Original Article

Experimental murine acremoniosis: an emerging opportunistic human infection

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Abstract

Acremonium is an emerging fungal pathogen causing severe infections. We evaluated the virulence of three clinically relevant species within the genus, i.e., Acremonium kiliense (currently Sarocladium kiliense), Acremonium sclerotigenum-A. egyptiacum complex and Acremonium implicatum in a murine model of disseminated infection. Both immunocompetent and immunosuppressed mice were infected with two inocula concentrations (2 × 10⁶ and 2 × 10⁸ conidia/animal) of two strains of each species. Tissue burden, mortality rate, histopathology and levels of (1→3)-β-D-glucan were used as virulence markers. None of the species of Acremonium tested was able to cause infection in immunocompetent mice. Conversely, severe infections were produced in immunocompromised mice, the spleen being the most affected organ. In general, the virulence of the Acremonium species tested was low, S. kiliense being the most virulent species.

Key words: virulence, immunosuppressed mice, mycosis, fungal infection, Acremonium.

Introduction

Opportunistic fungal infections have increased dramatically in recent years and are a leading cause of death in immunocompromised patients [1]. Although Acremonium is less common than other opportunistic molds, such as Aspergillus, Fusarium or Scedosporium, some species can cause invasive infections in humans [2,3]. Acremonium is a cosmopolitan environmental fungus commonly found in soil, plant debris and rotting mushrooms, as well as some species having been reported to be pathogens of plants, insects and mammals [4,5]. In humans, mycetoma, keratitis and onychomycosis are the most common clinical presentations of Acremonium infections [3,6,7], although disseminated infections have also been reported in immunosuppressed patients [8–11] and rarely in immunocompetent patients [12]. Acremonium is a complex and polyphyletic genus comprising approximately 150 species. Summerbell et al. [15] recently carried out a comprehensive review of the genus based on the ribosomal DNA sequences of a large number of species which resulted in A. kiliense being relocated to the genus Sarocladium. S. kiliense (Acremonium kiliense) is the most commonly recovered from clinical samples followed by A. sclerotigenum-A. egyptiacum complex and A. implicatum [13,14]. Due to the difficulty in identifying Acremonium isolates morphologically and to the general absence of reliable sequences in public databases, the etiological agent was not identified or at least wrongly identified in most clinical cases [16,17]. Therefore, the real

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incidence of the involvement of *Acremonium* spp. in the clinical setting is unknown.

The use of molecular tools in recent years has enlarged the range of fungal species that can be identified in a clinical situation. Concerning *Acremonium*, a recent molecular study investigated its prevalence in a large set of clinical isolates from the USA, which resulted in the finding that the most common were *S. kiliense*, *A. sclerotigenum-A. egyptiacum* complex, *A. implicatum*, *A. persicinum* and *A. atrogriseum* [14]. Since there are no recommended therapies for *Acremonium* infections and their resistance to the available drugs [3], it is crucial to develop appropriate models to evaluate possible therapies. In this study, we have investigated the virulence of the most clinically relevant species of *Acremonium* in mice by developing experimental models of acrémoniosis.

### Materials and methods

Six clinical isolates of the most common *Acremonium/Sarocladium* species were included in the investigation, i.e., *S. kiliense*, *A. implicatum* and *A. sclerotigenum-A. egyptiacum* complex (Table 1). They were grown on potato dextrose agar (PDA) at 25°C for 7 days, with the plates then flooded with sterile saline solution, the surface scraped with a culture loop and the resulting suspension collected with a sterile Pasteur pipette to prepare the inocula suspensions. The latter were transferred to 100 ml of potato dextrose broth, incubated at 25°C for 7 days, filtered twice through several layers of sterile gauze to remove large clumps of hyphal fragments and centrifuged at 3000 rpm for 20 min. The resulting pellets were suspended in sterile saline and the conidial inocula concentrations adjusted through counts made with a haemocytometer. The viability of the components of the inocula was confirmed by growing 10-fold dilutions on PDA.

Both immunocompetent and immunosuppressed four-week-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain), weighing 28–30 g, were used. The animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Animals were immunosuppressed by the administration of a single intraperitoneal injection of cyclophosphamide (200 mg/kg) plus i.v. injection of 5-fluorouracil (150 mg/kg) one day before infection [18,19].

In a preliminary study on immunosuppressed animals, three different inocula were assayed (2 × 10⁶, 2 × 10⁷ and 2 × 10⁸ conidia/animal) of *S. kiliense* UTHSC 03-3197 to establish the inoculum that could cause acute infection, with animals succumbing within 10 days (data not shown). All infections were established by administering 200 µl of the inoculum via the lateral tail vein of groups of 10 animals. For the survival study, animals were checked daily for 30 days, with survivors and animals meeting the criteria for discomfort euthanized by CO₂ inhalation.

