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Research Article
Upsurge in *Curvularia* Infections and Global Emerging Antifungal Drug Resistance

[1,2]Louis Bengyella, [3]Laban E. Yekwa, [2]Sayanika D. Waikhom, [4]Kiran Nawaz, [4]Sehrish Iftikhar, [1]Teboho S. Motloi, [5]Ernest Tambo and [6]Pranab Roy

[1]Department of Biotechnology, Faculty of Applied and Computer Sciences, Vaal University of Technology, Vanderbijlpark, South Africa
[2]School of Basic and Biomedical Sciences (SBBS), The University of Health and Allied Sciences, Ho, Volta Region, Ghana
[3]Division of Medical Virology, Stellenbosch University, South Africa
[4]Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan
[5]Department of Biochemistry and Pharmaceutical Sciences, Université des Montagnes, Bangangté, Cameroon
[6]Department of Biotechnology, Haldia Institute of Technology, 721657 Haldia, West Bengal, India

Abstract

Background and Objective: *Curvularia* species not only cause disease in plants but have emerged in the last decade as a human pathogen causing mild, febrile, to life-threatening illness if not well-treated. Because of *Curvularia's* interlocking lifestyle on plants, animals and human and increased use of azole fungicides, there is emerging evidence of upsurge in resistance to antifungal drugs, a major public health burden. The objective of this study was to evaluate the genetic diversity of *C. lunata* from plant origin relative to clinical strains and to profile the current literature on the global emerging antifungal drug resistance associated with *Curvularia* infections. **Materials and Methods:** In this study, the glyceraldehyde-3-phosphate dehydrogenase (GPDH) locus was used to illustrate the genetic diversity between *C. lunata* of clinical and plant origin. Tajima's X^2 test statistics was performed in MEGA6.1 phylogenetic software to investigate the diversity between sequences. **Results:** The results showed large genetic distance (~0.275±0.041) between lineages of *C. lunata* of clinical and plant origin. Even though no optimal antifungal therapy for *Curvularia* infections has been established for elite drugs like triazoles-itraconazole, voriconazole and posaconazole, it is cogently presented herein cases of successful site-specific treatment of infections caused by *C. lunata*. **Conclusion:** It is found that *C. lunata* from plant and clinical origins are genetically diverse and azole-fungicides exert selective pressure that accelerates evolution. Importantly, effective management of *Curvularia* infections is via combination therapy and regardless of the age and infected organ, treatment that last for at least 3 months is recommended.

Key words: *Curvularia*-resistance, GPDH loci, amphotericin B, *Curvularia lunata*, eumycetoma, azole-fungicides, keratitis

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Corresponding Author: Louis Bengyella, Department of Biotechnology, Faculty of Applied and Computer Sciences, Vaal University of Technology, Vanderbijlpark, South Africa Tel: 0027 712781091

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fungal pathogens contribute significantly to global human suffering and the worst hit regions of the world include Brazil (with affected population estimated at 1.7%), Ireland (~1.9%) and the prevalence is believed to be highest in China[1,2]. In recent years, a sharp increase in morbidity and mortality associated with invasive fungal infections has been observed. Studies revealed over 1.2 billion people worldwide suffered from fungal diseases[3,4]. Previously, it was shown that 1.5-2 million people died of fungal infections each year, far surpassing those killed by either tuberculosis or malaria[5]. This high mortality burden is based on lead human fungi such as *Cryptococcus neoformans*, *Aspergillus* spp. and *Candida* spp. Moreover, other fungal infections have witnessed a sharp increase in the last decade such as coccidiomycosis caused by *Coccidioides immitis* and *C. posadasii* [6] and species in the genera *Cochliobolus*, *Bipolaris* and *Curvularia* [7]. Species in these genera have not only developed the abilities to adapt to host tissues and counterattack the defense mechanisms, but they caused devastating diseases in several plant and animal species[8-10]. Thus, these fungi are often referred to as cross-kingdom pathogens.

