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The importance of appropriate processing and direct microscopic examination for the timely diagnosis and management of invasive infections caused by filamentous fungi

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Abstract

The gold standard for diagnosis of invasive fungal infections caused by filamentous fungi remains the visualization of fungal elements in fluids, and biopsy/tissue collected from a normally sterile body site. Parallel recovery of viable fungus from the sample subsequently permits antifungal susceptibility testing of the individual isolate. Central to both processes is the appropriate processing of tissue specimens to avoid damaging fungal elements and optimize viable organism recovery. Historically, mycologists have proposed that homogenization (grinding or bead-beating) of tissue should be avoided in cases of suspected fungal infection as it likely damages hyphae, instead preferring to chop tissue into small portions (dicing) for direct microscopic examination and culture. Here, we have compared the two processes directly on material from clinical patient cases of mucoromycosis and invasive aspergillosis. Representative portions of fresh biopsy samples were processed in parallel either by chopping (dicing) in the mycology reference laboratory or by bead-beating in the adjoining general microbiology laboratory. Aliquots of the samples were then cultured under identical conditions and subjected to direct microscopic examination. The results demonstrated that tissue homogenization significantly reduced (i) organism recovery rates in cases of both mucoromycosis and invasive aspergillosis and (ii) the number of fungal elements detectable upon direct microscopic examination. To our knowledge, this is the first study to directly compare these alternative processing methods and despite only employing a limited number of samples the data presented here, provide support for the perceived mycological wisdom that homogenization of tissue samples should be avoided when filamentous fungal infections are suspected.

Lay Summary

The gold standard for diagnosis of fungal infections remains the visualization of fungal elements in samples from usually sterile sites. Here we show that certain methods employed for processing biopsy samples significantly impact the ability to detect and grow fungi from genuine cases of infection.

Keywords: biopsy, tissue processing, *Aspergillus*, mucoraceous moulds, filamentous fungi, culture, microscopy, diagnosis of fungal infections

Introduction

Invasive fungal infections (IFIs) caused by filamentous fungi (moulds) continue to cause significant morbidity and mortality in immunocompromised patients.[1,2] However, diagnosis of IFIs remains challenging, despite a growing plethora of non-culture-based diagnostic strategies (reviewed in[3,4]). The gold standard for the diagnosis of invasive fungal infections due to filamentous fungi remains the visualization of fungal elements in tissue from a normally sterile site.[5–8] The sensitivity of culture of the causative organism from such specimens is notoriously poor, probably due to sub-optimal sampling and sample processing coupled with difficulties with interpretation of culture results, and the time-to-result is often protracted.[7,9–11] In contrast, microscopic examination of a fresh biopsy or tissue samples can be performed rapidly, and when fluorescent enhancers are employed is more sensitive than culture.[12] Moreover, microscopic inspection of the nature of fungal elements can also allow differentiation between several different fungal diseases, e.g., between hyalohyphomycosis (infection with narrow, septate, acutely branching hyaline hyphae), phaeohyphomycosis (infection with narrow, septate, darkly pigmented hyphae), and mucoromycosis (infection with broad, pauciseptate, ribbon-like hyphae), with the latter representing a medical emergency due to its rapid progression.[13–15] Subsequent recovery of the responsible fungus in the culture, if successful, would also allow antifungal susceptibility testing of the individual isolate, to aid optimization of antifungal treatment strategies.[6,16]

In most general microbiology laboratories, tissue samples are routinely processed by homogenization (using tissue grinding or in some cases bead-beating) to help liberate bacterial pathogens and aid their microscopic detection and subsequent recovery in culture. For many years, the perceived mycological wisdom has been that tissue/biopsy specimens

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Table 1. Summary of the results of parallel processing of tissue samples by dicing versus bead-beating.

