Nereis. Revista Iberoamericana Interdisciplinar de Métodos, Modelización y Simulación	12	99-108	Universidad Católica de Valencia San Vicente Mártir	Valencia (España)	ISSN 1888-8550

Isolation and characterization of extracellular vesicles in *Candida albicans*

Aislamiento y caracterización de las vesículas extracelulares en ***Candida albicans***

Fecha de recepción y aceptación: 21 de abril de 2020, 24 de abril de 2020

DOI: 10.46583/nereis_2020.12.611

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ABSTRACT

Background: The occurrence of systemic infections due to *C. albicans* has increased especially in critically ill patients. In fungal infections, secretory mechanisms are key events for disease establishment. Recent findings demonstrate that fungal organisms release many molecular components to the extracellular space in extracellular vesicles.
Aims: We develop a method to obtain exosomes from yeast cultures of the *Candida albicans*.
Methods: Yeast strains used in this work were *C. albicans* SC5314, *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258). Yeasts were grown at 37.º in liquid YPD medium. The cell cultures were centrifuged and the supernatant filtered through sterile nitrocellulose. Filtrates were concentrated and centrifuged using an ultracentrifuge. The sediment was analyzed by electron microscopy of transmission.
Results: The transmission of electron microscopy and nanoparticle tracking analysis confirmed the presence of extracellular vesicles (exosomes) of sizes between 100 and 200 nm and the absence of cellular contaminants. This was ratified by the characterization of proteins performed through the western blot technique, where the absence of cell contamination in the preparations was assessed.
Conclusions: The method proves to be highly effective due to the homogeneity and purity of the obtained microvesicles. The protocol developed in this paper proves to be effective for obtaining exosomes of other *Candida* species, which will allow future studies to determine its protein composition and the role that these vesicles can play.

KEYWORDS: *exosomes, extracellular vesicles*, Candida albicans, *tetraspanins*.

RESUMEN

Contexto: La aparición de infecciones sistémicas por *C. albicans* ha aumentado sobre todo en pacientes graves. En las infecciones fúngicas, los mecanismos de secreción son eventos clave para que el establecimiento de la enfermedad. Hallazgos recientes demuestran que los organismos fúngicos liberan muchos componentes moleculares al espacio extracelular en vesículas extracelulares.
Objetivos: Desarrollamos un método para obtener exosomas de cultivos de levadura de *Candida albicans*.
Métodos: Las cepas de levadura que se usaron en este trabajo son *C. albicans* SC5314, *C. parapsilosis* (ATCC 22019) y *C. krusei* (ATCC 6258). Las levaduras se cultivaron a 37.º C en un medio YPD líquido. Los cultivos de células fueron centrifugados y el sobrenadante, filtrado por medio de nitrocelulosa estéril. Los filtrados se concentraron y centrifugaron usando una ultracentrifugadora. El sedimento fue analizado por un microscopio electrónico de transmisión.
Resultados: La microscopía electrónica de transmisión y el análisis de nanopartículas confirman la presencia de vesículas extracelulares (exosomas) de un tamaño entre 100 y 200 nm, así como la ausencia de contaminantes celulares. Esto se ratificó mediante la caracterización de proteínas obtenidas por medio de la técnica de Western blot, donde se evaluó la ausencia de contaminación celular en las preparaciones.
Conclusiones: El método es altamente eficaz dada la homogeneidad y la pureza de las microvesículas obtenidas. El protocolo desarrollado en este artículo demuestra ser efectivo para obtener exosomas de otras especies *Candida*, lo que permitirá que en futuros estudios se determine su composición proteica y el papel que estas vesículas pueden desempeñar.

PALABRAS CLAVE: *exosomas, vesículas extracelulares*, Candida albicans, *tetraspaninas*.

INTRODUCTION

Candida albicans, a polymorphic fungus with yeast and mycelia as predominant morphological forms, is an opportunistic pathogenic; the increase of immunodeficiency patients of different etiologies has caused a significant rise in the incidence of systemic infection caused by this fungus. Although *C. albicans* is usually found in human microbiome, in healthy individuals *C. albicans* generally remains benign as commensal, although it can occasionally cause a variety of infections, ranging from superficial relatively mild infections of skin or mucosa, to potentially deadly systemic infections. The occurrence of systemic infections due to *C. albicans* has increased especially in critically ill patients as a consequence of increasing immune-deficiencies of different origins, such as AIDS, antitumor treatment or therapeutic immune suppression in transplant patients [1-9].

