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Short Communication

In vitro Arthrographis kalrae biofilm formation: Scanning electron microscopy and cytotoxic analysis

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Abstract To our knowledge, this study represents the first demonstration of *Arthrographis kalrae* biofilm formation *in vitro* by scanning electron microscopy and the distinct cytotoxic activity between planktonic and biofilm extracts on RAW 264.7 cell line. Higher activity was observed with biofilm. It could impact host immune response, that require furthers study.
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Introduction

Arthrographis kalrae is a thermally dimorphic fungus that is considered a rare and opportunistic human pathogen. However, infections have been reported in both immunodeficient and immunocompetent individuals.[1,2] Persistent *A. kalrae* infections cases have been reported, such as cases of keratitis,[3] knee joint infection,[4] meningitis and also a case of fatal stroke and cerebral vasculitis due to *A. kalrae*.[1,2] De

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Diego Candela et al. (2010) reported a case of endocarditis in which the patient underwent several surgical procedures due to the recurrence of *A. kalrae* infection and died during surgery.[5] As the patient used a mechanical prosthesis, it was suggested that *A. kalrae* biofilm could have been the cause of the severity and recurrence of the infection.

Biofilm is a microbial community associated with a surface that is capable of producing its own extracellular matrix (ECM), has a distinct phenotype from its cells in planktonic forms and is a relevant virulence factor of microorganisms.[6] Biofilm formation impacts not only antifungal drug resistance, but also host immune response. According to Kernien et al. (2018), biofilms can protect fungal pathogens from the innate immune system, by suppressing, killing or by modulating the monocyte/macrophage cytokine profile.[7] As it is widely known that the innate response modulates adaptive response, biofilm also impacts the host adaptive immune response.

To our knowledge, there are no data regarding *A. kalrae* biofilm in the literature. Therefore, the current work investigated the biofilm formation by *A. kalrae* and its cytotoxic activity.

Methods

Microorganism and cell line

The fungus *A. kalrae* IFM55165 (provided by the Research Center for Pathogenic Fungi and Microbial Toxicosis at the University of Chiba, Japan) was maintained at 36 °C in a 4% Sabouraud dextrose agar culture medium, with peaks every five days. The RAW 264.7 cells (murine macrophage cell line) were obtained from Dr. Fábio Henrique Kwasniewski, State University of Londrina, Londrina, Paraná, Brazil.

Biofilm assay

A. kalrae yeast-like (1×10^8 cells/mL) in RPMI 1640 supplemented with 2% glucose and 10% fetal bovine serum (FBS) were incubated for 4 (pre-adhesion), 12, 24, 36 and 48 h in 96-well polystyrene microplates at 37 °C and 5% CO_2. For total biofilm biomass determination, the supernatant was removed, the biofilm was washed and fixed. After drying, the crystal violet solution (0.1%) was added and removed after 15 min. After washing, acetic acid (33%) was added to release the dye and the solution transferred to another microplate and read in a spectrophotometer (Multiskan EX, Uniscience-Labsystems, Helsinki, Finland). The results are expressed in optical density (O. D.) at 550 nm. Two independent experiments were carried out under the same conditions.

Scanning electron microscopy (SEM)

For the analysis of *A. kalrae* biofilm, the biofilm assay was performed as described above on 10×10 mm polystyrene pieces deposited in 24-well plates. Biofilms of 48 h of growth were analyzed. After these incubation period, the RPMI was removed, the wells were washed twice with phosphate-buffered saline (PBS) and the biofilm was left to dry at 25 °C. Next, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) was added to the wells at 25 °C for 2 h to fix the biofilm. After this period, the biofilm was washed with 0.1 M sodium cacodylate buffer and dehydrated in increasing concentrations of ethanol (30, 50, 70, 80, 90, and $3 \times 100\%$). The samples were then dried using the hexamethyldisilazane reagent and metallized with gold. Visualization was performed in the FEI SCIOS high resolution scanning electron microscope.

