Prevalence and Antifungal Susceptibility of *Cryptococcus neoformans* Isolated from Pigeon Excreta in Chon Buri Province, Eastern Thailand

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ABSTRACT

The prevalence of cerebral meningitis caused by *Cryptococcus neoformans* in HIV-infected patients in Eastern Thailand is high. However, little is known about the occurrence of this pathogenic yeast in the environment of this region.

The aim of our study was to characterize the prevalence of *C. neoformans*, its serotypes and antifungal drug susceptibilities in environmental isolates from Chon Buri, Eastern Thailand.

*C. neoformans* was isolated from 10% of fifty pigeon excreta examined from this province. All *C. neoformans* isolates were of serotype A and although the isolates displayed slightly decreased susceptibility towards fluconazole, all tested sensitive to amphotericin B, fluconazole and itraconazole. This study is the first report of the occurrence of *C. neoformans* in pigeon excreta in eastern Thailand.

Key words: *Cryptococcus neoformans*, pigeon excreta, antifungal drug susceptibility, Thailand.

Introduction

*Cryptococcus neoformans* is a pathogenic yeast that causes cryptococcosis in humans¹,². It has historically been divided into three varieties of five serotypes based on antigenicity of the capsule: *C. neoformans* var. *grubii* (serotype A), var. *gattii* (serotypes B and C), var. *neoformans* (serotype D), and one hybrid (serotype AD)³,⁴. In 2002, *C. neoformans* var. *gattii* (serotypes B and C) was awarded species status and renamed *Cryptococcus gattii*.⁵ The different types of *C. neoformans* are found worldwide in environmental samples such as soil, bird excreta, or in the case of *C. gattii* on surfaces of eucalyptus or other tropical trees³,⁴,⁶⁻⁸.

The distribution of serotypes among the reservoirs may be region-specific⁵,⁶ and reflect into the clinic⁵. Pigeons are found nearly ubiquitously in populated areas and their excreta probably serve as the most important reservoir for *C. neoformans* of Thailand.

*C. gattii* is the most virulent of these serotypes and readily infects immunocompetent hosts. Although serotypes A and AD are mostly only found in immunocompromised patients, serotype A is the most frequently found in clinical samples⁶. The resistance rate to common antifungal drugs of *C. neoformans* is increasing, especially in AIDS patients⁶. However, there is little information concerning the rate of antifungal drug resistance to *C. neoformans* isolated from environmental sources in Thailand. Also, general data on the prevalence and distribution in the environment of...
C. neoformans serotypes in Southeast Asia is limited.

The aim of this study was to investigate C. neoformans prevalence, its serotypes as well as antifungal drug susceptibilities in environmental samples isolated in tourist areas with high HIV prevalence in Chon Buri, Southeast Bangkok and to compare the antifungal susceptibility of environmental strains to clinical strains.

Materials and Methods

Sample collection

Fifty pigeon excreta samples were collected from three districts (Mueang, Sri Racha and Pattaya) along the coastline of Chon Buri. Thirty samples obtained in Mueang district were collected from three different sites.

C. neoformans identification and serotyping

Pigeon excreta samples were suspended in 0.85% NaCl at a ratio 1:10 (W/V) and allowed to settle for 10 min. One ml of suspension was diluted 10 fold and 0.1 ml of diluted and undiluted suspension were spread onto Sabouraud’s dextrose agar (SDA) containing 0.4 mg/ml of penicillin G and 0.05 mg/ml of chloramphenicol. SDA plates were incubated at 37°C for 2–7 days. The candidate yeast colonies (round, smooth and white colored) were examined by India ink, phenol oxidase and urease test. Isolates positive in all three tests were assumed to be C. neoformans6,11).

The serotypes of assumed isolates of C. neoformans were determined by rapid slide agglutination using Crypto Ckeck (Iatron Laboratories, Tokyo, Japan) according to the manufacturer’s protocol.

Antifungal susceptibility test

Susceptibility testing was performed according to the reference method for broth dilution antifungal susceptibility testing of yeast M27–A2 of the National Committee for Clinical Laboratory Standards (NCCLS)12). Briefly, yeast suspensions of 0.5–2.5 × 10⁷ CFU/ml were tested to amphotericin B (AMB) and itraconazole (ITZ) at a concentration range of 0.03–16 μg/ml and fluconazole (FLZ) at a concentration range of 0.125–64 μg/ml. The 96 well plate was incubated at 37°C for 48 hours and then the plate was taken to measure absorbance value by spectrophotometer at 420 nm7,13). For FLZ and 5FC the MIC was defined as IC80, for AMB as IC100.

Results

Environmental prevalence of C. neoformans in Chon Buri

From the 50 pigeon excreta samples of three districts in Chon Buri, C. neoformans was found in five of them (10%) (Table 1). The highest Cryptococcus prevalence of Chon Buri was Pattaya (Table 1). Of the isolates studied by the agglutination test, 100% of C. neoformans isolated from pigeon excreta were of serotype A (Table 2).

