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Epidemiology, treatment options and outcome of invasive infections caused by Aspergillus section Usti

Glampedakis Emmanouil

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Service des maladies infectieuses

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THESE

préparée sous la direction du Docteur Frédéric Lamoth

et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

Emmanouil GLAMPEDAKIS

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Epidemiology, treatment options and outcome of invasive infections caused by Aspergillus section Usti

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pour Le Doyen
de la Faculté de Biologie et de Médecine

Monsieur le Professeur John Prior
Vice-Directeur de l'Ecole doctorale

L'aspergillose invasive (AI) est une importante cause de mortalité qui touche principalement les patients avec de cancers hématologiques et les transplantés. Parmi les différentes espèces d'*Aspergillus*, *A. fumigatus* et *A. flavus* causent la majorité des cas. Les *Aspergilli* de la section *Usti* (groupe *ustus*) sont des rares causes d'AI récemment rapportés comme émergents notamment parmi les transplantés recevant des prophylaxies antifongiques, surtout du posaconazole. Ce dernier médicament est prescrit en Suisse à titre préventif pour empêcher le développement d'aspergillose chez les transplantés de moelle.

Le but de cette thèse était de décrire l'épidémiologie européenne des AI par *Aspergillus* gr. *ustus*, leurs caractéristiques cliniques, les traitements administrés et leur outcome. Au niveau microbiologique, l'objectif était de décrire les espèces responsables d'AI, leur profil de susceptibilité aux antifongiques et de tester le synergisme de combinaisons antifongiques.

Nous avons identifié des cas où un *Aspergillus* groupe *ustus* a été retrouvé dans un prélèvement clinique en Suisse et dans d'autres pays européens (19 hôpitaux, 8 pays) pendant les dernières 10 années (2007-2018). Parmi 90 cas identifiés, 27 remplissaient les critères d'une AI probable ou prouvée (critères EORTC/MSG). Des cas additionnels (n=45) ont été identifié après une recherche systématique de la littérature. L'analyse poolée de ces 72 cas d'AI a montré que, dans la majorité de cas, les patients atteints étaient des transplantés de moelle osseuse (47%) ou d'organe solide (33%) et seulement 8% étaient neutropéniques. Quasi la moitié (47%) recevait une prophylaxie antifongique, principalement du posaconazole. Le poumon était l'organe principal touché mais des disséminations secondaires notamment au niveau cutané et cérébral étaient présentes dans 1/3 de cas. En ce qui concerne la prise en charge thérapeutique, l'antifongique principal administré (premier antifongique administré pour minimum 10 jours) était pour la majorité de l'amphotéricine B (39%) et du voriconazole (36%). La mortalité globale à 24 semaines était 58%. La mortalité était significativement plus haute chez les transplantés de moelle, quand l'infection était prouvée, et chez les receveurs d'amphotéricine B. Néanmoins les patients traités par amphotéricine B étaient plus immunosupprimés (plus de transplantés de moelle) avec des infections plus sévères (plus de cas prouvés et disséminés dans ce groupe), ce qui pourrait expliquer l'excès de mortalité dans cette catégorie.

Parallèlement, pour les cas pour lesquels la souche était disponible, celle-ci a été envoyée à l'Institut de Microbiologie du CHUV à Lausanne. L'identification des souches au niveau de l'espèce (séquençage des gènes de la β-tubuline et la calmoduline) a montré que la principale espèce du groupe *ustus* causant des IA était de loin *A. calidoustus* suivie par des cas isolés de *A. pseudodflectus* et *A. ustus sensu stricto*. Quant à la susceptibilité aux antifongiques testés par méthode CLSI, l'amphotéricine B était le médicament antifongique le plus actif *in vitro*. Les azoles étaient moins actifs, avec cependant une meilleure activité de l'isavuconazole, suivi par le voriconazole et le posaconazole. Pour une partie de souches d'*Aspergillus calidoustus* un synergisme a été démontré entre le voriconazole et le terbinafine *in vitro* (checkerboard microdilution method) validé *in vivo* dans un modèle d'infection de larves de *Galleria mellonella*.

En conclusion, les AI par *Aspergillus* groupe *ustus* surviennent dans un contexte post-transplantation, en général hors agranulocytose et souvent sous prophylaxie de posaconazole. La dissémination extra-pulmonaire est fréquente. Le principal pathogène du groupe est *A. calidoustus*. Parmi les options thérapeutiques, l'amphotéricine B montre la meilleure activité *in vitro* mais les résultats issus de cette thèse montrent que les azoles (notamment l'isavuconazole ou le voriconazole seul ou en combinaison avec la terbinafine) restent une alternative thérapeutique à considérer pour des cas sélectionnés (AI probables, non-disséminées, chez des hôtes moins immunosupprimés).

Clinical Infectious Diseases

MAJOR ARTICLE

Invasive Aspergillosis Due to *Aspergillus* Section *Usti*: A Multicenter Retrospective Study

Emmanouil Glampedakis,[1] Sophie Cassaing,[2] Arnaud Fekkar,[3] Eric Dannaoui,[4] Marie-Elisabeth Bougnoux,[5] Stéphane Bretagne,[6] Dionysios Neofytos,[7] Peter W. Schreiber,[8] Christophe Hennequin,[9] Florent Morio,[10] Olga Shadrivova,[11] Felix Bongomin,[12] Mario Fernández-Ruiz,[13] Anne Pauline Bellanger,[14] Sevtap Arikan-Akdagli,[15] Veronique Erard,[16] Maria Aigner,[17] Michela Paolucci,[18] Nina Khanna,[19] Eléna Charpentier,[2] Christine Bonnal,[20] Sophie Brun,[21] Frederic Gabriel,[22] Arnaud Riat,[23] Reinhard Zbinden,[8] Patrice Le Pape,[10] Nikolai Klimko,[11] Russel E. Lewis,[24] Malcolm Richardson,[12] Ahmet Cagkan İnkaya,[25] Alix T. Coste,[26] Pierre-Yves Bochud,[1] and Frederic Lamoth[1,26]; on behalf of the Fungal Infection Network of Switzerland (FUNGINOS)

[1]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland, [2]Department of Parasitology and Mycology, Toulouse University Hospital, Paul Sabatier University, Toulouse, France, [3]Groupe Hospitalier Pitié-Salpêtrière, Service de Parasitologie-Mycologie, Paris, France, [4]Paris-Descartes University, Faculty of Medicine, AP-HP, European Georges Pompidou Hospital, Parasitology-Mycology Unit, Paris, France, [5]Department of Microbiology, Necker-Enfants malades Hospital, AP-HP, Paris Descartes University, Paris, France, [6]Université de Paris, Parasitology-Mycology Laboratory, AP-HP, Groupe Hospitalier Saint-Louis-Lariboisière-Fernand-Widal, Paris, France, [7]Infectious Disease Service, Department of Internal Medicine, Geneva University Hospital, Geneva, Switzerland, [8]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich and University of Zurich, Zurich, Switzerland, [9]Sorbonne Université, Inserm, Centre de Recherche Saint-Antoine, CRSA, AP-HP, Hôpital Saint-Antoine, Paris, France, [10]Parasitology and Medical Mycology Laboratory, Nantes University Hospital, Nantes, France, [11]Mechnikov North-Western State Medical University, St Petersburg, Russian Federation, St Petersburg, Russia, [12]Mycology Reference Centre-Manchester, ECMM Center of Excellence in Clinical and Laboratory Mycology and Clinical Studies, Wythenshawe Hospital, Manchester University NHS Foundation Trust, Manchester, United Kingdom, [13]Unit of Infectious Diseases, Hospital Universitario "12 de Octubre," Instituto de Investigación Hospital "12 de Octubre" (imas12), Madrid, Spain, [14]Parasitology-Mycology Department, University Hospital, Besancon, France, [15]Mycology Laboratory, Department of Medical Microbiology, Hacettepe University Medical School, Ankara, Turkey, [16]Clinique de Médecine et Spécialités, Infectiologie, HFR-Fribourg, Fribourg, Switzerland, [17]Institute for Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria, [18]Unit of Clinical Microbiology, S. Orsola-Malpighi University Hospital, Bologna, Italy, [19]Division of Infectious Diseases and Hospital Epidemiology, University and University Hospital of Basel, Basel, Switzerland, [20]Parasitology Mycology Laboratory, Bichat Claude Bernard Universitary Hospital, Paris, France, [21]Parasitology-Mycology Department, Avicenne University Hospital, AP-HP, Bobigny, France, [22]CHU Bordeaux, Department of Parasitology and Mycology, Bordeaux, France, [23]Service of Laboratory Medicine, Department of Diagnostic, Geneva University Hospitals and Geneva University, Geneva, Switzerland, [24]Infectious Diseases Unit, S. Orsola-Malpighi Hospital, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy, [25]Department of Infectious Diseases, Faculty of Medicine, Hacettepe University, Ankara, Turkey, [26]Institute of Microbiology, Department of Laboratories, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland

Background. *Aspergillus* spp. of section *Usti* (*A. ustus*) represent a rare cause of invasive aspergillosis (IA). This multicenter study describes the epidemiology and outcome of *A. ustus* infections.

