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The nematode *Caenorhabditis elegans* displays a chemotaxis behavior to tuberculosis-specific odorants

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ABSTRACT

A simple, affordable diagnostic test for pulmonary tuberculosis (TB) is urgently needed to improve detection of active *Mycobacterium tuberculosis*. Recently, it has been suggested that animal behavior can be used as a biosensor to signal the presence of human disease. For example, the giant African pouched rats can detect tuberculosis by sniffing sputum specimens while trained honeybees respond to three of the volatile organic compounds (VOCs) detected in the breath of TB positive patients by proboscis extension. However, both rats and honeybees require animal housing facilities and professional trainers, which are outside the scope of most disease testing facilities. Here, we report that the innate olfactory behavioral response of the roundworm nematode *Caenorhabditis elegans* can be used to detect the TB-specific VOCs methyl *p*-anisate, methyl nicotinate, methyl phenylacetate and *o*-phenylanisole, in chemotaxis assays. Dauer larvae, a long-lived stress resistant alternative development state of *C. elegans* in which the animals can survive for extended periods of time in dry conditions with no food, were also demonstrated to detect the VOCs. We propose that exposing naive dauer larvae to TB-related VOCs and recording their response in this behavioral assay could lead to the development of a new method for TB diagnostics using breath as the sample type.

1. Introduction

The World Health Organization's Millennium Development Goal of halting and reversing the tuberculosis (TB) epidemic by 2015 has been met. TB incidence has fallen by an average of 1.5% per year since 2000 and is now 18% lower than the level of 2000. Despite these gains, TB remains one of the leading causes of morbidity and mortality globally with an estimated 9.6 million people falling ill and 1.5 million people dying from TB in 2014 [1]. Accurate and timely diagnosis is the first step in providing care to individual patients and to prevent the spread of TB. Each year, it is estimated that one third of the infected population are not diagnosed or notified of their disease state which corresponds to 3 million people who do not have access to treatment for this curable disease.

Traditionally, the diagnosis of pulmonary TB relies on a combined approach of clinical symptoms, a chest X-ray and sputum based laboratory testing including smear microscopy, bacterial culture and molecular methods [2]. Increased access to these traditional tests plus novel diagnostic approaches are required to increase access to care. Recently, the tuberculosis community identified the highest priority diagnostic needs to reach the "missing 3 million", and detailed target product profiles (TPP) were defined [3]. One of the TPPs described the requirements of a non-sputum based biomarker test capable of detecting *Mycobacterium tuberculosis* via biomarkers or biosignatures that would be preferably available at the point-of-care (POC) [3]. Human breath is an attractive alternative to sputum as the specimen for a noninvasive diagnostic test. The use of exhaled breath to either detect the bacilli DNA using the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) [4,5] or volatile organic compounds (VOCs) produced specifically to *M. tuberculosis* [6] have been explored. VOCs detection seems like the more promising approach, having been used in proof-of-concept field tests in South Africa that used a nanomaterial-based sensor [7]. Other methods could also be used for breath collection and its concentration, such as solid-phase microextraction (SPME), in which the VOCs of interested are extracted, concentrated using a silica fiber coated with a polymeric stationary phase and then assayed by gas chromatography or mass spectrometry [8].

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Volatile organic compounds (VOCs) in the breath or as body odors have long been associated with human disease [9], and it has been suggested that the field of "volatolomics" could help advance the production of inexpensive, non-invasive diagnostics [10–12]. In fact, VOCs present in the human breath of patients with specific maladies have been proposed as biomarkers for cancer [13], infectious diseases [14] and diabetes [15], among other diseases. *M. tuberculosis* produces an array of different VOCs, of which four have been described as exclusive to this organism: methyl nicotinate, methyl *p*-anisate, methyl phenylacetate and *o*-phenylanisole [6]. Giant African pouched rats (*Cricetomys gambianus)* have been trained to recognize TB-infected sputum samples and also have been shown to respond to a combination of VOCs including methyl nicotinate, methyl *p*-anisate and *o*-phenylanisole [16,17]. Honeybees have also been trained to extend their proboscis in response to TB-specific VOCs [18]. The use of animals with superior olfactory systems to detect *M. tuberculosis* may hold promise, but rats and bees would both require animal housing facilities and professional trainers which may limit the accessibility of this technology to specialized centers [19].