		Mycology		Microbiology	
Patient	Sample	Microscopy[a]	Culture	Microscopy[b]	Culture
1	Tissue, thigh	Large amounts pauciseptate hyphae (5 h)	*Mucor circinelloides* (d4)	Not performed	*M. circinelloides* (d4)
1	Mouth swab	Moderate amounts pauciseptate hyphae (6 h)	*Mucor circinelloides* (d2)	Not performed	*M. circinelloides* (d2)
2	Left nasal mucosa	Moderate amounts pauciseptate hyphae (2 h)	*Lichtheimia corymbifera* (d4)	Gram + ve cocci	Mixed bacteria *L. corymbifera* (d5)
2	Nasal septum	Large amounts pauci septate hyphae (2 h)	*Lichtheimia corymbifera* (d4)	Not performed	Not performed
2	Nasal septal cartilage	Large amounts pauciseptate hyphae (2 h)	*Lichtheimia corymbifera* (d4)	Gram + ve cocci	Mixed bacteria
2	Left inferior turbinate	Moderate amounts pauciseptate hyphae (2 h)	*Lichtheimia corymbifera* (d4) + MICs (d6)	Negative	Mixed bacteria
3	Brain biopsy	Large amounts of septate filamentous fungal hyphae (2 h)	*Aspergillus fumigatus* (d5)	Negative	*Corynebacterium propinquum* (d2)
4	Left middle turbinate	Large amounts of septate filamentous fungal hyphae (4 h)	*Aspergillus fumigatus* (d5) Azole-resistant (d8)	Negative	*Enterococcus faecium* (d7)

[a]Microscopy performed using calcofluor white and fluorescent microscopy.
[b]Microscopy performed after Gram-staining.

destined for mycological investigations should be prepared for microscopy and culture by chopping (dicing) into small pieces rather than by homogenization, to avoid damaging fragile fungal hyphae and consequently reducing the ability to detect and recover viable organism.[17–19] Obviously, for yeasts and particularly bacteria, this is not such consideration as they are smaller, less susceptible to mechanical disruption and each cell is a potentially viable propagule. However, as pointed out in a recent Commentary by the Fungal Diagnostics Laboratory Consortium in the USA, formal studies that compare the effect of tissue homogenization (grinding or bead beating) versus chopping (dicing) on the recovery of filamentous fungi, and members of the *Mucoromycota* in particular, are generally lacking.[20]

Here, we have attempted to address this issue by directly comparing the results of both processing methods on clinical patient samples from proven cases of both invasive aspergillosis and mucoromycosis. Representative portions of fresh, microscopy-positive tissue samples were processed by chopping (dicing) at the UKHSA National Mycology Reference Laboratory (MRL) and also by bead-beating following standard protocols in the neighboring general bacteriology laboratory at North Bristol NHS Trust (NBT), the geographic site at which the MRL is physically embedded. Processed samples were cultured in parallel using standard methodologies and also subjected to direct microscopic examination. The results of these comparisons provide preliminary evidence to support pre-existing mycological wisdom that homogenization of tissue samples could significantly impede the detection of fungal elements and recovery of viable fungi in cases of both mucoromycosis and aspergillosis.

Materials and Methods

Clinical samples

Biopsy samples and tissue samples recovered during serial debridement surgeries from four patients with invasive fungal infections (two patients with proven mucoromycosis [patients 1 and 2] and two patients with invasive aspergillosis [patients 3 and 4]) were included in this study (Table 1). All patients were immunocompromised and all samples were taken as part of routine diagnostic algorithms to determine the extent of infection and whether each successive debridement had been sufficient to remove all fungal biomass. Since all four patients were being treated in hospitals local to the Bristol area, tissue samples were received at the MRL and in parallel at NBT on the day of surgery.

Clinical sample processing at the MRL

Tissue samples were prepared for direct microscopic examination using calcofluor fluorescent enhancer (Fig. 1) and cultured at the MRL by chopping (dicing) into ~2 mm^3 pieces using a sterile disposable surgical scalpel in a sterile petri dish in a class II microbiological safety cabinet (MSC) at containment level 3, which is our usual procedure for processing tissues from any deep site. Between 6 and 10 representative 2 mm^3 pieces were then placed into two sterile 25 cm^2 vented tissue culture flasks that had been prepared in-house to contain Oxoid Sabouraud dextrose agar containing 0.5% (wt/vol) chloramphenicol (Unipath Limited, Basingstoke, UK) (Fig. 1a). Flasks were incubated at 30 and 37 °C for 2 weeks, with the daily examination. The remaining pieces from each sample were softened by incubation on a heat block at 50 °C for 15 min in an Eppendorf tube containing a few drops of 10% w/v KOH. For direct microscopic examination, the portions of softened tissue were placed on a sterile glass slide, covered by a drop of calcofluor fluorescent enhancer,[21] and squashed under a sterile coverslip to produce a monolayer of cells. Microscopic examination of softened fragments was performed using a fluorescent microscope fitted with a V-2A filter, with an excitation wavelength of 380–420 nm, a 430 nm dichromatic mirror, and a 450 nm barrier filter.

