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Effect of Inoculum Size and Culture Age on the Cellular Properties and Host-Pathogen Interactions of *Cryptococcus neoformans*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PVCY and KKC designed the experiments. Author KKC performed all experiments and analysis, interpreted data, and wrote the manuscript. Author PVCY supervised development of work, assisted in data interpretation, manuscript preparation, and acted as corresponding author. Authors PPC and ASHH supervised development of work and assisted in manuscript evaluation. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: *Cryptococcus neoformans* is a fungal pathogen which infection caused devastating morbidity and mortality in immunocompromised patients. The present study investigated the effect of culture starting inoculum size and culture age towards cellular properties of *C. neoformans* and its interactions with mammalian host alveolar epithelial cells.
Methodology: *C. neoformans* H99 was cultured at different starting inoculum sizes and collected at varied culture ages to examine the morphology of the yeast cells and agar invasion property. The interaction with host alveolar epithelial cells was assessed *in vitro* using A549 cells as the host cell model.
Results: Visual observation demonstrated that cryptococci cultured with higher starting inoculum sizes and longer incubation periods displayed flocculation properties, aberrant morphologies with

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lysed cell structure attached to intact yeast cells, release of capsule material to the culture medium, as well as changes in FITC staining of cell surface proteins. The changes in cryptococcal cellular morphology did not affect agar invasion, as the encapsulated cryptococci did not invade agar under all conditions tested. Lysed cell material on cryptococcal cells adhered to host alveolar epithelial cells, which induced localised actin reorganisation at the host-pathogen interface.

Conclusion: Our results indicate that the differences in starting inoculum size and culture age of *C. neoformans* H99 resulted in yeast cells with distinctive morphologies, which affected he pathogen's association with host alveolar epithelial cells.

Keywords: Culture age; starting inoculums; agar invasion; capsule; adherence; actin.

1. INTRODUCTION

Cryptococcus neoformans is an encapsulated facultative intracellular fungus that caused devastating cryptococcosis mainly in immunocompromised patients. It was estimated that the global burden of HIV-associated cryptococcal meningitis reaches 957,900 cases per year, accounting for 624,700 deaths annually [1]. Primary infection is contracted upon inhalation of infectious particles in forms of dehydrated haploid yeast cells or basidiospores into the lungs [2]. In immunocompromised patients, primary pulmonary cryptococcal infection can disseminate to various organs, with predilection to the central nervous system (CNS) leads to the highly fatal meningoencephalitis [3,4]. To allow haematogenous dissemination, *C. neoformans* was either recruited to extrapulmonary sites through migration within cryptococci-infected macrophages, or by direct invasion of the epithelium followed by passage through endothelial cells [5,6].

The adherence and internalisation of *C. neoformans* into alveolar epithelial cells have been investigated in several studies [7–12]. Although all studies generally agreed that *C. neoformans* adheres to epithelial cells, there are discrepancies reported on the degree of interaction with the host cells. Some studies demonstrated extensive adherence and internalisation of *C. neoformans* into alveolar epithelial cells [8,10,11], yet the report of Guillot and coworkers [12] showed that encapsulated cryptococci did not adhere to epithelial cells. Several factors such as assay methods and analysis, host and pathogen models, as well as culture age and density can lead to the mentioned discrepancies. In fact, previous studies have demonstrated that adherence of *C. neoformans* to primary rat lung cell culture and human alveolar epithelial cells is affected byculture age, where an older culture age generally confers cryptococci higher adherence efficacy to the host cells [10,11]. *Cryptococcus* species are usually presented as spherical-to-oval budding yeast cells ranging from 4-20 μm in clinical studies, yet unusual morphologies in forms of pseudohyphae, chains of budding yeast cells, poorly encapsulated cells, as well as structure resembling germ tubes were previously documented [13]. In light that cryptococcal cells do not always exhibit the typical encapsulated yeast cell-like forms, it is important to study factors leading to these structures and its implication toward pathogenesis.

