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Short communication

Smelling *Pseudomonas aeruginosa* infections using a whole-cell biosensor – An alternative for the gold-standard culturing assay

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ABSTRACT

Improved easy-to-use diagnostic tools for infections are in strong demand worldwide. Yet, despite dramatic advances in diagnostic technologies, the gold-standard remains culturing. Here we offer an alternative tool demonstrating that a bacterial biosensor can efficiently detect *Pseudomonas aeruginosa* infections in patients suffering from otitis externa. Detection was based on specific binding between the biosensor and 2-aminoacetophenone (2-AA), a volatile produced by *P. aeruginosa* in high amounts. We collected pus samples from ears of 26 subjects exhibiting symptoms of otitis externa. Detection of *P. aeruginosa* using the biosensor was compared to detection using gold-standard culturing assay and to gas-chromatograph–mass-spectrometry (GC–MS) analyses of 2-AA. The biosensor strain test matched the culture assay in 24 samples (92%) and the GC–MS analyses in 25 samples (96%). With this result in hand, we designed a device containing a whole-cell luminescent biosensor combined with a photo-multiplier tube. This device allowed detection of 2-AA at levels as low as 2 nmol, on par with detection level of GC–MS. The results of the described study demonstrate that the volatile 2-AA serves as an effective biomarker for *P. aeruginosa* in ear infections, and that activation of the biosensor strain by 2-AA provides a unique opportunity to design an easy-to-use device that can specifically detect *P. aeruginosa* infections.

1. Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen capable of infecting various tissue types (Bjarnsholt et al., 2009; Gómez and Prince, 2007; Streeter and Katouli, 2016; Wang et al., 2005). It is notorious for its persistent antibiotic-resistant mucoid biofilms, and it is well established that early aggressive antibiotic treatment of its infections is imperative for successful treatment (Bjarnsholt et al., 2009). Currently, routine detection of *P. aeruginosa* infections is carried out by culture inoculation. This method, which can detect *P. aeruginosa* in one to three days, requires analyses of samples in specialized laboratories and is prone to false results (Brown and Lowbury, 1965; Xu et al., 2004). The necessary growth step of bacteria in the culturing method can generate false-positive results due to contamination of plates and medical devices, as well as false-negative results due to overgrowth by other bacteria in a mixed-species infection (Verenkar et al., 1993; Burman and Reves, 2000; Xu et al., 2004). Therefore, new tools for faster and more reliable culture-independent detection of *P. aeruginosa* are in demand, and methods based on polymerase chain reaction (PCR), serological analysis or detection of volatile compounds emitted by *P. aeruginosa* have been examined (Goeminne et al., 2012; Héry-Arnaud et al., 2016; Savelev et al., 2011; Spilker et al., 2004; Tramper-Stranders et al., 2006; Xu et al., 2004). Though these methods may be more accurate than the gold-standard culturing assay, they all require specialized equipment, laboratories and personnel, and therefore cannot be easily implemented in clinics.

Several studies reported that the production of 2-aminoacetohenone (2-AA), a low-weight volatile compound produced by *P. aeruginosa* in high amounts, could be used as a biomarker for the presence of *P. aeruginosa* in infected tissues (Preti et al., 2009; Scott-Thomas et al., 2010). Detection of 2-AA and other *P. aeruginosa* volatiles has thus far been carried out by elaborate gas-chromatograph mass-spectrometry

Abbreviations: 2-AA, 2-aminoacetophenone; PMT, photo multiplier tube; GC–MS, gas chromatography mass–spectrometry

