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TYPING OF CRYPTOCOCCUS NEOFORMANS ISOLATES RECOVERED FROM DROPPINGS OF PIGEONS, PARROTS AND CANARIES

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SUMMARY

Biotyping of 9 isolates of *Cryptococcus neoformans* recovered from bird droppings was carried out on Canavanine-glycine-bromothymol blue (CGB) and Glycine-cycloheximide-phenol red (GCP) media. Only one isolate was identified as *C. neoformans var gattii* (serotype B-C) because it produced cobalt-blue colour on CGB medium and red colour on GCP medium. Five isolates were considered as *C. neoformans var neoformans* (serotype A-D) as they produced no change in colour on both media, while 3 isolates were suspected to be *C. neoformans var neoformans*, as they produced light blue colour on CGB medium, but no colour on the GCP medium. Polymerase chain reaction (PCR) was used for identifying and biotyping of six *C. neoformans* isolates, where two sets of base pairs C. *neoformans biotypes* specific were applied. The PCR results revealed that 5-isolates, which were identified and biotyped by conventional methods as *C. neoformans var neoformans*, in addition to the standard strain responded to the first primer of 695 bp, i.e. they were identified as serotype A. Only one isolate, which was identified as *C. neoformans var gattii* was amplified by the second primer pair of 448 bp that confirmed this isolate as serotype B.

INTRODUCTION

For the serotyping of *Cryptococcus* isolates, Evans and Kessel (1951) depended on antigenic differences that were detected with rabbit adsorbed sera in classification of *C. neoformans*

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into four serotypes. From the epidemiological point of view, it is important to identify *C. neoformans* isolates up to varieties and serotypes. According to Kwon-Chung et al. (1982) and Salkin and Hurd (1982), *C. neoformans* species are classified into two varieties; *C. neoformans var neoformans* (serotypes A and D) and *C. neoformans var gattii* (serotype B and C).

The biotyping of *Cryptococcus* species is based primarily on colour changes on two media, namely, *Canavanine glycine* bromothymol-blue (CGB) (Kwon-Chung et al., 1982) and Glycine cycloheximide phenol-red medium (GCP) (Salkin and Hurd,1982). *C. neoformans var neoformans* doesn't change the colour of both media, while *C. neoformans var gattii* induces colour changes into cobalt blue in the first medium and red in the second one.

Kreger-Van-Rij (1984) studied the urease activity for most of yeast species and she stated that urease activity is one of the most important biochemical proprieties for members of the genus *Cryptococcus* and basidiomycetous yeast. Kwon--Chung et al. (1987) reported that there are significant differences between two varieties of *C. neoformans* especially with regards to the nature of their urease activity. They concluded that yeast cells grown on yeast extract peptone glucose agar (YEPG) medium showed a high level of urease activity in both varieties, while cells grown on the YEPG agar with 100μM EDTA (Ethylenediamine-tetra-acetic acid) showed a marked reduction and inhibition of urease synthesis in *C. neoformans var gattii.* They recommended the CGP, GCP, YEPG media with and without EDTA for the biotyping of *Cryptococcus* isolates. Kabasawa et al. (1991) used commercial monoclonal antibodies to *Cryptococcus* capsular epitopes for serotyping of *C. neoformans* isolates into A, B, C, D, and AD serogroups. On the other hand, Boekhout et al. (2001) used amplified fragment length polymorphism (AFLP) to clarify variable status of serotype AD strain from the other three-varieties of *C. neoformans.*

The aim of the present work was to determine the biotypes and serotypes of *Cryptococcus* isolates recovered from droppings of parrots, canaries and pigeons.

MATERIAL AND METHODS

1. Cryptococcus isolates:

Nine isolates of *Cryptococcus* and one isolate *Candida albicans* were obtained from the culture collection of the Mycology Laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University. These isolates were previously isolated by Heidy Abo El-Yazeed et al. (2006) from droppings of parrots, canaries and pigeons. A standard strain of *C. neoformans* (ATCC 90112 CSF Pennsylvania isolate) was used as a positive control.

