Diagnosis of tuberculosis by trained African giant pouched rats and confounding impact of pathogens and microflora of the respiratory tract

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Running title: Detection of smear-negative tuberculosis by rats

Keywords: Tuberculosis, early diagnosis, respiratory tract, African giant rats, smear-negative, smear-positive sputum.

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Abstract

Trained African giant-pouched rats (*Cricetomys gambianus*) can detect *Mycobacterium tuberculosis* (*Mtb*), and show potential for diagnosis of tuberculosis (TB). However, rats’ ability to discriminate between clinical sputum containing other *Mycobacterium* spp. and nonmycobacterial species of the respiratory tract is unknown. It is also unknown whether nonmycobacterial species produce odour similar to *Mtb* and thereby cause detection of smear-negative sputum. Sputa from 289 subjects were analyzed by smear microscopy, culture, and rats. *Mycobacterium* spp. were isolated on Lowenstein Jensen medium, and nonmycobacterial species were isolated on four different media. Odour from nonmycobacterial species from smear- and *Mtb*-culture-negative sputa detected by ≥2 rats (rat-positive) was analyzed by gas-chromatography/mass-spectrometry and compared to *Mtb* odour. Rats detected 45 of 56 confirmed TB; 4 of 5 suspected TB, and 63 of 228 TB-negative subjects (sensitivity=80.4%, specificity=72.4%, accuracy=73.9%, positive predictive value=41.7%, negative predictive value=93.8%). Thirty-seven (78.7%) of 47 mycobacterial isolates were *Mtb* complex, with 75.7% from rat-positive sputa. Ten isolates were nontuberculous mycobacteria; one *M. intracellulare*, *M. avium* subsp. *hominissuis* and 8 were unidentified. Rat-positive sputa with *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus* spp., and *Enterococcus* spp. were associated with TB. *Rhodococcus* spp., *Nocardia* spp., *Streptomyces* spp., *Staphylococcus* sp., and *Candida* spp. from rat-positive sputa did not produce *Mtb*-specific volatiles (methyl nicotinate, methyl *para*-anisate, and *ortho*-phenylanisole). Prevalence of mycobacteria-related *Nocardia* and *Rhodococcus* in smear-negative sputa did not equal that of smear-negative mycobacteria (44.7%) of which 28.6% was rat-positive. These findings, and absence of *Mtb*-specific volatiles in nonmycobacterial species, indicate rats can be trained to specifically detect *Mtb*.
Introduction

Novel methods for rapid diagnosis of tuberculosis (TB) are urgently needed to complement smear microscopy, which has low sensitivity (20), and culture, which is slower and requires specialized laboratory conditions not available in resource-constrained settings. Trained African giant pouched rats (Cricetomys gambianus) possess profound potential for rapid detection of TB with higher sensitivity and specificity (38). An increase in TB case detection rate of 43–44% is achieved when Cricetomys rats are used as second-line screening tool, after smear microscopy (18, 25). Rats have a highly developed sense of smell among mammalian species (22), which renders them trainable to specifically sense TB odour in sputum samples with a broad range of acid-fast bacilli (AFB) counts (see Materials and methods). These rats also detect smear-negative sputa, which may contain few acid-fast bacilli missed by microscopy. Moreover, smear-negative results may persist in thorough re-examination of smears made after rat results.

In this study, we investigated the extent to which TB detection rats can discriminate between clinical sputa with different Mycobacterium spp. [Mycobacterium tuberculosis (Mtb) and nontuberculous mycobacteria (NTM)] and other microorganisms of the respiratory tract (Nocardia spp., Rhodococcus spp., Streptomyces spp., Moraxella spp., Candida spp., and Streptococcus pneumoniae), which can also be found in sputum. We also investigated whether the nonmycobacterial species found in sputum produce odour compounds similar to Mtb odour, which could cause false detection of smear-negative sputum without Mtb.
Materials and methods

Specimens

Sputa (n=514) from 289 individuals presenting for TB diagnosis in six selected Tanzanian TB clinics (i.e., Dar Es Salaam = 5; Morogoro = 1) were analyzed April–June 2009 (252 sputa from 161 individuals) and July 2010 (262 sputa from 128 individuals). Sputum aliquots (1 ml) for cultures were aseptically transferred into sterile screw-capped microtubes. The remaining volume (> 3 ml) in polypropylene sputum containers with lid was processed for TB detection by sniffer rats. Processing of sputa for rats included adding 5 ml of phosphate-buffered saline (PBS) to increase volume and avoid drying of sputum during inactivation. Sputum samples (>8 ml, with PBS) were heat-inactivated at 90°C for 30 min, cooled to room temperature and stored at −20°C until later use in TB diagnosis by the rats at Sokoine University of Agriculture, and Anti-Persoonmijnen Ontmijnende Product Ontwikkeling (SUA-APOPO) laboratory, Morogoro.