Total extract of *A. kalrae*

To obtain total extract (TE), *A. kalrae* yeast-like grown on Sabouraud dextrose agar 4% at 36 °C and cultured biofilm cells (according to the biofilm assay described above) were collected and suspended in distilled water with 2.5 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor). Next, the cells were macerated in a mortar with acetone and liquid nitrogen and the suspension was centrifuged at $1512\times g$ for 10 min at 25 °C. The supernatant was centrifuged at $16{,}128\times g$ at 4 °C for 10 min and the resulting supernatant (total extract) was aliquoted and its protein concentration determined in a NanoDrop Lite UV–Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Aliquots were stored at −80 °C until use.

Cytotoxicity assay

RAW 264.7 cell line (1×10^6 cells/mL) was seeded on 96-well plates, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, in a final volume of 100 μL, and incubated at 37 °C and 5% CO_2 for 24 h. After this period, the cells were washed and incubated with TE from planktonic and biofilm cells of *A. kalrae* (100, 400, and 800 μg/mL) at 37 °C and 5% CO_2 for 72 h. The cytotoxic effect was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with a Promega kit (Cell Titer 96®, Non-Radioactive, Cell Proliferation Assay) according to the manufacturer's instructions.

Statistical analysis

In the analysis of total biofilm biomass, the Kolmogorov–Smirnov test was used, followed by one-way ANOVA with Tukey's test for multiple comparisons between the groups evaluated. In the cytotoxicity assay, the Shapiro–Wilk normality test was used, followed by two-way ANOVA with Tukey's test for multiple comparisons between the groups evaluated. A p-value <0.05 was assumed to be significant. The analyses were performed using GraphPad Prism 6.01 software (GraphPad Software, San Diego, California, USA).

Results

A. kalrae total biofilm biomass and SEM

The results of total biomass with crystal violet solution demonstrated a progressive increase in *A. kalrae* biomass during 12, 24, 36, and 48 h of incubation (Fig. 1A), with