Methods. Patients with *A. ustus* isolated from any clinical specimen were retrospectively identified in 22 hospitals from 8 countries. When available, isolates were sent for species identification (*BenA*/*CaM* sequencing) and antifungal susceptibility testing. Additional cases were identified by review of the literature. Cases were classified as proven/probable IA or no infection, according to standard international criteria.

Results. Clinical report forms were obtained for 90 patients, of whom 27 had proven/probable IA. An additional 45 cases were identified from literature review for a total of 72 cases of proven/probable IA. Hematopoietic cell and solid-organ transplant recipients accounted for 47% and 33% cases, respectively. Only 8% patients were neutropenic at time of diagnosis. Ongoing antimold prophylaxis was present in 47% of cases. Pulmonary IA represented 67% of cases. Primary or secondary extrapulmonary sites of infection were observed in 46% of cases, with skin being affected in 28% of cases. Multiple antifungal drugs were used (consecutively or in combination) in 67% of cases. The 24-week mortality rate was 58%. *A. calidoustus* was the most frequent causal agent. Minimal inhibitory concentrations encompassing 90% isolates (MIC_{90}) were 1, 8, >16, and 4 μg/mL for amphotericin B, voriconazole, posaconazole, and isavuconazole, respectively.

Conclusions. *Aspergillus ustus* IA mainly occurred in nonneutropenic transplant patients and was frequently associated with extrapulmonary sites of infection. Mortality rate was high and optimal antifungal therapy remains to be defined.

Keywords. *Aspergillus ustus*; *Aspergillus calidoustus*; *Aspergillus pseudodeflectus*; *Aspergillus puniceus*; *Aspergillus insuetus*.

Invasive aspergillosis (IA) is an important cause of morbidity and mortality among severely immunocompromised hosts, such as patients with hematologic cancer or transplant recipients [1–3]. The genus *Aspergillus* includes more than 300 species, but only few of them are common pathogens for humans. *Aspergillus fumigatus* is the main causal agent of IA, followed by *A. flavus*, *A. niger*, and *A. terreus* [1–3]. *Aspergillus* spp. of section *Usti* (group *ustus*, further referred to as *A. ustus*) consist of about 20 species and represent a rare cause of IA [4–6]. However, they have been increasingly reported as potential pathogens over the last decade, especially in the setting of antimold active azole prophylaxis [7–9].

Aspergillus ustus IA is associated with high mortality rates [10]. The optimal antifungal treatment of these infections is

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Correspondence: F. Lamoth, Infectious Diseases Service, Lausanne University Hospital (CHUV), Rue du Bugnon 46, 1011 Lausanne, Switzerland (frederic.lamoth@chuv.ch).

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not well defined. Because these fungi exhibit decreased in vitro susceptibility to voriconazole and other mold-active azoles, amphotericin B formulations are recommended [11].

In this study, we report the results of a multicenter epidemiological study of IA caused by *A. ustus*. These cases were pooled with those previously reported in the literature.

METHODS

Study Design and Data Collection

This was a retrospective multicenter study. In order to recruit participating centers, a call for collaboration was launched via different networks including the following: (1) the Fungal Infection Network of Switzerland (FUNGINOS), the French National Reference Center for Invasive Mycoses and Antifungals ("Centre National de Référence des Mycoses Invasives et Antifongiques"), and the European Conference of Medical Mycology. Collaborators were asked to screen their local microbiology database and to report all cases for which an *Aspergillus* group *ustus* was isolated by culture in any clinical specimen. A standardized clinical report form (CRF) including demographic data, underlying diseases, immunosuppressive conditions, history of previous antifungal prophylaxis, characteristics of fungal infection, results of radiological and microbiological exams, antifungal treatment, and outcome was fulfilled for each case by the local investigator. Cases were classified as proven or probable IA or "colonization" (absence of clinical/radiological signs of IA) according to the criteria of the European Organization of Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) [12].

Microbiological Analyses

When available, clinical isolates were sent to the reference laboratory of the study (Lausanne University Hospital, Switzerland) as a spore suspension or subculture on solid agar medium. Identification of the fungus as an *Aspergillus* group *ustus* (section *Usti*) according to the taxonomic classification of Samson et al [13] was confirmed and determined at the species level by partial sequencing of the B-tubulin (*BenA*) and calmodulin (*CaM*) genes, as previously described [14]. Identity was obtained using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information and was validated if results obtained by both approaches (*BenA* and *CaM*) were concordant. Antifungal susceptibility testing for azole drugs (voriconazole, posaconazole, and isavuconazole) and amphotericin B was performed at the reference laboratory for all available isolates by broth microdilution method according to the protocol of the Clinical and Laboratory Standards Institute [15]. Only 1 culture isolate per patient was tested. *Aspergillus fumigatus* specimen 4147 (UK Neqas for Microbiology, London, UK) served as a quality-control strain. Strains were incubated at 37°C (*Aspergillus calidoustus*, *Aspergillus pseudodeflectus*) or 30°C for those that were not able to grow at 37°C (all other species). Reading was performed at 48 hours and the minimal inhibitory concentration (MIC) was defined as the concentration at which no residual growth was observed. MIC_{50} and MIC_{90} were defined as the MIC values encompassing 50% and 90% of the isolates, respectively.

Literature Review

A systematic review of the literature was performed to identify all published case reports and case series describing IA cases attributed to *A. ustus* until 31 December 2018. A search was performed in PubMed database (www.ncbi.nlm.nih.gov/pubmed) using the terms "*Aspergillus ustus*" and all the individual names of the 21 species belonging to group *ustus* [13]. Cases were included if meeting the following criteria: (1) articles written in English, (2) presence of sufficient clinical data to classify the case as proven or probable IA according to EORTC-MSG criteria [12], and (3) presence of data on antifungal therapy and outcome. Cases considered as localized fungal infections in immunocompetent patients (ie, ocular or cutaneous infections) were excluded. The clinical and microbiological data of the cases from the literature review were collected using the standardized CRF of the study.

Epidemiological and Outcome Analysis

Characteristics of patients and proven/probable IA were described for the present case series and for cases of the literature review in separate and pooled analyses. The main antifungal therapy was defined as the first antifungal drug that was administered for at least 10 consecutive days following IA diagnosis. The response to antifungal therapy was assessed according to the EORTC-MSG clinical and radiological criteria, with success defined as complete or partial response and failure as stable disease, progression, or death [16]. All-cause mortality was recorded for up to 24 weeks after IA diagnosis. The timing for the endpoint of response to therapy and mortality/survival was considered as the latest assessment occurring within the period of 24 weeks following IA diagnosis. The contribution of IA to death was considered as probable in case of progressive or stable infection (ie, failure) according to EORTC-MSG assessment of therapeutic response at time of death.

Statistical Analysis

Fisher's exact test and nonparametric test (Mann-Whitney) were used for the comparison of categorical and continuous variables, respectively. A 2-sided P value $\leq .05$ was considered as statistically significant.

Ethical Statement

The study was approved by Swissethics (project 2017-01562). All the CRFs and clinical samples were assigned a study code by the local investigator and sent anonymously to the reference center of the study (Lausanne University Hospital).

