Research Article

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Antifungal sensitivity profile of Fusarium spp. resulting keratitits

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Abstract
Fungal keratitis is an important cause of visual impairment and blindness. Genus Fusarium is a leading cause for fungal keratitis and it has higher degree of resistance to antifungal agents. Our objectives were to identify Fusarium spp. isolated from corneal specimens (received at Dept. of Mycology - MRI from 2013-2016) up to species level and to determine antifungal susceptibility pattern of them.

All Fusarium isolates (51) obtained from specimens of patients with keratitis were included in the study. Speciation was done using morphological characteristics of fungi. Antifungal sensitivity testing was done according to CLSI M 51- A guideline, against amphotericin B (10 µg), itraconazole (10 µg). and voriconazole (1 µg).

Majority of the isolates were F. solani complex (n=24). Three isolates were difficult to speciate morphologically. Significant number of Fusarium isolates had inhibitory zone diameters (IZD) less than tentative zone diameter epidemiological cut off values (TZD ECVs) for both itraconazole and amphotericin B, indicating emergence of resistant strains against these drugs. Forty five isolates (97.82%) had IZD more than corresponding TZD ECV for voriconazole. All F. solani complexes had IZD less than TZD ECVs for itraconazole.

Morphological identification cannot be used as the only method for speciation of Fusarium isolates. Antifungal sensitivity testing should be done for Fusarium isolates from keratitis patients as emergence of resistance strains is not uncommon against commonly used antifungal agents.

Keywords: Fungal keratitis, Fusarium spp., Antifungal resistance, Sri Lanka.

Introduction
Fungal keratitis is an important, sight-threatening problem occurring word wide, however it is more prevalent in tropical and subtropical areas [1,2]. Fungal keratitis accounts for 1-44% of all microbial keratitis and the incidence of fungal keratitis has increased in recent years [2,3].

It can be due to fungi from different classes of moulds and yeasts and the isolates vary with the geographic area [1,2]. The most frequently isolated fungi are Fusarium spp., Aspergillus spp., Curvularia spp., Bipolaris spp. and Candida spp. [2]. These fungi invade traumatized or immunologically compromised corneas and cause corneal inflammation [4]. It will manifest as dry, raised lesion with crenate or feathery borders, with satellite lesions and a hypopyon [2].

Genus Fusarium is a leading cause for the fungal keratitis [4]. The incidence of Fusarium keratitis has increased over the past 4 decades and it is estimated that approximately 50% of all microbial keratitis cases in tropical countries are due to genus Fusarium [5]. This increased incidence may be due to increased awareness as well as due to changes in risk factor profiles, including an increase in the use of topical steroids, increase in surgical procedures, increase in contact lens use and chronic ocular surface diseases [5]. F. solani is the most frequently isolated Fusarium species from the eye and it is followed by F. oxysporum, F. dimerum, F. incaratumequiseti, and Gibberella fujikuroi [5].

Because genus Fusarium is a leading cause for fungal keratitis, it is important to know the antifungal sensitivity profile of the clinical isolates of keratitis. The multi-resistant nature of this genus has led a continuous challenge to ophthalmologists treating Fusarium keratitis. Since the susceptibility profile is isolate dependent, identification at species level and anti-fungal susceptibility testing should be performed for any Fusarium involved in an invasive fungal infection including keratitis.

Although microdilution method is the gold standard for antifungal sensitivity testing it is cumbersome and time consuming method. The CLSI disk diffusion method (M51-A and supplement M51-S1) provides tentative zone diameter epidemiological cut-off values (TZD ECV) for non-dermatophyte filamentous fungi and it is faster and simpler method than the CLSI reference microdilution method (Alastruey-izquierdo et al. 2015). Since TZD ECVs do not based

on findings of the clinical outcome studies or pharmacology of the antifungal agents they are not used to categorize a fungal isolate in to susceptible or resistant [6]. However these TZD ECV could aid in the early identification of strains with acquired resistance mechanisms [6] . Our aim of this study was to identify Fusarium isolates, causing fungal keratitis from corneal specimens received at Department of Mycology, Medical Research Institute (MRI) from 2013-2016, to species level and to determine antifungal susceptibility pattern among those Fusarium isolates.

