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Introduction. Extracellular vesicles (EVs) are a powerful communication tool in many biological systems and in the last years they have been characterized in several fungi, where they contribute in the transport of molecules across their complex cell wall. In pathogenic species, these vesicles can carry molecules which exert an active role in virulence, like polysaccharides and proteins and RNA. Objective of our study is a first characterization of extracellular vesicles in the model black yeast *Exophiala dermatitidis*, a poly-extremophilic species and an emerging fungal pathogen in Europe. The fungus indeed, although mostly involved in cutaneous and subcutaneous mycosis and deep neurotropic infections in Asia, is more and more present in clinical reports in the West, especially in those regarding cystic fibrosis patients. We present here a study of the vesicles of *E. dermatitidis* (CBS 525.76), isolated in Japan from a male patient with chromoblastomycosis.

Methods. The fungus has been grown in liquid culture of 2% MEA for one week at 37°C. The vesicles have been then isolated with the ultracentrifugation method according to da Silva et al. [1] with some modifications. The culture was first centrifuged to remove the biomass, the supernatant was sterile filtrated centrifuged to remove cellular debris and finally ultra-centrifuged to obtain the vesicular fraction. The EVs have been analyzed by mean of transmission electron microscopy (TEM). Moreover, RNA and proteins have been isolated with the kit Total Exosome RNA & Protein Isolation Kit (ThermoFisher) and the RNA sequenced with the Ion Torrent technology on PGM. Proteins have been identified following LC-ESI-MS analysis of peptides. To test the vesicles for their virulence effect, we injected them into the larvae of the model host *Galleria mellonella* (honeycomb moth) which after 24h were infected with 1×10^7 yeast cells/larva. The experiment lasted 7 days at the temperature of 30°C.

TEM analysis and protein cargo of EVs. TEM analysis reveals that the vesicles of *E. dermatitidis* have a quite homogenous size, ranging between 30 and 80 nm (Figure 1) and that they strongly pigmented, with melanin mainly present on the outer membrane. This was confirmed by the results from the Nanoparticle Tracking analyzer (Zetaview®) (data not shown).

The identified vesicular proteins mainly belong to the following categories:

- Cell wall proteins [Endo-glucanase; GPI anchored cell wall beta];
- Oxidoreductases [aryl-alcohol and choline dehydrogenase]
- Fungal virulence factor [laccase, 3-phytase, aspergillopepsin I];
- Uncharacterized protein.

Fig.1 *E. dermatitidis* EVs visualized by TEM.

Fig.2 Left: Total RNA and EVs-RNA pattern. Right: PGM Sequencer.

Virulence tests with *G. mellonella*. The injection of *E. dermatitidis* EVs into the host resulted in a rapid killing of the larvae within 24h, while the fungus infection led to a total larvae mortality in longer time (Fig. 3).

The injection of *E. dermatitidis* EVs into the larvae of *G. mellonella* before infection with the yeast itself decreased the host survival (data not shown), suggesting that, differently from what observed in *C. albicans*, the EVs of *E. dermatitidis* do not trigger a protective host response.

Fig.3 Left: *G. mellonella* injection with EVs. Right: Kaplan-Meier curve of survival.

In conclusion, this first characterization of *E. dermatitidis* extracellular vesicles opens new perspectives to the comprehension of the pathobiology of the black yeasts. The molecular cargo of the vesicles gives a hint of their role in fungal virulence and it is currently investigated in human fibroblast and keratinocytes. Moreover, the vesicles seems to have a role in fungal virulence in the model host *G. mellonella*, as already observed in *C. albicans* and further experiment on the dose-dependent effect are currently being performed. The function of EVs needs to be further investigated in other experimental models, such as human cell lines that mimic pathogenic scenarios of the fungus.

[1] da Silva, R.P. et al. Extracellular vesicle-mediated export of fungal RNA. Sci. Rep. 5, 7763; DOI:10.1038/srep07763 (2015).

[2] Liu et al.: Identification and characterisation of non- coding small RNAs in the pathogenic filamentous fungus *Trichophyton rubrum*. BMC Genomics 2013 14:931

[3] Poyntner C, Blasi B, Arcalis E, Mirastschijski U, Sterflinger K, Tafer H (2016) The Transcriptome of *Exophiala dermatitidis* during Ex-vivo Skin Model Infection. Front. Cell. Infect. Microbiol. 6:136. doi: 10.3389/fcimb.2016.00136