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ORIGINAL RESEARCH

Comparison of Morphological, Virulence, and Antifungal Susceptibility Characteristics Within *Aspergillus Lentulus*

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Background: The emerging pathogenic species *Aspergillus lentulus*, within the *Aspergillus fumigatus* complex, poses a significant threat to patient health owing to its high rates of drug resistance and mortality. The aim of this study was to characterize the intraspecies morphology, virulence, and in vitro antifungal susceptibility of *A. lentulus*.

Methods: We cultured *A. lentulus* isolates from different sources (three clinical isolates and one environmental isolate), along with *A. fumigatus* (n=1) and *Aspergillus fumigatiaffinis* (n=1), to observe colony color, diameter, sporulation timing, and spore production. Virulence was assessed using a *Galleria mellonella* infection model, and survival curves were generated to evaluate strain pathogenicity. Antifungal susceptibility was determined using the colorimetric microdilution method with Sensititre YeastOne® panels.

Results: Compared to *A. fumigatus* and *A. fumigatiaffinis*, *A. lentulus* exhibited whitish colonies with pale green overtones, delayed sporulation initiation, and reduced spore yield. The four *A. lentulus* isolates showed intrastrain variability in the timing of colony color transition, growth rates, sporulation onset, and spore quantification. Virulence experiments demonstrated that all *A. lentulus* isolates successfully infected *G. mellonella* larvae and exhibited concentration- and time-dependent survival patterns. Clinical isolates consistently showed significantly lower larval survival rates than the environmental isolates at all infection concentrations. Antifungal susceptibility testing revealed the following minimum inhibitory concentration (MIC) ranges for *A. lentulus*: posaconazole 0.5–2 μg/mL, itraconazole 1–2 μg/mL, and voriconazole 2–8 μg/mL. Specifically, the clinical isolate *A. lentulus*-10199 and environmental isolate *A. lentulus*-10201 exhibited elevated voriconazole MICs (8 μg/mL). All strains demonstrated high MICs for amphotericin B (≥4 μg/mL) and caspofungin (≥8 μg/mL).

Conclusion: *Aspergillus lentulus* exhibited both interstrain and intrastrain variations in growth rate and sporulation characteristics. Clinical isolates demonstrated greater virulence potential than environmental isolates. *Aspergillus lentulus* displayed favorable susceptibility to posaconazole but reduced susceptibility to voriconazole, amphotericin B, and caspofungin.

Keywords: *Aspergillus lentulus*, virulence, antifungal susceptibility testing, azole, *Aspergillus fumigatus* complex

Introduction

Invasive fungal infections pose a severe threat to human health and are related to an estimated annual global mortality rate of 3.75 million people. *Aspergillus fumigatus* remains one of the primary pathogens causing invasive aspergillosis, which results in an alarming mortality rate of up to 50%.[1–3] The complexity of antifungal treatment and patient mortality significantly increases when infections are caused by azole-resistant strains or accompanied by delayed and missed diagnoses.

Molecular taxonomic studies have revealed that *A. fumigatus* actually constitutes a multispecies complex (the *A. fumigatus* complex),[4,5] comprising species such as *A. fumigatus*, *A. lentulus*, *A. fumisynnematus*, *A. fumigatiaffinis*, *Neosartorya fischeri*, and *A. novofumigatus*.[5] Among these, *A. lentulus* was first identified through molecular characterization in 2005[6] and has since been reported in multiple countries, including China.[7–15] However, biological characteristics and pathogenicity data for this species remain limited.

