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Germination dynamics of allergenic fungal spores in respiratory mucus

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Abstract Mucus and body temperature serve as the first lines of defense against invading pathogens. When the inhaled particles are deposited in the nose, the respiratory mucus and body temperature potentially impact fungal spore germination and prevent pathogen infections. We hypothesized that respiratory mucus and body temperature impact fungal spore germination. This study aimed to investigate the temporal germination dynamics of allergenic *Cladosporium, Aspergillus*, and *Penicillium* spp. in respiratory mucus ex situ and the effect of the temperature (28 °C and 37 °C in the upper and lower respiratory tract, respectively) on fungal germination. Fungal spore germination was inhibited at 37 °C in *C. oxysporum* and *C. cladosporioides*, but not *A. flavus*, *A. brunneoviolaceus*, *A. protuberus*, *P. citrinum*, and *P. oxalicum. Aspergillus flavus* and *A. brunneoviolaceus* exhibited a high germination rate in the mucus at 28 °C and 37 °C. This indicated that their germination was not limited in the mucus in the upper or lower respiratory tract, where spores of these fungi can overcome the inhibition of germination as in invasive aspergillosis pathogens. The germination rate of *A. protuberus* in the mucus was very low at both 28 °C and 37 °C. Spore germination of *P. citrinum* and *P. oxalicum* occurred at 37 °C (normal body temperature) but was inhibited by the mucus.

Keywords Fungal allergy · *Cladosporium* · *Aspergillus* · *Penicillium* · Viability · Mucus

1 Introduction

Pathogenic fungi cause various diseases including allergies, asthma, and pulmonary aspergillosis (Agarwal and Gupta 2011). Fungal sensitization is associated with a reduction in lung functions and the exacerbation of allergic diseases (Chen et al. 2014). More than 6,500,000 individuals have severe asthma with fungal sensitization because of the inhalation of a complex mixture of hyphal fragments and fungal spores (Agarwal and Gupta 2011). Some fungi, most notably *Aspergillus* spp., can germinate in the lungs, potentially causing allergic and infectious manifestations of allergic bronchopulmonary aspergillosis (Bains and Judson 2012; Janahi et al. 2017).

More than 180 fungal species have been reported to induce hypersensitivity in susceptible individuals, among which *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium*, and *Candida* spp. are considered

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important for clinical diagnosis (Achatz et al. 1995; Horner et al. 1995; Kurup et al. 2000). In Taiwan, *Cladosporium* is the most predominant airborne fungal genus (Hsu et al. 2011; Lin et al. 2018; Woo et al. 2013), followed by *Aspergillus* and *Fusarium* (Lin et al. 2018; Wei et al. 1993; Wu et al. 2004). Although allergic sensitization has been reported in more than 80 fungal species, only a few have been studied in any detail.

Fungi produce spores and conidia, which potentially contain various allergenic substances. When the spores or conidia are released, they remain dormant until they are ready to germinate. Most fungal allergens are released during (Mitakakis et al. 2001) and after spore germinate (Green et al. 2003) because the spores are covered by a protective hydrophobic layer facilitating immune evasion. Non-culturable or non-viable spores may still be allergenic and cause health problems (Kozak Jr et al. 1980).

These small particles can easily become airborne and be inhaled. According to Reponen (1995), 30–50% of fungal particles have been predicted to be deposited in the nose during inhalation. Fungi are a normal component of nasal mucus in healthy individuals (Lackner et al. 2005). The temperature and mucus in the nasal cavity potentially influence fungal spore germination, thus impacting antigen release.

Laboratory investigations have revealed that temperature, nutrients, and humidity influence fungal growth or germination. For example, the optimal growth temperature of mesophilic fungi, such as *Alternaria* and *Cladosporium* spp., are typically 20–30 °C. By contrast, thermotolerant fungi, such as *Penicillium*, and *Aspergillus* spp., can grow at human body temperature (37 °C). However, although numerous studies have investigated the germination dynamics on synthetic media, they have not considered the effect of respiratory mucus.