RESULTS

Characteristics of Patients and Invasive Aspergillosis

Ninety CRFs of patients for which an *Aspergillus* group *ustus* was isolated from a clinical specimen were obtained from 22 hospital centers in 8 countries from 2007 to 2018 (Supplementary Table 1). Two cases were withdrawn from the analysis because of a lack of clinical data. Of the 88 remaining cases, 27 fulfilled the EORTC-MSG criteria of proven (n = 9) or probable (n = 18) IA.

Our search in the medical literature identified 45 cases of proven (n = 28) and probable (n = 17) IA due to *A. ustus* from 29 publications, including 24 single case reports and 5 case series (from 2 to 6 cases) from 1974 to 2018 [5, 7–10, 17–40].

Characteristics of patients and infections of all proven/probable IA cases (n = 72) are shown in Tables 1 and 2. Compared with the present case series (n = 27), cases reported from the literature (n = 45) included a higher proportion of proven IA (62% vs 33%, $P = .02$). Other comparisons between both groups did not show statistically significant differences. Overall, most patients were hematopoietic cell transplant (HCT) or solid-organ transplant (SOT) recipients (47% and 33%, respectively) (Table 1). A majority of patients (84%) were receiving immunosuppressive therapies, and only 8% of patients were neutropenic at time of IA diagnosis. Ongoing prophylaxis or treatment with antimold active azoles for at least 7 days before IA diagnosis was present in 47% of cases (consisting of posaconazole and voriconazole in 62% and 24% of them, respectively). Characteristics of IA episodes are described in Table 2. The lung was the primary site of infection in 76% of cases. Primary or

Table 1. Epidemiological Characteristics of Patients with *Aspergillus ustus* Proven/Probable Invasive Aspergillosis

	Current Study (n = 27)	Literature Review (n = 45)	Overall (n = 72)
Demographic characteristics			
Age, years	55 (19–73)	48 (9–77)	48 (9–77)
Female	8 (30)	15 (33)	23 (32)
Underlying host conditions			
Hematologic malignancy	17 (63)	24 (53)	41 (57)
Acute leukemia	6 (35)	11 (46)	17 (41)
Myelodysplastic syndrome	2 (12)	3 (12)	5 (13)
Lymphoproliferative disorder	8 (47)	1 (4)	9 (22)
Other/not specified	1 (6)	9 (38)	10 (24)
Hematopoietic cell transplantation	14 (52)	20 (44)	34 (47)
Allogeneic	11 (78)	19 (95)	30 (88)
Graft-vs-host disease	10 (91)	15 (79)	25 (83)
Autologous	3 (22)	1 (5)	4 (12)
Solid-organ transplantation	7 (26)	17 (38)	24 (33)
Lung	1 (15)	8 (47)	9 (37)
Heart	4 (56)	4 (23)	8 (34)
Kidney	2 (29)	2 (12)	4 (17)
Liver	0 (0)	2 (12)	2 (8)
Lung and heart	0 (0)	1 (6)	1 (4)
Solid tumor	1 (3)	2 (4)	3 (4)
Autoimmune disease	1 (3)	0 (0)	1 (1)
Immunosuppressive conditions			
Neutropenia (PMNs <500/μL)	1 (4)	5 (11)	6 (8)
Immunosuppressive therapy	19/23 (82)[a]	34/40 (85)[a]	53/63 (84)[a]
Including corticosteroids	16 (84)	31 (91)	47 (89)
Including calcineurin inhibitors	10 (53)	23 (68)	33 (62)
Including mycophenolate mofetil	4 (21)	15 (44)	19 (36)
Ongoing antifungal therapy[b]			
Antimold active azole	13 (48)	21 (47)	34 (47)
Posaconazole	8 (61)	13 (62)	21 (62)
Voriconazole	5 (39)	3 (14)	8 (24)
Itraconazole	0 (0)	5 (24)	5 (14)
Other	0 (0)	5 (11)[c]	5 (7)[c]

Numbers represent absolute numbers (%) for proportions or medians (range) for continuous variables.

Abbreviation: PMN, polymorphonuclear neutrophil.

[a]Data not available for all cases.

[b]For ≥7 days before and ongoing at diagnosis.

[c]Caspofungin (2), amphotericin B (3).

Table 2. Characteristics of *Aspergillus ustus* Proven/Probable Invasive Aspergillosis

	Current Study (n = 27)	Literature Review (n = 45)	Overall (n = 72)
Type of infection			
EORTC-MSG classification			
Proven/probable cases	9 (33)/18 (67)	28 (62)/17 (38)	37 (51)/35 (49)
Extent of infection			
Single site/disseminated (>1 site)	21 (78)/6 (22)	27 (60)/18 (40)	48 (67)/24 (33)
Sites of infection[a]			
Lung	24 (88)	31 (69)	55 (76)
Skin/soft tissue	4 (15)	16 (35)	20 (28)
Brain	3 (11)	7 (15)	10 (14)
Bone	2 (7)	2 (4)	4 (5)
Other[b]	1 (4)	4 (8)	5 (7)
Aspergillus spp.			
A. calidoustus	19 (70)	13 (29)	32 (45)
A. pseudodeflectus	2 (7)	1 (2)	3 (4)
A. ustus	1 (4)	0 (0)	1 (1)
A. granulosus	0 (0)	2 (4)	2 (2)
Group *ustus*[c]	5 (19)	29 (65)	34 (48)
Concomitant infections			
All coinfections[a]	14 (52)	18 (40)	32 (44)
Fungal[d]	8 (57)	5 (28)	13 (41)
Bacterial	7 (50)	8 (44)	15 (47)
Viral	3 (21)	8 (44)	11 (34)
Management of IA			
Antifungal treatment	22 (81)	42 (93)	64 (89)
Multiple antifungal drugs[e]	12 (55)	31 (74)	43 (67)
Main antifungal therapy[f]			
Voriconazole	8 (36)	15 (36)	23 (36)
In combination with echinocandin	0 (0)	9 (60)	9 (39)
Amphotericin B	9 (41)	16 (38)	25 (39)
Lipid formulation	9 (100)	10 (62)	19 (76)
Deoxycholate	0 (0)	6 (38)	6 (24)
In combination with echinocandin	3 (33)	6 (38)	9 (36)
Amphotericin B and voriconazole	2 (9)	6 (14)	8 (12)
Other[g]	3 (14)	5 (12)	8 (12)
Surgery	4 (15)	7 (15)	11 (15)
Outcome			
Treatment failure	14 (52)	26 (58)	40 (55)
24-Week mortality	14 (52)	28 (62)	42 (58)
Probable contribution of IA to death	12 (86)	22 (78)	34 (81)

Numbers represent absolute numbers (%) for proportions or medians (range) for continuous variables.

Abbreviations: EORTC-MSG, European Organization for Research and Treatment of Cancer/Mycoses Study Group; IA, invasive aspergillosis.

[a]More than 1 possible.

[b]Other sites (>1 possible): liver, cardiac valve, myocardium, thyroid, eye.

[c]Isolates not available for sequencing (phenotypic identification at section/group level only).

[d]*A. fumigatus, A. flavus, A. nidulans, A. versicolor, A. melleus, A. tamarii, A. novofumigatus, Mucorales, Geotrichum* spp., *Scopulariopsis* spp., *Homographiella verticillata.*

[e]Multiple antifungal drugs used in combination or consecutively.

[f]First antifungal drug administered for ≥10 consecutive days following IA diagnosis.

[g]Posaconazole (n = 4), itraconazole (n = 2), micafungin (n = 2).

secondary extrapulmonary sites of infection were observed in 46% of cases. Lesions of skin/soft tissue and brain were present in 28% and 14% of cases, respectively. Disseminated infection (>1 organ affected) was observed in 33% of cases. In 18% of cases, IA was considered as mixed with another documented pathogenic mold (Table 2). Data on serum galactomannan were available for 33 patients, and the test was positive (optical density ≥0.5) in 28 (85%) of them. Results of galactomannan test in bronchoalveolar lavage fluid were available for 10 patients, of which 7 (70%) were positive (optical density ≥0.5).