Methodology

All Fusarium isolates (51) obtained from specimens of patients with keratitis received at Department of Mycology, MRI, Sri Lanka from January 2013 to March 2016 were included in the study. These 51 isolates were stored at -80˚C till process.

Morphological identification

Those isolates were identified according to the conventional morphological criteria. Both macroscopic and microscopic characteristics were used in identification and speciation of Fusarium isolates.

All 51 isolates were sub-cultured on potato-dextrose agar (PDA) to obtain pure growth. Plates were incubated for two weeks at 26˚C and the morphological identification was done by growth characteristics of cultures Eg; colour of the colonies (obverse & reverse), consistency of the colonies & growth rate. All cultures were mounted with lacto phenol cotton blue (LPCB) and examined for microscopic characteristics at the end of day 14. Slide cultures were done for all isolates to induce sporulation and incubated for 14 days prior to examine the characteristics of the sporulation and chlamydospores.

All morphological characteristics were confirmed by comparing them with the characters given in the "Atlas of clinical fungi" [7]. Table 1 shows the Key morphological features used to identify different Fusarium species.

Table 1:The Key morphological features used to identify different *Fusarium* species

Fusarium species	Key morphological features
F. chlamydosporum	Colonies grow with abundant aerial mycelium, deep pink, red or ochraceous to brownish, reverse carmine red or tan to brown. Conidiophores scattered over the aerial mycelium, branched, polyblastic conidiogenous cells numerous. Microconidia rarely produced and appearing only on sporodochial philides. Chlamydospores abundant roughened.
F. dimerum	Colonies are orange to apricot. Conidiophores loosely branched, often swollen phialides. Macroconidia strongly curved and pointed at the apex, mostly 1-3 septate. Microconidia were absent. Chlamydospores mostly intercalary, spherical to ovoid, single or in short chains, smooth walled.
F. nygamai	Colonies vinaceous to violet Microconidia are abundant, lateral on hyphae, cylindrical, on false heads or in short chains Macroconidial conidiophores arranged in dense sporodochia .falcate , with 3 to 5 septates, straight to slightly curved Chlamydospores were mostly abundant, single, in chains or in clusters, sub hyaline, smooth or roughened.
F. proliferatum	Colonies were white becoming vinaceous to purple. Conidiophor arising laterally from aerial hyphae, densly branched. Polyphialides abundant. Microconidia clavate with truncate base Macroconidia are abundant, with distinct foot cell , 3 to 5 septates, straight to slightly curved Chlamydospores were absent.
F. solani complex	Colonies are white to cream coloured. Reverse usually colourless Conidiophoe arising laterally from aerial hyphae. Macroconidia produced on shorter, branched conidiophores which soon form sporodochia. Usually moderately curved, with short, blunt apical, mostly 3 septate. Microconidia usually abundant, chlamydospores frequent.

Antifungal sensitivity testing method

Antifungal sensitivity testing was done according to CLSI M 51- A, Disk diffusion susceptibility testing of non-dermatophyte filamentous fungi, 2010 guideline (CSLI 2010).

Briefly, all the *Fusarium* isolates were grown on PDA plates and incubated for 48 hrs at 37 °C and then until day 14 at 26°C for sporulation. For each isolate, a spore suspension was prepared by covering the colonies with 0.9% sterile normal saline and then probing with a sterile pipette. The final spore inoculum was adjusted to a density of $1.0 - 5.0\times10^4$ spores per ml. Then the spore suspension was inoculated on the dried surface of a sterile non-supplemented Muller-Hinton agar by evenly streaking a swab, to have even distribution of inoculum.

Antifungal disks (Amphotericin B (10 µg), Itraconazole (10µg) and Voriconazole (1 µg)) were placed aseptically within 15 minutes of inoculation. Inoculated plates were incubated at 37°C for 72 to 96 h. Zone diameters were measured to nearest whole millimetre and compared with tentative zone diameters ECVs given in M 51 informational supplement (CLSI, 2010).

Emergence of strains with reduced susceptibility to antifungal agents was considered when inhibitory zone sizes are less than given tentative zone diameter ECVs given in the M 51 informational supplement. Quality control for the test was done with *Candida krusei* ATCC 6258.
The results were analysed by manually and SPSS software.

