

MATING TYPE DETERMINATION OF *CRYPTOCOCCUS NEOFORMANS* ISOLATES OBTAINED FROM FAECAL SAMPLES OF VETERINARY ANIMALS IN BOTSWANA

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ABSTRACT

Cryptococcus neoformans/gattii species complex cause cryptococcosis, a fatal condition in susceptible humans and animals. This paper aims to find the association of *C. neoformans* with veterinary excreta, and to carry out a mating type determination of the isolated strains. Two hundred sixty seven veterinary excreta samples from cattle, goats and poultry (chickens) were analysed for the presence of *C. neoformans*. Morphological and biochemical characteristics were used to identify *C. neoformans*, and mating type-specific PCR amplification was utilized to determine the mating type of the isolates. A total of 72 (26.9%) isolates were found positive for *C. neoformans*, mostly isolated from cattle (14.9%), followed by poultry (9.4%) and goats (2.6%). However, the isolation rates of the organism from veterinary animals were not statistically significant ($p < 0.05$). Mating type analysis of the 72 positive isolates revealed the presence of *MATa*, *MATa/MATa* (hybrids) and *MATa* in cattle (31.9%, 4.2%, 19.4%) > poultry (19.4%, 2.8%, 12.5%) > and goats (8.3%; 0%, 1.4%), respectively. The frequency of isolation of different mating types was not influenced by the veterinary animals ($p < 0.05$) or sampling location ($p < 0.05$). The results of our study expand the ecological niche of *C. neoformans* in Botswana and highlight the need to mitigate the dissemination of the pathogen to the population at risk.

Keywords: *Cryptococcus neoformans*, Veterinary Animals, Mating Type, *MATa*, *MATa*, Hybrid Genotypes

INTRODUCTION

Cryptococcus neoformans causes one of the deadliest infections known as cryptococcosis. The disease is believed to be acquired from the environment through the inhalation of infectious basidiospores or desiccated yeast cell and commonly presents as meningoencephalitis and pulmonary infection (Velagapudi *et al.*, 2009).

The causative agents comprise the *Cryptococcus neoformans/gattii* species complex, and *Cryptococcus neoformans* has two varieties; *Cryptococcus neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (Frazot *et al.*, 1999). *Cryptococcus gattii* (serotypes B and C), the second etiologic agent, was previously classified as *C. neoformans* var. *gattii* but a plethora of morphological, biochemical and molecular methods have been utilized to discriminate it into a discrete species (Kwon-Chun *et al.*, 2002; Bovers *et al.*, 2008a).

Furthermore, molecular typing studies have identified eight genotypes within the *Cryptococcus neoformans-gattii* species complex. Genotypes VNI to VNIV belong to *Cryptococcus neoformans* serotypes A, D, and AD hybrids whilst *Cryptococcus gattii* comprises VGI to VGIV (Bovers *et al.*, 2008b). In addition, a ninth genotype VNB exists among serotype A isolates from Botswana (Litvintseva *et al.*, 2006).

The two etiologic agents of cryptococcosis differ in ecology, pathogenicity, epidemiology and susceptibility to antifungal agents (Kwon-Chung *et al.*, 2002). *Cryptococcus gattii* is considered a primary pathogen as it infects immune-competent people (Cassedavall and Perfect, 1998; Litvintseva *et al.*, 2005a), although it has also been recovered from AIDS patients in some African countries (Sorrel, 2001; Litvintseva *et al.*, 2005b). *Cryptococcus neoformans* on the other hand is an established opportunistic pathogen and is responsible for the majority of cases of cryptococcosis in AIDS patients (Cassedavall and Perfect, 1998; Litvintseva *et al.*, 2005a).

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Cryptococcus neoformans is noted for its close association with pigeon excreta (Sorrel and Ellis, 1997) while *C. gattii* is associated with several tree species (Sorrel and Ellis, 1997; Kidd *et al.*, 2007). From these sources, humans are thought to become infected after exposure and inhalation of infectious basidiospores (Velagapudi *et al.*, 2009). Cryptococcosis is also known to cause diseases in domestic animals (Malik *et al.*, 2003).