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Existing studies demonstrate that, compared to *A. fumigatus sensu stricto*, *A. lentulus* typically exhibits white colonies with pale green hues, delayed sporulation, small columnar conidial heads, and the ability to produce ascospores (sexual reproductive structures),[16] while being unable to grow at temperatures >48°C.[6] *Aspergillus lentulus* and *A. fumigatus* can be distinguished rapidly by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) and surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS).[17,18] Clinical reports indicate that *A. lentulus* primarily infects immunocompromised patients, causing invasive pulmonary aspergillosis.[7–15] The high mortality rate associated with *A. lentulus* infections is hypothesized to correlate with host immunosuppression, strain virulence, and drug resistance.[7] However, conflicting evidence exists: some studies using *G. mellonella* infection models report significantly lower virulence levels than *A. fumigatus*,[19,20] while others observe intraspecific virulence variations among *A. lentulus*.[21] Clinical data confirm reduced susceptibility of *A. lentulus* to most antifungals, particularly azoles.[6,7,22,23] Studies have confirmed that the mechanism of azole resistance in *Aspergillus* is mainly point mutations or overexpression in the CYP51A gene, and tandem repeat (TR) mutations in the promoter region.[24,25] Non-CYP51A mutant azole-resistant strains are mainly related to the overexpression of the efflux pump ABC transporter.[26] Notably, previous research has focused exclusively on clinical isolates, neglecting environmental strains. There is a need to increase vigilance regarding threats to human health posed by *A. lentulus* originating from natural environments, enhancing clinical and laboratory alertness to *A. lentulus* infection. Therefore, this study investigated four *A. lentulus* (clinical and environmental) isolates, along with *A. fumigatus* and *A. fumigatiaffinis* from the *A. fumigatus* complex. Through a comparative analysis of the morphological characteristics, virulence profiles, and antifungal susceptibility patterns, we aimed to comprehensively elucidate the biological features and pathogenic mechanisms of *A. lentulus*.

Materials and Methods

Strains

The strain details are listed in Table 1. The test strains included *A. lentulus* (clinical strain, n=3; environmental strain, n=1), *A. fumigatus* (n=1), and *A. fumigatiaffinis* (n=1). Prior to this study, all of the strains were identified using molecular biology methods to amplify the partial β-tubulin gene and sequence it. Among them, *A. lentulus-10198* was isolated from the sputum of a patient with chronic obstructive emphysema and named *A. lentulus* PWCAL1 or 2429-Palide, we submitted this strain to the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/default.htm) with accession number LC187284. *A. spergillus lentulus*-10199, *A. lentulus*-10200, and *A. lentulus*-10201 were provided by the Medical Mycology Research Center of Chiba University, Japan.

Colony Color, Growth Rate, and Sporulation Time

Strains were cultured on 9 cm potato dextrose agar (PDA) plates at 27°C for 14 days. Colony diameter was measured daily, and color changes were observed. The colony diameter was measured using the average of two perpendicular diameters. Fungal immunofluorescence staining and microscopic examination were used to assess the sporulation time and spore quantity for each strain, and count the number of spores per field to evaluate total spores. Each experiment was independently repeated three times, and the results are expressed as mean ± standard deviation.

Table 1 Strain Information on *Aspergillus* in this Study (n=6)

Species	Number in This Study	Original Number	Origin	Source
A. lentulus	10198	PWCAL1=2429-Palide	Xinjiang, China	Patient sputum
A. lentulus	10199	IFM62135	Japan	Patient sputum
A. lentulus	10200	IFM54703/ATCC MYA-3566	Japan	Patient sputum
A. lentulus	10201	IFM62627	Japan	Soil
A. fumigatus	–	–	Xinjiang, China	Patient sputum
A. fumigatiaffinis	–	–	Xinjiang, China	Patient sputum

Galleria Mellonella Infection and Survival Curve

Galleria mellonella larvae were purchased from Huiyude Biotechnology Co. Ltd (Tianjin, China). Larvae exhibiting creamy-colored cuticles, active motility, and 7-day-old larvae were selected and maintained in a 37°C incubator in the dark for 24 h prior to infection. The test strains comprised both clinical (n=3) and environmental (n=1) *A. lentulus*. The strains were cultured on PDA medium at 27°C for 7 days to promote adequate sporulation. Colonies were washed with phosphate-buffered saline (PBS) to elute spores, and fungal suspensions were filtered through glass wool to remove hyphae. Spore concentrations were adjusted to 1×10^4, 1×10^5, and 1×10^6 conidia/mL using a hemocytometer. For each concentration, 20 larvae were inoculated by injecting 40 μL of spore suspension into the right proleg using a micro-syringe. The control larvae were treated with PBS only. After infection, the larvae were incubated at 37°C in the dark, and survival was monitored daily for 7 days.

