

1       **THE FIRST REPORT ON CRYPTOCOCCUS PROFILES OF ISOLATES FROM**  
2       **PATIENTS ATTENDING DR GEORGE MUKHARI ACADEMIC HOSPITAL,**  
3       **SOUTH AFRICA**

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20  
21       **ABSTRACT**

22       **Introduction:** Cryptococcosis is a fungal opportunistic infection that is vastly diagnosed  
23       among immune-compromised patients. Reduced susceptibility on commonly used antifungals  
24       is of concern. In the communities served by Dr. George Mukhari Tertiary (DGMT-  
25       Laboratory) Laboratory is not available.

26  
27       **Methodology:** E-test method was used to determine if isolates with reduced susceptibility to  
28       antifungals fluconazole, voriconazole and amphotericin-B had emerged. A multiplex  
29       Polymerase Chain Reaction (PCR) method was used to further identify serotypes that are  
30       circulating at Dr. George Mukhari Tertiary (DGMT-Hospital) Hospital.

31  
32       **Results:** E-test strips were interpreted as resistance, intermediate or susceptible in relation to  
33       each serotype identified. Of the 50 incident isolates tested, 100% were inhibited by both  
34       voriconazole and amphotericin-B. Fluconazole was resistance to 50% of incident isolates.

35  
36       **Conclusion:** *C. neoformans* serotype A is the predominant serotype in the area served by  
37       DGMT-Laboratory, accounting for 96% of the isolates. It is important for public health to  
38       continuously monitor resistance emergence.

39  
40       **Keywords:** cryptococcosis, serotypes

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## BACKGROUND

Cryptococcosis is amongst the leading and life-threatening opportunistic infection<sup>[1]</sup>. The disease is caused by *Cryptococcus neoformans* (*C. neoformans*) and *Cryptococcus gattii* (*C. gattii*), which are vastly diagnosed among immune-compromised patients<sup>[2]</sup>. Mainly *C. neoformans*, with three recognized serotypes which are acknowledged as serotype A, serotype D and the hybrid-AD<sup>[3]</sup>. Previously, these serotypes were identified and differentiated by the phenotypic approach<sup>[4]</sup>. Lately, are identified by PCR assays<sup>[5]</sup>. More methods of molecular assays are used to classify *C. neoformans* serotypes<sup>[6]</sup>. The classification is based on antigenic metamorphoses in the polysaccharide capsule associated with virulence factors<sup>[3,7]</sup>. Prevalently found circulating globally is serotype A, whereas serotype D and AD hybrid circulating in truncated numbers<sup>[1-3]</sup>. Serotype A account for more than 90% cases of cryptococcosis in South Africa owing to the extraordinary occurrence of HIV/AIDS<sup>[2,8,9,10]</sup>.

To treat cryptococcosis, the most widely used antifungal agents are amphotericin-B, flucytosine, voriconazole and fluconazole<sup>[9,11]</sup>. Amphotericin-B is used as the first line treatment but limited by toxicity that requires laboratory monitoring, voriconazole is limited to private sectors in Africa<sup>[9-12]</sup>. The amalgamations of antifungals are recommended for induction but flucytosine is not available in resource-poor countries<sup>[12]</sup>. Unfortunately, these are countries with a high incidence of cryptococcosis<sup>[10-12]</sup>. All these antifungals are also limited by emerging resistance mechanisms such as the antigenic polysaccharide capsule tolerance, the mating gene types, the acid tolerant abilities, and spores switching<sup>[12]</sup>. Globally, 20–58% of resistance cases are reported on cryptococcosis by means of diverse studies, focusing on fluconazole<sup>[8-</sup>