Invasive Aspergillosis Management and Outcomes

Patients were treated with multiple antifungal drugs (either sequentially or consecutively) in 67% of cases (Table 2). Of 64 evaluable cases, the main antifungal therapy (as defined above) was an amphotericin B formulation in 39% of cases and voriconazole in 36% of cases. Compared with patients receiving voriconazole as the main treatment (n = 23), those treated with amphotericin B (n = 25) were predominantly HCT patients (64% vs 30% in the voriconazole group, $P = .02$), with a higher proportion of proven (72% vs 26%, $P = .003$) and disseminated (56% vs 13%, $P = .002$) IA.

Combination therapies were administered to 26 of 64 (41%) patients and consisted of an echinocandin in addition to voriconazole (n = 9) or amphotericin B (n = 9) or a combination of amphotericin B and voriconazole (n = 8).

The overall response rate to antifungal therapy was considered as a failure (ie, stable disease or progression) in 55% of cases. Forty-two (58%) of the 72 patients with proven/probable IA died within 24 weeks (Table 2). The contribution of IA to death was considered as probable in 81% of cases.

Overall mortality was higher among patients with HCT versus non-HCT (79% vs 47%, $P = .01$), in IA classified as proven versus probable (77% vs 47%, $P = .01$), and in those receiving amphotericin B as the main antifungal regimen versus another drug (80% vs 46%, $P = .02$).

Microbiological Data

Aspergillus ustus isolates were obtained for 22 of 27 (81%) proven/probable IA cases of the present case series. Multilocus sequencing (*BenA* and *CaM*) identified *A. calidoustus* as the pathogenic species in 19 of 22 (86%) cases. *Aspergillus pseudodeflectus* (n = 2, probable pulmonary IA) and *Aspergillus ustus sensu stricto* (n = 1, proven soft-tissue IA) accounted for the remaining cases. For the IA cases of the literature review, identification was available only at the section level (*Aspergillus* group *ustus*) in 29 (64%) cases. Identification at the species level (using various sequencing approaches) was provided for 16 (36%) cases: 13 *A. calidoustus*, 2 *Aspergillus granulosus*, and 1 *A. pseudodeflectus*.

Table 3. Results of Antifungal Susceptibility Testing for the Different *Aspergillus ustus* Species

	MIC_{50}/MIC_{90}; Range, μg/mL			
	Voriconazole	Posaconazole	Isavuconazole	Amphotericin B
A. calidoustus (n = 44)	8/8; 2–16	16/>16; 4 to >16	2/4 2/4; 0.5 to >16	0.5/1; 0.25–2
A. pseudodeflectus (n = 5)	4–16	4 to >16	2–8	0.25–1
A. ustus (n = 2)	2	8	2–4	0.5
A. insuetus (n = 2)	4–8	8 to >16	4	0.25
A. keveii (n = 1)	4	8	8	0.25
A. puniceus (n = 1)	4	8	2	0.25
All group *ustus* (n = 55)	8/8; 2–16	16/>16; 4 to >16	2/4; 0.5 to >16	0.5/1; 0.25–2

For isolates with n ≤ 5, only ranges were provided.
Abbreviations: MIC_{50}, minimal inhibitory concentration encompassing 50% of isolates; MIC_{90}, minimal inhibitory concentration encompassing 90% of isolates.

Among the 61 cases of the present series that were classified as colonization, 35 isolates were available for sequencing. *Aspergillus calidoustus* was the predominant species (80%), while several other species (*A. pseudodeflectus*, *A. ustus*, *Aspergillus insuetus*, *Aspergillus puniceus*, *Aspergillus keveii*) were also isolated from respiratory samples.

Antifungal susceptibility testing was performed for 55 isolates of the present case series (including 22 proven/probable IA cases and 33 cases of colonization). MIC_{50}, MIC_{90}, and MIC ranges for all species and antifungal drugs are shown in Table 3. Amphotericin B was the most active drug in vitro (MIC_{50}/MIC_{90}: 0.5/1 μg/mL). Among triazoles, isavuconazole showed somewhat better activity (2/4 μg/mL), compared with voriconazole (8/8 μg/mL) and posaconazole (16/>16 μg/mL). No notable differences were observed across species.

DISCUSSION

Invasive aspergillosis due to *A. ustus* are rare infections. Our review of the literature identified 45 cases published as individual case reports or small cases series. The present study describes 27 additional cases of proven or probable IA, which represents the largest case series until now. These cases identified via a screening of microbiological databases may be more representative of the actual epidemiology of *A. ustus* infections compared with selected individual case reports. However, the proportion of proven IA was lower. In order to address this potential bias, characteristics of patients and infections have been provided separately for the 2 approaches in addition to the pooled analysis of all cases. It is also important to mention that our enrollment strategy could not be representative of the global epidemiology of *A. ustus* IA, with a majority of cases from Europe (mainly France and Switzerland).

Overall, *A. ustus* IA cases were mainly observed in nonneutropenic transplant (HCT or SOT) patients receiving long-term immunosuppressive therapy. Our analysis confirms the propensity of these molds to cause extrapulmonary IA affecting mainly the skin/soft tissues and brain. About half of cases were breakthrough IA among patients receiving antimold active azoles. The emergence of *A. ustus* in the setting of azole prophylaxis has been suggested in a recent report [7]. Our study does not provide data about the actual incidence or proportion of *A. ustus* IA but suggests that these infections remain rare events.

Despite the existence of about 20 species in section *Usti*, phylogenetic analyses have suggested that most cases of infection were actually due to *A. calidoustus* [4]. While *A. calidoustus* represented the main offending pathogen in our case series, some other species of section *Usti* were also documented as the cause of IA. Similar to *A. calidoustus*, *A. pseudodeflectus* is able to grow at 37°C and was the cause of 2 probable IA cases. This species was previously documented as a causal agent of IA in a single case report [17]. *Aspergillus ustus sensu stricto*, which is not thermotolerant at 37°C, was shown for the first time to be the cause of a proven infection limited to the skin and soft tissues. The different species of section *Usti* exhibited similar antifungal susceptibility profiles, with amphotericin B being the most active drug in vitro, as previously reported [41]. In addition, our study provides data about the novel triazole isavuconazole, for which MICs were somewhat lower compared with those of voriconazole or posaconazole.

Our results show that the rates of therapeutic failure and overall mortality of *A. ustus* IA are high. Interestingly, while these *Aspergillus* species exhibit some intrinsic level of resistance to azoles, voriconazole was considered as the main antifungal therapy in a substantial proportion of cases. Despite a relatively favorable outcome in this subgroup, these patients were mainly non-HCT patients with apparently less severe disease according to the classification of IA (probable only) and the extent of infection (not disseminated), in comparison to patients treated with an amphotericin B formulation who exhibited a lower survival rate. Because of the small number of cases, the retrospective design, the large time window of inclusion (1973–2018), and the absence of case matching for underlying diseases and severity of infection, a comparative analysis of drug efficacy was

not possible. Our previous analysis in an invertebrate model of *A. calidoustus* infection showed that amphotericin B and voriconazole were equally effective in rescuing infected *Galleria mellonella* larvae [42]. Only the combination of voriconazole and terbinafine demonstrated some synergism in vitro and in vivo (Galleria model), but this combination is marginally used in clinical practice. Therefore, the optimal antifungal therapy for *A. ustus* IA remains uncertain in the absence of a prospective randomized matched-control trial, which seems very difficult to carry out. Experts usually recommend the use of an amphotericin B formulation [11]. Because these infections are often breakthrough IA under posaconazole and voriconazole prophylaxis, continuation with an azole drug should be avoided in these cases. However, voriconazole with or without the addition of terbinafine might still have a place for the management of selected cases with less severe immunosuppression and disease or those who are intolerant to amphotericin B. The role of isavuconazole, exhibiting somewhat lower MICs compared with other azoles, remains also to be explored.