Mucus and body temperature are the first line of defense against invading microbes. When the inhaled particles are deposited in the nose, the respiratory mucus and body temperature potentially impact fungal spore germination and prevent pathogen infections. We hypothesized that respiratory mucus and body temperature affected fungal spore germination. Therefore, this study aimed to examine the ability of seven fungal spores to germinate in the mucus and at high temperature. Water (containing no nutrients) and potato dextrose broth (containing full nutrients) were used as negative and positive controls, respectively. We monitored the germination dynamics of seven allergenic fungal species in respiratory mucus and investigated the effect of temperature (28 °C and 37 °C representing the upper and lower respiratory tract, respectively) on fungal spore germination.

2 Materials and methods

2.1 Fungal isolates

Seven fungal isolates, namely *Cladosporium oxysporum* (BCRCFU30954), *C. cladosporioides* (BCRCFU30955), *Aspergillus protuberus* (BCRCFU31465), *A. flavus* (BCRCFU31464), *A. brunneoviolaceus* (BCRCFU 31463), *Penicillium citrinum* (BCRCFU31462), and *P. oxalicum* (BCRCFU30957), were collected from airborne fungal flora in Taichung (Lin et al. 2018) and their allergenicity was confirmed via the skin prick test (Shih et al. 2017). Briefly, proteins were extracted from the seven fungal species. A total of 49 subjects were included in the skin prick test, which was performed on healthy skin on the volar side of the forearm. After cleaning the skin with alcohol, a drop from each extract was applied to the skin and then the skin was pricked through each drop using a sterile lancet. Histamine hydrochloride (10 mg/mL) and glycerol saline were used as positive and negative controls, respectively. After 20 min, the area of wheal and flare was measured and recorded, as previously described (Dreborg 1989). These isolates were cultured on potato dextrose agar (PDA, BD Pharmingen) at 25 °C.

2.2 Collection and preparation of respiratory mucus media

The study subject was a healthy 20-year-old woman without allergic reaction to fungi. Respiratory mucus was collected from her nasal cavity via pipetting and diluted in physiological saline at a ratio of 1:2. After homogenization, the solution was sterilized twice through 0.2-μm membrane filters (Pynnonen et al. 2011). The filtrates were used as respiratory mucus media and preserved at − 20 °C for study.

2.3 Assessment of spore germination and statistical analysis

Spores were harvested using an inoculating needle from 8-day-old cultures and suspended in media including respiratory mucus medium, potato dextrose broth (containing all nutrients as the positive control), and sterile deionized water (containing no nutrients as the negative control). The hanging-drop assay was performed to observe spore germination. Each spore suspension was placed on a coverslip and then inverted to produce a hanging drop in the depression of the slide. The slides were sealed in plastic bags separately and incubated at 28 °C and 37 °C. The percentage germination rate was determined by counting at least 100 spores per treatment in triplicate every 12 h. Spore morphology during germination was observed microscopically.

Differences in the germination rates for different temperatures, media, and times were assessed using repeated measures ANOVA in R (R Core Team 2013).

3 Results

3.1 Spore germination rate at 28 °C

The spore germination rates of the seven fungal species in mucus, positive control (PDB), and negative control (water) at 28 °C and 37 °C after incubation for 48 h are presented in Table 1. At 28 °C, the germination rate of the seven fungal species in PDB was approximately 99.5%, indicating that all spores were viable and grew well in media containing all nutrients.

In water, these fungal spores exhibited various germination rates. *Cladosporium oxysporum* and *Aspergillus protuberus* presented the highest (99.5%) and lowest (1.4%) germination rates, respectively. The present results indicate that given a lack of nutrients, the spores of *A. protuberus* remained dormant; however, those of *C. oxysporum* still germinated well. The germination rates of *C. cladosporioides*, *A. flavus*, *A. brunneoviolaceus*, *Penicillium citrinum*, and *P. oxalicum* in water were 62.0%, 48.7%, 18.7%, 67.7%, and 31.3%, respectively (Table 1). In physiological saline, they exhibited similar germination rates, with only *C. oxysporum* displaying a higher germination rate (91.7%) compared with that in water (78.5%) at 12 h. This finding suggested that the germination potential of these fungi were reduced by the lack of nutrients.