In the current manuscript, we demonstrate that cryptococcal culture starting inoculum size and culture age affects the cellular morphology and cell surface protein distribution of *C. neoformans*. These changes in *C. neoformans* do not affect the invasive nature of cryptococci on agar, yet an aberrant lysed cell structure on the yeast cells facilitated the adherence of *C. neoformans* to mammalian epithelial cells and induced localised host actin reorganisation.

2. MATERIALS AND METHODS

2.1 *C. neoformans* Isolate and Cultivation Conditions

C. neoformans H99 (ATCC 208821, Serotype A, Mating type α) was purchased from the American Type Culture Collection (ATCC, USA). The yeast cell stock was stored at -80°C in Sabouraud dextrose broth (SDB) (BD, USA) with 10% (v/v) glycerol (Merck, Germany). Yeast cells were subcultured onto Sabouraud's dextrose agar (SDA) (BD, USA) and incubated at 37°C. A seeder culture was obtained by inoculating 10mL of SDB culture in a 50mL conical centrifuge tubes with colonies from agar, followed by incubation at 37°C with continuous agitation at 240 rpm for 24 hours. The density of the seeder culture was subsequently enumerated using a haemocytometer, adjusted to the desired yeast cells starting inoculum sizes (10^4-10^7 cells/mL)

(10 mL culture in 50 mL conical centrifuge tubes), and cultured for a period of 24-72 hours.

2.2 Microscopic Observation of Cellular Morphology of *C. neoformans*

To observe cellular morphology of *C. neoformans*, 10 µL of *C. neoformans* H99 culture grown at different starting inoculum sizes and culture period was placed on a glass slide with a coverslip mounted on top. Changes in cellular properties such as shape, sizes and aberrant morphologies were observed and captured using the inverted microscope Eclipse Ti-S equipped with digital camera DS-Ri1 (Nikon, Japan).

2.3 Agar Invasion Assay

To assess invasive growth, agar invasion assay was performed as reported previously [14]. Briefly, 1-10 µL of culture with different starting inoculum sizes and culture ages were spotted on SDA. After 3 days incubation at 37°C, the plates were photographed using a gel documentation system (UVP Bioimaging Systems, USA). Excess water were added to the agar plates, and cells were grown on the agar surface were gently removed with a cell scraper. Pictures of the plates were taken again and analysed for the presence yeast cells retained within the agar.

2.4 Staining of *C. neoformans* with India ink, Fluorescein Isothiocyanate (FITC) and Calcofluor White (CFW)

India ink staining was performed to observe cryptococcal capsule, as the polysaccharide capsule exhibit a halo around the yeast cells against the dark background created by the dye. Briefly, 1 part of the culture were stained with 5 parts of India ink (Merck, Germany), on a glass slide with a coverslip mounted on top. FITC is a dye commonly used for yeast and bacteria cells labelling, which targets surface protein [15]. For FITC (Sigma-Aldrich, USA) staining, $5x10^7$ yeast cells were pelleted and suspended in 50 µg/mL FITC in PBS for 15 minutes at room temperature. The stained cells were washed twice with PBS to remove unbound stain. CFW binds to cellulose and chitin, which are present on yeast cell wall [16]. The cell wall of the yeast cells was counterstained using 0.1 mg/mL CFW at room temperature for 15 minutes. The light and epifluorescence images were obtained on an inverted microscope Eclipse Ti-S equipped with digital camera DS-Ri1 (Nikon, Japan).

2.5 Adherence of *C. neoformans* to A549 Alveolar Epithelial Cells

A549 (human type II alveolar epithelial-like carcinoma) was purchased from ATCC (Rockville, USA), and routinely maintained in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (JRS, USA) and 100 U/mL penicillin/streptomycin (Gibco, USA) at 37°C in a 5% CO2 humidified incubator. For co-incubation study, cells were grown to confluent on coverslips in 24-well plates and co-incubated with FITC-stained *C. neoformans* H99 suspended in RPMI media for 4 hours. After the co-incubation period, the non-adherent yeast cells were removed and 0.1 mg/mL CFW was added for 3 minutes followed by three PBS washes. The cells were fixed using 3.75% paraformaldehyde (PFA) for 15 minutes followed by permeabilisation with 0.1% Triton X-100 for 3 minutes. Host actin was stained using 5 units/mL CF555 Phalloidin (Biotium, USA) according to the manufacturer's instructions. Image acquisition was performed as previously mentioned.