In conclusion, this analysis provides the largest dataset about the epidemiology of *A. ustus* IA with some clinically relevant information about the categories of patients at risk and the clinical presentation of disease. While our results outline the particularly high rate of therapeutic failure and mortality of these infections, the optimal therapeutic approach remains to be defined.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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SUSCEPTIBILITY

Efficacy of Antifungal Monotherapies and Combinations against *Aspergillus calidoustus*

E. Glampedakis,[a] A. T. Coste,[b] M. Aruanno,[b] D. Bachmann,[b] E. Delarze,[b] V. Erard,[c] F. Lamoth[a,b]

[a]Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Lausanne, Switzerland
[b]Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland
[c]Clinic of Medicine, Fribourg Hospital, Fribourg, Switzerland

ABSTRACT Invasive fungal infections due to *Aspergillus calidoustus* with decreased azole susceptibility are emerging in the setting of azole prophylaxis and are associated with poor outcomes. We assessed the *in vitro* activity of antifungal drugs used alone or in combinations against *A. calidoustus* and found a synergistic effect between voriconazole and terbinafine at concentrations within the therapeutic range. An invertebrate *Galleria mellonella* model of *A. calidoustus* infection tended to support the potential benefit of this combination.

KEYWORDS *Aspergillus ustus*, *Galleria mellonella*, *Usti*, amphotericin B, invasive fungal infections, synergism, terbinafine, voriconazole

Invasive aspergillosis is a severe infection affecting immunocompromised hosts. While *Aspergillus fumigatus* remains the predominant pathogenic species, *Aspergillus calidoustus* (section *Usti*) is an emerging pathogen exhibiting some degree of intrinsic azole resistance and causing breakthrough infections in patients receiving antimold prophylaxis (1–3). Dissemination to soft tissues or the brain is frequently observed in *A. calidoustus* infections, which are often refractory to antifungal therapy and associated with high mortality rates (3). As a result, many patients are treated with combinations of antifungals expecting some synergistic interactions, which has not been demonstrated. The aim of this study was to assess the *in vitro* and *in vivo* efficacy of antifungal monotherapies and combinations against *A. calidoustus*.

(Preliminary results of this research were presented as a poster at the 28th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Madrid, Spain, 21 to 24 April 2018 [4]).

Ten clinical isolates of *A. calidoustus* were selected for this study. Species identification was confirmed by partial sequencing of the beta-tubulin (*BenA*) and calmodulin (*CaM*) genes, as previously described (5). Drugs were obtained as powders (Sigma-Aldrich, St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO) for a stock concentration of 5 mg/ml, and stored at −20°C. *In vitro* antifungal susceptibility testing was performed according to the M38-A3 Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (6). Plates were incubated at 35°C and read by visual inspection at 48 h. Synergy testing was performed by the checkerboard dilution method as previously described, with interactions defined as synergistic, indifferent, or antagonistic for a fractional inhibitory concentration index (FICI) of ≤0.5, >0.5 to 4, and >4, respectively (7). Amphotericin B, voriconazole, posaconazole, isavuconazole, and terbinafine were tested alone and in combinations. Experiments were performed in duplicates. In the case of discordant results (FICI difference of >0.05), a third replicate was performed, and the result was expressed as the FICI for which two concordant results were obtained.

An invertebrate model of *A. calidoustus* infection was performed in *Galleria mello-*

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Address correspondence to F. Lamoth, Frederic.Lamoth@chuv.ch.

E.G. and A.T.C. contributed equally to this article.

TABLE 1 *In vitro* activity of antifungal drugs alone and combined against 10 *Aspergillus calidoustus* strains

Strain	MIC (μg/ml) for antifungal drugs[a]:					MICs (μg/ml) for VOR-TBF[b]	FICI for VOR-TBF[b]
	AMB	VOR	POS	ISA	TBF		
1	1	4	8	2	0.5	1, 0.12	0.5
2	1	4	8	2	1	1, 0.25	0.5
3	1	8	>16	2	0.5	1, 0.25	0.6
4	0.5	4	>16	4	1	1, 0.25	0.5
5	0.5	8	>16	4	1	2, 0.25	0.5
6	0.25	8	>16	4	0.5	2, 0.12	0.5
7	1	2	8	2	0.5	0.5, 0.12	0.5
8	1	2	8	2	1	0.25, 0.25	0.4
9	2	8	4	2	1	2, 0.25	0.5
10	0.5	8	>16	4	0.5	2, 0.12	0.5

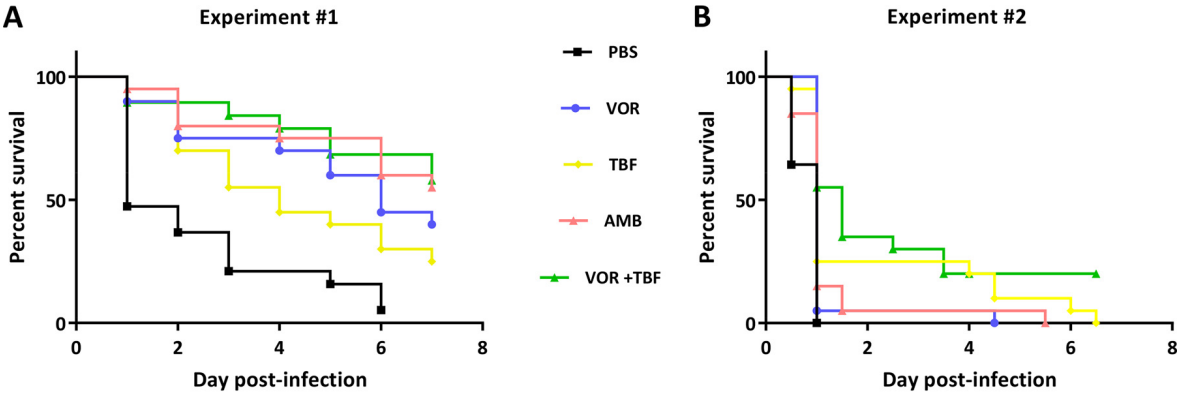
[a]AMB, amphotericin B; VOR, voriconazole; POS, posaconazole; ISA, isavuconazole; TBF, terbinafine.
[b]MIC of voriconazole and terbinafine when used in combination. FICI, fractional inhibitory concentration index.

nella larvae (Bait Express GmbH, Basel, Switzerland) as previously described (8). Groups of 20 larvae with a weight ranging from 350 to 400 mg were infected with a single dose of 3×10^6 spores in 40 μl phosphate-buffered saline (PBS). Antifungal drugs were injected 2 h later as a single 40-μl dose of amphotericin B (5 mg/kg), voriconazole (10 mg/kg), terbinafine (5 mg/kg), or a combination of voriconazole (10 mg/kg) and terbinafine (5 mg/kg). Drug doses were selected based on previous pharmacokinetic models in *G. mellonella* or extrapolated from human dosage (9, 10). Larvae were incubated at 37°C, and survival was assessed twice daily during 7 days postinfection. Performing a single injection of antifungal treatment 2-h postinfection, we analyzed the survival curve by the Gehan-Breslow-Wilcoxon test with GraphPad Prism software, giving more power to deaths at early time points.

MICs of amphotericin B, voriconazole, posaconazole, isavuconazole, and terbinafine are shown in Table 1. The combination of voriconazole and terbinafine was tested on all strains, with a synergistic effect (FICI, $\leq$0.5) in 9/10 strains (Table 1). Other drug combinations were tested on strains 1 and 2, with the following FICI results: posaconazole-terbinafine, 0.5 (synergistic); isavuconazole-terbinafine, 0.4 and 0.6 (synergistic and indifferent), amphotericin B-voriconazole, 3 (indifferent); and amphotericin B-terbinafine, >4 (antagonistic). Caspofungin, exhibiting only very modest fungistatic activity against *A. calidoustus*, showed indifferent interactions with either voriconazole or amphotericin B (FICI, 2).

To further assess the efficacy of antifungal drugs against *A. calidoustus*, we tested the different monotherapies and the voriconazole-terbinafine combination in a *G. mellonella* infection model using *A. calidoustus* strain 1. The experiment was performed in duplicates (Fig. 1A and B). Each individual drug (amphotericin B, voriconazole, and terbinafine) demonstrated a significant effect compared with the untreated group, but there was no superiority of one drug compared with another. The voriconazole-terbinafine combination was associated with a markedly improved survival compared with the untreated group in both experiments ($P < 0.0001$). Compared with monotherapies, the superiority of the combination therapy reached statistically significant *P* values against terbinafine alone in the first experiment and against both voriconazole and amphotericin B in the second experiment.