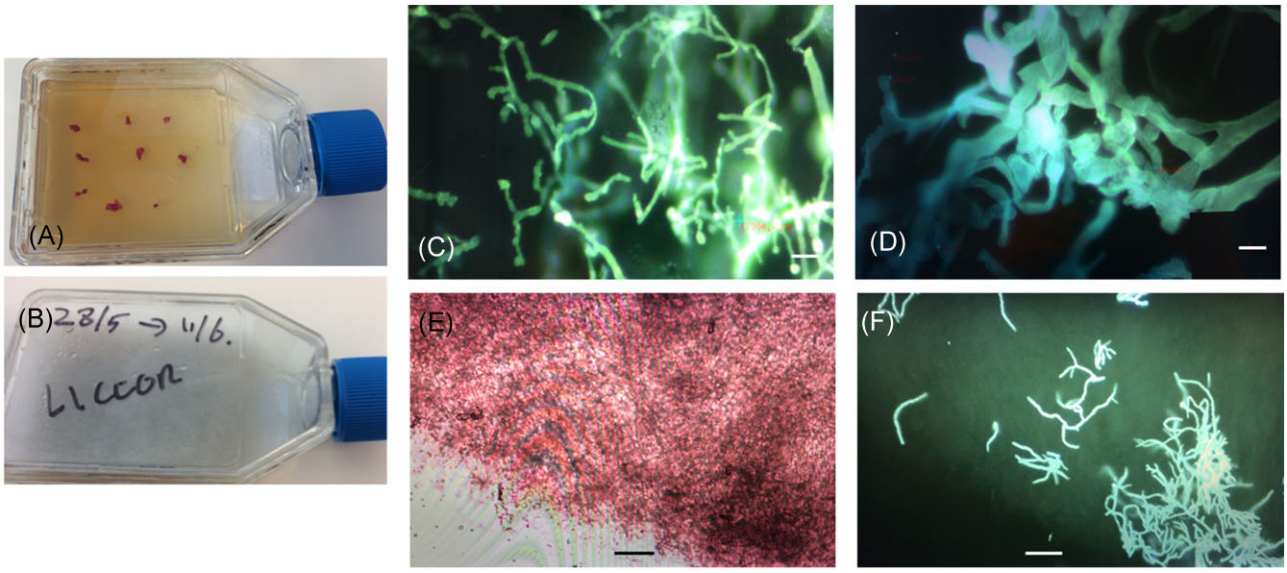

Figure 1. Sample processing and microscopy at the MRL. Panels A and B: Tissue samples from patient 2, post chopping (A) and after 4 days of incubation at 37°C (B). Tissue sample from patient 2 post KOH digestion, calcofluor treatment and microscopic examination under UV illumination, revealing broad, irregular, folded, ribbon-like, pauci-septate hyphae typical of the agents of mucoromycosis. Tissue sample from patient 4 post digestion in KOH and calcofluor, observed under normal and UV light (panels E and F, respectively), showing narrow, regular, dichotomously branched hyphae, typical of the agents of hyalophyphomycosis. Scale bars = 100 μm (Panels C, E and F) and 10 μm (panel D).