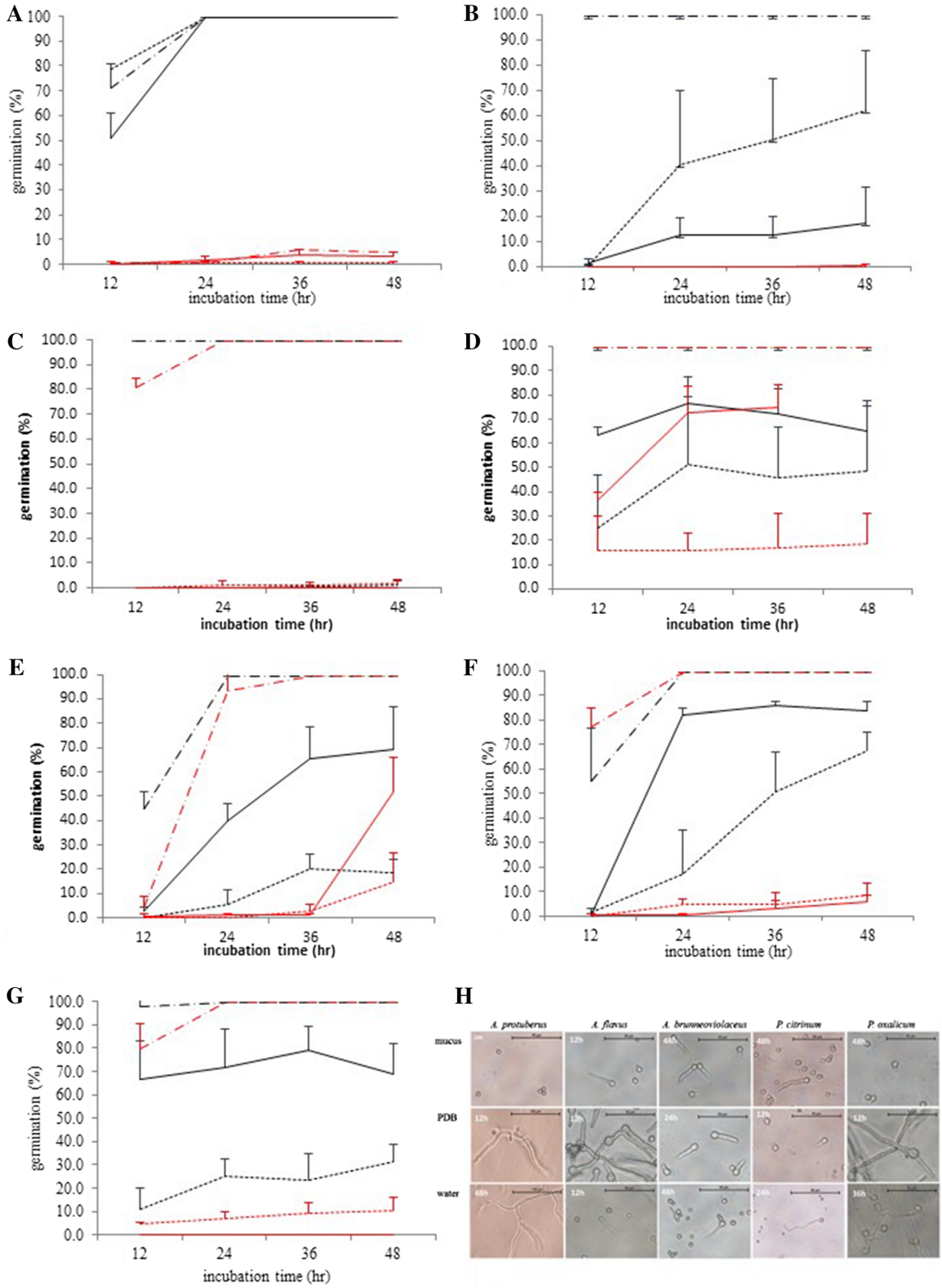
Cladosporium, *Aspergillus*, and *Penicillium* spp. displayed different germination rates in respiratory mucus. At 28 °C, the spore germination rate of *C. cladosporioides* was decreased by mucus (17.3%); however, that of *C. oxysporum* remained unaffected (99.5%) (Table 1). Changes in the germination rate of two *Cladosporium* spp. after 12–48 h of incubation are presented in Fig. 1A, B. At 28 °C, the germination rate of *A. protuberus* in respiratory mucus was 0.1% at 48 h (Table 1; Fig. 1C). Both mucus and the lack of

