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Extracellular vesicles of *Mucor lusitanicus* reveal virulence-associated protein cargo

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ABSTRACT

In this study, we report the first proteomic characterization of extracellular vesicles (EVs) in a Mucorales species. EV cargo of *Mucor lusitanicus* contains multiple virulence-associated and biomarker-relevant proteins, supporting a potential role for EVs in host-pathogen interaction and providing a basis for future diagnostic and functional studies.

1. Introduction

Mucormycosis is a life-threating fungal infection caused by members of the order Mucorales, with increasing incidence and limited diagnostic options [1]. WHO classified Mucorales fungi as high-priority group pathogens in 2022, highlighting the urgent need for improved research, diagnostics, and public health intervention [2].

Mucor lusitanicus (formerly *Mucor circinelloides* f. *lusitanicus*), a frequently used model organism, has been involved in several studies addressing the role of RNA interference in fungal cells, the mechanisms and role of morphological dimorphism, the production of enzymes, carotenoids and other metabolites, and the genetic and molecular background of pathogenicity in Mucorales fungi [3]. *Mucor lusitanicus* is clinically relevant Mucorales species that can cause opportunistic mucormycosis, particularly in severely immunocompromised or metabolically dysregulated patients, and represents a secondary etiologic agent of mucormycosis after *Rhizopus* spp [1].

Extracellular vesicles (EVs) are key mediators of intercellular communication and have been implicated in fungal virulence through the transport of bioactive molecules, including proteins involved in host interaction and immune modulation [4]. EV proteomes have been described in several fungal pathogens such as *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus flavus*; however, no such data are currently available for Mucorales [4,5]

Here, we provide the first proteomic characterization of EVs in *Mucor lusitanicus*, with a focus on identifying proteins associated with virulence and potential diagnostic relevance.

2. Methods

2.1. Strains and growth conditions

The wild-type *Mucor lusitanicus* strain CBS277.49 was used as the reference strain [3]. The MS12 strain is a double auxotrophic derivative of CBS277.49, carrying the leuA$^-$ and pyrG$^-$ mutations, which confer leucine and uracil auxotrophy, respectively [6]. The MS12+*pyrG* strain is an MS12 derivative in which the uracil auxotrophy was complemented by the introduction of a functional *pyrG* gene [3]. Strains were plated onto a solid minimal medium (YNB; 10 g glucose, 0.5 g yeast nitrogen base without amino acids [Sigma Aldrich, St. Louis, MO, USA],