91<sup>10,13,14]</sup>. Emerging resistance on the other  
92 cryptococcosis antifungals was not  
93 reported<sup>[9,15]</sup>. Furthermore, intrinsic and  
94 acquired resistance mechanisms are all  
95 associated with Cryptococci and the drugs  
96 proneness to those resistance  
97 mechanisms<sup>[12,16-18]</sup>.  
98  
99 The widespread use of fluconazole may  
100 lead to the emergence of reduced  
101 susceptibility<sup>[19,20]</sup>. Thus the development  
102 of resistance to fluconazole is devastating  
103 to the treatment of cryptococcosis, and it is  
104 necessary to know if there is cross-  
105 resistance with voriconazole which could  
106 be an alternative agent. It is important for  
107 institutions to monitor for changes in  
108 susceptibility profiles of isolates  
109 circulating in their areas in order to update  
110 the treatment regimens. Our aim in this  
111 study was to identify circulating serotypes  
112 and determination of the susceptibility  
113 profiles of *Cryptococcus* isolates to  
114 fluconazole, voriconazole and  
115 amphotericin-B antifungals form clinical  
116 specimens sent to the DGMT-Hospital  
117 NHLS.

## METHODS

**Ethical consideration:** Ethics were sorted from Medunsa Research Ethics Committee. Permission to obtain isolates was sorted from the DGMTL and NHLS managers. Clinical isolates were delinked from identifiers to ensure confidentiality.

**Sample size:** Epi Info version 3.5.3 (Centre for Disease Control and Prevention) was used to calculate the sample size. The required sample size in this study was 50. This was calculated at an estimated frequency of 50%, power of 80% and the confidence interval of 95%.

**Demographics:** Demographic data including age, sex and clinical diagnosis of patients from whom the isolates were isolated was obtained from the Laboratory Information Systems (LIS) in the laboratory.

140 **Collection and storage of isolates:** 177  
141 Clinical isolates were conveniently 178  
142 collected from the laboratory after 179  
143 processing for patient management was 180  
144 completed. The isolates were collected 181  
145 from February-July 2014, on a day to day 182  
146 basis until the sample size was reached. 183  
147 These isolates were already identified by 184  
148 the NHLS as *Cryptococcus* and stored in a 185  
149 - 4°C fridge. 186

150  
151 **Sub-culture of isolates:** The stored 188  
152 isolates were sub-cultured on Sabouraud 189  
153 dextrose agar (SDA) plate as described by 190  
154 Govender et al. 2011<sup>[9]</sup>. 191



155  
156 **Figure 1:** Muroid colonies on SDA  
157 medium

158  
159 **Confirmation and identification of** 208  
160 ***Cryptococcus*:** Gram staining was done to 209  
161 confirm the morphology of yeast cell 210  
162 according to Chayakulkeeree (2007) 211  
163 description<sup>[21]</sup>. India ink (negative stain) 212  
164 was done as described by Ogundeji (2013) 213  
165 to verify the presence of capsule<sup>[22]</sup>. The 214  
166 isolates were further inoculated to urease 215  
167 broth media test in a slant position as a 216  
168 confirmation test according to Gazzoni 217  
169 (2014) methods<sup>[23]</sup>. 218

170 **Susceptibility Testing:** After sub-culturing 220  
171 on SDA (**Figure 1**), susceptibility testing 221  
172 was achieved according to Clinical and 222  
173 Laboratory Standards Institution (CLSI) 223  
174 outlines of 2007<sup>[24]</sup> and as described by 224  
175 Govender et al. 2011, 2013<sup>[9,25]</sup>. 225  
176 226

177 **DNA Extraction and Sample Preparation**  
178 **for Multiplex PCR:** Genomic DNA was  
179 extracted from the clinical isolates using  
180 the commercial kit (ZR fungal/bacterial  
181 DNA MiniPrep kit) in accordance with the  
182 manufacturer's instructions (Zymo  
183 research group). The kit has been  
184 optimized for removal of PCR inhibitors  
185 and maximal recovery of pure DNA  
186 without RNA contamination. The  
187 extraction of DNA from the isolates was  
188 done using the protocol, "Biological  
189 liquids and cell suspensions"<sup>[26]</sup>.

191 **Primers selection:** The primers used were  
192 synthesized by Inqaba Biotechnical  
193 Industries (Pty) Ltd, Muckleneuk, and  
194 Pretoria. The serotypes specific primers  
195 were designed to target the Mating -  $\alpha$   
196 gene and Mating - a gene of both serotypes  
197 A and D<sup>[27]</sup>. Primers targeting for genes  
198 confirming *C. neoformans* serotypes are  
199 listed in **table 3**.