Population characteristics

The age of sputum donors varied from < 1 year to 86 years (mean ± standard deviation, 32 ± 3 years). The gender ratio (150 males: 139 females) was 1.08. The subjects were classified into three TB diagnostic categories: (i) confirmed TB: individuals with two smear-positive (AFB+) sputa, and/or positive mycobacterial (Mtbc) culture; (ii) suspected TB: individuals with only one smear-positive culture-negative sample (Mtbc); (iii) non-TB (negative): individuals with smear-negative and Mtbc culture-negative sputum. Individuals with NTM isolates were classified in the non-TB category. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of Mtbc detection by rats were determined as described elsewhere (13) using confirmed TB and non-TB subjects. Suspected TB cases were
excluded in the subsequent evaluation. Microorganisms from smear-negative, *Mtb*
culture-negative but rat-positive sputa were used to determine odour profiles and
confounding impact of microbes to TB diagnosis by rats.

Isolation and identification of mycobacteria

A total of 380 sputa from 289 subjects (1–2 samples per subject) were cultured to
isolate mycobacterial species. After processing, 252 sputa samples from 161 donors
were inoculated on Lowenstein Jensen medium (LJ) with pyruvate and LJ with
glycerine, and 128 sputa (128 donors) on LJ with glycerine only. Processing included
decontamination with 4% sodium hydroxide added to sputum in 1:1 ratio, mixing well
and leaving to stand for 45 min. The mixture was centrifuged at 3000 g for 20 min,
supernatant decanted, and sediment was neutralized with 14% potassium dihydrogen
phosphate. Cultures were incubated at 37°C for a minimum of 8 weeks with weekly
examination for growth (44). Isolates were stained using the Ziehl-Neelsen (ZN)
method and DNA was extracted from all AFB by the bead beating method (34).

Multiplex real-time PCR for genus *Mycobacterium*, *M. tuberculosis* sp. complex
(MTC) and *M. avium* complex (MAC) was performed as previously described (26,
31). A conventional multiplex PCR for this genus was also performed (42). Isolates
negative for PCR were identified by 16S rRNA gene sequencing (1). MTC isolates
were subjected to multispacer sequence typing (MST) (11).

Nonmycobacterial respiratory tract microbes

Chocolate agar, sabouraud dextrose agar, buffered charcoal yeast extract agar, and
paraffin agar were used to isolate nonmycobacterial respiratory tract microorganisms
from 394 sputa (289 donors). Cultures were incubated at 37°C for 6 weeks, with
isolates preliminarily identified by colony and cell morphology, and biochemical
tests. Moraxella sp. and Streptococcus sp. were subjected to PCR for M. catarrhalis and S. pneumoniae (15). Nocardia sp., Rhodococcus sp., and Streptomyces sp. were identified by growth characteristics in different media including opacification of Middlebrook 7H11 medium and formation of chalky white colonies (12). Nocardia isolates were further subjected to specific PCR (5, 14). 16S rRNA gene sequencing using fD1 and rP2 universal primers (39) was used to identify Rhodococcus sp., Enterococcus sp., and Staphylococcus sp.