2. Media used for biotyping of *Cryptococcus neoformans* :

The following media were used:

* Yeast extract-peptone-glucose agar with (YEPG) or without EDTA (YEPGE) (Kwon-Chung et al., 1987),
* Rapid urea hydrolysis broth (RUH broth) 2X concentration: (Roberts et al., 1978),
* Canavanine glycine bromothymol blue medium (CGB) (Kwon-Chung et al., 1982),

Glycine-cycloheximide-phenol red medium (GCP) (Salkin and Hurd, 1982).

3. Buffers and solutions used for DNA extraction from all *C. neoformans* isolates was prepared according to Sambrook et al. (1989)

METHODS

1. Biotyping of *Cryptococcus* isolates using;

a) Canavanine-glycine-bromothymol blue agar (CGB) (Kwon-Chung et al., 1982):

A loopful from 48-hours old culture of the tested *Cryptococcus* isolates was streaked on the CGB plate. The positive result was detected by change of pH from 5.8±0.1 (greenish yellow) to at least 7.0 (cobalt blue). The test is based on the ability of *C. neoformans var gattii* (serotypes B and C) isolates to grow in the presence of L-canavanine and to utilize glycine as a sole source of carbon, so the colour of CGB medium change from greenish yellow to blue green within 2-5 days at 25°C, while *C. neoformans var neoformans* (serotypes A and D) failed to grow when cultured on a medium containing glycine and canavanine.

b) Glycine-cycloheximide-phenol red medium (GCP) (Salkin and Hurd, 1982):

C. neoformans isolates were cultured on SDA medium for 48-hours, then a portion of their growth was removed with a sterile transfer loop and streaked over the surface of GCP medium and then were incubated at 27°C. The culture was checked each day for growth and colour change. *C. neoformans var neoformans* (serotypes A and D) did not grow within an experimental period of 5-days, while *C. neoformans var gattii* (serotypes B and C) grew and their growth was indicated by changing medium colour from yellow orange to bright red after 2-5 days at 20°C.

c) Urease inhibition by addition of EDTA to YEPG agar (Kwon-Chung et al., 1987) :

The *C. neoformans* isolates were grown on SDA slant for 48 - hour at 30°C. A loopful of cells was obtained from 48-hour slant culture and streaked on YEPG agar or YEPG agar with 100 M EDTA (YEPGE). The cells were incubated for 48-hour at 30°C, then it was tested for urease activity on RUH broth. *C.neoformans var neoformans* (serotype A and D) isolates produced positive urease activity within 1- to 3-hour incubation period at 37°C, while *C. neoformans var gatt* (serotype B and C) don't produce a positive urease activity on RUH broth during the same period.

d) Rapid urea hydrolysis broth (RUH broth) (Roberts et al., 1978) :

RUH broth was prepared in 2X concentration with urea (4.0 g). A loopful of 48-hour *Cryptococcus* colonies grown at 30°C on YEPG and YPEGE, was suspended in 2ml of sterile distilled water. The cell count in the suspension varied from 1 X 10^8 to 2 X 10^8 /ml. The cell suspension was vortexed and 1 ml of the suspension was added to 1 ml of 2 X RUH broth. The RUH broth with the cell suspension mixture was incubated at 37°C in a shaker water bath (40 Oscillation per min.). The tubes were examined every hour for 4-hours. Positive urease activity was indicated by magenta red colour, while negative one by orange yellow colour.

3. Serotyping of Cryptococcus neoformans isolates using polymerase chain reaction (PCR):

Cryptococcus neoformans DNA extraction was done according to Frederick et al. (1987) and amplification of the specific DNA fragments was done according to Aoki et al. (1999). The following primers were used:

PCR primers used for amplification

Serotype PCR primer pairs (base sequence)	DNA band size
Serotype A: CNa-70S (5'-ATTGCGTCCACCAAGGAGCTC-3') CNa-70A (5'-ATTGCGTCCATGTTACGTGGC-3')C	***C. neoformans var neoformans*** serotype A (695 bp)
Serotype B: CNb-49S (5'-ATTGCGTCCAAGGTGTTGTTG'3) CNb-49A 5"-ATTGCGTCCATCCAACCGTTATC-3')	***C. neoformans var gattii*** serotype B (448 bp)

RESULTS

1- Biotyping of *Cryptococcus neoformans* isolates:

a. Biotyping of Cryptococcus neoformans isolates based on colour change on CGB and GCP media:

As shown in Table (1) it is clear from the results that only isolate No. 4-p was identified as *C. neoformans var gattii* (serotype B or C) because it produced a cobalt-blue colour on CGB medium and red colour on GCP medium. The remaining 8-isolates were considered as *C. neoformans var neoformans* (serotypes A and D) as they produced no change of colour in both media.

