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Genomic Characterization of Recurrent Mold Infections in Thoracic Transplant Recipients

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Conflicts of Interest

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Abstract

Invasive mold disease in thoracic organ transplant recipients is a well-recognized complication, but the long-term persistence of molds within the human body and evasion of host defenses has not been well-described. We present two cases of invasive mold disease (*Verruconis gallopava* and *Aspergillus fumigatus*) in thoracic transplant recipients who had the same mold cultured years prior to the invasive disease presentation. The paired isolates from the index and recurrent infections in both patients were compared using whole genome sequencing to determine if the same strain of mold caused both the index and recurrent infections. In Case 1, the isolates were found to be of the same strain indicating that the initial colonizing isolate identified pre-transplant eventually caused invasive mold disease post-transplant while in Case 2, the two isolates were not of the same strain. These results demonstrate the distinct possibility of molds both persisting within the human body for years prior to invasive mold disease or the long-term risk of recurrent, persistent infection with more than one strain. Further studies of long-term molecular epidemiology of IMD and risk factors for mold persistence in transplant recipients are encouraged.

1. Introduction

Invasive mold disease (IMD) is relatively common in patients who have undergone thoracic solid organ transplantation. For instance, invasive aspergillosis occurs at rates reported between 6.5–14% in orthotopic heart transplant recipients.^{1,2} Within the lung transplant population, IMD has been reported in up to 12% of patients, with *Aspergillus* species being the major pathogen (72.7%).³ In the case of aspergillosis, immunosuppressive therapies for solid organ transplantation increase the risk of IMD by inhibiting T-cell activation and response to cytokines, hindering host defense to *Aspergillus* species at multiple levels.⁴ Theoretically, the risk of recurrent or persistent IMD continues as ongoing immunosuppressive therapy is vital for maintaining the longevity of the allograft. However, the persistence of invasive fungal infection with molds is not as well documented as with cryptococcosis and the endemic mycoses.

While we have insight into clinical (host) risk factors for IMD, we are still learning about the microorganism's ability to adapt and persist within the human body. This adaptation likely involves a combination of physiologic and genetic events.⁵ Next-generation sequencing of mold isolates can provide precise insights into the genetic identification and adaptation of the mold within the body. For instance, it can determine whether the same strain of mold can persist for years in the host and then cause recurrent disease. Here, we present two cases of thoracic organ transplant recipients who were known to have respiratory mold colonization followed by invasive disease due to the same mold species many years later.

Case 1

A 65-year-old man with a history of Hodgkin's lymphoma, a 30-pack/year smoking history, and idiopathic pulmonary fibrosis requiring bilateral orthotopic lung transplantation (2014) presented with subacute hoarseness and dysphagia in 2016. His pre-transplant course was notable for a bronchoalveolar lavage (BAL) culture that had grown *Verruconis gallopava* (formerly known as *Ochroconis*) prompting prophylactic voriconazole for two months

preoperatively. Notably, his serial BAL cultures post-transplant did not grow *V. gallopava* again until 2015, which prompted four more months of voriconazole therapy.

The patient's post-transplant course was complicated by recurrent acute cellular rejection, first treated with a corticosteroid burst and then with rabbit anti-thymocyte globulin. Despite no further evidence of rejection on allograft biopsies, the patient's pulmonary function tests continued to decline, and the patient had ongoing dyspnea and new hypoxia in 2016. He then presented with a cough productive of tan sputum in addition to dysphagia and hoarseness. Physical examination showed a chronically ill-appearing man with a mild increase in work of breathing and coarse breath sounds. Notable laboratory values included a white blood cell count of 4.9/L (normal range $3.2\text{--}9.8 \times 10^9/\text{L}$), CD4 count 39 LYM/uL (11% lymphocytes), hemoglobin of 8g/dL (13.7–17.3g/dL), platelets of 182 ($150\text{--}450 \times 10^9/\text{L}$), and a serum creatinine of 1.3mg/dL (0.6–1.3mg/dL). His immunosuppressive regimen consisted of tacrolimus 0.5mg daily, azathioprine 100mg daily, and prednisone 5mg daily.

