

## EVALUATION OF THE MICRODILUTION METHOD FOR DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF TERBINAFINE AGAINST *TRICHOPHYTON RUBRUM*

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### Abstract

*Trichophyton rubrum* is the leading cause of dermatophytosis with a global prevalence of 50–90%. *Terbinafine* is a first-line therapy that works by inhibiting the squalene epoxidase enzyme in ergosterol biosynthesis. Accurate determination of the minimum inhibitory concentration (MIC) using a standardized susceptibility test method is necessary to ensure the effectiveness of therapy. This study aims to standardize the concentration of *terbinafine* using the microdilution method based on CLSI M38-A2 guidelines against *Trichophyton rubrum* ATCC MYA-4438. The test was carried out using an inoculum concentration of  $1 \times 10^3$ – $3 \times 10^3$  CFU/mL with varying *terbinafine* concentrations, then incubated at 28–30°C for 3–4 days. The results showed MIC values of 0.125 µg/mL; 0.25 µg/mL; and 0.125 µg/mL, respectively. An essential agreement (EA) value of 100% indicates high method precision. Morphological analysis revealed changes in hyphal structure in the form of fragmentation, a decrease in the number of conidia, cytoplasmic clumping, and uneven cell walls due to squalene accumulation. Thus, the microdilution method proved valid and reproducible for determining the MIC of *terbinafine* against *T. rubrum*.

**Keywords:** *Trichophyton rubrum*, *terbinafine*, microdilution, MIC, antifungal susceptibility testing

### INTRODUCTION

Dermatophytosis is a superficial infection caused by dermatophytes, the most common pathogenic filamentous fungi with an infection rate of 20-25% worldwide. Dermatophyte fungi can infect nails, skin, and hair, causing various infections such as *tinea capitis*, onychomycosis, *tinea corporis*, and *tinea pedis*. *Trichophyton rubrum* is one of the causative species of dermatophytosis and is responsible for 50-90% of dermatophytosis worldwide [1]. As a keratinophilic fungus, symptoms are characterized by the presence of papulonodular lesions, the presence of fungal folliculitis (*granuloma majocchi*), and require oral therapy [2]. Transmission can occur from person to person and animal to animal, rarely from soil to person. Most people do not show clinical symptoms, but patients with dermatophytosis may experience impaired T-cell responses due to changes in local defenses (such as trauma with vascular disorders) or from primary (hereditary) or secondary (such as diabetes, HIV) immunosuppression [3].

Based on the source of infection, dermatophytes are classified into anthropophilic, zoophilic, and geophilic. *T. rubrum* is included in the anthropophilic group which is mainly transmitted between humans, either through direct contact or indirectly through contaminated objects. Infection by *T. rubrum* is more often found in tropical and subtropical areas with high humidity, including Indonesia. In addition, this infection tends to be chronic and persistent if not treated adequately, thus reducing the patient's quality of life and increasing the risk of transmission [4]. *T. rubrum* infection causes mild to moderate dermatological symptoms, with varying degrees of severity of infection. The response is triggered by keratinocytes as the first line of defense against *T. rubrum*. Several *Toll-like receptors*, such as TLR2, TLR4, TLR6, and Human Beta Defensin (HBD)-1, HBD-2, IL-1B, and IL-8 will be expressed as the initial host defense. A unique characteristic of *T. rubrum* is its ability to trigger both immediate hypersensitivity (IH) and delayed type hypersensitivity (DTH). IH will give a response in the form of local swelling and redness, while DTH will give a response in the form of induration. This reaction depends on the patient's immunity due to the different enzyme profiles produced by *T. rubrum* such as lipase, alkaline phosphatase, esterase, etc. [5].