Determining microorganisms in rat-positive sputum

Processed sputa (n=514) in polypropylene containers were analyzed by a group of 10 rats selected from 22 qualified rats. which consistently detected more than 80% of known smear-positive sputa (TB-positive). Detection procedures described by Weetjens et al. (38), (see: http://www.youtube.com/watch?v=KoRvdyuHxdE) (37) were used. Briefly, rats were rewarded with food if they paused for at least 5 s to sniff TB-positive sputum confirmed by smear-microscopy and/or culture. Smear-positive sputa consisted of various AFB counts: 1–9 AFB, 1+, 2+ to 3+, whereby 1–9 AFB refers to 1–9 AFB per 100 microscopy fields; 1+ is 10–99 AFB per 100 fields; 2+ is 1–10 AFB per field, and 3+ is more than 10 AFB per field (8). Rats were not rewarded on pausing at TB-negative samples. On average, one rat analyzed the set of 70 samples at a rate of 8 min per session. Each rat analyzed the 70 sputa twice (thus two sessions = 16 min). A sample was considered TB-positive if a minimum of two rats gave a positive signal on it. Therefore, consensus results (two rats × two sessions each) of 70 samples were obtained in 32 min. The training session was conducted by two teams of trainers handling five rats each, in 95 min. This duration includes time for changing rats, cleaning/wiping of the training cage floor with 70% ethanol to remove odour residues between rat sessions, and changing of metal bars containing
the 70 sputa. Detection of negative sputa by two rats, based on microscopy and/or culture, was indicative of \textit{Mtb} (rat-positive) and the sputum was subjected to thorough investigation.

\textit{Odour analysis by gas chromatography/mass spectrometry}

Volatile compounds of the microorganisms from AFB smear-negative, \textit{Mtb} culture-negative, and rat-positive sputa were identified using gas chromatography/mass spectrometry (GC/MS). Analyses were carried out on an Agilent 7890A GC system connected to an Agilent 5975C inert mass detector fitted with an HP-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.25 \textmu m film; J&W Scientific, USA).

Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 mL min$^{-1}$; injection volume: 2 \textmu l; transfer line: 300°C; electron energy: 70 eV. The GC program was set as follows: 5 min at 50°C, increasing with 5°C min$^{-1}$ to 320°C, operated in the splitless mode (60 s valve time); He carrier gas flow was 1.2 ml min$^{-1}$.

Briefly, selected bacteria and yeast isolates were grown on suitable media and headspace samples were collected for 24 h using a closed loop stripping apparatus (CLSA) as described (28), fitted with an activated charcoal filter (Chromtech; Precision Charcoal Filter, 5 mg). The collected volatiles were eluted from the filter for GC/MS analysis using 30 \textmu l of dichloromethane (Suprasolv, Merck, Germany).

Compounds were identified by comparison of GC/MS retention indices with those of mass spectral libraries and comparison with synthetic reference compounds. Retention indices $I$ were determined from a homologous series of \textit{n}-alkanes (C$_8$-C$_{35}$) (35).
Statistical analysis

The Fisher’s exact test was used to determine whether the distribution of rat-positive and -negative sputa with *Mtb* was different from that of sputa with NTM and nonmycobacterial species, with a *P* value < 0.05 for statistical significance.

Results

Detection of TB in sputum by rats

There were 56 confirmed TB cases based on smear microscopy (n=19) and culture (n=37), 228 TB-negative and 5 suspected TB cases. Rats detected 45 (true-positive) of the 56 confirmed TB cases and 63 (false-positive) of the 228 negative subjects. Four (80%) of the five suspected TB cases with one AFB-positive sputum were detected by rats (rat-positive). Sensitivity and specificity were 80.4% and 72.4%, respectively. Positive predictive value (PPV) was 41.7% and negative predictive value (NPV) was 93.8%, with an accuracy of TB diagnosis of 73.9%.

Mycobacterium sp. isolation

*Mycobacterium* spp. were isolated from 47 out of 289 patients (16.3%). Thirty-seven isolates from 37 patients were MTC (78.7%) based on specific multiplex PCRs for the *Mycobacterium* genus and MST analyses. Details of genotypic analyses (MST) of MTC isolates will be reported separately (Mgode, G.F. et al. *unpublished data*). The majority of MTC (75.7%) were from rat-positive sputa (Table 1, Fig. 1).

