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In vitro Susceptibility Test of Antifungal Drugs for Isolated *Aspergillus* Species from Aspergillosis Patients in Riyadh City, Saudi Arabia

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The purpose of this study was to isolate and identify the most important species belonging to *Aspergillus* genus that cause aspergillosis disease in Riyadh city, Saudi Arabia and determine the best specimens taken from patients to isolate the *Aspergillus,* as well as to specify the best antifungal drugs for its inhibition, also to identify which of these isolate species are more resistant to these antifungal agents. A total of 53 samples was collected from patients initially diagnosed as infected with Aspergillosis disease. These samples were from sputum, tracheal aspirate, nasopharyngeal aspirate, bronchoaveolar lavage and lung tissue. Direct examination, culturing process, isolation of the fungus were performed to identify the isolates by their micro- and marocharacteristics. To confirm the identification, the isolates were sent to Assiut University Mycological Center (AUMC) in Egypt. In vitro susceptibility test of different antifungal (Amphotericin B, Anidulafungin, Caspofungin, Fluconazole, 5-Flucyosine, Itraconazole, Micafungin, Posaconazole and Voriconazole) for isolated *Aspergillus* was carried out. The results revealed that the most fungal isolates were obtained from sputum samples followed by aspirate samples from trachea. The cause of aspergillosis disease was confined to the five following species: *A. fumigatus, A. flavus, A nidulans*, *A. niger* and *A. terreus*. The most frequent isolate was A. fumigatus. The antifungal caspofungin and anidulafungin were the most effective ones, whereas fluconazole and 5-flucyostine were the least effective antifungal drugs. It was also found that *A. fumigatus* was the most resistant species among all studied isolates.

Key words: *Aspergillus*, *Aspergillosis*, *Fumigatus*, *Caspofungin*, *Anidulafungin*.

Aspergillus fungi that causing aspergillosis have a global spread in most environments. It includes a variety of saprophytic filamentous fungi. About 200 species belonging to the genus *Aspergillus* had been identified, from which 20 species are capable of causing certain diseases to humans and animals. *Aspergillus fumigatus* is considered as the major reason of increasing cases of invasive aspergillosis. The other species of this genus include the fungi *A. flavus, A. terreus, A. niger* and *A. nidulans*. It is evident that all of them are opportunistic pathogenic fungi where they are active when the human immune system is weakened[1-3]. In 2009, Montenegro and his colleagues have published a first report of *A. lentulus* strain isolated from a patient with probable invasive aspergillosis[4]. Some researchers reported that *Aspergillus fungi* caused excessive allergic broncho-pulmonary cases in some individuals suffering from asthma and cystic

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fibrosis, also this type of fungus causes non-invasive aspergillosis for patients with tuberculosis diseases[5,6].

This undertaken disease threatens patients who are suffering from tumors, leukemia and patients of stem cell transplantation, organ transplants, those treated with cortical adrenal steroids for long duration, those who under treatment of reducing and curbbing the immune system, patients with genetic immunodeficiency as well as those with chronic granulomatous disease and human immune- virus patients[7, 8].

Since there is a some sort of scarcity of studies investigating the most important species of *Aspergillus* that causing aspergillosis disease in Suadi Arabia, this research was undertaken aiming at isolating and identifying these fungi in patients from Riyadh region diagnosed clinically as aspergillosis suffering patients, hence, investigating the sensitivity of the isolated species to the most important and commercially available antifungal drugs. Then, a comparison was performed among the collected samples from sputum, tracheal aspirate, nasopharyngeal aspirate, bronchoaveolar lavage and lung tissue to determine the best one for isolation and diagnosis of Aspergillus fungi.

MATERIALS AND METHODS

Sample collection

Samples were collected from patients receiving treatment in the hospitals who were infected with aspergillosis, depending on the log and diagnostic specialists at the hospital. Sample collection was done throughout a period of 8 months from several hospitals in Riyadh region, Saudi Arabia including King Saud Hospital for Chest Diseases, The Central Hospital in Riyadh, Prince Salman Hospital, King Fahd Medical City and King Khalid University Hospital. Samples were collected by a specialized medical team which included sputum, tracheal aspirate, nasopharyngeal aspirate, bronchoaveolar lavage and lung tissue. Samples were immediately transferred to the hospital laboratory for preliminary examinations.