Table 1 Germination rates of seven fungal species after 48 h of incubation in water, potato dextrose broth (PDB) and mucus

Temperature	28 °C			37 °C		
Medium	PDB	Water	Mucus	PDB	Water	Mucus
C. oxysporum	99.5 ± 0.0[a]	99.5 ± 0.0	99.5 ± 0.0	5.0 ± 0.0	0.4 ± 0.5	3.0 ± 1.7
C. cladosporioides	99.5 ± 0.0	62.0 ± 23.8	17.3 ± 14.2	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.5
A. protuberus	99.5 ± 0.0	1.4 ± 1.5	0.1 ± 0.0	99.5 ± 0.0	1.7 ± 1.5	0.1 ± 0.0
A. flavus	99.5 ± 0.0	48.7 ± 26.7	65.0 ± 12.5	99.5 ± 0.0	18.7 ± 12.4	75.0 ± 8.9[a]
A. brunneoviolaceus	99.5 ± 0.0	18.7 ± 5.1	69.0 ± 17.7	99.5 ± 0.0	14.7 ± 12.0	51.7 ± 14.6
P. citrinum	99.5 ± 0.0	67.7 ± 7.2	83.7 ± 3.8	99.5 ± 0.0	8.5 ± 5.0	5.7 ± 2.9
P. oxalicum	99.5 ± 0.0	31.3 ± 7.2	68.7 ± 13.5	99.5 ± 0.0	10.4 ± 7.3	0.11 ± 0.2

Water (containing no nutrients) and potato dextrose broth (containing all nutrients) were used as negative and positive controls, respectively

[a]Indicates the germination rate after 36 h of incubation


A
germination (%)
incubation time (hr)
B
germination (%)
incubation time (hr)
C
germination (%)
incubation time (hr)
D
germination (%)
incubation time (hr)
E
germination (%)
incubation time (hr)
F
germination (%)
incubation time (hr)
G
germination (%)
incubation time (hr)
H
A. protuberus
A. flavus
A. brunneoviolaceus
P. citrinum
P. oxalicum
mucus
PDB
water

◀**Fig. 1** After 12–48 h, the germination rates of *Cladosporium oxysporium* (**A**), *C. cladosporioides* (**B**), *Aspergillus protuberus* (**C**), *A. flavus* (**D**), *A. brunneoviolaceu* (**E**), *Penicillium citrinum* (**F**) and *P. oxalicum* (**G**) in different media at 28 °C and 37 °C and their morphology and germination are displayed (**H**). Black and red lines represented data obtained at 28 °C and 37 °C, respectively, and solid, dashed and dot-dashed lines represented the data obtained from respiratory mucus, water (negative control), and potato dextrose broth (positive control), respectively

nutrients significantly inhibited the germination of *A. protuberus* ($p < 0.0001$). Despite belonging to the same genus, *A. flavus* and *A. brunneoviolaceus* displayed different germination rates from that of *A. protuberus*. *Aspergillus flavus* and *A. brunneoviolaceus* presented a higher germination rate in respiratory mucus than in water (Fig. 1D, E). Compared with the positive control (PDB), germination of *P. citrinum* in respiratory mucus was delayed, and its rate increased from 0.4 to 82.0% from 12 to 24 h of incubation at 28 °C (Fig. 1F). By contrast, > 60% of *P. oxalicum* spores had already germinated in the respiratory mucus at 12 h at 28 °C (Fig. 1G). Both of these *Penicillium* spp. presented higher germination rates in respiratory mucus than in water at 28 °C (Table 1).

