Case-controlled Study

Evaluation of fluconazole, itraconazole, and voriconazole activity on *Candida albicans*: A case control study

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A R T I C L E   I N F O

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- Candida albicans
- Infectious disease
- Time-kill curve

A B S T R A C T

Background: Azole antifungals are the most commonly used antifungals. The high use of azoles for long-term therapy and prophylaxis is prone to cause resistance. Thus, it is necessary to evaluate the antifungal activity against Candida albicans.

Objectives: Analyzing the comparison of antifungal exposure on the time-kill curve to Candida albicans.

Method: A case-control study was conducted with a posttest control group design. This study used Candida albicans clinical and ATCC isolates exposed to antifungal solutions with 1 ×, 4 ×, and 16 × minimum inhibitory concentrations (MIC). Antibiotics used included fluconazole, itraconazole, and voriconazole. Candida albicans isolates were incubated with MIC, and the number of colonies was counted at 0, 2, 4, 8, 12, 24, and 48 h. The number of colonies that grew every hour of observation was included in the time-kill curve. The data were then analyzed using an ANOVA test with $p < 0.05$.

Results: The antifungals (fluconazole, itraconazole, and voriconazole) showed fungistatic activity against Candida albicans clinical and ATCC isolates. There was a significant comparison between the antifungal group and the control group at 12, 24, and 48 h. The most significant difference between antifungal and control group was found at 24 h where fluconazole had 95% CI = 0.807–2.061 ($p < 0.001$), itraconazole 95% CI = 0.722–1.976 ($p < 0.001$), and voriconazole 95% CI = 0.807–2.062 ($p < 0.001$).

Conclusion: Fluconazole, itraconazole, and voriconazole were effective in inhibiting the growth of Candida albicans. Maximum inhibition in vitro occurs after 12 h of antifungal exposure.

1. Introduction

Pathogenic fungi can cause invasive infections, chronic disease and recurrent superficial infections. Invasive infections caused by *Candida* species are often associated with higher mortality rates and extended hospital stays. *Candida* spp. are generally normal skin, mucosa, and gastrointestinal tract flora and are the most common cause of fungal infections. In immunocompromised patients, the fungus is capable of causing disease in multiple sites, whereas in immunocompromised individuals, the infection is localized. There are at least 15 *Candida* species that can cause human disease, but the following five pathogens cause more than 90% of invasive infections: *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis,* and *Candida krusei* \cite{1–5}.

Based on the SENTRY antifungal surveillance program from 1997 to 2016, of the 20,788 isolates admitted, 46.9% were *Candida albicans*, 18.7% *Candida glabrata*, 15.9% *Candida parapsilosis*, 9.3% *Candida tropicalis*, 2.8% *Candida krusei*, and 6.5% *Candida* spp., \cite{6,7}. A study in Asia found that *Candida albicans* caused the highest incidence of candidemia with a total of 41.36%, followed by *Candida tropicalis* at 25.45%, *Candida glabrata* at 13.93%, and *Candida parapsilosis* at 12.15%. Resistance to fluconazole was found in *Candida albicans* (10–13%), *Candida tropicalis* (5–19%), and *Candida glabrata* (36%) \cite{6–8}.

