Thesis progression

Thesis title: DNA barcoding markers for routine diagnosis of pathogenic yeasts

Thesis progression title: Screening antifungal susceptibility pattern of pathogenic yeast by using disk diffusion method

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1. Rationale and background

A growing population of immunosuppressed patients has resulted in increasingly frequent diagnoses of invasive fungal infections, including those caused by unusual yeasts. Infections by *Candida* species, especially candidemia, are currently one of the most common causes of nosocomial infection. The incidence of non-albicans species of *Candida* is increasing compared with that of *Candida albicans*, and several species, such as *Candida glabrata* and *Candida krusei*, may be resistant to azole antifungal therapy. Invasive infection caused by antifungal-resistant *Candida* spp is an emerging problem. Fluconazole resistance among *Candida albicans* isolates is estimated between 0% and 5% (1,2,3), with the highest rate reported in South Africa. Fluconazole resistance is a much bigger problem among non-albicans spp and ranges between 5% and 65%, with the highest rate reported in Denmark (4,5). Fluconazole resistance is problematic because it is the only antifungal drug available for the treatment of *Candida* infections in many parts of the world (6,7). Echinocandin resistance has also been reported in some settings; in the United States, approximately 6% of *Candida glabrata* isolates are resistant to echinocandins (7). Generally, these infections are associated with high mortality, and successful clinical outcome requires early diagnosis and effective antifungal therapy. Yet, antifungal options are few, with chemical classes for invasive disease treatment limited to azoles, echinocandins, polyenes, and flucytosine.

Over the past decade, the development of less toxic drugs, which can be applied safely in a range of patients with various conditions, has contributed to the expansion of antifungal use for prophylaxis, and empirical and directed therapy, which has in turn led to increased drug resistance. The use of medically related antifungal drugs in agriculture has resulted in environmental reservoirs for some drug-resistant pathogens (8). The emergence of drug resistance to any one drug class severely limits therapy because so few treatment options are

available. Multidrug resistance can eliminate treatment options, which has a devastating effect on patient outcomes (9).

Early initiation of the correct antifungal therapy has been demonstrated to have a direct impact on the patient's outcome. Consequently, a rapid, reproducible method of antifungal susceptibility testing would be useful in determining the epidemiology of and optimal treatment for infection with resistant isolates (10).

Screening clinical yeast isolates for decreased susceptibility to antifungal drugs has been difficult (11). Methodologies such as the standard National Committee for Clinical Laboratory Standards (NCCLS) broth macrodilution test and alternative methods such as the E test or microdilution adaptations of the NCCLS method generally compare favorably for determining MICs for isolates; however, these are not easily adapted to the screening of yeasts for antifungal susceptibility.

Therefore, this study aimed to determine the efficiency of the disk diffusion method in screening the antifungal susceptibility pattern of pathogenic yeast isolated in Khon Kaen, Thailand.

2. Objectives

2.1. To determine the prevalence, and species distribution of pathogenic yeast isolated in Khon Kaen, Thailand.

2.2. To investigate antifungal susceptibility patterns of pathogenic yeast isolated in Khon Kaen, Thailand.

3. Materials and Methods

3.1. Isolation of pathogenic yeast strains

A total of 150 isolates of pathogenic yeasts were enrolled for identification and antifungal susceptibility testing. These included 114 clinical isolates collected from blood and body fluid specimens at the Microbiology laboratory unit, Srinagarind Hospital, Khon Kaen University, Thailand, and 36 environment isolates collected from the domestic environment in Khon Kaen municipality, Khon Kaen, Thailand.

The yeast isolates were identified with the classical methodology, based on macro- and micro-morphological characteristics, cultured on chromogenic agar (Brilliance Candida Agar, Oxoid) following manufacturer's instructions, and further identified by MALDI-TOF MS, (MALDI Biotyper®, Bruker, Germany).

Each isolate was subcultured at least twice on Sabouraud dextrose agar and incubated at 35°C before testing to ensure purity and optimal growth.

3.2. Inoculum suspensions

Yeast inoculum suspensions were prepared as described for the NCCLS M27-A2 method. The turbidity was measured with a spectrophotometer at 530 nm and was adjusted to match a 0.5 McFarland density standard, resulting in a concentration of 1×10^6 to 5×10^6 yeast cells/ml. This inoculum was used directly for the inoculation of agar plates.

3.3. Antifungal agents

Paper disks were manufactured by Liofilchem, Inc, United States including Amphotericin B(10µg), Caspofungin (5µg), Fluconazole (25µg), Voriconazole (1µg) and Itraconazole (8µg).

3.4. Media and susceptibility testing method

Antimicrobial susceptibility screening testing for all yeast strains was performed by disk diffusion method on Mueller-Hinton agar (MH) (Himedia Laboratories, Maharashtra, India) with modification by adding 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB Medium).

A sterile cotton swab is dipped into the standardized inoculum suspension, rotated several times, and pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid. The entire dried agar surface is evenly streaked in three different directions, swabbing the rim of the plate as the final step. The lid of the plate should be left ajar to allow the agar surface to dry for no more than 15 min. Disks (6 mm) must be pressed down onto the inoculated agar surface to ensure complete contact with the agar and distributed evenly so they are not closer than 24 mm from center to center. After 20 to 24 h of incubation at 35°C, the resulting inhibition zones should be uniformly circular and a confluent lawn of growth should be present. The zone diameters surrounding the disks should be measured to the nearest whole millimeter at the point at which there is a prominent reduction in growth. If growth is insufficient, the plates should be read at 48 h. Quality control isolates *Candida albicans* ATCC 90028 and *C. krusei* ATCC 6258 were included in all runs, and all results were within published limits.