Rather than using trained animals, we propose exploiting the innate sensing capabilities of the nematode *C. elegans. C. elegans* is a commonly used model organism for genetic studies [20] and has been demonstrated to respond to over 100 VOCs with either a concentration-dependent attraction or repulsion [21,22]. Its genome contains over 1000 uncharacterized candidate G-protein coupled chemosensory receptors indicating a very large genetic potential to respond to VOCs [23–25]. Microfluidic devices have been engineered to detect and quantify *C. elegans* behavior [26,27], and Liao and co-workers showed that *C. elegans* recognized VOCs associated with different explosives and that genetic mutants could be used to finely tune this response [28]. Dauer, an alternative development state of *C. elegans* resistant to starvation and dehydration would eliminate the need for animal husbandry and feeding in a future to-be-developed test [29].

In this report, we demonstrate that both wild type and dauer forms of *C. elegans* display behavioral responses to the above-described TB-specific VOCs. These results showcase the potential for *C. elegans* to be incorporated into a new and affordable diagnostic test device for TB and, perhaps, other disease states, utilizing the olfactory response of *C. elegans*.

2. Methods

2.1. Chemicals

The VOCs methyl nicotinate (M59203), methyl *p*-anisate (253,146) and methyl phenylacetate (W273309) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and methyl *o*-phenylanisole (L01063) was purchased from Alfa Aesar (Haverhill, MA, USA). The controls used were: isoamyl alcohol (W205702) as an attractant, 1-octanol (472,328) as a repellent and ethanol (E7023) as a negative [21]. The paralyzing reagent was sodium azide (S2002) [21]. All controls and the paralyzing agent were obtained from Sigma-Aldrich (St. Louis, MO, USA)

2.2. C. elegans *strains*

Wild type N2 Bristol and DR1572 *daf-2*(*e1368*) strains were obtained from the *C. elegans* Genetic Center (CGC). Nematodes were cultured on Nematode Growth Media (NGM) plates seeded with OP50 *Escherichia coli* as a food source, according to standard methods [20]. Wild type N2 animals were grown at 20 °C, while *daf-2*(*e1368*) animals were maintained at 15 °C (permissive temperature). Synchronization of the nematodes for the chemotaxis assays was performed as previously described in the literature [30]. *daf-2(e1368*) embryos resulting from synchronization were cultured at 25 °C (nonpermissive temperature) to induce the dauer phenotype.

2.3. Chemotaxis assays

The chemotaxis assay protocol was adapted from Margie et al. [31]. Briefly, the assay was performed in 5 cm Petri dishes containing 12 ml of NGM. During the assay, 8ul of wild type or *daf-2*(*e1368*) nematodes in M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl) were transferred to a ¼ in. radius inner circle region of the plate, after which the chemicals were added to the appropriate quadrants of the plate (Fig. 1a). Wild type animals were allowed to roam for a minimum of 60 min before quantifying the results. Chemotaxis assays of wild type animals were scored the following day in our experiments, as we observed no change in CI value between one hour and 24 h assay time. The *daf-2*(*e1368*) nematodes, which move differently and are physiologically distinct from wild type, were assayed after 120 min. Animals were excluded from the analysis if they failed to clear the inner circle, were sitting on the marked lines or less than 2 body lengths away from those lines. Animals that were located on the edges or walls of the plates were counted as part of the corresponding quadrant. For each experiment, a chemotaxis index (CI) was calculated using the following formula: CI = (number of animals in both test quadrants – number of animals in both control quadrants)/(total number of scored animals). A + 1.0 score corresponds to maximal attraction, whereas a −1.0 score means total repulsion, and results close to 0 indicate a lack of response to the compound. For the dilution curve assays, compounds were diluted to 100, 10, 1 or 0.1 ng/μl in 95% ethanol. Chemotaxis assays were performed in triplicate for each condition unless otherwise indicated. Final datasets presented are the mean of three (wild type) or four (dauer) independent trials with at least 59 worms per trial.

2.4. Data analysis

Chemotaxis indexes for each replicate were calculated. The average of all replicates for each condition and for each strain was determined along with the standard error of the mean. *P* values were determined using a two-tailed Student *t* test (unequal variance).

