

First isolation of *Ascotricha chartarum* from bronchoalveolar lavage of two patients with pulmonary infections

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Abstract

Ascotricha chartarum is a rare human pathogen. We describe the isolation and characterization of *A. chartarum* from bronchoalveolar lavage samples of two patients with underlying pulmonary infections. The identity of both isolates was established by typical phenotypic characteristics and by sequencing of the internal transcribed spacer region and D1/D2 domains of recombinant DNA and β -tubulin gene fragment. The demonstration of branched, septate hyphae in direct microscopic examination of both the specimens and isolation of the fungus in pure cultures suggest its aetiologic role in the disease process. Because of phenotypic similarities of *A. chartarum* with *Chaetomium* spp. and other *Chaetomium*-like fungi, the application of molecular methods is needed for its accurate identification. Although in the absence of histopathologic evidence the aetiologic role of *A. chartarum* could not be established unequivocally, nonetheless, in view of the rarity of its isolation from clinical specimens and demonstration of hyphal elements in bronchoalveolar lavage sample, this report assumes considerable significance. It serves to create awareness about environmental fungi that previously have missed attention but may play a role in respiratory infections.

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Introduction

The genus *Ascotricha* was erected to accommodate *A. chartarum* by Berkeley in 1838 [1]. The genus belongs to the family *Xylariaceae* in the class *Ascomycetes* [2]. So far, 16 phenotypically distinct species have been recognized [3]. *Ascotricha* forms only a small proportion of environmental mycobiota, and its occurrence in clinical specimens is rare. The clinical significance of *A. chartarum* as a human pathogen remains confined to a single case of maxillary sinusitis in a female Indian subject [4]. The diagnosis was established by demonstrating the presence of

phaeoid fungal elements in nasal biopsy material and by isolating it in culture.

Here we describe two possible cases of respiratory infections by *A. chartarum*. The presence of hyphal elements was seen in direct microscopic examination of bronchoalveolar lavage (BAL) specimens; the fungus was also isolated in culture and identified by PCR sequencing of multiple phylogenetic markers.

Patients and methods

Case summaries

Case 1. A 53-year-old Sri Lankan man was admitted to hospital in October 2017 with complaints of shortness of breath, cough and fever. Chest X-ray revealed bilateral infiltrates. He was empirically prescribed therapy with ceftriaxone and clarithromycin. However, his condition deteriorated as he developed severe acute respiratory distress syndrome. He was

intubated and kept on 100% FiO₂. Follow-up chest X-ray revealed bilateral infiltrations involving the quadrants of the chest, and a presumptive diagnosis of interstitial lung disease was made. On day 10 of admission, the patient was moved to another hospital for venovenous extracorporeal membrane oxygenation (ECMO) insertion. On day 12, he tested positive for influenza A (subtype H1N1) and was prescribed Tamiflu along with Tazocin (piperacillin and tazobactam) and linezolid. The following day he became haemodynamically stable and afebrile. On day 15 he was weaned from ECMO. Soon after decannulation the patient again developed acute respiratory distress syndrome and was intubated and ventilated. Echocardiogram performed at this time was unremarkable.

Microscopic examination of the BAL sample obtained on day 10 (before ECMO placement) showed many septate hyphal elements, and BAL culture yielded several colonies of a filamentous fungus on Sabouraud dextrose agar (SDA) in addition to *Pseudomonas aeruginosa*. The fungal isolate (lab accession no. Kw277/17) was later identified as *A. chartarum* by phenotypic and molecular characteristics. No other fungal pathogen was isolated. The patient was prescribed colistin, trimoxazole, levofloxacin and amphotericin B. The serum sample obtained on day 15 was negative for *Aspergillus* galactomannan antigen and also for fungal DNA by panfungal PCR. On day 18, the patient developed left-sided pneumothorax. On day 20, he again required venovenous ECMO. Because he became anuric and hyperkalemic with high serum creatinine, he was placed on haemodialysis. On day 30, extended-spectrum β -lactamase-positive *Klebsiella pneumoniae* was isolated from blood culture. The patient became leukopenic with increased serum procalcitonin levels. Although central lines were changed and therapy with vancomycin was initiated, *K. pneumoniae* continued to be found in blood cultures. The BAL sample and central venous portal tip also yielded extended-spectrum

β -lactamase-positive *K. pneumoniae* and multidrug-resistant *Acinetobacter baumannii*.