Table (1) Biotyping of *Cryptococcus neoformans* isolates based on colour change on CGB and GCP media

Isolates Code No.	Colour change		*Cr. neoformans* biotype
	CGB	GCP	
1-P	-	-	*C. neoformans var neoformans* (A-D)
2-C	-	-	*C. neoformans var neoformans* (A-D)
3-C	-	-	*C. neoformans var neoformans* (A-D)
4-P	++	$+^1$	*C. neoformans var gattii* (B-C)
5-C	+	-	*C. neoformans var neoformans* (A-D)
6-P	-	-	*C. neoformans var neoformans* (A-D)
8-C	-	-	*C. neoformans var neoformans* (A-D)
11-Pi.	+	-	*C. neoformans var neoformans* (A-D)
12-Pi.	-	-	*C. neoformans var neoformans* (A-D)
(+ve)*control	-	-	*C. neoformans var neoformans* (A-D)
(-ve) control	-	-	*Candida albicans*

P= Parrot, C= Canary, Pi= Pigon

+ : Light blue $+^1$: red (+) : Weak red ++: Cobalt blue

***(+ve) control = the standard *C. neoformans strain*,**

Table (2) Results of urease test on *Cryptococcus neoformans* isolates grown on YEPG medium without EDTA

The used isolates	No. of positive isolates at different incubation times (hr)					
	1hr	2hr	3hr	4hr	5hr	6hr
C. neoformans var neoformans (A-D) 8-isolates	2/8	4/8	4/8	5/8	6/8	8/8
C. neoformans var gattii (B-C) 1-isolate	1/1	1/1	1/1	1/1	1/1	1/1
(+ve control) C. neoformans var neoformans (A-D)	1/1	1/1	1/1	1/1	1/1	1/1
(-ve control) (Candida albicans) 1-isolate	0/1	0/1	0/1	0/1	0/1	0/1

Table (3) Results of urease test on *Cryptococcus neoformans* isolates grown on YEPG medium with 100µM EDTA

The used isolates	No. of positive isolates at different incubation times (hr)					
	1hr	2hr	3hr	4hr	5hr	6hr
C. neoformans var neoformans (A-D) 9-isolates	1/8	2/8	4/8	4/8	8/8	8/8
C. neoformans var gattii (B-C) 1-isolate	0/1	0/1	0/1	0/1	0/1	0/1
(+ve control) C. neoformans var neoformans (A-D)	1/1	1/1	1/1	1/1	1/1	1/1
-ve control *(Candida albicans)* 1-isolate	0/1	0/1	0/1	0/1	0/1	0/1

b. Biotyping of *C. neoformans* isolates on the basis of urease inhibition by EDTA:

As shown in Tables (2 and 3), all isolates previously biotyped as *C. neoformans var neoformans* were positive in urease test, when grown on yeast extract peptone glucose agar medium with or without EDTA, the same as the standard *C. neoformans* strain. On the other hand, the isolate No. 4-p, which was biotyped as *C. neoformans var gattii* was negative on urease test as the enzyme synthesis was inhibited on YEPGE medium. This confirms the afore-mentioned biotyping. The Tables also indicate that the suitable incubation time to get a reliable result was 6-hr.

2. Amplification by polymerase chain reaction:

Six *Cryptococcus* isolate were tested by PCR using primer pairs specific for both serotypes A and B. The first pair was used for amplification of a DNA sequence of 695 bp, specific for *C. neoformans var neoformans* serotype A and the second primer pair was used for amplification of a DNA sequence of 448 bp, specific for *C. neoformans var gattii*, seroptype B.