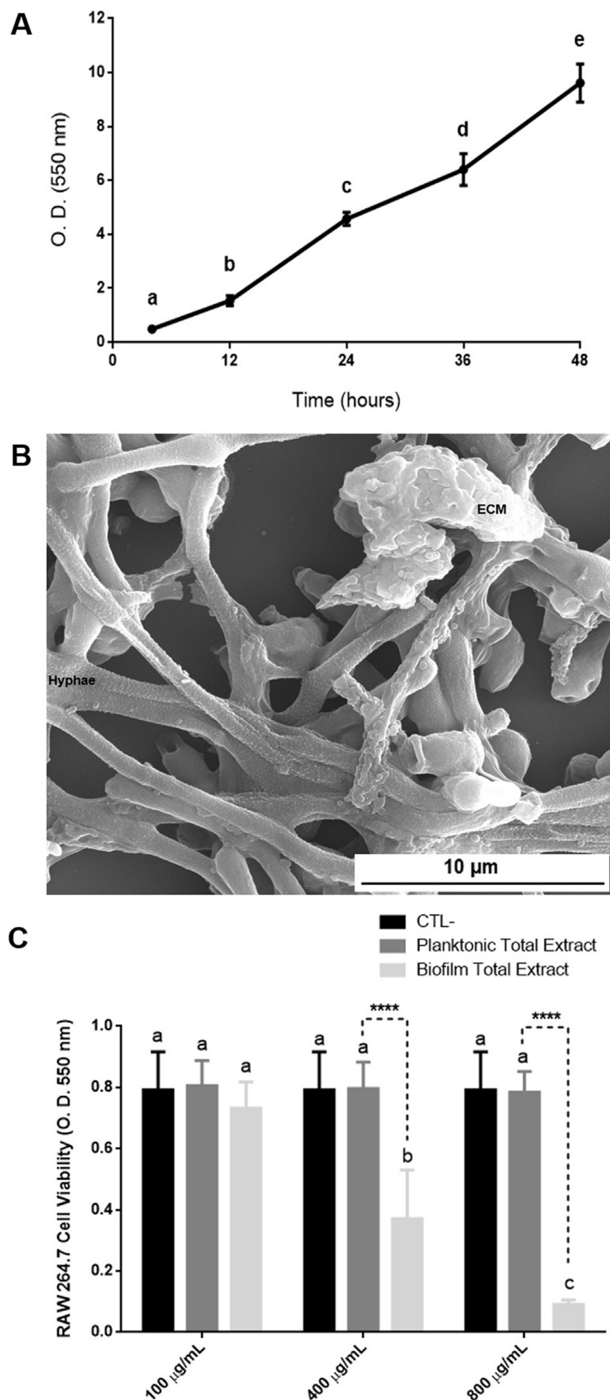

Fig. 1. **A.** Kinetics of biofilm formation by *A. kalrae* on polystyrene microplates determined by crystal violet staining. The values expressed in O. D. represent the average of two independent experiments. The letters indicate statistical difference between all analyzed times (a x b x c x d x e = $p < 0.0001$). **B.** Scanning electron microscopy of the *A. kalrae* biofilm grown for 48 h. ECM = extracellular matrix. Magnification: 10,000 x. **C.** Viability of RAW 264.7 cells treated with total extract of *A. kalrae* planktonic and biofilm cells. The letters indicate statistical difference in relation to the control and the asterisks indicate statistical difference between the groups ($p < 0.0001$).

statistical difference between all analyzed times ($p < 0.05$). The results of scanning electron microscopy demonstrated the presence of an ECM, a typical biofilm structure, with aspect of strong cohesion between hyphae (Fig. 1B), suggest the presence of mature biofilms on 48 h.

Cytotoxicity of planktonic and biofilm total extract on RAW 264.7 cells

A progressive decrease in cell viability of RAW 264.7 cells with increased concentration of TE from biofilm was observed. This decrease was statistically different from control without extract ($p < 0.05$). No cytotoxic activity was observed with extract from planktonic cells ($p > 0.05$) (Fig. 1C).

Discussion and conclusion

The presence of ECM, a typical biofilm structure, detected by scanning electron microscopy on polystyrene, suggests the ability of *A. kalrae* to form biofilm. The increase in total biomass in 24—48 h of culture and strong aggregation of fungi with a dense ECM area at 48 h, suggest mature biofilm production by *A. kalrae* in the short-term. This short-term, could also be due to the presence of serum in *A. kalrae* biofilm culture. According to Frade and Arthington-Skaggs (2011), the presence of serum accelerates the *Candida albicans* biofilm formation process.[8] The *in vitro* mature biofilm formation in 48 h is in accordance with other fungi such as *C. albicans* and *Cryptococcus neoformans*.[9,10] However, the aspect of ECM observed with *A. kalrae* was similar to ECM from *Aspergillus fumigatus* biofilms, and different to *C. albicans* ECM.[6]

Biofilm formation impacts not only antifungal drug resistance, but also host immune response. According to Kernien et al. (2018), biofilms can protect fungal pathogens from the innate immune system, by suppressing neutrophils and monocytes killing or by modulating the monocyte/macrophage cytokine profile.[7] As it is widely known that the innate response modulates adaptive response, biofilm also impacts the host adaptive immune response.

In previous works, it was evidenced that *A. kalrae* in their planktonic form present cytotoxic activity to mouse myeloma cell line (P3U1).[11] In this work, it was evidenced that the murine macrophage cell line (RAW 264.7) is also susceptible to *A. kalrae* cytotoxicity, but only with extract from biofilm and not with planktonic form. This result suggests that the formation of biofilm by the fungus *A. kalrae* can also negatively impact innate immunity, in agreement with Kernien et al. (2018).[7]

As in the case of endocarditis that *A. kalrae* biofilm formation may have been the possible cause of the infection's severity and recurrence reported by De Diego Candela et al. (2010),[5] other cases such as keratitis and knee joint injuries caused by *A. kalrae*, that was refractory to antifungal therapy and resolved by arthroplasty,[3,4] points to possible participation of biofilm.

The current work demonstrates for the first time that *A. kalrae* presents the capacity to form biofilm *in vitro* and that the biofilm product present higher cytotoxic

activity to murine macrophage cell line, suggesting expression of this factor when in the biofilm form. Possibly, *A. kalrae* forms biofilm *in vivo* and implies in persistent of infection by modulating innate immune response and inducing higher resistance to antifungal agents, that requires future studies.

Declaration of competing interest

The authors declare no conflict of interest.

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