On day 43, he became haemodynamically unstable and thrombocytopenic, and he died of profuse bleeding and sudden cardiac arrest.

Case 2. A 53-year-old Bangladeshi man with a history of hypertension and ischaemic heart disease was admitted to the medical ward for chest pain accompanied by a productive cough and shortness of breath of 1.5 months' duration. At admission, chest X-ray revealed hilar congestion and bilateral lung lesions of unknown aetiology. Culture and GeneXpert MTB/RIF test of sputum sample were negative for tubercle bacilli; however, T-spot test was positive. The patient was prescribed antituberculosis therapy comprising isoniazid, rifampicin, ethambutol and pyrazinamide. Bronchoscopy did not reveal any endotracheal lesions. Direct microscopy was positive and culture of BAL on SDA yielded several colonies of a filamentous fungus which was later identified as *A. chartarum* (lab accession no. Kw192/18) by typical phenotypic characteristics and molecular characterization. Although BAL was positive for galactomannan (index value of 0.65), the serum was negative. No antifungal treatment was prescribed.

The patient was discharged after receiving 1 week of anti-tuberculosis therapy and was lost to further follow-up. The precise aetiology of the lung lesions remained unknown.

Isolation and identification

The BAL specimens were received by the Mycology Reference Laboratory, Faculty of Medicine, Kuwait University, as part of the routine diagnostic service for fungal infections. Both specimens showed septate hyphal elements when examined with potassium hydroxide–calcofluor (Fig. 1(a)) and grew several pale-coloured colonies with white aerial mycelium after 5 days'

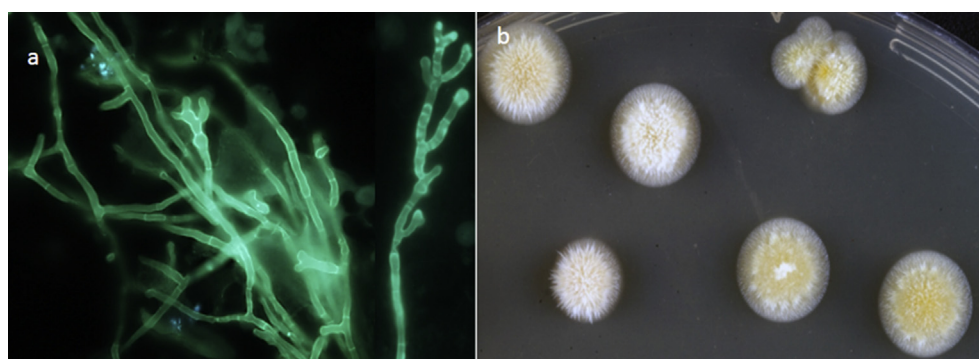


FIG. 1. (a) Potassium hydroxide–calcofluor mount showing branched septate hyphae of *Ascotricha chartarum* in bronchoalveolae of patient 1. (b) Culture of bronchoalveolar lavage of patient 1 yielding several yellow-coloured colonies of *A. chartarum* (Kw277/17) on Sabouraud dextrose agar after 5 days of incubation at 30°C.

incubation at 30°C on Sabouraud dextrose agar (Oxoid, Basingstoke, UK) supplemented with chloramphenicol (50 mg/L) (Fig. 1(b)).

Genomic DNA from the two isolates was prepared as described previously [5]. The internal transcribed spacer (ITS) region and D1/D2 domains of recombinant DNA (rDNA) were amplified by using panfungal primers and were sequenced with internal primers, as described in detail elsewhere [6,7]. The variable region of β -tubulin gene was amplified by using BTUBF (5'-TGGTAACCAAATCGGTGCTGCTT-3') and BTUBR (5'-GCACCCTCAGTGTAGTGACCCT-3') primers, and the amplicons were sequenced by using internal primers (BTUFS, 5'-TAACCAAATCGGTGCTGCTTTCTG-3' and BTURS, 5'-CCTCAGTGTAGTGACCCTTGGC-3'), as described previously [8]. GenBank Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches were performed for species identification. The DNA sequences for type and well-characterized strains of several *Ascotricha* spp. already available in GenBank were retrieved. The gene sequences were analysed individually, or nucleotide sequences of ITS region of rDNA and β -tubulin gene fragment were used for combined analysis.

