APOPO's tuberculosis research agenda: achievements, challenges and prospects

NEGUSSIE BEYENE[1*], AMANDA MAHONEY[1,2], CHRISTOPHE COX[1], BART WEETJENS[1], GEORGE MAKINGI[3], GEORGIES MGODE[4,5], AMY DURGIN[1,2], DIAN KUIPERS[1], MAUREEN JUBITANA[1], SAIDI EGWAGA[6], DEUS KAMARA[6], FRED LWILLA[6,8], SAYOKI G. MFINANGA[7], AMOS KAHWA[7], ROBERT MACHANG'U[4], RUDOVICK KAZWALA[3], KLAUS REITHER[8,9], STEFAN H.E. KAUFMANN[5] and ALAN POLING[1,2]

[1]*Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling, Sokoine University of Agriculture, P.O. Box 3078, Morogoro, Tanzania*

[2]*Department of Psychology, Western Michigan University, Kalamazoo, Michigan, USA*

[3]*Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, Morogoro, Tanzania*

[4]*Pest Management Centre, Sokoine University of Agriculture, Morogoro, Tanzania*

[5]*Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany*

[6]*National Tuberculosis and Leprosy Programme, Ministry of Health and Social Welfare, Dar es Salaam, Tanzania*

[7]*National Institute for Medical Research, Muhimbili Research Centre, Dar es Salaam, Tanzania*

[8]*Ifakara Health Institute, Dar es Salaam, Tanzania*

[9]*Swiss Tropical and Public Health Institute, Basel, Switzerland*

_____

Abstract: This article describes Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling (APOPO) recent use of specially trained African giant pouched rats as detectors of pulmonary tuberculosis in people living in Tanzania. It summarizes the achievements and challenges encountered over the years and outlines future prospects. Since 2008, second-line screening by the rats has identified more than 2000 tuberculosis-positive patients who were missed by microscopy at Direct Observation of Treatment – Short Course centres in Tanzania. Moreover, data that are reviewed herein have been collected with respect to the rats' sensitivity and specificity in detecting tuberculosis. Findings strongly suggest that scent-detecting rats offer a quick and practical tool for detecting pulmonary tuberculosis and within the year APOPO's tuberculosis-detection project will be extended to Mozambique. As part of its local capacity building effort, APOPO hires and trains Tanzanians to play many important roles in its TB detection project and provides research and training opportunities for Tanzanian students.

_____

Introduction

Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling (in English, Anti-Personnel Landmines Detection Product Development), characteristically referred to by its acronym, APOPO, is a Belgian non-profit humanitarian organization that started its preliminary research on the sniffing capacity of African giant pouched rats (*Cricetomys gambianus*) in Antwerp in 1997. In 2000, APOPO signed a partnership agreement with Sokoine University of Agriculture (SUA), Tanzania, Antwerp University (AU), and the Tanzanian Peoples Defence Force (TPDF) which let it build its headquarters, as well as its training and research facilities, on the premises of SUA under the auspices of the Pest Management Centre.

_____

* Correspondence: Negussie W. Beyene; E-mail: negussie.beyene@apopo.org

Initially, rats were collected from the outskirts of Morogoro municipality and from the slopes of Uluguru Mountains using Havahart® traps (Havarhart, Woodstream Corp., PA, USA) and then transferred to breeding kennels at the project site. The breeding kennels were constructed to imitate the rats' natural environment and now provide a continuous supply of rats for the project.

Cricetomys for Landmine Detection

African giant pouched rats, which are native to sub-Saharan Africa, are nocturnal and omnivorous members of the *Nesomyidae* family within the *Muroidea* superfamily. They are large colony-dwelling rodents, with adult body lengths of 25-45cm and tail lengths of 35-45cm. Adult females typically weigh 1-1.5kg. Males are somewhat larger. *Cricetomys* can live up to eight years in captivity. At four weeks of age, APOPO's rats are weaned and thereafter are housed in pairs in cages with unlimited access to water and a nest box. The rats are fed a varied diet of fruits, vegetables, grains, and commercial rodent chow. During weekdays they receive the majority of their food during training sessions so that they are mildly food-deprived during training and testing. A veterinarian, who provides health care as needed, regularly examines the rats.

From four weeks to approximately six weeks of age the rats are handled three times a day by trainers and other people, who expose them to a wide variety of objects, sights, sounds, and smells and hand-feed them preferred foods such as bananas and peanuts. These experiences constitute what is often termed "environmental enrichment," which is known to improve several aspects of the neurochemistry and behaviour of domestic laboratory rats (*Rattus norvegicus*), including their ability to learn and to remember (Hutchinson *et al.*, 2005; Nithianantharajah & Hannan, 2005; Van Praag *et al.*, 2000).

Initially, APOPO focused on training rats to detect landmines. Over time, successful training procedures were devised and *Cricetomys* currently are being used as accredited mine detection animals in Mozambique, where APOPO's team has cleared more than 2 million square meters of land. These training procedures and the rat's performance in operational demining are described elsewhere (Poling *et al.*, 2010a, 2010b, 2010c, 2011b).