The widely used antifungals are currently limited to three classes: azoles, echinocandins, and polyenes. The azole antifungals, especially fluconazole, are the most frequently used because of their high effectiveness, low toxicity, and immunomodulatory capacity and they can be used orally. This excess causes high use of azoles for long-term therapy and prophylaxis in high-risk patients, leading to resistance to azoles and less sensitive strains. In addition, using antifungals for agricultural activities creates a reservoir of drug-resistant pathogens in the...
Long-term therapy and repeated treatment with fluconazole can lead to resistance. Resistance to azole antifungals continues to grow, becoming more widespread in patients with severe clinical conditions. After the sensitivity test results indicate that the isolate is sensitive to fluconazole, the therapy could be changed to fluconazole. Systemic Candida infection may be suspected in patients with neutropenia who remain febrile after broad-spectrum antibiotic therapy. The Infectious Disease Society of America (IDSA) recommends the antifungal caspofungin, amphotericin B, or intravenous voriconazole as empiric therapy. Alternatively, fluconazole or itraconazole may be used [1,11]. Fluconazole has a broad spectrum, high efficiency, and good bioavailability and safety profile, so it is recommended as the first choice for treating Candida spp infections. Fluconazole and voriconazole have bioavailability ranging from 90%, while itraconazole has the lowest bioavailability. Fluconazole is the only azole antifungal eliminated via the kidney, and other azole antifungals are eliminated via the liver. Based on their ability to penetrate the CSF, the order of the best is fluconazole, itraconazole, and voriconazole. In patients with resistance to fluconazole, voriconazole, and itraconazole, fluconazole can be used as alternative therapy [12]. Fluconazole is good for treating infections caused by Candida spp., while itraconazole and voriconazole have antifungal activity against Candida spp. and Aspergillus spp. [13].

Long-term therapy and repeated treatment with fluconazole can lead to resistance. Resistance to azole antifungals continues to grow, becoming an essential problem in treating Candida spp infections. Several molecular resistance mechanisms have been identified, including changes in the gene encoding the ERG11 target enzyme or overexpression of efflux pump genes (CDR1, CDR2, and MDR1). Cross-resistance was observed with the antifungal (fluconazole, clotrimazole, itraconazole, and ketoconazole) [4,11,14].

Because of their availability, fluconazole and itraconazole are first-generation triazoles used in Asia. At the same time, voriconazole is the second-generation triazole which was first marketed with a broad-spectrum activity against fungal pathogens. With the increasing resistance to fluconazole, it is necessary to evaluate the antifungal activity of azoles against Candida albicans [15]. One of the evaluations of antifungal activity can be conducted with a Time-kill assay (TKA) which will provide information on the speed and antifungal activity, as well as pharmacodynamic characteristics (i.e. the relationship between drug concentration and its effect on fungal colony growth). These data can determine the dynamic relationship between antimicrobial agents and their effects on microbes. The TKA method counts the number of fungal isolates that grow at certain intervals after exposure to antifungals with specific concentrations. This method is based on the broth dilution technique [4]. This study aimed to analyze fluconazole, itraconazole, and voriconazole on the growth of Candida albicans with an in vitro study.

2. Methods

This study used a case-control study with a posttest control group design. This study reported the data based on strengthening the reporting of cohort studies in surgery (STROCSS) 2021 guidelines [16]. Subjects in this study consisted of Candida albicans clinical and American-type culture collection (ATTC) 14053. This study was to test the sensitivity of antifungal drugs to Candida albicans. The antifungals used were fluconazole (generic manufacturer), itraconazole (generic manufacturer), and voriconazole (generic manufacturer). This study was conducted from June 2021–May 2022. Each antifungal was exposed to 1 minimum inhibitory concentration (MIC), 4 MIC, and 16 MIC, where 1 MIC dose of fluconazole was 0.5 p/mL, itraconazole was 0.05 p/mL, and voriconazole 0.12 p/mL. While at 4 MIC = 4 × 1 MIC and at 16 MIC = 16 × 1 MIC.

The procedure in this study included: each Candida albicans isolate was given an antifungal exposure according to the MIC dose and added 1 more isolate that was not exposed to the MIC dose (control group). Subjects were also assessed for time-kill curves at the 0, 2nd, 4th, 8th, 12th, 24th, and 48th hours. Exposure to Candida albicans isolates with antifungals was repeated 6 times, and the results used were the average of these 6 results. Before the research, the isolate was confirmed to be not bacterial or sterile, and Candida albicans isolate indicated sensitivity to antifungal manually.