The potential of *C. albicans* to infect different host niches is sustained by various virulence factors such as morphological transition between yeast and hyphal forms, the expression of adhesins and invasins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes (proteases, phospholipases and lipases) [10]. In fungal infections, secretory mechanisms are key events for disease establishment [11-13] and virulence of *C. albicans* could be associated with some secretion mechanism. Recent findings show that fungal organisms release many molecular components to the extracellular space in extracellular vesicles (EV) [13-16].

Exosomes were discovered nearly 30 years ago, secreted by most cell types and being considered as simple garbage containers to transport non-desired components outside the cell. However, increasing evidence presented these containers as vehicles of information from cell to cell and tissues, altering the function and physiology of the receiver, and being implicated in tissue repair, neural communication or transfer of virulence factors in pathogenesis [17].

Eukaryotes protein secretion is driven by a signal peptide that directly secreted proteins through endoplasmic reticulum and Golgi with a concomitant protein glycosylation. However, the presence of non-glycosylated proteins lacking signal peptide outside the cell has remained unknown. Recently, lots of evidence demonstrated that cells produce vesicles that traverse the cell surface to release a wide range of cellular components into the extracellular space. Yeast cells also produce exosomes like vesicles that can play different roles in intercellular communication and are the vehicles for secretion of non-signal peptides and proteins [18].

Exosomes could play a role in infection by *C. albicans*, identify the components involved in the process, and it might help elucidate possible therapeutic targets and develop potential vaccines or immunotherapy against this infection. In this context, to know the molecules transported by *Candida* exosomes could bring important information about their potential role in *Candida spp.* infections. The main aim of this work was to develop a highly effective method for obtaining exosomes of *C. albicans* with a high degree of purity and free of cellular contamination and to show the possibility of extending the method to other *Candida* species.

MATERIALS AND METHODS

Strains, media and growth conditions

Yeast strains used in this work were *C. albicans* SC5314, *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258). Yeasts were grown at 37 ºC in liquid YPD medium (1 % of yeast extract, 2 % of peptone, and 2 % of glucose). Three flasks of one liter YPD medium were inoculated with 1 ml from previous 5 ml pre-culture inoculated with an isolated colony. Cultures were incubated in an orbital shaking (150 rpm) at 37 °C for 48 hours.

Obtaining yeast-free supernatant

Three liters of cell cultures were centrifuged at 4000xg for 15 min and supernatant filtered through sterile nitrocellulose, 0.45 µm pore size, membrane filters (Sartorius®).

Filtrates were concentrated by two hours of crossflow filtration using a Sartorius Vivaflow® 50R with an input stream of 200 to 400 mL/min and a pressure output of 2.5 bar, obtaining a final volume of 50 mL, which was subjected to a further filtration through pore size of 0.20 µm (Sartorius®) membrane sterile filters to ensure sterility and absence of other cellular elements but exosomes in the sample.

Ultracentrifugation

Concentrated filtered samples were centrifuged at 200000xg for 2 h at 4 °C, using an ultracentrifuge Beckman Coulter Optima® MAX. Sediment was suspended in PBS buffer (Phosphate Buffer Saline: Na_2HPO_4 and KH_2PO_4 0.01 M; 0.138 M NaCl; 0.0027 M KCl) pH 7.4, to a final volume of 1 mL. Aliquots of 100 µL were taken and kept frozen at -30 °C. One aliquot was processed immediately without freezing, adding 400 µL of Karnovsky solution (2.5 % paraformaldehyde and glutaraldehyde 0.5 % in PBS buffer), and processed by an electron microscopy of transmission.

Obtaining yeast cellular extracts

Yeast cells from 1 mL of YPD liquid culture were collected by centrifugation (8000 xg, 10 min). Then they were washed with sterile PBS buffer containing 1 mM of phenylmethylsulfonyl fluoride (PMSF), and re-suspended in 0.5 mL PBS-PMSF. After that they were dispensed in 2 mL tubes, the same volume of glass beads (425-600 µm of diameter, Sigma®) were added and subjected to four 20 s agitation cycles in a FastPrep FP120 cell disruptor. Cell extract was recovered, centrifuged at 8000xg for 10 minutes and divided in 100 µL aliquots and stored frozen.