Lessons learned in developing effective landmine-detection procedures were put to good use in training the rats to detect the presence of *Mycobacterium tuberculosis*, the microorganism that causes tuberculosis (TB), in human sputum samples and to evaluate their ability to do so. This work was possible due to a 2004 partnership agreement between APOPO, SUA, AU, the Tanzanian National Institute of Medical Research (NIMR), and the Tanzanian National Tuberculosis and Leprosy Programme (NTLP), which jointly committed to developing a scent-detection technology for diagnosing human TB. Their goal was to develop a quick, inexpensive, and accurate tool for diagnosing pulmonary tuberculosis to benefit people living in resource poor areas in sub-Saharan Africa and around the world.

Preliminary Investigations

Initially, a pilot study was conducted to determine the resistance of African giant pouched rats to TB by putting them in a specially designed cage that was placed in a biosafety cabinet and exposing the rats to aerosols from live *M. tuberculosis* broth. Eight rats (four adults and four young of both sexes) were used in this investigation. Four test rats received Bacilli's Calmette-Guerin (BCG) vaccinations while the remaining four served as controls and were

not vaccinated. Both groups were inoculated over an 8-h period by subjecting them to an aerosol containing 10^6 to 10^8 CFU/ml of *M. bovis* and *M. tuberculosis*. After an eight-week observation period these animals and four additional rats recently captured from the wild were euthanatized. Specimens from test, control, and wild rats were examined for pathological and molecular evidence of mycobacterial infection. No lesions or traces of mycobacteria (by PCR) were found in samples obtained from the test, control, or wild rats. These data suggested that *Cricetomys* are resistant to mycobacterial infection and could be safely used as TB-detection animals.

In the second stage of this preliminary investigation, five rats were selected and vaccinated with BCG. All of them were subjected to odour discrimination training for a period of eight weeks using inactivated culture isolates in a broth as the training stimulus. After this period they were split into two groups. Three rats were further trained to detect mycobacteria culture isolates and two rats were trained to detect *M. tuberculosis* in human sputum samples. The culture isolates were grown and processed at the Microbiology Laboratory of the Faculty of Veterinary Medicine at SUA and sputum samples were collected from Direct Observation of Treatment-Short Course (DOTS) centres at the Morogoro Regional Hospital and at the Muhimbili National Hospital in Dar es Salaam. After four weeks of training, all five rats were able to indicate consistently on the target TB odour from the culture isolates as well as from the sputum samples. They did so by pausing reliably at holes above pots containing known TB-positive samples and not pausing above holes containing known TB-negative samples. They were trained to respond in this way by consistently reinforcing (rewarding) with food progressively longer pauses above TB-positive samples and never reinforcing pauses above TB-negative samples. Such training is technically described as "operant discrimination training" and is the basis of all scent detection applications with animals, including the use of dogs and pouched rats to detect explosives.

Proof of Principle Study

Although the preliminary study suggested the rats are resistant to TB, a decision was made to inactivate microbes in the sputum samples so as to protect personnel and the rats from all possible infectious agents. For this proof of principle study, about 300 sputum sample sets were collected per week from selected DOTS centres in Dar es Salaam and Morogoro, using World Health Organization (WHO) recommended sputum containers (wide-mouthed container made of clear thin plastic that were disposable, unbreakable, and leak proof). All the samples were already examined by Ziehl-Neelsen (ZN) smear microscopy at the DOTS centres and stored in a freezer until they were collected and shipped to APOPO's facility in Morogoro. Each sample came from the DOTS centre with information about the sex, age, and identification number of the patient who produced it and the sample's ZN microscopy status (i.e., TB-positive or TB-negative).

One ml of each sputum sample was aseptically set aside for culturing and after the required sample processing procedure it was inoculated on solid Löwenstein-Jensen medium, incubated at 37°C and monitored for six weeks. Smears were prepared for microscopic examination, ZN, from the remaining portion of the sputum, at APOPO's laboratory so that DOTS results could be confirmed. Sterile phosphate-buffered saline (PBS, 5 ml) was added to the remaining portion of the sputum sample, which was heat-inactivated at 90°C in a water bath for 30 min (Doig *et al*. 2002). All samples were then frozen at –20°C

until the day of evaluation by the rats. During evaluation, sputum samples were presented to individual rats in a long metal and plastic cage with 10 holes equally spaced along the centreline of the floor. A sputum sample contained in a pot was placed below each hole and the rat's response at each hole was recorded. Pausing for at least five seconds was recorded as an indication that the sample contained TB, whereas pausing for a shorter time was recorded as an indication that the sample did not contain TB.