Results

Fifty-one Fusarium (51) isolates were sub cultured from stock cultures to obtain pure cultures. Five isolates out of 51 isolates were contaminated and they were excluded from the study. Figure 1 depicts the sample distribution of the study.

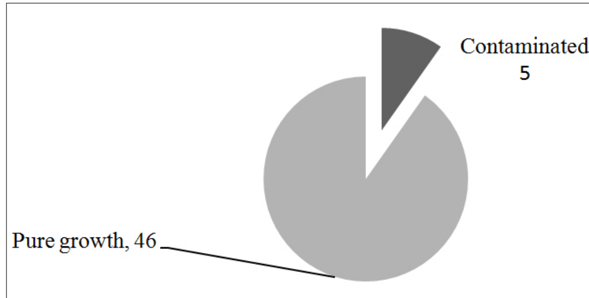

Figure 1: The sample distribution of the study

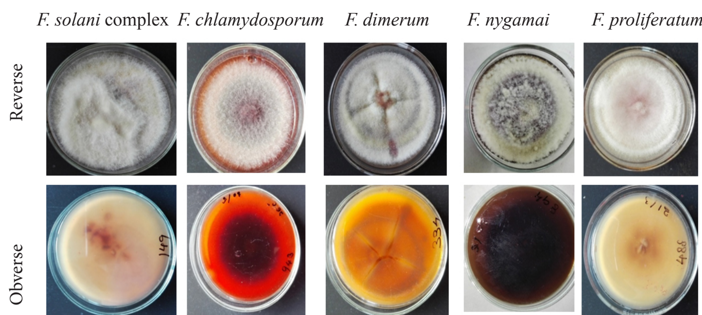

Figure 2: The colony morphology of different species

Forty three Fusarium isolates were morphologically identified as F. solani complex (n=24), F. chlamydosporum (n=15), F. dimerum (n=2), F. nygamai (n=1) and F. proliferatum (n=1).
Three isolates were difficult to speciate morphologically. However, based on the colony morphology, presence of characteristic microconidia and macroconidia they were tentatively identified as genus Fusarium. **(Table 2)** shows the species distribution of the isolates.

Table 2: Species distribution of different isolates

	Species	Number of isolates
1	F. solani complex	24
2	F. chlamydosporum	15
3	F. dimerum	2
4	F. nygamai	1
5	F. proliferatum	1
6	Difficult to speciate	3
	Total	46

Inhibitory zone diameters of different Fusarium isolates against amphotericin B, itraconazole and voriconazole were measured and compared with tentative zone diameters ECVs(TZD ECV) given in M 51 informational supplement. The zone diameter ranges given for different antifungals are given in **(Table 3)**.

Table 3: The zone diameter ranges given for itraconazole, voriconazole and amphotericine B by different Fusarium Species

Fusarium species	Number of isolates	ZD range mm Itraconazole	ZD range mm Voriconazole	ZD range mm Amphotericine B
F. solani complex	24	6-12	6-32	6-20
F. chlamydosporum	15	6-17	21-38	9-20
F. dimerum	2	6-14	21-40	10-15
F. nygamai	1	20	30	20
F. proliferatum	1	12	31	12
Undifferentiated	3	6-18	25-36	6-12

The distribution of inhibitory zone diameters of different Fusarium isolates against amphotericin B, itraconazole and voriconazole TZD ECVs are shown in **(Table 4)**.

Table 4: The distribution of inhibitory zone diameters of different Fusarium isolates against amphotericin B, itraconazole and voriconazole TZD ECVs

Fusarium species	Amphotericin B		Itraconazole		Voriconazole	
	IZD < TZD ECV	IZD> TZD ECV	IZD< TZD ECV	IZD> TZD ECV	IZD< TZD ECV	IZD>TZD ECV
F. solani complex (n=24)	n=16	n=8	n=24	n=0	n=1	n=23
F. chlamydosporum (n=15)	n=12	n=3	n=14	n=1	n=0	n=15
F. dimerum (n=2)	n=1	n=1	n=2	n=0	n=0	n=2
F. nygamai (n=1)	n=0	n=1	n=0	n=1	n=0	n=1
F. proliferatum (n=1)	n=1	n=0	n=1	n=0	n=0	n=1
Tentatively identified (n=3)	n=2	n=1	n=2	n=1	n=0	n=3

Forty three (93%) isolates and 32 isolates (70%) had less inhibitory zone diameters compared to tentative zone diameter ECVs for itraconazole and amphotericn B respectively.