Measurement data were collected and analyzed using statistical product and service solution (SPSS) software version 21.0 (IBM Corp., Armonk, NY, USA). In addition, the analysis was assisted by GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA). The statistical analysis used in this study was the ANOVA test, in which the analysis results were declared significant if \( p < 0.05 \).

3. Results

3.1. Fluconazole

The time-kill curve of fluconazole against clinical isolates of Candida albicans showed that concentrations of 1, 4, and 16 MIC could inhibit or even reduce colony growth in the first 8 h. In the next hour, a gradual increase in growth was observed until the 48th hour. The lowest decrease was found at 16 MIC at 8 h with a colony decrease of 3.64 log10 CFU/mL. Meanwhile, Candida albicans ATTC 14053 had conditions similar to clinical Candida albicans and the lowest decrease was found at 16 MIC at 8 h (3.64 log10 CFU/mL; Fig. 1).

3.2. Itraconazole

The time-kill curve of itraconazole against clinical isolates of Candida albicans showed growth inhibition by a concentration of 1 MIC at 4–8 h, while concentrations of 4 and 16 MIC could reduce the number of colonies by 3.64 log10 CFU/mL.

![Fig. 1. Time-kill curve of fluconazole on Candida albicans clinical and ATCC 14053.](image-url)
colonies at that hour. In the next hour, there was an increase in colony growth; there was minimal growth for a concentration of 16 MIC since the 24th hour (Fig. 2). Itraconazole decreased *Candida albicans* colony isolates at 4 MICs (3.86 log 10 CFU/mL) and 16 MICs (3.65 log 10 CFU/mL) at 8 h. Meanwhile, *Candida albicans* ATCC 14053 showed similar conditions (Fig. 2).

### 3.3. Voriconazole

The time-kill curve of voriconazole against *Candida albicans* showed inhibition of colony growth by concentrations of 1, 4, and 16 MIC at 4–8 h; the next hour, there was an increase in the number of colonies. A similar condition occurred with *Candida albicans* ATCC 14053 (Fig. 3).

### 3.4. Comparison of fluconazole, itraconazole, and voriconazole on *Candida albicans* isolates

Data on the difference in the number of colonies between control isolates and research isolates had different antifungal concentrations at each hour of observation. Maximum growth inhibition at each concentration was shown in bold. Most antifungals at a concentration of 1 MIC reached maximum inhibition at 24 h in both clinical and ATCC isolates; except for voriconazole in clinical isolates, maximum inhibition was observed at 48 h. An increase in inhibitions does not always accompany the increase in observation time, but the more significant dose is accompanied by an increase in the number of inhibitions (Table 1).

There was a significant comparison between the antifungal group and the control group at 12, 24, and 48 h. Meanwhile, there was no significant difference between antifungals in decreasing the number of *Candida albicans* colonies. The most significant difference between antifungal and control groups was found at 24 h where fluconazole had 95% CI = 0.807–2.061 (p <0.001), itraconazole 95% CI = 0.722–1.976 (Table 2).

### 4. Discussion

This study’s results follow that of Burgess et al., who stated that fluconazole concentrations below the MIC had minimal effectiveness (slightly inhibited growth), while concentrations equivalent to MIC or more suppressed growth in the first 8 h. At 12 h, there was little growth, and at 48 h, the number of colonies increased in all concentration groups. In contrast to this study, at 12 h, the colony increase was relatively high [17]. Burgess et al., also tested fluconazole with *Candida albicans* isolates categorized as susceptible-dose dependent (SDD) and resistant. In isolates with the SDD category, the greater the concentration, the longer the inhibition time. In resistant isolates, all groups of fluconazole concentrations could not restrain the growth rate of isolates [18]. Lee and Lee’s study showed growth inhibition mainly at 4–8 h, followed by growth at 12 h and a flattened curve [19].