A barium swallow exam was performed and aborted due to frank aspiration and visualization of a mass in the area of the tracheal cartilage with apparent narrowing of the proximal airway. A CT of the neck confirmed a heterogeneous mass in the glottis with severe mass effect on the adjacent trachea resulting in focal occlusion (Figure 1a).

The patient underwent tracheostomy for airway protection and biopsy of a transglottic mass (Figure 1b). Due to concern for a malignancy, no operative cultures were sent from the mass. The pathology, however, was consistent with invasive fungal infection showing numerous dematiaceous hyphae. Expecterated sputum cultures from this time grew *V. gallopava* (voriconazole minimum inhibitory concentration [MIC] 0.25mcg/mL and posaconazole MIC 0.06mcg/mL; susceptibility testing on 2013 isolate had not been performed). The patient was treated with voriconazole for suspicion of IMD due to *Verruconis*. Unfortunately, he suffered septic shock from a healthcare-associated pneumonia thought not to be directly related to IMD and passed away six weeks later.

Case 2

In 2014, a 61-year-old man with ischemic cardiomyopathy status-post orthotopic heart transplantation in 2001, chronic kidney disease, and Type 2 Diabetes Mellitus developed a spontaneous ecchymosis along the left upper quadrant of his abdomen with subsequent formation of a pustule. He had a history of an epicardial device placed in the left upper quadrant abdominal wall in 1988. Due to proximity to the phrenic nerve, one of the leads was left in place at the time of his heart transplantation. In 2004, the patient had sinusitis due to *Aspergillus fumigatus* requiring sphenoidostomy with mucosal removal and maxillary enterostomy followed by 9 months of voriconazole therapy. During that evaluation, the patient had a non-contrasted CT scan of his chest which revealed an opacity in the left lower lung lobe adjacent to a pericardial patch from his retained epicardial lead (Figure 2). Findings were concerning for a chronic patch infection. Therefore, he underwent surveillance imaging 6 months later, and this showed improvement but not complete resolution of the left lower lobe opacity. As the patient was asymptomatic, no further interventions were attempted.

When the pustule started draining hemopurulent fluid, the patient sought medical care. At presentation, he denied fevers, chills, malaise, cough, sinus congestion, shortness of breath, abdominal pain, or unintentional weight loss. His immunosuppression regimen included cyclosporine 100mg in the morning and 75mg in the evening, mycophenolate mofetil 1500mg twice daily, and prednisone 2.5mg daily.

The physical Examination was notable for fullness in the left upper quadrant with a scab without drainage but mildly tender to palpation. Laboratory values were notable for a baseline serum creatinine of 2.7 (normal range 0.6–1.3mg/dL) and platelets of 99 ($150\text{--}450 \times 10^9/\text{L}$). Two sets of blood cultures were negative. A non-contrasted CT scan of the chest showed a left lower lobe atelectasis likely related to the epicardial pacing pad but no evidence of sternal or anterior mediastinal infection. However, the cutaneous lesion appeared to be directly overlying the retained epicardial lead.

Consequently, the patient underwent a redo-thoracotomy with epicardial lead removal via a left lateral incision in 2014. Intraoperatively, an intra-pericardial abscess was found encompassing the posterior lead patch. The posterior patch was removed and sent for culture, and the abscess was surgically drained. KOH stains performed on the operative specimens revealed septate fungal hyphae. In response to this result and review of the patient's prior history of invasive aspergillosis, the patient was treated with posaconazole. Operative cultures ultimately grew *Aspergillus fumigatus*. Susceptibility testing to voriconazole and posaconazole was performed on the *Aspergillus* isolate: voriconazole and posaconazole MICs were both 0.25g/mL (susceptibility testing of *A. fumigatus* isolated from sinus cultures in 2004 had not been performed).