3.2 Spore germination rates at 37 °C

At 37 °C, the germination rates of *A. protuberus*, *A. flavus*, *A. brunneoviolaceus*, *P. citrinum*, and *P. oxalicum* after 24 h of incubation were approximately 99.5% in PDB (Table 1); however, the spores of *C. oxysporum* and *C. cladosporioides* rarely germinated in PDB, water, or mucus (Table 1). The germination rates of *C. oxysporum* and *C. cladosporioides* were significantly lower at 37 °C than at 28 °C ($p < 0.0001$). Body temperature (37 °C) inhibited the germination of *Cladosporium* spores.

The spores of three *Aspergillus* spp. in PDB germinated well at 37 °C, with germination rates of 100% at 12–24 h (Fig. 1C–E). Normal body temperature (37 °C) did not inhibit spore germination for *A. protuberus*, *A. flavus*, or *A. brunneoviolaceus*. However, the germination rate of *A. protuberus* in water, physiological saline (data not shown), and respiratory mucus was < 2% (Table 1). Nutrients and mucus significantly affected the germination rate of *A. protuberus* ($p < 0.0001$). Despite belonging to the same genus, *A. flavus* and *A. brunneoviolaceus* displayed different germination rates in respiratory mucus from that of *A. protuberus*. In respiratory mucus, the spores of *A. flavus* germinated faster than those of *A. brunneoviolaceus* (Fig. 1D, E). Both these species displayed significantly lower germination rates in water (Table 1) and physiological saline (0%). Respiratory mucus and lower nutrients delayed their germination.

The germination rates of both *P. citrinum* and *P. oxalicum* were 99.5% in PDB (Table 1). Spores of *P. citrinum* and *P. oxalicum* exhibited limited germination in water and mucus, indicating that their germination was inhibited by low nutrients and mucus (Table 1; Fig. 1F, G).

In summary, spore germination in *C. cladosporioides* and *C. oxysporum* was significantly inhibited by body temperature (37 °C) in PDB (positive control), mucus, and water (negative control). Moreover, at 28 °C, spore germination of *C. cladosporioides* was inhibited by respiratory mucus; however, this was not observed in *C. oxysporum.* Spores of three *Aspergillus* spp. and two *Penicillium* spp. germinated well in PDB at 28 °C and 37 °C. At both 28 °C and 37 °C, respiratory mucus could not inhibit germination in *A. brunneoviolaceus* or *A. flavus*, and it exclusively inhibited *A. protuberus*. Spore germination in *P. citrinum* and *P. oxalicum* was inhibited upon incubation in respiratory mucus at 37 °C.

4 Discussion

Fungi are ubiquitous in various environments, and we are exposed to thousands of their spores every day. However, not all fungi are pathogenic. Spore germination and fungal developmental stages are important factors influencing the outcome of the encounter (Sephton-Clark and Voelz 2018). Subsequently, the host immune status also influences the outcome of a fungal infection (Perfect 2012). Furthermore, the germination dynamics of fungal spores in the host environments is a potentially critical factor influencing fungal invasion.

In this study, the high germination rates of *C. cladosporioides* and *C. oxysporum* in water at 28 °C suggested that nutrient scarcity did not limit their germination. However, on mucus treatment, *C.*

oxysporum demonstrated a similar germination rate to that of the positive control (PDB); however, the germination rate of *C. cladosporioides* was significantly decreased by respiratory mucus. The components of nasal mucus, including lysozyme, lactoferrin, mucus glycoproteins, secretory leukoprotease inhibitor, uric acid, peroxidase, aminopeptidase, immunoglobulins, and neutral endopeptidase, mediate innate mucosal host defense (Kaliner 1991, 1992). These components potentially help inhibit spore germination in *C. cladosporioides*.