Direct microscopic test

Direct microscopic examination of the samples was carried out by making a smear of the sample on a slide after adding a drop of potassium hydroxide(10 %)[9]

Isolation of the fungi

The isolation process was conducted according to[10, 11] where 3-5 ml of the liquid samples (sputum, trachea and pulmonary bronchi lotion) were placed in sterile plastic container (10 ml), then samples were diluted with an equal volume of sterile saline solution thereafter, they were mixed well using vortex for 10 min. Samples were then centrifuged (1500 rpm) for 10 min. The suspension was disposed and 2 ml of sterile saline solution was added to the sediment and mixed well. 0.2 ml of mixture was transferred into new tube where 0.8 of sterile saline solution was added. One drop was then placed in the middle of a Petri dish containing sabouraud agar. Concerning the lung tissue, it was cut into very small pieces using a sterile medical scalpel, then each piece was placed on the surface of the sabouraud agar. All Petri dishes were incubated at 30 ºC for 5 days. The pure (single) cultures were obtained by making subcultures.

Identification of fungal isolates

The pure fungal isolates were identified by macro- and micro-characteristics according to[12, 13] where the following types of media were used: Mycology Broth (MB), Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), Corn Meal Broth (CMB), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), Malt Extract Agar (MEA), Corn Meal Agar (CMA) and Czapek Dox Agar (CzDA). Growth rate, the optimum temperature for growth, shape and color of the colony and reverse color were determined. The microscopic characteristics were studied by light microscope following staining the samples with lactophenolglycerol stain. These characteristics included hyphae, conidiophore, conidia, vescicle, phialides and sclertia. Upon completion of the identification, the isolates have been deposited into the culture collection of Assiut University Mycological Center (AUMC), Arab Republic of Egypt.

In vitro susceptibility test of different antifungals for isolated aspergillus species

A sensititre yeast one in vitro diagnostic kit(Trek Diagnostic System Limited, U.K.) was used to determine the minimum inhibitory concentration (MIC) from the following antifungal drugs Amphotericin B, Anidulafungin, Caspofungin,

Fluconazole, 5-Flucytosine, Itraconazole, Micafungin, Posaconazole and Voriconazole. The sensititre yeast one in vitro diagnostic kit contains sequential twofold dilutions of Amphotericin B (0.12 to 8 µg/ml) Anidulafungin (0.015 to 8 µg/ml), Caspofungin (0.008 to 8 µg/ml), Fluconazole (0.12 to 256 µg/ml), 5-Flucytosine (0.06 to 64 µg/ml), Itraconazole (0.015 to 16 µg/ml), Micafungin (0.008 to 8 µg/ml), Posaconazole (0.008 to 8 µg/ml) and Voriconazole (0.008 to 8 µg/ml). The suspension of isolates were prepared to a final turbidity of 0.15 McFarland standards. The inoculums and inoculation were prepared and perform within 15 min, also inoculation was performed within 15 min. The procedures used to accomplish this part of work was according to[14,15]. Directions of kit manufacturer were followed in preparing and adding the inoculums, then test plates were incubated in a vertical position at 35 ºC without CO_2 for 24 hrs. The color within the wells was observed in which the blue color indicates no growth whereas the pink red color indicates the presence of fungal growth.

RESULTS

Samples

Fifty three samples were collected during the study period, including 22 samples of sputum, 11 of tracheal aspirate, 13 of nasopharyngeal aspirate, 5 of bronchoalveolar lavage and 2 of lung biopsy.

Direct microscopic test and isolation

Table 1 shows that the number of samples gave a preliminary positive result through direct microscopic examination (10% KOH) was 9 samples with a percentage of 16.9 % of the total samples, these positive samples included 3 samples from sputum, 3 from tracheal aspirate, 2 from nasopharyngeal aspirate, one sample from bronchoalveolar lavage whereas no fungal elements were exhibited in lung tissue samples.