Fungal load and (1→3)-β-D-glucan serum levels were determined only in the group of immunosuppressed animals infected with each of the two inocula. Additionally a control group of uninfected mice was included. Mice were anesthetized by sevoflurane inhalation (Sevorane; Abbott, Madrid, Spain), blood collected by cardiac puncture, centrifuged at 3500 rpm and the serum obtained stored at −80°C until its use for determining (1→3)-β-D-glucan levels. Next, animals were euthanized and brain, kidneys, liver, lung and spleen were aseptically removed. Approximately half of each organ was weighed, mechanically homogenized in 1 ml of sterile saline and serially 10-fold diluted. Dilutions were inoculated onto PDA plates and incubated at 25°C for 7 days in order to determine the colony forming units (CFU) per gram. Serum levels of (1→3)-β-D-glucan were determined using the Fungitell kit (Associates of Cape Cod, East Falmouth, MA, USA) following the manufacturer’s instructions. The other half of each organ was fixed with 10% buffered formalin, dehydrated, paraffin-embedded, sliced into 2 µm sections, stained with haematoxylin and eosin (H-E), periodic acid-Schiff and Grocott methamine silver and examined in blinded fashion by light microscopy.

Mean survival time (MST) was estimated using the Kaplan-Meier method and compared among groups with the log rank test. Differences in levels of (1→3)-β-D-glucan between species and inocula were determined by the ANCOVA test using the SPSS program. When multiple comparisons were carried out, the Bonferroni correction was used to avoid an increase in type I error. Organ burden data was analyzed using the Mann-Whitney U-test using GraphPad Prism 5 for Windows. *P*-values ≤ 0.05 were considered statistically significant.

### Table 1. Clinical isolates of *Acremonium* spp./*Sarocladium* included in this study and their origins.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sarocladium kiliense</em></td>
<td>UTHSC 03-3197</td>
<td>Vitreous humor</td>
</tr>
<tr>
<td></td>
<td>UTHSC 07-550</td>
<td>Blood</td>
</tr>
<tr>
<td><em>Acremonium sclerotigenum-A. egyptiacum complex</em></td>
<td>UTHSC 01-194</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>UTHSC 05-2270</td>
<td>Blood</td>
</tr>
<tr>
<td><em>Acremonium implicatum</em></td>
<td>UTHSC 07-3260</td>
<td>Bone</td>
</tr>
<tr>
<td></td>
<td>UTHSC 07-3667</td>
<td>Bronch wash</td>
</tr>
</tbody>
</table>
Results

The inocula escalation study carried out on *S. kiliense* strain UTHSC 03-3197 showed that $2 \times 10^6$ and $2 \times 10^8$ conidia/animal were both able to cause fatal acute infections in mice (data not shown). We therefore used these inocula levels for challenging both immunocompetent and immunosuppressed mice with the six fungal strains included in the studies. None of the infected immunocompetent animals succumbed to the infection or showed clear signs of disease.

Fig. 1 shows that at low inocula there were no observable differences in survival rates between strains of the same species ($P > 0.05$), although there were significant differences in MST. The infection with *A. implicatum* strains killed 30% of the animals with an MST of 22.5–23.7 days, demonstrating less virulence than infections caused by *S. kiliense* or the *A. sclerotigenum-A. egyptiacum* complex, which killed 70% of the animals with MST ranges of 14.1–14.4 and 14.7–16.1 days, respectively. Infections at a high inocula resulted in the death of all animals regardless of species, with a MST range of 4.6–6.2 days (Table 1).

As there were no signs of disease or death in immunocompetent mice, we determined the fungal load and levels of (1→3)-β-D-glucan in immunosuppressed animals infected with both previously tested inocula. Viable fungal cells were recovered from all the studied organs and the number of CFUs recovered from depended on inocula concentrations with significantly higher counts found in animals receiving high doses than in those challenged with low inocula levels (Fig. 2). In mice challenged with low inoculums numbers there were no significant differences in fungal load between the two strains of the same species ($P > 0.05$) (Fig. 3), i.e., the lungs were the most affected organs in those animals infected with *A. implicatum* or *A. sclerotigenum-A. egyptiacum* complex and the spleen in those infected with *S. kiliense* (Fig. 2). When high inoculum levels were used, the liver was the most affected organ in animals infected with *A. implicatum* and the spleen in those challenged with *S. kiliense* (Fig. 2). Regardless of inoculum size and strain, the brain was the least affected organ (Fig. 2). With the high inoculum, significant differences between the strains of *A. implicatum* were found in brain ($P = 0.0001$), liver...
Figure 3. Quantitative fungal recovery from spleen (A), brain (B), liver (C), lung (D) and kidney (E) from immunosuppressed mice infected with low (2 × 10^6 conidia/animal) (white box) and high (2 × 10^8 conidia/animal) (gray box) inocula of Sarocladium kiliense (S. k), Acremonium sclerotigenum-A. egyptiacum complex (A. s-e) and Acremonium implicatum (A. i). *significant differences between strains of the same species at high inoculum (P ≤ 0.05). Horizontal lines indicate the mean.

