Isolation and Identification of Dermatophytes (Tinea corporis and Tinea cruris) and Evaluating Their Sensitivity to Antifungals by Using Epsilometer Test.

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Abstract:

Background: Dermatophytoses have been considered to be a major public health problem in many parts of the world.

Aims of the study: to determine the causative agents of dermatophytoses in Al-yarmook teaching Hospital and evaluating their antifungal susceptibilities by using E-test.

Materials and methods:

1. **Media and chemicals**: preparation and sterilization were done according to manufacturer's instructions.
2. **Specimens:** All microorganisms were clinical isolates obtained from a variety of specimens including skin, from groin and trunk, were identified to species level on the basis of standard biochemical tests, microscopy, and colony characteristics.
3. **Reagents:** Lactophenol Aniline Blue and Potassium Hydroxide (KOH) were used for direct microscopic preparations of fungal specimens and isolated molds to distinguish fungal elements for the purpose of identification.
4. **Culture media:** Sabouraud's dextrose agar (SDA) and dermatophyte test media (DTM) were used for primary isolation of dermatophytes, prepared by the addition of antibiotics for more selective identification.
5. **Antifungal susceptibility by using E-test:** The Epsilometer test (usually abbreviated E-test) is a laboratory test used by microbiologists to determine whether or not a specific strain of bacterium or fungus is susceptible to the action of a specific antibiotic or antifungal [1].

Results: *Trichophyton mentagrophytes* was the most frequent dermatophyte isolated 35(41%) followed by *T. rubrum* 18(21%), *Epidermophyton floccosum* 11(12.9%), *Microsporum canis* 6(7%), *T. violaceum* 1(1.17%), *T. verrucosum* 1(1.17%), and *Microsporum ferrugineum* 1(1.17%). The species of dermatophytes showed similar patterns of susceptibility to each antifungal agent tested with the exception of *Trichophyton rubrum* species which showed low suscebtibility that deserves to be mentioned.

Conclusions: E-test seems to be a reliable methodology for susceptibility testing for dermatophytes as well as for yeasts. Specific test conditions for the E-test, including the appropriate medium for the test, are required to correctly assay the susceptibility of dermatophytes to antifungal drugs.

Key words: Dermatophytes, E-test, antifungal susceptibility.

Introduction

Fungal infections are common skin diseases, affecting millions of people worldwide [2]. The high prevalence of superficial mycotic infections shows that 20-25% of the world's population has skin mycoses, making these one of the most frequent forms of infection. Most mycotic infections are superficial and limited to a depth of 1-2 mm. the fungi that usually cause only superficial infection in skin, hair, nails are called dematophytes [3]. The dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair, and nails) of humans and other animals to produce infections. Infection is generally cutaneous and restricted to the nonliving cornified layers because of the inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts [4]. Because dermatophytes require keratin for growth, they are restricted to hair, nails, and superficial skin. Thus, these fungi do not infect mucosal surfaces. Many studies that have been conducted throughout the world revealed the increased prevalence and shift in the epidemiology of dermatophytes [5]. Geographic locations are important factors in the type of infection seen and tend to be epidemic and endemic in areas where crowding and imperfect conditions for personal hygiene seem to favor its spread and persistence within the community [6]. Development of standardized antifungal susceptibility testing methods has been the focus of intensive research in the last 15 years. The increasing incidence of opportunistic severe fungal infections has greatly enhanced the interest in novel methods for *in vitro* antifungal susceptibility testing; the establishment of a standardized broth reference method for antifungal susceptibility testing of yeasts has opened the door to a number of interesting and useful developments. Also, the availability of reference methods provides a useful touchstone for the development of commercial products that promise to be more users friendly and to further improvement of test standardization. Incorporation of antifungal susceptibility testing methods into the clinical trials of new antifungal agents will facilitate the establishment of clinical correlates and further enhance the clinical utility of antifungal susceptibility testing, E-test assay has widely been used for determination of minimal inhibitory concentration (MIC) to antifungal drugs of the filamentous fungi, but dermatophytes were not yet included in most of these studies [7], [8]. Only a few studies [9], [10] have focused the MIC determination of antifungal agents to dermatophytic fungi by E-test. This technique has proven to be a simple straightforward method for determining *in vitro* susceptibility of fungal pathogens; E-test is an attractive alternative method as it is easy to perform, exhibits good correlation with the reference methods

and gives reproducible results, but is relatively expensive.