Virulence in *C. neoformans* is due to possession of several factors, one of which is melanin production (Salas *et al.*, 1996) that protects the yeasts from host immune defences. Cryptococcal virulence has also been linked to a bipolar mating type (*MAT*) locus (Nielsen *et al.*, 2005) consisting of *MATa* and *MATb*. Globally, most environmental and clinical isolates are *MATa* (Halliday *et al.*, 1999) and they have been shown to be more virulent than congenic *MATb* strains (Nielsen *et al.*, 2005). In Botswana, both mating types have been recovered from environmental and clinical isolates (Litvintseva *et al.*, 2003; Litvintseva *et al.*, 2006).

The possession of both mating types among isolates in Southern Africa implies sexual reproduction and recombination hence the higher genetic diversity associated with African populations of *Cryptococcus neoformans* and *Cryptococcus gattii* (Litvintseva *et al.*, 2012).

Various studies have characterized *Cryptococcus neoformans* isolates from environmental (Litvintseva *et al.*, 2011) and clinical (Litvintseva *et al.*, 2005b) sources. However, the prevalence of *Cryptococcus neoformans* in veterinary animals in Botswana remains to be determined. This paper, therefore, aims to isolate *Cryptococcus neoformans* from faecal samples of veterinary animals, and determine the mating types among the isolates.

MATERIALS AND METHODS

Sampling

Faecal samples were collected from three towns; Serowe and Palapye in Northern Botswana and Gaborone in Southern Botswana from October 2015 to March 2016. Aged faeces from cattle, goats and poultry were collected according to a modified protocol of Ellis and Pfeifer (1990). Aseptic techniques were used to collect the faecal matter into sterile 30ml centrifuge tubes (Corning, New York, USA) which were then placed in a cooler and transported to the laboratory. Upon arrival at the lab, 2 g of the solid excreta was dissolved in 20ml sterile phosphate buffered saline (Oxoid, Basingstoke, UK). A further dilution of 1:100 was made and both diluents were shaken at room temperature on an orbital shaker (Innova, New Brunswick, USA) set at 150rpm for 2h. All samples were processed within 3 days after collection.

Isolation and Identification of *Cryptococcus neoformans*

After shaking, duplicate inocula of 50µl of undiluted and 1:10 and 1:100 dilutions were then spread plated on benomyl Niger seed agar (Pham *et al.*, 2014) supplemented with 0.4g chloramphenicol (Sigma-Aldrich, St. Louis, USA) and 0.025g getamicin (Sigma-Aldrich) per litre. Samples thus plated were incubated for 3 to 4 days at room temperature. Brown yeast colonies characteristic of *Cryptococcus neoformans* were then purified on Niger seed agar (without benomyl and antibiotics).

Purified isolates were observed microscopically for typical cryptococcal cells and identified biochemically for urease activity and assimilation of potassium nitrate.

Isolates that showed urease activity and did not reduce nitrate were tentatively identified as *Cryptococcus neoformans*/*Cryptococcus gattii* species complex. The identified isolates were preserved at -80°C in tryptose soy broth (Merck, Darmstadt, Germany) containing 15% glycerol until used in the subsequent steps.

DNA Extraction and Mating Type Determination

Each isolate was cultured on Yeast-extract peptone dextrose agar (Oxoid, London, UK). A small portion was then collected directly from the solid medium for the extraction of genomic DNA using a sterile wooden applicator stick. Extraction and purification of genomic DNA was done using the Master Pure Yeast DNA purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to manufacturer's instructions.

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Mating type was determined using a Gene AMP PCR system (Applied Biosystems, Carlifornia, USA. *Cryptococcus neoformans/gattii* universal primer per sets: STE20-Aa-f, 5'-CTAACTCTACTACACCTCACGGCA-3' (forward primer) and STE20-Aa-r, 5'-CGCACTGCAAAATAGATAAGTCTG-3' (reverse primer); STE20-Aα-f, 5'-GGCTGCAATCACAGCACCTTAC-3' (forward primer) and STE20-Aα-r, 5'-CTTCATGACATCACTCCCCTAT-3' (reverse primer) (Li *et al.*, 2012) were used to determine the mating type of each isolate.