Clinical sample processing at NBT

At NBT, for processing of small samples (less than the size of a garden pea), Ballotini beads in sterile distilled water were added directly to the original sample container and homogenized by mixing on a vortex mixer for 1 min. For larger samples, representative pieces were cut from the main tissue using a disposable scalpel and disposable forceps in a petri dish and added to Ballotini beads with 5 ml of sterile water, prior to vortex mixing for 1 min. All tissue processing was performed in a class II MSC in a containment level two laboratory. After homogenization by bead-beating, a drop of the homogenized tissue sample was transferred to a clean microscope slide, spread with a sterile loop to make a thin smear, and then Gram-stained using standard procedures.[22] In parallel, homogenates were cultured on Blood agar, Chocolate agar, and Cysteine-lactose-electrolyte-deficient (CLED) plates at 37 °C for 48 h, fastidious anaerobic agar at 37 °C for 5 d, and two Sabouraud dextrose agar plates containing 0.5% (wt/vol) chloramphenicol, incubated at 30 and 37 °C for 7d (all media Oxoid, Unipath Limited, Basingstoke, UK).

Quantification of comparative fungal hyphal yield using each processing method

Quantification of the quantities of fungal hyphae detectable after calcofluor staining of processed samples was performed using the online version of Image J (ImageJ.JS (imjoy.io) exactly as described previously (https://www.unige.ch/medecine/bioimaging/files/1914/1208/6000/Quantification.pdf).
Briefly, .jpeg versions of images were imported into Image J, converted from RGB to grayscale prior to background subtraction using the 3D-rolling ball, threshold setting, and then pixel number/intensity quantification using 'analyze particles'.

Results

Between March 2019 and May 2021, invasive fungal infections were suspected in four independent patients from hospitals in the Bristol region where tissue samples were available to aid in definitive diagnoses. For all four patients, portions of biopsy material//tissue samples were sent in parallel to the MRL for detailed mycological analyses and to NBT to be processed using general bacteriology techniques. Since the MRL employs chopping (dicing) of tissue, calcofluor staining and culture on mycological media and NBT process tissue samples using general bacteriology techniques (tissue homogenization by bead-beating, gram staining and culture on various media including Sabouraud agar), comparison of the results of the microbiological investigations in the two departments allowed an appraisal of the effects of tissue processing method on diagnostic success (positive direct microscopic examination and also recovery of the viable organism in culture).

As can be seen from Table 1, all eight tissue samples from the four independent patients were direct microscopy positive using MRL techniques. Moreover, careful microscopic examination of KOH-digested, calcofluor-stained tissue allowed the rapid diagnosis of mucoromycosis in two cases and hyalohyphomycosis (aspergillosis) in the other two cases, based on the appearance and size of hyphal fragments visualized in the tissue samples (Fig. 1c–e). These preliminary diagnoses based on microscopic examination were made 2–5 h after receipt of the tissue/biopsy samples (Table 1). These initial diagnoses were confirmed by recovery of the viable organism in culture in all cases 2–5 d post receipt of the tissue sample: two patients had mucoromycosis (one case of disseminated infection due to *Mucor circinelloides*, and one case of invasive sinusitis due to *Lichtheimia corymbifera*; patients 1 and 2, respectively; Table 1; Fig. 1b) and two had invasive aspergillosis (one case of cerebral infection and one case of invasive sinusitis; patients 3 and 4, respectively; Table 1). Furthermore, antifungal susceptibility testing of the isolate of *A. fumigatus* from

patient 4 using CLSI broth microdilution methodologies[22] demonstrated that this isolate was resistant to itraconazole, voriconazole, posaconazole and isavuconazole on day 8 after tissue sample receipt, a clinically important result that was only possible because an isolate had been recovered in culture for susceptibility testing. Subsequently, we undertook molecular confirmation of the presence of the L98H mutation associated with the finding of environmentally acquired azole resistance in this isolate (data not shown). In contrast, the presence of fungal elements was not reported in any of the eight samples processed at NBT using bead-beating of tissue/Gram stain (where performed), and viable fungi were recovered in culture from only 3/8 samples. A total of 2/8 samples grew the mucoraceous mould *Mucor circinelloides* which is well-known for producing oidia within the hyphae, each of which can act as a viable propagule, thus making it less susceptible to complete loss of viability due to the homogenization process. It is one of the few mucoraceous moulds known to do this and in our patient incidentally allowed the hematogenous dissemination of infection from a primary oral mucosal site to cutaneous lesions of the arm and thigh. This is very unusual for mucoraceous mould infections which normally demonstrate rapid spread to contiguous sites. Indeed, the MRL received a total of 12 independent tissue samples for patient 2, all of which were direct microscopy positive, and 8 of which yielded *L. corymbifera* in culture. NBT received parallel aliquots of five of those samples, only one of which yielded viable organisms. Similarly, the MRL received 10 independent samples for patient 4: six were direct microscopy positive, and seven were positive after culture. For the five parallel samples processed at NBT, all were both microscopy- and culture-negative. These anecdotal results provide strong suggestive evidence that bead-beating or homogenization of tissue samples is deleterious to the detection and recovery of *Aspergillus* spp. and the agents of mucoromycosis in tissue biopsies and also underline the reduced sensitivity of Gram-staining as compared with calcofluor and fluorescent microscopy for the detection of fungal elements.