Sixteen rats evaluated a total of 2,597 samples (345 were smear-positive and 2,252 smear-negative at the DOTS centres) over a period of 35 days. The same sets of samples were presented twice to all rats. The average daily results for the rats as a group ranged between 72.4 and 99.5% true-positive and 0.6 and 8.1% false-positive when DOTS-centre evaluations were used as the reference standard. Average scores for all the rats during the entire 35-day trial were 87.8% true-positive and 4.1% false-positive. The true-positive level defines the sensitivity of a diagnostic test and 1 - the false-positive level defines its specificity. Between the 21st and 32nd days (inclusive), blind samples (i.e., samples for which the results of microscopy were not revealed to the rat handlers) were included in the daily sample set. The rats were not rewarded when they indicated on these blind samples. The true-positive rate for the entire group varied between 82.3 and 100% on the blind positive samples, with an overall average score of 93.1%. During the 12 days of blind testing, the rats consistently identified seven DOTS-negative samples as TB-positive. A second ZN analysis at APOPO's laboratory confirmed that the rats' evaluation of these samples was correct.

Another series of blinded tests was performed on 7 consecutive days by two rats on a total of 819 sputum samples, of which 67 were confirmed positive and 752 were confirmed negative by culture. The cumulative results for the two rats revealed a sensitivity of 86.5% and a specificity of 89.1%. The average scores and false-positive results per rat were respectively 73.1% and 6.7%, while the cumulative scores for both rats were respectively 86.5% and 10.9%. This study clearly demonstrated the potential usefulness of African giant pouched rats as TB detectors and the publication describing it (Weetjens *et al.* 2009a) was the first report of using animals in general, and rats in particular, for TB detection.

Second-line Screening

The promising results of the proof of principle study encouraged members of APOPO's research team to evaluate the possibility of using *Cricetomys* for second-line screening of sputum samples previously evaluated by microscopy at the DOTS centres. The purpose of this project was, first, to improve new-case detections and thereby save lives and, second, to evaluate the rats performance with respect to their potential for use in first-line screening. Although sputum-smear microscopy characteristically has excellent specificity and is commonly used in resource poor countries in sub-Saharan Africa and elsewhere, the method's sensitivity varies greatly and is often relatively low (Dye *et al.*, 2005; Steingart *et al.*, 2006). If second-line screening by APOPO's rats detects a significant number of cases of TB missed by microscopy, then combining the two techniques could be of substantial clinical benefit. Three published studies have evaluated this possibility (Weetjens *et al.* 2009b; Poling *et al.* 2010; Mahoney *et al.* 2011).

In the first, the rats screened sputum samples collected from 15,041 patients from four DOTS centres in Dar es Salaam in the period January 2008 to May 2009 and found 577 additional cases beyond the 1,838 detected at the centres, resulting in a 31.4% increase in case detection (Weetjens *et al*. 2009b). Similarly, screening of samples collected from 10,523

patients who visited seven DOTS centres in 2009 revealed an additional 620 cases beyond the 1403 cases found at the centres, i.e. a 44% increase in detection (Poling *et al.*, 2010). Likewise, samples collected from 12,329 patients have been screened by the rats in the period January to December 2010 resulting in 716 new cases over the 1671 identified at the centres, which is a 42.8% increase in new-case detection (Mahoney *et al.*, 2011). These data provide substantial evidence that *Cricetomys* are of clinical value as second-line screens used in combination with first-line screening by smear microscopy. To evaluate further the value of pouched rats in this context, rats and personnel from APOPO will soon begin a second second-line screening project in Maputo, Mozambique. A major goal of APOPO and its partners is to expand the use of TB-detection rats as widely as possible in the interest of reducing human suffering and saving lives.

Improving Sample Processing

One of the advantages of using African giant pouched rats as TB detectors is their speed in analyzing sputum samples. As described elsewhere (Weetjens *et al.*, 2009; Poling *et al.*, 2010), a single rat can process many samples per day, which allows multiple rats to evaluate each sample and offers the possibility of a quick and inexpensive diagnostic tool. In our work to date, the time required for technicians to prepare samples, not the time required for rats to analyze them, limited the number of samples that can be processed. Sample preparation basically involves addition of phosphate-buffered saline and heat-inactivation using an autoclave. Developing alternative methods for preparing and presenting samples could dramatically improve APOPO's TB-diagnostic product.

One possible strategy to reduce the work and time associated with sample processing is to have the rats evaluate microscope slides, which are always prepared in smear microscopy. Data recently collected demonstrate that the rats can detect the presence of TB on prepared slides, but their accuracy in doing so was substantially lower than when they evaluated sputum presented in pots (Mahoney *et al.*, 2011b). Although the possibility of using slides operationally has not been dismissed entirely, alternative strategies for sample preparation are currently being evaluated. One is using Virkon® S (a new formulation of the oxidant-based chemical disinfectant Virkon,® produced by E. I. DuPont de Nemours and Company, Wilmington, DE, USA), rather than autoclaving, to inactivate samples. If Virkon® S kills infectious microorganisms with a brief contact time and does not affect the rats' ability to detect the presence of TB; it will be used as the inactivation agent instead of the phosphate-buffered saline addition and heat-inactivation process currently in use.