200  
201 **Amplification of genes:** This was done on  
202 the extracted DNA using specific primers  
203 (**Table 3**). Two master-mixtures (MM)  
204 were prepared. Reagents were obtained  
205 from Bioline Meridian Life Science  
206 Company (UK), each PCR assay was set-  
207 up with nuclease-free water as the negative  
208 control (Bioline, UK), and positive  
209 controls were not included due to financial  
210 constraints. MyTaq™ HS DNA-  
211 Polymerase (Bioline, UK) was used in the  
212 PCR reactions.

214 For each sample, a 50  $\mu$ l reaction MM was  
215 prepared following the manufacturer's  
216 instructions (Bioline, UK).

217  
218 Briefly: 10  $\mu$ L x MyTaq™ HS buffer, 1  
219  $\mu$ L of each of 2 primers, 5  $\mu$ L of the  
220 template, 0.5  $\mu$ L MyTaq™ HS DNA-  
221 Polymerase (Bioline, UK) (5 U/ $\mu$ L), and  
222 32,5  $\mu$ L nuclease-free water. Two sets of  
223 MM were used, in MM1 contained alpha-  
224 Aa-D primer set and the MM2 contained  
225 a-Aalpha-D. The 0.2 mL PCR tubes each  
226 containing 50  $\mu$ L placed into a reaction

227 was allowed to take place in a GeneAmp  
228 PCR System 9700 (MTHE 01326 PE  
229 Applied Biosystems) thermocycler for 3  
230 hours; succeeding PCR temperatures as  
231 described by Saiki (1999)<sup>[28]</sup>.

232  
233 **Detection of amplified products:**  
234 Electrophoresis was performed on all  
235 samples using 2,0% agarose gel (Crystal  
236 TBE, Bioline, UK) for 40 minutes at 100  
237 V, with ethidium bromide and UV  
238 transilluminator (Gel Doc™ EZ System).  
239 The 1 kb molecular weight marker  
240 (HyperLadder IV, Bioline, UK) was used  
241 in together with the amplified products.  
242 The photographic copy was taken using a  
243 Gel Doc EZ imager and the results were  
244 recorded as representative of serotype-A $\alpha$ ,  
245 D $\alpha$  or A-a, D-a genes. For expected bands  
246 see **table 3**.

247  
248 **Capturing of data:** Microsoft Excel  
249 (Microsoft Office, 2010) was used to  
250 analyze data and the captured data was  
251 double-checked to ensure reliability; Epi  
252 Info version 3.5.3 (Centers for Disease  
253 Control & Prevention). Descriptive  
254 statistical analysis was performed based on  
255 ANOVA excel, 2010. Measures of central  
256 tendency and dispersion were calculated  
257 for continuous variables (e.g. age);  
258 frequencies and proportions of categorical  
259 data (e.g. serotypes) were calculated.

260  
261 **Reliability, Validity, and Objectivity:** All  
262 tests were performed according to  
263 recognized, accredited standard operating  
264 procedures as well as to the instructions of  
265 the manufactures in the case where  
266 commercially available kits were used.  
267 Molecular size markers were used during  
268 agarose gel electrophoresis.

269  
270

## RESULTS

271 **Study population:** There were 50  
272 *Cryptococci* isolates collected from  
273 different clinical specimens sent to the  
274 DGMT-Laboratory during the study  
275 period, June to October 2014 (5 months).

276 Eleven (22%) isolates were from blood  
277 specimens and 39 (78%) from Cerebral  
278 Spinal Fluid (CSF).

279  
280 **Table 1: Demographics of the patients:**  
281 Only 41 of the 50 patients from where the  
282 clinical specimens were sent had complete  
283 information from the laboratory  
284 information system.

Females	Males	Unknown
30 (60%)	11 (22%)	9 (18%)

285  
286  
287 The ages of the 41 patients analyzed  
288 ranged from 15 to 86 with the majority  
289 being between 35 and 45.

