = 0.86$) whereby all smear-positive samples and few smear-negative culture-positive sputum samples were detected by rats (Table 1). The percentage of smear positivity in the study population was 17.4 % (28 out of 161), and 13 individuals were smear-positive, culture-negative (8.1 %). Rats detected a large number of isolates belonging to different MST patterns. The detection rate of sputum with *M. tuberculosis* genotypes ranged from 50 to 100 % (Table 2) excluding MST 3 and MST 59 that were not detected.

Discussion

This study identifies 10 distinct MST genotypes of *M. tuberculosis* in Dar es Salaam's four selected DOTS centres, which are located in different geographical settings.

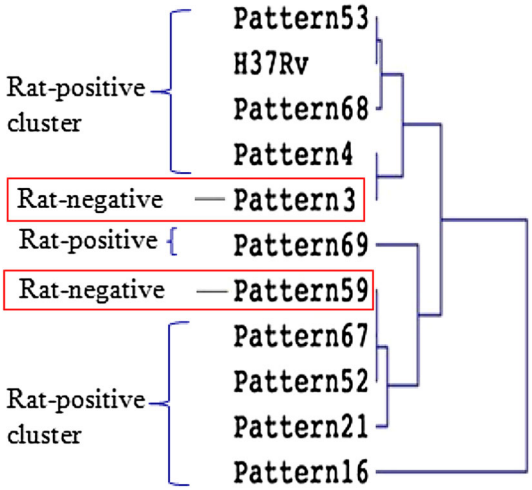


Fig. 2 Dendogram showing relationship of different multispacer sequence typing (MST) patterns of *Mycobacterium tuberculosis* detected by trained rats. MST 3 and MST 59, which are from rat-negative sputum samples, are genetically related to genotypes (MST patterns) detected by trained rats

Table 2 *Mycobacterium tuberculosis* genotypes and nontuberculous mycobacteria with respective sputum smear and trained rats' results

Isolates—MST pattern	Total no.	Smear-positive (*n*)	Rat-positive (*n*)	Rat detection (%)
MST 3	1	0	0	0
MST 4	3	2	3	100
MST 16	3	2	2	66.6
MST 21	1	1	1	100
MST 52	1	1	1	100
MST 53	2	0	1	50
MST 59	2	0	0	0
MST 67	10	7	7	70
MST 68	2	2	2	100
MST 69	1	1	1	100
NTM	2	0	0	0

MST multispacer sequence typing, *NTM* nontuberculous mycobacteria

The high diversity of MST genotypes of *M. tuberculosis* observed in this study corroborate previous spoligotyping findings, which also show high diversity of *M. tuberculosis* genotypes with three most prevalent families in Dar es Salaam, namely: CAS (37 %), LAM (22 %) and EIA (17 %) [8]. In this study, 5.8 % of mycobacterial isolates from patients suspected of pulmonary TB were not MTC organisms. This is not significantly different from the NTM prevalence of 0.3 % previously reported in Dar es Salaam by Mattee et al. [10]. In their study, the 31 NTM isolates were not further characterised, whereas the present investigation identified two different MAC species. Although information on the clinical manifestation or history of patients is not reported here, the identification of MAC-infected patients indicates the need for further studies and consideration of MAC for TB diagnosis in Tanzania. The increasing occurrence of NTM in Dar es Salaam corroborates recent reports on the occurrence of NTM species in invasive diseases in northern Tanzania [4, 5].

In this study, genotyping of *M. tuberculosis* isolates was performed using MST [6], which revealed three new profiles. We observed a significant correlation between MST 67 and the geographic origin of a patient in one of the four DOTS clinics. This suggests the presence of a single *M. tuberculosis* clone in this DOTS centre and potential of spreading in the same location [7]. Further investigations are needed to determine whether MST 67 is indeed a single clone or is due to cross-contamination in the DOTS centre. However, in both cases these data further support the need for appropriate prevention measures against transmission of TB in Dar es Salaam, including isolation measures in the DOTS centres. Our data suggest that this *M. tuberculosis* clone is not widespread in Dar es Salaam, in agreement with the large diversity of MST genotypes identified, which could reflect the multiplicity of infected clusters in this cosmopolitan city.