Improving Rats' Detection when Few Bacilli are Present

As described previously, APOPO's rats are performing an operant discrimination when they detect TB. Because historically they have been reinforced only when they emit an identification response (pause for five seconds) at a sample containing *M. tuberculosis*, they learn to emit the identification response only when they encounter the odour characteristic of the bacillus. There is a large body of scientific literature on discrimination learning, showing that the intensity of the stimuli (e.g., concentration of bacilli in our case) which the animals are initially trained to identify, affects their subsequent performance (Catania, 1999; Chance, 2003). We observed this phenomenon when rats were trained to detect 2,4,6-trinitrotoluene (TNT), which is the main explosive charge in the vast majority of landmines.

Those rats trained and reinforced to detect a low concentration of TNT (10ng/L) easily detected different concentrations of TNT (10 and 100 ng/L); whereas rats trained only to detect a high concentration did not detect TNT 10ng/L reliably. A similar phenomenon was observed when we examined routine training data for the month of August 2009, during which TB-detection rats were trained on sputum samples graded as AFB (scanty), +1, +2 and +3 at the DOTS centres (Table 1).

Table 1: Sensitivity of rats on sputum samples with different concentration of bacilli

Rat name	Sensitivity (% of samples identified as TB-positive by the rat)			
	AFB (scanty)	+1	+2	+3
Allen	12.5	82.4	97.2	90
Brown	70	84.3	93.8	100
Bruce	50	75.9	93.8	95.5
Laila	50	82.2	98	100
Onur	46.2	85.8	100	90.9
Patron	50	79.5	92.9	90.9
Queen	43.8	72.2	100	100
Richard	54.5	79.9	90.3	100

Each of the eight rats examined detected a substantially lower percentage of AFB samples than of higher-concentration samples. We believe the main reason for this is that very few low concentration (AFB or scanty) samples are detected at the DOTS centres, therefore our rats are primarily trained to detect high-concentration samples. Such training may well limit their tendency to emit identification responses to low-intensity stimuli that is, to samples containing low levels of *M. tuberculosis*, even if the rats can reliably detect the odour of those bacilli.

Because microscopy is especially likely to misidentify (i.e., designate as TB-negative) smears that properly should be considered AFB (Fujika, 2005), when the rats are used in second-line screening it is especially important that they reliably emit an identification response to such low-intensity samples. It is, of course, also important that they consistently identify high-concentration samples. A logical strategy to pursue in increasing the likelihood that rats will reliably identify both low- and high-concentration samples is to train them only with low-concentration samples. Such training is currently underway and it is anticipated that rats specifically trained to identify low-concentration samples will be more sensitive in identifying low-concentration sputum samples than rats not given such training, and similarly sensitive in identifying higher-concentration samples.

Improving Confirmatory Methods

From the beginning, the actual status of sputum samples identified as TB-negative by DOTS centres' microscopy and TB-positive by APOPO's rats has been determined by a second microscopy (ZN or more recently fluorescent) performed in APOPO's laboratory. Although fluorescent microscopy often is more sensitive than ZN light microscopy, neither have consistently high sensitivity (Steingart, 2006). Therefore, a rat-positive sample found not to contain *M. tuberculosis* by two microscopies may not be a true negative, especially if few bacilli are present. From a scientific perspective, the result is an inflated false-positive rate for the rats and from a clinical perspective the result is a failure to detect and treat some cases of TB correctly identified by the rats.

To deal with these issues, we are exploring the possibility of using an automated polymerase chain reaction (PCR) analysis performed by a Cepheid GeneXpert (Xpert MTB/RIF, Sunnyvale, CA) as the final confirmation of sample status. Although the GeneXpert has high sensitivity and specificity (Boeme *et al.*, 2010), it is rather slow. Moreover, each test is relatively expensive (c. US$20). Using the GeneXpert only to evaluate microscopy-negative, rat-positive samples may be viable in both research and operational contexts, however, because relatively few such samples appear, which minimizes overall cost and processing time.

With the exception of limited data for two rats in the study by Weetjens *et al.* (2009a), the performance of APOPO's rats has been determined by comparison to the results of microscopy, which characteristically has high specificity but relatively low (and variable) sensitivity (Dye *et al.*, 2005; Steingart *et al.*, 2006). The low sensitivity of smear microscopy makes it a poor reference standard from a scientific perspective although it is imperative to compare the rats' performance to that of microscopy, because the goal is to use them as an adjunct to (in second-line screening) or alterative to (in first-line screening) that method.