The results of amplification are shown in Table (4). It is clear that 5 isolates identified by the conventional methods as *C. neoformans* var neoformans, in addition to the standard strain responded to the first primer of 695 bp, i.e. they can be identified as serotype A. Only isolate No. 4-p, which was identified as *C. neoformans* var gattii was amplified by the second primer pair of 448 bp and this could be confirmed as serotype B (Fig. 1).

Table (4) Results of polymerase chain reaction for the examined *Cryptococcus neoformans* isolates

Isolates Code No.	Serotype and PCR Primer pair	Expected DNA band size (bp) by PCR
1-P	(CNa-70-S / CNa-70-A)	695
	(CNb-49S / CNb-49A)	Negative
2-C	(CNa-70-S / CNa-70-A)	695
	(CNb-49S / CNb-49A)	Negative
3-C	(CNa-70-S / CNa-70-A)	695
	(CNb-49S / CNb-49A)	Negative
4-P	(CNa-70-S / CNa-70-A)	Negative
	(CNb-49S / CNb-49A)	448
5-C	(CNa-70-S / CNa-70-A)	695
	(CNb-49S / CNb-49A)	Negative
6-P	(CNa-70-S / CNa-70-A)	695
	(CNb-49S / CNb-49A)	Negative
Positive control	(CNa-70-S / CNa-70-A)	695
	(CNb-49S / CNb-49A)	Negative
Negative control	(CNa-70-S / CNa-70-A)	Negative
	(CNb-49S / CNb-49A)	Negative

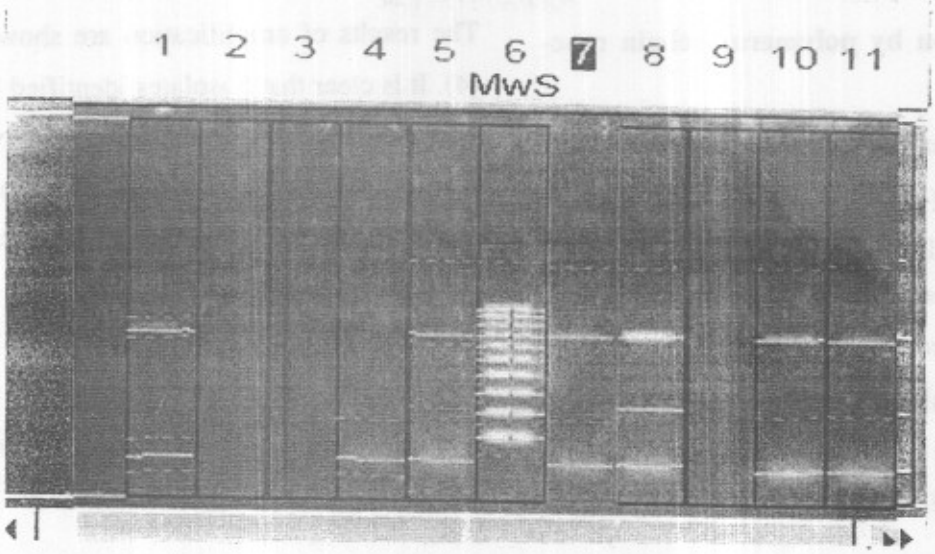

***Fig.(1)* : Results of analysis of Electrophoretic profile of PCR products from different yeast isolates amplified using the primer (CNa-70-S / CNa-70-A) by gel documentation system SYNGENE, GENE GENIUS, BIO IMAGING SYSTEM EV 700. Gateway computer program.**