3. Results

Young adult *C. elegans* were introduced into the center of a chemotaxis plate (Fig. 1a) that has been divided into quadrants: 2 containing the chemical of interest and 2 containing the solvent control (ethanol). The animals were exposed to range of concentrations (100 ng/μl, 10 ng/μl, 1 ng/μl or 0.1 ng/μl) of methyl nicotinate, methyl *p*-anisate, methyl phenylacetate or *o*-phenylanisole. On separate chemotaxis plates, we assayed an attractant control (isoamyl alcohol) and a repellent control (1-octanol) [21] to ensure assay reproducibility. The animals were allowed to roam for at least 60 minutes, then were counted in the 4 quadrants to calculate the chemotaxis index (CI). Fig. 1b illustrates how a chemotaxis plate looks as the assay is initiated with the addition of the animals to the center (inset at $t = 0$) and at the end before the animals are counted ($t = 1$ h, the areas of the plate where compounds were added are shown).

C. elegans displayed a significant behavioral response to at least one concentration for each of the four VOCs (Fig. 2). The animals were repelled by 100 ng/μl and 10 ng/μl of methyl nicotinate (CI = −0.4), but did not respond to 1 ng/μl or less. They were attracted to 100 ng/μl, 10 ng/μl and 1 ng/μl of *o*-phenylanisole (CI = 0.3), but did not respond to 0.1 ng/μl (Fig. 2a). The strongest