Antifungal susceptibility

The Etest (bioMérieux, Marcy l'Etoile, France) was used to determine the MIC values of five antifungal agents, as described previously [9]. Briefly, the test was performed on RPMI 1640 medium supplemented with 2% glucose, and the pH was adjusted to 7.0 with 0.165 M morpholinepropanesulfonic acid buffer. The isolate was cultivated on a potato dextrose agar slant (Difco; Becton Dickinson, San Diego, CA, USA) for 7 days at 30°C. Conidia were collected in 2 mL sterile normal saline, and clumps were allowed to settle. The plates were inoculated

by dipping a sterile swab into the conidial suspension and streaking it uniformly over the agar surface. Plates were allowed to dry at room temperature for 15 minutes before Etest strips were applied. MICs were read after 72 hours of incubation at 35°C where the border of the inhibition ellipse intersected the scale on the antifungal strip.

Results

Phenotypic identification

Both isolates (Kw277/17 and Kw192/18) showed moderate growth on SDA and potato dextrose agar at 30°C, attaining a diameter of about 25 mm in 10 days. The colonies were initially white with yellowish felt covered white aerial mycelium (Fig. 2(a)). On ageing, greyish black sectors appeared on the surface with presence of abundant ascomata (Fig. 2(b)). The texture was floccose with reverse black. The colonies appeared rugose at 37°C, attaining a diameter of 20 mm in 15 days (Fig. 2(c)). The growth was inhibited at 40°C, and the isolates failed to grow at 42°C. Conidiophores were erect and dichotomously branched, regularly septate and brown at the lower part, becoming colourless above. Conidiophores were smooth or slightly roughened, colourless but sometimes becoming pale with age. Conidia were irregularly ellipsoid to pyriform, $5.5\text{--}6 \times 3.5\text{--}4 \mu\text{m}$, smooth to rough (Fig. 3). Ascomata were ostiolate, dark brown to black, globose to subglobose below, $70\text{--}110 \times 50\text{--}90 \mu\text{m}$ with a distinct neck; terminal hairs were erect and branched, septate, dark brown to black (Fig. 4). Asci were cylindrical, thin walled and deliquescent after the spores had matured, mainly $60\text{--}70 \times 10 \mu\text{m}$ in size, containing eight ascospores and arranged in a single row (Fig. 4). Ascospores appeared olive brown when mature and were ellipsoid ($7\text{--}10 \times 6\text{--}8 \mu\text{m}$) with an equatorial germ furrow.

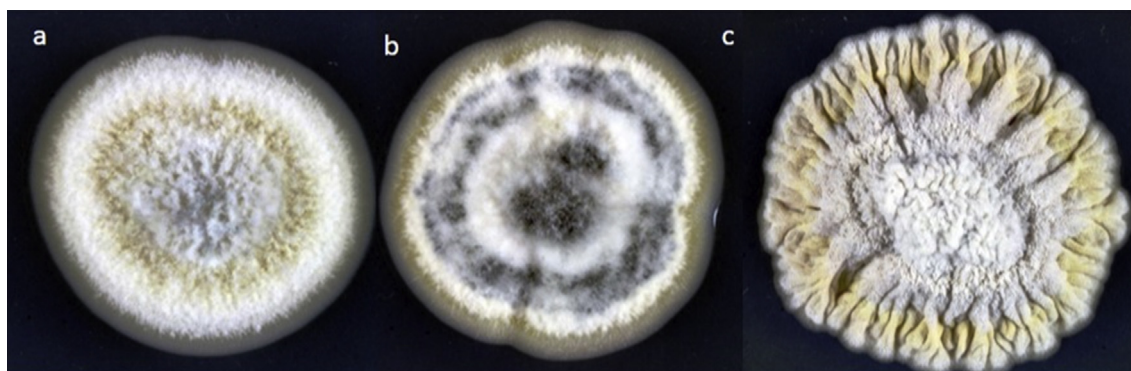


FIG. 2. Colony characteristics of isolate Kw277/17 on potato dextrose agar after incubation for 7 days (a) and 10 days (b) at 30°C, and after 15 days (c) of incubation at 37°C.