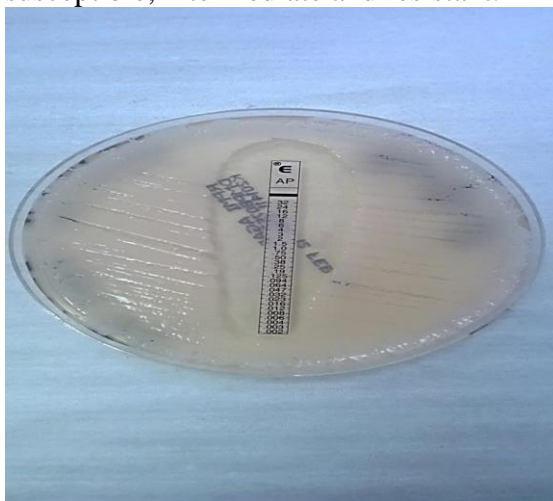
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291 **Biochemical test for species:** Urease slope  
292 was done to all 50 isolates. After a period  
293 of 24 hours incubation at 30°C, the color  
294 change was observed. The change of  
295 colorless broth to pink broth medium was  
296 confirming the presence of *C. neoformans*  
297 species (**figure 2**).



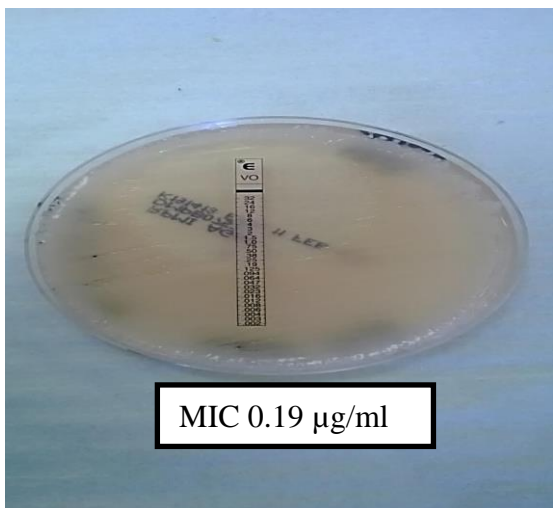
298  
299 **Figure 2: Urease slope of one of the**  
300 **isolates showing a colour change after**  
301 **24 hours incubation.**

302 **Susceptibility testing:** After the incubation  
303 period of the three antifungals E-test strips  
304 (bioMérieux S.A., Marcy l'Etoile, France)  
305 which were fluconazole, voriconazole, and  
306 amphotericin-B, results were then read  
307 following the CLSI<sup>[24]</sup>. The Minimum  
308 inhibitory concentration (MIC) values  
309 were read at the point of intersection  
310 between the zones of growth and the edge

311 of the strip. The amphotericin-B was read  
 312 at the point of complete inhibition (100%)  
 313 as shown in **figure 3**, both fluconazole and  
 314 voriconazole MICs were read at a point of  
 315 significant inhibition of growth, about  
 316 80% reduction of growth as shown in  
 317 **figure 4** and **5a-b**. MIC values were  
 318 documented on a data collection sheet. The  
 319 MIC values for fluconazole and  
 320 voriconazole were interpreted in  
 321 accordance with CLSI updated M27  
 322 breakpoints (2013) guideline and for  
 323 amphotericin B, according to NCCLS M27  
 324 guideline<sup>[24]</sup>. These were interpreted as  
 325 susceptible, intermediate and resistant.



