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ORIGINAL ARTICLE

Fungal microbiota dynamics as a postmortem investigation tool: focus on *Aspergillus*, *Penicillium* and *Candida* species

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Abstract

Aims: To investigate the presence of fungi during three human decomposition stages: bloated, putrefaction and skeletonization.
Methods and Results: The samples were gathered in the city of Fortaleza, Ceará, Brazil, from the public morgue and cemeteries. The material was submitted to conventional mycological procedures by direct examination and macro/micro morphological and biochemical analyses. The main fungi isolated were *Aspergillus* spp., *Penicillium* spp. and *Candida* spp. in the bloated stage ($n = 34$ cadavers) and in the putrefaction stage ($n = 6$ cadavers), while in the skeletonization stage ($n = 20$ cadavers), the main fungi were *Aspergillus* spp., *Penicillium* spp. and *Mucor* sp.
Conclusions: *Aspergillus*, *Penicillium* and *Candida* species were associated with decomposed human cadavers.
Significance and Impact of the Study: These findings enable tracing out the profile of fungal communities of human cadavers for the first time. However, much more research will be necessary to develop this new segment of mycology and to enable its routine use in forensic science.

Introduction

Forensic mycology is a relatively new term describing the study of the species of fungi present in cadavers. It can have application in forensic medicine, particularly determination of the fungal groups to help establish the time of death (Carter and Tibbett 2003).

Studies of the relevant role of fungi in postmortem decomposition have been increasing because the corpse is a plentiful source of organic material (Ishii 2006), with a rising number of experimental descriptions and case studies in forensic mycology (Carter and Tibbett 2003; Hitosugi *et al.* 2006). These studies have shown that certain groups of these micro-organisms can provide valuable clues for estimating the time of death.

Although there have been some descriptions published on the participation of fungi in the postmortem process (Hitosugi *et al.* 2006), few have focused on the species that are present at each stage of decomposition and the possible application of this information to forensic medicine. Besides this, the isolation of certain fungal species in determined geographical areas also helps in the characterization and classification of the typical regional micro-organisms, in view of the variation of species in contact with corpses under different growth conditions (Ishii *et al.* 2007).

In 2003, Carter and Tibbett demonstrated how and why the field mycology might provide a further tool towards the investigation of crime scenes in forest ecosystems. The fruiting structures of certain fungi, particularly the ammonia and postputrefaction fungi, have been recorded repeatedly in association with decomposed mammalian cadavers in different regions of the world (Carter and Tibbett 2003). Based on these reports, the

present study investigated the presence of fungi during three decomposition stages: the bloated, putrefaction and skeletonization stages.

Material and methods

Ethical aspects

The present study was previously submitted to evaluation by the research ethics committee of State University of Ceará and obtained approval under number 064969333-9.

Sample collection sites

The samples were gathered at the city morgue in Fortaleza, in the state of Ceará (northeastern Brazil), and from public cemeteries in that state. All the cadavers were examined between January and December 2007.

Study subjects

The samples were taken from human corpses in three stages of decomposition: bloating stage ($n = 34$), putrefaction stage ($n = 6$) and skeletonization stage ($n = 20$). Each cadaver was evaluated in advance to establish the postmortem interval. All subjects were between the ages of 18 and 60 at death, victims of unnatural death – homicide, suicide, accidental asphyxiation, poisoning, electrocution, traffic accidents and other mechanical traumas. In the bloating stages, all corpses were found at home. In the putrefaction stage, four corpses were examined after being exhumed and two were found in fields. In the skeletonization stage, all corpses were found in fields.

Biosafety

To preserve the integrity of the material collected and to protect the researchers' health, biological masks and 0disposable caps, gowns, slippers and gloves were worn at all times (Brunicardi *et al.* 2006).

Gathering the samples

The cadaver material was taken from the sites with the greatest probability of fungal growth: mouth, rectum, vagina, under the foreskin, lungs, skin, scalp hair, clothing and the surrounding area (grave soil and coffin fragments). The skin samples, approx. 1·0 cm in length, were taken by scraping with a sterile scalpel, and the hair samples, approx. five units with 1·0 cm in length, were obtained with sterile tweezers. These samples were decontaminated before collection with 70% isopropyl alcohol. Sterile swabs were used to collect material from the mucosas of the mouth, rectum, vagina and under the foreskin, using a rotating movement. At least two samples were collected from each site, one for direct examination and the other for culturing (Balows *et al.* 1992). In the case of the samples from the pulmonary sites, a biopsy was performed with a sterile scalpel, and a single fragment 1·0 cm in length was retrieved from the main bronchial tube and one from the peripheral bronchial tube. For characterization of the cadaver environment in the skeletonization stage, when organic material is scanty, samples of approx. 1·0 cm in length from clothing and coffins and 1·0 g of grave soil were collected. The samples were placed in a test tube with 3 ml of saline solution at a temperature of 25–28°C and sent to the Specialized Medical Mycology Center (CEMM) of the Department of Pathology and Legal Medicine of Ceará Federal University.

