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Short Communication

Anti-*Candida* and anti-quorum sensing activity of airborne microorganisms detected by a rapid method

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Abstract

Introduction: Introducing new antibiotics to the clinic is critical. **Methods:** We adapted a plate method described by Kawaguchi and coworkers in 2013[1] for detecting inhibitory airborne microorganisms. **Results:** We obtained 51 microbial colonies antagonist to *Chromobacterium violaceum*, purified and retested them, and of these, 39 (76.5%) were confirmed. They comprised 24 bacteria, 13 fungi, and 2 yeasts. Among the fungi, eight (61.5%) produced active extracts. Among the bacterial, yeast, and fungal strains, 17 (44.7%) and 12 (31.6%) were active against *Candida albicans* and *Candida parapsilosis*, respectively. **Conclusions:** The proposed screening method is a rapid strategy for discovering potential antibiotic producers.

Keywords: *Candida* sp. *Chromobacterium violaceum*. Quorum sensing. Antimicrobials.

According to Lewis[2], the rise and spread of antimicrobial resistance presents a unique challenge to both science and medicine. Resistance to antimicrobials has led to the selection of a list of six priority pathogens (two gram-positive and four gram-negative) termed ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which require rapid development of new antibiotics (reviewed in[3]). In case of fungal infections, invasive candidiasis is increasingly caused by non-*albicans Candida* intrinsically resistant to antifungals[4].

The crisis in antimicrobial discovery, has prompted experts to call for the revival of natural product drug discovery[2]. However, the enormous background of known and/or nuisance compounds presents a serious barrier to discovery[2]. Despite this, recent results like those presented by Maffioli et al.[5] bring hope to the search for new leads from microbial-extract screening.

An alternative to looking for drugs that kill bacteria is to search for molecules that target virulence factors with minimal to no effect on growth[6].

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Bacteria coordinate gene expression as a function of cell density in a communication mechanism named quorum sensing (QS)[6]. Therefore, bacteria are capable of performing tasks as a group, including migration to more favorable environments, biofilm formation, virulence gene expression, bacteriocin and antibiotic production, bioluminescence, and pigment production, among others[6,7]. Considering the importance of QS, the search for inhibitors against this mechanism has become a research target[8].

In QS, signaling molecules are usually called autoinducers and QS inhibition can be accomplished by interfering with the synthesis, secretion and degradation of these molecules, as well as by hampering their recognition by receptor proteins such as LuxR type homologs[6,7]. Nowadays, despite promising results[9] (also reviewed in depth in[6]), according to Defoirdt[6], it will probably take many more years before these agents can be used in the clinic.

In 2013, Kawaguchi and coworkers[1] proposed a supposedly more efficient and faster method to select candidate fungal producers of anti-*Candida albicans* antibiotics. In this study, we adapted this method using the strain *Chromobacterium violaceum* ATCC 12472, which naturally produces a violet pigment (violacein), to screen for antibiosis, anti-quorum sensing activity, and for anti-*Candida* spp. activity. Considering the scarce number of studies involving microorganisms collected from the air in antimicrobial activity screening[10], we decided to

collect air samples in the municipality of Itacoatiara, Amazonas, Brazil.

The air sampling of microorganisms was preferably performed in places with high circulation of people, such as a university campus and municipal fairs, particularly in places with selling grain products, such as flour. Considering the high amount of fungi, the air in the municipal dump was also sampled. Collection was also carried out in forest environments around the municipality of Itacoatiara. The twelve locations of air sampling and their geographic coordinates were as follows: Private Land in Itacoatiara (S 03º04'31.0''; W 058º27'38.4''), Canaçari District 1 (S 03º03'56.6''; W 058º27'13.1''), Canaçari District 2 (S 03º03'40.6''; W 058º26'33.4''), Canaçari District 3 (S 03º03'09.5''; W 058º26'01.1''), University Restaurant (S 03º08'34.746''; W 058º25'55.258''), Parking Lot of the University Campus (S 03º8'35.678''; W 58º25'52.231''); University Campus Autoclave Room (S 03º8'33.439''; W 58º25'53.976''); University Campus Cleaning Room (S 03º8'33.626''; W 58º25'53.368''), University Campus Tree 1 (S 03º8'35.678''; W 58º25'52.231''), Itacoatiara Municipal Dump (S 03º8'50.744''; W 58º25'39.727''), University Campus Tree 2 (S 03º8'33.77''; W 58º25'52.35''), and Municipal Flour Market (S 03º8'33.77''; W 58º25'52.35'').