3.5. Interpretation of the breakpoints

The clear zone diameter of each disk will be measured and the breakpoints will be interpreted according to the guideline modified from antifungal disk diffusion susceptibility testing of yeasts M44-A.

4. Result

4.1. Identification of yeast isolated

A total of 150 yeast isolates were obtained including 36 isolates from the environment and 114 isolates from clinical samples.

No	Species	Env sp	Clinical sp	Total
		N (%)	N (%)	N (%)
1	*C. albicans*		53 (46.49%)	53 (35.33%)
2	*C. tropicalis*		28 (24.56%)	28 (18.67%)
3	*C. parapsilosis*	5 (13.88%)	8 (7.02%)	13 (8.67%)
4	*C. glabrata*		11 (9.65%)	11 (7.33%)
5	*C. krusei*		4 (3.5%)	4 (2.67%)
6	*C. metapsilosis*	1 (2.78%)	1 (0.88%)	2 (1.33%)
7	*C. guilliermondii*		2 (1.75%)	2 (1.33%)
8	*Kodamaea ohmeri*		2 (1.75%)	2 (1.33%)
9	*C. catenulata*		1 (0.88%)	1 (0.67%)
10	*C. ciferrii*		1 (0.88%)	1 (0.67%)
11	*C. lusitaniae*		1 (0.88%)	1 (0.67%)
12	*C. orthopsilosis*		1 (0.88%)	1 (0.67%)
13	*C. rugosa*		1 (0.88%)	1 (0.67%)
14	*Cystobasidium minutum*	1 (2.78%)		1 (0.67%)
15	*Filifactor villosus*	1 (2.78%)		1 (0.67%)
16	*Rhodotorula mucilaginosa*	1 (2.78%)		1 (0.67%)
17	No organism identification possible/ No peak found	27 (75%)		27 (18%)
Total		36	114	150 (100%)

Based on macro- and micro-morphological characteristics, chromogenic agar, and MALDI-TOF MS methods, sixteen yeast species were identified, the most common yeast was *C. albicans* (53 isolates, 35.33%) followed by *C. tropicalis* (28 isolates, 18.67%), *C. parapsilosis* (13 isolates, 8.67%), *C. glabrata* (11 isolates, 7.33%). However, 27 yeast isolates from environment samples could not identified.

4.2. Antifungal susceptibility result by Kirby-Bauer disk diffusion method

No	Species	N	Antifungal drugs								
			Fluconazole (n,%)			Voriconazole (n,%)			Caspofungin (n,%)		
			S	SDD	R	S	I	R	S	I	R
Antifungal Disc Interpretative Criteria											
1	*C. albicans*		≥ 17	14-16	≤ 13	≥ 17	15-16	≤ 14	≥ 17	15-16	≤ 14
2	*C. tropicalis*		≥ 17	14-16	≤ 13	≥ 17	15-16	≤ 14	≥ 17	15-16	≤14
3	*C. parapsilosis*		≥ 17	14-16	≤ 13	≥ 17	15-16	≤ 14	≥ 13	11-12	≤ 10
4	*C. glabrata*		-	≥15	≤ 14	-	-	-	-	-	-
5	*C. krusei*		-	-	-	≥ 15	13-14	≤ 12	≥ 17	15-16	≤ 14
6	*C. guilliermondii*		-	-	-	-	-	-	≥ 13	11-12	≤ 10
Result											
1	*C. albicans*	53	53 100%	-	-	53 100%	-	-	50 94.4%	-	3 30%
2	*C. tropicalis*	28	21 75%	1 3.6%	6 21.4%	22 78.6%	-	8 28.6 %	28 100%	-	-
3	*C. parapsilosis*	13	12 92.3%	-	1 7.7%	12 92.3%	1 7.7%	-	6 46.1%	1 7.7%	6 46.1%
4	*C. glabrata*	11	-	8 72.7%	3 27.3%	-	-	-	-	-	-
5	*C. krusei*	4	-	-	-	3 75%	1 25%	-	3 75%	-	1 25%
6	*C. guilliermondii*	2	-	-	-	-	-	-	2 100%	-	-
Total		111	86 77.5%	9 8.1%	10 9%	90 81%	2 1.8%	8 7.2%	89 80.2%	1 0.9%	10 9%

Among *C. albicans*, all 53 isolates were 100% susceptible to fluconazole and voriconazole, 94.4% were susceptible to caspofungin. There were 105 yeast isolates tested with fluconazole, in which 81.9 % of yeast isolates showed susceptibility, 9 isolates (8.57%) showed susceptible dose-dependent, those were *C. glabrata* (8 isolates) and one isolate of *C. tropicalis*, and 10 fluconazole-resistant isolates (9.52%) were *Candida tropicalis* (6), *C. glabrata* (3), and *C. parapsilosis* (1).

5. Conclusion

The disk diffusion test is easy to perform and requires minimal labor and training for test performance for a small number of clinical isolates; however, endpoints can be difficult to determine. It is cost-effective because it usually provides qualitative results 24 h sooner than the standard broth dilution methods.

Reports correlating disk diffusion results with M27-A methods have concluded that the disk diffusion methodology does not adequately separate some fluconazole-resistant from fluconazole susceptible dose-dependent isolates. Therefore, MIC determination may be needed for those strains that have intermediate or resistant zones by the diffusion method.

6. References

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