Curvularia lunata (teleomorph sexual state-*Cochliobolus lunatus*) is the lead causal agent of diseases in both plants and animals within the genera *Curvularia*. The fungus *C. lunata* abundantly produces 1-7 celled-conidia during sporulation and survives higher temperature of ~40°C[8,9] and principally transmitted via air. Known clinical manifestations include cutaneous and subcutaneous infections, keretomycosis, cerebral phaeohyphomycosis, allergic bronchopulmonary mycosis and endophthalmitis[10-12]. The level of severity of *Curvularia* infections varies among patients, making it a public health burden[10-12].

Previous studies aiming at identifying aggressive and novel species causing mycotic diseases in the genera *Curvularia*[7,13,14] revealed important genetic diversity. However, a fundamental problem in *Curvularia* infections is the risk of transferring a genetically evolved isolate from farms (where azole fungicides are used) to humans. Such transferability through human-plant interaction or intake of contaminated air could cause resistance to antifungal drugs since field isolates suffer from fungicide selective pressure and undergo virulence differentiation to adapt to adverse conditions. For instance, eumycetoma caused by *C. lunata* in farmers in India, South-East Rajasthan and Chandigarh responded poorly to treatment and needed long-term therapies[15,16]. Most agricultural regions are known for the use of azole fungicides such as metalaxyl, imidazole, propiconazole and tebuconazole formulation that poses selective pressure on cross-kingdom pathogens and impact on antifungal drug resistance[5,7]. Acquired resistance via azole fungicides exposure and extreme genetic variations poses a critical public health problem because of the ease of resistance build-up to antifungal therapy. Importantly, the current status of *Curvularia* infections, management and global emerging antifungal drug resistance is comparatively examined in this study.

MATERIALS AND METHODS

Study area and sampling: Based on previous report of black-to-black leaf spot disease of potato (*Solanum tuberosum* L.) caused by *C. lunata*[17], a routine survey was performed in 5 potato plantations of Burdwan District (23°14′ N, 87°51′ E, altitude 150 m, 102.1 km from Kolkata), West Bengal, India, during the winter month of December to March of 2010, 2011 and 2012. The potato fields often receive two episodes spray of metalaxylmanocozeb fungicide during farming season. Potato leaves showing brown-to-black spot disease were excised and treated with 2% NaClO solution for 2 min and rinsed in sterile water with three changes. The leaf pieces were aseptically plated on V8 agar medium (HiMedia®, Mumbai, India) and incubated at 25°C in the dark. The colonies that developed after 7 days were transferred to fresh V8 agar plates in order to purify the cultures and isolates were morphologically identified based on standard monograph taxonomic keys[18]. Pathogenicity test was previously done and confirmed aggressive against potato[19].

DNA extraction and polymerase chain reaction: The genomic DNA was isolated from fungal isolates grown in potato dextrose broth (PDB; HiMedia®, Mumbai, India). Total genomic DNA was extracted from mycelium mat using UltraClean™ Microbial DNA isolation kits (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) as described by the manufacturer. The quality and quantity of the DNA was determined using a 1% agarose gel electrophoresis and a nanodrop spectrophotometer (BioSpec-nano, Shimadzu®, Japan), respectively. For genetic diversity studies, specific primers (forward: 5-CGATATGCGGCATATGCA-3; reverse: 5-ACCTACGCATTGCGGAA-3) were designed for glyceraldehyde-3-phosphate dehydrogenase (GPDH) gene using reference *C. lunata* (GenBank: Gb|X58718) in Integrated DNA Technology (IDT) primer designer software. Amplification of GPDH was performed as follows. The PCR mix contained 11 ng genomic DNA, 5 µL Green GoTaq® reaction buffer (Promega®, Madison, WI, USA), 0.2 mM each of

deoxyribonucleoside triphosphate (dNTP), 0.2 μM of each primer and 1.1 U of GoTaq® DNA polymerase in a total reaction volume of 25 μL in triplicates (PCR conditions: 5 min at 95°C, 35 cycles of 1 min at 94°C, 1 min annealing at 53°C, 2 min for extension 72°C and a final 5 min extension at 72°C). The quality of the amplicon was checked by performing agarose gel electrophoresis. The PCR products were purified and sequenced and sequences were assigned to taxa based on 98-100% sequence similarity threshold in DNA Data Base of Japan (DDBJ: AB859034, AB859035, AB859036, AB859037 and AB859038).