*Terbinafine* is a commonly used antifungal drug in Indonesia and is the gold standard for treating superficial fungal infections. *Terbinafine* works by disrupting the early stages of ergosterol biosynthesis by inhibiting the enzyme squalene epoxidase (SQLE) and preventing squalene oxidation. This results in the accumulation of toxic squalene and the depletion of ergosterol in the fungal membrane, which disrupts membrane integrity and function, ultimately leading to fungal cell death. However, when using the antifungal drug *terbinafine*, antifungal susceptibility testing must be performed to prevent antifungal resistance in dermatophytosis patients. Antifungal resistance will lead to higher infection rates and contribute to the spread of fungal infections globally. Global prevalence indicates a 20-25% chronic nature of dermatophytosis, causing treatment failure and antifungal drug resistance. Based on research conducted by Connie F. Cannete-Gibas et al (2023) in 2021-2023, the prevalence of antifungal resistance reached 18.6%. *Terbinafine* resistance can occur due to point mutations in the squalene epoxidase gene, amino acid substitutions, and overexpression of the *T. rubrum* gene which makes *terbinafine* ineffective as a therapy [6-9].

*Terbinafine* was first discovered in 1983, belonging to the allylamine group. *Terbinafine* can be absorbed 70%-80% orally and shows linear absorption up to the ideal dose (250 mg), total body exposure is directly proportional to the dose. Meanwhile, the absorption of *terbinafine* topical cream and gel formulations is in the range of 746-949 ng/cm. Topical treatment increases in the stratum corneum by 15% and AUC increases by up to 40% in less than 1 week [10]. To ensure that *terbinafine* can be used as an effective therapy for dermatophytosis patients and prevent the occurrence of resistance, antifungal susceptibility testing is carried out using the broth microdilution method based on the *Clinical and Laboratory Standards Institute* (CLSI) M38-A2 guidelines. This method is widely used because it has advantages, including a relatively simple procedure, does not require special equipment, and produces quantitative data in the form of minimum inhibitory concentration (MIC) values. The MIC value provides an indication of the level of effectiveness of the antifungal in inhibiting the growth of test microorganisms *in vitro*. Furthermore, this method is also known to have a good level of reproducibility when carried out according to standards [11]. Variations in test procedures, such as inoculum concentration, incubation conditions, and antifungal concentration range, can affect the results obtained. Therefore, evaluation of the microdilution method in determining the MIC of *terbinafine* on *Trichophyton rubrum* is important to ensure the accuracy, precision, and consistency of test results. Based on this, this study aims to evaluate the performance of the microdilution method based on CLSI M38-A2 guidelines in determining the MIC value of *terbinafine* against *Trichophyton rubrum* ATCC MYA-4438.

## METHOD

### a) Preparation of antifungal solution

*Terbinafine* (pure powder) was accurately weighed and dissolved in DMSO ( *dimethyl sulfoxide* ) to obtain a stock solution with a concentration of 6400 µg/mL. This stock solution was then used as the *intermediate concentration* (IC).

The IC solution was then diluted using RPMI-1640 medium (with MOPS buffer, pH 7.0) in *two-fold serial dilutions* to obtain a final concentration range of 128 µg/mL - 0.0019 µg/mL. All solutions were prepared aseptically and used immediately or stored at 2–8°C in a light-protected condition until use.

### b) *T. rubrum* inoculum suspension

*Trichophyton rubrum* ATCC MYA-4438 isolate was grown on *Sabouraud Dextrose Agar* (SDA) medium and incubated at 28–30°C for 4–5 days until *mature colonies formed*. A total of 3–5 mL of sterile 0.85% NaCl solution was added to the surface of the colony, then the colony was gently scratched using a sterile loop to release the conidia. The resulting suspension was left to settle for 10–20 minutes.

The supernatant containing conidia was then collected and counted using a hemocytometer to obtain an inoculum concentration of  $1 \times 10^3$  -  $3 \times 10^3$  CFU/mL. This inoculum suspension was used immediately in the assay.