Protein quantification

Quantification was carried out using the method of Bradford [19] and the reagent provided by Bio-Rad®.

Transmission electron microscopy (TEM)

Exosome pellets were fixed with 2.5 % of glutaraldehyde and washed with distilled water. After that, Exosomes were post fixed with 2 % of osmium tetroxide and dehydrated in increasing concentrations of ethanol. Embedding was performed with LR-White resin at 60 ºC for 24-48 h. Ultrathin sections were contrasted with 2 % of uranyl acetate and observed in a JEM 1010 TEM (JEOL) electron microscope at 60 kV.

Exosomes size determination

The size distribution of exosomes was measured using NanoSight LM10 (Malvern, Worcestershire, UK) and analyzed with the Nanoparticles Tracking Analysis software (Malvern) according to the manufacturer's protocol. Before each experiment, the machine was calibrated for nanoparticle size and quantity using standardized nanoparticle dilutions provided by the manufacturer.

Protein gel electrophoresis and Western blot techniques

SDS-PAGE electrophoresis was performed as described elsewhere [20] in 10 % (w/v) acrylamide gels loaded with 20 µg protein. The gels were stained with Coomassie brilliant blue or electro-blotted onto polyvinylidene difluoride (PVDF) membrane and immune detected with antibodies anti-Calnexin, anti-CD9, anti-CD63, anti-CD81, anti-Enolase, anti-Hsp70 (System Biosciences, with a dilution of 1:2500) and anti-candida cell wall (our group). Detection was carried out with peroxidase marked goat anti-rabbit antibodies (Sigma-Aldrich, with dilution 1:20000) by the enhanced chemiluminescent (ECL) method from Amersham Biosciences, following the manufacturer's instructions [21,22].

Treatment with Concanavalin A-ferritin

Each sample, with the sediment containing the exosome fraction, was resuspended in a solution composed of 0.6 M KCl, 0.5 M NaCl and 2 mM in $MnCl_2$, $MgCl_2$ and $CaCl_2$ (solution A), subsequently adding Concanavalin A-ferritin to a final concentration 0.5 mg/mL and incubating for 30 min at room temperature. Each sample was washed twice with solution A, using ultracentrifugation at 200000 xg for 70 min at 4 °C, both times. The sediment obtained was resuspended with PBS buffer pH 7.4 and fixed with formaldehyde/glutaraldehyde for inclusion in resins and subsequent processing for observation by transmission electron microscopy.

RESULTS AND DISCUSSION

Samples of exosomes obtained from *C. albicans* SC5314 were subjected to an analysis of determination of the total protein value, obtaining 0.166 µg/µL.

To check the purity of the sample a fraction of exosomes was processed by transmission electronic microscopy. Figure 1(A) shows two exosomes from *C. albicans* SC5314, in which there are vesicular structures with characteristic double membrane, sizes ranging from 100 to 200 nm. Figure 1(B) shows a preparation of exosomes not filtered through a 0.20 µm pore size, membrane filters, in which one can see exosome structures mixed with some yeast cells.

The images obtained by transmission electron microscopy have allowed us to validate the method of obtaining exosomes, ratifying the purity of the preparations with double membrane extracellular vesicles with sizes between 100 and 200 nm. Similar results were obtained with yeast cultures of *C. krusei* and *C. parapsilosis*, which proves the validity of this method to obtain samples of purified microvesicles without cellular contamination (figures 1C and 1D).