***In silico* mining of DNA databases:** The overarching dataset was obtained by using key words such as *Cochliobolus*, *Curvularia*, *Cochliobolus*- and *Curvularia*- infections for interrogating PubMed database and Google-scholar to retrieve current literature and accession numbers. Herein, both *Cochliobolus* and *Curvularia* were used to avoid missing out information following recent nomenclatural changes such as the recommendations of International Commission on the Taxonomy of Fungi (ICTF) that *Curvularia* should be considered over *Pseudocochliobolus* and protect *Bipolaris* Shoemaker 1959 (A) over *Cochliobolus* Drechsler 1954 (S)[20]. Using GenBank® BLAST search tool, a studied set of GPDH sequences deposited in the last decade were collected based on the information associated with the sequences such as GC content, length (>200 bp), geographic origin of isolates and trimmed to unique sequence set using ElimDupes (available at http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html). The data set was further cleaned by eliminating sequences with 100% sequence similarity for isolates from the same host and geographical coordinates. A total of 52 unique sequences were aligned in Muscle program[21] and the best substitution model parameters were determined based on Akaike Information Criterion, corrected (AICc) and Bayesian Information Criterion (BIC).

Statistical analysis: The diversity analysis and sequence polymorphisms were performed in MEGA6 software[22]. The overall genetic diversity among *C. lunata* of plant and clinical origin was computed as previously described[23].

Ethical approval and informed consent: No animals were used. Only potato plants and *Cochliobolus lunatus* strains were used as living organisms. Permission to survey potato farms in Burdwan District, the main potato farming zone in West Bengal, India was granted by the Government of India, Ministry of Science and Technology through the Department of Biotechnology vide program number No.3240223450, for the period of five years starting from 2010 to 2015.

RESULTS AND DISCUSSION

Transferability of plant isolates of *C. lunata* to human is a high risk factor for resistance based on wide genetic distance: Morphologically, *C. lunata* produces varied sizes of conidia (Fig. 1a). From the 52 sequences computed, 284 patterns were found out of a total of 732 sites and 360 sites were without polymorphism (49.18%). The estimated average evolutionary divergence over all *Curvularia* species in the data set was 0.275±0.041 based on T92+G model[23]. The estimated transition-transversion bias (R) in the data set was 2.78, hallmarked by [A↔T] = 3.303, [C↔A] = 3.303, [A↔G] = 18.392, [T↔C] = 18.392, [C↔G] = 3.303 and [G↔T] = 3.303 at a discrete Gamma distribution ([+G], parameter = 0.1097). While novel *Curvularia* species isolated from clinical specimens[14] formed unique clusters, *C. lunata* from potato plants failed to cluster with clinical isolates (Fig. 1b). Considering the potato fields from which the *C. lunata* were located at about 1 km apart from each other, it is concluded that *C. lunata* are evolving divergently. In recent years, several previously known plant pathogens in the genus *Curvularia* such as *C. lunata*, *C. brachyspora*, *C. clavata*, *C. geniculata*, *C. inaequalis*, *C. pallescens*, *C. senegalensis* and *C. verruculosa* have developed capacities to invade human[7,14,24]. Among all the *Curvularia* species, *C. lunata* have shown high level genome plasticity[25]. For instance, whole genome sequencing of *Curvularia* species revealed that *C. lunata* is the most diverse species, bearing in mind that only ~20% of its genome aligned to the reference *Cochliobolus heterostrophus* C5 genome, compared to about 75% alignment observed to other *Curvularia* species[25]. Importantly, high genetic diversity of *C. lunata* is also attributed to its cross-mating abilities with other species[26,27].