Table. 1. Percentage of positive samples indicating direct microscopic test and fungal growth on saobourd dextrose agar (SDA) from Riyadh city patients with probable aspergillosis disease

Type of Sample	Number of Samples	Positive results with KOH (10%)	Positive results on SDA
Sputum	22	3	22
Tracheal Aspirate	11	3	11
Nasopharyngeal Aspirate	13	2	13
Bronchoalveolar lavage	5	1	5
Lung Biopsy	2	0	2
Total	53	9	53
%	100	16.9 %	35.8 %

When the collected samples were cultured on SDA medium, the percentage of positive samples was 35.8 % (19 out of 53 samples). The sputum samples comprised the greater number (7 out of 19) followed by samples of tracheal aspirate and nasopharyngeal aspirate (5 out of 19). No growth was observed in lung tissue samples.

Morphological identification of isolated fungi

Five species belonging to aspergillus genus were identified including *A. fumigatus, A. flavus, A. nidulans, A. niger* and *A. terreus*. The identification was based on the microscopic and cultural properties and growth rate of fungal isolates at different range of temperatures using SDA, PDA, CzDA, CMA, CYA and MEA media. Tables 2,3 and 4 include the micro-characteristics of aspergillus species using three types of media only SDA, PDA and CzDA. Table 5 shows frequency and identification number of each species that defined by Assiut University Mycological Center (AUMC), Arab Republic of Egypt. The obtained results revealed that *A. fumigatus* was the most frequent isolate whereas, *A. nidulans* was the least one.

***In vitro* susceptibility test**

Table 6 shows the minimal inhibitory concentration (MIC) from each antifungal drug for growth of isolated *Aspergillus* species from Aspergillosis patients in Riyadh city, Saudi Arabia. The obtained results revealed that the

Table 2. Macro- characteristics, micro- characteristics and growth rate of isolated *Aspergillus* species from aspergillosis patients in Riyadh city on saobourd dextrose agar(SDA).

		A. flavus S.NO.* 3, 8. 14, 19	*A .fumigatus* S.NO.2, 4, 7, 9. 12, 15, 18	*A. nidulans* S.No.10	*A. niger* S.No.1, 5, 11, 13, 17	*A. terreus* S. No. 6, 16
Growth	25 °C	1.2	0.9	0.9	1.7	0.8
Rate cm/24h	30 °C	1.2	1.1	1.0	2.3	1.2
	35 °C	1.4	1.3	0.9	2.6	1.6
Macroscopic characters	Colony color	Yellow, green	White to blue green	Creamy buff to dark green	Charcoal black	Pale buff to brown
	Reverse color	Pale yellow	Yellow	Yellow to brown	Yellow	Pale yellow to brown
	Mycelium	White to green Fluffy	White to green Velvety And flat	White to green Velvety and flat	White to yellow Granular, flat	White Velvety and folded
Microscopic characters	Sclertia	Globose brownish	Nil	Globose dark redbrown	Nil	Nil
	Hyphae	Septate	Septate	Septate	Septate	Septate
	Conidio--phore	Colorless to pale brown, Roughened	Colorless, Smooth	Brownish Smooth	Colorless, Smooth	Colorless, Smooth
	Conidia	Pale green, Globose, Smooth to finely	Colorless to Grayish, Globose rough	Colorless, Pale green, Globose roughwalled	Colorless to dark Globose smooth	Colorless to yellow Globule to ellipsoidal smoothwalled
	Vescicle	Globose	Flask	Hemispherical	Round	Flask
	Phialides	Uniseriate	Uniseriate	Biseriate	Uniseriate/ Biseriate	Biseriate

Table 3. Macro- characteristics, micro- characteristics and growth rate of isolated *Aspergillus* species from aspergillosis patients in Riyadh city on potato dextrose agar (PDA)