All primers were obtained from Inqaba Biotec, Pretoria, South Africa. Each PCR reaction mixture consisted of 12.5µl of 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 1µl each of reverse and forward primers, 2µl of genomic DNA and the mixture made up to 25µl with sterile nuclease-free water.

Two strains; *Cryptococcus neoformans* MOL 19 strain (mating type a) and *Cryptococcus neoformans* H99 strain (mating type α), both obtained from the National Health Laboratory, Gaborone, Botswana were used as positive controls.

The PCR amplification protocol was as follows; initial denaturation was done at 95°C for 5 minutes, followed by 34 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, amplification at 72°C for 30 seconds and final extension at 72°C for 5 minutes. The PCR amplicons were electrophoresed on 1.2% agarose gel (Biorad, Hercules, California, USA) and then visualized with the Syngene gel documentation system (Syngene, Cambridge, UK).

Statistical Analyses

Graphpad Prism 7 (GraphPad Software Company, LaJolla, CA, USA) was used to analyze the statistical significance of the data. One-way ANOVA was employed to separate the means of occurrence of *C. neoformans* in different veterinary animals and locations investigated in this study. Fisher's exact tests were used to analyze the frequency of mating types in farm animals in Serowe, Palapye and Gaborone.

RESULTS AND DISCUSSION

Results

Out of 267 veterinary faecal samples only 72 samples (26.9%) tested positive for *Cryptococcus neoformans* (Table 1). Most isolates that tested positive were from cattle (14.9%), followed by poultry (9.4%) and goats only accounted for 2.6% of the isolates. However, the isolation rates of *C. neoformans* in the veterinary animals were not statistically significant (p<0.05).

Table 1: Number and Percentage of Veterinary Isolates of *C. neoformans* in Three Different Localities in Botswana

Location	Veterinary Animals		
	Cattle	Poultry	Goat
Serowe	19 (7.1) ^{#a}	10 (4.0)	4 (1.5)
Palapye	13 (4.9)	8 (3.0)	1 (0.4)
Gaborone	8 (3.0)	7 (2.6)	2 (0.7)
Total positive isolates	40	25	7

[#]Numbers in parentheses indicate percentages.

^aThe data are not significantly different at 95% (p<0.05).

Most isolates that were positive were cattle isolates from Serowe (7.1%) and the neighbouring town of Palapye (4.9%). *Cryptococcus neoformans* was also identified from 10 (4.0%) poultry isolates from Serowe.

Goat samples recorded the fewest number of positive isolates in the three towns, accounting for 0.7% and 0.4% in Gaborone and Palapye respectively. Notably, statistical analysis revealed that the location of sampling did not have any influence on the isolation of *C. neoformans* from different veterinary animals (p<0.05).

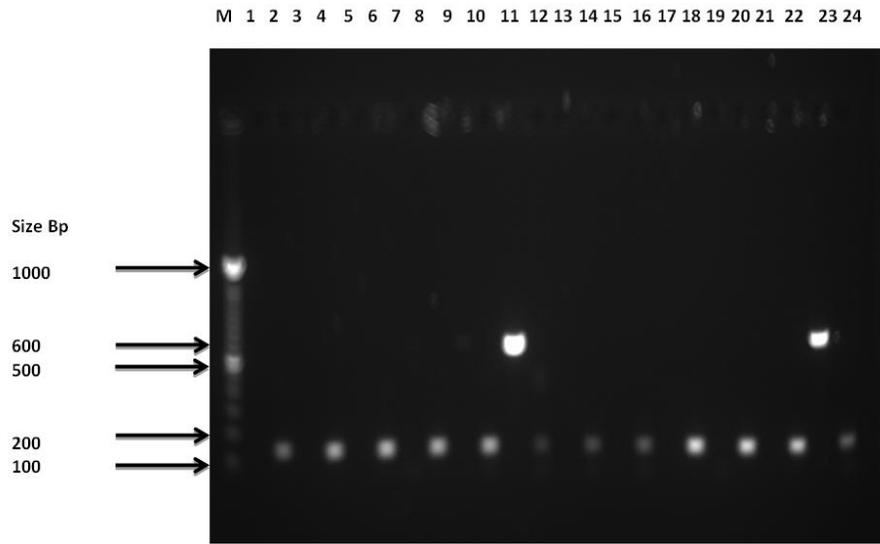


Figure 1: Mating Type Determination of *C. neoformans/gattii* Species Complex in Botswana