To test equality of evolutionary rate between lineage of *C. lunata* from plant and clinical origin, Tajima's test[28] for *C. lunata* (AB859034, AB859035 and AF081394) was performed at X^2 test statistic cut-off 0.5 (p = 0.479 with 1 degree of freedom). Unique differences for *C. lunata*-AB859034 was 5, *C. lunata*-AB859035 was 3, *C. lunata*-AF081394 was 204, identical sites for all three sequences was 221 and divergent sites in all three sequences was 6. Thus, rejecting the null hypothesis that lineages of *C. lunata* from clinical and plant origins undergoes equal rate of evolution (Fig. 1b). This divergent evolution could pose a severe difficulty in management should *C. lunata* exposed to azoles fungicides jump host (or transferred) from plants to humans. Since it is discerning to assess the probable future burden of the implication of fungicides used for controlling *Curvularia* plant diseases in parallel with antifungal drug resistance now, rather than when the problem becomes severe.

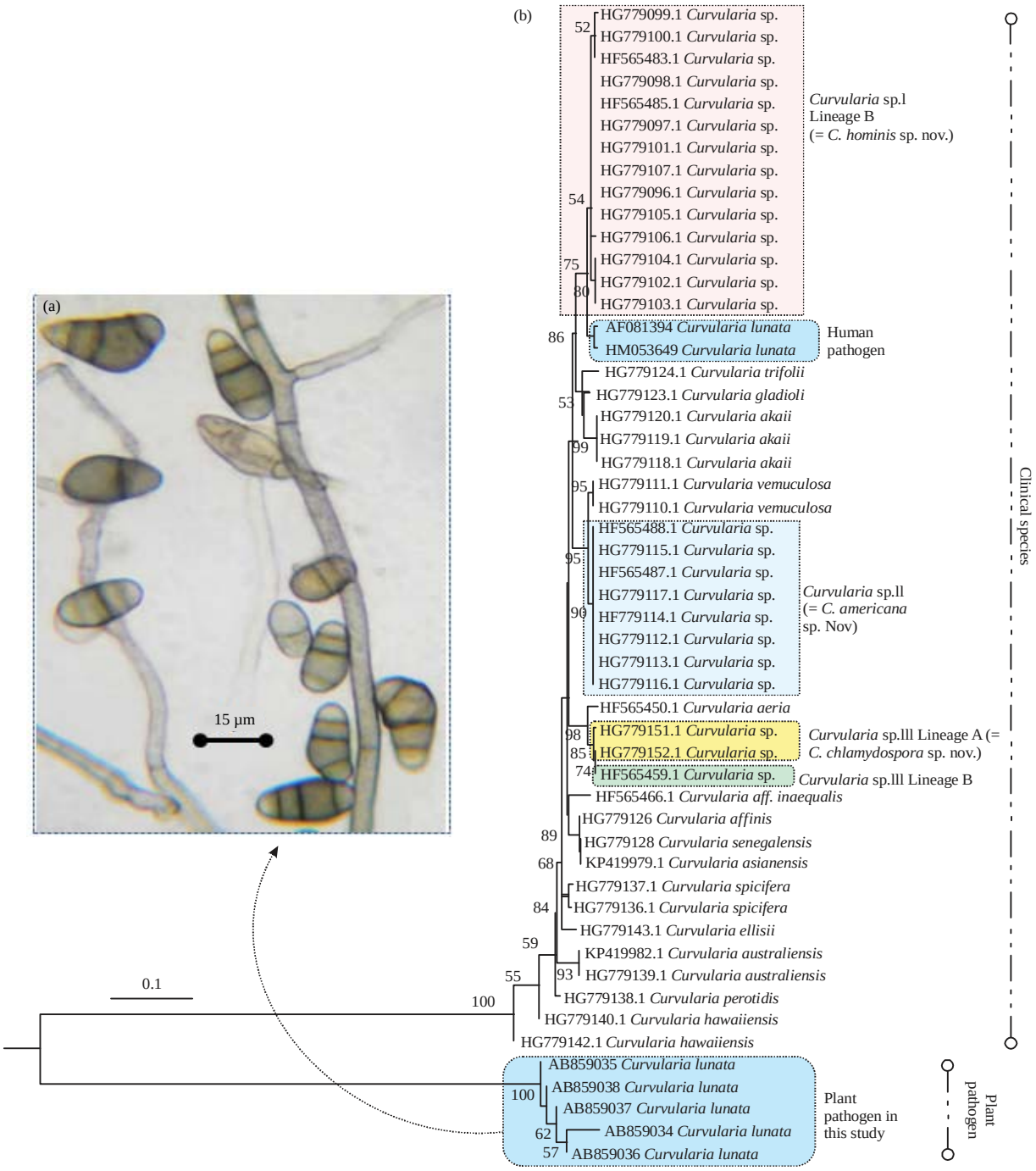

Fig. 1(a-b): Taxonomic placement of *C. lunata* based on TN92+G substitution model[29] and the tree was rooted as previously described[19]

Hypothetical interlocking lifestyles and fungicide pressure on *Curvularia* species enhances the emergence of resistance: *Curvularia* species profusely sporulates under adverse condition of high temperatures in or on putative hosts and conidia are easily dispersed in air. Thus, suggesting that *Curvularia* inoculum might be contracted by a healthy individual via breathing, interaction with infected crops, vegetables and scenario of direct interaction of a healthy individual with turfgrasses or ornamental plants in playgrounds. This is exemplified by footballers and farmers

contracting *Curvularia* diseases caused by *C. lunata*[15,16,30]. Introduced in around late-1960's, the use of azole-fungicides (such as imidazoles, propiconazole and tebuconazole) in farms and turfgrasses in playgrounds acts as selective pressure, creates fungal ecological imbalances, may promote virulence differentiation and in this situation, increases public health risk and burden.

Curvularia species are mostly sensitive to triazoles drugs and the interplay between azole-fungicide and a genetically evolved *Curvularia* species that is transferred from plant to man could hypothetically lead to resistance development in immunocompromised patients receiving treatment. Fundamentally, azole drugs likewise azole-fungicides inhibit the activity of lanosterol 14α-demethylase and disrupt the production of ergosterol, a major component of cell membrane and impair fungal growth. First and foremost, azoles are highly stable molecules that persist actively in water, soil, fruits and vegetables for months[31-34]. Thus, patients under long-term