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DIAGNOSTICS

Ability of *Cricetomys* rats to detect *Mycobacterium tuberculosis* and discriminate it from other microorganisms

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SUMMARY

Trained African giant pouched rats (*Cricetomys gambianus*) have potential for diagnosis of tuberculosis (TB). These rats target volatile compounds of *Mycobacterium tuberculosis* (*Mtb*) that cause TB. *Mtb* and nontuberculous mycobacteria (NTM) species are related to *Nocardia* and *Rhodococcus* spp., which are also acid-fast bacilli and can be misdiagnosed as *Mtb* in smear microscopy. Diagnostic performance of *C. gambianus* on *in vitro*-cultured mycobacterial and related pulmonary microbes is unknown. This study reports on the response of TB detection rats to cultures of reference *Mtb*, clinical *Mtb*, NTM, *Nocardia*; *Rhodococcus*; *Streptomyces*; *Bacillus*; and yeasts. Trained rats significantly discriminated *Mtb* from other microbes ($p < 0.008$, Fisher's exact test). Detection of *Mtb* cultures was age-related, with exponential and early stationary phase detected more frequently than early log phase and late stationary phase ($p < 0.001$, Fisher's test) (sensitivity = 83.33%, specificity = 94.4%, accuracy = 94%). The detection of naturally TB-infected sputum exceeded that of negative sputum mixed with *Mtb*, indicating that *C. gambianus* are conditioned to detect odours of TB-positive sputum better than spiked sputum. Although further studies on volatiles from detectable growth phases of *Mtb* are vital for identification of *Mtb*-specific volatiles detected by rats, our study underline the potential of *C. gambianus* for TB diagnosis.

1. Introduction

Trained African giant pouched rats (*Cricetomys gambianus*) can detect *Mycobacterium tuberculosis* (*Mtb*) in sputum samples from humans with confirmed pulmonary tuberculosis (TB).[1] These rats target volatile compounds (odours) specific to *Mtb*, the causative agent of TB. *Mtb* and nontuberculous mycobacteria (NTM) species belong to the genus *Mycobacterium*, which is related to the genus *Nocardia* and *Rhodococcus*. Some NTM and members of the genera *Nocardia* and *Rhodococcus*, which are also acid-fast bacilli, are increasingly recognized as pathogens of the respiratory tract[2–4] and can be misdiagnosed as *Mtb* in smear microscopy during TB diagnosis. Misdiagnosis of pulmonary nocardiosis and/or *Rhodococcus* infection as TB may lead to unnecessary treatment with anti-TB drugs.

The diagnostic performance of trained *C. gambianus* on pure cultures of mycobacterial species and related microbes, which may be present in sputum samples, is unknown. This study aims to determine how *C. gambianus*, trained to detect TB-positive sputum samples, react to pure cultures of reference *Mtb*, NTM, clinical *Mtb* isolates; *Nocardia* spp.; *Rhodococcus* sp.; *Streptomyces* spp.; *Bacillus* sp.; *Candida* sp.; and *Saccharomyces* sp.

2. Material and methods

2.1. Microorganisms

Thirty-eight bacterial and yeast strains from the genera *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Bacillus*, *Candida* and *Saccharomyces* obtained from various culture reference centres were used. These strains originated from the Belgian Coordinated Collections of Microorganisms (BCCMtm/LMG), Gent, Belgium (http://bccm.belspo.be); the German Collection of Microorganisms and Cell Cultures (DSMZ) (http://www.dsmz.de); the Max Planck Institute for Infection Biology, Berlin, Germany, and Sokoine University of Agriculture, Morogoro, Tanzania (Table 1).

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Table 1
Microorganisms (20 bacterial and 2 yeast species) tested by trained TB detection rats.