Laboratory processing

Each specimen was clarified in 40% potassium hydroxide (KOH) and placed between slides and slide covers for microscopic examination at 100× and 400×. Along with direct examination, an aliquot of the material was inoculated at three points in dishes containing 2% glucose Sabouraud agar, Sabouraud agar with vancomycin plus polymixin B (to inhibit the growth of Gram-positive and Gram-negative contaminating bacteria, respectively) and Sabouraud agar with vancomycin plus polymixin B and cycloheximide (to inhibit contaminating airborne fungal strains). The dishes were incubated at 25–28°C in the dark for up to 20 days, with daily observations until fungal growth was detected. The fungi were identified by phenotypical analyses, comprising macromorphology (texture, surface and diameter of the colony as well as the presence of pigmentation), micromorphology (size, surface and pigmentation of conidia and morphology of conidiogenic cells) and biochemical tests (e.g. nitrogen assimilation test and carbohydrate fermentation test) (De Hoog *et al.*, 2000).

Results

Clinical specimen inserts were carried out in 234 direct examinations from corpses in the bloated stage, 42 in the putrefaction stage and 120 in the skeletonization stage. None of them produced results of any diagnostic value because the intense bacterial growth prevented visualization of the fungal structures at this stage of the study. The morphological analyses were performed for *Aspergillus* spp. and *Penicillium* spp. identification. In this way, a set of morphological characteristics allowed unambiguous identification of the few species isolated. These characteristics were as follows: *Aspergillus flavus* – radiating

conidial heads, uni- and biseriate conidiogenous cells, conidiophore hyaline, echinulate conidia, 3·5-μm-diameter, yellowish-green colonies; *Aspergillus niger* – radiating conidial heads, smooth-walled conidiophores, 50–100-μm-diameter vesicles, biseriate conidiogenous cells, metulae twice as long as the phialides, brown and ornamented conidia with warts, 3·5–5·0 μm in diameter; *Penicillium piceum* – colonies growing moderately, rapidly in Czapek Dox agar, velutinous to floccose, pale to bright yellow, with olivaceous green conidiation and orange-brown to dark brown reverse, thin- and smooth-walled conidiophores 15–22 um long, biverticillate penicillin cells, metulae in dense whorls, 7–12 × 3–4 μm, each metulae with 3–8 phialides, smooth-walled conidia, ellipsoidal 3·0–3·5 × 2·2–2·5 μm; *Penicillium rugulosum* – restricted velutinous colonies, yellow-green to dark green in colour, attaining <12-mm diameter in 7 days, conidiophores with 70–100-μm-long stipes and with smooth walls, usually biverticillate penicillin cells, metulae 10–15 μm long, ellipsoidal conidia, 3·0–3·5 × 2·5–3·0 μm; *Penicillium verruculosum* – velutinous or floccose colonies, attaining over 18-mm diameter in 7 days, white to bright yellow mycelium, green conidial mass, smooth-walled conidiophores with stipes 150–250 μm long, phialides with 7–10 whorls, 8–15-μm long, spherical conidia with roughened walls, 3·0–3·5 μm in diameter, good growth at 37°C; *Candida albicans* – cream-coloured colonies, pseudomycelium emerging from cellular clump and Chlamydoconidia on Tween-80 agar and germ tubes with human serum and growth at 37°C; *Candida parapsilosis* – cream-coloured colonies, pseudomycelium present, mostly abundant with giant cell, with width 8–10 μm and length 15–20 μm (De Hoog *et al.* 2000).

From the 34 cadavers in the bloated phase, we collected 234 samples for laboratory analysis, distributed among mucosas (n = 102), hair (n = 64), skin (n = 34) and

Table 1 Species of fungi isolated from the hair, skin, mucosas and lungs of corpses in the bloated, putrefaction and skeletonization stages