This *in vitro* and *in vivo* analysis of the activity of antifungal drugs used alone or in combination against *Aspergillus calidoustus* lead to some interesting conclusions. First, despite better *in vitro* activity, amphotericin B did not demonstrate superiority over voriconazole in an invertebrate model of infection. Indeed, voriconazole demonstrated a significant *in vivo* efficacy against this fungus despite MIC values that are usually considered at the limit or above the therapeutic range of concentrations. The addition of terbinafine to voriconazole or other mold-active azoles (posaconazole and isavuconazole) resulted in a moderate positive interaction at the limit of the synergistic criteria (FICI approximately 0.5). Low concentrations of terbinafine (0.12 to 0.25 μg/ml) were sufficient to lower the voriconazole MIC from 4 to 8 μg/ml to 1 to 2 μg/ml, which



C

	Gehan-Breslow-Wilcoxon test	
	Experiment #1	Experiment #2
PBS vs AMB	**** p<0.0001	ns p=0.069
PBS vs VOR	*** p =0.0005	** p=0.0037
PBS vs TBF	** p= 0.0055	** p=0.0048
PBS vs VOR + TBF	**** p<0.0001	**** <0.0001
AMB vs VOR	ns p=0.34	ns p=0.69
AMB vs TBF	ns p=0.051	ns p=0.19
VOR vs TBF	ns p=0.26	ns p=0.21
VOR + TBF vs AMB :	ns p=0.85	** p=0.0027
VOR + TBF vs VOR :	ns p=0.28	****p=0.009
VOR + TBF vs TBF:	* p=0.029	ns p=0.097

FIG 1 Efficacy of antifungal drugs in a *Galleria mellonella* model of *A. calidoustus* infection. Survival curves of *G. mellonella* larvae injected with 3×10^6 spores of *A. calidoustus* and a single dose of antifungal drug 2-h postinfection. Larvae were monitored for survival twice daily. Groups of 20 larvae were used each time. (A and B) The results of two independent experiments. (C) Data were analyzed by Gehan-Breslow-Wilcoxon test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. PBS, phosphate-buffered saline (no drug); AMB, amphotericin B; VOR, voriconazole; TBF, terbinafine.

corresponds to targeted therapeutic serum concentrations for the treatment of invasive aspergillosis (11, 12). The invertebrate model of *A. calidoustus* infection tended to support the potential benefit of the voriconazole-terbinafine combination despite some discrepant results between the two experiments. Such variability of the fitness of the *Galleria* spp. larvae is frequently observed (8), which may be due to their heterogeneous genetic background, age and stage of seasonal cycle, or mode of preservation. Despite variability, the voriconazole-terbinafine combination was associated with the highest survival rate compared with the untreated group in both experiments ($P <$ 0.0001) and was the only treatment arm showing some significant improvement compared with the others. The second experiment, associated with a higher early mortality rate, may be more representative to assess significant differences between treatment arms. In this last experiment, the voriconazole-terbinafine combination was significantly more effective compared with both voriconazole or amphotericin B alone. Albeit not tested in the *Galleria mellonella* model, other drug combinations, such as amphotericin B associated with voriconazole or terbinafine, resulted in indifferent or even deleterious interactions *in vitro*.

Topical or systemic terbinafine has been anecdotally used as adjunctive antifungal treatment for primary cutaneous infections due to *A. calidoustus* in the past (13, 14). Terbinafine is known for its accumulation in soft tissues and can also penetrate the hematoencephalic barrier (15); thus, it represents an interesting adjuvant therapy for *A. calidoustus* infections that frequently affect the soft tissues and brain (29% and 12% of cases, respectively) (3). The combination of voriconazole and terbinafine has already

demonstrated some synergism against other *Aspergillus* spp. or other molds (e.g., *Scedosporium* spp.) and has been used for the treatment of invasive scedosporiosis (16–18).

A. calidoustus infection remains a rare but often fatal disease, for which the optimal antifungal treatment is still debated. Because of the intrinsic level of azole resistance of this fungus, amphotericin B is usually the preferred first-line antifungal therapy, but its use is limited by nephrotoxicity and the lack of an oral formulation for prolonged therapy. Although this study was performed with a limited number of isolates, our results showed that, despite better *in vitro* antifungal activity, amphotericin B did not demonstrate better efficacy compared with voriconazole in a *Galleria mellonella* model of infection. Terbinafine may be considered as an adjunctive therapy to voriconazole for its *in vitro* synergistic effect and possibly improved *in vivo* efficacy; however, combination with amphotericin B should be avoided because of *in vitro* antagonistic interactions.

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Review

Clinical Relevance and Characteristics of *Aspergillus calidoustus* and Other *Aspergillus* Species of Section *Usti*

Emmanouil Glampedakis [1], Véronique Erard [2] and Frederic Lamoth [1,3,*]

[1] Infectious Diseases Service, Lausanne University Hospital and University of Lausanne, 1011 Lausanne, Switzerland; emmanouil.glampedakis@chuv.ch
[2] Clinique de Médecine et spécialités, infectiologie, HFR-Fribourg, 1708 Fribourg, Switzerland; Veronique.Erard@h-fr.ch
[3] Institute of Microbiology, Lausanne University Hospital and University of Lausanne, 1011 Lausanne, Switzerland
* Correspondence: frederic.lamoth@chuv.ch; Tel.: +41-21-314-1010

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Abstract: The *Aspergilli* of section *Usti* (group *ustus*) are represented by over 20 species, of which *Aspergillus calidoustus* is the most relevant human pathogen. Invasive aspergillosis (IA) caused by these fungi is rare but could represent an emerging issue among the expanding population of patients with long-term immunosuppression receiving antifungal prophylaxis. Clinicians should be aware of this unusual type of IA, which often exhibits distinct clinical features, such as an insidious and prolonged course and a high occurrence of extra-pulmonary manifestations, such as skin/soft tissue or brain lesions. Moreover, these *Aspergillus* spp. pose a therapeutic challenge because of their decreased susceptibility to azole drugs. In this review, we outline the microbiological and clinical characteristics of IA due to *Aspergillus* spp. of section *Usti* and discuss the therapeutic options.

Keywords: *Aspergillus ustus*; *Aspergillus pseudodeflectus*; *Aspergillus granulosus*; *Aspergillus insuetus*; *Aspergillus puniceus*; *Aspergillus keveii*; invasive aspergillosis

1. Introduction

Fungi of the genus *Aspergillus* represent the most important pathogenic molds for humans, causing invasive aspergillosis (IA) in patients with impaired immune defenses. While over 300 *Aspergillus* spp. have been described, the vast majority of IA cases are attributed to less than five species, consisting mainly of *A. fumigatus* (60–80% cases) and *A. flavus*, *A. niger* (or related cryptic species) and *A. terreus* for most of the remaining cases [1–4]. A recent study however suggested that epidemiology of IA may evolve as a consequence of the widespread use of anti-mold azole prophylaxis (i.e., posaconazole or voriconazole) with emergence of *Aspergillus* of section *Usti* (group *ustus*) exhibiting natural resistance to these antifungals [5]. This section includes over 20 species that are ubiquitous molds found in the indoor and outdoor environment [6]. Notably, they were the most frequent *Aspergillus* spp. found in drinking water distribution systems in Norway, including from hospital tap water [7]. *A. ustus* and related species were also frequently recovered from water-damaged buildings and from caves affected by human activities [8,9]. While the first case of IA due to *Aspergillus* of section *Usti* was described in 1974 [10], these infections have been increasingly reported in the literature since 2000 [11,12].

The aim of this review is to provide a practical summary of what infectious diseases specialists and microbiologists should know about *Aspergillus* spp. of section *Usti* for their daily practice.