No.	Species	Strain	Source	Samples tested (n)*
1	*Mtb*	H37Rv	Laboratory strain	9
2	*Mtb*	Beijing 2	Netherlands	14
3	*Mtb*	Beijing 3	South Korea	4
4	*Mtb*	n/a	Netherlands	3
5	*Mtb*	Beijing 5	South Africa	39
6	*Mtb*	Beijing 6	Mongolia	8
7	*M. smegmatis*	MC^2155	n/a	25
8	*M. avium* subspecies avium	n/a	n/a	5
9	*M. scrofulaceum*	n/a	n/a	5
10	*M. vaccae*	n/a	n/a	5
11	*M. aichiense*	LMG 19259	Soil	15
12	*M. alvei*	LMG 19260	Water	2
13	*M. aurum*	LMG 19255	Soil	19
14	*M. neoaurum*	LMG 19258	Soil	20
15	*M. peregrinum*	LMG 19256	Human	7
16	*M. bovis*	BCG – Pasteur	n/a	7
17	*M. bovis*	BCG-Copenhagen	n/a	7
18	*Streptomyces antibioticus*	LMG 5966	Soil	11
19	*S. griseoflavus*	LMG 19344	Soil	20
20	*S. griseoluteus*	LMG 19356	Soil	6
21	*S. coelicolor*/ *S. albidoflavus*	DSM 40233	n/a	8
22	*Nocardia lutea*	LMG 4066	Soil	4
23	*N. uniformis*	LMG 4082	Soil	3
24	*N. asteroides*	LMG 4062	n/a	7
25	*Mtb* complex, clinical isolate	N 185/08	Human-Tanzania	12
26	*Mtb* complex, clinical isolate	RT 1340	Human-Tanzania	5
27	*Mtb* complex, clinical isolate	N 1283/08	Human-Tanzania	3
28	*Mtb* complex, clinical isolate	RT 1284/08	Human-Tanzania	9
29	*Mtb* complex, clinical isolate	N 1080/08	Human-Tanzania	9
30	*Mtb* complex, clinical isolate	BR 30	Human-Tanzania	9
31	*Mtb* complex, clinical isolate	RT 1104	Human-Tanzania	2
32	*Mtb* complex, clinical isolate	N 194/08	Human-Tanzania	4
33	*M. bovis* clinical isolate	KP 20	Human-Tanzania	6
34	*Mtb*	H37Ra	Laboratory strain	4
35	*Rhodococcus equi*	n/a	Laboratory strain	4
36	*Bacillus subtilis*	n/a	Local isolate	4
37	*Candida albicans*	n/a	Human-Tanzania	5
38	*Saccharomyces cerevisiae*	n/a	Baker's yeast	5
Total number of microorganism samples tested				334

Mtb Mycobacterium tuberculosis, n/a not applicable.
* Different age-based cultures of same species/strain, including same-age cultures, tested repeatedly on different days (technical replicates).

2.2. Cultivation and inactivation of microorganisms

Lyophilized bacterial strains were reconstituted according to supplier's instructions and inoculated into 14–20 ml of Middlebrook (7H9) liquid medium containing albumin dextrose catalase (ADC) enrichment without Tween and Glycerol. Cultures were incubated at temperature ranges of 28–30 °C and at 37 °C for a period of 4 days–9 weeks for slow-growing species, under appropriate biosafety conditions. A loopful of culture was then inoculated on Luria/Miller (LB) agar and incubated at 37 °C to check for purity. Viable colony forming units (CFUs) of liquid cultures were counted after diluting cultures serially and plating 100 μl of each dilution on Middlebrook 7H11 and LB agar, which were incubated at suitable temperature (28–30 °C and at 37 °C, respectively). Growth was also determined by measuring optical density (OD_{580nm} or OD_{600nm}) using a UV/Visible spectrophotometer (Amersham Biosciences, Uppsala, Sweden). For *Mtb*, an OD_{580nm} of 0.1 was equal to 5×10^7 bacterial cells/ml.

