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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