Since it remained possible (though highly unlikely) that the above anecdotal results stemmed from uneven distributions of fungal elements in the samples submitted to MRL and NBT, we assessed the issue of the processing method on hyphal integrity directly. For three tissue samples from serial debridement surgeries from patient 2 (Table 1), sufficient material remained after primary processing to allow direct, in parallel comparisons of the effects of the processing method on fungal hyphal integrity. All three biopsy samples from this patient were shown to harbor high burdens of fungal hyphae on the processing of small, representative portions of each sample at the MRL (data not shown). Thus, representative, randomly selected portions of these three tissue samples (A, B and C; Fig. 2) were prepared for processing by chopping (dicing) at the MRL and bead-beating at NBT and then returned to the MRL for microscopic examination after KOH treatment and calcofluor staining to allow estimation of the quantity and integrity of fungal hyphae post-processing. While large quantities of broad pauci-septate fungal hyphae were visible in all three samples processed by chopping (dicing) at the MRL (Fig. 2d–f), microscopic examination of the equivalent aliquots processed by homogenisation at NBT revealed scant, short, heavily damaged fragments of hyphae only, with an estimated ~75% reduction in hyphal quantities (Fig. 2g–i; residual hyphae quantified by Image J of 22.6%, panel G compared to D; 25.4%, panel H compared to panel E, and 22.2%, panel I compared with panel F). Since the three starting samples had been stored frozen at −20 °C after primary processing, which is known to deleteriously impact fungal recovery,[23] culture of the post-processed samples was not attempted in this part of the study.

Discussion

The gold standard for the diagnosis of invasive fungal infections caused by filamentous fungi is the visualization of fungal elements in clinical material collected aseptically from a normally sterile site.[5–8] However, it is generally accepted that the sensitivity of culture from such specimens is notoriously poor, at least in part due to issues with optimal approaches to sample processing, and microscopic examination when fluorescent enhancers are employed has proven more sensitive than culture.[7,9–12] Despite these findings, as pointed out recently, formal studies that compare the effect of tissue homogenization (grinding or bead beating) versus chopping (dicing) on the recovery of filamentous fungi are to date lacking.[20] This study has at least partially addressed this issue, and demonstrated that homogenization of tissue samples by bead-beating as anticipated substantially reduces the ability to detect and recover viable fungus.

The methods employed for tissue sample processing at the MRL (chopping of tissue into ~2 mm^3 sections, some of which are used for KOH digestion/calcofluor, other portions of which are used for protracted culture on mycological media) significantly improved the detection and culture of *Aspergillus fumigatus* and also two agents of mucoromycosis from clinically relevant diagnostic tissue samples, when compared with bead-beating and gram stain/culture on a variety of bacteriological and mycological media. The deleterious effects of tissue homogenization on the recovery of *A. fumigatus* were somewhat surprising given the septate nature of *A. fumigatus* hyphae, where the presence of regular septation could be predicted to wall-off regions of damage and protect from leakage of adjoining hyphal contents, thereby leaving at least some potentially viable propagules. In contrast, the devastation to the delicate mucoraceous mould mycelium could be predicted by the lack of regular cross-walls as isolates are pauci-septate, thus damage to one area of mycelium can have devastating consequences to much larger areas due to leakage of cellular contents. This is often the explanation of why hyphae may be seen on direct microscopic examination or histology but there is still failure to grow in culture or even to isolate DNA for molecular diagnostic tests as all the cytoplasm and nuclear contents have leaked out leaving only the fragmented cell walls. This is the reason why when we process fixed tissue samples from wax blocks for molecular examination, we request 6–10 thick cut slices to try to maximise the chances of isolating DNA. The anecdotal results obtained from parallel processing of samples submitted to MRL and NBT, which suggested that tissue homogenisation damages fungal hyphae, were confirmed using aliquots of repeat biopsy samples that were chopped or homogenised and then examined by KOH/calcofluor, which demonstrated that homogenisation resulted in a ~75% reduction in the detection of intact hyphal elements. To our knowledge, this is the first study of this kind that directly examines the effects of tissue processing method on fungal viability and hyphal integrity.