All test organisms, except *Mtb*, were heat-inactivated in a 90 °C water bath for 30 min and left to cool at room temperature before being stored at −20 °C until later use. Aliquots of 4 ml of *Mtb* culture in secured screw-capped plastic vials were inactivated on a dry heating block at 100 °C for 1 h, in a biosafety level 3 facility. The level of *Mtb* culture in vials did not exceed the part of vial that was inside the holes of dry heat block to ensure contact with heated area. The efficiency of dry heat inactivation of *Mtb* was assessed by culturing aliquots of inactivated cultures in 7H9 and 7H11 media incubated at 37 °C for 3 months while checking eventual growth at 7-day intervals.

2.3. Spiking sputum samples with inactivated microorganisms

Negative sputum samples from TB clinics in Dar es Salaam, Tanzania, were used for spiking test microorganisms. The negative status of these samples has been confirmed by smear microscopy [Ziehl Neelsen (ZN)], fluorescent microscopy (FM), mycobacterial culture and by TB detection rats (*C. gambianus*). About 10 ml of heat-inactivated negative sputum with saline were spiked with 100 μl, 500 μl and or 1000 μl of bacterial culture. A minimum of two replicate samples of each test microorganism were tested by a minimum of six rats, performing two test sessions each per day. The positive control consisted of confirmed TB-positive sputum samples ($n = 7$), which were mixed with sterile medium. For negative controls, confirmed TB-negative sputum mixed with sterile medium was used. One day was skipped between subsequent tests to allow the rats to perform routine TB detection. The experiment was conducted over a period of 94 days (January–April 2009). Microorganisms detected in initial tests were further presented to rats to confirm findings. Further tests included culture with different ages (growth phases) to determine the most detectable phase since different volatiles can be produced by the same microorganism in different growth stages and culture conditions.

The rats' training procedure and judging of positive detection is described in detail elsewhere.[1,5] Briefly, during training sessions, rats were rewarded with food (mashed banana mixed with crushed commercial rat food) when they paused for 5 s at known TB-positive sputum samples. They did not receive food for pausing at known TB-negative samples. With extensive training the rats learnt to consistently pause at TB-positive samples but not at TB-negative samples. During the reward condition in the present research, identification responses to the seven TB-positive sputa (reward samples) were followed by food delivery. During the no-reward condition, food was never presented especially on indication of any of the spiked samples. The National Institute for Medical Research (NIMR) of Tanzania granted ethical clearance for this APOPO-TB detection rats study.

2.4. Assessment of growth phases of detected microorganisms

Reference species *Mtb* and *Mycobacterium smegmatis* (representing pathogenic and NTM species) were grown in Middlebrook 7H9 broth and incubated at 37 °C with shaking. Culture samples (4 ml) were heat-inactivated after 10, 21, 30 and 41 days, whereas *M. smegmatis* cultures were further sampled at 65 days of incubation. CFUs were measured as described above. Three replicates

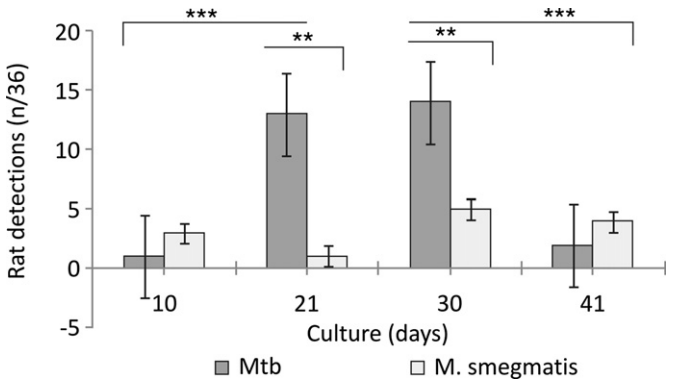

Figure 1. Detection of different growth phases of *Mtb* and *M. smegmatis* cultures by rats. *Mtb* was more frequently detected in exponential (log phase) and early stationary phase cultures (21–30 days) than in early log phase (≤10 days) and late stationary phase cultures (≥41 days) (three asterisks, $p < 0.001$, Fisher's exact test). *Mtb* detection was significantly different compared to *M. smegmatis* in exponential (log phase) and early stationary phase cultures (21–30 days) (two asterisks, $p < 0.008$, Fisher's exact test). Error bars represent standard error (SE) of detections (n/36 expected detections).