Materials and methods

Materials:

Media and chemicals: provided by the department of Microbiology in the College of medicine in Al-Mustansiriya University, preparation and sterilization was according to manufacturer's instructions, which include Sabouraud's dextrose agar, dermatophyte test medium, and other common media. RPMI 1640 media (Roswell Park Memorial Institute medium) was used for determination of (MIC) minimal inhibitory concentration by using E-test for antifungal drugs; fluconazole, itraconazole, voriconazole and amphotricin B**.**

Methods:

Reagents: Lactophenol Aniline Blue and Potassium Hydroxide (KOH) were used for direct microscopic preparations of fungal specimens and isolated molds to distinguish fungal elements for the purpose of identification.

Culture media: Sabouraud's dextrose agar and dermatophte test medium (DTM) were used for primary isolation of dermatophytes, prepared by the addition of antibiotics for more selective identification.

Collecting of samples: All 85 dermatophytes isolates used in this study were taken from:

1- Fifty patients diagnosed with tinea corporis (Skin scraping from the body trunk).

2- Thirty five patients diagnosed with tinea cruris (Skin scraping from the groin region).

All microorganisms were clinical isolates obtained from a variety of specimens including skin, from groin and trunk, were identified to species level on the basis of standard biochemical tests, microscopy, and colony characteristics. Patient's skin was disinfected with 70% alcohol prior to sample collection. If a characteristic dermatophyte "ring" was present on the skin, scrapings were collected from the outer margin of the ring at its junction with the normal skin. Otherwise, samples were collected from areas where the skin appears to be scaling. The skin scrapings were either placed in a sterile Petri dish or in clean paper envelopes; using closed tubes was avoided, as retained moisture may result in overgrowth of contaminants [11]. Or scrapings were placed between two clean glass slides, placed in a clean envelope labeled with the patient's data. Transport kits that have black background (scrapings may be easily visualized) are recommended. Ideally, samples should be delivered to the laboratory within 2 hours of collection as fungal viability may decrease with prolonged specimen storage. Collecting samples using a moistened cotton swab is an easy, reproducible method of obtaining a fungal culture sample, especially from small children who may be frightened by other methods [12].

Maintenance of the samples:

Methods used in this study:

1. Sabouraud agar slopes refrigerated at +8 +/- 1 degree
2. Sterile distilled water.

Cultivation of samples:

Sabouraud Dextrose Agar is a general-purpose medium developed by Sabouraud for the cultivation of dermatophytes [13]. The low pH of approximately 5.6 is favorable for the growth of fungi, especially dermatophytes, and inhibitory to contaminating bacteria in clinical specimens. The acidic pH of the medium may, however, also inhibit some species of fungi. Emmons modified the original formulation by reducing the dextrose content and adjusting the pH to near neutral to increase the recovery of fungi. The addition of antimicrobial agents improves the recovery of pathogenic fungi from specimens heavily contaminated with bacteria and saprophytic fungi [14]. Most laboratories use at least 2 different media (selective and non-selective) for fungal culture, which are incubated at 25-30˚C and examined weekly for up to 4 weeks before issuing a negative report [15]. Most dermatophytes lose their distinctive cultural and microscopical features when kept for a long time in culture. Fungal culture medium is used for positive identification of the species. Usually fungal growth is noted in 5 to 14 days, Microscopic morphology of the micro and macroconidia is the most reliable identification character, but a good slide preparation is needed, the stimulation of sporulation in some strains is also important. Culture characteristics such as surface texture, topography and pigmentation are variable so they are the least reliable criteria for identification. Clinical information such as the appearance of the lesion, site, geographic location, travel history, animal contacts and race is also important, especially in identifying rare non-sporulating species like *Trichophyton concentricum, Microsporum audouinii* and *Trichophyton schoenleinii.*

Inoculum preparation for dermatophyte: stock inoculum suspensions were prepared as described in the E-test Technical Guide [11]. For each isolate, to induce conidium formation only, the surface of the colony was gently scraped off and transferred to a sterile flask containing sterile 200 ml of Sabouraud's glucose broth with chloramphenicol and cycloheximide. For isolates that do not readily produce conidia (*T. violaceum*), a small portion of mycelial growth was gently scrapped with the loop bent , ground with sterile glass rod in the bottom of sterile flask then 50 ml of sterile broth was added . The flask was incubated for 72 hours at room temperature with continuous shaking. After that , 1 ml of the suspension was transferred to sterile tube , the larger hyphal fragments were allowed to settle for 10 minutes , while the supernatant was standardized and adjusted to an optical density (OD)

that ranged from 0.09-0.11 at wave length 530 nm (80-82% transmitance) [16].