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(GC–MS) or selected-ion flow-tube mass-spectrometry (SIFT-MS) (Carroll et al., 2005; Goeminne et al., 2012). These methods are costly and require technical experience, both often lacking in local medical centers. Here we suggest an alternative method for detection of 2-AA. We have previously reported that 2-AA specifically activated the LuxR quorum sensing (QS) receptor of the marine bacterium *Aliivibrio fischeri* (Kviatkovski et al., 2015). We found that 2-AA activated the LuxR receptor of both wild type *A. fischeri* MJ-1 and the genetically modified *Escherichia coli* MJ109/pSB401 strain. The latter, which was originally constructed by Winson et al. (1998) to respond to acyl-homoserine lactone (the non-volatile cognate signal of LuxR), harbors the pSB401 plasmid encoding the LuxR receptor in conjugation to the luciferase encoding operon. We showed that the *E. coli* MJ109/pSB401 strain (thereafter named the biosensor strain), was activated upon exposure to *P. aeruginosa*'s volatile bouquet and specifically to 2-AA (Kviatkovski et al., 2015). According to our previously reported experiments and *in silico* docking analysis, the specific activation of the LuxR receptor by 2-AA was dependent on the positions of the amine and ketone in the 2-AA ring and was based on the interaction of 2-AA with seven amino acids within the binding pocket of the receptor (Kviatkovski et al., 2015).

Activation of LuxR by 2-AA was deemed highly specific because (i) LuxR did not respond to 2-AA homologs, (ii) 2-AA did not activate other LuxR-like receptors, and (iii) volatiles of 2-AA deficient *P. aeruginosa* mutants did not activate the LuxR biosensor strain (Kviatkovski et al., 2015).

In the present study we describe the use of a LuxR-harboring whole-cell biosensor strain to specifically detect *P. aeruginosa* infections, via binding of 2-AA, in patients suffering from otitis externa.

2. Material and methods

2.1. Preparation of biosensor cells and 2-AA samples

E. coli/pSB401 (the biosensor strain) was grown with shaking (180 rpm) in Luria-Bertani (LB) broth supplemented with 20 μg/ml of tetracycline at 37 °C. Following overnight incubation, the cells were washed in 0.9% NaCl saline solution and diluted 100 fold in LB to obtain a concentration of approximately 10^6 cells/ml. Ten microliter of the biosensor cells were immobilized on a solid medium (described below) in the inner part of a 1.5 ml Eppendorf tube cap. Five hundred microliters of the analyzed sample, either 2-AA solution (Sigma-Aldrich, St. Luis, MO, USA) or pus sample (provided by the Edith Wolfson Hospital), suspended in Ringers lactate buffer (Hartmann's solution, Teva Medical; 0.2 g/l calcium chloride, 3.1 g/l lactic acid, 0.3 g/l potassium chloride and 6 g/l sodium chloride), were placed within the Eppendorf tube. In this setting there was no direct contact between the sample at the bottom of the Eppendorf tube and the biosensor strain in its cap, thus allowing activation only through air-borne substances. The tubes were closed and incubated for 18 h prior to measurement of the luminescence produced by the biosensor strain. The luminescence produced by the biosensor strain was measured either in a Photo Multiplier Tube (PMT) based plate reader or by our PMT-based compact biosensor device, as described below.

2.2. Long-term viability of the biosensor strain on different solid media

In order to evaluate the long-term stability of the biosensor strain we examined three types of solid media polymers for immobilizing the bacterial biosensor: water-agar, LB-agar and sodium alginate. For preparation of water agar-immobilized biosensor, overnight grown and washed cells were mixed 1:1 with 3% agar at 40 °C. Then, 250 μl of the mix were transferred to the cap of an Eppendorf where they were allowed to solidify. For preparation of LB-agar-immobilized biosensor, 10 μl of the cells were plated on 250 μl LB-agar matrix (1.5%), which was allowed to solidify within the cap of the Eppendorf tube. For preparation of alginate-immobilized biosensor, washed cells were mixed 1:1 with 3% sodium alginate solution in water. 250 μl of the mix were added to the cap of the Eppendorf. Further, 10 μl of 1 M $CaCl_2$ were added to the mix for solidification and encapsulation of the biosensor cells.