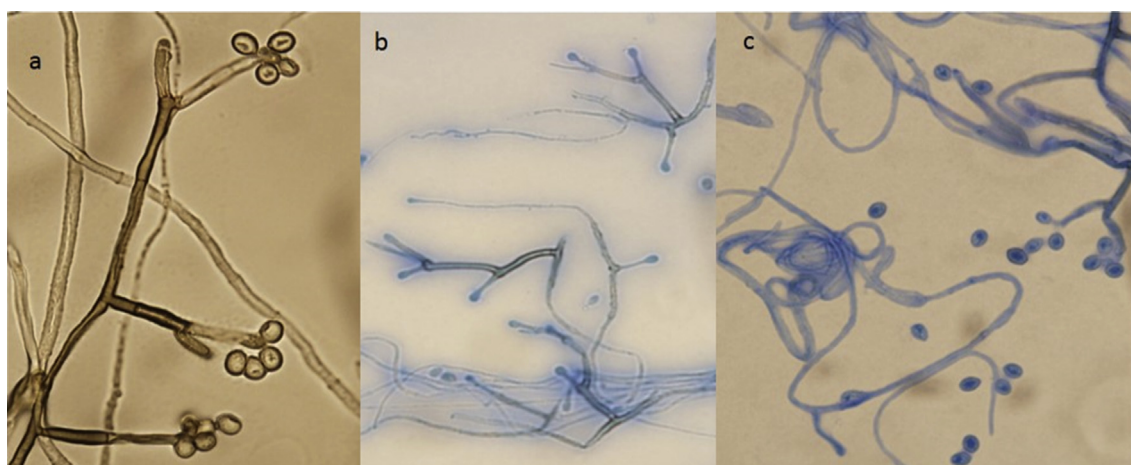


FIG. 3. Slide culture preparation of isolate Kw192/18 (a) lactophenol cotton blue mount (b, c) showing conidiophores with smooth to slightly rough ellipsoid to pyriform conidia.

Molecular characterization

The nucleotide sequences of the ITS region and D1/D2 domain of rDNA for both the isolates were identical. Based on nucleotide sequence comparisons with available sequences in the GenBank, the ITS region sequences showed 0 to 4 (<1%) nucleotide differences with strains of several *Ascotricha* species. The DNA sequence comparisons of the D1/D2 domain of rDNA were not very informative because sequence identities of <98% were observed (>99% identity is required for species identification [10, 11]) due to nonavailability of sequences from *Ascotricha* spp. for this gene in the GenBank database. The DNA sequence comparisons of the D1/D2 domain of rDNA with MycoBank database (<http://www.mycobank.org/BioLMICSSequences.aspx?expandparm=f&file=ALL>) showed nearly 100% identity with the corresponding sequences from *A. lusitanica* CBS 462.70 as well as from *A. chartarum* CBS 859.95 and CBS 657.95. Consequently, the β -tubulin gene fragment was sequenced. The data showed complete identity with the

corresponding sequence from *A. chartarum* but not with other *Ascotricha* spp. The ITS region of rDNA and β -tubulin gene fragment sequences from several *Ascotricha* species available from GenBank were retrieved [3]. Multiple sequence alignments were performed with Clustal Omega. The phylogenetic tree was constructed with combined ITS region of rDNA and β -tubulin gene fragment sequence data with MEGA 6.1 software by using the neighbour-joining method with the Kimura two-parameter model. The robustness of tree branches was assessed by bootstrap analysis with 1000 replicates. The dendrogram showed that both of our isolates were most closely related with *A. chartarum* and not with *A. bosei* or *A. lusitanica* or other *Ascotricha* spp. (Fig. 5). Thus, on the basis of the combined ITS region of rDNA and β -tubulin gene sequence comparisons, the identity of both of our isolates was established as *A. chartarum*.