	Stage of decomposition		
Site	Bloated stage (n = 143)	Putrefaction stage (n = 12)	Skeletonized stage (n = 26)
Hair	*Aspergillus flavus* (17) *Aspergillus niger* (14) *Penicillium rugulosum* (9) *Penicillium piceum* (2) *Candida albicans* (4) *Candida parapsilosis* (5) *Trichosporon* sp. (4) *Acremonium* sp. (1) *Mucor* spp. (2) *Geotrichum* sp. (1)	*A. flavus* (1) *A. niger* (1) *Penicillium* sp. (1) *Acremonium* sp. (1)	*A. flavus* (5) *A. niger* (7) *Penicillium* sp. (4) *P. rugulosum* (3) *P. piceum* (1) *Trichosporon* sp. (1) *Mucor* sp. (4) *Trichoderma* sp. (1)
Skin	*A. flavus* (7) *A. niger* (3) *P. rugulosum* (3) *P. piceum* (1) *C. albicans* (5) *C. parapsilosis* (5) *Mucor* spp. (3)	*A. flavus* (1) *C. albicans* (1)	–
Mucosas	*A. flavus* (11) *A. niger* (5) *P. rugulosum* (3) *P. piceum* (2) *C. albicans* (10) *C. parapsilosis* (6) *Trichosporon* sp. (1) *Acremonium* sp. (1) *Mucor* sp. (1) *Fusarium* sp. (1)	*P. piceum* (1) *C. albicans* (2) *Candida guilliermondii* (2)	–
Lungs	*A. flavus* (5) *A. niger* (3) *Penicillium* sp. (1) *P. rugulosum* (1) *C. albicans* (2) *C. parapsilosis* (3) *Trichoderma* sp. (1)	*C. albicans* (1)	–

lungs (n = 34). The quantitative association between the fungal genera and external collection sites (hair and skin) in the bloated stage was as follows: hair – *A. flavus* = 17, *A. niger* = 14, *P. rugulosum* = 9, *P. piceum* = 2, *C. albicans* = 4 and *C. parapsilosis* = 5, *Trichosporon* sp. = 4, *Mucor* sp. = 2, *Geotrichum* sp. = 1 and *Acremonium* sp. = 1; skin – *C. albicans* = 5 and *C. parapsilosis* = 5, *A. flavus* = 7, *A. niger* = 3, *P. rugulosum* = 3, *P. piceum* = 1 and *Mucor* sp. = 3. The results in the oral, genital and rectal mucosas were as follows: *A. flavus* = 11, *A. niger* = 5, *C. albicans* = 10 and *C. parapsilosis* = 6, *Penicillium* sp. = 5 *P. rugulosum* = 3, *P. piceum* = 2, *Acremonium* sp. = 1, *Fusarium* sp. = 1, *Trichosporon* sp. = 1 and *Mucor* sp. = 1. The following fungi were found in the lungs: *A. flavus* = 5, *A. niger* = 3, *C. albicans* = 2, *C. parapsilosis* = 3, *Penicillium* sp. = 1, *P. rugulosum* = 1 and *Trichoderma* sp. = 1 (Table 1).

The samples taken from the external sites (hair and skin) of the six corpses examined in the putrefaction stage yielded the following genera: hair – *A. flavus* = 1, *A. niger* = 1, *Penicillium* sp. = 1 and *Acremonium* sp. = 1 and skin – *A. flavus* = 1 and *C. albicans* = 1. The fungi present in the oral, genital and rectal mucosas were as follows: *Candida guilliermondii* = 2 and *C. albicans* = 2; *P. piceum* = 1. Only one *C. albicans* strain was found in the lungs (Table 1). Finally, the samples taken from the 20 corpses evaluated in the skeletonization stage yielded 90 isolates of filamentous fungi and three isolates of yeasts. The sites of the corpses themselves (hair and bone) produced the following genera: hair – *A. niger* = 7, *A. flavus* = 5, *P. rugulosum* = 3, *P. piceum* = 1, *Mucor* sp. = 4; *Trichoderma* sp. = 1 and *Trichosporon* sp. = 1 and bone – *Penicillium* spp. = 10 e *P. verruculosum* = 1, *A. flavus* = 6, *A. niger* = 3 and *Mucor* sp. = 3 (Table 1).

The nearby sites examined (clothing, soil and coffins) yielded the following genera: clothing – *A. niger* = 7, *A. flavus* = 5, *Penicillium* sp. = 9, *P. piceum* = 1; *Mucor* sp. = 4 and *Acremonium* sp. = 1; soil – *A. flavus* = 4, *A. niger* = 3, *Penicillium* sp. = 4, *P. piceum* = 1; *Mucor* sp. = 3 and *Trichosporon* sp. = 1; and coffins – *C. albicans* = 1 (Table 2). After identification, the fungi were deposited in the collection of the Specialized Medical Mycology Center (CEMM, Ceará Federal University).

Discussion

Because of the many factors – environmental and individual characteristics of corpses – that affect the growth of fungi, it is not surprising that there are differences between the few reports available to forensic mycology researchers (Ishii *et al.* 2007) and the findings described in regions with peculiar characteristics, such as north-eastern Brazil.

One point stands out in results of fungal isolation in all the stages evaluated (bloated, putrefaction and skeletonization), namely that direct mycological examination is not fruitful. The bacterial kinetics promotes faster division of the prokaryotic cells in comparison with the eukaryotic fungal cells. The latter also require a narrower range of temperature, pH, moisture and luminosity conditions for adequate division and population dynamics (Murray *et al.* 1990). Therefore, direct mycological examination by optical microscope is of negligible value to identify the postmortem fungi.