The distribution of inhibitory zone diameters of all Fusarium isolates against all three antifungals are shown in **(Figure 4)**.

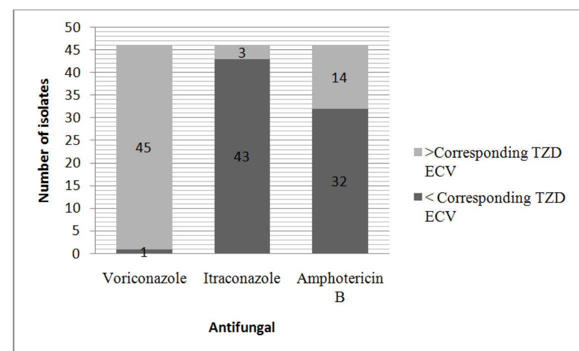

Figure 4: The distribution of inhibitory zone diameters of all Fusarium isolates against all three antifungal agents.

The distribution of inhibitory zone diameters of different Fusarium spp. isolates against all three antifungal agents were evaluated. Majority of F. solani complex , F.proliferatum and F. chlamydosporum had lower inhibitory zone sizes compared with TZD ECV for amphotericin B indicating the presence of isolates with acquired resistance among

F.solani,F.chlamydosporum & F.proliferatum.

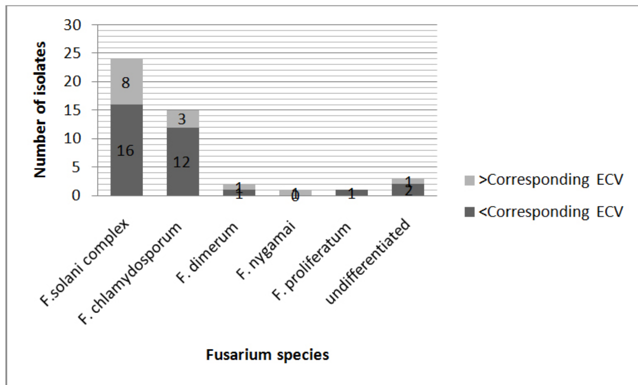

Figure 5: The distribution of inhibitory zone diameters for Amphotericin B at the species level.

All F. solani complexes and 14/15 of F. chlamydosporum, all F. dimerum had inhibitory zone sizes less than TZD ECVs for itraconazole indicating the presence of isolates with acquired resistance among F.solani,F.chlamydosporum,F.dimerum & F.proliferatum.

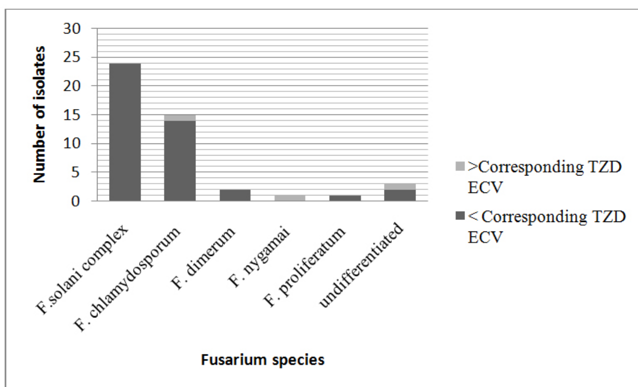

Figure 6: The distribution of inhibitory zone diameters for Itraconazole at the species level.

However, all most all Fusarium species had larger inhibitory zones compared with tentative zone diameter ECVs for voriconazole, except one isolate of Fusarium solani complex. This finding indicates the presence of isolates with acquired resistance to voriconazole among F.solani complex.

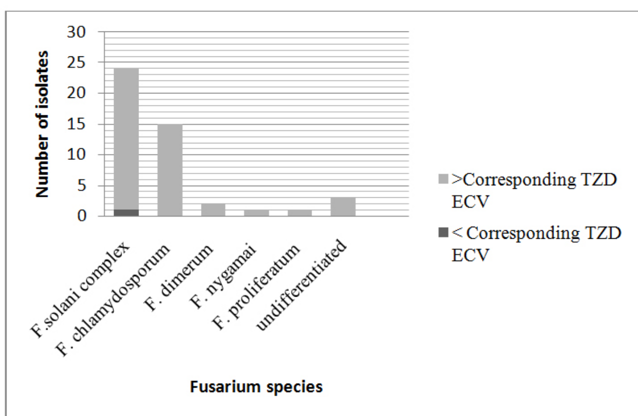

Figure 7: The distribution of inhibitory zone diameters for voriconazole at the species level.