		A. flavus S.NO.* 3, 8. 14, 19	*A .fumigatus* S.NO.2, 4, 7, 9. 12, 15, 18	*A. nidulans* S.No.10	*A. niger* S.No.1, 5, 11, 13, 17	*A. terreus* S. No. 6, 16
Growth	25 °C	1.2	0.7	0.8	1.2	0.9
Rate cm/24h	30 °C	1.4	1.2	1.0	1.9	1.3
	35 °C	1.5	1.3	1.0	2.0	1.5
Macroscopic characters	Colony color	Bright, green	blue green	dark green	Charcoal black	Pale buff to brown
	Reverse color	yellow	Yellow to brown	Yellow to brown	Yellow	Pale yellow to brown
	Mycelium	White to green Fluffy	White to green Velvety And flat	White to green Velvety and flat	White to yellow Granular, flat	White Velvety and folded
Microscopic characters	Sclertia	Globose	Nil	Globose	Nil	Nil
	Hyphae	Septate	Septate	Septate	Septate	Septate
	Conidio--phore	Colorless to pale brown, Roughened	Colorless, Smooth	Brownish Smooth	Colorless, Smooth	Colorless, Smooth
	Conidia	pale green Globose, Smooth to finely	Colorless to Grayish, Globose rough	Colorless, Pale green, Globose roughwalled	Colorless to dark Globose smooth	Colorless to yellow Globule to ellipsoidal smoothwalled
	Vescicle	Globose	Flask	Hemispherical	Round	Flask
	Phialides	Uniseriate	Uniseriate	Biseriate	Uniseriate/ Biseriate	Biseriate

Table 4. Macro- characteristics, micro- characteristics and growth rate of isolated *Aspergillus* species from aspergillosis patients in Riyadh city on Czapek dox agar (CzDA)

		A. flavus S.NO.* 3, 8. 14, 19	*A .fumigatus* S.NO.2, 4, 7, 9. 12, 15, 18	*A. nidulans* S.No.10	*A. niger* S.No.1, 5, 11, 13, 17	*A. terreus* S. No. 6, 16
Growth	20 °C	0.9	1.0	0.7	0.7	0.6
Rate cm/24h	30 °C	1.0	1.1	1.1	1.1	0.9
	35 °C	1.1	1.3	1.0	1.1	1.1
Macroscopic characters	Colony color	White to green	Blue green to grayish	Pain green	Dark brown to black	Cinnamon buff to sand brown
	Reverse color	White	Pale yellow to brown	Purple brown	Colorless to white	Pale yellow to white
	Mycelium	White to green Fluffy Granular	White to gray velvety and flat	White to green velvety and flate	White to yellow Granular flat	White velvety and folded
Microscopic characters	Sclertia	Nil	Nil	Globose	Nil	Nil
	Hyphae	Septate	Septate	Septate	Septate	Septate
	Conidio--phore	Colorless, Roughened	Colorless, smooth	Brownish smooth	Colorless, smooth	Colorless, smooth
	Conidia	pale green Globose, Smooth to finely	Colorless to Grayish, Globose rough	Colorless, Pale green, Globose roughwalled	Colorless to dark Globose smooth	Colorless to yellow Globule to ellipsoidal smoothwalled
	Vescicle	Globose	Flask	Hemispherical	Round	Flask
	Phialides	Uniseriate	Uniseriate	Biseriate	Uniseriate/ Biseriate	Biseriate

Table 5. Percentage of frequency *Aspergillus* species isolated from aspergillosis patients in Riyadh city and the identification number in the culture collection of Assiut University Mycological Center (AUMC), Arab Republic of Egypt

Aspecies	Number of isolates	Total isolates	Percentage %	AUMC No.
A. fumigatus	7	19	36.8	8794
A. flavus	4	19	21	8774
A. nidulans	1	19	5.3	8776
A. niger	5	19	26	8777
A. terreus	2	19	10.5	8778

concentration 0.5 - 2 mg/L from amphotericin B led to growth inhibition of 50 % for all fungal isolates. However, 1-4 mg/L was required to inhibit 90 % of fungal growth. It was also observed that the fungal isolates *A. fumigatus 6 , A. flavus 1, A. flavus 3, A. flavus, A. terreus 1* and *A. terreus 2* were the most resistant ones to amphotericin B. The results also showed that anidulafungin and micafungin were the most effective antifungal drugs against majority of fungal isolates where their MIC 50 was 0.015 - 0.03 mg/L and MIC 90 was between 0.03 to 0.06 mg/L. whereas the least effective antifungal agent was fluconazol. It could be reported that the isolate number of 6 identified as *A. fumigatus* was the most resistant one to the most tested antifungal drugs.