We have recently completed a study (Mahoney *et al.*, 2012a) in which the results of evaluation by 10 rats and by microscopy conducted at DOTS centres were compared to the results of culturing and PCR analysis of 910 sputum samples. In this research, an M.Sc. student in Applied Microbiology at the Faculty of Veterinary Medicine of SUA and his colleagues compared rats' evaluations with the results of ZN microscopy, culture on solid Lowenstein-Jensen (LJ) media whose isolates were checked by ZN microscopy, and PCR analysis of the culture isolates. Samples collected from 456 patients were analyzed. When three rats were randomly assigned to form a group, and when a sample was considered as TB-positive if 2 of the 3 rats in the group emitted an indicator response to that sample, an average sensitivity of 72% and specificity of 94% was achieved with the PCR results as the reference standard. When also compared to PCR results, the sensitivity of DOTS microscopy was 48% and the specificity was 98%. Moreover, the rats identified 23 TB-positive cases that were not identified by microscopy, that is, they increased the case detection rate by 48.9%. These results provide further evidence that pouched rats are a valuable adjunct to, and may be a viable substitute for, sputum smear microscopy as a TB diagnostic in resource poor countries.

Although culturing (often followed by PCR analysis) is a well-accepted reference standard for TB detection, it can be difficult to perform accurately, especially in operational settings in resource poor area, and should not be viewed as an infallible index of the presence or absence of *M. tuberculosis* in tested patients (Kivihya-Ndugga *et al.*, 2004; Mendoza *et al.*, 1993; Parvez *et al.*, 2003). APOPO's research team and its partners have successfully cultured *M. tuberculosis*, but it is a difficult and expensive task in our operational setting and not feasible as a consistent reference standard. Moreover, it may not be as sensitive as one would hope.

For example, we recently used a GeneXpert to analyze 60 sputum samples that were negative by microscopy as well as culture but indicated as positive by multiple rats. Eighteen of them (30%) were found to be positive for *M. tuberculosis*. We also analyzed 100 samples that were negative by all techniques including the rats and found only 8% of them positive by Xpert MTB/RIF (Unpublished result). This result clearly suggests that under the conditions where APOPO operates, the automated molecular technique is more sensitive than other available methods. As noted previously, however, the GeneXpert (like other molecular techniques) is too expensive for routine screening in resource poor countries.

Moreover, some experts argue that every TB diagnostic test should be considered as only one element in the diagnostic algorithm and patient follow-up and drug-response/treatment success should be taken as the best reference, especially in areas where the HIV prevalence is high (Apers *et al.*, 2004).

In view of the considerations just raised, it is difficult to ascertain the true accuracy of the rats as TB detectors. To provide a more accurate index, APOPO is collaborating with the Swiss Tropical and Public Health Institute and the Ifakara Health Institute on a cohort study that collects a wide range of diagnostic data, specifically rats' evaluations, ZN microscopy results, fluorescent microscopy results, chest X-ray findings, culture analysis, standard PCR analysis, GeneXpert PCR analysis, patient follow-up evaluations, and drug-response ratings. This study will also enable us to evaluate the rats' performance in patients who are and are not known to be HIV-positive. To date, because permission to know patients' HIV status was only recently granted, we have been unable to analyze data separately for HIV-positive and HIV-negative patients. Doing so is obviously important, because TB is a leading cause of death in HIV-positive people and it can be especially difficult to diagnose in this population (Smith *et al.*, 1994; Kivihya-Nduga *et al.*, 2004; Mtei *et al.,* 2005; Cain *et al.*, 2010).

Automated Cage Assessment

Until very recently, all of the TB-detection research was done in manually operated training cages. With these cages, trainers (typically three for each cage) place the rat in the cage, observe it as it moves along the line of holes sniffing the sputum samples, record all identification responses, and reinforce (i.e., delivery of food immediately following) identification responses to samples known to contain *M. tuberculosis*. In blinded testing, the DOTS-centre status of all training samples are known to the trainers, but the status of test samples are unknown. Although the manually operated cages are workable, as evident in the data reported by Weetjens *et al.* (2009a, 2009b), human error is a real and significant problem with all such systems (Poling *et al.*, 1995).

Despite APOPO's confidence in its trained and certified rodent trainers, mistakes occur. For example, appropriately reinforcing correct identification responses requires trainers to be aware of which pots are known to contain *M. tuberculosis*, to ascertain when a rat has paused for five seconds at a sniffing hole immediately above such a pot, and to deliver mashed bananas as soon as the five-second criterion is met. Each rat evaluates many samples per day (140 in current second-line screening work, but this number could increase substantially in other applications) and the location of reinforcement samples (those known to contain the bacillus) changes each time a new set of 10 samples is presented. Even if procedures are quite standardized, the possibility of errors related to the location of reinforcement samples could lead to a failure to reinforce a correct identification or, more seriously, to reinforce an identification response to a sample that does not contain *M. tuberculosis*. Moreover, if trainers know that a given sample is a reinforcement sample, this may affect how they evaluate the rat's behaviour (e.g., they may consider a four-second pause as meeting criterion) or lead them to provide cues (e.g., leaning forward) that come to control the rat's behaviour. Such actions are unconscious and almost universal and in no sense constitute cheating (Poling *et al.*, 1995).

