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SHORT COMMUNICATION

First report of amphotericin B resistant *Candida haemulonii* isolated from the ICU of a referral hospital in Indonesia

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ABSTRACT

Background and Objectives: Amphotericin B is a broad-spectrum antifungal agent commonly used to treat *Candida haemulonii* infection. *C. haemulonii* was isolated from patients reported to be intrinsically resistant to amphotericin B, encoded by the ERG2 and ERG11 genes. However, there have been limited studies concerning amphotericin B-resistant *C. haemulonii* in Indonesia. The objective of this study is to explore the phenotypic and genotypic characteristics (ERG2 and ERG11) of *C. haemulonii* isolated from the ICU of a referral hospital in Indonesia.

Materials and Methods: Identification and susceptibility tests were conducted using VITEK2. Thereafter, DNA was extracted and amplified using conventional PCR followed by DNA sequencing (Sanger method).

Results: The results of the phenotypic susceptibility test showed that all *C. haemulonii* were resistant to amphotericin B. ERG2 and ERG11 sequences showed the same amino acid sequence and corresponded to references that are resistant to amphotericin B.

Conclusion: The resistant properties of *C. haemulonii* against amphotericin B found in this study require further exploration, including comparing resistant and sensitive *C. haemulonii* to amphotericin B. In addition, it is necessary to analyze other genes besides ERG2 and ERG11.

Keywords: Amphotericin B; *Candida*; Environment; Gene; Healthcare; Resistance

INTRODUCTION

An acute infection known as invasive candidiasis has a crude mortality rate of 40-55% in intensive care units (ICU) (1). Previous studies have identified several cases of invasive candidiasis in the paediatric ICU (PICU), neonatal ICU (NICU), and operating rooms (2-4). Invasive candidiasis is primarily caused by *Candida albicans* and *Candida* non-*albicans*, such as *Candida parapsilosis, Candida tropicalis* and *Candida glabrata* (5). In addition to these species, *Candida haemulonii* can cause invasive candidiasis (6), with the number of infections reported to be increasing and spreading across South America, Asia, the Middle East and Europe (7).

Hospitalized immunocompromised patients are exposed to candidiasis due to the transmission of pathogens originating from health workers, facilities, or

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other patients (8). Contamination of damp surfaces in the hospital environment such as sink is considered to be a potential cause of the spread of *Candida* spp. (9).

Candidiasis caused by *Candida* species can be treated with antifungals. One of the antifungals commonly used to treat candidiasis is amphotericin B, which is a broad-spectrum antifungal agent (10, 11). Amphotericin B resistance is still extremely rare. *C. haemulonii* is among the *Candida* species reported to be multidrug-resistant (MDR), including resistance to amphotericin B (7). All *C. haemulonii* species complex isolates examined showed high MIC values for amphotericin B and flucytosine, according to 11 years of study on *C. haemulonii* in Brazil (12). Resistance to amphotericin B can occur through various factors. One of the factors that can cause this resistance is mutations of the ERG genes. Of these genes, ERG2 and ERG11 were mostly reported to affect resistance to *Candida* species (13, 14).

To the best of our knowledge, there was no report of *C. haemulonii* resistance to amphotericin B from Indonesia, which is associated with ERG2 and ERG11. Therefore, we conducted this study to investigate *C. haemulonii* isolated from an ICU environment of a referral hospital in Indonesia. These data are important for clinicians in patient management infected with *C. haemulonii*.

MATERIALS AND METHODS

We selected six isolates stored in the Clinical Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital. Samples were collected from environmental swabs taken from the Intensive Care Unit (ICU), from March to November 2021. Swabs had been moistened with NaCl and directly inoculated onto CHROMagar Candida™ medium. Isolates were incubated at 35°C for 24-48 hours and then stored in the freezer at 4°C before being sub-cultured (15). Subculture was carried out on SDA (Sabouraud Dextrose Agar) and incubated at 35°C for 48 hours, henceforth the confirmation test is carried out for identification using VITEK®2 (BioMerieux) and molecular analysis (16). The purity of the cultures was checked by seeing the morphology of colonies, Gram staining, and a confirmation test using VITEK®2 (BioMerieux) for identification and resistance.

