

## *Colletotrichum truncatum*: an Unusual Pathogen Causing Mycotic Keratitis and Endophthalmitis<sup>∇</sup>

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**In recent years, the well-known plant pathogens of the *Colletotrichum* genus were increasingly reported to cause ophthalmic infections in humans. Among 66 species in the *Colletotrichum* genus, only a few are known to be pathogenic for humans. We report here five cases of ophthalmic infections due to *Colletotrichum truncatum*, a species never reported earlier to cause human infection. The isolates were identified by morphological characteristics and the sequencing of internal spacer regions of ribosomal DNA. The progress of lesions in those patients was slow compared to that of lesions caused by *Aspergillus* or *Fusarium* infections. The surgical management included total penetrating keratoplasty in patients with keratitis and pars plana vitrectomy in endophthalmitis. Two patients were treated additionally with intravitreal amphotericin B deoxycholate, one patient with oral itraconazole, and another patient with oral and topical fluconazole therapy. The present series therefore highlights the expanding spectrum of agents causing eye infections and the inclusion of *C. truncatum* as a human pathogen.**

*Colletotrichum* species are well-known plant pathogens in tropical and subtropical countries. The taxonomy and nomenclature of *Colletotrichum* species are confusing, even to scientists working in this field (11). Initially, the species were identified by the characteristic host specificity, location of isolation, and conidial morphology. Subsequently, based on morphological characteristics, host range (of plants), and phylogenetic analysis, the fungi in the genus *Colletotrichum* were systematically classified. Currently there are 66 species, including 19 species for which the identification is doubtful (11).

Human infection due to *Colletotrichum* spp. is rare and has been reported from ophthalmic and subcutaneous infections (5, 8, 10, 13–15, 17–24, 26, 30, 32, 38, 41). The latter group of infections generally are reported in immunocompromised hosts (4). The species implicated in human infections include *C. dematium* (5, 8, 10, 13, 15, 17, 21, 32), *C. coccodes* (reported as *C. atramentarium*) (18, 24, 27), *C. gloeosporioides* (8, 20, 23, 24, 30, 40), *C. graminicola* (26, 41), *C. crassipes* (4), *C. capsicii* (38) (currently named *C. truncatum*) (7), and an unknown species (8, 22). Only a few cases of *C. dematium* eye infections are reported from India (5, 13–15, 21, 32). In all of those reports, the fungus was identified on the basis of morphological features. Here, we report for the first time five cases of ophthalmic infections due to *C. truncatum*, which initially were misidentified morphologically as *C. dematium*. The study also emphasizes the need of nucleic acid sequence analysis for the accurate identification of these difficult-to-identify fungi.

### MATERIALS AND METHODS

The records of all patients with culture-proven fungal eye infections during 2003 to 2009 at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, were retrospectively reviewed. The clinical details of patients with eye infections due to *Colletotrichum* species were noted. The age, sex, eye involved, risk factors, antibiotic and antifungal agents used for treatment, clinical course, and outcome for each patient were recorded.

**Mycological investigation.** The direct microscopy of corneal scrapings from patients with corneal ulcers and vitreous/aqueous aspirate from patients with endophthalmitis were performed under a fluorescent microscope (Olympus BX51; Tokyo, Japan) using 1% calcofluor (Sigma-Aldrich, St. Louis, MO) and 10% KOH. Sabouraud dextrose agar (SDA) (Hi Media, Mumbai, India) was used for the primary isolation of the fungi. The morphological features of the isolates were recorded after growth on potato dextrose agar (PDA) and oatmeal agar. The isolates are deposited at the National Culture Collection of Pathogenic Fungi (NCCPF), PGIMER, Chandigarh, India.