Ten mycobacterial isolates were NTM of which two were *M. intracellulare* and *M. avium* subsp. *hominissuis*. Eight NTM (17%) of 47 mycobacterial isolates were not identified to species level. Four of the eight NTM were from rat-positive sputa, of which one was smear-positive. The detection trend for sputa with NTM (including *M.*
avium subsp. hominissuis and M. intracellulare) was marginally different from
detection of sputa with Mtb ($P = 0.054$, Fisher’s exact test) (Fig. 1). Isolates identified
as M. avium subsp. hominissuis and M. intracellulare were from rat-negative sputa
(Table 1). Nine of the 10 NTM were from smear-negative sputa corroborating
previous reports showing increasing occurrence of NTM in clinical samples (7, 10).
Overall, 21 (44.7%) of all mycobacterial isolates (n=47) were from smear-negative
sputa, revealing that a significant proportion of smear-negative sputa contained
mycobacterial species, which were probably the cause of detection of these sputa by
rats. Indeed, six (28.6%) of the 21 mycobacterial isolates from smear-negative sputa
were rat-positive indicating increased detection rate of smear-negative TB by > 28%.

Isolation and species distribution of opportunistic pathogens of the respiratory tract
Among the four media used, paraffin agar improved isolation success of species,
which included Nocardia sp., Streptomyces sp., Candida sp., and one NTM. These
were identified by their characteristic colony morphologies and pigmentation in this
medium. Rhodococcus spp. were isolated on chocolate agar and buffered charcoal
yeast extract agar, whereas Moraxella spp., Streptococcus sp., and Enterococcus spp.
were isolated on chocolate agar. Yeast species were isolated on all four media used.

Streptococcus spp. were the most abundant among the respiratory tract
nonmycobacterial isolates (n=69). Thirteen isolates from rat-positive sputa were
identified as S. pneumoniae by specific PCR (15) and the remaining streptococcal
isolates were assigned to S. pneumoniae based on colony and cell morphology, which
were similar to 13 isolates identified by PCR. Other microorganisms from sputum are
shown in Table 2. Two of the three Nocardia isolates were identified as N. farcinica
(5, 14), of the N. asteroides complex. Rhodococcus spp., Enterococcus spp.,
Staphylococcus succinus and other Staphylococcus spp. were identified by 16S rRNA gene sequence (39). Candida spp. were identified by Gram stain. Nonmycobacterial microorganisms co-occurred in some individuals. For example, Streptococcus spp. co-occurred with Candida spp. (n=5), and with M. catarrhalis (n=3), Streptomyces spp. (n=3), Rhodococcus spp. (n=1), and Nocardia spp. (n=1). Co-occurrence was also found in Nocardia spp. and Streptomyces spp. (n=2), Candida spp., and M. catarrhalis (n=4). Nonmycobacterial species also co-occurred with Mtb (Table 2 and Fig. 3). Rat-positive sputa with Staphylococcus spp. and Enterococcus spp. were also either smear-positive or mycobacterial culture-positive. Rats’ detection of sputum containing mycobacterial and nonmycobacterial species is presented in Tables 1 and 2, and Figures 1–3.

Comparison of rat-positive (detected) and rat-negative (undetected) sputa with Mtb (Table 1) versus sputa with nonmycobacterial species (Table 2) revealed that detection of sputa with M. catarrhalis, S. pneumoniae, Candida spp., Enterococcus spp., Staphylococcus succinus and other Staphylococcus spp. was significantly different from Mtb (P < 0.05) (Table 2, Fig. 1). Thus, nonmycobacterial species did not cause detection of sputum by rats compared to Mtb. The distribution of rat-positive and -negative sputa with Rhodococcus spp., Nocardia spp., Streptomyces spp., and few unknown microorganisms was not significantly different from that of Mtb. However, these species were not as abundant in detected sputa as Mtb (Tables 1 and 2, Fig. 1) and have low prevalence.

Odour analysis

An odour analysis was performed on selected isolates from Mtb smear-negative, culture-negative, rat-positive sputa, namely, Rhodococcus sp., Candida sp., and
Staphylococcus sp. isolates, as well as reference strains of Nocardia spp. (N. asteroides and N. africana) and Streptomyces spp. (S. coelicolor, S. griseoflavus, and S. antibioticus). Table 3 depicts compounds repeatedly found in these strains. Methyl nicotinate, methyl para-anisate, ortho-phenylanisole, and methyl phenylacetate were predominant in Mtb, as reported by Syhre and Chambers (32). A wide variety of compounds occurring in Mtb strains may serve as basis for the odour detection. We identified volatiles shared by Mtb, Nocardia spp., Streptomyces spp., and Rhodococcus sp., for example, 2-phenylethanol or 2-hydroxy-3-pentanone (Table 3), which are also produced by other microbial species (28, 29, 36, 41) and hence cannot be regarded as specific markers for Mtb. For example, aciphyllene, which is a known sesquiterpene from the endophytic fungus Muscodor albus (3), is a typical volatile compound of Nocardia spp. Candidate TB markers of Mtb were not found in nonmycobacterial species, which had a distribution of rat-positive and -negative sputa similar to sputa with Mtb (P > 0.05) (Table 2).