azole exposure either from environmental contamination or treatment are prone to develop resistance[35]. Since *Curvularia* infections are often times not acute, associated with non-specific symptoms and signs, consequently, many people contract *Curvularia* diseases unaware and undiagnosed. Considering the worldwide distribution of *Curvularia* diseases wherever maize, sorghum, millet, sugarcane and rice are farmed and the enormous use of fungicides to manage the diseases, it is tempting to hypothesize that by 2047, *Curvularia* antifungal drug resistance would be a critical public health problem. This is because by 2047, most *Curvularia* species infecting humans could have evolved to azoles resistant phenotypes due to azoles fungicide pressure. Taken together, fungicides selective pressure coupled with mutation and recombination event would consequently increases the likelihood of selecting resistance *Curvularia* species exhibiting resistance to azoles antifungal drugs.

***Curvularia* species infects immunocompetent and immunocompromised individuals:** It has been revealed that common sites of *Curvularia* infections in humans are the nasal cavity, ocular, skin and nails, respiratory tract, eyes, in that order[7,36-39] and in deep tissue such as central nervous system[40,41]. Although attention has been on a small cohort of fungi genera such as *Cryptococcus*, *Aspergillus*, *Pneumocystis* and *Candida*, there are increasing reports of *Curvularia*-mediated phaeohyphomycosis in human and animal[10,42]. Phaeohyphomycosis refers to cutaneous and systemic diseases caused by melanised fungi that often develop dark-wall-septate mycelia in host tissues. *Curvularia lunata* efficiently triggers subcutaneous phaeohyphomycosis hallmarked by granulomas in the adipose panniculus[10]. Eumycetoma caused by *C. lunatus* in back-shoulder of a female patient that lasted for 12 years was characterized by multiple swellings and discharging wounds[43]. However, Shinde *et al.*[43] remarked that the rare *Curvularia* mediated-mycetoma was associated with Gram positive *Staphylococcus aureus* possibly occurring as opportunistic infections. In this study[43] antibiotic therapy did not lead to improvement, but the patient responded well with itraconazole 200 mg twice daily. *C. lunata* mediated-eumycetoma appears to be common in Africa, India, Mexico and South America[16]. Recent cases of eumycetoma[44-53] caused by *C. lunata* and treatment regimens are summarized (Table 1). Previously, Rinaldi *et al.*[40] found that in a sample size of 24 patients infected by *Curvularia* species, only two patients were systemically immunosuppressed. The results indicated that *Curvularia* species causes diseases both in immunocompetent and immunocompromised individuals at different levels of severity.