Laboratory test: Many typical isolates of common dermatophytes can be identified directly from primary isolation media, particularly, Sabouraud dextrose agar and potato dextrose agar.

Identification characters include colony pigmentation, texture, and growth rate and distinctive morphological structures, other dermatophytes need more tests which help in identification depending on physical reaction of the dermatophytes such as pigment production of some species on (PDA) potato dextrose agar and cornmeal agar also hair perforation test and urease test when heavy inoculums were used from a pure 18 – 24 hour culture, Inoculated by streaking back and forth over the entire slant surface. Stabbing was avoided because it serves as a color control, tubes were incubated with loose caps at 35 ± 2°C, reactions were observed after 6 and 24 hours, and for the next 6 days, longer periods of incubation may be necessary.

Antifungal susceptibility by using test E-test: The E-test is basically an agar diffusion method. The E-test utilizes a rectangular strip that has been impregnated with the drug to be studied. A lawn of organisms was spread and grown on an agar plate, and the E-test strip was laid on top; the drug diffused out into the agar, producing an exponential gradient of the drug to be tested. There is an exponential scale printed on the strip. After hours of incubation, an elliptical zone of inhibition was produced and the point at which the ellipse meets the strip gives a reading for the minimum inhibitory concentration (MIC) of the drug. The test has been validated for many organisms against the broth/agar dilution method and shown to have excellent correlation.

Medium preparation: 500 ml RPMI 1640 supplemented with L- glutamine (broth), 65 gm/L Sabouraud Dextrose Agar, 500 ml Distilled water

For agar diffusion E-test, the suspension was applied to the agar surface with a cotton swab. The plates were allowed to dry for 15 min before the E-test strips were applied. The MICs endpoints were read after 48h and 72h of incubation for Candida and dermatophytes respectively .

E-Test reading : The MIC was determined after 2-3-day incubation at 28°C. The E-test drug concentrations ranged from 0.016 to 256 µg/ml for fluconazole and from 0.002 to 32 µg/ml for itraconazole, voriconazole, and amphotericin B. E-test MIC was determined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip.

Results:

Among the eighty five cases of dermatophytes (all directly examined), fifty cases of tinea corporis 37(74%) were positive by both direct KOH mount smear and by culture, 9(18%) were negative in direct KOH mount smear but positive in culture and the remaining four cases (8%) gave a positive direct KOH mount smear but negative in culture. Thirty five cases of tinea cruris 20(57.1%) were positive by both direct KOH mount smear and by culture, 7(20%) were negative in direct KOH mount smear but positive in culture and the remaining eight cases (22.9%) gave a positive direct KOH mount smear but negative in culture. Table (1).

Table-1- Direct KOH mount smear and culture of 85 cases of dermatophytes.

Examined cases	Tinea corporis		Tinea cruris	
	No	%	No	%
D+ , C+	37	74	20	57.1
D- , C+	9	18	7	20
D+ , C-	4	8	8	22.9
Total	50	100.0	35	100.0

D+ = Positive direct KOH examination, D- = Negative direct KOH examination.
C+ = Positive culture, C- = Negative culture.

Identification of dermatophytes species from clinical isolates:

Laboratory findings include hair perforation test which was used for further species identification, this test was used to differentiate certain species of *T. mentagrophytes*, which has the ability to penetrate the hair in vitro, from *T. rubrum*, which does not have such ability. Penetrated hair segments were identified by wedge-shaped perforations. The other biochemical test like urease test also differentiates *T. mentagrophytes* from *T. rubrum*, by color change; the first changes the medium to the red while the second does not. Corn meal agar and rice grain medium were used according to Fisher and Cook [17]. Sterile rice grain medium was used to distinguish *M. audouinii* from *M. canis. M. canis* has the ability to produce yellow pigment on sterile Rice Grain medium, while *M. audouinii* fails to produce this pigment and at best it produces a light brown discoloration of rice. Figure (1).