To obtain anemophilous (airborne) microorganisms, three Petri dishes with a modified Luria Bertani (LB) agar supplemented with 5 g/L NaCl instead of 10 g/L were used. This modification was made considering a single observation where we noticed a more diverse growth of filamentous fungi with 5 g/L and no apparent alterations in violacein production by *C. violaceum* (data not shown). The plates were opened for 15 min with the lid off and faced downwards, at a height of 1.2 m from the soil, using a flat support; presence of walls was considered and an attempt was made to collect samples across the sampled area. After exposure, the plates were transported to the laboratory and incubated at 30°C for up to three days, with 12-hour dark/light cycles (in order to better simulate day and night conditions), to form visible colonies. In parallel, a *C. violaceum* cell suspension was prepared from overnight grown cells and adjusted to OD_{600} = 0.15, and approximately 60 µL was sprayed on the plates using an atomizer (the spray was applied 3 to 4 times in order to achieve this volume) after the three days of growth, as described by Kawaguchi[1], and shown in **Figure 1**. Precautions were taken such that the suspension completely covered the plate. After 15 minutes of drying at room temperature by opening the plates in a laminar flow bio-safety cabinet, the plates were incubated in inverted position at 28°C for 24-36h. Inhibition of QS was indicated by an opaque halo around the fungal colonies and classic antibiosis was indicated by a clear halo[7].

Potential inhibitory microorganisms that formed a halo of inhibition were repeatedly subcultured on potato dextrose agar (PDA) plates to obtain pure cultures and these were used to repeat the test against *C. violaceum* (**Figure 2**).

The anemophilous microorganisms were first characterized as filamentous fungi, yeasts, or bacteria based on their macroscopic and microscopic morphology. For the filamentous fungal genus identification, the evaluated macromorphological characteristics included colony size, texture, relief, and pigmentation. Micromorphological analyses were performed using the micro-cultivation technique in PDA to identify the vegetative structures and especially the specific reproductive structures of the genus, as described by Watanabe[11].

To obtain filamentous fungal extracts, the 13 isolated fungi were grown on PDA for three days. After that, six plugs of 6 mm in diameter were cut out of the agar and added to 30 mL of Czapeck-Dox broth and six plugs were added to