3. RESULTS AND DISCUSSION

3.1 Effect of Culture Age and Cell Density on Morphology and Invasive Property of *C. neoformans* H99

C. neoformans H99 was suspended in SDB at inoculum sizes of 10^4, 10^5, 10^6, 10^7 cells/mL and cultured for a period of 24, 48 and 72 hours. The cultures were subsequently stained with India ink and viewed under light microscope. Visual observations showed that the starting inoculum sizes and culture ages did not dramatically affect the cell and capsule sizes of the cryptococci. Cells that were grown in low concentration (10^4 and 10^5 cells/mL) displayed common morphology of single budding cell at all culture ages. Cells that were grown in high concentration (10^6 and 10^7 cells/mL) whereas displayed flocculation phenotype by 72 hours, and early cell lysis was apparent, Capsule materials were evidently released in bulk to the culture medium, where India ink was repelled and formed clump upon contact with the spent culture medium (Fig. 1A, arrows). This observation is consistent with *in vivo* cryptococcal infection, where *C. neoformans* capsular polysaccharide is abundantly released into the tissue [17].

A close examination of the culture with starting inoculum size of 10^7 cells/mL at 72 hours demonstrated that most yeast cells displayed aberrant morphologies, with irregular shaped cells rather than the universally rounded shape in earlier culture ages. We observed attachment of lysed cells or small budded daughter cells to intact yeast cells (Fig. 1B). This is likely induced by limitation of nutrients and acidification of the culture medium. In fact, acidification of medium caused by production of organic metabolic acid and proton extrusion in glucose-rich medium is a common phenomenon in yeast cells culture [18,19]. The study conducted by Farkas et al. [20] and Yoshida et al. [21] demonstrated that growth of *C. neoformans* in synthetic medium with 1.0-3.0% glucose often results in spontaneous acidification of the medium from an initial pH value of 5.5 to 2.5 when the yeast cells growth progresses into stationary phase. This resulted in cryptococci death as a consequence of autolytic erosion of the yeast cell wall induced by endogenous β-1,3-glucanase [20,21]. Yoshida et al. [21] noted two fatal alterations to cryptococci under the mentioned conditions: first, release of cytoplasm and protein-rich dead cell structure attached to the surviving cells, and second, cryptococci shrunk distinctly with no sign of cell rupture. Similar aberrant structures were noted in the current study, and they could not be removed by extensive washing. These aberrant structures were exposed to interact with the host cells, thus we subsequently investigated their possible role facilitating invasion or adherence properties of the pathogen.

The cultures of different starting inoculum size and culture ages were directly spotted onto SDA and cultured at 37°C. The invasive growth was assayed after 3 days post-incubation. The result showed that *C. neoformans* does not invade the culture agar under all conditions tested (Fig.1C). The invasive growth, defined as the ability of microorganism to penetrate solid media and grow within the matrix [16], could potentially represent the ability of microorganism to invade host tissues, such as traversing biological barrier including epithelial and endothelial cells. A previous study has demonstrated that the encapsulated yeast form of *C. neoformans* does not manifest agar invasion growth at 30°C [22]. The result in our study is in agreement with the mentioned study and we further demonstrated that *C. neoformans* grown at physiological temperature (37°C) does not invade the agar surface regardless of starting inoculum size or culture age, despite displaying obvious changes in cellular morphology.