**Phylogeny and molecular identification.** Molecular identification was done by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). The genomic DNA from the mycelia was extracted by following a modified protocol of Lee and Taylor (16). Briefly, *Colletotrichum* spp. were allowed to grow in potato dextrose broth at 37°C on a rotary shaker (Innova, Ependroff, Hamburg, Germany) at 120 rpm for 3 to 5 days. The growth was filtered, and the harvested mycelial mat was washed with sterile normal saline and crushed in the presence of liquid nitrogen. The powder was transferred to a 1.5-ml microcentrifuge tube containing 600  $\mu$ l of lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 3% SDS). After a brief vortexing, proteinase K (Sigma St. Louis, MO) was added at a final concentration of 20  $\mu$ g/ml, and the solution was incubated at 56°C for 1 h. The DNA was extracted using a phenol-chloroform extraction procedure. The precipitation of DNA was done with an equal volume of isopropanol in the presence of 3 M sodium acetate. The pellet was washed with 70% alcohol and dissolved in 100  $\mu$ l of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at –20°C until further use.

The amplification of the ITS region of the rRNA gene was performed by PCR. Amplification reactions were done as a 10- $\mu$ l reaction mixture in the presence of 2 mM MgCl<sub>2</sub>, 200  $\mu$ M each deoxynucleotide triphosphate (dNTP), 0.25  $\mu$ M each primer (ITS1, GCATATCAATAAGCGGAGGAAAAG; ITS4, GGTCGGTGT TCAAGACGG) (39), 0.25 U of *Taq* polymerase (Bangalore Genei, Bengaluru, India), and 5 to 10 ng of fungal genomic DNA. The PCR cycling conditions consisted of an initial denaturation step for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 5 min. The gene fragments were visualized on an agarose gel and excised with a clean sharp scalpel, and the DNA was

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retrieved by using a Q/A quick-spin column (QIAquick; Qiagen, Hilden, Germany) per the manufacturer's instructions. DNA sequencing was performed with the primers described above using a BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. The sequencing reactions were analyzed by an ABI Genetic Analyzer (Applied Biosystems, Foster City, CA).

For each isolate, the DNA sequences obtained from forward and reverse primers were used to obtain consensus sequences using the Seqman software (DNA Star; Lasergene, Madison, WI). The isolates were identified by blasting the sequences in the NCBI GenBank. Based on the ITS sequence data from the ex-type or ex-epitype cultures of different *Colletotrichum* species, a phylogenetic tree was constructed (2). DNA sequences obtained from the database and the sequences of our isolates were aligned using Clustal X (37), and gaps were treated as missing data. Phylogenetic analysis was carried out by MEGA4 (35) based on the neighbor-joining method (28). The evolutionary distances were computed using the maximum composite likelihood method and were expressed as numbers of base substitutions per site (36). All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (using the pairwise deletion option). The resulting tree was evaluated with 1,000 bootstrap replications to test the stability.

**Nucleotide sequence accession numbers.** The sequences of all of the isolates determined in the course of this work were deposited in NCBI GenBank, and the accession numbers are given in Table 1.

**RESULTS**

During the 7-year study period (2003 to 2009), 717 ophthalmic samples (from 608 patients with fungal keratitis and 109 patients with endophthalmitis) were positive for the isolation of fungi. Only six isolates (0.83%) were identified as *Colletotrichum* spp., out of which four were isolated from corneal scrapings (0.65%) and two from vitreous tap (1.83%). The age of these patients ranged from 18 to 70 years; five were male and one was female. A history of trauma was recorded for four patients due to wooden stick injury for two patients, fall of insect for one patient, and trauma due to vegetable matter for one patient. One patient had the subconjunctival injection of dexamethasone, which might be a risk factor for the development of *Colletotrichum* infection. No obvious history of trauma could be elicited for the sixth patient. As the patient was 70 years old, trivial unnoticed trauma could not be excluded. The visual acuity of the affected eye was less in all patients at the time of presentation than that of the unaffected eye. The clinical details of these six patients are summarized in Table 1.