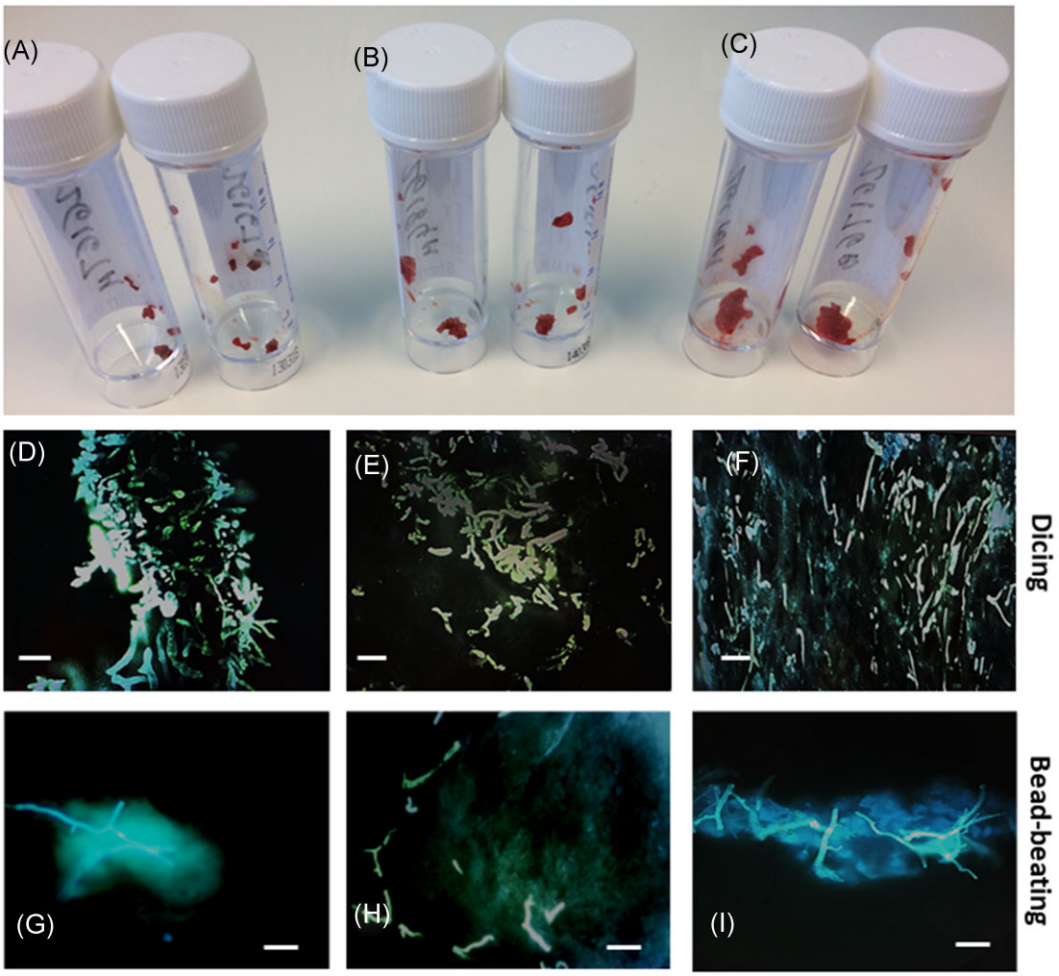

Figure 2. Bead-beating reduces hyphal integrity detection post-processing. Parallel processing of 3 representative samples from patient 2 (Panels A–C) by chopping (dicing), KOH digestion and calcofluor staining (Panels D–F ; corresponding to samples A–C, respectively) or bead-beating, KOH digestion and calcofluor staining (Panels G–I; corresponding to samples A–C, respectively). All microscopy was performed under UV illumination. All scale bars = 100 μm. Photographs corresponding to minced versus bead-beaten samples are indicated on the right-hand side.