Sixty seven percent (16/24) and 100% (24/24) of F.solani complexes had less inhibitory zone diameters than TZD ECV for amphotericin B and itraconazole respectively. However 95.8% of F.solani complexes had larger inhibitory zone diameters than TZD ECV of voriconazole.

Discussion

The genus Fusarium could be isolated from soil, plant and manmade habitats globally [7]. Most of the human infections are acquired by inoculation via contaminated thrones or plant leaves in otherwise healthy individuals [7].

It is important to identify Fusarium isolates up to the accepted species complex (SC) or species level however it is a significant challenge to the laboratory [6]. As a result, in most clinical cases the etiological agent is reported as Fusarium species.

The morphological characteristics of cultures have been used to identify fungal isolates and it is used in many laboratories [8].The morphological characteristics ,both macroscopic features (colour of the colonies (obverse & reverse), consistency of the colonies , growth rate) and microscopic features (sporulation and chlamydospores) are used for the laboratory identification [7].

The genus was organized into sixteen section with 65 species according to these morphological differences by Wollenweber and Reinking [7]. We have used the morphological identification methods to identify Fusarium isolates, causing fungal keratitis from corneal specimens to species level in our study. We were able to identify those 46 isolates as F. solani complex (52%), F. chlamydosporum (33%), F. dimerum (4%), F. nygamai(2%) F. proliferatum (2%.) and 3 isolates of genus Fusarium(7%) (tentatively identified).

Majority (52%) of isolates in our study were belonged to F. solani complex. This feature was shared by other similar studies. It is reported that among the clinical isolates, Fusarium solani as the most frequently found species [5]. Lalitha, P. et al., 2008 has also indicated that "F. solani was the most common Fusarium species isolated from specimens of fungal keratitis" in her study findings [9].

The next common species was F. chlamydosporum (33%) and it was followed by F. dimerum 4%, F. nygamai 2% and F. proliferatum 2% in the current study. However, the distribution of species in current study is different from other studies. For example, Tortorano et al. 2008 has showed that F. solani complex was followed by next frequently isolated species, F. oxysporum (20%), F. verticillioides (10%), and Fusarium moniliforme (10%) [10]. According to a recent study, F. solani was the most frequently isolated Fusarium species from the eye and it was followed by F. oxysporum, F. dimerum, F. incaratumequiseti, and Gibberella fujikuroi [5]. This may represent the species variation according to different geographic locations.

However, most of the isolates shared common features and 3 isolated were difficult to identify only using morphological features. Although it is accurate in classification at genus level, the morphologic classification of Fusarium isolates to the species level is inconsistent due to high degree of morphologic variability demonstrated at different growth stages [5]. The growth rate and colony morphology are influenced by the media and growth conditions [8]. The characteristic morphological structures will take weeks and may influence by different induction factors [8]. Many Fusarium species are appeared

similar in culture and represent species complexes instead of single species [8]. As a result molecular methods are used to identify Fusarium species and more than 200 species have been identified in 22 species complexes based on molecular sequencing recently [7].

Although members of this genus are relatively resistant to most antifungal agents, different Fusarium species show different susceptibility profiles. So, it is important to detect antifungal sensitivity of the Fusarium species isolated from clinical specimens [10].

The standardized methods of antifungal sensitivity testing have been published by both European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI). Although micro-dilution methods are the gold standards or reference method for antifungal sensitivity testing, though it is cumbersome and time consuming method (Alastruey-izquierdo et al. 2015). The CLSI disk diffusion method (M51-A and supplement M51-S1) for non-dermatophyte filamentous fungi (Alternaria spp., Aspergillus spp., Bipolaris spp., Fusaium spp., Paecilomyces spp. Rhizopus oryzae, other mucoraceous mould species, Pseudallescheria boydii species complex and Scedosporium prolificans) provides qualitative results within 8-24 h and this is faster and simple than the CLSI reference microdilution method (Alastruey-izquierdo et al. 2015). CLSI M 51 A guideline provides tentative epidemiological cut-off values (TZD ECV) for caspofungin, triazoles (voriconazole, itraconazole, posaconazole), and amphotericin B.