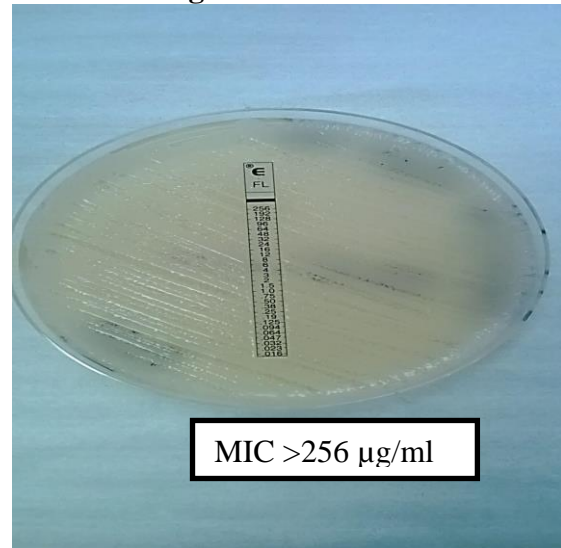
326  
 327 **Figure 3: Amphotericin-B point of**  
 328 **100% inhibition of growth**  
 329



330  
 331 **Figure 4: Voriconazole point of 80%**  
 332 **inhibition of growth**  
 333



334  
 335 **Figure 5a: Fluconazole point of 80%**  
 336 **inhibition of growth**



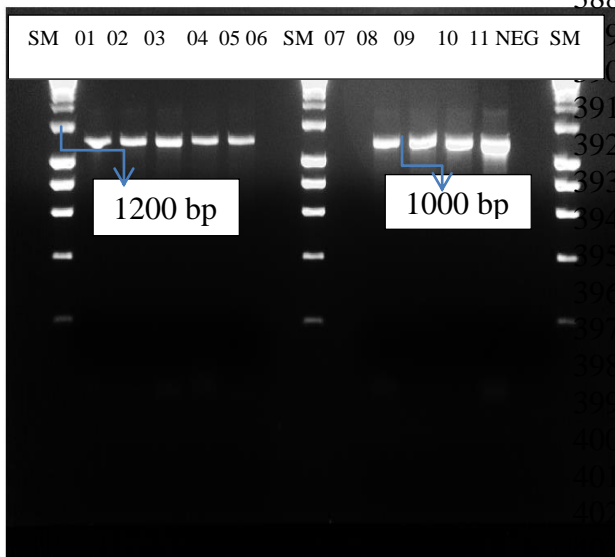
337  
 338 **Figure 5b: Fluconazole point of 0%**  
 339 **inhibition of growth**

340  
 341 The MICs were determined in all isolates.  
 342 Voriconazole and amphotericin-B were  
 343 susceptible to all isolates as presented in  
 344 **table 4** above.

345  
 346 **Table 2: Molecular confirmation of**  
 347 **serotypes:** PCR for serotyping of *C.*  
 348 *neoformans* was performed on all 50  
 349 isolates.

Master-mix 1 (MM1)			Master-mix 2 (MM2)		
Serotypes					
A- $\alpha$	D-a	AD- $\alpha\alpha$	A-a	D- $\alpha$	AD- $\alpha\alpha$
48(96%)	-	-	-	2(4%)	-

350 The agarose gel picture below, show  
351 representatives of PCR results on an  
352 agarose electrophoresis



353  
354 **Figure: Representative Agarose gel**  
355 **electrophoresis.** Where: Lane 1-11:  
356 Clinical isolates; Neg: Negative control;  
357 SM: 1000 bp (1 kd) (size markers); Lane  
358 number: 2-5; 8-11 represent serotype Aα  
359 mating genes; Lane number: 6-7 negative  
360 results.

## 362 DISCUSSION

363 Resistance to antifungal agents used  
364 against cryptococcosis is globally  
365 reported<sup>[9,16,29-31]</sup>. In Africa, cryptococcosis  
366 epidemiology data is scarce but  
367 accumulated evidence in South Africa,  
368 makes it apparent that resistance  
369 development to commonly used antifungal  
370 agents is of concern<sup>[8-10,25]</sup>. Therefore,  
371 monitoring the susceptibility of these  
372 commonly used antifungal agents in  
373 different geographical areas is essential.

374  
375 Data on circulating serotypes responsible  
376 for cryptococcosis in communities served  
377 by DGMT-Laboratory is not available.  
378 This study serves to profile the  
379 susceptibility and to identify the  
380 circulating serotypes of *Cryptococcus* at  
381 DGMT-hospital, in South Africa.