3.2 Effect of Starting Inoculum Size and Culture Age on FITC and CFW Labelling of *C. neoformans*

Subsequently, *C. neoformans* with starting inoculum size of 10^7 cells/mL were stained using FITC at different culture ages (24, 48 and 72 hours) to assess changes in cell surface protein distributions. The isothiocyanate group of FITC confers negative charges to the dye, which enables it to crosslink with the positively charged peptide group on microbial surface proteins [15]. It was noted that the 24 hours culture were minimally stained by FITC, with signals localised mainly to the cell wall. At 48 hours and 72 hours of incubation, most cells exhibited stronger FITC staining signal, which is prominent in the whole cell body (Fig. 2A). The degree of FITC staining is likely not affected by the size of the capsule, as visual observations through India ink staining did not detect drastic changes on the capsule sizes. Alteration in FITC staining signals in *C. neoformans* at different culture ages signifies likely alteration in cell surface protein distribution and permeability of the cells.

A close examination of cryptococcal cells with aberrant morphologies showed that lysed cell structures attached to intact cells were strongly stained by FITC but not CFW (Fig. 2B). The result indicated that the structure might be cytoplasmic origin without the components of a cell wall. This lysed cell attachment is likely due to the rapid and strong precipitation of cytoplasmic proteins caused by low pH of the medium [21].

3.3 Cryptococcal Lysed Cell Structures Mediated the Adherence to A549 Alveolar Epithelial Cells and Induced Host Cell Actin Reorganisation

To study the effect of aberrant cellular morphology towards the host-pathogen interaction, we compared the adherence and host actin organisation of A549 cells upon co-incubation with cryptococcal cells displaying normal morphologies (starting inoculum size of 10^6 and culture age of 48 hours) with cryptococcal cells displaying aberrant morphologies (starting inoculum size of 10^6 and culture age of 72 hours). It was generally noted that cryptococcal cells with normal round, non-

aggregating morphology barely adhered to A549 cells, and adherence was usually were not accompanied by actin reorganisation of the host cells (Fig. 3A). In contrast, in culture with aberrant cryptococcal morphologies, most cryptococci adhered to the host cells through the lysed cell structures, and induced localised actin reorganisation beneath the structure (Fig. 3 B-D). A rare case of germ tube-like structure was noted to extend towards the host cell, enclosed by host actin (Fig. 3D).

It is well-established that reorganisation of actin is required for the internalisation of several bacterial and fungal pathogens into host cells [23–27]. Thus, the actin reorganisation observed is likely a mechanism induced by *C. neoformans* to mediate its own uptake by the host cell. This observation might possibly explain the reason why cryptococcal cells with older culture ages were reported to be more efficient at adhering to alveolar epithelial cells. The relevance of this aberrant structure in pathogenesis is unknown, yet unusual cryptococcal morphologies resembling those observed in this study have been reported in clinical settings [13]. Moreover, the lungs have relatively low nutrient level compared to serum, [28] which might led to formation of aberrant yeast cells as observed in the current study. Future investigation in this aspect is warranted.