In Vitro Antifungal Susceptibility Testing

Antifungal susceptibility was determined using the Sensititre YeastOne® kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Four *A. lentulus* strains were tested against three classes of antifungal agent: (a) polyenes (amphotericin B); (b) azoles (itraconazole, voriconazole, posaconazole, fluconazole); and (c) echinocandins (caspofungin). Final concentration ranges were: amphotericin B (0.12–8 μg/mL), caspofungin/voriconazole/posaconazole (0.008–8 μg/mL), itraconazole (0.015–16 μg/mL), and fluconazole (0.125–256 μg/mL). Minimum inhibitory concentrations (MICs) were interpreted using epidemiological cutoff values (ECVs) for filamentous fungi according to the Clinical Laboratory Standards Institute (CLSI) (M27, M38, M44, M51, M57) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) 2022 guidelines. CLSI ECVs (μg/mL) for *A. fumigatus* were as follows: amphotericin B (2), itraconazole (1), caspofungin (0.5), and voriconazole (≤0.5 susceptible, 1 intermediate, ≥2 resistant). EUCAST ECVs (μg/mL) were as follows: amphotericin B (1), itraconazole (1), posaconazole (0.25), and voriconazole (1). *Candida krusei* ATCC 6258 was used as the quality control strain. The inoculated plates were incubated at 35°C for 48 h before MIC determination.

Statistical Analysis

Statistical analyses were performed using SPSS version 24.0. Intergroup comparisons were made using analysis of variance (ANOVA). Statistical significance was set at a p-value of 0.05.

Results

Colony Color

During the first 4 days of cultivation, all four *A. lentulus* strains exhibited white colonies (Figure 1 and Table 2). Notably, *A. lentulus*-1019 and *A. lentulus*-10201 developed a whitish-green coloration on days 5 and 6, respectively, whereas *A. lentulus*-10199 and *A. lentulus*-10200 turned whitish-green on days 9 and 10, respectively. The timing of the color transition of *A. fumigatiaffinis* was similar to that of *A. lentulus* (Table 2). With prolonged growth, colonies of both *A. lentulus* and *A. fumigatiaffinis* remained predominantly white. In contrast, *A. fumigatus* showed the earliest color change, displaying whitish-green colonies by day 3 and fully developing a smoky-green hue by day 8.

Growth Rate

As illustrated in Figure 2, on PDA medium after 1 week of cultivation, the colony diameters of three *A. lentulus* strains (*A. lentulus*-10198, *A. lentulus*-10200, and *A. lentulus*-10201) reached 7.5 cm, whereas *A. lentulus*-10199 measured 5.0 cm. *Aspergillus fumigatiaffinis* colonies grew to 3.2 cm after 1 week and to only 4.8 cm after 2 weeks.

Sporulation Time

Among the four *A. lentulus* strains, *A. lentulus*-10199 and *A. lentulus*-10201 began sporulation on day 5, *A. lentulus*-10198 on day 7, and *A. lentulus*-10200 on day 12 (Figure 3). In comparison, the control strain of *A. fumigatus* initiated sporulation on day 2, and *A. fumigatiaffinis* on day 4.