382  
383 Based on our study, the susceptibility of  
384 the amphotericin-B, fluconazole, and

385 voriconazole was profiled; resistance to  
386 fluconazole was of foremost concern  
387 (**table 4**).

388  
389 It was not surprising to see that half of our  
390 isolates were completely resistant to  
391 fluconazole. Our results were in keeping  
392 with multiple studies of diverse geographic  
393 areas, such that Arsenijevic et al (2014) in  
394 Serbia revealed 60% resistance of clinical  
395 isolates<sup>[32]</sup>, and that of 63% by Favalessa  
396 et al (2014) in West Brazil patients<sup>[33]</sup>.  
397 Furthermore, a South African report of  
398 Govender et al (2011) and (2013) showed  
399 58% resistance to fluconazole<sup>[9,25]</sup>.

400 Fluconazole resistance is based on the *C.*  
401 *neoformans* mechanisms of action<sup>[8,16,-18]</sup>.  
402 The other factors that contribute to the  
403 recurrence of cryptococcosis among South  
404 African patients are limited access to  
405 treatment and inadequate treatment<sup>[8-10,25]</sup>.

406  
407 Furthermore, isolates of our study were  
408 highly susceptible to voriconazole and  
409 amphotericin-B. Our findings were not  
410 different but comparable to the studies of  
411 Arsenijevic et al (2014)<sup>[32]</sup>, Govender et al  
412 (2011) and (2013), they all reported 100%  
413 susceptibility on voriconazole and  
414 amphotericin-B<sup>[9,25]</sup>. There was no cross-  
415 resistance between amphotericin-B,  
416 voriconazole, and fluconazole on in-vitro  
417 testing. It will, however, be important to  
418 assess this based on clinical outcome in  
419 patients.

420  
421 Unfortunately, Amphotericin-B had no  
422 breaking-points according to CLSI  
423 updated M27 break-points document of  
424 2013, we, therefore, interpreted our results  
425 in accordance with NCCLS M27-A  
426 guideline document (NCCLS M27-A  
427 guideline, 2000)<sup>[24]</sup>. Fortunately Govender  
428 et al (2011) also, however, indicated the  
429 challenges of performing susceptibility  
430 testing for amphotericin-B because of the  
431 absence of CLSI break-points<sup>[9]</sup>.

434 Molecular-based, our study confirmed that 480  
435 *C. neoformans* serotype A is predominant 481  
436 in our setting. Accumulated evidence 482  
437 showed that serotype A has been reported 483  
438 as more virulent and prevalent than the 484  
439 other serotypes<sup>[32-36]</sup>. Likewise, Lugarini et 485  
440 al (2008) in Brazil, reported a prevalence 486  
441 of 53% serotype A  $\alpha$ -mating gene types 487  
442 circulating across the country<sup>[34]</sup>. A similar 488  
443 study by Favalessa et al (2014) in Midwest 489  
444 Brazil also reported serotype A making 490  
445 63% of the isolates from HIV/AIDS 491  
446 patients<sup>[33]</sup>. Khayhan et al (2013) also 492  
447 confirmed serotype A as the most 493  
448 prevalent serotype in Asia Phayoa<sup>[35]</sup>. In 494  
449 our study, we didn't manage to find the 495  
450 HIV status of our patients. Our study was 496  
451 in keeping with a systemic review study of 497  
452 Litvintseva et al (2011) which was 498  
453 conducted in African countries, reported 499  
454 serotype A specifically the  $\alpha$ -mating gene 500  
455 types to account for 79% of the isolates<sup>[36]</sup>, 501  
456 and according to our study in South 502  
457 Africa, serotype A is the commonest 503  
458 circulating serotype across our setting, 504  
459 counting for 96%  $\alpha$ -mating gene types. 505