Body temperature (37 °C) inhibited spore germination in *C. oxysporum* and *C. cladosporioides* but not in *A. flavus*, *A. brunneoviolaceus*, *A. protuberus*, *P. citrinum*, or *P. oxalicum.* The present results indicated that temperature was the critical factor limiting spore germination in *Cladosporium* spp. The air temperature in the human nasal cavity and upper respiratory tract varies from ambient temperature to 33.9 °C (Ma et al. 2018). Body temperature (37 °C) inhibited spore germination of *C. cladosporioides* and *C. oxysporum*, suggesting that *Cladosporium* spores could not germinate in the lower respiratory tract or invade human tissue. The allergen of *Cladosporium* could be detected around the spores (Green et al. 2009). After inhalation of their spores, the spore surface allergen might cause an immediate type of hypersensitivity reaction. *Cladosporium oxysporum* could germinate in the upper respiratory tract but could not colonize the lower respiratory tract of the host.

Aspergillus spores are common in air. Sercombe et al. (2006) reported that for the *Aspergillus* group, germinated and ungerminated spores were present in the nasal cavity after indoor and outdoor exposure. Compared with *Cladosporium* and *Penicillium* spp., *A. flavus* and *A. brunneoviolaceus* exhibited higher germination rates in mucus at both 28 °C and 37 °C, indicating that their germination was not limited by the mucus in upper and lower respiratory tract. *Aspergillus flavus* and *A. brunneoviolaceus* are associated with invasive aspergillosis in immunocompromised patients (Hedayati et al. 2007; Perrone et al. 2013). For *Aspergillus* spp., several toxins released by hyphae (Mitchell et al. 1997; Kamei and Watanabe 2005) and the production of toxins may help *Aspergillus* colonize and invade the respiratory epithelium (Paulussen et al. 2017). The high germination rates of these two *Aspergillus* spp. in respiratory mucus may contribute to their virulence.

Spores germination in *A. flavus* and *A. brunneoviolaceus* was not inhibited at 37 °C or by the antimicrobial substances in respiratory mucus, which was required for their colonization in the respiratory tract and growth in human bodies. Furthermore, Tomee and Kauffman (2000) suggested that the ability of *Aspergillus* to invasively grow in the respiratory tract is mediated by its germination potential physiological temperatures. Significant exposure to *A. flavus* could increase susceptibility to invasive aspergillosis (Rocchi et al. 2014).

Aspergillus protuberus, which is a recently accepted species distinct from *Aspergillus* section *Versicolores* (Jurjevic et al. 2012), has been frequently isolated from indoor air samples in the USA (Jurjevic et al. 2012) and Taiwan. Although *A. protuberus* reportedly causes vaginitis in Turkey (Borsa et al. 2015) and kerion-type scalp mycosis in China (Jia et al. 2019), its germination rate in respiratory mucus was very low in this study.

This study reported not only that spores of *A. flavus* and *A. brunneoviolaceus* germinated at 37 °C in respiratory mucus ex situ, but also that *A. flavus* and *A. brunneoviolaceus* extended their hyphae at 37 °C in respiratory mucus. The ability of these *Aspergillus* spp. to colonize the host and cause invasive aspergillosis (Hedayati et al. 2007; Perrone et al. 2013) is mediated by the ability to germinate at physiological temperatures in respiratory mucus. More than 60% of the spores of *C. oxysporum*, *P. oxalicum*, and *P. citrinum* germinated at 28 °C in the respiratory mucus, whereas less than 10% of their spores germinated at 37 °C. The reduction in the germination rate could decrease their potential to cause lung infection.