Intrinsic resistance and current challenges in the treatment of *Curvularia* infections: *Curvularia* species are known to abundantly produce melanin[24], coloured metabolites and secretes diverse secretome during infection[54]. One key attribute of melanin is that it protects the microbes from host defenses, increases resistance to phagocytosis *in vitro* and *in vivo*[55,56], confer a survival advantage in the environment[57] and have the ability to bind amphotericin B and caspofungin[58]. Thus, melanin make fungal clearance hard in infected tissues. *Curvularia* species are dark-brown and highly pigmented molds and constitutively secretes melanin during colonisation of putative host, as a result, treatment with current antifungal drugs is challenging.

The optimal antifungal therapy for the treatment of *Curvularia* infections is still unknown, amphotericin B deoxycholate (which targets ergosterol and disrupting plasma membrane), azoles derivatives viz., miconazole, ketoconazole and itraconazole (which targets ergosterol biosynthesis at 14-α-demethylase) and terbinafine (which inhibits ergosterol synthesis at the level of squalene epoxidase) have actively been used in the treatment of *Curvularia* infections producing variable results[53]. Occasional resistance to amphotericin B has been observed in *Curvularia* spp., *Chaeromium* spp., *Phialemonium* spp. and *Exophiala* spp., which are heavy melanin producing fungi[59]. Echinocandins has been found clinically irrelevant to heavy melanin producing fungi[57], nonetheless, azoles show broader *in vitro* activity against *Curvularia* species[43,45,46]. Interestingly, voriconazole and itraconazole showed poor activity to most frequently

Table 1: History of *C. lunata* infections indicate that it affects all age groups and many parts of the body

Diseases	Treatment regimes	Sites	Sources
Phaeohyphomycosis	Oral itraconazole and topical lanoconazole for 9-weeks healed lower leg lesions in an 80 years-old immunocompetent subject	Leg	Lee *et al.*[44]
Eumycetoma	Back-shoulder infection in a female, treated with itraconazole 200 mg twice daily	Back-shoulder	Shind *et al.*[43]
Keratitis	Topical natamycin 4 mg mL^{-1} was given to 27 years-old man for 4 months without improvement, followed by oral itraconazole 200 mg day^{-1} for two months	Eye (corneal ulcer)	Banukumar *et al.*[45]
Onychomycosis	Oral itraconazole 200 mg day^{-1} was given to a 25 years-old female for 6 months	Toe-hallux	Fraenza *et al.*[46]
Pneumonia	A 65 years-old female was treated with fluconazole 150 mg day^{-1} for 3 weeks		Dharmic *et al.*[47]
Allergic fungal rhinosinusitis	Oral itraconazole 200 mg twice a day (BID) for 4 weeks with mometasonefurate nasal spray (100 mcg BID for 6 months) and normal saline nasal irrigations	Nasal cavity	Cavanna *et al.*[48]
Phaeohyphomycosis	Oral itraconazole 200 mg day^{-1} for 8 months in a 25 years old male, renal transplant recipient who developed leg ulcer	Leg ulcer	Vasquez-del-Mercado *et al.*[49]
Peritonitis	A 55 years-old housewife was treated with oral voriconazole 400 mg twice a day loading dose and maintained at 200 mg day^{-1} for 3 weeks	Peritoneal cavity	Kalawat *et al.*[50]
Locally invasive phaeohyphomycosis (Invasive tuneaunguium)	Oral itraconazole 400 mg mL^{-1} daily for a 41 years-old immunosuppressed man undergoing stage III accelerated chemotherapy	Great toe	Safdar *et al.*[51]
Multicentric paranasal sinusitis	Oral itraconazole at 600 mg daily dose for 5 days, continued at 400 mg subsequently. Blood level of itraconazole and hydroxyitraconazole were maintained between 2.2 and 2.8 μg mL^{-1} and 3.6 and 3.8 μg mL^{-1}, respectively, for 4 months	Nasal cavity	Safdar *et al.*[51]
Cerebral phaeohyphomycosis	Aggressive antifungal therapy for a 21 years-old male consisting of: • Oral amphotericin B, vancomycin and ceftriaxone prior to fungal identification • Liposomal amphotericin B, flucytosine and clindamycin were added when body temperature rose	CNS	Carter and Boudreaux[41]
Keratitis (Co-infection by *Acanthamoeba-Cochliobolus*)	• Eye drops of - lomefloxacin, tobramycin, natamycin and cyclopentolate • Eye drops of 1% of propamidineisothionate, natamycin, fluconazole, neosporin and cycloplegic	Eyes	Gupta *et al.*[52]
Eumycetoma	Foot infection in a 65 years-old male farmer was treated with oral itraconozale 200 mg twice daily	Foot	Garg *et al.*[16]
Eumycetoma	Foot infection was treated with oral ketoconazole 200 mg daily for 2 months in a 27 years-old male	Foot	Janaki *et al.*[53]