The direct microscopy of the samples revealed the presence of septate, acutely angled branching hyphae. Fungal growth on SDA had pinkish aerial mycelia that became gray-black with dark brown on the reverse. Colonies on oatmeal agar were flat, and the entire margin did not have any aerial mycelia; the surface was buff color and covered with olivaceous gray to iron gray acervuli, with the reverse side buff to pale olivaceous gray (Fig. 1A and B). The examination of the slide cultures after 7 days of growth at 25°C revealed hyaline, septate hyphae. Conidiomata were acervular, with 2 to 5 septate setae appearing hyaline to pale brown and smooth to verruculose, with tapering toward the tip. Conidia were hyaline, smooth-walled to verruculose, aseptate, and long, and the central part was slightly curved with parallel walls, ending abruptly at the round and truncated base, with tapering toward the acute and more strongly curved apex. Appressoria were light brown, with the entire lobed edge having a roundish to ellipsoidal outline (Fig. 1C and D). The culture isolates were presumptively identified as *C. dematium* based on these morphological findings. Subsequently, BLAST results of the sequences showed 99 to 100%

TABLE 1. Clinical and mycological details of cases with ophthalmic infections caused by *Colletotrichum*<sup>a</sup>

Case no.	NCCPF	GenBank accession no.	Age/sex	Mode of infection	Clinical specimen type	Direct smear type	Presumptive morphological identification	Identification by ITS sequencing	Treatment	Outcome
1	112002	HO437661	60/F	Trauma with wooden stick	Corneal scraping	Septate hyphae	<i>Colletotrichum dematium</i>	<i>C. truncatum</i>	Therapeutic keratoplasty, oral and topical fluconazole	Atrophic bullitis with staphylooma
2	112003	HO437662	38/M	Subconjunctival injection of dexamethasone	Vitreous tap	Septate hyphae	<i>Colletotrichum dematium</i>	<i>C. truncatum</i>	Amphotericin B, dexamethasone, itraconazole, pars plana vitrectomy	Clinical improvement
3	112004	HO437663	70/M	No trauma	Corneal scraping	Septate hyphae	<i>Colletotrichum dematium</i>	<i>C. truncatum</i>	Therapeutic keratoplasty	Clinical improvement
4	112006	ND	18/M	Vegetable matter	Corneal scraping	Septate hyphae	<i>Colletotrichum dematium</i>	ND	Therapeutic keratoplasty	Clinical improvement
5	112007	HO437660	58/M	Insect fall	Corneal scraping	Septate hyphae	<i>Colletotrichum dematium</i>	<i>C. truncatum</i>	Therapeutic keratoplasty	Corneal opacity
6	112008	HO437664	50/M	Trauma with wooden stick	Vitreous tap	Septate hyphae	<i>Colletotrichum dematium</i>	<i>C. truncatum</i>	Amphotericin B, pars plana vitrectomy	Partial improvement in CF (1.5 m)

<sup>a</sup> F, female; M, male; ND, not done because the strain could not be revived from culture collection; CF, counting finger (value represents the maximum distance at which the patient was able to count fingers on a hand).

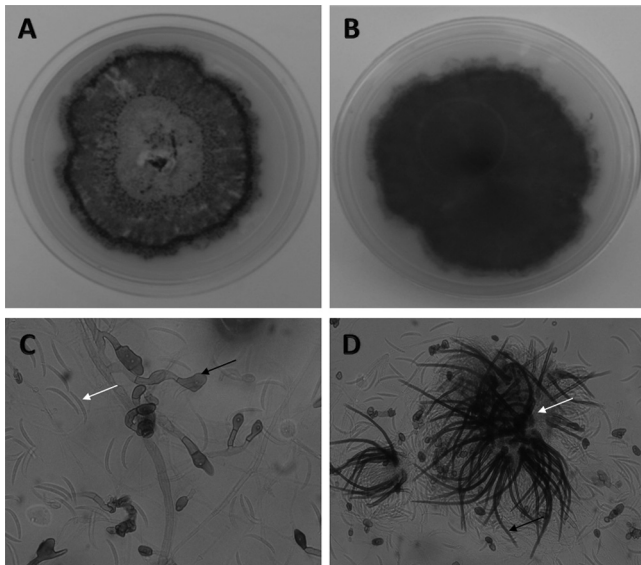


FIG. 1. Colonies of *Colletotrichum truncatum* (NCCPF 112007) as noted on oatmeal agar after 10 days of incubation. (A) Obverse image; (B) reverse image. (C) Lactophenol cotton blue mount of *Colletotrichum truncatum* (NCCPF 112007) showing the appressoria (black arrow) and falcate nonseptate conidia (white arrow). (D) Acervuli (white arrow) containing setae (black arrow).

identity with the *C. truncatum* isolated from the environment in India (GU227878, CBS 172.48). The phylogram constructed by using the ITS data showed our strains clustering with the ex-type strains of *C. truncatum* (CBS 151.35 and CBS 120709) (Fig. 2). The molecular identification of one strain was not possible, as the strain could not be revived and the stored culture was contaminated.