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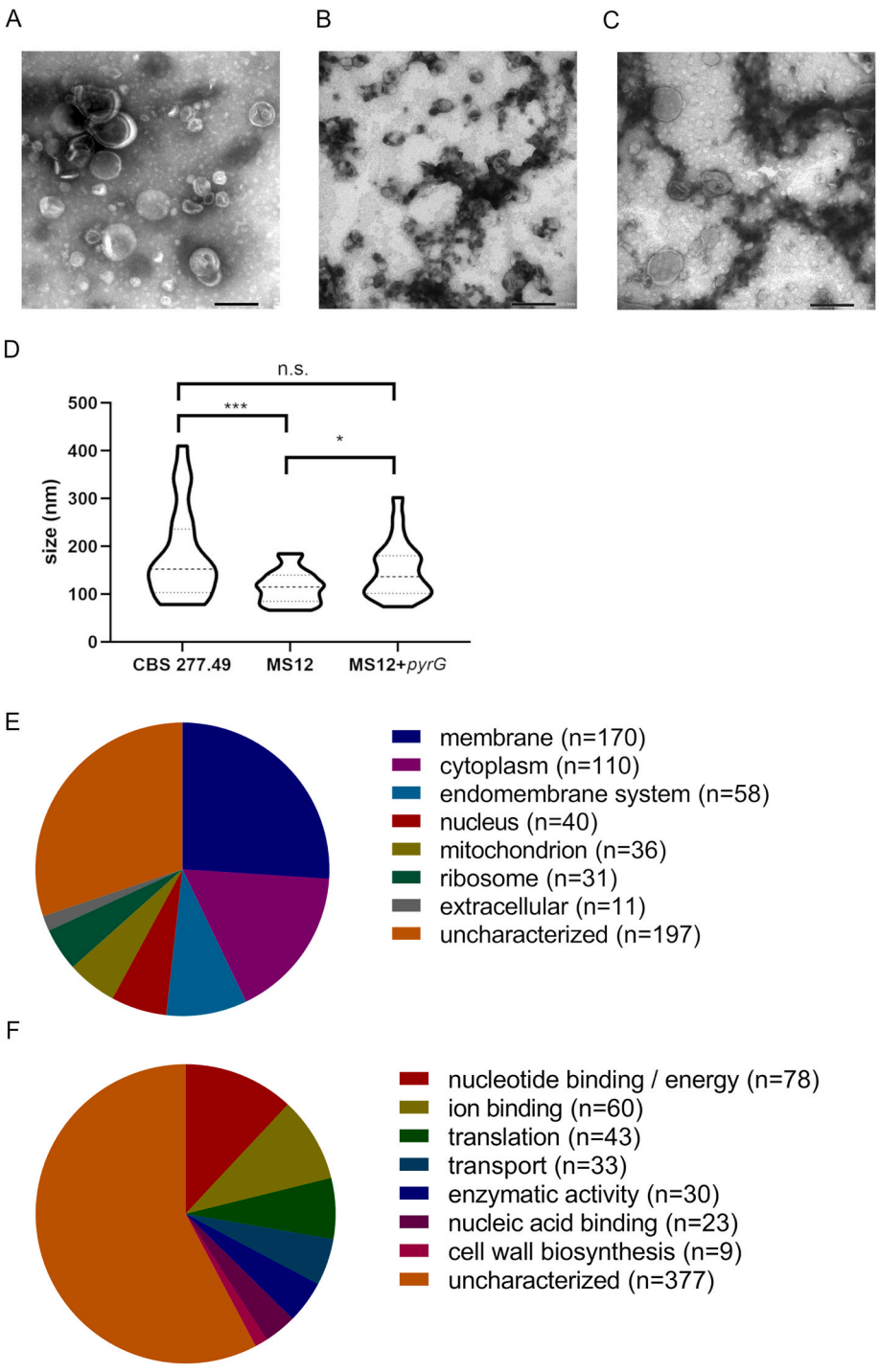

Fig. 1. TEM of EVs secreted by *M. lusitanucus* (CBS 277.49) **(A)** MS12 **(B),** and MS12+*pyrG* **(C)** EVs. Scalebar: 200 nm. Violin plots show the size distributions of isolated extracellular vesicles (n = 200) **(D)** The identified proteins (n = 653) were classified based on Gene Ontology (GO) annotations, and redundant terms were consolidated into broader categories according to cellular component **(E)** and molecular function **(F).**

1.5 g $(NH_4)_2SO_4$, 1.5 g sodium glutamate, 0.5 g uracil and/or leucin if necessary and 20 g agar per liter) incubated for four days at 25 °C.

2.2. Isolation of EVs

To purify EVs, fungal spores (100 µl of 10^6 spores/ml) were plated onto cellophane-coated YNB solid minimal medium. Fungal cultures grew for four days at 25 °C. After four days, the cellophane was washed in 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter, pH 7.2-7.4). The PBS solution was centrifuged at 3380 x g for 20 min at 4 °C. The supernatant was centrifuged at 10,000 x g for 20 min at 4 °C. After 20 min, the supernatant was filtered through a 0.45 µm filter (Millipore Burlington, MA, USA) and ultracentrifuged at 100,000 x g at 4 °C for 1 h. The pellet was washed with cold 1X PBS and ultracentrifuged again at 100,000 x g for 1 h at 4 °C. The pellet was resuspended in 100 µl 1X PBS and stored at 4 °C.

2.3. Transmission electron microscope (TEM) analysis of EVs

Purified EVs were analyzed with a JEM-1400 Flash TEM (JEOL,

Tokyo, Japan) for morphological characterization. To obtain the negatively stained samples, 10 µl of the EV extract was mounted on a 150-mesh formvar-coated copper grid (Electron Microscopy Sciences, Hatfield, PA, USA). After 1 min, the excessive fluid was blotted away with the edge of a filter paper. Subsequently, the samples were contrasted with 10 µl 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA) in 50% ethanol for 2 min (2 times). After the removal of the excessive staining solution, samples were dried under a Petri dish for at least 2 h before the TEM evaluation. Negatively stained samples were recorded at 30,000–80 000× magnification with a 16 MP Matataki Flash camera (JEOL, Tokyo, Japan). Average of the longest diameter and the longest perpendicular to the first diameter was calculated based on the line measurements of the built-in tool of the TEM.