2. Taxonomy and Microbiology

Based on phylogenetic analyses, there are currently 26 recognized *Aspergillus* species belonging to section *Usti* (Table 1) [6,8,13]. Most of them, including *A. ustus* sensu stricto, are unable to grow at 37 °C and therefore are not considered as human pathogens. Actually, most cases of human infections that were attributed to *A. ustus* in the literature, were secondarily reassigned to a novel distinct species, *A. calidoustus*, which is able to grow at 37 °C [14]. Two other closely related species, *A. pseudodeflectus* and *A. granulosus*, are also thermotolerant at human body temperature and were also found to be able to cause invasive infections in humans [11,15–17]. *A. ustus* sensu stricto was also isolated from a patient with aspergillosis localized to the skin and soft tissue [11]. Other non-thermotolerant species of section *Usti* that were isolated as colonizers or contaminants from clinical specimens include *A. insuetus*, *A. puniceus* and *A. keveii* [11].

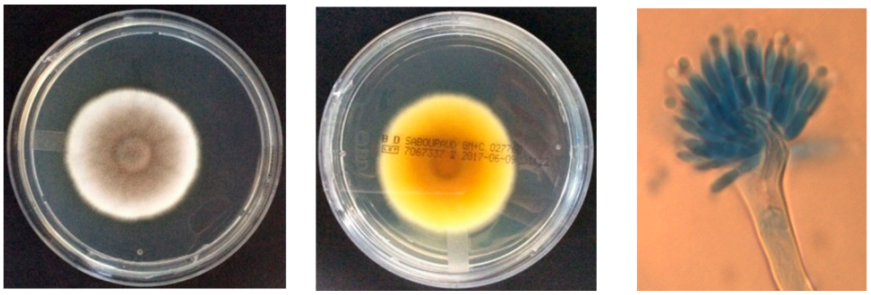
Table 1. The 26 *Aspergillus* species of section *Usti* and their pathogenic role in humans.

Isolated in Clinical Specimens	Environmental Samples Only	
Proven/probable IA [1] cases	*A. amylovorus*	*A. turkensis*
A. calidoustus [2]	*A. asper*	*A. germanicus*
A. pseudodeflectus [3]	*A. baeticus*	*A. heterothallicus*
A. granulosus [4]	*A. californicus*	*A. kassunensis*
A. ustus [5]	*A. carlsbadensis*	*A. lucknowensis*
Colonization only	*A. cavernicola*	*A. monodii*
A. insuetus	*A. collinsii*	*A. pseudoustus*
A. keveii	*A. deflectus*	*A. subsessilis*
A. puniceus	*A. egyptiacus*	*A. thessauricus*
	A. elongatus	

IA: invasive aspergillosis. [1] Proven probable invasive aspergillosis according to the criteria of the European Organization for Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) [18]. [2] Major cause of IA in humans [11]. [3] Three reported cases of probable IA [11,15]. [4] Two reported cases of proven IA [16,17]. [5] Single reported case of proven soft tissue IA [11].

Morphological characteristics of *Aspergillus* spp. of section *Usti* are usually reliable for identification at the section level. However, species identification would require partial sequencing of the beta-tubulin (*BenA*) or calmodulin (*CaM*) genes, which is not routinely available in most diagnostic microbiology laboratories [11]. Standard sequencing methods targeting the internal transcribed spacer (ITS) or 26-28S rDNA are not reliable enough for identification beyond the section level. Experience with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is limited for these rare species and misidentification has been reported [19].

Most species of section *Usti* will grow at 25–30 °C. Higher temperature (37 °C) allows distinguishing *A. calidoustus* or other thermotolerant species (e.g., *A. pseudodeflectus*, *A. granulosus*) from nonpathogenic species. Colonies are usually apparent between 2 and 5 days of growth. Macroscopic aspects on standard fungal culture media (e.g., Czapek yeast extract agar, Sabouraud or potato dextrose agar) show velvety greyish to brown cinnamon colonies (Figure 1, left) [6,20]. The yellowish reverse color with presence of yellow-brown soluble pigment is typical but can be absent for some species and/or according to the culture medium (Figure 1, middle). Under the microscope, conidial heads are usually short and loosely columnar with biseriate phialides (Figure 1, right) [6,20]. Conidia typically harbor rough ornamentation. Some specific characteristics (Hülle cells, Ehrlich reaction, growth on creatine, production of extrolites) may help distinguishing the different species, but these methods require the expertise of reference laboratories [6,20].



Typical features of *Aspergillus* of section *Usti*

Colony morphology	Grey to brown (top), yellowish (reverse). Yellow-brown pigment
Conidial head	Short, loosely columnar, biseriate phialides
Conidia	Round, brownish with rough ornementation

Figure 1. Morphological aspects of *Aspergillus calidoustus*. Macroscopic aspect of the colony on Sabouraud dextrose agar medium, top (**left**) and reverse (**middle**). Microscopic aspect (1000 ×) of a conidial head (staining: lactophenol blue) (**right**). Note: rough ornamentation of conidia is not visible here and could better visualized by scanning electron microscopy.

Antifungal susceptibility testing shows very similar profiles across species [11,21]. Amphotericin B is the most active drug in vitro with minimal inhibitory concentration encompassing 50% (MIC_{50}) and 90% (MIC_{90}) isolates of 0.5 and 1 µg/mL, respectively [11]. Azoles exhibit in vitro activity at concentrations that are usually at the upper limit or beyond the therapeutic range of concentration. Notably, isavuconazole displays somewhat higher activity compared to voriconazole and posaconazole (this latter one being the less active): MIC_{50}/MIC_{90} of 2/4 µg/mL, 8/8 µg/mL and 16/>16 µg/mL, respectively [11]. The fungistatic activity of echinocandins is comparable to that against other *Aspergillus* spp. with micafungin and anidulafungin exhibiting lower MIC compared to caspofungin [21]. The novel long-lasting echinocandin rezafungin (CD101) and the glucan synthase inhibitor ibrexafungerp (SCY-078) are also active against *Aspergillus* of section *Usti* [22,23]. Terbinafine has good in vitro activity (MIC 0.25 to 1 µg/mL), and its combination with voriconazole was synergistic in vitro and in a *Galleria mellonella* model of infection [24].

3. Epidemiology and Clinical Characteristics

IA caused *by Aspergillus* section *Usti* (further referred as *A. ustus* IA) remains a rare disease. In a cohort of 218 culture positive IA from the Transplant-Associated Infection Surveillance Network (TRANSNET), *A. ustus* complex species were the fifth cause of IA being responsible for 2.7% of all cases [25]. In the Prospective Alliance Therapy (PATH) registry, these species accounted for 0.8% of cases (rank 6th) [4]. In a single center study of 24 microbiologically documented breakthrough invasive mold infections, *A. ustus* accounted for 12.5% of all episodes and 43% of IA [5]. Outbreaks of *A. ustus* IA have been reported among hematopoietic stem cell transplant (HSCT) or solid-organ transplant (SOT) recipients [26,27]. While these data are mainly derived from North American cohorts, the incidence of *A. ustus* IA in other regions of the world is not well described.

The largest epidemiological description of proven/probable *A. ustus* IA included 72 cases, of which 45 were obtained from previous published case reports or small case-series (1974–2018) and 27 were collected via a screening of microbiological databases of 22 European hospital centers (2007–2018) [11]. Most patients were non-neutropenic transplant recipients (47% HSCT and 33% SOT recipients) receiving long-term immunosuppressive therapy (anti-calcineurin drugs and/or corticosteroids). About half of them (47%) had ongoing anti-mold azole prophylaxis (mainly posaconazole) at time of diagnosis. This observation is consistent with the above mentioned epidemiological studies suggesting

a higher prevalence of *A. ustus* IA among transplant patients and those receiving anti-mold azole prophylaxis [5,26,27].

A. ustus IA were disseminated (i.e., more than one organ affected) in 33% cases. While the lung was affected in 76% cases, primary or secondary extra-pulmonary sites of infections were frequently observed. Skin and/or soft-tissue lesions were present in 28% cases and cerebral aspergillosis in 14% cases [11]. Serum galactomannan was positive in 85% patients. Overall mortality was high (58% at 6 months, with IA being considered as a major or partial cause of death in 81% of cases) [11].

In summary, *A. ustus* IA exhibit some distinct clinical features compared to other IA, as they seem to affect mainly non-neutropenic transplant patients receiving anti-mold active prophylaxis and have a propensity to cause primary or secondary skin lesions or other extra-pulmonary foci of infection. The clinical case presented in Box 1 is illustrative of these characteristics and shows the insidious course of this fungal disease with notably the positive galactomannan in serum preceding the clinical signs of infection by several weeks or months.