Follow-up CT chest imaging performed two months post-operatively revealed almost complete resolution of left lower lobe opacity. The patient was treated with posaconazole for approximately two years. He had radiographic resolution of infection but passed away two years later due to complications from pneumonia thought not to be directly related to IMD.

Table 1 compares the characteristics of the two cases of IMD.

2. Materials and Methods

Paired mold isolates from each patient were retrieved from our clinical microbiology laboratory biorepository. The isolates sequenced from Case 1 came from expectorated sputum cultures (2013 and 2016). The isolates sequenced from Case 2 came from operative fungal sinus culture (2004) and intra-pericardial abscess (2014). Additional methods for DNA extraction and whole genome sequencing can be found in Supplementary Data.

3. Results

For the patient with invasive *Verruconis* (Case 1), the isolates were predicted to be of the same strain indicating that the initial colonizing isolate pre-lung transplant eventually, despite several courses of azole therapy, caused IMD post-transplant. In fact, the two isolates had few genetic differences despite their presence in the host for at least three years. Both isolates differed from the CBS 43764 assembly by a similar number of SNPs (37,277 for

106.16-D and 38,218 for 107.16-D). Of positions with a non-ambiguous call in both isolates, only 114 positions varied between the two isolates; of these, only six were mapped to coding regions, including one synonymous variant and five missense variants in genes, including a glycosyl transferase (PV09_00857), a chromatin assembly factor (PV09_02174), a Vps54-like protein (PV09_07092), a diphthamide synthesis protein (PV09_02469), and a heterokaryon domain protein (PV09_09211). Compared to the reference genome, both isolates contained a predicted missense variant (V65A) in the *cyp51a* gene (PV09_06865), which is the target of azole binding. However, this change is unlikely to increase drug resistance as it is a conservative change, and the isolate phenotypically did not appear to be resistant to azoles *in vitro*.

In the patient with recurrent invasive aspergillosis (Case 2), the two isolates were not genetically related. Each of the two isolates differed from the Af293 reference genome by a similar number of SNPs (55,695 for Af-M1509-D and 53,081 for M5960-D); for positions with a non-ambiguous call in both isolates, 18,368 SNPs differed between the isolates. Therefore, we suspect that the patient's cardiac device infection was either due to a new exposure to *Aspergillus* or a different *Aspergillus* strain that colonized the epicardial lead during the patient's original presentation with invasive aspergillosis. It is also possible that the original strain from the sinus cultures was not the same strain that produced the radiographic infiltrate in the lung.

4. Discussion

We present two challenging cases of IMD in thoracic transplant recipients including the first report of relapsed infection due to *V. gallopava* resulting in an invasive transglottic mass and a cardiac device infection due to *A. fumigatus*. Whole genome sequencing was utilized to precisely determine if this was due to persistence of the same fungal strain. In Case 1, the strains of *Verruconis* were essentially the same, but in Case 2, the strains of *Aspergillus* were different. In the second case, the mold infection was either secondary to a new or recurrent strain. However, in Case 1, the mold progressed from colonization to disease despite voriconazole treatment as the patient experienced augmentation of immunosuppression for treatment of rejection.

One possible explanation of how a mold can cause recurrent infection or even persist within the host for years is the concept of adaptation. Adaptation is defined by a microorganism's ability to change its phenotype to improve its fitness when challenged by new environmental conditions.⁵ It is postulated to occur within a spectrum of four events: 1) physiologic changes in which the microorganism responds to environmental changes and host stressors; 2) epigenetic changes (e.g. DNA methylation) to create a molecular memory of the physiologic changes; 3) DNA-copy number adaptations (e.g. deletions or duplications); and 4) genomic mutations, which represents the ultimate level of adaptations where specific genetic changes are stored and thus inherited for prolonged periods of time. In our experience with recurrent cryptococcal yeast isolates that have been compared from the same patient, we have observed both genetic changes which may promote fitness or drug resistance and epigenetic factors.⁶