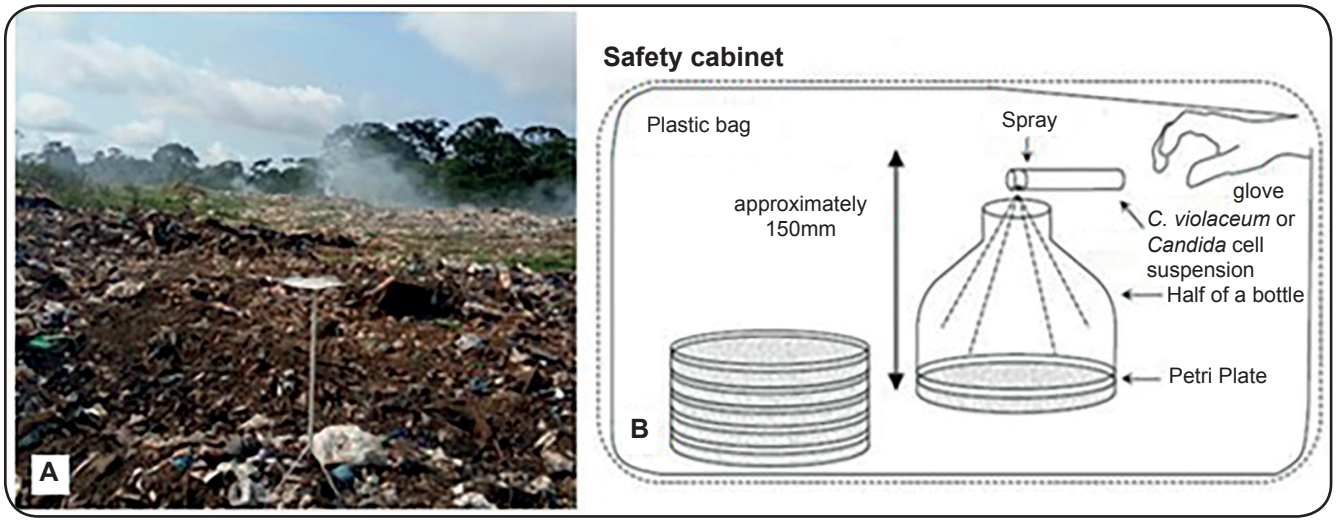

FIGURE 1: Example of a collection site for airborne microorganisms in the municipal dump of Itacoatiara, Amazonas. Three Luria-Bertani Petri dishes supplemented with 5 g/L sodium chloride were fully opened and exposed for 15 minutes at a height of 1.2 m from the soil (A)(see materials and methods). In (B), method for the inoculation of *Chromobacterium violaceum* or *Candida* sp. cell suspensions in Petri dishes with previously collected air microorganisms. A cell suspension of approximately 60 µL was sprayed on the air collection plates kept in a plastic bag installed in a biological safety cabinet. Adapted from Kawagushi et al.[1].

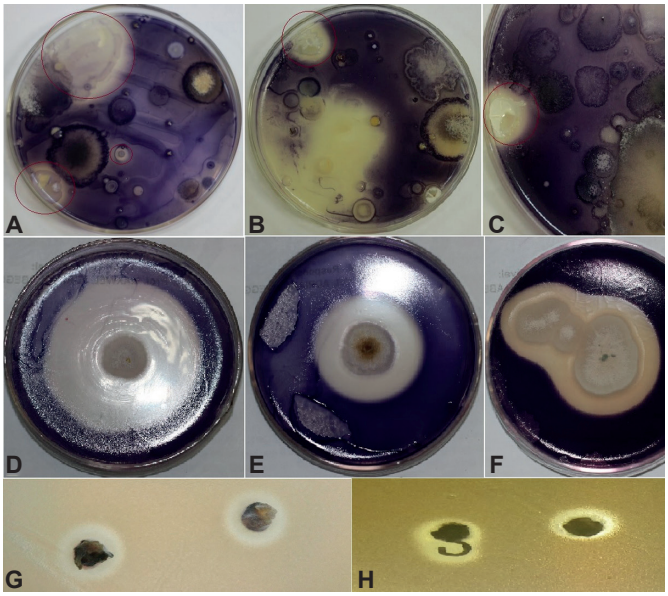

FIGURE 2: Plates illustrating the results of direct tests with the rapid method of airborne microorganisms against *Chromobacterium violaceum* applied by spraying. The red circles demonstrate colonies with inhibitory activity in A, B, and C. D, E, and F, demonstrate the inhibitory activity against *C. violaceum* by microorganisms isolated from colonies from the rapid method plates. In G, a halo of inhibition against *C. albicans* and in H, a halo of inhibition against *C. parapsilosis* is observed in the plug diffusion test.