Trained rats detected sputa with *M. tuberculosis* independent from genotype. Overall, rats detected 8 out of 10 (80 %) MST genotypes. Rats did not detect two genotypes, namely MST 3 and MST 59 with 1 and 2 isolates. There was a strong correlation between rats' detection of sputum and sputum smear positiveness ($r = 0.86$). This correlation was expected because training of these rats includes detection of clinical sputum samples with different levels of acid-fast bacilli count ranging from 1 AFB to +3. Rats also detected few smear-negative culture-positive sputum samples. This corroborates a previous report that trained rats can detect 28 % of smear-negative TB [11]. In Dar es Salaam, both the percentage of smear-positive mycobacterial infections is lower (17.4 %) and the percentage of culture positivity are lower (21.1 %). Smear microscopy and conventional culture limit stringent evaluation of TB detection by rats. Thus, more extensive clinical diagnosis and other confirmatory tests such as interferon gamma release assays should be emphasised in future evaluations of TB diagnosis. Further studies are also needed to determine the detection threshold of the rats for all genotype isolates using a larger sample size. This can be achieved by modifying the culturing method in order to isolate non-replicating (dormant) *M. tuberculosis* in addition, which exist in sputum samples of TB patients [9]. In the present study, only 34 isolates were cultured from 161 individuals seeking TB diagnosis. Thirteen individuals (8.1 %) had smear-positive, culture-negative findings indicating potential limitations of conventional culture particularly in

recovering dormant *Mycobacterium* organisms [14]. Hence, this is likely an underestimate of actual culture-positive TB due to the failure of dormant *M. tuberculosis* to grow in conventional medium lacking resuscitation-promoting factors required for its reactivation and growth [14]. Transcriptomic studies [9] have also revealed dormant *M. tuberculosis* in sputum samples of TB patients.

The potential impact of cross-contamination of MST 67 to the performance of the rats could not be elucidated in this study because 6 out of 7 (85.7 %) detected sputa with MST 67 genotype were smear-positive in the TB clinic, suggesting that patients with this genotype indeed suffered from TB. The sputum with MST 67 genotype specimens detected by rats ($n = 7$) was collected over three different periods [(March ($n = 2$), April ($n = 3$) and June ($n = 1$)] from one clinic, while one MST 67 isolate (June 2009) was from a different clinic. Three smear-negative sputa with MST 67 isolates (30 %) were not detected by rats, whereas two other smear-negative sputa (2/8; 25 %) with two different genotypes (MST 4 and MST 53) were detected by rats, indicating that typical TB smear-negative sputa with different MST genotypes are detected by the rats. Recent studies on detection of TB in clinical samples by rats show that 28 % of the smear-negative, culture-positive TB are detected by these rats [11], and screening by rats of sputum samples previously examined by direct microscopy in DOTS centres increases case detection by 44 % [17].

In conclusion, the *M. tuberculosis* strains identified herein comprise a large diversity of genotypes (n = 10) not widely circulating in Dar es Salaam, as well as two NTM species. This study allowed a preliminary assessment of the ability of TB detection rats to diagnose TB caused by different *M. tuberculosis* genotypes, which can differ in virulence and induced pathology [3, 13]. Rats detect 80 % of sputa with *M. tuberculosis* MST genotypes found in Dar es Salaam. This corroborates a recent report that different strains of *M. tuberculosis* from Africa, Asia and Europe produce a similar blend of specific volatile compounds, which are detected by trained rats [12]. Our data emphasise the potential value of trained rats for more accurate and rapid diagnosis of pulmonary TB in resource-limited countries.

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Conflict of interest The authors declare that they have no conflict of interest regarding this work.

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