To minimize human error and unintentional cues to the rats, APOPO developed a fully automated cage. It consists of a rectilinear aluminium and plastic chamber 205-cm long x 55-cm wide x 55-cm high. The floor of the chamber contains 10 2.5-cm diameter sniffing

holes spaced equidistantly along the centreline. Pots containing sputum can be placed immediately below these holes by means of a sliding bar with 10 appropriately spaced holes. A photocell directs a beam of light immediately below each hole and a detector unit is activated when the beam is broken, indicating that a rat has placed its nose (or potentially some other body part) in the sniffing hole. A commercial food dispenser located at one end of the chamber allows for the delivery of food (three 45-mg Noyes banana-flavoured rat pellets; P.J. Noyes, Lancaster, NH) and computer-directed equipment presents food automatically when an indication response (pausing, hence breaking the beam) above a TB-positive sample occurs. A Tanzanian Ph.D. student is studying the behaviour of the rats when working in this fully-automated cage and it is anticipated that future TB detection will be conducted in such cages. APOPO intends to produce an exportable TB-detection technology with specifiable and repeatable characteristics, and automating that technology as much as possible is a long step in that direction.

Assessment of Common Bacilli in Sputum and Rats' Reaction to Them

A series of projects designed to evaluate the bacilli that are commonly found in the sputum samples evaluated by APOPO's rats, and the rats' reaction to those bacilli, were recently completed by a Tanzanian student as his Ph.D. research at the Max Planck Institute of Infection Biology (Mgode *et al*., 2011a, b). In one study, he and his colleagues compared the diagnostic performance of the TB-detecting rats on cultures of reference *M. tuberculosis*, clinical *M. tuberculosis*, non-tuberculous mycobacteria species (NTM), *Nocardia*, *Rhodococcus*, *Streptomyces*, *Bacillus*, *Candida*, and *Saccharomyces* species. *M. tuberculosis* and NTM are related to *Nocardia* and *Rhodococcus* species, which are also acid-fast bacilli and can thereby be misdiagnosed as *M. tuberculosis* in smear microscopy. Trained rats distinguished *M. tuberculosis* from other microbes (p=0.008, Fisher's exact test). The detection of naturally TB-infected sputum exceeded that of negative sputum mixed with pure cultures of *M. tuberculosis*, indicating that rats are highly conditioned to detect odours of spiked TB-positive sputum rather than spiked TB-negative sputum (Mgode *et al*., 2011a).

Another study compared the odours (volatiles) of microbiota from smear-negative and *M. tuberculosis* culture-negative, but rat-positive, sputa to the odours of *M. tuberculosis*. Rat-positive sputa containing *Streptococcus pneumoniae*, *Staphylococcus* spp. and *Enterococcus* spp. were associated with TB-positivity. However, analysis of volatiles from *Streptomyces* spp., *Candida* spp., *Rhodococcus* spp. and *Nocardia* spp., representing microbiota from smear-negative and *M. tuberculosis* culture-negative but rat-positive sputa revealed that these microbes do not produce *M. tuberculosis*-specific volatiles. Furthermore, the prevalence of mycobacteria-related *Nocardia* and *Rhodococcus* spp. is substantially lower than the percentage of smear-negative sputa detected by rats, of which 44.7% were *M. tuberculosis* culture-positive. Therefore, the rats are reacting to *M. tuberculosis*-specific odour compounds but not to odours from other respiratory tract microbiota (Mgode *et al*., 2011b). It is noteworthy that several volatile compounds characteristic for the TB odour have been identified and it is clear that TB-specific odour is not due to a single compound but rather to a combination of different volatiles. This combination is characteristic for *M. tuberculosis* and allows discrimination from odours produced by other related pathogenic and apathogenic microbes of the respiratory tract (Nawrath *et al*., in press).

Patient Follow-up

Every Friday, a laboratory technician from APOPO visits each partnering DOTS centre to collect sputum samples and deliver a list of sputum samples that were found TB-negative by microscopy at that centre but found positive by the rats and confirmed by a second microscopy (or a GeneXpert analysis) at APOPO. In principle, the patient should be contacted through the telephone number provided during submission of the sputum sample, asked to return to the DOTS centre, and given treatment as deemed necessary by the attending physician. APOPO has reported more than 2,000 additional TB-positive cases to the DOTS centres, but information about the treatments they received and their response to these treatments could not be obtained. Therefore, the actual clinical benefit derived from the use of rats in second-line screening is unclear.

This issue was the focus of a coordination meeting recently held by APOPO, SUA, NIMR, and NTLP. Members of these organizations agreed that a sound strategy for maximizing clinical benefit is obtaining from NTLP accreditation for APOPO's microscopy (both bright-field and LED) and GeneXpert (i.e., Xpert MTB/RT) facility as a TB diagnostic in Tanzania. Following accreditation, APOPO's laboratory will participate in all official quality control schemes conducted by NTLP, which will maintain the quality of diagnostic results. The anticipated outcome of this effort is to have the second microscopy (or automated PCR analysis) performed at APOPO officially recognized by NTLP, which at the DOTS centres will prompt immediate patient recall for possible clinical action.