30 mL of 2% malt extract broth. The fungi were then grown at ambient temperature for three days in a shaker at 150 rpm. The culture was mixed with 15 mL of absolute ethanol and mixed for one day. The extracts were dried in aseptic conditions and resuspended to a final concentration of 10 mg/mL with sterile distilled water. The extracts were then tested against *C. violaceum* using the agar well diffusion test applying 50 µL of extract to each hole[12]. The tests were performed in triplicate with sterile distilled water as the negative control (**Table 1**).

The tests of antagonism for the fungi and yeasts against *Candida albicans* ATCC 18804 and *Candida parapsilosis* ATCC 22019 were performed using the agar plug diffusion method. According to Balouiri et al.[12], this method is often used to highlight the antagonism between microorganisms. For this, the fungi and were previously grown on PDA plates on yeast extract-peptone-dextrose media (YPD), respectively, for three days and the bacteria were grown on LB media for 36 hours; 6 mm plugs were aseptically cut with a sterile cork borer and transferred to YPD plates previously inoculated by the pour plate technique along with 100 µL suspension of fresh *Candida* sp. cells at OD_{600} = 0.15. Measurements such as inhibition halos were performed after 24–48h of incubation at 30°C.

By opening three plates in twelve different locations, 51 microbial colonies that manifested antagonism against *C. violaceum* were obtained in the proposed rapid screening method. These colonies were isolated in pure culture and retested against the same strain, and 39 cultures were found to manifest halos of inhibition whereas 12 did not show any inhibition (**Figure 2**), resulting in false positives. Strains 7.3 (*Aspergillus* sp.) and 7.4 (also an *Aspergillus* strain) produced the largest halos of inhibition. Of the 39, the halos were turbid in five cases, and showed the presence of microorganisms when observed through the microscope, suggesting an anti-QS activity at first.

In the case of 13 isolates of the 39, which were identified as filamentous fungi, fermentation was carried out as explained

TABLE 1: Inhibitory activity of anemophilous fungi isolated by a rapid screening method against *Candida* sp. and *Chromobacterium violaceum*.

Strain Number/Fungal Genus or Yeast	Site of Isolation	Activity on *Candida albicans**	Activity on *Candida parapsilosis*	Activity of extracts/ Media≠
1.1Unidentified fungi	Private Land	-	-	-
2.1Unidentified fungi	Canaçari District 1	N.T.	N.T.	-
2.4 *Paecilomyces* sp.	Canaçari District 1	-	-	-
2.5 Unidentified fungi	Canaçari District 1	+	+	+/2% Malt
5.3 Unidentified fungi	University Restaurant	-	-	+/2% Malt
5.5 *Aspergillus* sp.	University Restaurant	-	-	+/2% Malt; Czapeck
5.8 *Curvularia* sp.	University Restaurant	+	+	+/2% Malt; Czapeck
6.1 *Aspergillus* sp.	University Campus	-	+	+/2% Malt; Czapeck
7.1 *Aspergillus* sp.	Autoclave Room	-	-	+/2% Malt; Czapeck
7.3 *Aspergillus* sp.	Autoclave Room	-	-	-
7.4 *Aspergillus* sp.	Autoclave Room	+	+	+/2% Malt
8.2 *Fusarium* sp.	Cleaning Room	-	-	-
8.4 *Penicillium* sp.	Cleaning Room	-	-	+/2% Malt; Czapeck
10.3 Yeast	Campus Trees	-	-	N.T.
11.4 Yeast	Municipal Dump	+	+	N.T.

Legend: *activity determined by a plug diffusion assay (see methods); ≠activity against *Chromobacterium violaceum* ATCC 12472 determined by a hole test in LB agar (see methods). **N.T.:** not tested (the fungal colonies did not develop sufficiently to perform the test).

above. For eight filamentous fungal strains, the extract of at least one medium was active against *C. violaceum* (**Table 1**). It was observed that in most cases, strains with active extracts in one of the media also showed activity in the other media tested. Despite the apparent conidiation of the fungi in PDA by means of the reference used[11], we could not attribute four fungi to any given genus with an acceptable level of confidence (**Table 1**).