## DISCUSSION

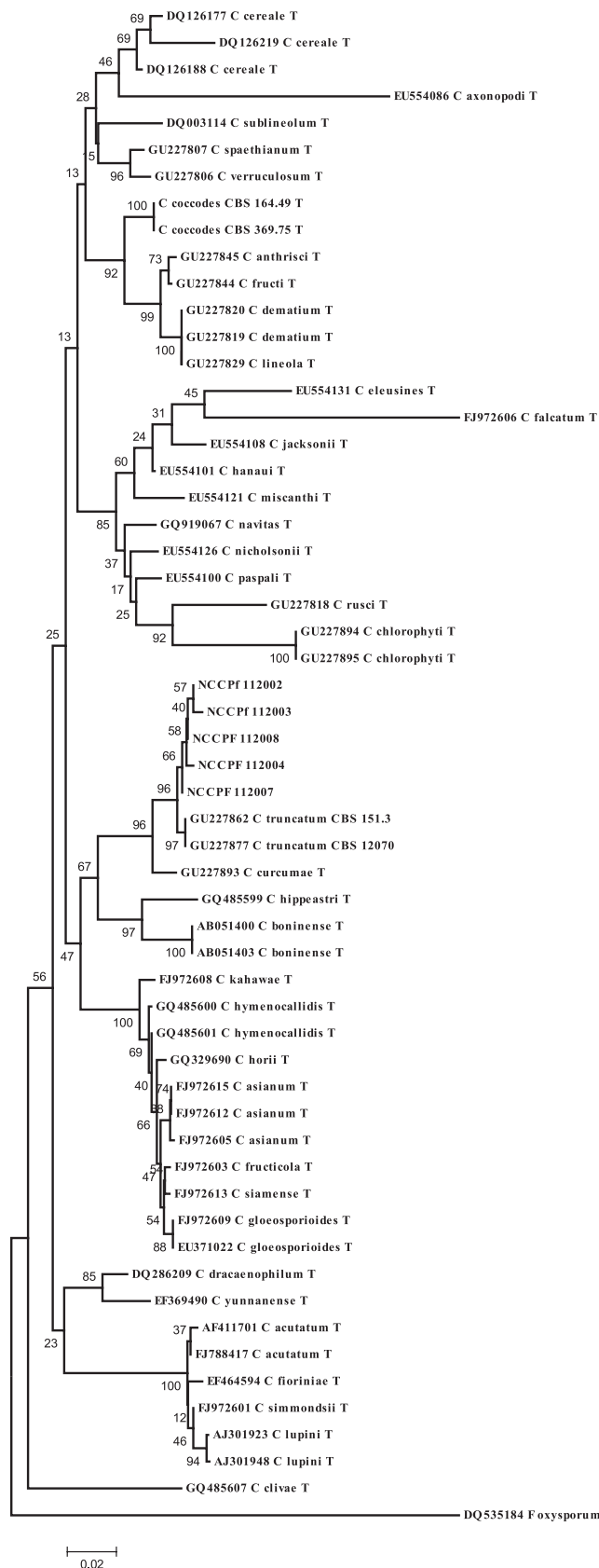
Although *Colletotrichum* spp. are considered important plant pathogens, the taxonomy of this group of fungi is confusing even to plant pathologists. Several efforts have been made to revise the classification by using a polyphasic approach incorporating morphological, biochemical, physiological, and phylogenetic characteristics (2). The epitypification of this fungus is important, as the type strain might have been lost or might be in poor condition (12, 29). Certain positive developments in the epitypification has helped to solve many taxonomic problems and the understanding of species, genera, families, or orders of this group of fungi (6, 11, 12, 25, 29). Presently, *Colletotrichum* spp. have gained importance as human pathogens causing keratitis (8, 13–15, 17, 21, 23, 26, 27, 30, 38, 40, 41). Of 66 currently known species, *C. dematium* has been reported from most human cases (5, 8, 10, 13, 15, 17, 21, 32). Other rare species implicated in human infections include *C. gloeosporioides* (20, 23, 40), *Glomerella cingulata* (teleomorph state of *C. gloeosporioides*) (30), *C. graminicola* (26, 41), and *C. coccodes* (18, 24). Eye infections due to *Colletotrichum* spp. have been reported in many countries, including *C. gloeosporioides* (8), *C. graminicola* (26), *C. dematium* (8, 10), and unknown *Colletotrichum* spp. (8, 19) in the United States, *C. gloeosporioides* (40, 20, 23) in Japan, *C. dematium* (13–15,