We would also like to highlight several additional features of sample processing at the MRL that might improve the diagnosis of invasive fungal infections if adopted in general microbiology laboratories. Samples, after processing are cultured in vented medical flat tissue culture flasks poured in-house with Sabouraud agar. In addition, when samples are processed a separate 'sterility test' flask is always included, and left lid-off for the duration of the entire processing session. The use of medical flats rather than conventional Petri dishes is an important consideration when processing samples that might contain dimorphic, Hazard Group 3 (HG3) fungi, as they limit the risks of exposure of laboratory staff to such high-risk pathogens. In some laboratories with very high sample throughput the use of tissue culture flasks may be cost-prohibitive and Petri dishes more cost-effective. In such scenarios, we would still encourage the introduction of measures designed to reduce exposures of laboratory staff to HG3 fungi, including sealing of inoculated plates/dishes with tape, or bagging of individual dishes. It is also worth underscoring here that all samples from sterile sites processed at the MRL are incubated in parallel at 30 and 37 °C, to capture the thermally dimorphic forms of such fungi. Additionally, the use of closed flasks and the inclusion of a sterility test reduce the risk of recovery of contaminating environmental fungi, and the possibility to detect environmental contamination, respectively, thus precipitating deep cleaning as required. Finally, the culture of tissue samples as visible, discrete, chopped 2 mm^3 pieces of tissue regularly placed across the culture flask surface also facilitates decisions regarding whether any fungi recovered in culture were actually from one of the inoculum sites, or rather an accidental bystander.

The major limitation to the current study involves the relatively small sample size (eight tissue samples from four patients, encompassing two independent infections with *A. fumigatus* and two further infections with different members of the Mucoromycota). This limitation stems entirely from the difficulty in acquiring appropriate human clinical patient samples from proven cases of invasive fungal disease in sufficient quantities to allow parallel comparisons of processing approaches. First, tissue-based diagnosis of invasive fungal infections, whilst the gold standard, is most often achieved using small quantities of sample acquired via minimally invasive techniques (e.g., need-guided biopsies), meaning that inadequate material is available after initial diagnostic work-up to permit comparisons of the type described here. Additionally, samples with homogenous and high tissue burden are relatively rare,

and the use of samples from infections with low tissue burdens increases the likelihood that aliquots used for any comparison are not representative. Finally, the use of animal models of fungal infection, or artificial infection of autopsy material (both of which could facilitate comparisons with larger numbers of samples and different fungal organisms) are unlikely to faithfully reproduce the complete pathological processes inherent to human invasive fungal infections. In summary, the results obtained with all eight tissue samples examined in the current study do lend support to the conventional mycological wisdom that homogenization of tissue samples should be avoided if fungal infections are suspected. However, further studies will be required to definitively confirm this hypothesis.

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Declaration of interest

None.