In the present study the identification of dermatophytes isolates from the all cases revealed that the *Trichophyton mentagrophyte* was the predominant species, this result was in agreement with Mohsen *et al.* [18]. In tinea corporis cases the

number of *Trichophyton mentagrophyte* was 22(44%), followed by *Trichophyton rubrum* 13(26%), *Microsporum canis* 6(12%), *Epidermatophyton floccosum* 4(8%), and *Microsporum ferrugineum* 1(2%).

While the identification of dermatophytes isolates from the cases of tinea cruris revealed that *Trichophyton mentagrophyte* was also the predominant species 13(37.1%), followed by *Epidermatophyton floccosum* 7(20%), *Trichophyton rubrum* 5(14.3%), *Trichophyton violaceum* 1(2.9%), and *Trichophyton verrucosum* 2(2.9%). Table (2).

Fig -1- *Culture of Microsporum canis on rice grain medium used to distinguish it from Microsporum audouinii*

Table-2- species of dermatophytes isolated from 85 cases (tinea corporis and tinea cruris).

Diagnosis	Tinea corporis		Tinea cruris	
	No	%	No	%
E. floccosum	4	8.0	7	20.0
M. canis	6	12.0	-	-
T. mentagrophyte	22	44.0	13	37.1
T. rubrum	13	26.0	5	14.3
M. ferrugineum	1	2.0	-	-
T. verrucosum	-	-	1	2.9
T. violaceum	-	-	1	2.9
Negative	4	8.0	8	22.9

According to Falahati *et al.*, [19], tinea corporis was the main infection of dermatophytosis (31.4%) followed by tinea cruris (20.7%). This finding coincides with our study. Those results revealed that Trichophyton species were responsible for maximum cases of dermatophytes (62%). In Iraq Abass (1995) [20], found that (61%) of human dermatophyte cases were infected with Trichophyton species. Also Sudad and Mohammed mentioned that (70%) of human dermatophyte cases in the year 2011 were infected with the Trichophyton species [21].

Antifungal susceptibility testing of isolates by using E-test:

MICs of antifungal agents for 85 dermatophyte isolate could be determined by E-test after 72 hour when incubated at 28˚C. The determination of the isolates as susceptible or resistant is complex and not yet been established for dermatophytes by this test, There is a scarcity of reports using E-test for filamentous fungi [7], [8]. There are nearly no reference methods to test antifungal drugs' susceptibilities for dermatophytes. The E-test used in this study required specific medium and practicing to determine the end point of inhibition zone; sometimes there was trailing end point of some azoles, particularly fluconazole; exhibit a phenomenon known as trailing. Trailing occurs when the turbidity continually decreases as the drug concentration increases but the suspension fails to become optically clear (partial inhibition of growth over an extended range of antifungal concentrations).

For most isolates, the difference between readings at 24 hours versus 48 hours is minimal and will not alter the interpretative category, i.e. does not change whether the isolate would be read as susceptible or resistant. In our work, the evaluation of *in vitro* susceptibility by using E-test showed that the antifungal drugs tested, with exception of amphotericin B, displayed good activity against dermatophytes. It is worth mentioning that voriconazole and itraconazole had the lowest MIC values and similar results have been verified by other authors that showed that these drugs had low MICs against dermatophytes [21], [22]. These low MICs found for these two drugs can help to explain the promising results obtained for the treatment of dermatophytosis with these antifungal agents.

Although amphotericin B has showed the highest MIC values of all the antifungal agents tested, Voriconazole was the most active drug in our study, with significant result, Mean±SD for tinea corporis and tinea cruris 0.026, 0.046 ($p < 0.05$) respectively,as shown in Table (3). These data are in agreement with researchers who reported higher activity of voriconazole in comparison to the others azole derivates [23]. This drug has been tested against dermatophytes by Serrano-Martino et al. by using the microdilution method CLSI M38-A. This high-susceptibility profile of dermatophytes to voriconazole could be promising in the treatment of dermatophytosis. *T. rubrum* showed the lowest susceptibility to this drug for both tinea corporis and tinea cruris, Figure (2), (3) comparing to *T. mentagrophytes*, Figure (4).