Discussion

Our study reveals that trained African giant pouched rats (C. gambianus) target Mtb in sputa, and not other microorganisms of the respiratory tract. Mtb was the most frequently detected species among the microorganisms isolated from sputa of suspected cases (Fig. 2). Most of the rat-positive sputa containing opportunistic pulmonary pathogens also contained Mtb as confirmed by either smear microscopy or culture. Rats’ detection of smear- and culture-negative sputa containing other pulmonary pathogens, such as M. catarrhalis, S. pneumoniae, Candida spp., Enterococcus spp., Staphylococcus succinus, and other Staphylococcus spp. appears to be due to Mtb, which could be below the detection thresholds of microscopy and culture (19). The statistically significant difference found in the distribution of rat-
positive and -negative sputa with *Mtb* and nonmycobacterial species (*P* < 0.05) indicates that trained rats do not false-detect sputa with these microorganisms as *Mtb*-positive samples (Tables 1 and 2). Sputa with these species alone, excluding those with *Rhodococcus* spp., *Nocardia* spp., and *Streptomyces* spp. were less frequently detected (Table 2, Fig. 2). Sputa containing *Enterococcus* spp. and *Staphylococcus* spp. were also detected in the presence of *Mtb*. The detection of sputa with *Rhodococcus* spp., *Nocardia* spp., and *Streptomyces* was not significantly different from that of sputa with *Mtb* (*P* > 0.05), which suggests that sputa with these mycobacteria-related species could be false-detected by rats as samples with *Mtb*. However, analysis of odour compounds revealed that these species do not produce candidate odour markers for TB diagnosis produced by *Mtb* (Table 3). This can be taken as evidence for presence of *Mtb* bacilli in sputa with these species, which were not detected by culture or microscopy.

The prevalence of mycobacterial-related *Nocardia* spp. and *Rhodococcus* spp. in Tanzania was lower (1%) compared to 4–5% reported in other sub-Saharan countries (16). Hence, these species cannot account for the high proportion of smear-negative rat-positive sputa of which 28.6% were TB cases detected by rats and culture. Smear-negative, culture-positive *Mycobacterium* spp. contributed to 44.7% of the total mycobacterial isolates. This enabled the evaluation of causes of detection of smear-negative sputa and extent of detection of sputa with different mycobacterial species. However, the present study used randomly chosen sputa based on sample volume rather than patients’ symptoms, such as bronchopneumonia, which increases the isolation rate of *Nocardia* spp. (23). While the prevalence of *Nocardia* sp. in this study could be an underestimate, the low prevalence of *Rhodococcus* sp. cannot be adequately discussed since there are no other data from Tanzania regarding this...
The prevalence of *Streptomyces* spp. and *Candida* spp. was higher (7.3% and 7.8%, respectively) but can also not account for the proportion of smear-negative, rat-positive sputa. The detection of sputa with NTM as well as smear-negative, *Mtb* culture-negative sputa could be due to low *Mtb* abundance, loss (death) of the few *Mtb* during decontamination and neutralization or co-occurrence and competition between fast-growing NTM and slow-growing *Mtb* in culture. The prevalence of NTM is increasing (7, 10) especially in smear-negative TB/HIV co-infected patients associated with low *Mtb* load in sputum (9). Dormant *Mtb* bacilli also cause culture-negativity (21). Further studies targeting isolation of dormant *Mtb*, for example, by incorporation of resuscitation promoting factors (*rpf*) in medium (21) are needed to further determine the rate of detection of *Mtb* by rats in smear-negative samples, and the extent of detection of sputa with a high proportion of NTM. The geneXpert MTB/RIF for *Mtb* (4) could assist in revealing *Mtb* in smear and culture-negative, rat-positive sputa and provide deeper insight into the false-positive rate of the rats.