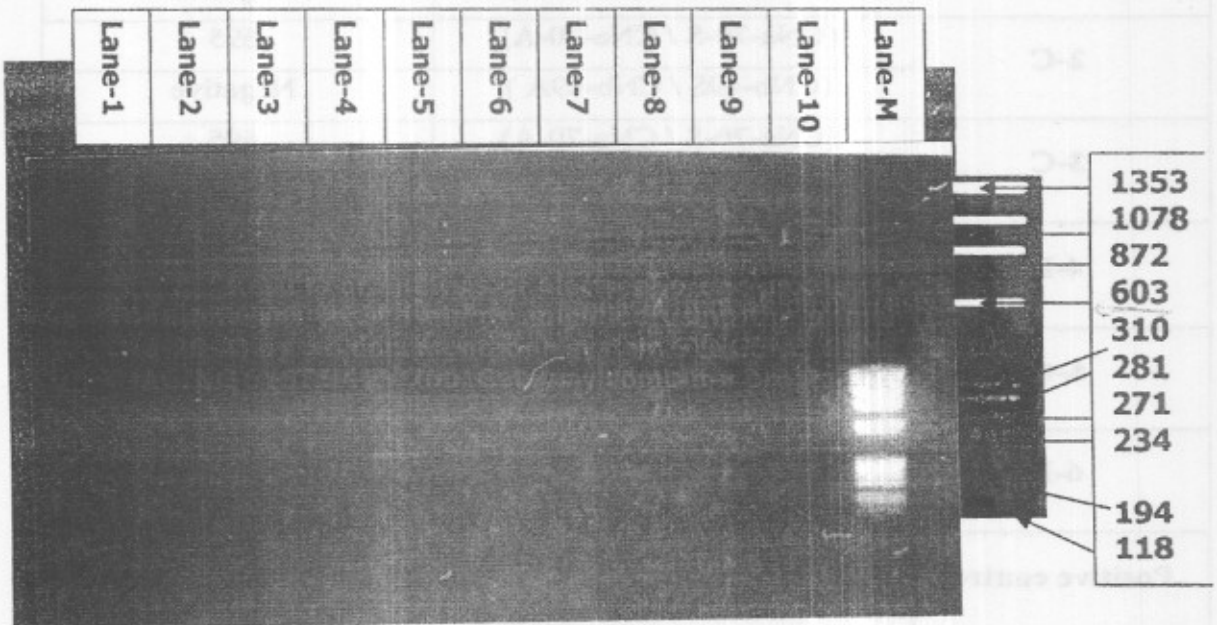

Fig. (2): Electrophoretic profile of PCR products from different yeasts isolates amplified using the primer (CNb-49S/CNb-49A): Lane (4) Isolate 4-P. Lane (M) X174 DNA- Hae III Digest used as molecular marker.

while the isolate biotyped as *C. neoformans* var gattii didn't produce a fragment with CNa-70S and CNa-70A primer pair. On the other hand, CNb-49S and CNb-49A primer pair amplified a 448 bp fragment only from this isolate, which confirm its identification as *C. neoformans var gattii* (serotype B).

If the classification mentioned by Franzot et al. (1999), who grouped the pathogen into 3 varieties, namely *C. neoformans var neoformans* (serotype D and AD), *C. neoformans var gattii* (serotype B and C) and C. neoformans var grubii (serotype A) is considered, then the isolates recovered in the present work may be classified on the basis of the PCR amplification of the specific primers into *C. neoformans var grubii* and *C. neoformans var gattii.*

Meyer et al. (1999) reported that PCR fingerprinting could be the major typing technique for global molecular epidemiological survey of *C. neoformans* and divided more than 4000 clinical and environmental isolates into 8 major molecular types VNI and VNII (var grubii, Serotype A), VNIII (Serotype AD), VNIV (var neoformans, Serotype D) VGI, VGII, VGIII and VGIV (var gattii, Serotyoes B and C).

In the study of *C. neoformans* ecology, it was found that the great differences between *C. neoformans* varieties are mainly in its geographical distribution and habitat (Kwon-Chung and Bennett, 1984, Levitz, 1991, Kwon-Chung and Bennett, 1992, Sorrell and Ellis, 1997 and Casadevall and Perfect, 1998). *C. neoformans var gattii* is restricted mainly in tropical and subtropical regions and commonly occurs in patients with normal immune status (Rozenbaum and Gonçalves, 1994 and Speed and Dunt, 1995), whereas *C. neoformans var neoformans* and *C. neoformans* var grubii are distributed throughout the world (Bennett et al., 1977) and are usually the causative agent of cryptococcosis in patients affected with AIDS or imunocompromised persons due to other reasons (Bottone et al., 1987).