21) and *C. graminicola* (41) in India, *C. dematium* (17) in China, *C. coccodes* (18) in South Africa, and *C. capsicii* (38) in Nepal. To the best of our knowledge, no infection in humans due to *C. truncatum* had been reported before the present series. However, *C. capsicii* isolated from a patient with keratitis (38) was later identified as *C. truncatum* based on molecular methods (7). The present series describes five cases of ophthalmic infection due to *C. truncatum*.

The identification of *Colletotrichum* to the species level may be important for epidemiological investigations. However, morphological identification on the basis of the appearance of conidia, appressoria, and acervuli on routine medium is both time-consuming and technically challenging. To induce characteristic morphological features, such as aseptate conidia, appressoria, and acervuli, growth on PDA is useful. Once the culture is actively sporulating, plugs of around 4 mm are cut from the actively sporulating areas and are grown on PDA plates under alternating 12 h of UV light and 12 h of no light (33). Still, the major difficulty for morphological identification is the close resemblance of the curved conidia of *Fusarium* and *Colletotrichum* spp. However, *Colletotrichum* spp. can be identified by nonseptate conidia, the presence of appressoria, and (in the later stage) acervuli with setae.

Due to the difficulty of the morphological identification of *Colletotrichum* spp. in the diagnostic laboratory, the molecular approach seems to be more applicable and accurate. Phylogenetic analysis based on the nucleic acid sequence of the internal transcribed spacer region of the rDNA is the most suitable method for the identification of *Colletotrichum* isolates to the species level, although many sequences might have been deposited under different names in GenBank due to the repeated revisions of the taxonomy of *Colletotrichum* spp. Concerns were expressed occasionally when using ITS sequence data alone for the identification of *Colletotrichum* spp. (2). Crouch et al. reported a high error rate (86%) for comparing ITS sequence data within the *C. graminicola* species complex (6). Similarly, more than 86% evolutionary divergence was noted from type species of *C. gloeosporioides* for the analysis of 343 ITS sequences (3). In spite of this limitation, only sequences of the ITS region of the rDNA are available for all of the ex-type and epitype cultures of *Colletotrichum* spp. Hence, we attempted to compare the sequences of our strains to the typified and epitypified strains of the recently described *Colletotrichum* spp. In the phylogenetic study, all of our strains clustered together with *C. truncatum* (CBS 151.3 and CBS 120709) with 96% bootstrap agreement. *C. curcuma* is the only species that is closely related to *C. truncatum* (7). The need for molecular techniques for species identification is emphasized despite the limitation of the present database, as we misidentified all of our strains as *C. dematium* due to its close morphological resemblance to *C. truncatum*. Note that all *C. dematium* clinical isolates from India were identified by morphological characteristics only, and no sequence study was attempted.