Phenotypic identification and antifungal susceptibility. The fungal susceptibility test to amphotericin B and phenotypic identification was performed using an inoculum suspension adjusted to McFarland 1.8-2.2 in a VITEK®2 system (BioMerieux) (17).

DNA extraction and PCR amplification. DNA extraction was performed using a kit extraction (QIAamp®DNA Mini Kit, 51304, Germany) on 48-h-old sample culture isolates according to the manufacturer's instructions, with a final elution volume of 50 μL (18). The purity of the extracted DNA was checked using NanoDrop Microvolume Spectrophotometers (19). DNA amplification occurred by using a pair of ERG2 and ERG11 primers designed with the PerlPrimer software application. The ERG2 primers were F1 (5'-ATG AAC TTC TGG CTT AAA CTT A-3') and R1 (5'-TTG GCC AAG GGT AAG TTC TTA G-3'), while the ERG11 primers were F1 (5'-ATG GCC TTG AAA GAC TAT CTC GT-3') and R3 (5'-AGG GAG ACA TGC GTG TAC TAA-3'). The amplification process was performed using conventional PCR with a total reaction volume of 20 μL and run on the PCR System 9700 Applied Biosystem. The PCR was carried out based on an optimization procedure that involved initial denaturation at 94°C for 4 minutes; 25 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds (ERG2); annealing at 53°C for 30 seconds (ERG11) and elongation at 72°C for 1,10 minutes; and a final extension at 72°C for 10 minutes. The PCR product was analyzed on 1,5% agarose gel and visualised using UV fluorescence gel doc tools.

DNA sequencing and sequence editing. DNA sequencing was performed using overlapping primers. Overlapping editing using SeqScape v.7.0 (Applied Biosystem) and BioEdit was used to analyzed the DNA sequencing result (20). The primers used for DNA sequencing are the same as those used for PCR amplification. For ERG11, alongside the primers used for PCR, there were additional primers within the gene to amplify the whole gene: R1 (5'–GAA CGT AAC AAC GGT GTT GC–3'), F2 (5'–ACT TCA AAG CTT GTT CTG AG–3'), R2 (5'–GAC GAT TTG CAG AAG ATG CC–3') and F3 (5'–CCT CCA AAG GTG GTA GCT TAA AGG–3').

Analysis of amino acid changes. The analysis was performed on the six sample sequences and

one resistant reference sequence (accession number NW_020289902.1 for the ERG2 gene and MZ711435.1 for the ERG11 gene) using BioEdit to determine any amino acid changes in these sequences (20).

Ethical approval. The study was approved by the Ethics Committee, Faculty of Medicine, Universitas Indonesia (number KET-863/UN2.F1/ETIK/PPM.00.02/ 2022).

RESULTS

Based on the identification and susceptibility testing results, all samples isolated from the sink faucet showed the same sensitivity pattern: resistance to amphotericin B (Table 1). PCR results of the ERG2 and ERG11 genes from the six samples showed that the amplification product corresponded to the expected DNA fragment length (657 bp for ERG2 and 1575 bp for ERG11). The analysis of amino acid changes for both genes showed that the six samples had the same sequence as the resistant reference isolates (Figs 1 and 2). All sequence isolates analysed in this study have been submitted to GenBank under accession numbers OR463470 to OR463475 and OR506955 to OR506960.

DISCUSSION

In this study, all samples of *C. haemulonii* isolates were resistant to amphotericin B, as indicated by high MIC values of 4-8 µg/ml. Research from other countries found the same results and they classified *C. haemulonii* as intrinsically resistant (21-23). However, it is still questionable because a study found amphotericin B-sensitive *C. haemulonii* (24).