Althouh *C. neoformans var gattii* is restricted to some geographical areas, the determination of the main natural habitat of this variety remained unknown for a long time till 1990, when Ellis and Pfeiffer (1990-a and-b) established that *C. neoformans* appears to have a specific ecological association with *Eucalyptus camaldulensis*. Then in 1992 Ellis and Pfeiffer reported again the isolation of *C. neoformans var gattii* from Eucalyptus. It was found that *C. neoformans var gattii* has other sources, such as brown kiwi (Hill et al. 1995), African Grey parrot (Sorrell et al., 1996) bats, koal and other mammals (Ellis and Pfeiffe 1990-a and -b, Lazéra et al., 1993).

The recovery of only one isolate of *C. neofo mans var gattii* out of 8 isolates recovered fro parrot and canary droppings in Giza zoo does n substantiate the particular role of eucalyptus tre in the ecology of this variety, in as much as t

zoo is full of such types of trees. On the other hand, the high numbers of recovery of *C. neoformans var grubii* from birds in the zoo may be either due to the presence of carrier state among these birds or that the rich plants in the zoo provides a favouring ecology for this *C. neoformans* variety.

The results of serotyping obtained in the present work are substantiated by the findings published by other workers. Steenbergen and Casadevall (2000) analyzed 40 *C. neoformans* isolates from New York and their prevalence. Their study revealed that 39 strains were typeable strains, from them 85 % were *C. neoformans var grubii* (serotype A), 12.5 % were *C. neoformans var neoformans* (serotype D), and 2.5% were serotype AD. Boekhout et al. (2001) showed that most global worldwide variety was *C. neoformans var grubii*, 73.8% (n=251) followed by variety gattii, 20.3 % (n=69). Nishikawa et al. (2003) made a serotyping for 467 *C. neoformans* isolates from clinical and environmental sources from Brazil. The results of serotyping revealed a prevalence rate of 77.95% for serotypes A followed 18.2% for serotype B then serotype AD (1.3%), D (0.4%), C (0.2%) and untypeable (1.93%). Meyer et al. (2003) made a molecular typing for 340 *C. neoformans* isolates from nine-countries depended on PCR fingerprinting. They concluded that, 251/340 (73.68%) were *C. neoformans var grubii*, 6/340 (1.7%) were *C. neoformans var neoformans*, and 13/340 (3.8%) were AD hybrid isolates. The remaining 69/340 (20.2%) isolates were *C. neoformans var gattii.*

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تصنيف عترات الكربتوكوكس نيوفورمانس المعزولة من بزق الحمام والببغاوات وعصافير الكناريا

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اثبت التصنيف البيولوجى لعدد ٩ عترات من فطر الكربتوكوكس نيوفورمانس المعزولة من بزق الطيور والذى تم عمله باستخدام منابت ميكروبية متخصصة ان معزولة واحدة فقط تنتمي الى الكربتوكوكس نيوفورمانس فار جاتى (نوع سيرولوجي C-B) حيث ادى نموه علي المنبت الميكروبى CGB الى تكوين لون ازرق مخضر وانتج لون احمر علي المنبت الاخر GCP. عدد خمسة معزولات تم تصنيفها بنفس الطريقة الى الكربتوكوكس نيوفورمانس فار نيوفورمانس (نوع سيرولوجي D- A) حيث ادى نموها على المنابت السابقة الى عدم حدوث تغير في اللون. ثلاثة معزولات انتجت لون ازرق خفيف على منبت ال CGB ولم تنتج اى لون علي منبت ال GCP وقد ادي ذلك الى احتمالية تصنيفها علي اساس انها الكربتوكوكس نيوفورمانس فار نيوفورمانس. اثبت استخدام تفاعل البلمرة المتسلسل لعمل تصنيف دقيق الي ٦ من معزولات الكربتوكوكس نيوفورمانس ان خمسة معزولات من هذا الفطر والتى تم تصنيفها بالطرق التقليدية علي انها كربتوكوكس نيوفورمانس فار نيوفورمانس وكذلك العترة القياسية من هذا الفطر انها كلها تنتمي الي النوع السيرولوجي A بينما العترة الاخيرة والتي سبق تصنيفها علي انها الكربتوكوكس نيوفورمانس فار جاتي انها تنتمي الي النوع السيرولوجي B.