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Prevalence and virulence factors of *Candida* spp. associated with blow flies

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ABSTRACT

Objective: To investigate the prevalence of *Candida* spp. and the virulence factors of *Candida albicans* (*C. albicans*) isolated from external surfaces of blow flies collected from Mae Sot, Tak Province, Thailand.

Methods: The blow flies were collected by sterile sweep nets from three areas in Mae Sot. Yeast isolation was first performed on Sabouraud dextrose agar (SDA) supplemented with chloramphenicol and cycloheximide. The yeast isolates were then identified by using chromogenic agar, a yeast identification test kit, a germ tube formation test and a carbohydrate utilization test. The β-hemolysis was determined on 7% sheep blood agar, while phospholipase activity was measured on SDA agar supplemented with 10% egg yolk suspension. Antifungal susceptibility testing was determined by broth micro-dilution testing against ketoconazole and amphotericin B.

Results: The prevalence rate of *Candida* spp. on the external surfaces of the blow flies was 78.1%. All *C. albicans* isolated from the blow fly demonstrated β-hemolysin and potent phospholipase activities and 47.1% of *C. albicans* were resistant to ketoconazole with MIC values 128 μg/mL.

Conclusions: The results indicate that blow flies could play an essential role in the transmission of potentially pathogenic and antifungal resistant *C. albicans* into the environment. Further investigation on other virulence factors and genetic relatedness among isolates from the blow flies, the environment and clinical specimens is required to confirm this role.

1. Introduction

Candida infection is the most common cause of yeast infection worldwide of which *Candida albicans* (*C. albicans*) is the most frequent isolate from candidiasis and candidemia [1]. Most *Candida* infection is endogenous and caused by overgrowth of commensal *Candida* species. Although, the transmission of *Candida* spp. from external sources is uncommon, recent studies have shown that some species of *Candida* cause candidemia via exogenous origins by contamination of medical devices [2]. This ubiquitous organism possesses multiple virulence factors and thus people with weakened immune systems could become infected via direct contact with contaminated items or from other external sources [3,4].

Blow flies are recognized as mechanical carriers of various microorganisms and parasites as they feed and lay eggs on decomposing organic matter. Several investigations have focused upon and reported on their potential roles in the transmission of communicable diseases. These flies may carry up to 11 species of bacteria on their external surfaces and carry the highest number of microorganisms compared to other filth flies. The microorganisms associated with blow flies include various species of pathogenic and non-pathogenic bacteria, fungi and parasites [5,6]. However, there are no reports on the types of yeasts, especially *Candida* spp., carried on the external surfaces of blow flies.

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The purpose of this study was to investigate the prevalence of *Candida* spp. and virulence factors of *C. albicans* associated with blow flies. The results from this study will provide new information related to *Candida* spp. carried by blow flies and provide a link to the sources of *Candida* infection.

2. Materials and methods

2.1. Collection and identification of blow flies

Blow flies were collected by using sterile sweeping nets from three natural fly breeding areas in Mae Sot, Tak Province, Thailand; a seafood market along the Thai–Myanmar border, a main garbage dump area, and grove woods. The collected flies were immobilized by storing on ice while being transported to the laboratory. After isolation of yeasts, the fly specimens were dried at 50 °C for 24 h and identified according to the taxonomic identification key of Kurahashi and Bunchu [7].

2.2. Isolation and identification of Candida spp.

After collection, the flies were randomly selected and each fly was then placed in 3 mL peptone water and shaken for 1 min before spreading on the surface of Sabouraud dextrose agar (SDA) supplemented with 0.05 g/L chloramphenicol and 0.4 g/L cycloheximide. After incubation at 30 °C for 48 h, three colonies with typical yeast appearance were randomly isolated from each plate. The isolates were then streaked on chromogenic agar (CHROMagar® Candida, France) for *Candida* spp. and incubated at 30 °C for 48 h. Five standard strains *i.e. C. albicans* ATCC 10231, *Candida glabata* TISTR 5006, *Candida krusei* TISTR 5258, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 9968 were used to compare their morphology on this chromogenic agar. The green colonies on this agar, which are an indication of *C. albicans*, were further identified by using a yeast identification test kit (YT MicroPlate™, BiOLOG, USA) and by testing for germ tube formation. The carbohydrate (mannitol, maltose, cellubiose) utilization test was performed to confirm of other *Candida* species.