Aspergillus, in particular, possesses a biochemical versatility that allows adaptation within its environments and may result in fungal persistence within the host.⁷ Additionally, its genome encodes virulence factors, azole resistance mechanisms, and cell wall modifications that allow the mold to evade the host's immune system and antifungal therapy.⁷⁻⁹ Another study of serial *A. fumigatus* isolates from patients noted a non-synonymous mutation in the cyp51A gene of an itraconazole-resistant isolate as previously noted by targeted sequencing.^{10,11} We did not observe non-synonymous mutations in cyp51A or cyb51b in the two collected isolates in this study that match known resistance mutations, consistent with the lack of measured azole resistance *in vitro*.¹⁰

In Case 2, we hypothesize that this patient suffered from a chronic, smoldering epicardial lead infection due to *A. fumigatus* placed at the time of surgery and representative of the initial sinopulmonary inoculum of multiple *Aspergillus* strains and/or species in 2004. Additionally, if there was a heterogeneous population of *Aspergillus* causing infection, microbiologic sampling may have only captured one strain and/or species. Given the CT chest imaging findings of left lower lobe opacity adjacent to pericardial patch from the epicardial lead, we specifically suspect that the lead infection arose from dissemination of the previous episode of sinopulmonary aspergillosis in 2004. As the mold was not eradicated by the original 9-month course of voriconazole therapy or the host immune defense, we hypothesize that it persisted within a biofilm on the posterior epicardial lead and migrated up the lead and into the patch, forming the intra-pericardial abscess.

Biofilms are formed when microorganisms attach irreversibly to the surface of an anatomic site or medical device, grow, and produce extracellular polymers to facilitate matrix formation.¹² The formation of an extracellular matrix can alter the organism's phenotype by modifying growth rate and gene transcription, leading to decreased antimicrobial susceptibility. For example, within bronchial epithelial biofilms and among the *A. fumigatus* isolates persisting in these biofilms, MICs to voriconazole, posaconazole, and itraconazole were noted to increase from a range of 0.125–0.25g/mL to 1–2g/mL.¹³ The suspicion for biofilm formation and previous exposure to triazoles therapy in Case 2 prompted azole susceptibility testing on the second *A. fumigatus* strain. The MIC was 0.25mcg/mL, which was relatively susceptible, and this functional assay was supported by the whole genome sequencing analysis. Although there were non-synonymous sequence changes in the ERG11 gene (cyp51A) that is the target of azole drugs, there were no changes in the positions that have been associated with drug resistance.

Previous cases of *A. fumigatus* cardiac device infection have been reported in the literature with diagnosis ranging from two to seven years post-device implantation.^{14,15} Similar to Case 1, these published cases were associated with a delay in diagnosis likely due to the rarity of this pathogen causing cardiac device infection. Regardless of pathology, particularly in an immunosuppressed host, late cardiac device infection due to *Aspergillus* should be considered in the differential diagnosis due to the mold's ability to smolder within a biofilm on hardware for many years as demonstrated by Case 2.

In contrast to *Aspergillus*, less is known about the epidemiology and pathogenesis of *V. gallopava*. It is a dematiaceous, thermotolerant mold that causes zoonotic and human

infection with a predilection for central nervous system invasion. In humans, it tends to infect immunocompromised hosts likely through inhalation of spores with the potential for extrapulmonary dissemination.¹⁶ In a series of 10 cases from the University of Pittsburgh, investigators report that all cases occurred in solid organ transplant recipients, and most occurred late after transplantation with pulmonary manifestations.¹⁷ The attributable 6-month mortality rate was 20%. In our case, this mold likely colonized the patient's airway for years prior to becoming an invasive mold at the site of colonization during the iatrogenic immunosuppression. In fact, azole pre-emptive therapy did not seem to eliminate this mold unless the patient was re-infected from a similar environmental source over time, which seemed unlikely.