encounter isolates such as *Curvularia aeria*, *Curvularia geniculata/Curvularia senegalensis*, *C. lunata*, *Curvularia inaequalis*, *Curvularia verruculosa* and *Curvularia borreliae* in USA[36], suggesting that effective therapy could only be achieve via rigorous trials.

Till date, there is no WHO protocol or consensus on treatment guideline, type of antifungal drugs and treatment regime for *Curvularia* infections. On the basis that *C. lunata* indiscriminately infects all parts of the leg, peritoneal cavity, respiratory tract, central nervous system and the eye of human (Table 1) and more so, infections are treated through trials of different antifungal drugs, development of resistance is eminent in the near future. To assert this, Paredes *et al*.[60] used immunosuppressed murine models infected with *Curvularia* species to test the efficiency of amphotericin B, posanazole and voriconazole and found only the 2 azoles could decrease the fungal load. To achieve immunity suppression, 5-fluorouracil and cyclophosphamide were injected into the mouse. The outcome of amphotericin B resistance in immune-suppressed mouse was in line with Varughese *et al*.[61], who identified an amphotericin-resistant *C. lunata* that caused peritonitis. Efforts to outwit resistance in *C. lunata* have begun, notably, the identification of a new cytochrome P450, CYP53A15, largely assumes to be the target for natural antifungal compounds[62]. Although the incidence of antifungal resistance is low, evidence from Table 1 indicated that combine therapy is the most effective method for the treatment of *Curvularia* infections.

CONCLUSION

Curvularia species thrived on plant hosts as well as infect immunocompetent and immunocompromised individuals at variable degree of severity. *Curvularia lunata* from plant and clinical origin are genetically diverse based on GPDH locus. Treatment of *Curvularia* infection is hard to achieve and appropriate combination therapy last for at least twelve weeks. With the current use of azole-fungicides, it is herein predicted that by the year 2047 most *Curvularia* species infecting plants and humans may have evolved to azole-resistant phenotypes posing severe difficulties in the management of *Curvularia* diseases. Finally, strengthening legislation aimed at reducing the use of azole-fungicides could help reduce the selective pressure that contributes to genetic diversity of *Curvularia* species.

SIGNIFICANCE STATEMENTS

- *Curvularia* species resistance to azole antifungal drugs and fungicides showed global increase in reported cases
- Evidence rationale use of azole-fungicides in farms could reduce selective pressures that drive resistance in *Curvularia* species to antifungal therapy

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