DISCUSSION

Aspergillosis is considered as one of the most serious fungal infections that cause numerous health problems. The accurate identification at the level of the species is crucial as the first step in the treatment. Aspergillosis disease is spreading between the pulmonary area and trachea (a polyfocal disease within a specific system). The

Table 6. The Minimum inhibitory concentration (MIC) of antifungal drugs that inhibit 50 % (MIC_{50}) and 90 % (MIC_{90}) of aspergillus growth isolated from aspergillosis patients in Riyadh city, Saudi Arabia

Anti-fungals	MIC mg/mL	*A.fumigatus* 1	*A.fumigatus* 2	*A.fumigatus* 3	*A.fumigatus* 4	*A.fumigatus* 5	*A.fumigatus* 6	*A.fumigatus* 7	*A.flavus* 1	*A.flavus* 2	*A.flavus* 3
AB	MIC_{50}	0.5	0.5	0.5	0.5	0.5	2.0	0.5	1.0	1.0	1.0
	MIC_{90}	1.0	1.0	1.0	1.0	1.0	4.0	1.0	2.0	2.0	2.0
AND	MIC_{50}	0.015	0.015	0.015	0.015	0.015	0.03	0.015	0.015	0.015	0.015
	MIC_{90}	0.03	0.03	0.03	0.03	0.03	0.06	0.03	0.03	0.03	0.03
CAS	MIC_{50}	0.12	0.12	0.12	0.12	0.25	0.25	0.12	0.12	0.12	0.12
	MIC_{90}	0.25	0.25	0.25	0.25	0.5	0.5	0.25	0.25	0.25	0.25
FC	MIC_{50}	32	32	32	32	32	32	32	32	32	32
	MIC_{90}	64	64	64	64	64	64	64	64	64	64
FZ	MIC_{50}	64	64	128	128	64	128	64	64	64	64
	MIC_{90}	128	128	256	256	128	256	128	128	128	128
IT	MIC_{50}	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.25
	MIC_{90}	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.5	0.5
MF	MIC_{50}	0.015	0.015	0.015	0.015	0.015	0.03	0.015	0.015	0.015	0.015
	MIC_{90}	0.03	0.03	0.03	0.03	0.03	0.06	0.03	0.03	0.03	0.03
PZ	MIC_{50}	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.06	0.12
	MIC_{90}	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.12	0.25
VOR	MIC_{50}	0.5	0.5	0.5	0.5	0.5	1.0	0.5	0.5	0.5	0.5
	MIC_{90}	0.5	0.5	1.0	0.5	0.5	2.0	1.0	1.0	0.5	1.0
AB	MIC_{50}	1.0	1.0	0.5	0.5	1.0	0.5	1.0	2.0	2.0	
	MIC_{90}	2.0	2.0	1.0	1.0	2.0	1.0	2.0	4.0	4.0	
AND	MIC_{50}	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.03	
	MIC_{90}	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.06	
CAS	MIC_{50}	0.25	0.25	0.12	0.25	0.25	0.12	0.12	0.25	0.12	
	MIC_{90}	0.5	0.5	0.25	0.25	0.5	0.25	0.25	0.5	0.25	
FC	MIC_{50}	32	32	32	32	32	64	32	32	64	
	MIC_{90}	64	64	32	64	64	64	64	64	64	
FZ	MIC_{50}	64	64	64	64	64	128	64	64	128	
	MIC_{90}	128	128	128	128	128	256	128	128	256	
IT	MIC_{50}	0.25	0.25	0.5	0.5	0.5	1.0	0.5	0.12	0.25	
	MIC_{90}	0.5	0.5	1.0	1.0	1.0	2.0	1.0	0.25	0.25	
MF	MIC_{50}	0.015	0.015	0.015	0.015	0.015	0.03	0.015	0.015	0.03	
	MIC_{90}	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.06	
PZ	MIC_{50}	0.06	0.12	0.25	0.12	0.25	0.25	0.12	0.12	0.06	
	MIC_{90}	0.12	0.25	0.5	0.25	0.25	0.5	0.25	0.5	0.12	
VOR	MIC_{50}	1.0	0.12	0.5	1.0	0.5	0.5	0.5	0.5	0.5	
	MIC_{90}	1.0	0.25	1.0	2.0	1.0	0.5	1.0	1.0	1.0	

AB= Amphotericin B, AND= Anidulafungin, , CAS= Caspofungin, FC= 5-Flucytosine, FZ= Fluconazole, IT=Itraconazole, MF=Micafungin, PZ=Posaconazole, VOR=Voriconazole.