### c) Minimum inhibitory concentration (MIC) test

The MIC test was performed using the microdilution method in a 96-well U-shaped *microdilution plate* according to CLSI M38-A2 guidelines. A total of 100 µL of *terbinafine solutions* with various concentrations was added to each well, then 100 µL of the inoculum suspension was added to obtain a 1:1 ratio. The controls were prepared as follows:

- 1) *Growth control* (GC): contains medium and inoculum without antifungal
- 2) *Sterility control* (SC): contains medium without inoculum and without antifungal.

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The plates were then incubated at 28–30°C for 3–4 days. The results were read visually by observing the turbidity in each well. The MIC value was determined as the lowest concentration of terbinafine that indicated no fungal growth (wells appeared clear).

## d) Microscopic Test

Microscopic tests were performed to evaluate the morphological changes of *T. rubrum* after terbinafine exposure. The suspension from the MIC test wells was re-inoculated into SDA medium and incubated at 28–30°C for 3–4 days. The growing colonies were then observed microscopically using lactophenol cotton blue reagent. Parameters observed included hyphal structure, number and shape of conidia, and other morphological changes compared to the untreated control.

## RESULTS AND DISCUSSION

The test results were carried out in triplicate to ensure the consistency and accuracy of the method. Based on the results of visual observation of turbidity on the microdilution plate, the MIC values obtained in three consecutive replications were 0.125 µg/mL; 0.25 µg/mL; and 0.125 µg/mL. These values indicate that terbinafine has effective inhibitory activity against the growth of *T. rubrum* at low concentrations (Table 1).

Table 1. Results of Essential Agreement (EA) on KHM value

Antifungal	Treatment Isolate	Reference Method (MIC µg/mL)	Standardization of the MIC Method (MIC µg/mL)	Dilution Difference	EA Status
<i>Terbinafine</i>	I	0.25-0.5	0.125	-1 dilution	Agree
	II	0.25-0.5	0.25	0 (Exact)	Agree
	III	0.25-0.5	0.125	-1 dilution	Agree

From these results, the EA status was obtained as “agree,” with an exact match and a difference of one dilution lower (-1 dilution). From these results, the EA calculation was performed (Table 2), the results showing 100% reproducibility.

Table 2. Results of EA percentage calculations

Antifungal	Total Test Isolates	Total Isolates Included in ±1 dilution	EA Results
<i>Terbinafine</i>	3	3	100%

Essential agreement (EA) is a comparison of the percentage of isolates between MIC values with antifungal susceptibility test methods that are within the range of ±1 or ±2 Log<sub>2</sub> dilutions of the value obtained by the reference method. EA calculations are performed using CLSI M52, from the calculation, results are accepted if they fall within the range of ±1 Log<sub>2</sub> dilutions<sup>[13,14]</sup>. When the test MIC value is compared with the CLSI M38-A2 reference value range, the MIC results obtained are still within the acceptable range. In addition, the results of the essential agreement (EA) calculation show a value of 100%, which indicates that the method used has a level of suitability and accuracy between replications (Table 2). The essential agreement (EA) results in this study showed a value of 100%. EA is the conformity of the MIC test results that are in one double dilution stage (two-fold serial dilution). The reproducibility of the method is stated as good if the EA value is > 90%<sup>[14]</sup>. By obtaining an EA of 100%, it can be concluded that the microdilution method used has a very high level of precision and stable consistency of results between replications. This indicates that the work procedures, instruments, and analysis used have run well, so this method is suitable for use as a standard antifungal susceptibility test for *Trichophyton rubrum*. The MIC results of terbinafine in this study showed low values indicating strong antifungal activity against *T. rubrum*. This is related to the mechanism of action of terbinafine as an inhibitor of SQLE, a key enzyme in ergosterol biosynthesis in dermatophyte fungi. Inhibition of SQLE causes disruption of the conversion of squalene to lanosterol, resulting in the accumulation of toxic squalene and a decrease in ergosterol in the cell membrane. This condition causes membrane disorganization,