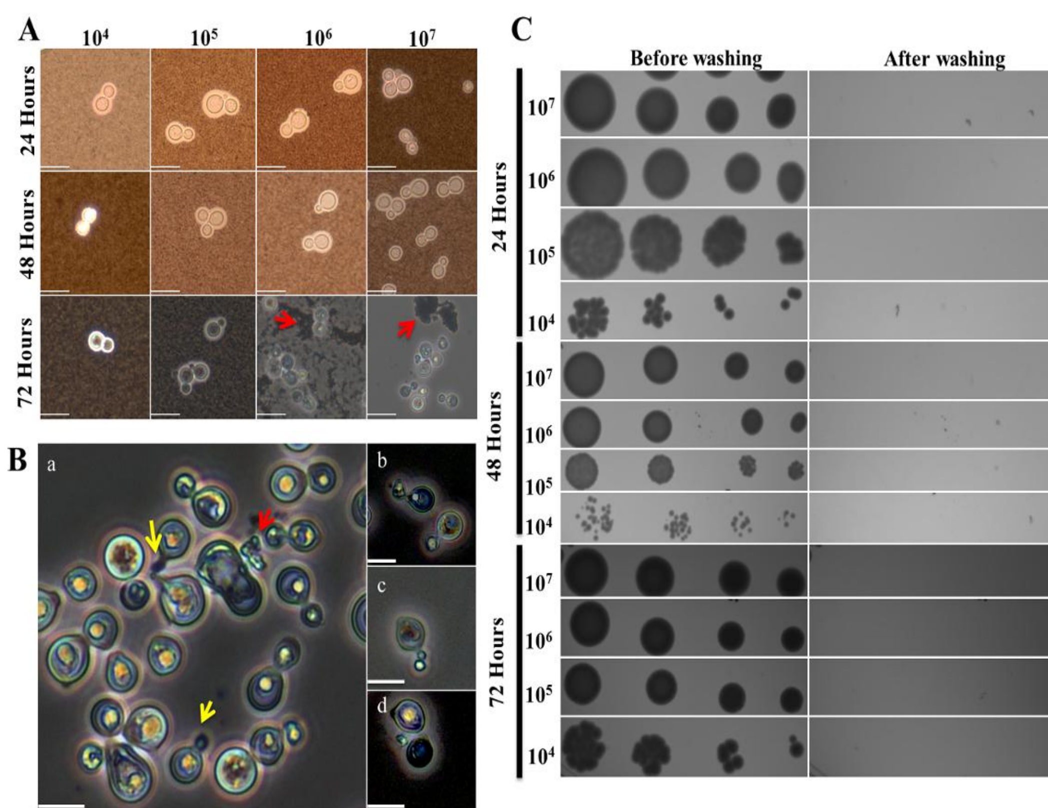

Fig. 1. Effect of starting inoculum size and culture age towards cellular morphology and agar invasion property of *C. neoformans*

A. *C. neoformans H99 was grown from starter cultures with different inoculum sizes (10^4, 10^5, 10^6, 10^7 cells/mL) and incubated for a different periods of time (24, 48, 72 hours). The cultures were stained with India ink and visualised through light microscope, arrow= ink aggregation.* ***B.*** *72 hours culture of H99 with an initial starting inoculum of 10^7 cells/mL. a. Dead cells remain attached to the outer surface of viable cryptococci (red arrow); Small budded yeast cells were noted in some cells (yellow arrow) b. Lysed cells remain bound to viable cells. c. Shrunken yeast cells/tiny budding cells. d. remnants of lysed cells firmly attach to intact cryptococci. Bar=20 µm.* ***C.*** *Comparison of agar invasive properties of C. neoformans H99 cultured with different starting inoculum sizes and culture ages. 1, 2, 5 and 10 µL of the cryptococcal cultures were directly spotted on SDA and agar invasion was assessed. C. neoformans H99 was non-invasive at all conditions tested*