2% Agarose gel electrophoresis of *MATa* and *MATα* locus amplification obtained with the primers (STE20-Aa-f; STE20-Aa-r and STE20-Aα-f; STE20-Aα-r). Lane M = 100 bp DNA ladder ((New England Biolabs, Ipswich, MA, USA); Lanes 1 to 12= cattle isolates, Lanes 13 to 18= poultry isolates, Lanes 20 to 22 = goat isolates. Lane 23 (*MATa* positive control), lane 24 (*MATα* positive control).

PCR amplification using STE20 universal primers revealed the distribution of *MATa* and *MATα* alleles among veterinary isolates in Botswana, including hybrid genotypes (Figure 1). A total of 72 isolates were obtained from Serowe, Palapye and Gaborone (Figure 2). Of the 72 isolates, 43 (59.7%) were *MATα* while 24 (33.3%) were *MATa*, and only 5 (6.9%) were hybrids (Figure 2). The percentage isolation of *MATα*, hybrids and *MATa* among the different animals were cattle (31.9%, 4.2%, 19.4%)> poultry (19.4%, 2.8%, 12.5%)> and goats (8.3%; 0%, 1.4%), respectively (Figure 2). Fisher's exact tests revealed that the frequency of isolation of different mating types was neither influenced by the location of sampling ($p < 0.05$) nor veterinary animals ($p < 0.05$).

Discussion

The present study detected *C. neoformans* in 72 (26.9%) of veterinary faecal samples in three localities, Serowe, Palapye and Gaborone in Botswana. Among the three animals investigated, *C. neoformans* was isolated more frequently from cattle (14.9%) than poultry (9.4%) and goats (2.6%) (Table 1). It is important to note that most studies on the isolation of *C. neoformans* from veterinary sources have tended to focus on case studies of animals with cryptococcosis (Faggi *et al.*, 1993; McGill *et al.*, 2009). The majority of cryptococcosis cases in veterinary animals have especially been observed in cats and dogs, where asymptomatic carriage in nasal cavities has been demonstrated (Malik *et al.*, 1997).

However, this study documents the occurrence of the pathogen in faecal samples of asymptomatic cattle, poultry and goats. Hassan *et al.*, (2013) in Egypt found 16% of cattle faecal samples to harbour *C. neoformans*, a finding which is comparable to the present study. Normally, *C. neoformans* is associated with avian droppings, including chickens, but more especially pigeons (Bovers *et al.*, 2008a). Therefore, the predomination of cattle excreta over poultry droppings in our study is in stark contrast to the previous studies.

It is important to note that previous studies (Litvintseva *et al.*, 2011; Chen *et al.*, 2015) have identified the indigenous tree species, *Colophospermum mopane* (*mopane*), as the primary ecological niche of *C. neoformans* in Botswana. The tree species is a source of timber for construction in Botswana, and it is also used for construction of livestock kraals (Makhado *et al.*, 2009). The higher recovery of *C. neoformans* in cattle could be as a result of their interaction with *mopane* during foraging and when they

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are fenced in kraals made from *C. mopane*. This could also be used to explain the higher frequency of the pathogen in Northern Botswana (Serowe and Palapye) than Gaborone which is located in the South. *Colophospermum mopane* is confined to Northern Botswana and does not exist in the South.