were collected at each of the four and five growth intervals of *Mtb* and *M. smegmatis,* respectively, and were tested by six trained rats without rewarding them upon detection of the test microorganisms.

2.5. Sample layout and presentation

The test layout involved 70 sputum samples of which 9 (12.85%) were test microorganisms; 7 (10%) were TB-positive controls for rewarding the rats; and 54 (77.14%) were negative controls mixed with sterile 7H9 medium. Positions for positive control sputa were randomly selected by a computer program to ensure random distribution across sample panels and, that samples were not located side by side. Negative sputum samples spiked with test microorganisms were also randomly distributed across sample panels in positions selected manually, such that samples were separated from like samples and from positive controls, in order to avoid bias and the occurrence of patterns that could easily be learnt by the rats. In the first sessions, the rats analysed samples in an A_{1-10} to G_{1-10} series, whereby A–G refers to codes of seven metal panels each with 10 holes for holding 10 sputum sample pots, and A_{1-10} is position 1–10 in the metal panel coded A. In the second sessions the A–G series was randomized again to avoid eventual memorization of the arrangement of samples. Samples detected by at least two rats (cut-off level) were considered significantly detected. These criteria were used during routine TB detection by the rats.

2.6. Statistical analysis

Fisher's exact test was used to determine significance between rats' detection of cultures of *Mtb* and NTM species. Odds ratio (OR) was used to determine whether the detection of cultures was better than random chance. A P value < 0.05 was used to establish the statistical significance of comparisons. Sensitivity, specificity and accuracy of the trained rats to detect cultures of *Mtb* and NTM (*M. smegmatis*) were determined using detection scores of different growth phases and combined detection of all growth phases of each species.

3. Results

3.1. Screening (detection) of microorganisms by rats

A total of 334 samples from 38 strains (20 bacterial and 2 yeast species) were tested by TB detection rats (Table 1). Initial testing of different culture batches of the same strain, but different incubation periods showed variable or inconsistent detections in certain batches. For example, some cultures of the reference species *Mtb* and the clinical isolate of this species coded N185/8 were detected more often than others. The rats occasionally detected *M. smegmatis*, *Mycobacterium neoaurum*, *Mycobacterium aurum* and other *Mtb* clinical isolates. Rats did not detect mycobacteria-related *Nocardia* spp., *Rhodococcus* sp. and the other microbes.

3.2. Detection of different growth phases of Mtb and M. smegmatis

Testing of various growth intervals of *Mtb* and *M. smegmatis* revealed that *Mtb* is more frequently detected in exponential (log phase) and early stationary phase cultures (21–30 days) than in early log phase (≤10 days) and late stationary phase cultures (≥41 days) ($p < 0.001$, Fisher's exact test) (Figure 1). The detection of nontuberculous *M. smegmatis* was random with no growth-related pattern. Four of the five interval samples of this species were each detected only once unlike *Mtb*. Comparison of the detections of the two species showed a significant difference with *Mtb* detected by more than two rats repeatedly as compared to *M. smegmatis*, which was detected only once in each of the four intervals ($p < 0.008$, Fisher's exact test) (Table 2, Figure 1). The detection of *Mtb* corresponded with the standard growth curve of this species constructed from CFU values obtained at each interval before inactivation of the culture (Figure 2).