Odour analysis from selected nonmycobacterial isolates from smear-negative, rat-positive sputa and reference *Nocardia* spp. and *Streptomyces* spp. (Table 3) showed that these microorganisms produced volatile compounds different from those reported for *Mtb* (32). Previous studies on volatile compounds of related nonmycobacterial organisms such as *R. fascians*, *S. epidermidis*, and *Streptomyces* spp. (17, 27, 36) reported volatiles not identical to the described *Mtb*-specific volatile compounds. Together, these findings indicate absence of *Mtb*-specific compounds from nonmycobacterial microorganisms.
The volatile compounds of a given isolate cultured on artificial medium can differ from those of the same isolate in the host tissue due to differences in growth substrates as reported for the fungus *Trichoderma* sp. (6, 40). *Mtb* bacilli grown in artificial medium also lack characteristic chemical compounds, such as phthioic acid, phthiocol, tuberculostearic acids and polysaccharides found in *Mtb* bacilli from host tissue (2). The lipid content of *Mtb* bacilli grown *in vitro* also differs from *Mtb* bacilli in tissue (30). These compounds, however, are not among the candidate odour markers of TB, which have recently been reported for *Mtb* in both artificial medium and in breath samples of TB patients (24, 32, 33). Absence of the candidate TB volatile compounds in nonmycobacterial species hence suggests that these species do not confound detection of sputum by trained rats. Further studies are needed to precisely determine whether the rats detect candidate volatile compounds of *Mtb* produced in artificial medium.

The sensitivity and specificity of TB diagnosis by rats in this study was high (80.4% and 72.4%, respectively), but lower than the previously reported 86.6% and 93.8%, respectively (38). The lower PPV (41.7%) and higher NPV (93.8%) obtained are largely affected by the prevalence of TB in a given population. Our study indicates that harnessing rats for early TB diagnosis could have a significant impact on TB control. This is supported by the higher NPV (93.8%), which indicates that individuals with rat-negative sputum have a 93.8% likelihood of not having active TB. Consensus results of two rats (two sessions each) are obtained in 32 min for the set of 70 sputa. This is faster than the smear microscopy in which one microscopist is recommended to analyze an average of 20 samples per day (43). The shorter time used by rats to detect TB could enable screening of a larger population and reduce new TB transmissions resulting from undetected TB cases and delayed diagnosis.
Further studies are needed to precisely identify the specific volatile compounds detected by rats and their occurrence in diverse *Mycobacterium* spp. The diagnosis of emerging *Nocardia* spp. and *Rhodococcus* spp. pathogens should also be considered, especially when TB is ruled out in patients with pulmonary disease symptoms. Our data further underline the potential value of conditioned rats for rapid, specific and sensitive diagnosis of TB.
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References


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Accessed 2 September 2011.


World Health Organization, Geneva, Switzerland.
Table 1. Mycobacterium spp. from smear-positive and -negative sputum samples (n=47) tested by trained Cricetomys gambianus rats.

<table>
<thead>
<tr>
<th>Mycobacterial designation</th>
<th>Number (n)</th>
<th>Distribution (%)</th>
<th>Rat-positive</th>
<th>Rat-negative</th>
<th>Detection (%)</th>
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<tbody>
<tr>
<td></td>
<td>(n)</td>
<td></td>
<td>Rat+</td>
<td>Rat-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Smear+</td>
<td>Smear-</td>
<td></td>
<td>Smear+</td>
<td>Smear-</td>
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<tr>
<td>Mtb</td>
<td>37</td>
<td>25</td>
<td>12</td>
<td>78.7</td>
<td>28</td>
</tr>
<tr>
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<td>8</td>
<td>1</td>
<td>7</td>
<td>17.0</td>
<td>4</td>
</tr>
<tr>
<td>M. avium subsp. hominissuis</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2.1</td>
<td>0</td>
</tr>
</tbody>
</table>