The DNA sequencing data for the two isolates described here have been submitted to the European Molecular Biology

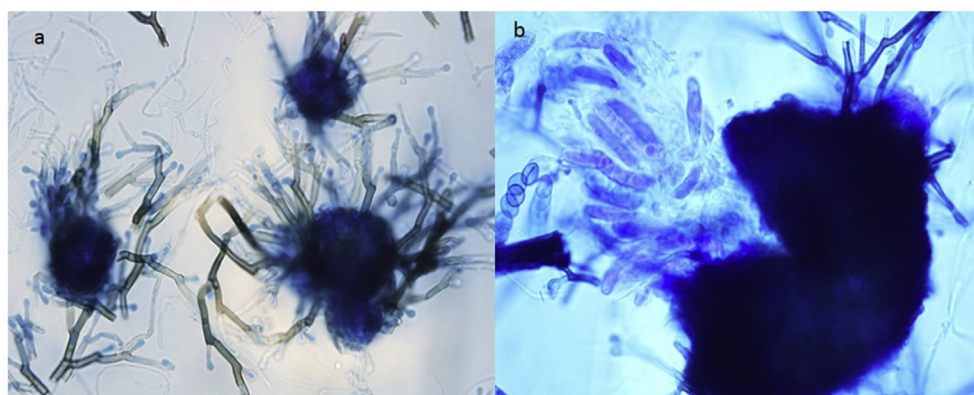


FIG. 4. Ascomata of *Ascotricha chartarum* (Kw192/18) in different stages of development: (a) young ascomata with peridial hair conidia and (b) mature ascomata showing asci filled with eight ascospores.

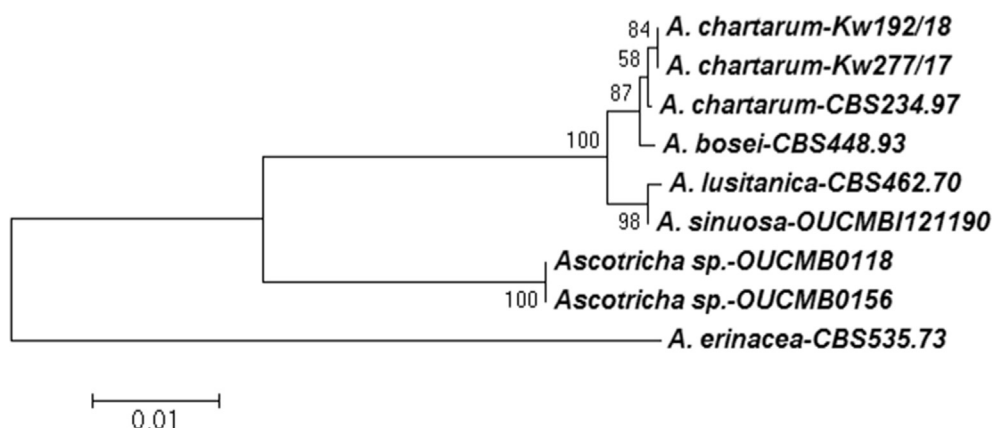


FIG. 5. Neighbour-joining phylogenetic tree based on combined internal transcribed spacer region of recombinant DNA β -tubulin gene sequence data for two *Ascotricha* species isolates (Kw277/17, Kw192/18) from Kuwait together with reference strains of several *Ascotricha* spp. Numbers on node branches are bootstrap frequencies.

Laboratory under accession numbers LS450960 to LS450963, LS 451020 and LS451021.

Antifungal susceptibility

Both strains (Kw122/18 and Kw277/17) appeared resistant to caspofungin and micafungin, with MICs of >32 $\mu\text{g/mL}$, but were susceptible to itraconazole (MICs of 0.008 $\mu\text{g/mL}$), posaconazole (MICs of 0.016 $\mu\text{g/mL}$ and 0.032 $\mu\text{g/mL}$), voriconazole (MICs of 0.002 $\mu\text{g/mL}$ and 0.004 $\mu\text{g/mL}$) and amphotericin B (MICs of 0.094 $\mu\text{g/mL}$ and 0.047 $\mu\text{g/mL}$).

Discussion

Although *A. chartarum* is capable of growing at 37°C , its role as a human pathogen is not well established. An early report considered *A. chartarum* var. *orientalis* Castell & Jackson as a cause of dermatoid infection [2]. So far, only one other report has described the aetiological role of *A. chartarum* in maxillary sinusitis [4]. The diagnosis was established by culture and by demonstrating the presence of pigmented fungal structures in a tissue biopsy sample. So far, *A. chartarum* has not been reported from respiratory or other clinical specimens, either as a contaminant or as a pathogen [12]. In this context, the present report of isolation of *A. chartarum* from BAL samples of two patients assumes considerable clinical significance because both of them had pulmonary disease or manifestations. Although the fungus was seen by direct microscopic examination of the BAL sample, its unequivocal aetiological role in the disease process remains to be established, as tissue biopsy samples for culture and/or histopathologic demonstration of the fungus were not available. Whether isolation of *A. chartarum* from BAL samples has similar clinical significance as for *Aspergillus* species or some