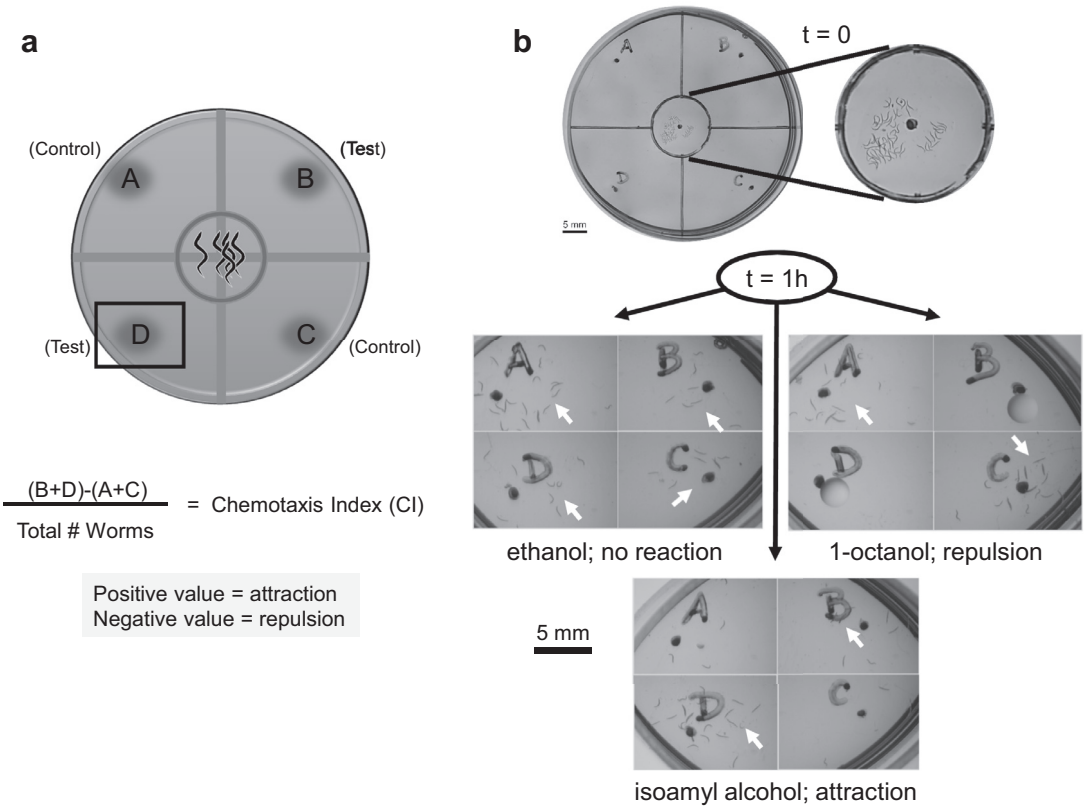

Fig. 1. *C. elegans* chemotaxis assay. (a) Schematic of assay. Test compound is spotted on 2 opposite quadrants of Petri dish; solvent control is spotted on the 2 remaining quadrants. All spots also contain an anesthetic to retain the worms. After at least 1 h, worms in each quarter of the plate are counted and a chemotaxis index (CI) is calculated. Positive CI values indicate an attraction response; negative values indicate repulsion. The black box in area D represents the regions pictured in (b). (b) Representative images of before ($t = 0$) and after ($t = 1$ h) chemotaxis assay with ethanol (negative control), isoamyl alcohol (attractant control) and 1-octanol (repellent control). Error bar is 0.5 mm. White arrows indicate areas where animals accumulated at the end of the assay.

response was observed with methyl *p*-anisate which acted as a repellent at all concentrations tested with a CI value of −0.9 at 100 ng/μl, 10 ng/μl and 1 ng/μl and a reduced CI value of −0.4 at 0.1 ng/μl (Fig. 2b). Interestingly, methyl phenylacetate acted as a strong repellent at 100 ng/μl (CI = −0.8), but this response was reversed to attraction at 10 ng/μl and 1 ng/μl (CI = 0.7) and no response was detected at 0.1 ng/μl (Fig. 2b). Similar reversals of the chemotaxis index with decreasing concentrations have been previously reported with hexanol and heptanol [21]. Taken together, the results indicate that adult wild type *C. elegans* are able to detect and respond to all 4 of the TB-specific VOCs. We speculate that the observed olfactory response to TB-specific VOCs may be employed to diagnose TB by detecting these VOCs in human breath.

A significant challenge to using living organisms in a diagnostic test is the care and feeding of the animals. Wild type *C. elegans* live, on average, approximately 3 weeks [30] resulting in a test with an impractical short shelf life. Furthermore, they go through a development cycle from oocyte to adult worm, and the olfactory response varies in the different development stages [32–34] (Fig. 3a). Dauer, an alternative development state which is resistant to starvation and harsh environmental conditions such as dehydration and can live up to 4 months [35,36] could provide a solution to this problem. To determine if the dauer form of *C. elegans* displays a response to the TB-specific VOCs, we tested animals that carry a temperature-sensitive mutation in the insulin-like receptor gene, *daf-2,* which leads to a constitutive dauer state when animals are grown at the nonpermissive temperature. Once dauer worms were generated, we performed the same chemotaxis assay using the highest concentration (100 ng/μl) for the 4 TB-specific VOCs. The measured response of the dauer animals was comparable to the CI values calculated for adult wild type nematodes (Fig. 3b). Dauer animals avoided methyl nicotinate, methyl *p*-anisate and methyl phenylacetate (CI = −0.6), while *o*-phenylanisole acted as a mild attractant (CI = 0.3). Our results indicate that the dauer form of *C. elegans* can be used in a behavioral assay to detect TB-specific VOCs.

It is noteworthy that the attractant control, isoamyl alcohol, generated an unexpected avoidance response in the dauer state (CI = −0.4). The bilateral AWC neurons, which are responsible for detecting isoamyl alcohol [21], have a different morphology in the dauer form [37]. We speculate that the altered morphology of the AWC neurons in dauer animals may be responsible for the change in behavioral response to isoamyl alcohol.

4. Discussion

The benefit of using exhaled breath as a noninvasive and easy to collect specimen is obvious. If a highly sensitive POC diagnostic test utilizing breath is developed, its use could help reduce the treatment gap of the “missing 3 million” patients and possibly be used in active case finding to detect patients before they present at clinics, thus interrupting disease transmission [38]. In this report, we have demonstrated the potential for exploiting the innate olfactory response of the soil nematode *C. elegans* to detect 4 VOCs that have been isolated from the breath of TB positive patients [6] and that this response could also be detected using the dauer larvae, an alternative development state. Because the dauer animals are nonfeeding and stress resistant, we propose that they can be directly incorporated into a microfluidic device specifically designed for chemotaxis behavior assays (Fig. 4). In our concept, the