The concentration of (1→3)-β-D-glucan in sera of uninfected mice were 54.04 ± 0.38 pg/ml in contrast to those obtained from mice infected with Acremonium spp. which were ≥80 pg/ml. These results were dependent on the species (P = 0.023) and the inocula concentrations (P < 0.0001), being significantly higher for high inoculum (P ≤ 0.001), with no interaction between them (P = 0.618) (Fig. 4). When the means for each species were evaluated there were significant differences between S. kiliense and A. sclerotigenum-A. egyptiacum (P = 0.020) but none between A. implicatum and S. kiliense or the A. sclerotigenum-A. egyptiacum (P > 0.05).

Organs from mice infected with any of the three species assayed were similar histopathologically, regardless of the inoculum size tested. In general, the infection did not produce a detectable inflammatory response, necrosis, edema or angioinvasion. However, in kidneys glomerular invasion by hyphae with no parenchyma destruction was observed (Fig. 5A) and focal and low presence of round shaped fungal cells was found in brain, lung, spleen and liver (Fig. 5B).

Discussion

We have developed a murine model of disseminated acremoniosis for evaluating and comparing virulence in the most clinically relevant species. In recent years, numerous animal models have been developed for common pathogenic yeasts and molds, which have been useful for
evaluating antifungal therapies [20]. However, despite an increase in invasive infections by Acremonium [2], no animal models are available for this fungus, probably because of the complex taxonomy of Acremonium [3,14,15], which makes it very difficult to determine representative isolates of clinical relevant species for studying in animal models.

In general, our results indicate a low virulence of the three species tested, S. kiliense being slightly more virulent than the others, at least at low inocula levels. All the immunocompetent mice survived the infection despite the high inocula concentrations employed, which mirrors the natural course of the infection if patients are not immunocompromised [2]. Disseminated infection by Acremonium has been reported to involve multiple organs, including liver, spleen, kidney and brain [8,9,11,21]. In our study, we recovered fungi from all organs studied, including brain at both inoculum in all the species tested.

To some extent, the clinical behavior and even the morphology of Acremonium are similar to that of Fusarium, both fungi producing a disseminated infection almost exclusively in neutropenic patients and being highly refractory to antifungal drugs [22]. One of the typical characteristics of Acremonium that it shares with Fusarium, Scedosporium and other genera, is its ability to sporulate in tissue, called ‘adventitious sporulation’, which explains the relative high frequency of recovering these fungi from the blood of infected patients [23]. In vivo sporulation has been described in a case of disseminated Acremonium infection at day 79 [8], but in our study we did not observe such characteristics, probably because the organs were removed too soon after infection. Moreover, since we did not attempt to culture blood we could not evaluate the presence of fungemia.

Current serological methods used in fungal diagnosis are generally based on the detection of cell-wall components [24]. We have evaluated the usefulness of (1→3)-β-D-glucan as a marker of infection and whether there are any significant differences among the species of Acremonium tested. Our results reveal high serum levels, even when low inocula were used. Significant differences were observed among the species when the mean value was calculated, with S. kiliense showing the highest values. Other authors obtained similar high values in experimental fungal infections, from mice and guinea pigs in invasive pulmonary aspergillosis in serum and BAL fluid, respectively [25,26]. Although those animal models were different from our study, they highlight the usefulness of (1→3)-β-D-glucan for evaluating the progression of infection and its response to antifungal therapies. Although we cannot confirm fungemia

Figure 4. Serum levels of (1→3)-β-D-glucan in mice infected with S. kiliense (S. k), A. sclerotigenum-A. egyptiacum complex (A. s-e), Acremonium implicatum (A. i) and uninfected mice as control group on day 5 post-infection. The horizontal line indicates the cut-off for positivity (≥80 pg/ml). *significant differences (P ≤ 0.05) between low (2×10^6 conidia/animal) and high (2×10^8 conidia/animal) inoculum.

Figure 5. Histological section at 5th day post-infection of kidney and liver of mice immunosuppressed infected with 2×10^8 conidia/animals of the strain UTHS 03-3197 of S. kiliense. (A) Kidney section showing glomerular invasion by hyphae cells (arrow) with no alteration of parenchyma or necrosis. (B) Liver section showing scarcity of round-shaped fungal cells (arrow). This Figure is reproduced in color in the online version of Medical Mycology.
because blood cultures were not carried out, the high levels of this antigen in serum obtained in our study might be related to the angioinvasive ability reported for *Acremonium*, which would facilitate its release directly into the bloodstream [23]. Therefore, although it must be taken into account that such antigen is a panfungal marker [27], detection of (1→3)-β-D-glucan could be considered a useful tool for the prompt detection of disseminated infections by uncommon fungi like *Acremonium* once aspergillosis has been discarded.

Murine models have been used extensively to test experimental antifungal therapy against clinical important fungi, and past experience demonstrated that efficacy in animal models is usually predictive of clinical efficacy [28]. To our knowledge, this is the first animal study that has explored the virulence of *Acremonium*, which could be useful for future studies directed to establish a suitable antifungal therapy for the management of these refractory infections.

In conclusion, our results demonstrate a low virulence of *Acremonium/Sarocladium*, even in immunosuppressed animals. *S. kiliense*, the most common species in the clinical setting was the most virulent here.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**References**