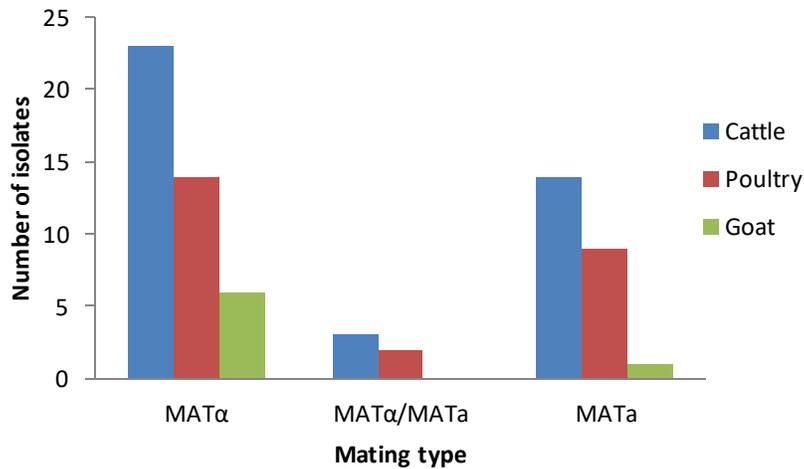


Figure 2: Distribution of Different Mating Types; *MATa*, Hybrid (*MATa/MATa*) and *MATa* in Isolates Obtained from the Faecal Samples of Veterinary Animals

Mating type analysis of veterinary isolates showed recovery of both mating types (*MATα* and *MATa*), including hybrids. These findings are consistent with other studies (Litvintseva *et al.*, 2003; Litvintseva *et al.*, 2006; Chen *et al.*, 2015) which also found both mating types in populations of *Cryptococcus neoformans* in Botswana but from environmental and clinical isolates. Globally, there is a dearth of the *MATa* allele (Litvintseva *et al.*, 2006; Litvintseva *et al.*, 2012). Isolates of *C. neoformans* from Botswana have also been found to be more genetically diverse than the global populations due to the higher frequency of the *MATa* allele (Litvintseva *et al.*, 2003; Litvintseva *et al.*, 2006) which increase the chances of sexual reproduction and recombination. Australian environmental isolates of both *Cryptococcus* mating types were found in almost equal proportions as is the case in recombining populations (Halliday *et al.*, 2003). However, genetic analyses of the isolates revealed a clonal population, with no evidence of sexual recombination. The Botswana environment has been found to harbour a unique *Cryptococcus neoformans* VNB genotype which contains a high proportion of the *MATa* allele that is also genetically diverse and rare globally (Litvintseva *et al.*, 2006). Since the veterinary isolates in the present study had not been genotyped, we cannot tell the genotypes or diversity associated with these strains. However, we cannot rule out the possibility of some of our isolates being the VNB clade which probably contributed to a higher proportion of the *MATa* genotypes.

Interestingly, 5 (6.9%) of the positive *C. neoformans* isolates were hybrid genotypes. Notably, hybrid genotypes were only identified from cattle and poultry isolates in this study. *Cryptococcus neoformans* hybrid genotypes have been previously reported in clinical and environmental studies in Botswana (Litvintseva *et al.*, 2006; Litvintseva *et al.*, 2007), so their isolation in this study was therefore, not unexpected. Hybrids of *C. neoformans* have been shown to be more resistant to physical factors such as UV irradiation and temperature (Litvintseva *et al.*, 2007) and hybrid genotypes most likely have enhanced fitness which allows the pathogen to expand to new environments. Besides the ability to expand to new niches, Boekhoet *et al.*, (2001) also proposed that the hybrids may have altered virulence and resistance to antifungals.

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In conclusion, the detection of *Cryptococcus neoformans* in veterinary animal excreta expands the ecological niche of the organism in Botswana.

It is important to note that the HIV/AIDS pandemic continues unabated in Botswana where the Botswana AIDS Impact Survey IV (Central Statistics Office, 2014) estimated prevalence rates of 18.5% in the adult population.

This essentially means that a significant number of immune-compromised people at the epicentre of *Cryptococcus neoformans* evolution are continuously exposed to these pathogens through contact with farm animals and their excreta. It would be most interesting for future studies to carry out the genetic diversity of *Cryptococcus neoformans* veterinary isolates in Botswana as this would unravel population genetic structure of the isolates.

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