The overall analyses show that the TB detection rats have a better detection rate than random (odds ratio) for *Mtb* [OR = 3.77 (95% CI: 2.34–5.94), $p < 10^{-5}$] but not for the NTM, *M. smegmatis* [OR = 1.42 (95% CI: 0.72–2.61), $p = 0.23$].

The sensitivity, specificity, and accuracy of detecting *Mtb* cultures in exponential and stationary phases were higher than for NTM (83.33%, 94.4% and 94%, respectively) (Table 2). Sensitivity value dropped to 50% when all growth phases, including the less-detected early and late cultures, were included in the analysis (Table 2). The specificity of the rats was unchanged by the inclusion of all *Mtb* cultures (growth phases).

Table 2
Detection of *Mtb* and *M. smegmatis* and sensitivity, specificity and accuracy of TB detection rats.

Species	Samples (n)	Rats positive response (n)	Rats negative response (n)	Sensitivity (%)	Specificity (%)	Rats accuracy (%)
Mtb: exponential and early stationary phase (21–30 days) cultures	6	5	1	83.33	94.40	94.00
Mtb: all growth phases (10–41 days)	12	6/12	6	50.00	94.40	91.30
M. smegmatis: all growth phases (10–65 days)	14	4/14*	10	28.50	94.40	88.60
Negative sputum	161	9/161	152	n/a	n/a	n/a

* The detection of *M. smegmatis* was random (not growth phase-related) whereas the four detections are sum of unrepeated single detection of four different growth phases.
N number, n/a not applicable.

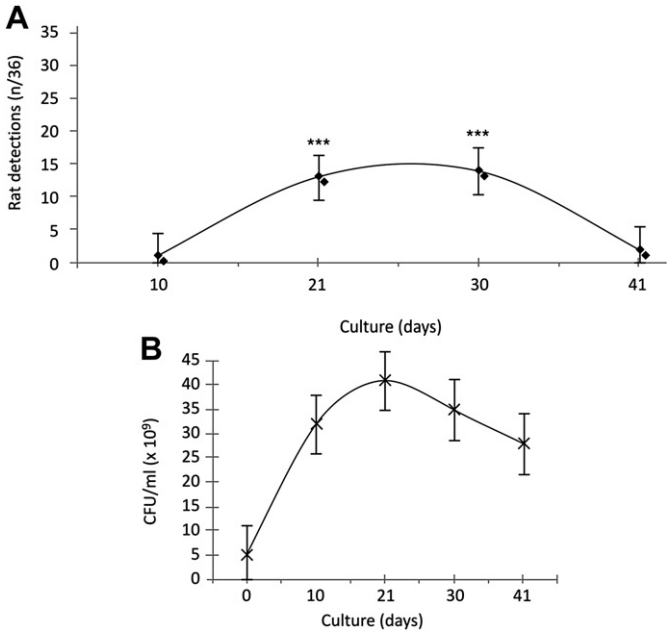

Figure 2. Detection of different growth phases of *Mtb* in 7H9 medium by rats and growth curve of respective *Mtb* culture. Upper curve (A) shows number of positive rat scores and lower curve (B) shows *Mtb* bacterial count established from OD_{580nm} values whereby an OD of 0.1 was equal to 5×10^7 mycobacteria cells/ml. Exponential and early stationary phase cultures (21–30 days) were detected more frequently than early log phase (≤10 days) and late stationary phase cultures (≥41 days) (three asterisks, $p < 0.001$, Fisher's exact test). Error bars represent standard error (SE) of rats' detections and CFU counts.