2.4. Protein content analysis of isolated EVs

The total protein content of the sample was determined using the BCA kit (Sigma Aldrich, St. Louis, MO, USA), and a sample portion containing 10 µg protein was prepared by centrifugal filter-assisted digestion protocol (FASP) on an Ultracel 30 kDa centrifugal filter. Briefly, the samples were denatured using sodium dodecyl sulphate (SDS, 2 m/V %) and dithiothreitol (DTT, 20 mM) and alkylated using iodoacetamide (IAA, 50 mM). Digestion was performed with 250 ng trypsin to which 1 w/v% sodium deoxycholate was added. After 18 h of digestion, the sodium deoxycholate was precipitated, the supernatant was removed by evaporation, then the precipitate was redissolved in the initial eluent, and 2 µl was injected into the LC-MS system.

A Thermo Orbitrap Exporis™ 240 mass spectrometer coupled to a Waters ACQUITY UPLC M-Class liquid chromatograph was used for the measurements. Water containing 0.1% formic acid and acetonitrile were used as eluents. The peptides were separated in an 85 min nanoflow liquid chromatography gradient and analyzed in data-dependent (DDA) data acquisition mode. Fragpipe software was used to identify the peptides and determine their relative abundance.

Functional classification of the identified proteins was performed using Gene Ontology annotations retrieved from UniProtKB/TrEMBL (https://www.uniprot.org/) for the corresponding protein accessions. The annotation table was generated in December 2025, corresponding to UniProtKB release 2025_04 and Gene Ontology release 2025-10-10. GO terms were categorized into biological process, cellular component, and molecular function classes. Redundant GO terms were grouped into higher-level functional categories. Proteins lacking GO annotation were classified as "uncharacterized". Percentages were calculated relative to the total number of identified proteins.

3. Results

3.1. The size and protein content of EVs produced by M. lusitanicus

TEM confirmed the presence of EVs with typical spherical morphology and heterogeneous size distribution in *M. lusitanicus* (Fig. 1A–C). Quantitative analysis (Fig. 1D) reveals a significant reduction in EV size (average 128 nm) in the uracil auxotrophic mutant strain (MS12) compared to the wild type (average 144 nm), while complementation partially restores the phenotype (average 146 nm).

Proteomic analysis of EV, produced by the wild type *M. lusitanicus* CBS 277.49 strain, identified a total of 653 proteins (Supplementary Table 1), representing the first EV proteome described in a Mucorales species. Among the identified proteins, membrane-associated (n = 170; 26%) and cytoplasmic (n = 110; 16.8%) components were predominant (Fig. 1E). Functionally, proteins involved in nucleotide binding (n = 78; 11.9%) and ion binding (n = 60; 9.2%) were highly represented.

EV cargo included multiple classes of proteins previously associated with fungal virulence. These encompassed enzymes involved in cell wall remodeling (e.g., chitin-related enzymes and enzymes involved in glucan biosynthesis), secreted proteases (e.g., aspartic proteases/rhizopuspepsins, secreted subtilisin-like serine proteases), and oxidative stress-related proteins (multicopper oxidases, heat shock proteins, catalase, superoxide dismutase). We also identified proteins that may play a role in determining EV content and may regulate their transport (ATP-grasp domain-containing protein, VPS10 domain-containing protein) [5]. Such factors are known to contribute to host tissue invasion (e.g., adhesin-domain protein), immune evasion, and survival under host-induced stress conditions. In addition, several proteins with potential diagnostic relevance were identified, including adhesin-like proteins, heat shock proteins, cell surface and membrane-associated proteins, which may implicate Mucorales pathogenicity and host cell

Table 1
The list of identified proteins that may play a role in host-pathogen interactions and are potential biomarkers for mucormycosis.