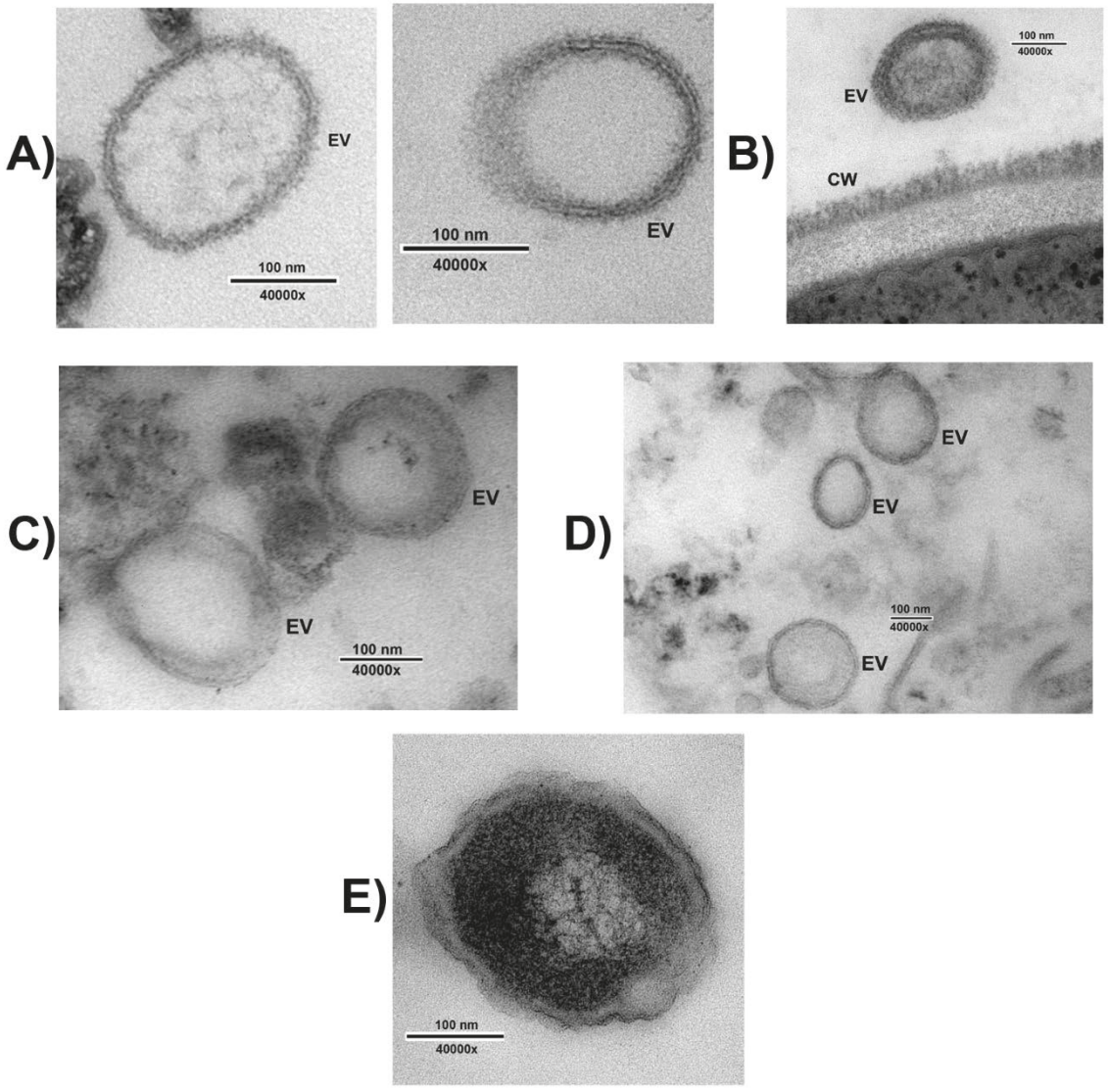

Fig. 1. Images obtained by transmission electron microscopy: (A) Extracellular vesicles of *C. albicans* SC5314 purified. (B) Exosomes together with yeasts in samples not filtered by 0.20 μm. (C) Extracellular vesicles of *C. krusei* and *C. parapsilosis* (D). (E) Purified exosomes of *C. albicans* SC5314 without treatment with Concanavalin A-ferritin.

C. albicans SC5314 exosomes' size and purity were evaluated in a Nano Team Sight®, by NTA. This technique allows us to determine the size and distribution of particles. The results highlight the high homogeneity of particle sizes. The highest concentrations of particles were those corresponding to the sizes of 125 nm and 165 nm, 146.1 nm being their average size. A presence of particles with size of 245 and 385 nm was observed, but they were not taken into account due to their low number. These results corroborate the efficacy of the method to obtain exosomes of high purity.

Exosome purity was evaluated by Western Blot (WB), using antibody against extracellular vesicles and markers of cytosolic fraction. Figure 2(A) shows the presence of vesicle markers CD9, CD63 and CD81 antigens epitopes in the fractions of exosomes, while reaction is negative in their respective cell lysates (L); on the other hand, cytoplasm marker calnexin was only detected in cell lysate. Moonlighting proteins were also identified in microvesicles, as one can see in Figure 2(B), enolase was only detected in microvesicles, while HSP70 was detected in both microvesicles and supernatant (SN) of the last ultracentifugation.