Antifungal drug susceptibility

Since no official interpretive breakpoints are available, guidelines for Candida are generally used for C. neoformans10). MIC values of C. neoformans to AMB, ITZ, and FLZ were in the ranges 0.125–0.5, 0.03–0.125, and 8–16 μg/ml, respectively. Our results indicated that all isolates were susceptible to the three antifungal drugs tested. However, FLZ MIC values were elevated on average by two log₂ dilutions in the environmental isolates as compared to isolates from Chon Buri patients, while values for AMB and ITZ were within the same ranges (Table 3).
Discussion

In this study, *C. neoformans* was found in five of the 50 (10%) environmental pigeon excreta samples (Table 1). The prevalence in Chon Buri was similar to that observed in urban Bangkok in the center of Thailand \(^6\) but higher numbers (16.4%) had previously been observed in rural Chiang Mai in northern Thailand \(^6\) (Table 2). Currently, there is no data on the prevalence of *C. neoformans* in the Southeast Asian environment other than from Thailand \(^6\) (Table 2). Therefore, Thai data should be considered representative for *C. neoformans* prevalence in Southeast Asia.

All *C. neoformans* isolates from pigeon excreta in this study were of serotype A (Table 2) and this agrees with data for pigeon excreta analyzed from Chiang Mai province \(^6\). Serotype A is predominant in all Southeast Asian countries studied so far (Table 2) and the distribution between environmental and clinical isolates from HIV + patients is similar \(^6\). While among clinical isolates of *C. neoformans* from Chiang Mai, only ~1% was non-serotype A \(^6\). Significantly higher numbers of other serotypes were found in non-HIV infected patients in Vietnam \(^7\) which is in concordance with the increased virulence of *C. gattii*.

These results correlated with previous reports that clinical *C. neoformans* isolates in Thailand were susceptible to these substances \(^8,9\). Studies in neighboring countries showed that although all clinical *Cryptococcus* strains were susceptible to AMB, voriconazole and posaconazole, there was an increasing trend towards FLZ resistance in Cambodia; approximately 20% of clinical isolates in Singapore were found to exhibit decreased susceptibility to FLZ \(^10\). Decreased susceptibility to FLZ was also observed in other

### Table 2. Prevalence of different *C. neoformans* serotypes across Southeast Asia

<table>
<thead>
<tr>
<th>Country</th>
<th>City</th>
<th>Source</th>
<th>N° samples</th>
<th>Serotype [%]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand</td>
<td>Chon Buri</td>
<td>pigeon excreta</td>
<td>50</td>
<td>100</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>Chiang Mai</td>
<td>pigeon excreta</td>
<td>55</td>
<td>100</td>
<td>6)</td>
</tr>
<tr>
<td></td>
<td>Bangkok</td>
<td>pigeon excreta</td>
<td>11</td>
<td>n.d.</td>
<td>15)</td>
</tr>
<tr>
<td></td>
<td>Chiang Mai</td>
<td>clinical</td>
<td>n.a.</td>
<td>98.7</td>
<td>6)</td>
</tr>
<tr>
<td></td>
<td>countrywide</td>
<td>clinical</td>
<td>n.a.</td>
<td>95.7</td>
<td>19)</td>
</tr>
<tr>
<td>Vietnam</td>
<td>Ho Chi Minh</td>
<td>HIV + patients</td>
<td>n.a.</td>
<td>100</td>
<td>17)</td>
</tr>
<tr>
<td></td>
<td>Ho Chi Minh</td>
<td>HIV − patients</td>
<td>n.a.</td>
<td>72.5</td>
<td>17)</td>
</tr>
<tr>
<td>Cambodia</td>
<td>Phnom Penh</td>
<td>HIV + patients</td>
<td>n.a.</td>
<td>98.5</td>
<td>20)</td>
</tr>
<tr>
<td>Malaysia</td>
<td>countrywide</td>
<td>clinical</td>
<td>n.a.</td>
<td>88.5</td>
<td>23)</td>
</tr>
</tbody>
</table>

*n.d.*, not determined; *n.a.*, not applicable.

### Table 3. Antifungal drug susceptibilities of *C. neoformans* isolates

<table>
<thead>
<tr>
<th>sample</th>
<th>MIC (μg/ml)</th>
<th>AMB</th>
<th>ITZ</th>
<th>FLZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Env07</td>
<td>0.25</td>
<td>0.03</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>Env18</td>
<td>0.25</td>
<td>0.06</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>Env27</td>
<td>0.25</td>
<td>0.06</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>Env32</td>
<td>0.5</td>
<td>0.125</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Env34</td>
<td>0.125</td>
<td>0.06</td>
<td>0.125</td>
<td>8</td>
</tr>
<tr>
<td>clinical isolates (^a)</td>
<td>0.25-1</td>
<td>0.03-0.125</td>
<td>1-4</td>
<td></td>
</tr>
</tbody>
</table>

Susceptibility to AMB, FLZ and ITZ was tested according to the CLSI M27-A2 reference method at concentrations ranging from 0.03-16 μg/ml (AMB and ITZ) and from 0.125-64 μg/ml (FLZ) \(^7,13\). 

\(^a\) unpublished data from 21 strains isolated at Queen Savang Vadhana Memorial Hospital, Chon Buri.
parts of the world. Our drug susceptibility data as well as data from other studies indicate that a trend towards decreasing FLZ susceptibility can be seen throughout Southeast Asia. Although true antifungal drug resistance of C. neoformans is rare in Thailand and was not observed among our isolates, its epidemiology should be monitored more closely to prevent emergence of antifungal drug resistance to this yeast.

Acknowledgement

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References


