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Screening and Selection of *Cryptococcus Neoformans* Using Urease Activity and Temperature Range

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ABSTRACT

Pigeon dropping has been known to be a reservoir for *Cryptococcus neoformans*. In this research, attempt has been made to screen and select for this life threatening organism using temperature (thermotolerance) and urease test sequentially. Fourteen (14) samples from pigeon droppings were collected from three markets in Benin City. Isolates obtained from these samples were subjected to urease and temperature test. The results showed that 10 isolates that were positive for temperature were equally positive for the urease tests, with highest growth rate 37°C recorded for samples obtained from New Benin (NB3). This experiment demonstrates the possibility of isolating C. *neoformans* at minimum temperature of 37°C thermotolerance and urease activity tests. Pathogenicity studies are recommended to further understand host – pathogen relationships.

Keywords: *Cryptococcus neoformans*, Pathogenic, Urease, Thermotolerance, Isolates

INTRODUCTION

Cryptococcus neoformans is a spherical, encapsulated, non myceliated, non-fermenting yeast like fungal cell (Okagaki *et al*., 2010). It is pathogenic to humans and animals. *C. neoformans* causes fatal meningitis primarily in immunosuppressed individuals (Cogliati, 2012).The major environmental sources of *C. neoformans* have been found to be soil contaminated with pigeon droppings, on Eucalyptus tree and decaying wood forming hollows in living trees (Chakrabarti *et al*., 1997). The organism exists as both free living and in association with other variety of hosts (Olszewski *et al*., 2010). The primary mode of transmission is inhalation of spores from the environment.

Infectious disease caused by *Cryptococcus neoformans* is called cryptococcosis. *C. neoformans* only have adverse effect on immunocompromised patients though it can persist in healthy individuals in the latent form. In humans, *C. neoformans* infection is spread by inhalation of aerosolized basidiospores and can disseminate to the central nervous system where it can cause meningoencaphalitis (Velagapudi *et al*., 2009). *C. neoformans* strains manifest antigenic differences that allow them to be grouped into five different serotypes (A, B, C, D, AD hybrids) as well as different varieties. Recognition of *C. neoformans* by gram staining is hampered by the presence of large gelatinous capsules (Bottone, 1980). However microscopic recognition of *C. neoformans* is made possible using India ink stain (Zerpa, *et al*., 1996) which is relatively expensive. This was the basis on which we decided to explore other methods for screeningfor *C. neoformans.*

Urease test is a routine test in the identification of *C. neoformans*, urease activity has also been linked with virulence in this fungus (Fu *et al*., 2018). In the bacteria *Helicobacter pylori* and *Proteus mirabilis*, urease activity has been found to be an important factor in pathogenicity of these organisms (Eaton *et al.*, 1991; Tsuda *et al*., 1994). With this background, we decided to use urease activity and temperature (thermotolerance) in screening for pathogenic *C. neoformans*. Probably, the two factors may be able to differentiate between pathogenic and non-pathogengeic *C. neoformans.*

MATERIALS AND METHODS

Collection of Samples:

Fourteen (14) samples of pigeon droppings were collected from 3 selected markets located in Benin City, Edo State markets. Accordingly, 4 samples were collected from Oba market, 5 samples from New Benin market and 5 samples

were collected from Uselu market. A sterilized spatula was used to transfer samples into McCartney bottles and properly labeled according to area of collection. The samples were taken to the laboratory in the Department of Plant Biology and Biotechnology, University of Benin, Benin City where the work was carried out.

Media

Sabouraud dextrose agar (SDA) was used to culture the initial samples and to store the verified isolates. Niger seed medium was used as a selective medium for the confirmation of the presence of *C. neoformans*. Urea base **(**Christensen) agar medium was used for urease test**.**

Preparation of Isolates

About 1 g of collected pigeon dropping samples were added to 10 ml of sabouraud dextrose broth, followed by mild shaking to allow the inoculum dissolve, then incubated at 25 °C for 24 hours. Approximately 100 µL sabouraud dextrose broth was spread on Sabouraud Dextrose Agar (SDA) medium and incubated using the Gallenhamp cooled incubator at 37°C for a period of 5 days. Isolates from the cultures were further subcultured on SDA to obtain contamination free single colonies. The resulting isolates were later cultured in Niger seed medium as a selective medium for *Cryptococcus neoformans*.

Urease Test Assay

Urease activity was carried out as described by Junior *et al*. (2013); i.e. 20% urea solution was added to already prepared Christensen agar medium. Afterwards, McCartney bottles with urease medium in slant were inoculated with *Cryptococcus neoformans* and left at 25°C for 48 hours. Observation was made for colour change. The initial color of the medium was yellow before incubation at 25°C. A color change from yellow to pink after incubation for a day indicates urease enzyme activity where urea is hydrolyzed to ammonia and carbamate (Casadevall and Steenberg, 2003).

Temperature Test

Poured plates with medium, were inoculated with isolates of *Cryptococcus neoformans* from the different samples and were incubated at 25ºC The procedure was repeated at 37ºC. At the end of 48 hour incubation period, the growth diameter for each isolates was measured in millimeter. From the above tests, those positive reflect the presence of the organism and passed both test, while negative revealed the absence of the organism. For a sample to be positive, it has to pass both test. The samples were tested thrice for the presence of *C. neoformans*.

Statistical Analysis:

Isolates that tested positive were analysed for thermotolerance difference, using student T-test (SPSS v 20). The mean diameter growth of the organisms were measured after 48 hours at incubation temperatures of 25ºC and 37ºC and the means compared for statistical differences at $p< 0.01$.