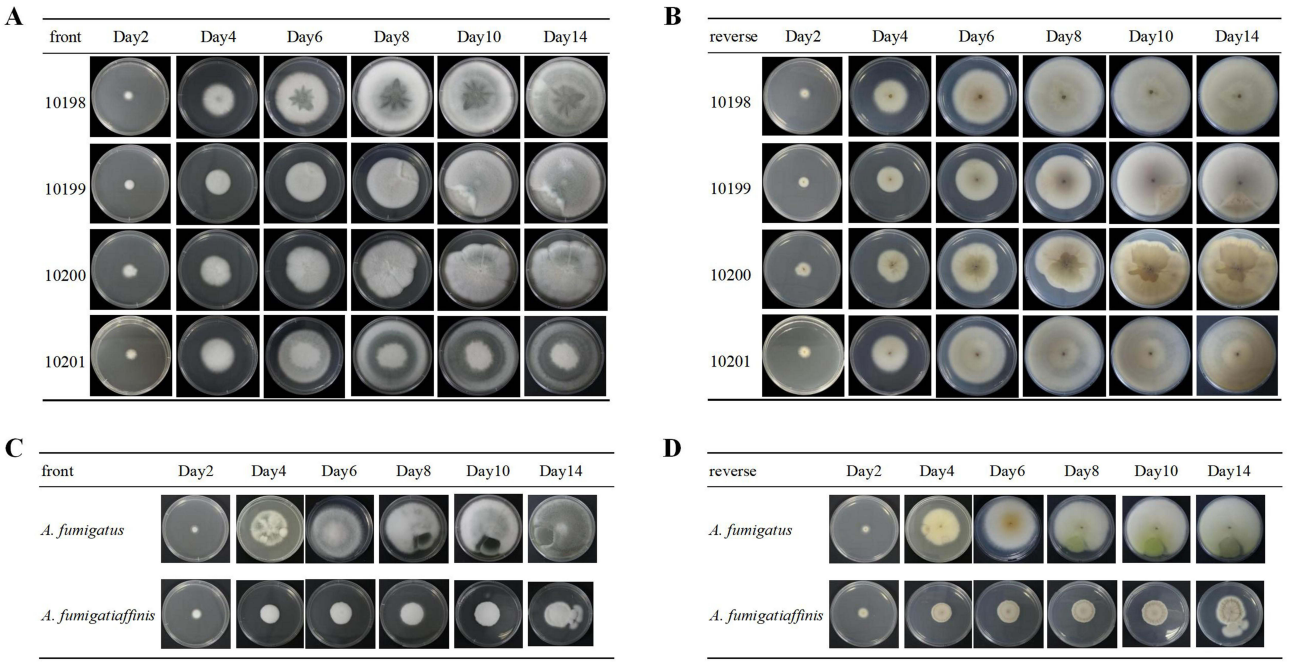

Figure 1 Colony characteristics of *Aspergillus lentulus* and control strains. (**A**) Colony characteristics of *A. lentulus*, PDA, 28°C, front. (**B**) Colony characteristics of *A. lentulus*, PDA, 28°C, reverse. (**C**) Colony characteristics of control strains, PDA, 28°C, front. (**D**) Colony characteristics of control strains, PDA, 28°C, reverse.

Galleria Mellonella Survival Curve Analysis

Larvae infected with the four *A. lentulus* strains showed increased mortality at higher fungal inoculum doses (Figure 4). At the highest dose (10^6 conidia/mL), the average 7-day mortality rates were 77.86%, 65.0%, 29.0%, and 0%, respectively. At the lowest dose (10^4 conidia/mL), the respective rates were 60.7%, 0%, 2.14%, and 0%. The *A. lentulus*-10198 clinical strain caused 70–90% larval mortality by day 2 at doses of 10^4–10^6 conidia/mL. At the highest dose, *A. lentulus*-10198, *A. lentulus*-10199, and *A. lentulus*-10200 induced 90%, 75%, and 100% mortality by day 7, with mortality rates of 70%, 0%, and 5%, respectively. In contrast, the *A. lentulus*-10201 environmental strain caused no larval mortality at any dose within 7 days.

In Vitro Antifungal Susceptibility

As shown in Table 3, the MIC ranges of five antifungal agents against the four *A. lentulus* were as follows: caspofungin ≥8 μg/mL, amphotericin B ≥4 μg/mL, posaconazole 0.5–2 μg/mL, itraconazole 1–2 μg/mL, and voriconazole 2–8 μg/mL. The MIC values of *A. lentulus* for three major classes of antifungal agents all exceeded the ECV of *A. fumigatus*, indicating reduced susceptibility or resistance.