Following immobilization of the biosensor strain in each of the three different media, all the treatments were stored at 4 °C. The detection of 2-AA was examined using (i) 24 h old immobilized biosensor matrix, (ii) two week old immobilized biosensor matrix and (iii) five week old immobilized biosensor matrix. 2-AA solutions of 2, 10 or 100 nmol were added to the Eppendorfs with the different matrices, and were incubated overnight at 37 °C, as described above. Eppendorfs containing Ringers lactate buffer without 2-AA served as control. Following overnight incubation, the bioluminescence produced by the biosensor was evaluated by transferring the biosensor cells into a 96-well plate and measuring luminescence using a PMT-based infinite-F200 plate reader (Tecan Trading AG, Switzerland).

2.3. Optimal incubation temperature for 2-AA detection

For evaluating the optimal temperature for the assay, the biosensor strain was prepared as described above and immobilized on LB agar within the cap of the Eppendorf tube. 2-AA was added in amounts of 10 or 100 nmol to the Eppendorf tubes, which were transferred either to 30 °C or 37 °C. Following incubation of 18 h the biosensors were analyzed in the PMT plate reader as describe above.

2.4. Analysis of pus samples

Pus samples were collected from the external ear canal of 26 subjects clinically diagnosed as suffering from external otitis. All subjects provided informed written consent for their samples to be tested according to procedures that were approved by the Edith Wolfson Hospital Helsinki Committee. The subjects were of both sexes, with varied ages, from a few month infants to 85 years old seniors. All samples were analyzed for the presence of *P. aeruginosa* using three different methods as follows: A small portion of the pus sample was used for routine culture assays, which included the following growth mediums: blood agar, chocolate agar, Thioglycolate broth, Phenylethyl alcohol, Sabouraud, CHROMagar Orientation and MacConkey agar. The remaining pus sample was divided to two equal subsamples: one was subjected to the dynamic head-space analyses using GC–MS for 2-AA detection and the other was analyzed by the biosensor. Pus volumes varied between the different patients reaching a maximal volume of about 1 ml. In order to assure equal volumes in all analyses, lower amounts of collected pus samples were supplemented to a final volume of 1 ml using Ringers lactate buffer. For dynamic headspace analysis, 500 μl of the pus suspended in Ringers lactate buffer were placed in 20 mm headspace vials. The headspace vial was agitated and incubated at 60 °C for 10 min. The headspace was continuously collected for 1 h using 500 ml of Helium at 20 ml/min, unto a Tenax TA tube. The Tenax tube was desorbed in a Thermal desorption unit (Gerstel TDU) for 4 min at 21 °C in splitless mode and vapors subsequently trapped in a programmed temperature vaporizing (PTV) injector (Gerstel CIS4) that was kept in −70 °C. After desorption was complete, the PTV was heated at 12 °C/sec to 300 °C and was held there for 4 min. The GC Column was Restek Rxi-XLB medium polarity column, 30 × 250 × 0.25. The flow was 1.1 ml/min, oven program was 40 °C for 3 min then 12.5 °C/min to 300 °C for 3 min. Mass spectra acquisition was done in selective ion monitoring (SIM) mode, for masses 92.0;120.0;135.0.

The second subsample was examined with the biosensor strain for activation of the LuxR receptor by 2-AA as follows: 500 μl of the pus sample, suspended in Ringers lactate buffer, were transferred to an Eppendorf tube containing the biosensor strain immobilized on LB-agar in the inner part of the Eppendorf cap as described in Section 2.2. The volume of the analyzed samples was chosen to be 500 μl as this was the maximal amount of pus enabling equal partitioning to both GC–MS