other known respiratory fungal pathogens is unclear [13]. Interestingly, both isolates were obtained within a period of 11 weeks from two patients, who were admitted in two different hospitals located about 20 km apart. It is unlikely that there was any common epidemiologic linkage with regard to isolation of *A. chartarum* from these two patients. Patient I was also positive for influenza A virus 2 days after *A. chartarum* was demonstrated in the BAL sample. It is possible that the patient already had influenza which predisposed him to *A. chartarum* infection. Several recent reports support an association of *Aspergillus* infection with ECMO and/or influenza [14–16].

The phenotypic identification of our isolates as *A. chartarum* is based on the presence of long ascomatal hairs with hyaline branches, brown ellipsoidal-shaped ascospores with an equatorial germ slit and an asexual stage characterized by conidiophores and conidia. The identity was subsequently confirmed by DNA sequence data for the ITS region of rDNA and β -tubulin gene fragment. Historically, Whiteside [17], on the basis of hymenial paraphyses, proposed that the genus *Ascotricha* was closely related to the *Xylariaceae* (*Xylariales*, *Sordariomycetes*). However, Hawksworth [2] revised *Ascotricha* and placed it in the *Chaetomiaceae* (*Sordariales*, *Sordariomycetes*), but subsequent scanning electronic microscopic studies revealed that the genus had strong affinities with the *Xylariaceae* [18]. This taxonomic status of *Ascotrichum* has recently been confirmed by phylogenetic analyses of multiple loci [3].

So far *A. chartarum* has remained unrecognized as a human pathogen. Information about its aerial/seasonal prevalence, virulence factors, susceptibilities to antifungal agents and experimental/animal studies to demonstrate its pathogenic potential is lacking. The species appears to have a wide geographic distribution and has also been reported from indoor environments [2,19]. *Ascotricha* species are known to possess

strong cellulolytic activity [20] and have been isolated from diverse environmental sources, such as lignum, damp sheet-rock paper, woody and straw materials or other cellulosic substrates [2,21]. Two new *Ascotricha* species have been isolated from marine habitat in association with algae, which may be its preferred niche [3]. *Ascotricha* species have been exploited for industrial usage because of their ability to produce novel compounds that may have commercial applications, such as those with antifungal or antiangiogenic properties. Because *A. chartarum* is capable of growing at 37°C and also contains melanin, a known virulence factor, it is possible that under certain conditions, like other *Chaetomium* or other *Chaetomium*-like fungi, it can become an opportunistic pathogen in immunocompromised patients [22]. Data on antifungal susceptibility of *A. chartarum* are lacking. Our isolates showed reduced susceptibility to echinocandins, which is also observed in *Chaetomium* and some other dematiaceous fungi [23].

In conclusion, we have described the isolation and characterization of *A. chartarum* from BAL samples of two patients with pulmonary infections. Because tissue biopsy samples were not available for culture and/or histopathology, the aetiologic role of *A. chartarum* in the disease process could not be unequivocally established. Nonetheless, in view of the rarity of isolation of *A. chartarum* from clinical specimens, this report assumes considerable significance. It serves to create awareness about environmental fungi that previously have missed attention but may play a role in respiratory infections.

Conflict of interest

None declared.