Table 2 Colony Color Changes After Culture of Different *Aspergillus* Strains

Species	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
A. lentulus-10198	White	White	White	White	White/green	White/green	White/green	White/green	White/green	White/green
A. lentulus-10199	White	White	White	White	White	White	White	White	White/green	White/green
A. lentulus-10200	White	White	White	White	White	White	White	White	White	White/green
A. lentulus-10201	White	White	White	White	White	White/green	White/green	White/green	White/green	White/green
A. fumigatus	White	White	White	White/green	White/green	White/green	White/green	Smoky green	Smoky green	Smoky green
A. fumigatiaffinis	White	White	White	White	White	White	White/green	White/green	White/green	White/green

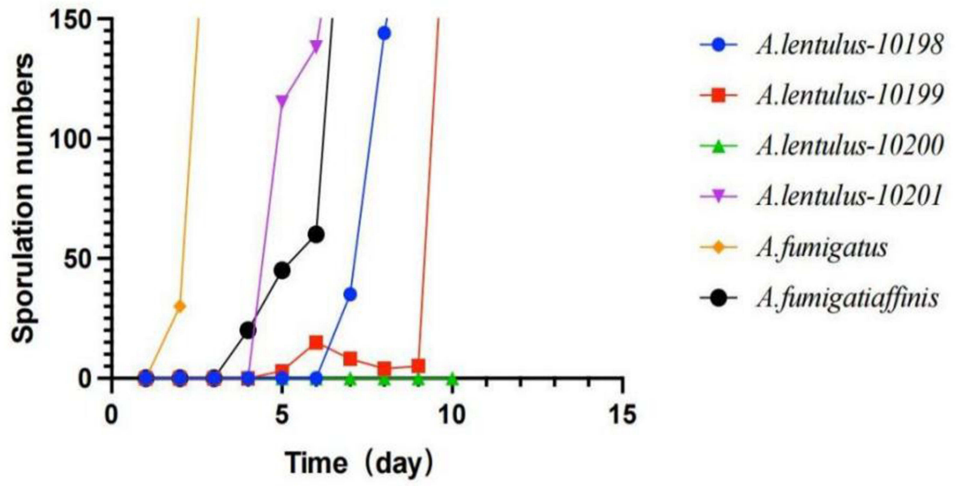

Figure 2 *Aspergillus lentulus* and control strains were cultured on PDA at 28°C for 2 weeks, and the growth diameters of colonies of different strains were observed.

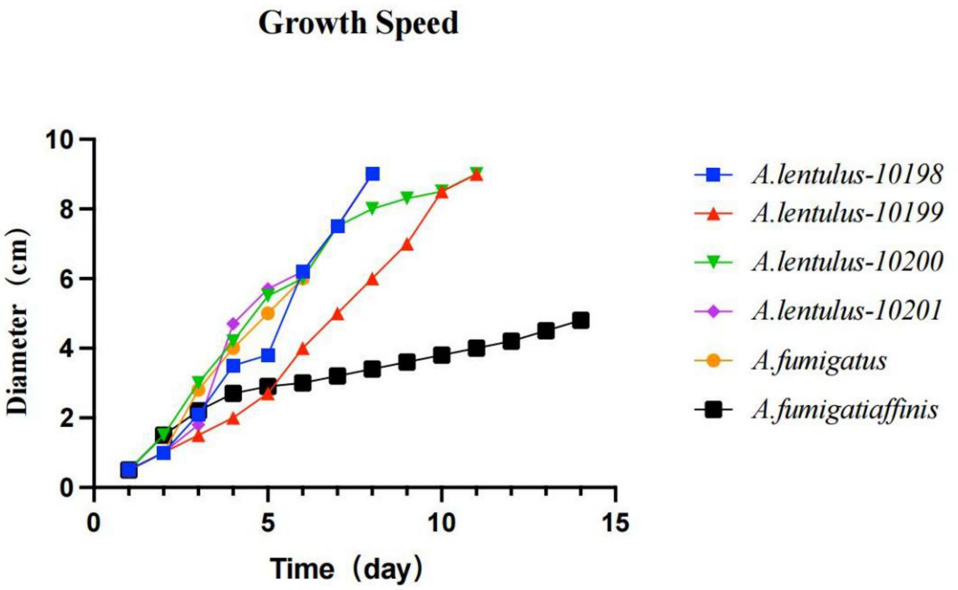

Figure 3 *Aspergillus lentulus* and control strains were cultured on PDA at 28°C, and spore production time and quantity were measured.