RESULTS

Tables 1 shows the results of screening for *C. neoformans* using urease and thermotolerance test sequentially. Samples from Oba market were negative while the highest number of isolates was obtained from samples obtained from New Benin and Uselu markets.

DISCUSSION

The isolation of *C. neoformans* from pigeon droppings in the sites visited confirms pigeon as a habitat for this opportunistic pathogen and has been confirmed also by many researchers in different part of the world. Pigeon droppings have been reported as important substrate for the presence and maintenance of *C. neoformans* in the environment (Cassadevall *et al*., 1996).

The presence of *C. neoformans* recovered in the environment is an important finding. This fungus has been reported as an agent of opportunistic infections such as meningitis, lung infections, fungemia, and abscess and skin infection mainly with patients with great deteriorations (Chang *et al*. 2004; Mitchel, and Perfect 1995).

Table 1: Urease and Thermotolerance Testof *C. neoformans*Isolated from Pigeon Droppings

Site name	Sample	First isolation	Second isolation	Third isolation
Uselu	Use 1	-	+	-
New Benin	NB 3	+	+	+
Oba market	Oba 2	-	-	-
Uselu	Use 3b	+	-	+
Oba market	Oba 3	-	-	-
Uselu	Use 2	-	+	-
New Benin	NB 1	-	+	-
Uselu	Use 2b	-	+	+
Uselu	Use 1b	+	-	+
Oba market	Oba 4	-	-	-
New Benin	NB 5b	+	+	+
New Benin	NB 5c	+	-	+
New Benin	NB 5d	+	-	+
Oba market	Oba 1	-	-	-

+ isolate present - isolate absent.

Figure 1 presents results of thermotolerance test which suggests no significant difference in the growth of isolates at 37 °C and 25 °C.

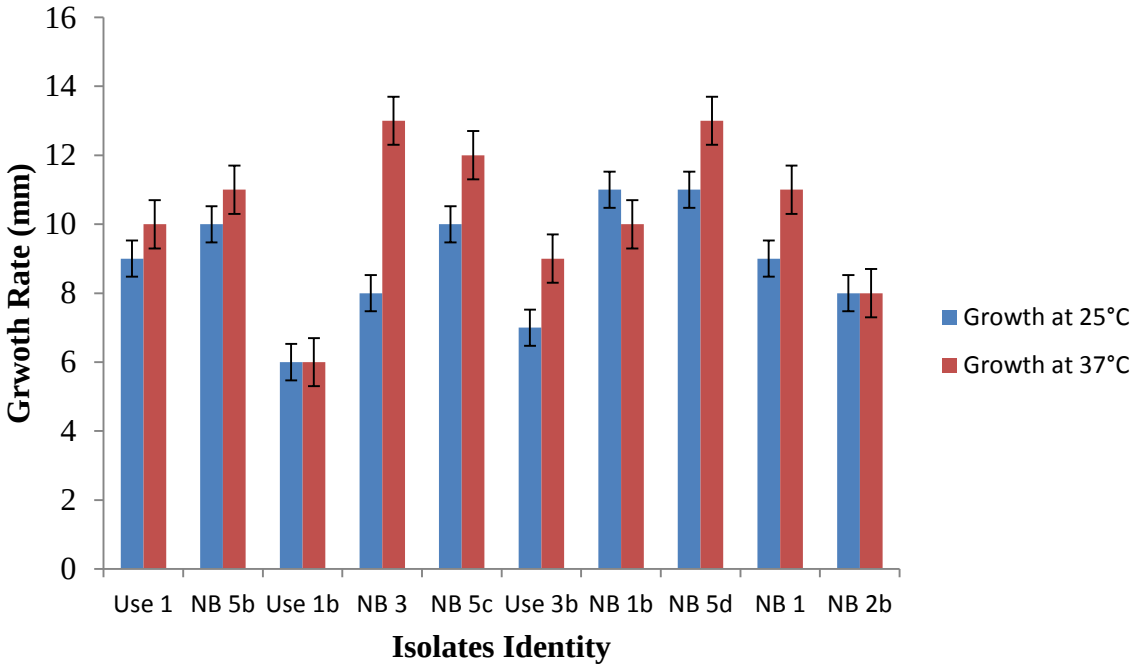

Figure1: Growth rate of *C. neoformans* isolates from Pigeon Droppings.

Several factors including urease activity have been associated with the virulence of *C. neoformans* (Cox *et al.,* 2000). Ability to grow at 37°C (Figure1) showed that thermotolerance is also a virulence factor for this organism. Ability to grow at 37°C is generally accepted as virulence factor uniquely for *C. neoformans* (Kwon-Chung, *et al*., 1982; Kwon-Chung and

Rhodes, 1986). The results of the screening showed that both urease and thermotolerance test can screen out samples that do not contain *C. Neoformans*. This study has attempted to match urease factor with that of temperature (ability to grow at 37°C) in screening and selection of pathogenic *C. neoformans*. According to Casadevall and Steenberg (2003) urease enzyme plays a major role as a virulence factor of several pathogenic agents such as *Cryptococcus neoformans* and *Cryptococcus gatti*. Interestingly, all the isolates that were positive at 37°C also tested positive for the urease test. This suggests that thermotolerance and urease test can serve as complement alternative for screening and selection of pathogenic *C. neoformans*.

CONCLUSION

In conclusion, this experiment demonstrates the possibility of isolating *C. neoformans* at minimum temperature 37°C thermotolerance and urease activity tests. Pathogenicity studies is recommended to further understand host – pathogen relationship.

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