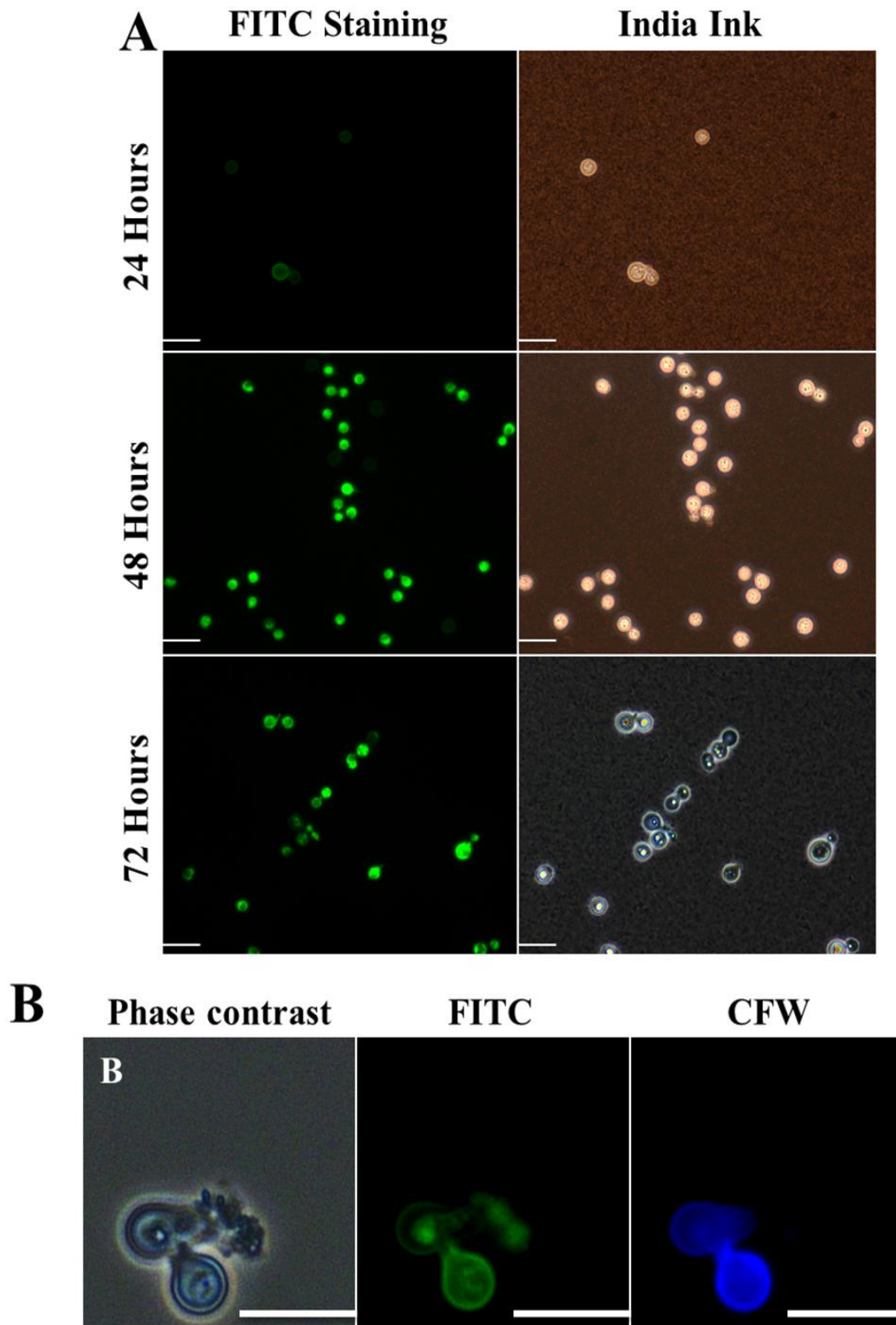

Fig. 2. Cellular morphology and FITC/CFW staining of *C. neoformans* H99 cultured at high starting inoculum size

C. neoformans H99 cultures were grown from starter culture of 10^7 cells/mL and incubated for 72 hours. ***A.*** *Comparison of cellular morphology and FITC staining of C. neoformans H99 at different culture ages. FITC staining of cryptococci was stronger with longer incubation period.* ***B.*** *Cells were pelleted and stained with FITC followed by counterstaining with CFW. FITC but not CFW stained the lysed cells structure. Bar = 20 μm*