Discussion

The mortality rate for invasive aspergillosis can be as high as 60%. When first-line treatments such as voriconazole and isavuconazole are administered, the mortality rate drops to approximately 20–30%. For refractory invasive aspergillosis, combination therapy with different classes of antifungal drugs is particularly important.

Although most pathogenic *Aspergillus* species are phenotypically distinguishable, infections can also be caused by phylogenetically related species that morphologically resemble *A. fumigatus*,[27] which may underlie refractory invasive aspergillosis. Key differences among these species lie in colony growth, stability of sporulation, conidial surface markers, and maximum growth temperature.[5] Within the *A. fumigatus* complex, *A. lentulus*, *A. fumigatus*, and *A. fumigatiaffinis* exhibited morphological similarities. However, *A. lentulus* and *A. fumigatiaffinis* colonies predominantly displayed a white coloration over extended growth periods, which may be correlated with their slower sporulation rates and poor conidiation. Specifically, *A. lentulus* strains demonstrated delayed and reduced sporulation, with the *A. lentulus*-10200 strain observed to have only one spore in the full-field view of the microscope by day 11. In contrast, the control

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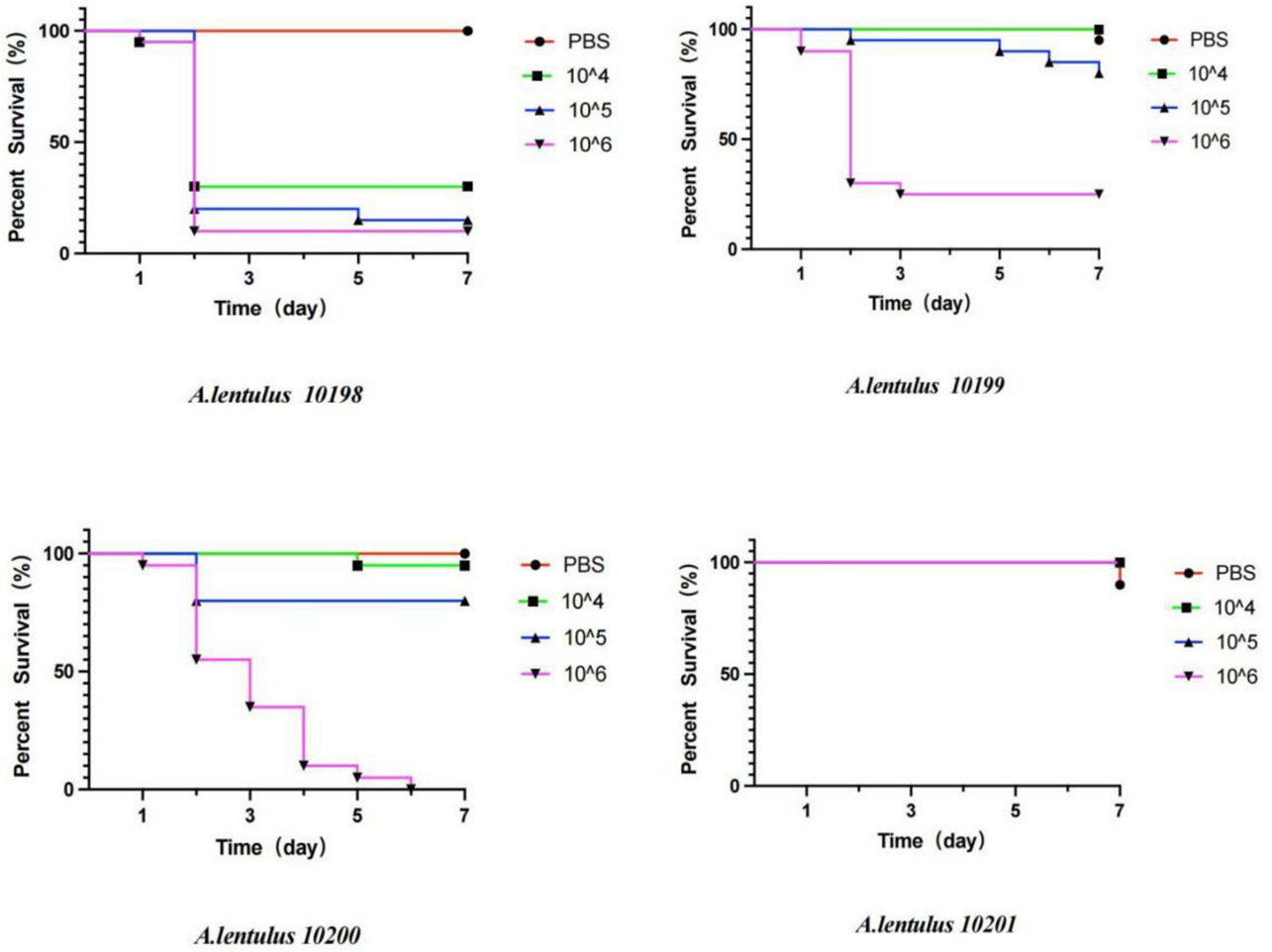