Table 2 Genera of fungi isolated from nearby sites examined (clothing, soil and coffins)

	Stage of decomposition		
Site	Bloated stage (n = 0)	Putrefaction stage (n = 0)	Skeletonized stage (n = 67)
Bones	–	–	*Aspergillus flavus* (6) *Aspergillus niger* (3) *Penicillium* spp. (10) *Penicillium verruculosum* (1) *Mucor* spp. (3) *A. flavus* (5)
Clothes	–	–	*A. niger* (7) *Penicillium* spp. (9) *Penicillium piceum* (1) *Mucor* spp. (4) *Acremonium* sp. (1) *A. flavus* (4)
Soil	–	–	*A. niger* (3) *Penicillium* spp. (4) *P. piceum* (1) *Trichosporon* sp. (1) *Mucor* spp. (3)
Coffins			*Candida albicans* (1)

Instead, it is essential in forensic mycology studies to use culture media and/or other complementary laboratory methods to identify the presence of fungi. Regarding the form of asexual fungal division, the filamentous form prevailed in the bloated and skeletonization stages. As known, the majority of fungi reproduce asexually in nature, and many of their representatives are airborne strains and can easily grow on practically any substrate (e.g. *Penicillium* spp. and *Aspergillus* spp.) (Sharma 1988).

The results found in this study may not reflect the diversity of fungi in each stage of decomposition. We believe that refinement of the method employed can have a great impact on the recovery of some fungal species. In this respect, it is possible that the inclusion of other substances to the isolation media, such as benomyl, capable of inhibiting the growth of *Aspergillus* sp. (Luz *et al.* 2007), will permit isolating species with less competitive power, such as the dematiaceous fungi.

The abundant growth of *Aspergillus* spp. and *Penicillium* spp. under the conditions employed might have impeded the isolation of typical soil species, such as the hyphomycetes, which were not recovered even with the addition of the inhibitor cyclohexamide to the culture media. To permit the growth of other fungal species not isolated in this work, we also suggest incubation of the material at a temperature of 37°C, because of the heat sensitivity of various species of *Penicillium* spp. (De Hoog *et al.* 2000).

In contrast, yeasts predominated in the putrefaction stage. In the oral, genital and rectal mucosas, the isolation of yeasts is more frequent because they are part of the normal microbiota of these areas. Until the present study, there were only a limited number of reports of the isolation of the fungal micro-organisms cited here under conditions of corpse putrefaction (Ishii 2006; Ishii *et al.* 2007). As a rule, this absence of information has been because of the failure to collect and investigate material under those conditions. Although the results demonstrated here are only descriptive, the mere presence of these fungi in these conditions is the reason for greater interest in fungi in forensic medicine, as a way to establish the time of death.

The external collection sites were more propitious than the internal ones for fungal growth for both airborne fungi and yeasts, especially the genera *Aspergillus* and *Candida*, respectively. This corroborates other works on the postmortem alterations caused by fungi (Collier 2005). The yeasts also grew more on the skin than in the hair region. This fact can be explained by the after-death rupture of the skin barriers and greater contact with mucous secretions, besides their possible presence in the skin microbiota.

Another interesting observation regarding our postmortem isolation of fungi is the absence of dematiaceous fungi (able to produce pigment, generally melanin) among those isolated. This fact may be because of the growth characteristics common to various representatives of this group, which usually grow slowly, producing lesions with chronic evolution. Also, the intense competition with bacteria, hyaline fungi and insects could have contributed to their absence in this study.

Although observation of the presence of fungi on the surface of corpses by forensic medical practitioners is not recent, the isolation of these organisms is not carried out routinely. Because their description can even give indications of the place of death, additional studies are necessary for the use of fungi as a forensic tool (Ishii 2006).

The present study is only a starting point, and the results are not yet sufficient to allow the presence of fungi to act as effective biological markers of the time of death. However, it does demonstrate that there are differences in the fungi isolated during the process of corpse decomposition, especially by the isolation of fungi such as *Aspergillus* spp., *Penicillium* spp. and *Candida* spp. These results indicate that the presence of fungi on, in and around cadavers can provide additional information to determine the time of death as accurately as possible. Further research with larger samples and more detailed description of conditions is necessary to ratify the findings presented here and to establish the real importance of mycology as a tool to assist with forensic medicine, as is already the case of forensic entomology (Menezes *et al.* 2007).

Conclusion

These findings already permit tracing out a horizon for deeper understanding of the subject. However, much more research will be necessary to develop this new segment of mycology, enabling the frequent use of its findings in forensic science, as is already the case with entomology.

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