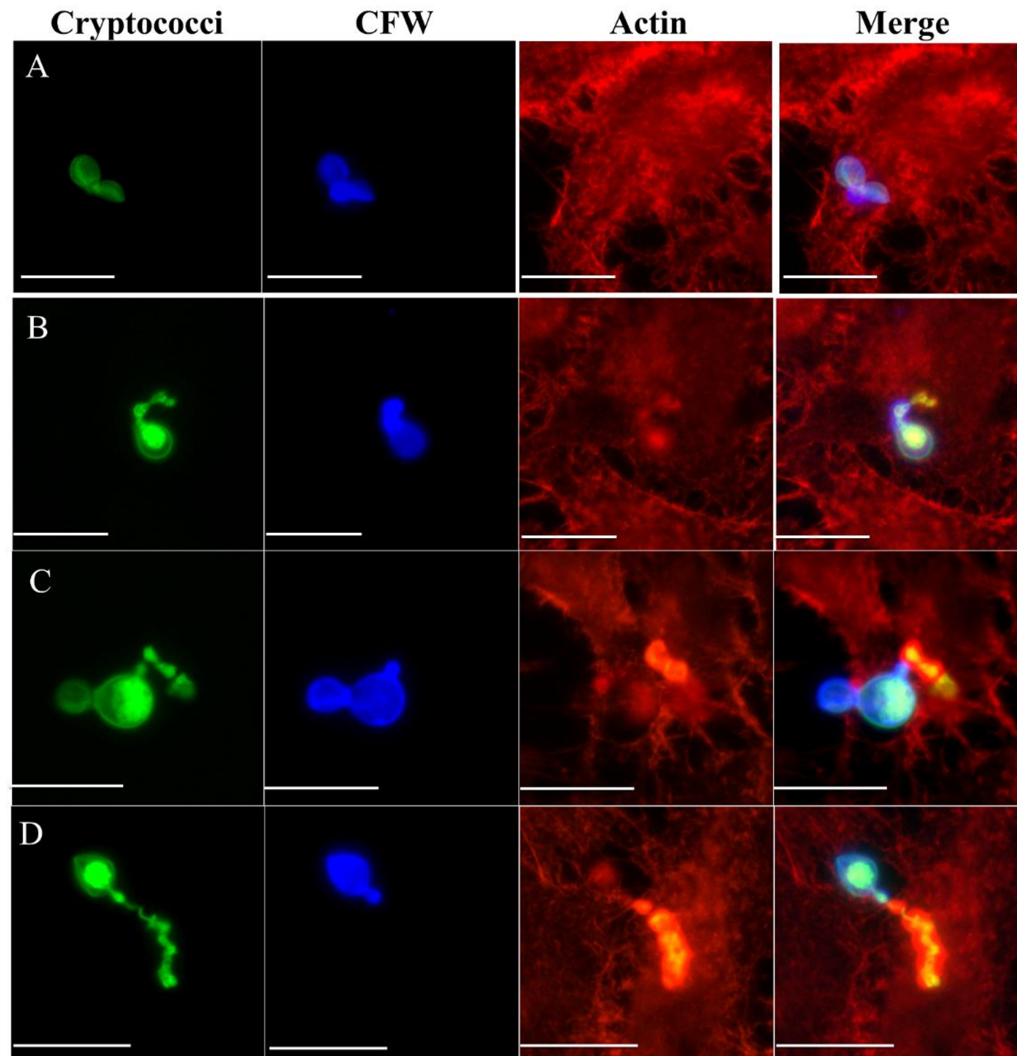

Fig. 3. Actin reorganization of A549 alveolar epithelial cells induced by adherence of FITC-labelled *C. neoformans* H99

A. *Cryptococcal cells with starting inoculum size of 10^6 cells/mL and incubated for 48 hours did not induced actin reorganization upon adherence.* ***B-D.*** *Cryptococcal cells with starting inoculum size of 10^6 cells/mL and incubated for 72 hours induced localised actin reorganization upon adherence. The lysed cryptococcal structure or germ-tube liked stricture adhered to viable cells, corresponded to strong actin localisation of A549 cells at the cryptococcal adherence site. Bar = 20 µm*

4. CONCLUSION

In summary, the present study showed that the inoculum size and culture age of *C. neoformans* H99 affected the cellular morphology, surface protein distribution and interactions alveolar epithelial cells, while not affecting the agar invasion property or the capsule size of the yeast cells. Our study indicates that the starting inoculum size and culture age of *C. neoformans* H99 culture should be taken into account in studying the host-pathogen interactions of *C. neoformans*. In addition, the implication of unusual morphologies of *C. neoformans* in the pathogenesis is unclear and warrants further investigation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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