Fluconazole showed higher MICs concentrations from other azole derivates, our results are similar to studies reported by Maria et al., and; Zafer et al.,[24], [25], showing significant results Mean±SD for tinea corporis but not for tinea cruris, 0.0001, 0.438 ($p < 0.05$) respectively, Table (3). Fluconazole, exhibit a trailing phenomenon as we mentioned above, but this phenomenon did not affect the MICs reading. *T. rubrum* also showed a lowest susceptibility for this drug followed by *Microsporum canis* and *Epidermatophyton floccosum*. *Microsporum ferrugineum* and *T. violaceum* also showed low susceptibility for this drug.

***Table 3*: MIC values of different antifungals for different species of dermatophytes.**

Drug	Species	Tinea corporis	Tinea cruris
		Mean±SD	**Mean±SD**
Fluconazole	E floccosum	17.3±8.9	19.9±21.1
	M canis	32.7±30.2	-
	T mentagrophyte	6.7±10.6	13.1±18.3
	T rubrum	52.9±38.2	34.4±40.0
	M ferrugineum	0±	-
	T verrucosum	-	0±
	T violaceum	-	0±
	P value	**0.0001***	**0.438**
Itraconazole	E floccosum	2.3±2.0	3.9±3.0
	M canis	2.7±2.7	-
	T mentagrophyte	3.3±4.4	4.8±3.1
	T rubrum	9.7±6.5	11.6±8.3
	M ferrugineum	5.0±	-
	T verrucosum	-	0±
	T violaceum	-	0±
	P value	**0.005***	**0.027***
Voriconazole	E floccosum	0.5±0.3	0.9±0.7
	M canis	1.0±0.9	-
	T mentagrophyte	1.3±2.9	1.4±0.9
	T rubrum	5.1±4.9	6.9±7.7
	M ferrugineum	0.9±	-
	T verrucosum	-	0.9±
	T violaceum	-	2.5±
	P value	**0.026***	**0.046***
Amphotericin B	E floccosum	11.5±8.4	6.1±5.1
	M canis	5.0±5.5	-
	T mentagrophyte	11.9±5.7	11.6±6.8
	T rubrum	10.4±6.1	5.4±8.7
	M ferrugineum	7.0±	-
	T verrucosum	-	0±
	T violaceum	-	7.0±
	P value	**0.170**	**0.215**

*Significant using ANOVA test for difference among means at $P < 0.05$ level

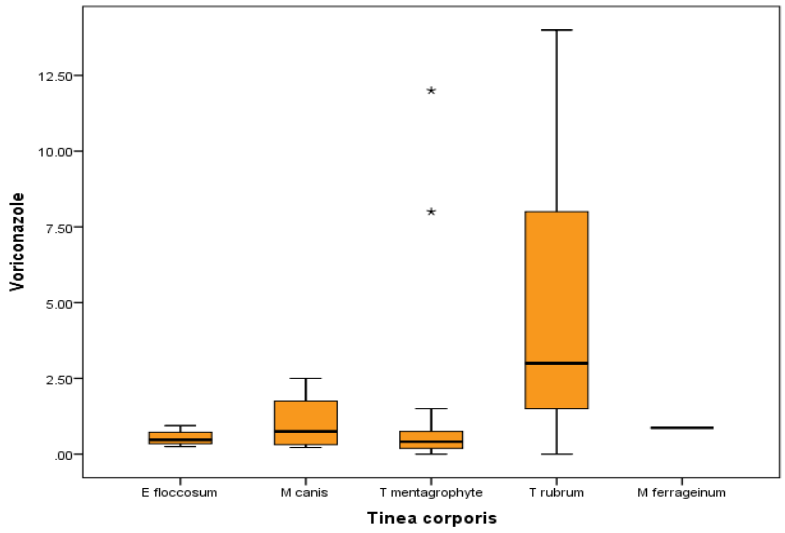

Fig -2- Distribution of mean for voriconazole MICs to dermatophytes species causing tinea corporis.