2.3. Determination of virulence factors

The 10 μL *Candida* suspensions with turbidity equal to McFarland standard No. 2 were used. For hemolysis activity, the suspensions were inoculated on surfaces of SDA agar supplemented with 7% sheep blood and 3% glucose and then incubated at 30 °C for 48 h. The β-hemolysis activity was observed as a translucent zone around the organism colonies and the hemolytic index (HI value) was estimated by dividing the total diameter of the translucent zone plus the colonies by the diameter of the colonies, as described by Wan *et al.* [8].

For extracellular phospholipase activity, the suspensions were inoculated on the surface of SDA agar supplemented with 5% NaCl, 0.05% $CaCl_2$ and 10% sterile egg yolk suspension. After incubation for 96 h at 30 °C, the precipitation zone was observed and the activity was evaluated as described by Price *et al.* [9]. The phospholipase activity was calculated by dividing the colony diameter by the diameter of the precipitation zone (Pz) around the colony. The activity was classified into 5 groups according to Pz values: 1 = no activity, 0.90–0.99 = weak activity, 0.80–0.89 = mild activity, 0.70–0.79 = moderate activity and ≤ 0.69 = potent activity.

2.4. Antifungal susceptibility testing

The minimum inhibitory concentration (MIC) against two antifungal agents, amphotericin B and ketoconazole, was determined by using the EUCAST method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts [10]. Briefly, antifungal susceptibility testing was performed in flat-bottom 96-well microtitre plates with RPMI 1640 medium supplemented with 2% glucose and antifungal agent concentrations ranging from 0.25 to 128 μg/mL. The inoculum size of 1×10^5 to 2.5×10^5 CFU/mL were then added to each well. MIC end points were determined at 48 h after incubation at 30 °C. The *C. albicans* ATCC 10231 was used in the test as a reference strain in the standard EUCAST method.

3. Results

The present study aimed to investigate the prevalence of *Candida* spp. on the external surfaces of blow flies collected from three areas in Mae Sot, Tak Province, Thailand. This study also investigated some characteristics of the *C. albicans* isolates involved in their virulence, which were hemolysis reaction, phospholipase activity and sensitivity to antifungal drugs. A total of 105 blow flies were collected and approximately 97% of them were *Chrysomya megacephala* (Diptera: Calliphoridae) and the others were *Achoetandrus* (*Chrysomya*) *rufifacies* and *Lucilia cuprina* (Diptera: Calliphoridae). The prevalence rate of *Candida* species carried on blow flies collected from these areas was 78.1%. Among these *Candida* isolates, 17 isolates were identified as *C. albicans* based on their characteristics on the chromogenic agar, the ability to form a germ tube and more than 90% correspondence to *C. albicans* on YT MicroPlate™. 20.0% of the blow flies collected from the seafood market carried *C. albicans*, while the prevalence rate of *C. albicans* on blow flies collected from the garbage dump area was 11.4% and from the grove woods 5.7%. Overall, the prevalence rate of *C. albicans* on the surfaces of the blow flies was 12.4%. *C. tropicalis*, *C. krusei* and *C. parapsilosis* were also identified based on their characteristics on the chromogenic agar and the ability to utilize carbohydrates. The total prevalence rate of *C. tropicalis* was 44.8, while it was 20.0% for *C. krusei* and only 1% for *C. parapsilosis* (Table 1). Other yeast species, *Saccharomyces* spp. and *Rhodotorula* spp. were also isolated from blow flies collected in this study.

Table 1
The prevalence rate of *Candida* species isolated on blow flies collected from the three areas in Mae Sot.

Collected area	Prevalence of *Candida* spp. (%)				
	C. tropicalis	*C. krusei*	*C. parapsilosis*	*C. albicans*	*Candida* spp.
Seafood market (n = 35)	42.8	22.9	–	20.0	85.7
Garbage dump (n = 35)	22.9	14.3	2.9	11.4	51.4
Grove woods (n = 35)	68.6	22.9	–	5.7	97.1
Total (n = 105)	44.8	20.0	1.0	12.4	78.1

All *C. albicans* isolates exhibited β-hemolysis reaction on blood agar with HI values ranging from 2.17 to 3.00 and showed potent phospholipase activity with Pz values ranging from 0.39 to 0.57. Antifungal susceptibility testing was performed against two antifungal drugs, amphotericin B from polyene class and ketoconazole from the imidazole class. Most isolates were sensitive to amphotericin B and ketoconazole, however 8 isolates (47.1%) exhibited high levels of resistance to both antifungal drugs tested with MIC values of equal or more than 128 μg/mL. The highest prevalence rate of resistant *C. albicans* isolates was found in the garbage dump area as three out of four isolates were resistant to ketoconazole, followed by the grove woods and the seafood market (Table 2).