Figure 4 *Aspergillus lentulus* clinical strain (10198, 10199, 10200) and environmental strain (10201) killed *Galleria mellonella* larvae at 10^4, 10^5, and 10^6 conidia/mL, and infected larvae for 7 consecutive days. The larvae mortality was dose dependent. The larvae were inoculated with PBS as a control.

strain *A. fumigatus* exhibited rapid and abundant sporulation, highlighting the interspecific and intraspecific variations in sporulation dynamics. *Aspergillus fumigatus* displayed the fastest growth, followed by *A. lentulus*, whereas *A. fumigatiaffinis* grew the most slowly, showing a significant divergence from the other two species. For *A. fumigatiaffinis*, we only observed its slowest growth on PDA medium. Our findings indicate that colony color, sporulation patterns, and growth rates may serve as morphological criteria for distinguishing *A. lentulus* from *A. fumigatus* and *A. fumigatiaffinis*. Although *A. lentulus* was initially regarded as a sibling species of *A. fumigatus*,

Table 3 Results of the Sensitivity Test to Antifungal Drugs In Vitro by *Aspergillus Lentulus* and Control Strains (MIC μg/mL)

Species / Drug	PZ	VOR	IZ	CAS	AB
A. lentulus-10198	0.5	2	1	≥8	≥4
A. lentulus-10199	0.5	8	1	≥8	≥4
A. lentulus-10200	1	2	1	≥8	≥4
A. lentulus-10201	2	8	2	≥8	≥4
A. fumigatiaffinis	0.5	2	1	1	≥8
A. fumigatus	0.015	0.03	0.015	0.008	0.25

phylogenetic analyses based on DNA sequencing and phenotypic traits have revealed substantial divergence between *A. lentulus* and *A. fumigatus*.[21] Both *A. lentulus* and *A. fumigatiaffinis* are recently identified species, with phenotypic analyses indicating close similarities between them,[21] a finding corroborated by our study. Therefore, routine implementation of fungal culture along with morphological and molecular biological identification of fungal species in the laboratory is essential for ensuring accurate clinical diagnosis and successful treatment.