Box 1. Illustrative case of *Aspergillus calidoustus* invasive aspergillosis.

A 64-year old woman underwent allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia. Three years later, she was treated by two chemotherapy cycles (FLAG and FLAG-IDA) for two consecutive relapses of the hematologic cancer, followed by maintenance therapy with azacitidine and sorafenib. She was receiving tacrolimus and corticosteroids for cutaneous and digestive graft versus host disease (GVHD). Antifungal prophylaxis with posaconazole was administered with appropriate trough concentrations (>0.5 mg/L). During follow-up, an increase in serum galactomannan was observed with a first positive value at 1.8 (optical density index), while she was asymptomatic. Three months later, she noticed painless skin nodules on her right leg, upper back and axillary hollow. Serum galactomannan at this time was persistently positive (6.43). Histopathological examination of the nodules revealed subcutaneous granulomas with mycelial elements. Cultures of skin biopsy grew a mold identified as an *Aspergillus* group *ustus* by sequencing of the 26-28S rDNA and identified at species level as *Aspergillus calidoustus* by partial sequencing of the beta-tubulin (*BenA*) and calmodulin (*CaM*) genes. Total body CT and ^{18}F-FDG PET/CT did not reveal any other lesion.

The patient received multiple antifungal treatment lines (liposomal amphotericin B with caspofungin, voriconazole with terbinafine, liposomal amphotericin B with caspofungin and terbinafine). Following surgical excision of all skin nodules, she experienced a recurrence of infection with suspected fungal arthritis of the right shoulder, which was treated by intra-articular injections of amphotericin B. A reduction of the immunosuppressive regimen was attempted, but the patient experienced a flare of GVHD and ultimately died. While all clinical foci of infection had resolved, serum galactomannan was persistently positive at time of death. Autopsy however did not reveal evidences of remaining invasive mold infection.

4. Treatment

As previously mentioned, the species of *Aspergillus* section *Usti* exhibit high MICs to the azole drugs, which represent the first-line antifungal therapy of IA [28]. As a result, current guidelines recommend the use of amphotericin B lipid formulations, which are the most active drug in vitro [28]. In practice, antifungal management is difficult with frequent use of multiple antifungal agents, either consecutively or in combination (Box 1) [11]. Interestingly, our analysis of the 72 *A. ustus* IA cases show that voriconazole was used as first-line therapy (i.e., first antifungal drug administered for at least 10 consecutive days) in a substantial proportion of cases [11]. These patients actually seemed to be less immunocompromised (non-HSCT recipients) and less severely ill with IA that were non-disseminated and classified as probable only, in comparison to those who were treated by amphotericin B. Not surprisingly, the mortality rate was significantly lower in this subgroup compared to amphotericin B-treated patients. Because of these evident biases in retrospective non-matched cohorts, it is not possible to draw conclusions about comparative drug efficacy. Nonetheless, it is noteworthy that voriconazole and amphotericin B were equally effective in a *Galleria mellonella* model of *A. calidoustus* infection [24]. The novel triazole isavuconazole seems to be somewhat more active than voriconazole in vitro, but clinical experience with this drug for the treatment of *A. ustus* IA is still very limited [29].

The potential benefit of drug combination is also debated. Indeed, an echinocandin, in combination with amphotericin B or voriconazole, was part of the first-line antifungal regimen in about one third of cases [11]. Some patients also received a combination of amphotericin B and voriconazole. Overall, mortality was high among patients receiving combination therapies, which may actually reflect the severity of the initial presentation of the disease in these cases. In vitro, these drug combinations were classified as indifferent [24]. Only the combination of voriconazole and terbinafine demonstrated a synergistic interaction in vitro and in the *Galleria* model [24]. While clinical experience with terbinafine for invasive mold infections is very limited, this drug may have an interest as adjunctive treatment for *A. ustus* IA because of its high penetration in skin and soft tissue and possibly in the brain [30]. Similarly, the combination of voriconazole and terbinafine has been used for other refractory mold diseases, such as scedosporiosis, although its benefit was not demonstrated [31].

From these observations, we can conclude that the optimal therapeutic approach of *A. ustus* IA would still deserve further investigations. Notably, this is another example that in vitro data do not necessarily correlate with clinical efficacy, as it has been previously shown for other difficult-to-treat mold infections [32]. Non-pharmacological parameters, such as recovery of the immune system or the initial severity of the disease with delay in diagnosis may represent the predominant predictors for outcome.

Our personal approach of *A.ustus* IA, as described in Table 2, is to consider liposomal amphotericin B as the first-line treatment, especially for severe cases. However, we consider that voriconazole or isavuconazole (this latter drug being even more active in vitro) alone or combined with terbinafine remain possible therapeutic options, in particular for less severe cases (localized and/or probable IA in patients with mild/moderate immunosuppression and in the absence of previous mold-active azole prophylaxis) or as second-line therapy in case of nephrotoxicity of amphotericin B or for maintenance therapy. Posaconazole should be avoided because of its quasi-lack of in vitro activity and the occurrence of breakthrough *A. ustus* IA with this drug. The role of echinocandins remains unclear, but this drug class could be used as adjunctive therapy in severe cases.

Table 2. Current antifungal therapeutic options against *Aspergillus calidoustus* and other *Aspergillus* spp. of section *Usti.*

Antifungal Drug Classes	Evidences	Comments
Amphotericin B	Relatively good in vitro activity (MIC 0.25–2 µg/mL) [11,21] Effective in a *Galleria* model [24]	Recommended as first-line on the basis of optimal in vitro activity (use lipid-based formulation)
Mold-active azoles	Relatively low in vitro activity (MIC 2–16 µg/mL): isavuconazole > voriconazole > posaconazole [11,21] Voriconazole effective in a *Galleria* model of infection [24] Caveat: breakthrough infections frequently reported (mainly under posaconazole, but also voriconazole)	Pre-clinical and clinical data suggest possible use in selected situations (e.g., less severe cases or second-line/maintenance treatment, absence of previous mold-active azole prophylaxis) Avoid posaconazole
Echinocandins	Fungistatic effect: micafungin/anidulafungin > caspofungin [21]	May be used in combination with either amphotericin B or triazoles despite no evidence of synergism Few experience as monotherapy, use only if no other alternatives (preferably micafungin or anidulafungin)
Terbinafine	Relatively good in vitro activity (MIC 0.25–1 µg/mL) [21,24] Effective in a *Galleria* model of infection [24] In vitro and in vivo (*Galleria*) synergism with voriconazole, posaconazole and isavuconazole [24] In vitro antagonism with amphotericin B [24] Accumulation in skin (no sustained levels in blood) [30]	May be combined with voriconazole (or isavuconazole) in selected situations (see above, possible interest in patients with skin lesions or alternative to amphotericin B in case of intolerance) Use as monotherapy not recommended

MIC: minimal inhibitory concentration, >: activity superior to.

Novel broad-spectrum antifungal agents are needed to treat *A. ustus* and other refractory mold infections. Some of them provided promising in vitro results. The Gwt1p inhibitor APX001A (E1210) and olorofim (F901318), an inhibitor of pyrimidine biosynthesis, show good activity against *Aspergillus* species of section *Usti* [33,34].

5. Conclusions

Aspergillus of section *Usti* (group *ustus*), in particular *A. calidoustus*, are increasingly recognized as causal agents of IA, as a possible consequence of the extent of the population of transplant patients with long-term immunosuppression and the widespread use of antifungal prophylaxis. This mold infection is challenging because of its insidious course, atypical presentation and multidrug resistance. Clinicians should suspect *A. ustus* IA in front of a transplant patient with persistently positive galactomannan despite no clinical or radiological evidence of IA or in the presence of skin lesions or soft tissue nodules. Optimal antifungal therapy still needs to be better defined. While amphotericin B is the most active drug in vitro, other antifungals or drug combinations (e.g., voriconazole or isavuconazole +/− terbinafine, adjuvant echinocandin) could be considered in selected situations.

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