LD_{50}= mg//L 0.5- 4 0.015 -0.03 0.12 – 0.25 32 – 64 64 – 256 0.12 – 1.0 0.015 – 0.03 0.06 – 0.25 0.12 – 1 LD_{90}= mg/L 1-4 0.03 -0.06 0.25 – 0.5 32- 6 4 128 – 256 0.25 – 2.0 0.03 – 0.06 0.12 – 0.5 0.25 - 2

mortality rate among humans due to this disease reaches to 90 % of the infected people[16-18] In the present study, samples were collected from different areas related to respiratory system from patients probable suffering from aspergillosis in some Riyadh city hospitals. The study can not rely solely on one area or direct microscopic method only in the diagnostic process, however, culturing and identification must be performed and this was assured by[19]. In this research, five species belonging to aspergillus genus were determined named *A. flavus, A. fumigatus, A. nidulans, A. niger* and *A. terreus*, which considered as the main reasons of aspergillosis in the Riyadh region, Saudi Arabia. These obtained results are consistent with those reported by[20].

Throughout the isolation process, *A. fumigatus* was found to be the most frequent one among recorded species, where it was isolated from sputum samples followed by tracheal aspirate in a greater proportion when compared with other samples. This results is supported by a report mentioned by[21]. It was found that *A. nidulans* was the least frequent isolate where it was obtained from a sample of tracheal aspirate, the same result had been reached by[20]. Determination of the appropriate antifungal drug is so crucial to aspergillosis patients particularly those who have problems in their immune system as a result of any known reason such as chemotherapy and/or organ transplantation. The sensititre yeast one in vitro diagnostic kit method appears to be comparable to the Clinical and Laboratory Standards Institute (CLSI reference) method for testing the susceptibility of *Candida* spp. to the echinocandins anidulafungin, caspofungin, and micafungin[22].

It was shown that anidulafungin and micafungin were the most effective ones against all the tested isolates, followed by caspofungin and posaconazole, whereas fluconazole and 5-flucyosine were the least effective ones. The isolate No. 6 which was isolated and identified as *A. fumigatus* was the most resistant species against the investigated antifungal drugs. The results showed that the *A. terreus* was the most resistant species against the amphotericin B that is considered the most established antifungal drugs used for treatment of invasive aspergillosis. Several researches reported that *A. terreus* was less susceptible to amphotericin B than other *Aspergillus* genus[23], the present study confirm those results. Some researchers suggested that the resistance is related to a lack of ergosterol[24] while other studies did not observe any relation[23].

Micafungin and andulafungin are pertaining to enchinocadin group which includes also caspofungin, this is a distinguished antifungal agent which characterized by inhibiting 1,3 b-glucan which is a component of the polysaccharide comprising the cellular wall[25]. Caspofungin is considered as the solely antifungal of that group which allowed to be used according to the American Food and Drug Organization. It was found that the combination between caspofungin and voriconazole supported the treatment efficiency in clinically diseased patterns[26].

It could be concluded that the best thing to do is to collect samples from various regions from patients infected with aspergillosis. Culture and isolation process were appropriate to diagnose the *Aspergillus* species. *A. fumagitus, A. flavuse, A. nidulans, A. niger* and *A. terreus* were the major fungal species responsible for occurrence of aspergillosis disease in Riyadh region, Saudi Arabia. *A. fumigatus* was the most resistant species to all investigated antifungal agents, whereas anidulafungin and micafungin were the most effective antifungal drugs tested experimentally in vitro, but 5-flucyostine and fluconazole were the least effective ones.

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