Given the functional parallels between the innate immune systems of *G. mellonella* larvae and mammals,[28,29] we employed a *G. mellonella* model to assess *A. lentulus* virulence. All clinical *A. lentulus* isolates successfully infected larvae, with survival rates varying according to inoculum concentration and infection duration. The clinical isolates exhibited markedly higher virulence than the environmental isolates, suggesting intraspecific virulence heterogeneity. Among the three clinical strains, *A. lentulus*-10198 was isolated from the sputum of a COPD patient. The patient died after unsuccessful antifungal treatment following a diagnosis of invasive pulmonary aspergillosis. The *G. mellonella* survival curve showed that *A. lentulus*-10198 demonstrated the strongest virulence, inducing 70–90% mortality at 10^4–10^6 conidia/mL by day 2 post-inoculation, compared to 0–70% and 0–45% for the *A. lentulus*-10199 and *A. lentulus*-10200 strains, respectively. The final mortality rates across concentrations ranged from 70–90%, 0–75%, 5–100%, and 0% for the environmental strain, *A. lentulus*-10201, showing negligible virulence (0% mortality). However, the relationship between *A. lentulus* virulence and its strain origin remains unclear. In rodent models, *A. fumigatus* environmental isolates exhibited lower virulence than their clinical counterparts.[30] Similarly, Alshareef and Robson reported higher virulence in clinical *A. fumigatus* isolates (n=10) than in environmental strains (n=20) in *G. mellonella*, alongside substantial intrasource variability.[31] Our findings align with these trends, demonstrating enhanced virulence in clinical *A. lentulus* isolates. However, conflicting studies exist: Cheema and Christians observed lower survival in larvae infected with environmental *A. fumigatus* isolates (n=8) compared to clinical strains (n=8),[32] while Knox et al reported greater virulence in environmental *A. fumigatus* strains from the International Space Station using a zebrafish model.[33] Dos Santos et al further documented significant interspecies and intraspecies virulence heterogeneity among *A. lentulus* (n=6), *A. fumigatus* (n=6), and *A. fumigatiaffinis* (n=4) in *G. mellonella*, with survival rates at day 10 ranging from 0% to >75%.[21] Our results concur, underscoring intraspecific virulence variability in *A. lentulus*.

In vitro antifungal susceptibility testing plays a critical role in guiding precise medication and fungal resistance surveillance. *Aspergillus lentulus* is notable for high MICs to all azoles and amphotericin B,[6,34,35] although one study reported susceptibility to isavuconazole (MIC 0.25 μg/mL).[36] In our study, four *A. lentulus* strains exhibited low MICs to posaconazole (0.5–2 μg/mL), suggesting the potential efficacy of less clinically utilized antifungals. Voriconazole MICs (2–8 μg/mL) exceeded those of itraconazole (1–2 μg/mL), implying reduced voriconazole activity. However, conflicting reports exist, with some strains showing higher itraconazole MICs.[37] Clinical breakpoints for *A. lentulus* remain undefined. Extrapolation from *A. fumigatus* criteria classifies *A. lentulus* as azole resistant, mediated by CYP51 mutations.[38] Notably, our environmental *A. lentulus* isolate displayed elevated MICs to voriconazole (8 μg/mL), itraconazole (2 μg/mL), and posaconazole (2 μg/mL), surpassing clinical strains. A study by Watanabe et al identified azole-resistant *A. lentulus* (MICs ≥4 mg/L to voriconazole) in Japanese environments,[39] while Colosi et al isolated a Romanian vineyard strain resistant to itraconazole (8 mg/L), voriconazole (4 mg/L), and posaconazole (4 mg/L).[40] The prevalence and mechanisms of environmental azole resistance in *A. lentulus* remain unclear.

Conclusion

This study delineated the phenotypic, virulence, and antifungal susceptibility profiles of *A. lentulus* within the *A. fumigatus* complex, thereby offering critical insights for clinical diagnosis and management. Limitations include a small sample size, necessitating expanded strain collection and deeper mechanistic investigations. Because of the general decrease in sensitivity of *A. lentulus* to azoles, more attention should be paid to the mechanism of azole resistance.

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki. The clinical samples in this study were acquired as part of the routine hospital laboratory procedure.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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