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References

- [1] Berkeley MJ. Notices of British fungi. *Ann Mag Nat Hist Ser I* 1838;1: 257–64.
- [2] Hawksworth DL. A revision of the genus *Ascotricha* Berk. *Mycol Pap* 1971;126:1–28.
- [3] Cheng X, Li W, Cai L. Molecular phylogeny of *Ascotricha*, including two new marine algae-associated species. *Mycologia* 2015;107:490–504.
- [4] Singh SM, Naidu J, Jain S, Nawange SR, Dhindsa MK. Maxillary sinusitis caused by *Ascotricha chartarum* Berk. (anamorph *Dicyna ampullifera* Boul.): a new phaeoid opportunistic human pathogen. *J Med Vet Mycol* 1996;34:215–8.
- [5] Khan ZU, Ahmad S, Theyyathel AM. Detection of *Aspergillus fumigatus*-specific DNA, (1–3)- β -D-glucan and galactomannan in serum and bronchoalveolar lavage specimens of experimentally infected rats. *Mycoses* 2008;51:129–35.
- [6] Khan ZU, Ahmad S, Mokaddas E, Chandy R, Cano J, Guarro J. *Actinomyces elegans* var. *kuwaitiensis* isolated from the wound of a diabetic patient. *Antonie Leeuwenhoek* 2008;94:343–52.
- [7] Khan ZU, Ahmad S, Hagen F, Fell JW, Kowshik T, Chandy R, et al. *Cryptococcus randhawai* sp. nov., a novel anamorphic basidiomycetous yeast isolated from tree trunk hollow of *Ficus religiosa* (peepal tree) from New Delhi, India. *Antonie Leeuwenhoek* 2010;97:253–9.
- [8] Khan Z, Ahmad S, Al-Ghimlas F, Al-Mutairi S, Joseph L, Chandy R, et al. *Purpureocillium lilacinum* as a cause of cavitary pulmonary disease: a new clinical presentation and observations on atypical morphologic characteristics of the isolate. *J Clin Microbiol* 2012;50:1800–4.
- [9] Khan Z, Al-Obaid K, Ahmad S, Ghani AA, Joseph L, Chandy R. *Acremonium kiliense*: reappraisal of its clinical significance. *J Clin Microbiol* 2011;49:2342–7.
- [10] Kurtzman CP, Robnett CJ. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Leeuwenhoek* 1998;73:331–71.
- [11] Schoch CL, Fungal Barcoding Consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci USA* 2012;109:6241–6.
- [12] Chowdhary A, Agarwal K, Meis JF. Filamentous fungi in respiratory infections. What lies beyond aspergillosis and mucormycosis? *PLoS Pathog* 2016;12, e1005491.
- [13] Bonnal C, Leleu C, Brugière O, Chochillon C, Porcher R, Boelle PY, et al. Relationship between fungal colonisation of the respiratory tract in lung transplant recipients and fungal contamination of the hospital environment. *PLoS One* 2015;10, e0144044.
- [14] Ajmal S, Mahmood M, Abu Saleh O, Larson J, Sohail MR. Invasive fungal infections associated with prior respiratory viral infections in immunocompromised hosts. *Infection* 2018;46:555–8.
- [15] Cavayas YA, Yusuff H, Porter R. Fungal infections in adult patients on extracorporeal life support. *Crit Care* 2018;22:98.
- [16] Rodriguez-Goncer I, Thomas S, Foden P, Richardson MD, Ashworth A, Barker J, et al. Invasive pulmonary aspergillosis is associated with adverse clinical outcomes in critically ill patients receiving veno-venous extracorporeal membrane oxygenation. *Eur J Clin Microbiol Infect Dis* 2018;37:1251–7.
- [17] Whiteside WC. Morphological studies in the *Chaetomiaceae* I. *Mycologia* 1962;54:152–9.
- [18] Hawksworth DL, Wells H. Ornamentation on the terminal hairs in *Chaetomium* Kunze ex Fr. and some allied genera. *Mycol Pap* 1973;134: 1–24.
- [19] Li DW, Yang CS. Notes on indoor fungi I: new records and noteworthy fungi from indoor environments. *Mycotaxon* 2004;89:473–88.
- [20] De Hoog GS, Guarro J, Gene J, Figueras MJ. Atlas of clinical fungi. 2nd ed. Centraalbureau voor Schimmelcultures/Universitat Rovira I Virgili; 2000.
- [21] Hanlin R. Illustrated genera of Ascomycetes. St Paul: APS Press; 1990.
- [22] Ahmed SA, Khan ZU, Wang XW, Moussa TAA, Al-Zahrani HS, Almaghrabi OA, et al. *Chaetomium*-like fungi causing opportunistic infections in humans: a possible role for extremotolerance. *Fungal Divers* 2016;76:11–26.
- [23] Chowdhary A, Meis JF, Guarro J, de Hoog GS, Kathuria S, Arendrup MC, et al. ESCMID and ECMM joint clinical guidelines for the diagnosis and management of systemic phaeohyphomycosis: diseases caused by black fungi. *Clin Microbiol Infect* 2014;20(Suppl. 3): 47–75.