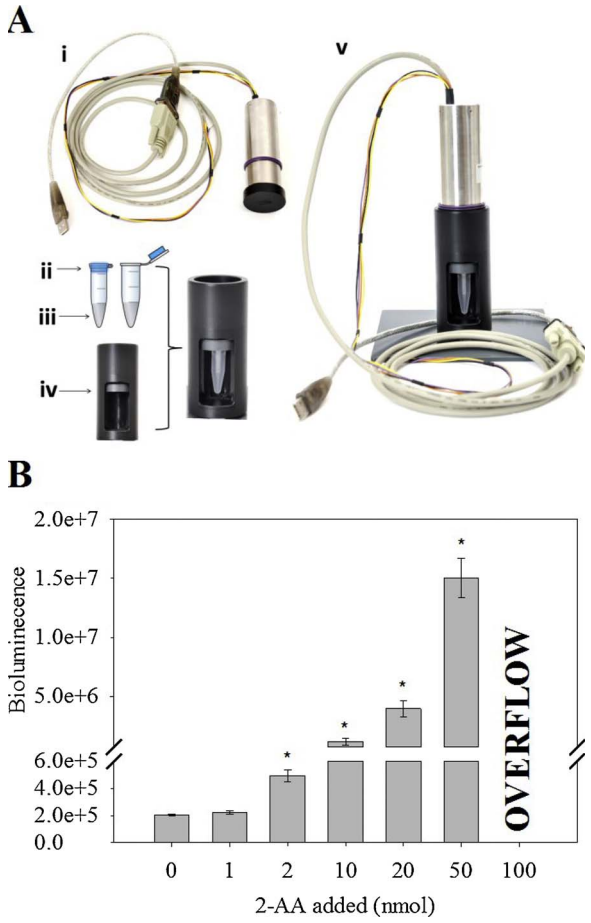

Fig. 1. Prototype biosensor device design and performance. A) Prototype PMT-based device. (i) PMT photodetector; (ii) cap containing the reporter strain; (iii) Eppendorf tube containing the pus sample; (iv) black plastic (ABS) chamber; (v) complete PMT and chamber setup. B) Detection limit of the PMT-based device. Luminescence levels of the biosensor exposed to different amounts of 2-AA, as measured using the PMT device. n = 6; Error bars represent standard error, asterisks indicate statistical difference ($P < 0.05$) between each of the 2-AA amounts and control, according to *t*-test.

analysis and biosensor assay. Lower volume of pus sample (e.g. 250 µl) resulted in a lower luminescent output from the biosensor strain (data not shown). The luminescence produced by the biosensor strain after 18 h of incubation was measured using the plate-reader as described above.

2.5. Design of the PMT-integrated biosensor device

We designed a plastic capsule (black ABS) installed in an opaque enclosure that can hold an Eppendorf tube and a PMT (Hamamatsu H9319-01 Photon Counting) equivalent to that used in the plate reader described above (Fig. 1A). In this prototype PMT-integrated biosensor device, an Eppendorf tube containing the biosensor strain and 2-AA sample (please see Section 2.1 for description), was fit for direct analysis (Fig. 1A). Blue light at a peak wavelength of 490 nm, emitted from the biosensor due to the activation of LuxR by 2-AA, was directly captured with an integration time of one second by the PMT photodetector, with a bandwidth of 300–650 nm.

The PMT transmitted digitally, via a USB hub, the number of detected photons within a specified period of time. Its high sensitivity of $4.1{\cdot}10^5$ [xp/sW] at 400 nm enabled detection of very low-intensity light samples but requires a light-tight enclosure to prevent activation from external photons. The transmitted digital data was collected by standard acquisition software (LabVIEW).

In order to evaluate the detection limit of the PMT-integrated biosensor device, different amounts of 2-AA were analyzed. Either 0, 1, 2, 10, 50 or 100 nmol of 2-AA, dissolved in 500 µl of Ringers lactate buffer, were added to Eppendorf tubes, which, following overnight incubation (18 h), were analyzed using the PMT-based device.