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increased cell permeability, and ultimately fungal cell death [15]. *Terbinafine* is effective for treating dermatophytosis due to the substitution of *tert-butyl acetylene* on the phenyl ring in the side chain of the molecule. *Terbinafine* has minimal drug interactions and small concentrations can inhibit 95% of squalene epoxidase activity [16]. In a study conducted by Subuhi Kaul et al (2017), *terbinafine* is superior to several other antifungal drugs. In systemic therapy, *terbinafine* is a commonly used drug with a cure rate of up to 87%. In elderly patients undergoing dual drug therapy for various comorbidities, oral *terbinafine* can be used to avoid long-term rhabdomyolysis. In children, *terbinafine* has demonstrated efficacy and safety, without clinically relevant liver function impairment [17]. The MIC test results were followed up with microscopic examination to observe changes in the morphology of *T. rubrum*. Microscopic observations showed significant morphological changes in *T. rubrum* after *terbinafine* exposure. The observed changes included hyphal fragmentation, a decrease in the number of conidia, cytoplasmic clumping, and changes in the cell wall structure, which appeared uneven and denser than the control (Figure 1).

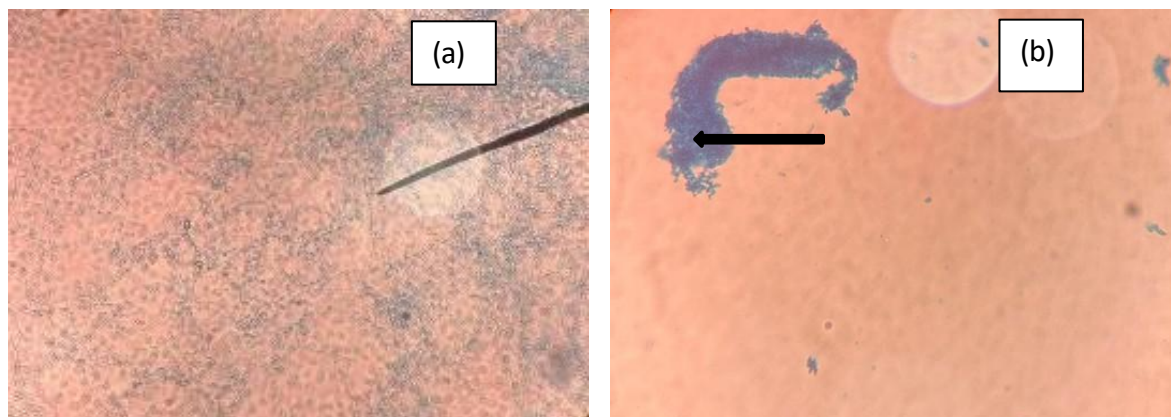


Figure 1. Morphological changes of *T. rubrum* before and after exposure to the antifungal *terbinafine* (40x objective).

- T. rubrum* before exposure to *terbinafine*,
- T. rubrum* after exposure to *terbinafine*. Clumping of *T. rubrum* hyphae and conidia and a decrease in the number of conidia