A study by Burgess et al., showed that itraconazole inhibited the growth of isolates in the first 12 h in almost all concentrations. In contrast to this study’s results, itraconazole inhibited the growth of isolates in the first 8 h [17,18]. In the study of Manavathu et al., the itraconazole time-kill curve showed an increasing pattern in the first 12 h and was followed by a decrease in the number of colonies at 24 h [20]. The results of this study are from those studies after the 8th hour. In the study of Li et al., using concentrations of 0.25 ×, 1 ×, 4 ×, and 16 × MIC, the time-kill curve showed an increase in the first 8 h, resembles the control curve. At concentrations of 1 ×, 4 ×, and 16 × MIC, the curve appeared to
Table 1

Differences in the decrease in the number of Candida albicans colonies after antifungal exposure.

| Antifungal       | MIC | Hours 2 | 4 | 8 | 12 | 24 | 48 |
|------------------|-----|---------|---|---|----|----|----|
| Candida albicans |     |         |   |   |    |    |    |
| Fluconazole 1    | 1   | -0.05   | -0.24 | -0.92 | -0.86 | -1.16 | -0.98 |
|                  | 4   | -0.03   | -0.21 | -1.28 | -1.09 | -1.39 | -1.28 |
|                  | 16  | -0.08   | -0.42 | -1.37 | -1.49 | -1.46 | -1.57 |
| Itraconazole 1   |     |         |   |   |    |    |    |
|                  | 4   | -0.02   | -0.24 | -1.15 | -1.14 | -1.27 | -0.97 |
|                  | 16  | -0.03   | -0.39 | -1.35 | -1.48 | -1.48 | -1.58 |
| Voriconazole 1   |     |         |   |   |    |    |    |
|                  | 4   | -0.05   | -0.22 | -1.16 | -1.79 | -1.70 | -1.43 |
|                  | 16  | -0.03   | -0.43 | -1.46 | -1.44 | -1.61 | -1.75 |
| Candida albicans ATCC 14053 |     |         |   |   |    |    |    |
| Fluconazole 1    | 1   | -0.04   | -0.16 | -0.48 | -0.51 | -1.14 | -1.11 |
|                  | 4   | -0.04   | -0.25 | -0.68 | -0.72 | -1.53 | -1.56 |
|                  | 16  | -0.04   | -0.28 | -0.92 | -1.00 | -1.92 | -2.18 |
| Itraconazole 1   |     |         |   |   |    |    |    |
|                  | 4   | -0.02   | -0.16 | -0.61 | -1.07 | -1.22 | -1.20 |
|                  | 16  | -0.05   | -0.48 | -0.98 | -1.25 | -1.84 | -1.82 |
| Voriconazole 1   |     |         |   |   |    |    |    |
|                  | 4   | -0.02   | -0.27 | -0.66 | -1.11 | -1.44 | -1.57 |
|                  | 16  | -0.05   | -0.48 | -1.02 | -1.34 | -1.94 | -1.89 |

Note: MIC = Minimum inhibitory concentration.

Table 2

Comparison of antifungal drug effectivities used for Candida albicans.

| Comparison          | 12 h      | 24 h        | 48 h       |
|---------------------|-----------|-------------|------------|
|                     | CI 95%    | P           | CI 95%     | P           | CI 95%     | P           |
| Control vs. fluconazole | 0.299-1.592 | 0.007*  | 0.807-2.061 | -0.001** | 0.709-2.183 | 0.001*     |
| Control vs. itraconazole | 0.356-1.650 | 0.005*  | 0.722-1.976 | -0.001** | 0.470-1.944 | 0.003*     |
| Control vs. voriconazole | 0.514-1.808 | 0.002*  | 0.807-2.062 | -0.001** | 0.715-2.189 | 0.001*     |
| Fluconazole vs. itraconazole | -0.399-0.515 | 0.793  | -0.359-0.829 | 0.690  | -0.282-0.760 | 0.345      |
| Fluconazole vs. voriconazole | -0.214-0.673 | 0.332  | -0.444-0.443 | 0.999  | -0.515-0.527 | 0.981      |
| Itraconazole vs. voriconazole | -0.399-0.616 | 0.474  | -0.358-0.529 | 0.689  | -0.276-0.766 | 0.334      |

Note: *Significant <0.05; **Significant <0.01.

decrease at 12 h and did not appear to increase until 72 h [21].