3. Results and discussion

In order to examine and optimize the viability and activity of the biosensor strain, three types of solid media were analyzed: Water-agar, LB-agar and sodium alginate. Biosensor strains were immobilized on each of these media types (thereafter termed biosensor matrix) and were stored for varying durations at 4 °C until exposure to 2-AA. The matrix supporting the most stable and sensitive reaction of the biosensor strain towards 2-AA was the LB-agar biosensor matrix. Following 24 h of storage the LB-agar biosensor matrix exhibited two-orders of magnitude increase in response to 100 nmol of 2-AA relative to non-exposed control (208-fold response) (Fig. S1). At this time point, an increase of only one-order of magnitude was exhibited by water-agar and alginate biosensor matrices relative to non-exposed control (8.5-fold and 19.5-fold, respectively) (Fig. S1). After one week of storage at 4 °C, the relative response of the biosensors to 100 nmol of 2-AA was still two orders of magnitude in LB-agar biosensor matrix, five-fold in the water-agar and one order of magnitude in the alginate (Fig. S1). After five weeks of storage at 4 °C, activation of the biosensor by overnight incubation with 100 nmol of 2-AA was observed in all three biosensor matrixes, with the strongest response recorded in LB-agar matrix (156 fold compared to control) (Fig. S1). Alginate and water-agar matrixes exhibited lower activation levels of 3.5 and 52 fold increase compared to control (Fig. S1). LB-agar-biosensor mix was also highly stable and sensitive to lower concentrations of 2-AA (Fig. S2). Upon addition of 2 and 10 nmol of 2-AA a two- and five-fold increase in luminescence was observed, respectively. This response remained stable at all three time points of storage. At these lower concentrations of 2-AA (2 and 10 nmol), neither the water-agar nor the alginate mix could efficiently detect 2-AA after prolong storage at 4 °C, exhibiting less than a 2-fold response (data not shown). These results indicate that the biosensor remains highly active in LB-agar, even after five weeks of storage at 4 °C, and that the LB-agar-immobilized biosensor strain is applicable for long-termed storage at 4 °C.

Additionally, we evaluated the optimal temperature for the assay. Incubation of the biosensor strain with two and 10 nmol of 2-AA at the temperature of 37 °C achieved a higher response to 2-AA, relative to incubation at 30 °C (Fig. S3). This indicates that 37 °C, which is the optimal temperature for *E.coli* growth, is also optimal for the biosensor assay.

In order to assess the diagnostic efficacy of the biosensor strain, we analyzed 26 pus samples from the external ear canal of subjects clinically diagnosed as suffering from external otitis. All samples were analyzed for the presence of *P. aeruginosa* using three different methods as follows: (i) detection of *P. aeruginosa* using culturing method, (i) detection of 2-AA using GC–MS and (iii) detection of 2-AA using the biosensor strain.

Results obtained from the biosensor test matched the results obtained from culture assay in 24 of 26 analyzed samples (92%) and to those from the GC–MS analyses in 25 of the 26 samples (96%) (Table 1, Fig. S4). The results reflect high accuracy (d' = 2.74, Zhang and Mueller Sensitivity = 0.95), which is significantly different from chance (binomial, $p < 10^{-6}$).

Eighteen of 26 pus samples were negative for *P. aeruginosa* infections as detected by culture assay and GC–MS (69.20%). Of these 18 negative samples, the biosensor strain detected 17 samples as negative (65%), whereas an additional sample (termed false positive) was positive for *P. aeruginosa* infection in the biosensor strain test but negative in the GC–MS analysis and culture assay. In this sample the culturing assay detected a mixed micro-flora. Seven samples (27%) were positive for *P. aeruginosa* infection as indicated by culture assays, biosensor test

Table 1
Detection of *P. aeruginosa* in otitis externa pus samples as analyzed using *E. coli* JM109/pSB401 biosensor and GC–MS, compared to culture analysis. Pus samples were taken from subjects suffering from otitis externa. Percent of positive and negative *P. aeruginosa* detected infections, as well as, false-positive and false-negative samples are shown, together with absolute numbers (in parentheses). In addition, non-parametrical signal detection statistics are shown for biosensor analysis – d-prime (d') and Zhang and Mueller Sensitivity (A) and p-value.