The present case series describes infections of the cornea and also endophthalmitis due to *C. truncatum*. No case of endophthalmitis due to *Colletotrichum* spp. was reported earlier. It would be pertinent to study the virulence properties of *C. truncatum*, as two patients developed endophthalmitis. *Colletotrichum* spp. have been reported to cause infections in skin, subcutaneous tissue, and eye, and it rarely causes systemic



disease in humans. The first case of keratitis due to *C. dematium* was reported in 1983, and subsequently the majority of infections due to *Colletotrichum* spp. were reported in the eye (5, 8, 13–15, 17–21, 23, 26, 27, 30, 32, 38, 40, 41). Although *Colletotrichum* eye infections are increasingly reported, the rate of isolation still is low (0.83% in the present series). However, it would require close observation, as the identification of the fungus is difficult and many centers may not attempt it. *Colletotrichum* spp. usually enter tissue by trauma caused by vegetable matter or the contamination of a wound with soil (8, 15, 20, 21, 24). The traumatic implantation is important for the initiation of infection, as the conidia are contained in the asexual fruiting body (acervular conidiomata) and are not freely released. Trauma/insect fall/iatrogenic injury were noted in affected eyes in all of our patients except one.

*C. truncatum* has ubiquitous distribution and is reported to cause the anthracnose of bean, soybean, peanut, chili pepper, and pepper. The agent has been isolated from the United States, India, and Thailand (2, 9, 25). In India, *C. truncatum* is known to infect kharif crops such as paddy, sugarcane, groundnuts, maize, and a variety of pulses, which are grown during kharif season (summer and rainy seasons) (9), and it is reported from the northwestern Himalayas of the Maratwada region of Maharashtra state and in Tamil Nadu (reported as *C. capsicii* in chili fruit) (29). The present cases, except one (case 6), also were from the northwestern part of India and were reported with eye infections at the end of the kharif season. Although no attempt was made to correlate environmental and clinical isolates in the present study, it would be important for a future detailed epidemiological study of these infections.

As is the case for infection with other types of fungal keratitis, patients with *Colletotrichum* spp. keratitis present with a history of pain, redness, decreased vision, photophobia, and discharge. The ulcers usually are central or paracentral in position with irregular or serrated edges. *Colletotrichum* keratitis progresses slowly compared to *Aspergillus* or *Fusarium* keratitis, and brown pigmentation may be noted in the ulcer bed as the fungus is a dematiaceous mold (1).

Like other fungal eye infections, *Colletotrichum* spp. infections are best managed by the surgical removal of the fungal mass and the infected tissue. The patients in the present series were treated by total penetrating keratoplasty for keratitis or pars plana vitrectomy for endophthalmitis. The majority (67%) of the patients had partial or complete clinical improvement. Two of the patients who had endophthalmitis were treated additionally with intravitreal amphotericin B deoxycholate. One of the *Colletotrichum* keratitis patients received oral and topical fluconazole. There is no consensus on the medical therapy of *Colletotrichum* ophthalmic infection in the literature (8, 14, 21). In a 21-year-long study, good response to natamycin was noted in nine patients despite exhibiting high minimum

FIG. 2. Phylogram generated using the maximum composite likelihood method based on rDNA ITS sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. *Fusarium oxysporum* was used as an outgroup.

inhibitory concentrations (MIC) by *in vitro* susceptibility testing. The *in vitro* amphotericin B MIC for all of those strains was uniformly low (8). Similarly, the antifungal susceptibility laboratory of the University of Texas reported that the amphotericin B MICs for most of their *Colletotrichum* spp. were low (34). In contrast, Shukla et al. have reported that *Colletotrichum* strains were more sensitive to clotrimazole and micanozole than amphotericin B (31). In the review by Kaliyamurthy et al., the complete resolution of *Colletotrichum* ulcers was reported for the combination of medical and surgical therapy. The average duration of therapy for complete resolution ranged from 2 to 6 weeks (14). It appears that the early removal of the fungal mass by keratoplasty or pars plana vitrectomy is the best management option available until a consensus develops on additional medical therapy.

In conclusion, although *Colletotrichum* infection in humans is rare, the current study highlights the expanding spectrum of infectious agents due to *Colletotrichum* spp. and reports *C. truncatum* as a human pathogenic species. It is too early to judge whether species identification is necessary for the management of this infection, but it may be important for the epidemiology of the disease. As the morphological identification of the agent is difficult, molecular techniques may help in accurate diagnosis. Future studies are required to understand its epidemiology as well as the management of this disease.

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