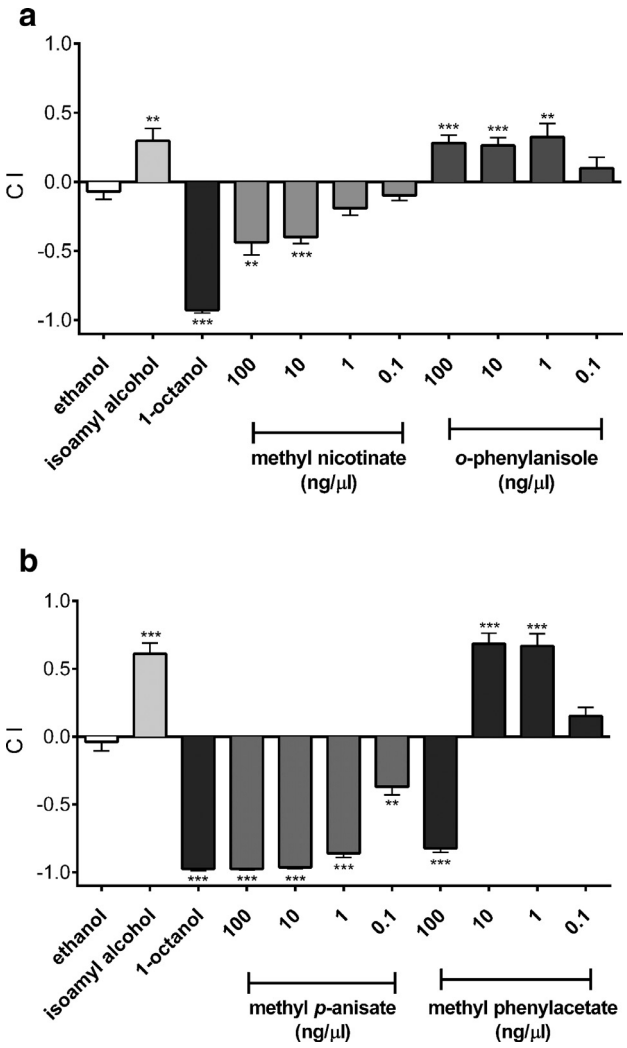

Fig. 2. Chemotaxis indexes calculated for wild type adult *C. elegans* exposed to different concentrations (100, 10, 1 and 0.1 ng/μl) of TB-specific VOCs – methyl nicotinate, *o*-phenylanisole (a), methyl *p*-anisate, methyl phenylacetate (b). Ethanol was used as the negative control, while isoamyl alcohol and 1-octanol were used as a known attractant and repellent, respectively. Bars represent the average of 3 independent experiments, each performed in duplicate or triplicate (7–9 assay plates per condition). Error bars = S.E.M. Starred bars indicate statistically significant differences in a 2-tailed Student t-test (unequal variance) when compared to the negative control ethanol (** $p < 0.01$; *** $p < 0.001$).

device would be provided to the user containing a defined number of dauer animals. Exhaled human breath from TB-suspect patients would be collected and concentrated via a specially designed collection system (such as solid-phase microextraction [8]) and these compounds would be introduced into the chemotaxis assay cartridge initiating the test. At the end of the test, the location of the animals would be detected and recorded by an optic reader measuring changes in optical density or fluorescence (nematode species can be easily engineered to express fluorescence proteins [39]) and the chemotaxis index would be calculated. Based on our results, an answer could be provided in 1 h (the time used for the chemotaxis assays), but miniaturization into a microfluidics device should allow to reduce the assay time; this would significantly reduce the turnaround time of most current TB diagnostics, and help tackle one of the major priorities defined by the WHO for their desired target product profiles [3]. Multiple animals will be used in each test, potentially increasing the accuracy of the result and its negative predictive value compared to other assays that rely on only one or a few animals per test [17,40].