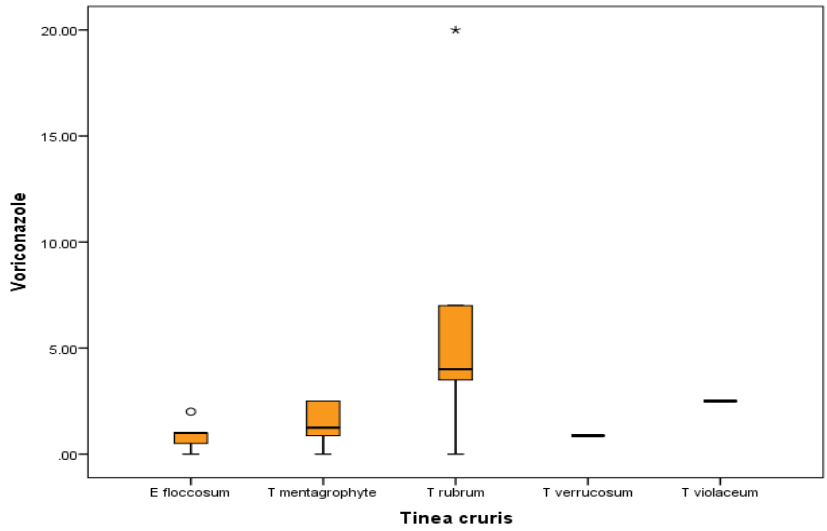

Fig -3- *Distribution of mean for voriconazole MICs to dermatophytes species causing tinea cruris.*

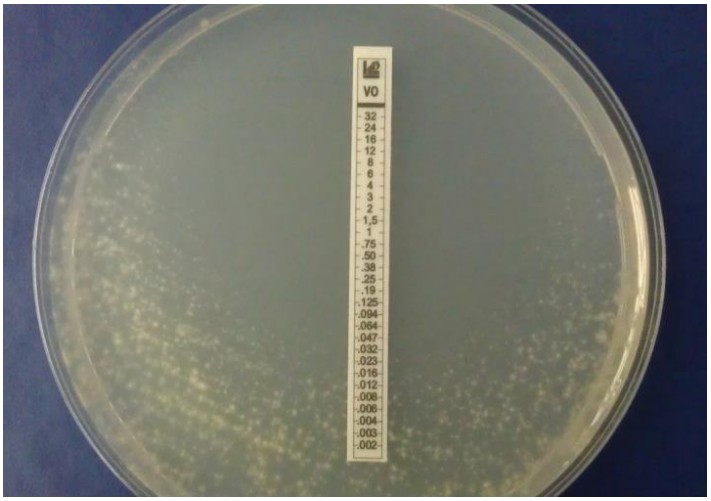

Fig -4- *E-test for T. mentagrophytes with voriconazole showed low MICs concentration (0.016 µg/ml).*

Discussion:

Voriconazole and itraconazole had the lowest MIC values, itraconazole had significant Mean±SD for tinea corporis and tinea cruris 0.005, 0.027 ($p < 0.05$) respectively. Table (3). This data is in agreement with researchers who reported high activity of this drug for dermatophytes [26]. Espinel [7] evaluated the *in vitro* activities of itraconazole and amphotericin B by E-test and broth microdilution method against 186 isolates of molds, and reported higher agreement between the itraconazole MICs obtained by both methods. In our study amphotericin B had shown the highest MIC values of all the antifungal agents tested, and did not display promising activity against dermatophytes. Amphotericin B has insignificant Mean±SD for tinea corporis and tinea cruris 0.170, 0.215 ($p < 0.05$) respectively, Table (3). Our data suggests a possible correlation between E-test and other methods, since MIC values for fluconazole were higher than MICs for all others azole derivates in most studies [27], [8], [9]. In general, our data are in agreement with the study of Maria [28] by using E-test to determine antifungal susceptibility and found that voriconazole was the most and fluconazole was the less-active drug from azole derivative drugs. Our results are in agreement with susceptibility data observed by researchers that used other methodologies.

Conclusions

1. *Trichopyton mentagrophytes* was the predominant species.
2. *T. violaceum* and *T. verrucosum* caused tinea cruris; this was not reported before as the causative agent of tinea cruris. *M. ferrugineum* was indicated as a causative agent of tinea corporis , which was also not reported before.
3. E-test seems to be a reliable methodology for susceptibility- testing for dermatophytes as well as for yeasts.
4. Specific test conditions for the E-test, including the appropriate medium for the test, are required to correctly assay the susceptibility of dermatophytes to antifungal drugs.
5. Amphotericin B has showed the highest MIC values of all the antifungal agents tested
6. Voriconazole was the most and Fluconazole was the less-active drug from azole derivative drugs.

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