After inhalation of fungal spores, cilia eliminate the spores and keep the airways clean, and the innate immune system constantly monitors and clears this threat (Tanaka et al. 2015). In vitro studies have reported that the fungal spores were destroyed in 8 h through macrophage- and neutrophil-mediated neutralization of fungal spores (Philippe et al. 2003; Levitz and Diamond 1985). Therefore, fungal spores cannot remain in the respiratory mucus for as long as 48 h in the upper respiratory system. However, fungal spore germination involves numerous steps including wetting of the spore, initiation of respiration, and detection of appropriate nutrients. Spores release enzymes to thin the outer cell wall and these enzymes induce inflammatory responses, thus compromising

mucociliary clearance and activating innate immune responses leading to the pathogenesis of asthma (reviewed by Pfavayi et al. 2020). Spores probably do not have to proceed to the germ tube stage to release allergens.

This study focused on two prominent diseases caused by airborne fungi: respiratory allergies and aspergillosis. Allergens are released during fungal spore germination (Green et al. 2003) and localize at the surface of the spore, the site of germination, or around ungerminated hyphal tips (Green et al. 2009). Inhalation of many ungerminated spores can potentially trigger allergic symptoms in a short period before being eliminated via mucociliary clearance. If the number of antigens around the fungal spore surface is limited and mucus inhibits spore germination, the antigen levels do not approach the threshold to trigger the allergic symptoms. However, if spore germination was not inhibited by mucus, a few spores could release antigens during germination. In such a situation, the antigen threshold level can be easily approached, potentially leading to sensitization and resulting in subsequent symptoms. Further studies are required to assess antigen levels on the spore surface or those released during spore germination. This study used respiratory mucus from a healthy individual, and the present results serve as a reference for respiratory allergies.

Among patients at a high risk of respiratory disease, the spores were not eliminated and destroyed through mucociliary clearance and by alveolar macrophages. Instead, they germinated and caused an invasive infection. However, the germination dynamic of fungal spores in respiratory mucus from these patients is unclear. We further intend to focus on this important issue.

Among patients with aspergillosis, germinating *Aspergillus* spores were not eliminated through mucociliary clearance and destroyed by alveolar macrophages and their hypha colonized patient tissue. In this study, the respiratory mucus was filtered through 0.2-μm membrane filters to eliminate cells. We did not assess macrophage- or neutrophil-mediated neutralization of fungal spores by primary human phagocytes. However, in patients with low mucociliary clearance (e.g., COPD), spores may have germinated, potentially resulting in issues. Some fungal species produce mycotoxin, impair the motile and chemosensory functions of airway cilia and cause the cilia to beat more slowly (Lee et al. 2016).

The limitation of this study was that respiratory mucus was obtained from only one individual. The antimicrobial activity of nasal secretions and their role in the prevention of microbial colonization were confirmed by Fleming (1922) and the present study. Cole et al. (1999) reported that nasal secretions have intrinsic antimicrobial activity; however, their activity varied between donors and between target bacteria. Subsequently, the host immune status influences the antimicrobial activity of nasal secretions. Further studies are required to elucidate the role of host immune status in the germination of fungal conidia and the outcome of fungal infections.

5 Conclusions

In conclusion, this study monitored the germination dynamics of allergenic fungal species in respiratory mucus ex situ and investigated the effect of temperature and mucus on their germination. The present data show that fungal spore germination was inhibited at 37 °C in *C. oxysporum* and *C. cladosporioides*, but not in *A. flavus*, *A. brunneoviolaceus*, *A. protuberus*, *P. citrinum*, or *P. oxalicum*. *Aspergillus flavus* and *A. brunneoviolaceus* displayed a higher germination rate in the mucus at 28 °C and 37 °C indicating that their germination was not limited by the mucus in the upper and lower respiratory tracts, where these fungal spores can overcome the inhibition of germination as invasive aspergillosis pathogens. For *P. citrinum* and *P. oxalicum*, spore germination was unaffected by temperature of 37 °C but was inhibited by respiratory mucus. The present results provide insights into the potency of airborne fungi as human pathogens. Further studies are required to investigate the role of host immune status in the germination of fungal conidia and the outcome of fungal infections.

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