The TZD ECV is the highest MIC that would categorize an isolate as wild type (WT) without known mechanisms of resistance [6]. The TZD ECV will distinguish wild type (WT) from non-WT isolates [6]. Non-wild-type isolates of fungi show less antifungal susceptibility and they often harbour molecular mechanisms of resistance (Alastruey-izquierdo et al. 2015, Espinel-Ingroff, A et al. 2016). TZD ECV is a sensitive indicator of the emergence of strains with reduced susceptibility and it is used to track the emergence of resistance strains among fungi [6].

Here in our study, we have performed the antifungal susceptibility profile of Fusarium spp. isolated from specimens from keratitis patients by CLSI disk diffusion method which provides tentative zone diameter ECV values for selected filamentous fungi (CLSI 2010).

Majority (70%) of Fusarium isolates of our study had lower inhibitory zone diameters compared with tentative zone diameter ECV for amphotericin B indicating the presence of isolates with acquired resistance. Our finding is different from the finding of the study of Alastruey-izquierdo et al. They have found that, amphotericin B was the only drug, that showed activity in vitro against all Fusarium species by CLSI micro dilution method.

Azole (Itraconazole,voriconazole etc) resistance is not uncommon among Fusarium species and this has been detected by other studies.In our study, ninety-three percent of all Fusarium isolates had lesser zone diameters compared to itraconazole tentative zone diameter ECV indicating the presence of isolates that are likely to have acquired resistance mechanisms against itraconazole. A study analysing the in vitro sensitivity pattern of different antifungal compounds against 67 clinical isolates of Fusarium spp. according to CLSI micro dilution reference method found that no in vitro activity of azoles (itraconzole,voriconazole, posaconazole) drugs against most of the isolates of Fusarium. However, ninety eight percent of all Fusarium isolates of our study had zone diameters more than that for corresponding tentative zone diameter epidemiological cut off value (ECV) for voriconazole indicating the lack of isolates that are likely to have acquired resistance mechanisms against voriconazole in our study sample . Only one isolate of Fusarium solani complex showed low inhibitory zone diameter (IZD) than TZD ECV. The study performed by Tortorano et al through CLSI broth microdilution reported voriconazole and posaconazole are active against Fusarium species except F.solani. [10]. In the similar study they report that F. proliferatum and F. oxysporum isolates had broad range of minimal inhibitory concentrate (MIC)s against voriconazole [10].

Majority of F.solani complex of our study had lower inhibitory zone sizes for itrconazole and amphotericine B compared with tentative zone diameter ECVs provided at CLSI disk diffusion method. Further, one isolate of Fusarium solani complex showed low inhibitory zone diameter than TZD ECV. Since emergence of reduced susceptibility to antifungal agents is considered when inhibitory zone sizes less than given tentative zone diameter ECVs, this may indicate emergence of resistance strains frequently among F.solani complex. In lituratue it is reported that F. solani is more resistance to commonly used antifungal drugs compared with non-solani spp. and accompanied with worse outcome compared with non-solani spp. [11]. Other studies also have reported less antifungal sensitivity of Fusarium species against many antifungals and F.solani being more resistant than other Fusarium species. A study conducted in Italy following CLSI broth micro dilution method on 75 Fusarium clinical isolates, conclude that majority of F.solani isolates from clinical specimens had high MIC for azole antifungals including voriconazole [10].

Conclusion

Fusarium solani complex, the most resistant type of Fusarium species, is more frequently present among our patient sample. Accurate identification of Fusarium isolates up to species level can aid in the choice of appropriate antifungal therapy because different Fusarium species show different antifungal sensitivity profiles. However morphological identification cannot be used as the only method for speciation of Fusarium isolates and may require other methods like molecular identification. Antifungal sensitivity testing should be done for Fusarium isolates from keratitis patients as emergence of resistant strains through Fusarium clinical isolates is not uncommon for commonly used antifungal agents [12-14].

Acknowledgment

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