References

1. Kontoyiannis DP, Marr KA, Park BJ et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin Infect Dis*. 2010; 50: 1091–1100.
2. Caira M, Girmenia C, Fadda RM et al. Invasive fungal infections in patients with acute myeloid leukemia and in those submitted to allogeneic hemopoietic stem cell transplant: who is at highest risk? *Eur J Haematol*. 2008; 81: 242–243.
3. Miceli MH, Maertens J. Role of non-culture-based tests, with an emphasis on galactomannan testing for the diagnosis of invasive aspergillosis. *Semin Respir Crit Care Med*. 2015; 36: 650–661.
4. Kidd SE, Chen SC, Meyer W, Halliday CL. A new age in molecular diagnostics for invasive fungal disease: are we ready? *Front Microbiol*. 2020; 10: 2903.
5. Dornbusch HJ, Groll A, Walsh TJ. Diagnosis of invasive fungal infections in immunocompromised children. *Clin Microbiol Infect*. 2010; 16: 1328–1334.
6. Arendrup MC, Bille J, Dannaoui E et al. ECIL-3 classical diagnostic procedures for the diagnosis of invasive fungal diseases in patients with leukaemia. *Bone Marrow Transplant*. 2012; 47: 1030–1045.
7. Borman AM, Johnson EM. Interpretation of fungal culture results. *Curr Fungal Infect Rep*. 2014;8: 312–321.
8. Lass-Flörl C. How to make a fast diagnosis in invasive aspergillosis. *Med Mycol*. 2019; 57: S155–S160.
9. Nguyen MH, Leather H, Clancy CJ et al. Galactomannan testing in bronchoalveolar lavage fluid facilitates the diagnosis of invasive pulmonary aspergillosis in patients with hematologic malignancies and stem cell transplant recipients. *Biol Blood Marrow Transplant*. 2011; 17: 1043–1050.
10. Arendrup MC, Chryssanthou E, Gaustad P, Koskela M, Sandven P, Fernandez V. Diagnostics of fungal infections in the Nordic countries: we still need to improve! *Scand J Infect Dis*. 2007; 39: 337–343.
11. Ruhnke M, Bohme A, Buchheidt D et al. Diagnosis of invasive fungal infections in hematology and oncology–guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). *Ann Hematol*. 2003; 82: S141–S148.
12. Pagano L, Pagliari G, Basso A et al. The role of bronchoalveolar lavage in the microbiological diagnosis of pneumonia in patients with haematological malignancies. *Ann Med*. 1997; 29: 535–540.
13. Son HJ, Song JS, Choi S et al. A comparison of histomorphologic diagnosis with culture- and immunohistochemistry-based diagnosis of invasive aspergillosis and mucormycosis. *Infect Dis*. 2020; 52: 279–283.
14. Chavez JA, Brat DJ, Hunter SB, Velazquez Vega J, Guarner J. Practical diagnostic approach to the presence of hyphae in neuropathology specimens with three illustrative cases. *Am J Clin Pathol*. 2018; 149: 98–104.
15. Rudramurthy SM, Hoenigl M, Meis JF et al. ECMM and ISHAM. ECMM/ISHAM recommendations for clinical management of COVID-19 associated mucormycosis in low- and middle-income countries. *Mycoses*. 2021; 64: 1028–1037.
16. Schelenz S, Owens K, Guy R et al. English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR). National mycology laboratory diagnostic capacity for invasive fungal diseases in 2017: evidence of sub-optimal practice. *J Infect* 2019; 79: 167–173.
17. CLSI. 2012. *Principles and practice for detection of fungi in clinical specimens: direct examination and culture. Approved guideline M54-A*. Clinical and Laboratory Standards Institute, Wayne, PA.
18. Leber A. *Clinical microbiology procedure handbook*, 4th ed, vol 2. ASM Press, Washington, DC.
19. Berkow E, McGowan K. Mycology: specimen collection, transport and processing, p 1016–1024. *In* Carroll K, Pfaller M Landry M (ed), *Manual of Clinical Microbiology*, 12th ed. ASM Press, Washington, DC.
20. Zhang SX, Babady NE, Hanson KE et al.Fungal Diagnostics Laboratories Consortium (FDLC). Recognition of diagnostic gaps for laboratory diagnosis of fungal diseases: expert opinion from the Fungal Diagnostics Laboratories Consortium (FDLC). *J Clin Microbiol*. 2021; 59: e0178420.
21. Haldane DJ, Robart E. A comparison of calcofluor white, potassium hydroxide, and culture for the laboratory diagnosis of superficial fungal infection. *Diagn Microbiol Infect Dis*. 1990; 13: 337–339.
22. Bartholomew JW, Mittwer T. The Gram stain. *Bacteriol Rev*. 1952; 16: 1–29.
23. Berikten D. Survival of thermophilic fungi in various preservation methods: a comparative study. *Cryobiology*. 2021; 101: 38–43.