Like other *Candida* species, amphotericin B resistance is associated with amino acid changes in ERG2 and ERG11 (13, 14, 25). The characteristics of these amino acid changes are also thought to occur in *C. haemulonii*. This was concluded from the characteristics of the sterol content in *C. haemulonii*, which is similar to other candida species that are resistant to amphotericin B (26). Therefore, ERG2 and ERG11 were examined to see whether there was any correlation between *C. haemulonii* resistance and ERG2 and ERG11.

All amino acid sequences from isolates analysed in this study are the same as the resistant reference sequences from GenBank. This indicates that all isolates were genotypically resistant, which was confirmed phenotypically. In order to determine the amino acid changes associated with resistance, the sequences of sensitive isolates should be compared.

Table 1. Results of identification and susceptibility test to amphotericin B using VITEK2

No.	Isolate	Identification	MIC (ng/µl)	Interpretation	Source
1.	CH3	*Candida haemulonii*	8	Resistant	Sink faucet
2.	CH5	*Candida haemulonii*	8	Resistant	Sink faucet
3.	CH7	*Candida haemulonii*	4	Resistant	Sink faucet
4.	CH81	*Candida haemulonii*	8	Resistant	Sink faucet
5.	CH82	*Candida haemulonii*	8	Resistant	Sink faucet
6.	CH12	*Candida haemulonii*	8	Resistant	Sink faucet

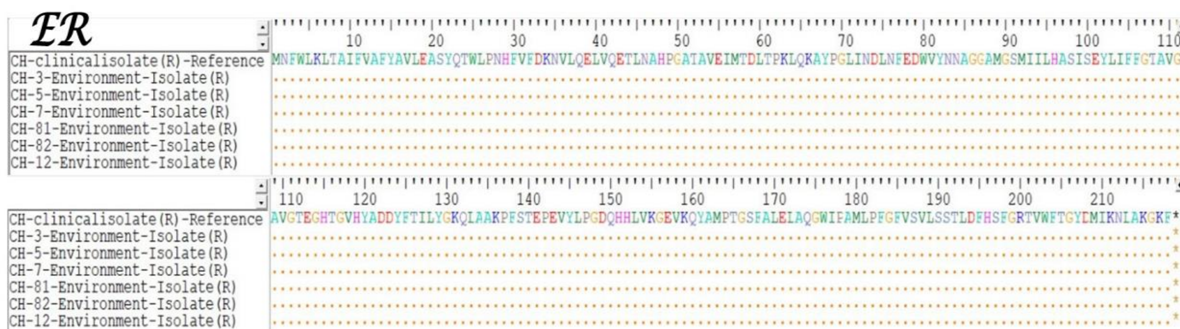

Fig. 1. Amino acid sequence comparison of ERG2 gene.

Fig. 2. Amino acid sequence comparison of ERG11 gene

However, we found no sensitive sequence in the public genetic database. Gade et al. (2020) reported sensitive *C. haemulonii* using the Etest method (24), but no gene sequence information exists. Therefore, it is necessary to carry out further studies regarding the sensitive sequences of *C. haemulonii* to enable comparison with the resistant sequences so that the amino acid positions in ERG2 and ERG11 which have an essential role in resistance to amphotericin B can be determined.

The limitation of this study is that we only used six isolates which were too small to be generalized into the population, and apart from ERG2 and ERG11, it is also necessary to analyze the ERG9 gene because the study conducted by Silva et al. (2020) reported that there was an accumulation of squalene when *C. haemulonii* was exposed to amphotericin B, which is synthesized by the ERG9 gene. This indicates an upregulation of the ergosterol biosynthetic pathway, which can strengthen membrane integrity and prevent ergosterol sequestration (26). In addition, the ERG3 and ERG6 genes also need to be analyzed because they have been reported to be associated with resistance in *C. albicans* and *C. glabrata* to amphotericin B (13, 26).

CONCLUSION

All *Candida haemulonii* isolated from the ICU in Indonesia were resistant to amphotericin B and had the same sequence of amino acids encoded by the ERG2 and ERG11 genes as reference sequences. The intrinsic resistance property of *C. haemulonii* against amphotericin B requires further exploration.

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