According to Pfaffer et al., an antifungal is said to have fungicidal activity if there is a decrease in the number of colonies by 99% or 3-log10 units in CFU/mL compared to the initial inoculum. Fungistic activity can be concluded if there is a decrease in the number of colonies by <99% or <3-log10 units in CFU/mL compared to the initial inoculum [22]. A study by Lee and Lee, which observed the growth of Candida albicans for 24 h using a time-kill curve, showed that fluconazole had fungistic activity at concentrations of 16 and 32 μg mL\(^{-1}\) and at concentrations of 64 and 128 μg mL\(^{-1}\) activity was observed fungicidal. Although this study did not reach the fungicidal level, voriconazole at concentrations of 4 × and 16 × MIC reduced the colony growth of clinical isolates and Candida albicans ATCC 14053 [19]. A study by Li et al., observing the activity of voriconazole against Candida albicans, Candida glabrata, and Candida parapsilosis, found that voriconazole in a concentration of 16 × MIC had fungicidal activity against Candida parapsilosis. For other isolates, voriconazole showed continued fungicidal activity for 72 h. The study also mentioned that the maximum inhibition of voriconazole at a concentration of 1 × MIC was -2.78 log, 4 × MIC -2.99 log, and 16 × MIC -4.15 log compared to control isolates [21]. The study by Burgess et al., calculated the reduction in colony growth of the control group from the fluconazole group. At 24 h, the total reduction of Candida albicans was 67% (1 × MIC). This result differed from this study, which found that the number of reductions at that hour was 18% [17, 18].

A study by Burgess et al., compared the area under the kill curve (AUKC) at 0–48 h with the MIC of each isolate (with different sensitivities), the antifungals used were amphotericin B, itraconazole, and fluconazole. The study found that the AUKC of amphotericin B was significantly lower than the other 2 antifungals in all isolates. While in fluconazole and itraconazole, there was no significant difference, but there was a prominent difference, such as the fluconazole curve for SDD isolates that resembled the curve for sensitive isolates. In contrast, the itraconazole curve for SDD isolates resembled the curve for resistant isolates [17,18].

The limitation of this study is that it only uses isolates sensitive to azole antifungals. Hence, it is not possible to know how the azole activity is on isolates that are categorized as SDD or resistant. Future studies hope that the azole antifungal group can be compared with other antifungal and fungal types, not only Candida albicans.

5. Conclusion

Fluconazole in all concentrations is fungistatic against clinical isolates of Candida albicans and isolates of ATCC 14053. Maximum inhibition occurs after isolates are exposed for 24 h. Itraconazole at all concentrations is fungistic against clinical isolates of Candida albicans and isolates of ATCC 14053. Maximum inhibition occurs after isolates are exposed for 12 h. Fluconazole, itraconazole, and voriconazole exposure do not significantly differ in the growth rate of clinical isolates of Candida albicans and ATCC 14053 in vitro.

Provenance and peer review

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Ethical approval

We have conducted an ethical approval base on the Declaration of Helsinki with registration research at the Health Research Ethics Committee in Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.

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Author contribution

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

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Declaration of competing interest

None.

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Agung Dwi Wahyu Widodo is the person in charge of the publication of our manuscript.

Consent

All participants are required to fill out an informed consent.

Registration of research studies

1. Name of the registry: Health Research Ethics Committee in Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.
2. Unique Identifying number or registration ID: 0341/KEPK/I/2022.
3. Hyperlink to your specific registration (must be publicly accessible and will be checked):-.

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Declarations of competing interest

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