Pharmacologically, *terbinafine* (*N*,6,6-trimethyl-*N*-(*naphthalen-1-ylmethyl*)hept-2-en-4-yn-1-amine) has fungicidal properties that work specifically against SQLE in dermatophytes. This mechanism explains why *terbinafine* showed a low MIC value in this study. This result is in line with the study of Ghannoum et al. (2004) who reported that most *T. rubrum* isolates had *terbinafine* MIC values  $\leq 0.25$   $\mu\text{g/mL}$ , indicating high sensitivity to this drug. In addition, Mukherjee et al. (2003) also reported that *terbinafine* remained effective against *T. rubrum*, although some isolates began to show an increase in MIC indicating the potential for early resistance [18,19]. Before exposure to *terbinafine* at various concentrations, *T. rubrum* microscopically had hyaline, septate hyphae, smooth surfaces, numerous branches, and microconidia attached to the hyphal surface. Microconidia were small and abundant along the hyphae, visible under a light microscope [20]. Microscopic examination results showed changes in fungal morphology, with the higher concentration of *terbinafine* antifungal causing hyphal growth to become smaller and undergo changes in shape, such as tortuous, and an increase in irregular branching at the hyphal tips. Based on research (Figure 1b), after exposure to *terbinafine* antifungal, cytoplasmic coagulation occurred or the cell contents appeared to be clumped and uneven (granulation). Hyphae were fragmented into small pieces and the number of conidia decreased. Cytoplasmic coagulation or cytoplasmic granulation is a characteristic feature of cellular damage caused by *terbinafine* exposure in dermatophyte fungi (Figure 1). This phenomenon was also reported by Osborne et al. (2006), which states that exposure to *terbinafine* in dermatophytes causes cytoplasmic disorganization, cell membrane damage, and progressive hyphal fragmentation. This strengthens the effect of *terbinafine*. not only seen in growth inhibition (MIC/MIC), but also in structural changes Microscopically, fungal cells [21]. Clear or smooth cytoplasm will appear mottled or rough, the hyphae will appear dark or denser, and are often accompanied by cell walls that appear uneven or cracked due to the accumulation of squalene which is toxic to the cell membrane [18]. Apart from the concentration aspect, the effectiveness of *terbinafine* is also influenced by character. its pharmacodynamics are *time-dependent*. Optimal activity is achieved when drug concentration is above the MIC for a certain duration (%T > MIC). In this condition, intracellular squalene accumulation increases with incubation time, so that the fungicidal effect becomes more pronounced. This parameter was also reported by Ryder (1992), who stated that the effect of *terbinafine* is greatly influenced by the duration of exposure due to the accumulation of the substrate toxicity occurs gradually before reaching fungal cell death [22]. The microdilution method based on CLSI M38-A2 in this study was proven to be able to produce accurate, consistent, and high-precision

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quantitative data. The advantages of this method are also supported by Pfaller and Diekema (2012), who stated that microdilution is the gold standard method for antifungal susceptibility testing because it has good reproducibility and can be used for filamentous fungi such as dermatophytes<sup>[23]</sup>. However, there are several limitations that need to be considered. The use of the standard isolate *T. rubrum* ATCC MYA-4438 provides controlled conditions, but does not fully reflect the sensitivity variations of clinical isolates in the field. Furthermore, this study focused on *in vitro* evaluation, so it cannot directly represent clinical responses in patients. The visual MIC reading method also has the potential for interpretation variation despite following standard CLSI guidelines. The CLSI M38-A2 method develops a reproducible procedure for antifungal susceptibility testing of filamentous fungi using the broth microdilution method. However, the MIC values provided are well standardized and can be used for the treatment of dermatophytosis patients<sup>[12]</sup>. Furthermore, this study did not include a comparison with other antifungals as a comparative control, nor did it thoroughly evaluate the pharmacodynamic relationship, such as %T > MIC, to clinical outcomes. Therefore, further research with a broader range of isolates and a comparative approach is expected to provide a more comprehensive picture of *T. rubrum* sensitivity to *terbinafine*. Overall, the results of this study indicate that the microdilution method is a valid, precise, and recommendable method for evaluating the sensitivity of *T. rubrum* to *terbinafine*, and supports the use of *terbinafine* as the primary therapy for dermatophytosis in a rational and evidence-based manner.

## CONCLUSION

The microdilution method based on CLSI M38-A2 guidelines has been proven to have high precision and reproducibility in determining the minimum inhibitory concentration (MIC) value of *terbinafine* against *Trichophyton rubrum* ATCC MYA-4438, as indicated by an essential agreement (EA) value of 100%. The MIC value obtained was in the range of 0.125–0.25 µg/mL, indicating strong antifungal activity of *terbinafine* against *T. rubrum*. In addition, microscopic tests showed morphological changes in the form of hyphal fragmentation, a decrease in the number of conidia, and damage to the fungal cell structure due to squalene accumulation.

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