Table 2

Precipitation zone (Pz), hemolysis index (HI) and MIC values of *C. albicans* isolated from blow flies collected from the three areas in Mae Sot.

Areas	Isolates	HI value	Pz value	MIC (mg/mL)	
				Ketoconazole	Amphotericin B
	C. albicans ATCC 10231	2.74	0.49	16	32
Seafood market	SM09	2.73	0.45	128	< 0.25
	SM10	2.82	0.47	128	< 0.25
	SM11	2.35	0.43	32	< 0.25
	SM14	2.72	0.53	64	< 0.25
	SM15	2.68	0.40	> 128	0.50
	SM17	2.59	0.39	32	< 0.25
	SM29	2.44	0.42	32	< 0.25
	SM31	3.00	0.52	32	< 0.25
	SM32	2.70	0.57	32	< 0.25
Garbage dump	GD08	2.60	0.46	128	< 0.25
	GD09	2.26	0.53	128	1.00
	GD11	2.58	0.49	32	< 0.25
	GD19	2.63	0.48	128	< 0.25
Grove woods	GW15	2.40	0.46	32	< 0.25
	GW16	2.17	0.48	128	< 0.25
	GW33	2.22	0.50	16	< 0.25
	GW34	2.39	0.51	> 128	< 0.25

4. Discussion

Mae Sot, from where the blow fly samples were collected, is a town on the Thai–Myanmar border with a dynamic population of refugees and economic migrants. This area has long been considered as a public health concern especially in communicable and emerging diseases. Most of the blow flies collected was *Ch. megacephala*, which is the most abundant blow fly species in Thailand [11]. There are a few reports on the types of yeasts carried on vector insects. Srivoramas *et al.* (2012) found that *Musca domestica* and *Ch. megacephala* collected from Northeastern Thailand carried yeasts at the prevalence rate of 11% [12]. However, the prevalence of *Candida* species on the external surfaces of the *Ch. megacephala* collected in this study was 78.1%. The differences might be due to different environments and the difference yeast isolation methods. The addition of cycloheximide, antifungal agents, into the isolation medium in this study may have increased the probability of yeast isolation.

Several virulence factors such as biofilm formation, proteinase, hemolysin and phospholipase contribute to the *Candida* pathogenicity. The β-hemolysis reaction is due to the production of β-hemolysin, a membrane pore toxin, caused toxicity to membrane of mammalian cells. Phospholipase activity and germ tube formation promote mucosa penetration, indicating invasiveness of the strain [13–16]. Pinto *et al.* (2008) observed that *C. albicans* from infection origins possessed higher phospholipase activity than from commensal isolates [17]. Previous studies showed that approximately 94% of *C. albicans* from blood cultures *i.e.* invasive infections was positive for phospholipase activity with a wide range of activity [18]. Therefore, the *C. albicans* isolates in this study could be potentially pathogenic and could cause invasive infection.

It is worth noting that the highest prevalence rate of ketoconazole resistant isolates was found in the blow flies collected from the garbage dump area. The area is a location for the disposal of waste materials and may contain huge quantities of toxic chemicals which may be toxic to many organisms, but potentially promote resistance. The antifungal resistance in *Candida* species is increasingly important. The Centers for Disease Control and Prevention (CDC) reported that the incidence rate of antifungal resistance in *Candida* invasive infection is increasing worldwide and approximately 30% of patients with bloodstream drug-resistant *Candida* infections (candidemia) die during their hospitalization [19,20]. Approximately 50% of the *C. albicans* isolates in this study was resistant to ketoconazole. Thus, the blow flies could also play a role in spreading and promoting of antifungal resistance.

This study demonstrates for the first time that blow flies are mechanical vectors for *Candida* species, including *C. albicans* and provides evidence on the prevalence of *C. albicans* in blow flies. This preliminary study shows that the *C. albicans* isolates are potential pathogens with β-hemolysis and high phospholipase activities. Most infection by *Candida* spp. is from endogenous sources, however recent studies have shown that transmission from external sources is possible. Therefore, the results from this study demonstrate that blow flies play an important role in spreading potentially pathogenic and antifungal resistant *Candida* species in the environment. Other virulence factors and genetic similarity between *Candida* isolates from blow flies, environment and clinical samples should be further investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

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