We acknowledge that that there are limitations and/or potential pitfalls to our proposed test design. The observed olfactory response to the TB-specific VOCs reported here demonstrates only initial feasibility. The animals were exposed to purified compounds in isolation. We will need to determine if the same response is obtained when the compounds are isolated from wild type *M. tuberculosis* and when spiked into human breath specimens [41]. The range of concentration for these four VOCs in the breath of TB patients is not known, and it is possible that they are present in too low of concentrations to be detected by *C. elegans*. We anticipate building a collection system that will concentrate multiple exhaled breaths thus increasing the amount of the VOCs of interest in the chemotaxis assay and, potentially, the sensitivity of the test [41]. We have not tested the TB-specific VOCs in combination with one another, nor have we tested if breath from healthy volunteers will also engender an olfactory response in worms. Therefore, at this moment, we do not know if nematodes would be attracted or repelled by a biologically relevant sample containing methyl nicotinate, methyl *p*-anisate, methyl phenylacetate and *o*-phenylanisole. This will be essential to determine before a diagnostic device can be created, since it is the combination of these 4 VOCs that is specific to *M. tuberculosis* [6].

Determining which neurons in the nematode are responsible for the detection of the TB-specific VOCs may contribute to the solution of this problem. Laser ablation of specific neurons or genetic mutants could be generated to only respond to a subset of VOCs. This could lead to the identification of the pathways involved in this response, and to a more finely tuned chemotaxis behavior that avoids potential confounding effects of other VOCs also present in human breath. A better understanding of these mechanisms would support the value of using the *C. elegans* chemotaxis response to VOCs as a diagnostic method for not only TB, but also other diseases such as cancer [9] and diabetes [15] that have been shown to display emission of various VOCs as biomarkers.

A key finding in this report is that dauers can be used to detect VOCs that are biomarkers for TB. The dauer neuroanatomy resembles non-dauers in many aspects, which suggests similar neural functioning. However, little is known about the neural circuitry or synaptic wiring of the dauer. In comparison to other tissues, the dauer nervous system expands and constitutes a proportionately larger share of the animal's total volume [32,37], and studies have shown that the behavioral repertoire of dauers differs in several aspects from directly-developing larvae and adults. This may explain why dauers responded aversely to the control attractant isoamyl alcohol. More specifically, the AWC neurons in the dauer animals enclose most of the nose circumferentially when compared to non-dauers [32]. The increase in volume and surface area of the AWC neuron may have heightened the sensitivity of the dauer to the volatile odorant, resulting in this avoidance behavior. Testing dauer response to isoamyl alcohol in a dose-dependent manner may address this possibility. The dauer form's anatomical differences from the other developmental stages of *C. elegans* in combination with its stress and starvation resistance may make it the ideal form to be used for VOC detection.

C. elegans is an anatomically simple organism with a well-defined nervous system that is tractable to molecular and classical genetic analysis [20,30]. It is a model organism that has contributed to our understanding of development, nervous system function, aging and protein folding disorders [20,42]. Here we report a new way to utilize *C. elegans* to detect TB-specific chemicals of interest. Their use could be expanded into detecting other diseases with distinct VOCs as biomarkers. The worms' olfactory response observed in this study is pre-wired and may be necessary for their survival [43]. Most attractant molecules identified thus far are natural products of bacterial metabolism and bacteria are the food source for *C. elegans*, which may limit the range of