460 Furthermore, our study showed that only a 506  
461 few isolates were confirmed to be serotype 507  
462 D  $\alpha$ -mating genes type. Those few isolates 508  
463 were from patients over the age of 65. 509  
464 Duke University in Durham previously 510  
465 reported that serotype D is very rare and 511  
466 less information is documented about the 512  
467 distribution of this serotype<sup>[37]</sup>, whereas 513  
468 Feretzaki et al (2014) in India reported that 514  
469 serotype D requires very high inoculum to 515  
470 disseminate and cause infections like 516  
471 meningitis<sup>[38]</sup>. There is no information or 517  
472 data documented about the distribution of 518  
473 serotype D  $\alpha$ -mating gene-types in South 519  
474 Africa and in our setting. Our two patients 520  
475 could have been more immune- 521  
476 compromised than the others because of 522  
477 their age. Furthermore, no study has been 523  
478 conducted according to our knowledge on 524  
479 serotypes and mating-genes in South

Africa, Pretoria, DGMT-Hospital. Our  
results highlight the importance of  
properly treating cryptococcosis.

## CONCLUSION

*C. neoformans* serotype A is a  
predominant serotype in the area served by  
DGMT Laboratory, accounting for 96% of  
the isolates. Fifty percent of the isolates  
were resistant to fluconazole while 100%  
of those tested were susceptible to  
voriconazole and amphotericin-B,  
suggesting a lack of cross-resistance on in-  
vitro testing.

The study had several limitations such as  
low population number and financial  
constraints. However, because of high  
fluconazole resistance suggested, the study  
recommends the routine performance of  
susceptibility testing to fluconazole. Cross-  
resistance with voriconazole and  
amphotericin-B is to be evaluated further.

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## Conflict of Interest

The authors declare no conflicts of interest  
with respect to authorship and/or  
publication of this article.

## Author Contributions

Conceived and designed the experiments:  
EZ Jiyane. Performed the experiments: EZ  
Jiyane, Analysed the data: EZ Jiyane,  
Contributed reagents/materials/analysis  
tools: VLIR, EZ Jiyane; Contributed to the  
writing of the manuscript: EZ Jiyane;  
critically reviewed the manuscript: EZ  
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527

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861 **Table 3: Combination and sequences of the primers used for the determination of**  
 862 **serotype and mating type of *C. neoformans* by PCR multiplex alpha-Aa-D and a-**  
 863 **Aalpha-D (N= 50)**  
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Gene alleles	Primers	Sequence 5'3'	PCR product - (bp)
<i>MAT<math>\alpha</math></i> serotype-A (MM1)	JOHE 7264 JOHE 7265	AGCTGATGCTGTGGATTGAATAC GTTCAATTAATCTCACTACCTGTAG	1200
<i>MAT<math>\alpha</math></i> serotype D (MM1)	JOHE 7273 JOHE 7275	GTTTCATCAGATACAGAGGAGTGG CTCCACTGTCAAACCTACGGC	870
<i>MAT<math>\alpha</math></i> serotype A (MM2)	JOHE 7270 JOHE 7272	ATCAGAGACAGAGGAGGAGCAAGAC TCCACTGGCAACCCTGCGAG	870
<i>MAT<math>\alpha</math></i> serotype D (MM2)	JOHE 7267 JOHE 7268	ATAGGCTGGTGTGCTGTGAATTAAG GTTCAAGTAATCTCACTACATGCG	1200

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866 **Table 4: MIC's of the isolates against common antifungals (N= 50)**  
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Antifungal drugs	Interpretation	MIC scales	Isolates numbers
**Fluconazole	Susceptible	$\leq 2 \mu\text{g/mL}$	13 (26%)
	Intermediate	$4 \mu\text{g/mL}$	12 (24%)
	Resistant	$\geq 8 \mu\text{g/mL}$	25 (50%)

**Voriconazole	Susceptible	$\leq 0.12 \mu\text{g/mL}$	50 (100%)
	Intermediate	$0.25 \mu\text{g/mL} - 0.5 \mu\text{g/mL}$	0
	Resistant	$\geq 1 \mu\text{g/mL}$	0
*Amphotericin-B	Susceptible	$\leq 0.5 \mu\text{g/mL}$	50 (100%)
	Intermediate	-	0
	Resistant	$\geq 2 \mu\text{g/mL}$	0

868 \*interpretation according to NCCLS M27-A document 2000

869 \*\*interpretation according to CLSI M27-A document 2013

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