A second strategy to maximize clinical benefit is to employ a postgraduate medical student to conduct a pilot study on the clinical follow-up of patients identified by APOPO's rats. This project will examine the rate of patient recalls, examine the relations between physicians' final diagnosis and various diagnostic indicators as available (e.g., rats' evaluations, clinical features, microscopy results, PCR results, and chest X-rays) and, where treatment is provided, ascertain the relation to the drug(s) prescribed and the patient's response to them. Steps currently are underway to implement both strategies for maximizing (and measuring) clinical benefit.

Future Prospects

APOPO intends to produce a reliable, fast, and simple alternative to the century-old smear microscopy for TB diagnosis in developing countries. The achievements so far are encouraging and the strategies implemented to overcome the challenges presented are promising. Based on results of all research components currently in progress and that of the cohort study planned in collaboration with the Swiss Tropical and Public Health Institute and the Ifakara Health Institute, APOPO may soon start using its detection rats' technology for first-line screening of TB in people who live in slums, prisons, and other historically underserved areas. In the meantime, the existing second-line screening program will be continued and replicated in Mozambique, which will help to ascertain the practicality and exportability of APOPO's TB-detection product. Although unconventional, that product shows promise and appears to merit further investigation and more widespread adoption in appropriate settings.

References

Apers, L., Wijarajah, C., Mustvangwa, J., Chigara, N., Mason, P. & van der Stuyft, P. (2004) Accuracy of routine diagnosis of pulmonary tuberculosis in an area of high HIV prevalence. *International Journal of Tuberculosis and Lung Disease* 8, 945-951.

Boehme, C.C., Nabeta, P., Hillemann, D., Nicol, M.P., Shenai, S., Krapp, F., Allen, J., Tahirli, R., Blakemore, R., Rustomiee, R., Milovic, A., Jones, M., O'Brien, S. M., Persing, D. H., Ruesch-Gerdes, S., Gotuzzo, E., Rodrigues, C., Alland, D. & Perkins, M.D. (2010). Rapid molecular detection of Tuberculosis and Rifampin resistance. *New England Journal of Medicine* 363, 1005-1015.

Cain, K.P., Kimberly, D., McCarthy, K.D., Heilig, C.M., Monkongdee, P., Tasaneeyapan, T., Kanara, N., Kimerling, M.E., Chheng, P., Thai, S., Sar, B., Phanuphak, P., Teeratakulpisarn, N., Phanuphak, N., Dung, N.H., Quy, H.T., Thai, L.H. & Varma, J.K. (2010) An algorithm for tuberculosis screening and diagnosis in people with HIV. *New England Journal of Medicine* 362, 707-716.

Catania, A.C. (1999) *Learning*. Upper Saddle River, NJ: Prentice-Hall.

Chance, P. (2003) *Learning and behavior*. Belmont, CA: Wadsworth.

Doig, C., Seagar, A.L., Watt, B., Forbes, K.J. (2002) The efficacy of the heat killing of *Mycobacterium tuberculosis*. *Journal of Clinical Pathology* 55, 778-779.

Dye, C., Watt, C.J., Bleed, D.M., Hosseini, S.M., Raviglione, M.C. (2005) Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. *Journal of the American Medical Association* 293, 2767-2775.

Fujika, A. (2005) *AFB microscopy training*. Tokyo: The Research Institute of Tuberculosis.

Hutchinson, E., Avery A. & VandeWoude, S. (2005) Environmental enrichment for laboratory rodents. *ILAR Journal* 48, 148-161.

Kivihya-Ndugga, L., van Cleeff, M., Juma, E., Kimwomi, J., Githui, W., Oskam, L., Schuitema, A., van Soolingen, D., Nganga, L., Kibuga, D., Odhiambo, J. & Klatser, P. (2004) Comparison of PCR with the routine procedure for diagnosis of tuberculosis in a population with high prevalences of tuberculosis and human immunodeficiency virus. *Journal of Clinical Microbiology* 42, 1012-1015.

Mahoney, A., Weetjens, B., Cox, C., Beyene, N., Mgode, G., Jubitana, M., Kuipers, D., Kazwala, R., Mfinanga, G., Durgin, A. & Poling, A. (2011) Using giant African pouched rats to detect tuberculosis in human sputum samples: 2010 findings. *The Pan African Medical Journal* 9, 28.

Mahoney, A., Weetjens, B., Cox, C., Beyene, N., Reither, K., Makingi, G., Jubitana, M., Mfinanga, G., Kahwa, A., Durgin, A., Poling, A. & Kazwala, R. (2012a) Detection of tuberculosis in human sputum: comparison to culturing and Polymerase Chain Reaction. *Tuberculosis Research and Treatment* (in press).