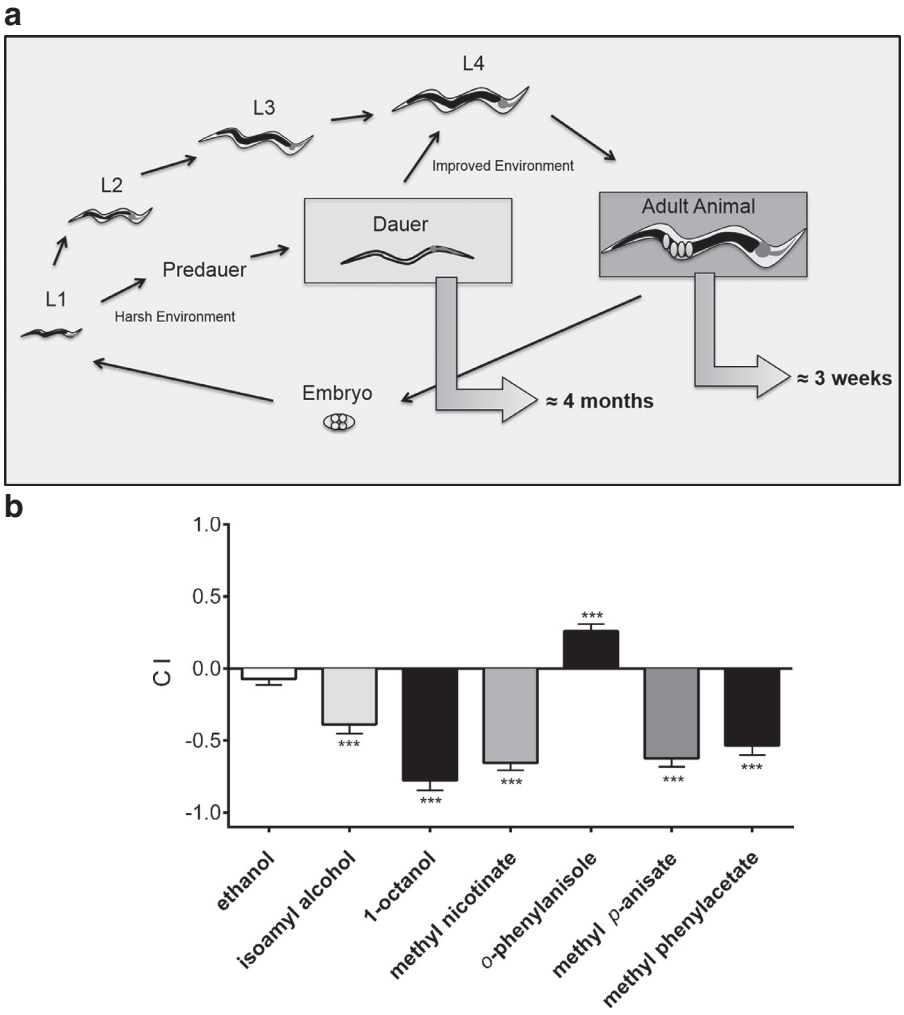

Fig. 3. (a) *C. elegans* life cycle. In favorable environmental conditions, nematodes develop through 4 larval stages (L1 to L4) until they reach adulthood (after 2 days at 20 °C), with a life span of approximately 3 weeks for the adult wild type. In harsh environmental conditions, such as starvation or changes in temperature, an alternative life cycle results in dauer larvae, which can survive in stasis up to 4 months or resume normal development (to the L4 stage) upon improvement of the growth conditions. (b) Chemotaxis indexes calculated for dauer *C. elegans* exposed to 100 ng/μl of the indicated TB-specific VOCs – methyl nicotinate, *o*-phenylanisole, methyl *p*-anisate, methyl phenylacetate. Ethanol was used as the negative control, while isoamyl alcohol and 1-octanol were used as a known attractant and repellent, respectively. Bars represent the average of 4 independent experiments, performed in triplicate (12 assay plates per condition). Error bars = S.E.M. Starred bars indicate statistically significant differences in a 2-tailed student *t*-test (unequal variance) when compared to the negative control ethanol (** $p < 0.01$; *** $p < 0.001$).

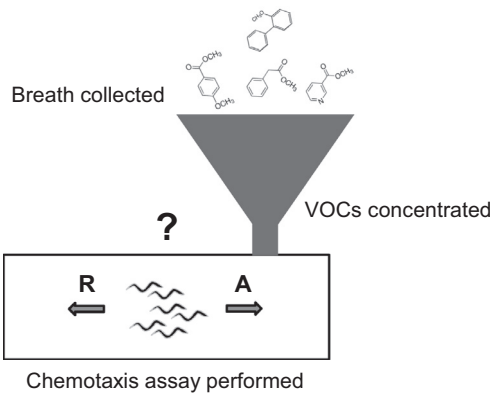

Fig. 4. Proposed workflow for a diagnostic test for TB-specific volatile organic compounds in a patient's breath. R = repellent; A = attractant.

VOCs detected. If the volatile target is not recognized by the nematodes, it may still be possible to use this system for detection by specifically engineering receptors to recognize the chemical target utilizing yeast for cloning and optimizing signaling [44] and then expressing the recombinant receptor in *C. elegans*. We are confident our modest report can be expanded in any number of directions using worms as detection tools and also in contributing to the greater understanding of the *C. elegans* olfactory system.

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