		Positive	Negative	False positive	False negative
Biosensor	Percent of total 26 samples	27%	65.40%	3.80%	3.80%
	(Number of samples)	(7)	(17)	(1)	(1)
	Non parametric signal detection statistics	d' = 2.74, A = 0.95, binomial, $p < 10^{-6}$			
GC	Percent of total 26 samples	27%	69.20%	0%	3.80%
	(Number of samples)	(7)	(18)	(0)	(1)

and GC–MS analysis. One sample (3.8%) was false negative, *i.e.* positive for *P. aeruginosa* infection according to the culture assay but was negative in the biosensor test and GC–MS analysis. This can be explained by either a *P. aeruginosa* variant that produced 2-AA amounts below the detection limit of our biosensor, or contamination of the culture plate by exogenous *P. aeruginosa* bacteria, a known drawback of the gold-standard culture-based method used today (Farmer et al., 1982; Siebor et al., 2007). The LuxR-based biosensor is much less susceptible to contaminations due to: (i) high specificity of the ligand-receptor interaction, (ii) no need in culturing and (iii) the volatile manner of the test, which avoids direct contact of samples and sampling tools with the biosensor strain.

Bacterial volatiles have already been reported as significant biomarkers for diagnostics (Zhu et al., 2011; Sohrabi et al., 2014). 2-AA is produced by *P. aeruginosa* in very high amounts, often reaching micromolar concentrations (Cox and Parker, 1979; Kesarwani et al., 2011). It was also detected, in lower amounts, in environmental bacterial strains such as the plant associated *Serratia plymuthica* and *Burkholderia thailandensis*, as well as members of the marine *Roseobacter* clade (Thiel et al., 2010; Blom et al., 2011). To the best of our knowledge, 2-AA has not been detected in related pathogens other than *P. aeruginosa* (Zhu et al., 2011), suggesting it could serve as a specific biomarker for *P. aeruginosa*. Indeed, in addition to the presented study, others have shown that 2-AA is indicative of *P. aeruginosa*'s presence in infected tissues (Preti et al., 2009; Scott-Thomas et al., 2010).

For simple on-site use of the biosensor strain in clinics, we designed a compact prototype biosensor device consisting of a plastic capsule that can hold an Eppendorf tube and a PMT (Hamamatsu H9319-01 Photon Counting) (Fig. 1A). The accuracy and sensitivity of the biosensor device was examined with synthetic 2-AA using amounts of 0, 1, 2, 10, 50 or 100 nmol per 500 µl of Ringers lactate buffer. The whole prototype device, consisting of PMT detector and biosensor strain was highly sensitive to 2-AA. The low limit for detection of 2-AA was 2 nmol of 2-AA in a sample (Fig. 1B). Notably, the detection limit of the biosensor was the same as the detection limit of the GC–MS, since 2-AA was not detected below 2 nmol using GC–MS as well (Fig. S5).

4. Concluding remarks

There is a worldwide growing demand for better and more accessible diagnostic tools for a variety of infectious diseases afflicting individuals. In this study we demonstrated that, through binding of a volatile biomarker, a whole-cell biosensor can efficiently diagnose *P. aeruginosa* infections in patients suffering from otitis externa.

We propose that the PMT-integrated biosensor device has advantages over the gold standard culturing method because it does not require a culturing step in diagnosis, and thus is less susceptible to false results. Moreover, the PMT-based device could be used in local clinics, thereby abolishing the need to refer the patients to analytic laboratories and providing faster results.

The use of whole-cell bacterial biosensors for the detection of various compounds is appealing since they offer the benefits of low cost and improved accuracy detection systems. Despite recent advances in the development of bacterial whole-cell biosensors for analyses of environmental samples (Belkin, 2003; Van der Meer and Belkin, 2010), their use for diagnosis of infections has yet to be established. Detecting volatile compounds using such biosensors could provide novel opportunities for specific and efficient detection of bacterial infections.

Conflict of interests

The office of Technology Licensing at Hebrew University in collaboration with the Office of Technology Licensing at the Weizmann Institute of Science filed a patent related to the results of this manuscript PCT/IL2015/050227.

Apart from this the authors declare no commercial or financial conflict of interests.

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Permission statement

All subjects provided informed written consent for their samples to be tested according to procedures that were approved by the Edith Wolfson Hospital Helsinki Committee.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiotec.2017.12.023.

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