Mahoney, A., Weetjens, B. J., Cox, C., Beyene, N., Jubitana, M., Kuipers, D., Kazwala, R., Mfinanga, G.S., Durgin, A. & Poling, A. (2011b) *The Psychological Record* (in press).

Mtei, L., Matee, M., Herfort, O., Bakari, M., Horsburgh, C.R., Waddell, R., Cole, B. F., Vuola, J. M., Tvaroha, S., Kreiswirth, B., Pallangyo, K. & Von Reyn, C. F. (2005) High rates of clinical and subclinical tuberculosis among HIV-infected ambulatory subjects in Tanzania. *Clinical Infectious Diseases* 40, 1500-1507.

Mendoza, M.T., Reyes, L.F., Pascual, M. & Tan-Torres, T. (1993) Culture isolation of *Mycobacterium tuberculosis* using Radiometric Bactec System compared with the conventional Lowenstein-Jensen Media. *Phillippine Journal of Microbiology and Infectious Diseases* 22, 17-22.

Mgode, G.F., Weetjens, B.J., Cox, C., Jubitana, M., Machang'u, R.S., Lazar, D., Weiner, J., Van Geertruyden, J.P. & Kaufmann, S.H. (2011a) Ability of Cricetomys rats to detect Mycobacterium tuberculosis and discriminate it from other microorganisms. *Tuberculosis*. doi.org/10.1016/j.tube.2011.11.008.

Mgode, G. F., Weetjens, B.J., Nawrath, T., Cox, C., Jubitana, M., Machang'u, R.S., Cohen-Bacrie, S., Bedotto, M., Drancourt, M., Schulz, S. & Kaufmann, S.H. (2011b) Diagnosis of tuberculosis by trained African giant pouched rats and confounding impact of pathogens and microflora of the respiratory tract. *Journal of Clinical Microbiology* 50, 274-280.

Nawrath, T., Mgode, G.F., Weetjens, B., Kaufmann, S.H.E. & Schulz, S. (2012) The volatiles of pathogenic and non-pathogenic mycobacteria and related bacteria. *Beilstein Journal of Organic Chemistry* (in press).

Nithianantharajah, J. & Hannan A.J. (2005) Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews of Neuroscience* 7, 697-709.

Parvez, M., Hasan, K.N., Rumi, M., Ahmed, S., Salimullah, M., Tahera, Y., Gomes, D.J., Huq, F. & Hassan, M.S. (2003) PCR can help early diagnosis of pulmonary tuberculosis. *Southeast Asia Journal of Tropical Medicine and Public Health* 34, 147-153.

Poling, A., Methot, L. & LeSage, M. (1995) *Fundamentals of Behaviour Analytic Research*. New York: Plenum Press.

Poling, A.,Weetjens, B., Cox, C., Mgode, G., Jubitana, M., Kazwala, R., Mfinganga, G.S. & 't Veld, D.H. (2010). Using giant African rats to detect tuberculosis: 2009 findings. *American Journal of Tropical Medicine and Hygiene* 83, 1308-1310.

Poling, A., Weetjens, B., Cox, C., Beyene, N., Durgin, A. & Mahoney, A. (2011) Tuberculosis detection by giant African pouched rats (*Cricetomys gambianus*). *The Behavior Analyst* 34, 47-54.

Smith, R.L., Yew, K., Berkowitz, K.A. & Aranda, C.P. (1994) Factors affecting the yield of acid-fast sputum smears in patients with HIV and tuberculosis. *Chest* 106, 684-686.

Steingart, K.R., Henry, M., Ng, V., Hopewell, P.C., Ramsay, A., Cunningham, J., Irbanczik, R., Perkins, M., Azia, M. & Pal, M. (2006) Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infectious Disease* 6, 570-581.

Swaminathan, S., Paramasivan, C.N., Kumar, S.R., Mohan, V., Venkatesan, P. (2004) Unrecognised tuberculosis in HIV-infected patients: sputum culture is a useful tool. *International Journal of Tuberculosis and Lung Diseases* 8, 896-898.

Van Praag, H., Kempermann, G. & Gage, F. H. (2000) Neural consequences of environmental enrichment. *Nature Reviews of Neuroscience* 1, 191-198.

Weetjens, B.J., Mgode, G.F., Machang'u, R.S., Kazwala, R., Mfinanga, G., Lwilla, F., Cox, C., Jubitana, M., Kanyhaga, H., Mtandu, R., Kahwa, A., Mwessongo, J., Makingi, G., Mfaume, S., Van Steenberge, J., Beyene, N.W., Billet, M. & Verhagen, R. (2009a) African pouched rats for the detection of pulmonary tuberculosis in sputum samples. *International Journal of Tuberculosis and Lung Disease* 13, 737-743.

Weetjens, B. J., Mgode, G. F., Davis, B. K., Cox, C. & Beyene, N. W. (2009b) African giant rats for tuberculosis detection: A novel diagnostic technology. *Global Forum Update on Research for Health* 6, 39-42.