Smear positive, smear+, smear negative, smear-. Combined rat-positive (detection) of sputa with NTM and M. avium subsp. hominissuis and M. intracellulare is 40%.
<table>
<thead>
<tr>
<th>Species</th>
<th>Number (n)</th>
<th>Rat-positive</th>
<th>Rat-negative</th>
<th>Smear positive detection (%)</th>
<th>Significance</th>
</tr>
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<tr>
<td>Moraxella catarrhalis</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>20.1%</td>
<td>66.7*</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>79.9%</td>
<td>NS</td>
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<tr>
<td>Nocardia (N. farcinica)</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>79.9%</td>
<td>NS</td>
</tr>
<tr>
<td>Streptomyces</td>
<td>10</td>
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<td>8</td>
<td>40.0%</td>
<td>66.7*</td>
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<tr>
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<td>21</td>
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<td>18</td>
<td>60.4%</td>
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<td>55</td>
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<tr>
<td>Staphylococcus</td>
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<td>0.0</td>
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<tr>
<td>Unidentified</td>
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<td>4</td>
<td>7</td>
<td>18.2%</td>
<td>3.85e-5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>139</td>
<td>28</td>
<td>111</td>
<td>(20.1%)</td>
<td></td>
</tr>
</tbody>
</table>

* Frequently detected microorganisms from smear-negative Mtb culture-positive sputum not detected by rats are not presented in this Table.

^ Significance in Fisher’s exact test as compared to distribution of rat-positive/rat-negative Mtb data (Table 1). Each row in Table 2 is compared with the first row of Table 1. Rat-positive not significantly different from sputa with Mtb is shown by NS.
Table 3. Volatile compounds of isolates from sputum samples and reference *Mtb*, *Nocardia* spp. and *Streptomyces* spp.

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<thead>
<tr>
<th>Compounds</th>
<th>Microorganisms species tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Mtb</em></td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>X</td>
</tr>
<tr>
<td>Dimethyl trisulfide</td>
<td></td>
</tr>
<tr>
<td>Dimethyl tetrasulfide</td>
<td></td>
</tr>
<tr>
<td>Methyl methanethiosulfonate</td>
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</tr>
<tr>
<td>2,3-Dimethyl-5-isopentylpyrazine</td>
<td>X</td>
</tr>
<tr>
<td>Unknown pyrazine</td>
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</tr>
<tr>
<td>Camphor</td>
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</tr>
<tr>
<td>Linalyl acetate</td>
<td></td>
</tr>
<tr>
<td>Isobornyl acetate</td>
<td>X</td>
</tr>
<tr>
<td>Aciphyllene</td>
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</tr>
<tr>
<td>Unknown diterpenoid</td>
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</tr>
<tr>
<td>2-Hydroxy-3-butanoic</td>
<td>X</td>
</tr>
<tr>
<td>2-Hydroxy-3-pentanone</td>
<td>X</td>
</tr>
<tr>
<td>2,5-Dimethylthiophene</td>
<td>X</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>X</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>X</td>
</tr>
<tr>
<td>4-Methyl-2-pentanone</td>
<td>X</td>
</tr>
<tr>
<td>4-Methylpent-3-en-2-one</td>
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</tr>
<tr>
<td>γ-Methylbutyrolactone</td>
<td>X</td>
</tr>
<tr>
<td>2-Phenylethanol</td>
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</tr>
<tr>
<td>Ethyl phenylacetate</td>
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</tr>
<tr>
<td>Methyl phenylacetate a</td>
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</tr>
<tr>
<td>Methyl nicotinate a</td>
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</tr>
<tr>
<td>Methyl para-anisate a</td>
<td></td>
</tr>
<tr>
<td>ortho-Phenylanisole a</td>
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</tbody>
</table>

*a* Syhre and Chambers. 2008
Fig. 1. Rat-positive (%) sputum samples with different individual microorganisms. Number of isolates of each species in detected sputum is indicated in brackets. Statistically significant difference ($P < 0.05$, Fisher’s exact test) between rat-positive sputa with *Mtb* (reference) and rat-positive sputa with nonmycobacterial species is shown by an asterisk. Rat-positive not significantly different from sputa with *Mtb* is shown by NS.
Fig. 2. Overall rat-positive and rat-negative sputum samples with different microorganisms. Nontuberculous mycobacteria (NTM).
Fig. 3. Patterns of rat positive and rat-negative in smear-positive (sm+) and smear negative (sm−) sputum samples with *Mycobacterium* and nonmycobacterial microorganisms. Smear-positive sputa with *Mtb* have higher frequency of rat-positive than smear-negative (100% vs. 28%, respectively).