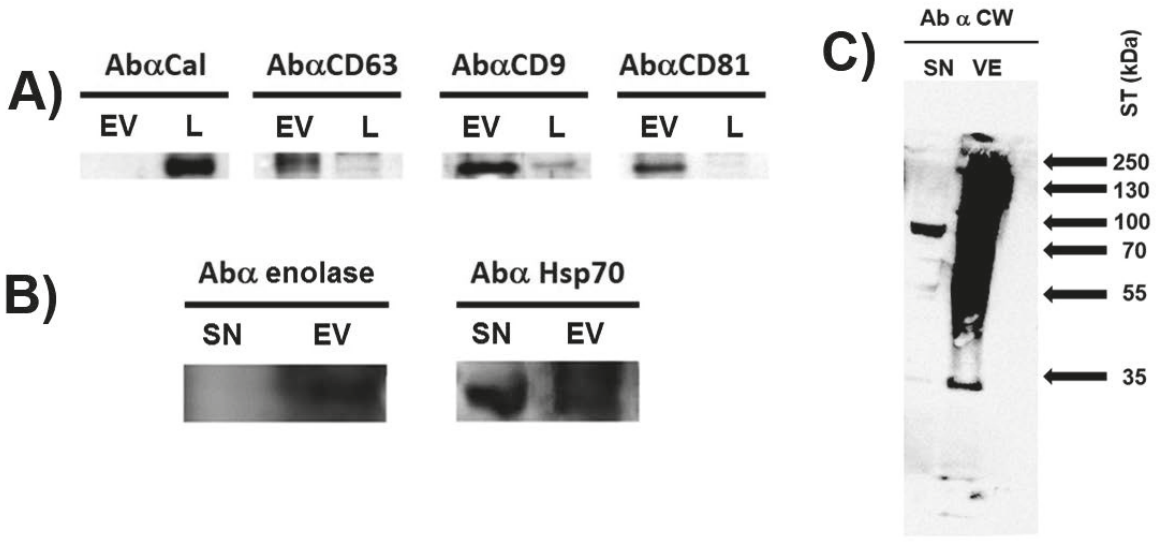

Fig. 2. Results corresponding to the Western blot performed in samples of exosomes of *C. albicans* using: A) Anti-Calnexin, anti-CD63, anti-CD9 and anti-CD81 antibodies in samples of EV and their corresponding (L). B) Anti-enolase, and anti-Hsp70 antibodies in samples of EV and their corresponding SN. C) Positive result with high polydispersity of the EV fraction versus the anti-Candida cell wall antibodies.

Tetraspanins such as CD9, CD63 and CD81, are considered to be biomarkers of extracellular vesicles by several authors [23-26]. The positive result in the EV fraction and their absence in the cell lysate ensures the purity of the obtained exosomes and the absence of cellular contaminants. Calnexin (a protein located in the endoplasmic reticulum) is not detected in EV sample, while it is clearly present in cell lysate fractions. This allows ratifying the results obtained with tetraspanins about the purity of the obtained exosomes and the absence of possible contamination with cellular fragments.

Purified exosomes were also tested for presence of mannoproteins; in order to achieve this we treated exosomes samples with Concanavalin A-ferritin. Transmission electron microscope images show black dotted spots inside the vesicles (Fig 1E), not observed in not treated samples (figure 1A), which indicates the presence of mannoproteins in the microvesicles and confirms the results obtained by WB of microvesicles proteins detected with anti-mannan (anti-Candida cell wall) antibodies (figure 2C).

The method developed introduces modifications to previous protocols to purify yeast microvesicles/exosomes which are free of pollution cytoplasmic contamination that might interfere with following studies to determine its protein composition and the role that these vesicles can play.

Transparency declarations

None to declare.

Financing

This work was partially supported by grant PI12/01797, MINECO integrated in the "Fondo Europeo de Desarrollo Regional (FEDER)", and cofinanced by Instituto de Salud Carlos III (ISCIII).

Acknowledgments

J. A. Castillo-Garit thanks the program "Estades Temporals per a Investigadors Convidats" for a fellowship to work at the University of Valencia in 2018.

Conflict of interest

The authors declare that they have no conflict of interest.

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