In reporting these cases with a corresponding whole genome sequencing investigation, we hope that other providers will recognize that molds can persist within the immunosuppressed host for years (Case 1) and cause invasive disease in the setting of augmentation of immunosuppression. Conversely, invasive mold disease can recur due to a re-infection event in a chronically immunosuppressed host or lay dormant within a biofilm for years (Case 2). We envision a larger study would need to encompass a long period of time to capture these events (in Case 1, up to 10 years) and characterize which mold species can cause persistent or recurrent disease. Use of next-generation sequencing techniques will help elucidate the molecular epidemiology of these pathogens and can precisely document infecting strains that evade host immune defenses.

As we do not know the mutation rate for these mold species within the host, we cannot declare the precise number of SNPs required to identify two isolates as being of the same or different strain. To determine this number, one would need an estimate of the number of generations per week or year in the host and then back calculate a rough estimate of the expected number of SNPs. After calculating the mutation rate, one would then apply a statistical test to determine whether the number of SNPs observed was significantly different than what one would expect by just the mutation rate. However, one could compare the mutation rates that have been measured in other fungal species. For example, the estimated mutation rate in *Saccharomyces cerevisiae* is 1.67×10^{-10} per base per generation.¹⁸ Furthermore, our work with serial isolates of *Cryptococcus* suggested that the mutation rate of this yeast in the cerebrospinal fluid of the human host was approximately one polymorphism change every 58 days.⁶ Interpreting our results for the *Aspergillus* cases based on these data, it is likely that the difference of 18,368 SNPs would require a mutation rate that is many orders of magnitude higher than those estimated for other fungi, so the *Aspergillus* infection would be inconsistent with a clonal infection in our opinion.

In conclusion, we report two cases of unusual manifestations of IMD in thoracic organ transplant recipients and characterize how molds can persist within the human body with use of whole genome sequencing. Whilst we cannot draw conclusions on optimal treatment of these infections, we suspect that Case 2 achieved cure with a combination of surgical resection of the pacemaker lead and prolonged antifungal therapy. Case 1's IMD was likely being controlled with antifungal therapy at the time of his death, but assurance of cure could not be determined. Finally, with more sequencing data from infecting strains and quantitative measurement of clinically important phenotypes, we might be able to find a

molecular blueprint of this mechanism. We also need ways to catalog and validate mutations that cause drug resistance, particularly in fungi like *V. gallopava* that are not widely studied. It is clear that fungi will microevolve to persist within the host, and we now have the molecular tools to provide a window into this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IMD	Invasive mold disease
WGS	Whole genome sequencing
MIC	Minimum inhibitory concentrations
BAL	Bronchoalveolar lavage
bp	Base pairs
rRNA	Ribosomal RNA
SNP	Single nucleotide polymorphism

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Figure 1.
Case 1. Radiographic and intra-operative visualization of suspected invasive mold disease due to *Verruconis gallopava*.

Figure 2.
Case 2. CT Chest without contrast from 2004 showing a persistent opacity in the left lower lobe adjacent to the pericardial patch (arrow).

Table 1

Characteristics of the presented cases.

Characteristic	Case 1	Case 2
Solid organ transplant	Lung	Heart
Age at the time of infection (years)	65	61
Infecting mold species	<i>Verruconis gallopava</i>	<i>Aspergillus fumigatus</i>
Years from first isolation of fungus to disease recognition	3	10
Years from transplant to disease	2	13
Surgical intervention	Tracheostomy, biopsy of trans-glottic mass	Incision and drainage, removal of retained epicardial lead
Antifungal treatment	Voriconazole, micafungin, inhaled amphotericin B lipid complex	Posaconazole delayed-release tablet
Treatment outcome	Could not determine but seemed to be controlled	Suspected cure but remained on extended suppressive posaconazole
Current status	Deceased from healthcare-associated pneumonia	Deceased from community-acquired pneumonia