In the plug diffusion test against *Candida* sp., among 13 fungi strains and two yeasts, four (33.3%) were active against *C. albicans*, and five (41.7%) against *C. parapsilosis* by exhibiting a clear halo of inhibition (**Figure 2**). Interestingly, in most cases, strains active against *C. albicans* were also active against *C. parapsilosis*. Of the 24 bacterial strains isolated with activity against *C. violaceum,* 13 strains (3.1, 4.4, 5.6, 5.7, 9.2, 10.1, 10.2, 10.5, 11.2, 12.1, 12.3, 12.4, and 12.5) (54.2 %) showed activity against *C. albicans* and seven strains (5.6, 9.2, 10.1, 10.4, 12.1, 12.2, and 12.4) (29.2 %) showed activity against *C. parapsilosis* on the agar plug diffusion test.

Although strains active against *C. violaceum* could be further tested against other bacteria, we chose to examine their activity in altering *Candida* growth considering the importance of *Candida* resistance[4], and a previous observation that a QS molecule (3-oxo-C12 homoserine lactone) could inhibit *C. albicans* filamentation[13].

There is some evidence in literature that activity against *C. violaceum* (a gram-negative species) can extend to other bacterial species, including gram-negative species, as shown by carvacrol[14] and other compounds[9]. We recognize that in future research, active strains and extracts must be further screened against additional bacterial species and a panel of ESKAPE species[3] and of pathogenic *Candida* species[4].

The apparent QS inhibitory activity of five strains that manifested turbid halos in the plate test employing *C. violaceum* ATCC 12472, can only be considered as a preliminary result, as colorless transparent halos can be difficult to discern from QS inhibition, which forms a turbid colorless halo[7]. In this case, strain extracts have to be further tested in a confirmatory violacein inhibition assay[7].

In QS inhibitory assays, an important limitation is that the QS-regulated phenotypes are often co-dependent on other factors and/or dependent on the metabolic activity of cells. Therefore, evidence of QS disintegration is not always strong and many compounds reported to inhibit QS have proven to be ineffective when studied in greater depth. To avoid this, the impact of the candidate on additional QS regulated phenotypes (mostly QS-regulated virulence factors) must be verified[6]. Additionally, it cannot be excluded that the QS inhibitory activity was due to enzymatic activity, as we did not treat the extracts with proteinase K. Burt et al.[14] pointed out that, carvacrol and many other phytochemicals initially target the bacterial membrane. At minimum inhibitory concentration (MIC) values, complete leakage and loss of membrane potential is observed. It is possible that lower concentrations of these compounds could have smaller destabilization effects on these membranes, which could reduce the QS capability of bacteria[14].

The process of discovering antibiotics usually includes several steps: (1) isolation of microorganisms (usually 2–3 days), (2) pure culture (3–5 days), (3) identification of isolated microorganisms to eliminate duplicate strains (14–21 days), (4) testing whether or not the culture broths or extracts of these microorganisms show antimicrobial activity (7–8 days) and (5) re-culture of candidate microorganisms to observe the reproducibility of antimicrobial activity (6–8 days). This traditional method usually takes 5–7 weeks[15]. The rapid method proposed by Kawagushi et al.[1] can probably accelerate the discovery of antibiotics, considering that prior isolation of microorganisms and a screening test for each isolated strain is unnecessary.

This study also indicates that it is possible to reduce the conventional procedure that takes five to seven weeks to a shorter time, with more hits considering the extracts obtained. Although there are quite a few methods for the *in vitro* evaluation of antimicrobial activity as recently reviewed by Balouiri et al.[12], in the adaptation proposed here, the high contrast brought by the violet pigment produced by *C. violaceum* seems facilitate identification of halos of inhibition in direct plate methods, and renders prior isolation of microorganisms unnecessary.

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Conflicts of interest: The authors declare that there are no conflicts of interest.

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