potential effects	identified proteins	GO biological process
host pathogene interaction	aspartic proteases/ rhizopuspepsins (n = 8) chitin deacetylases (n = 7) lipases (n = 5) mannosyltransferases (n = 5) adhesin-domain protein (n = 4) GAPDH (n = 3) CotH-domain proteins (n = 3) secreted subtilisin-like serine proteases (n = 2) multicopper oxidase (n = 1)	proteolysis [GO:0006508] carbohydrate metabolic process [GO:0005975]; fungal-type cell wall biogenesis [GO:0009272] lipid metabolic process [GO:0006629] cell wall mannoprotein biosynthetic process [GO:0000032]; mannosylation [GO:0097502; protein N-linked glycosylation [GO:0006487] unassigned glucose metabolic process [GO:0006006]; glycolytic process [GO:0006096] unassigned proteolysis [GO:0006508] cellular response to iron ion starvation [GO:0010106]; reductive iron assimilation [GO:0033215]
potential biomarkers	aspartic proteases/ rhizopuspepsins (n = 8) mannosyltransferases (n = 5) adhesin-domain proteins (n = 4) GAPDH (n = 3) CotH-domain proteins (n = 3) heat shock proteins (n = 3) thioredoxin-dependent peroxiredoxin (n = 2)	proteolysis [GO:0006508] cell wall mannoprotein biosynthetic process [GO:0000032]; mannosylation [GO:0097502]; protein N-linked glycosylation [GO:0006487] unassigned glucose metabolic process [GO:0006006]; glycolytic process [GO:0006096] unassigned apoptotic mitochondrial changes [GO:0008637]; chaperone cofactor-dependent protein refolding [GO:0051085]; mitochondrial unfolded protein response [GO:0034514]; positive regulation of interferon-alpha production [GO:0032727]; positive regulation of interleukin-6 production [GO:0032755]; positive regulation of T cell activation [GO:0050870]; positive regulation of type II interferon production [GO:0032729]; protein import into mitochondrial intermembrane space [GO:0045041]; protein refolding [GO:0042026]; response to heat [GO:0009408] cell redox homeostasis [GO:0045454]; hydrogen peroxide catabolic process [GO:0042744]; removal of superoxide radicals [GO:0019430]

interaction. The detection of CotH-domain proteins in EVs suggests a potential role for extracellular vesicle-mediated delivery in Mucorales-specific virulence mechanisms [7].

These findings indicate that EVs of *M. lusitanicus* carry a complex protein cargo enriched in virulence-associated factors (Table 1).

4. Discussion

This study provides the first proteomic characterization of EVs in Mucorales. The enrichment of virulence-associated proteins in the EV cargo supports a role for these vesicles in host-pathogen interactions. The presence of cell-surface proteins, as well as adhesion-like proteins, in EVs suggests that extracellular vesicle-mediated transport may play a potential role in *Mucorales*-specific virulence mechanisms, and possibly facilitates tissue adhesion and dissemination via interactions with host cell proteins [7]. In addition, the identification of proteins involved in the virulence of other human pathogenic fungi (e.g., *Candida* sp, *Aspergillus* sp, *Cryptococcus* sp.) and their interaction with the host, such as adhesins, proteases, and cell wall-modifying enzymes, indicates that EVs may represent a source of candidate diagnostic biomarkers [8–10].

Overall, these findings extend current knowledge of fungal EV biology to Mucorales and establish a foundation for future functional and translational studies.

The production of smaller extracellular vesicles by the uracil auxotrophic strain (MS12$^{)}$ indicates that uracil auxotrophy may influence not only fungal growth and virulence, as suggested by our previous results, but also EV formation [3]. Given that the proteomic analysis identified several EV-associated proteins linked to fungal virulence, these alterations in EV size may have functional consequences and could indirectly affect the pathogenic potential and overall fitness of the fungus.

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CRediT authorship contribution statement

Sándor Kiss-Vetráb: Data curation, Investigation, Methodology, Writing – original draft. **Áron Péter:** Data curation, Investigation, Methodology. **Kitti Bauer:** Investigation, Methodology. **Bernadett Vágó:** Investigation, Methodology. **Naomi Varghese:** Investigation, Methodology. **Tamás Ferenc Polgár:** Data curation, Methodology, Software. **Zoltán Kele:** Data curation, Methodology. **Gábor Kecskeméti:** Data curation, Methodology. **Csaba Vágvölgyi:** Writing – review & editing. **Tamás Papp:** Funding acquisition, Writing – review & editing. **Gábor Nagy:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2026.08.002.

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