4. Discussion

The present study shows that trained *C. gambianus* can distinguish *Mtb* from NTM and the related *Nocardia* and *Rhodococcus* species. The detection of pure cultures of *Mtb* is growth stage-dependent, which indicates that the specific odour of *Mtb* detected by these rats is either at levels that cannot be detected by the rats or is not produced in the undetected growth stages of this species. Hence not all cultures of *Mtb* can be used to determine the diagnostic performance of these rats. The trained rats repeatedly detected pure cultures of *Mtb* at exponential and early stationary phase more frequently than early- and late-phase (older) cultures ($p < 0.001$) (Table 2, Figures 1 and 2). The age-based detection of *Mtb* cultures was revealed when an attempt was made to establish the causes for variation in rats' response to cultures of the same strains, which were cultured and harvested at different time-points (days). These findings corroborate those of another study on *Mtb* volatiles which has revealed variations in odour profiles of *Mtb* related to growth phase and type of media used (G. Mgode, *unpublished data*). A difference in volatile profiles of bacteria that is related to growth phases was also recently reported on species of bacteria of human skin which produce mosquito-attractant odour.[6]

The present study attempted to mimic conditions under which rats are trained to detect TB in sputum samples by spiking TB-negative sputa, derived from different individuals, with test microorganisms. Rats were able to consistently detect *Mtb* organisms spiked in negative sputa. However, average positive scores on mimicked samples were slightly lower compared to those obtained from typical TB-positive sputa. This suggests that rats are highly conditioned to detect *Mtb* odour in naturally infected TB-positive sputum compared to mimicked sputa spiked with pure culture *Mtb*. The false-positive rate (detection of TB-negative sputum) in the present study was lower (5.59%), which also indicates the higher specificity of these rats to TB detection (94.4%). The slightly lower detection rate of the mimicked samples suggests differences in background odour of the typical TB-positive sputum and the simulated one. This is possible since *Mtb* inhabiting the host body can present different odour profiles from that of the *Mtb* grown in synthetic culture medium. The interaction of *Mtb* with host cells and that of *Mtb* in culture medium is different, as are the substrates that determine the type of volatiles produced in the two milieus. This phenomenon has also been reported for other microbial species such as *Trichoderma* fungi.[7,8]

The detection of some NTM, for example, *M. smegmatis*, *M. aurum* and *M. neoaurum*, which was not statistically significant [OR = 1.42 (95% CI: 0.72–2.61), ($p = 0.23$)], suggests an overlap of some odour components between *Mtb* and NTM species. However, previous studies have shown that *Mtb* produces specific volatiles that are potential markers for TB diagnosis,[9] and such specific compounds could be among what the rats use to discriminate *Mtb* from NTM as observed in the present study. The ability of these rats to distinguish *Mtb* from NTM and related *Nocardia* and *Rhodococcus* species, which are emerging pulmonary pathogens, shows the potential of these rats in TB diagnosis. NTM as well as *Nocardia* spp. and *Rhodococcus* spp. can be ruled out in sputum samples detected by trained rats.

The assessment of the diagnostic quality of TB detection rats based on the most detectable *Mtb* growth phase showed higher sensitivity, specificity and accuracy. For example, five out of six exponential and early stationary phase (21–30 days) *Mtb* cultures were detected with a sensitivity of 83.33%, specificity of 94.40% and accuracy of 94.00%. These are in accordance with the recently reported sensitivity value of 82.00% and specificity of 90.00% obtained from testing of clinical sputum samples from over 10,000 individuals using these rats.[5]

This study underlines the potential of African giant pouched rats for TB diagnosis. Further studies on volatile compounds of most detectable growth phases of *Mtb* and clinical sputum samples are vital for ultimate identification of specific target *Mtb* volatiles detected by these trained rats. Future studies of *Mtb*-specific odours and other biomarkers for rapid diagnosis of TB should target microorganisms in various growth stages to enhance the recovery of a wide range of potential biomarkers. The ability of these rats to discriminate *in vitro* cultures of *Mtb* from those of other microorganisms reinforces the usefulness of this novel technology in enhancing diagnosis of TB, particularly in resource-limited settings. The previously reported shorter time period of trained rats to diagnose TB (140 specimens analysed in approx. 40 min)[1] is an added advantage of this technology to accelerate detection of TB.

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