HHS Public Access

Author manuscript

Mycoses. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Mycoses. 2016 March ; 59(3): 151–156. doi:10.1111/myc.12444.

Comparison of biotyping methods as alternative identification tools to molecular typing of pathogenic *Cryptococcus* species in sub-Saharan Africa

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Summary

Cryptococcal meningitis is the leading fungal infection and AIDS defining opportunistic illness in patients with late stage HIV infection, particularly in South-East Asia and sub-Saharan Africa. Given the high mortality, clinical differences and the extensive ecological niche of *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes, there is need for laboratories in sub-Sahara African countries to adopt new and alternative reliable diagnostic algorithms that rapidly identify and distinguish these species. We biotyped 74 and then amplified fragment length polymorphism (AFLP) genotyped 66 *Cryptococcus* isolates from a cohort of patients with HIV-associated cryptococcal meningitis. *Cryptococcus gattii sensu lato* was isolated at a prevalence of 16.7% (*n* = 11/66) and *C. neoformans sensu stricto* was responsible for 83.3% (*n* = 55/66) of the infections. L-Canavanine glycine bromothymol blue, yeast-carbon-base-D-proline-D-tryptophan and creatinine dextrose bromothymol blue thymine were able to distinguish pathogenic *C. gattii sensu lato* from *C. neoformans sensu stricto* species when compared with amplified fragment length polymorphism genotyping. This study demonstrates high *C. gattii sensu lato* prevalence in Zimbabwe. In addition, biotyping methods can be used as alternative diagnostic tools to molecular typing in resource-limited areas for differentiating pathogenic *Cryptococcus* species.

Keywords

Biotyping; molecular typing; Cryptococcus gattii sensu lato; Cryptococcus neoformans sensu stricto

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Conflict of Interest

JFM received grants from Astellas, Basilea, and Merck. He has been a consultant to Astellas, Basilea and Merck and received speaker's fees from Merck, United Medical and Gilead Sciences. All other authors declare no conflict of interest.

Introduction

Cryptococcal meningitis is caused by pathogenic *Cryptococcus* species. It is a devastating AIDS defining opportunistic illness in patients with late stage HIV infection, particularly in South-east Asia and sub-Saharan Africa [1,2,3]. *Cryptococcus neoformans* and *C. gattii* are the most commonly isolated pathogenic *Cryptococcus* species in 99.9% of the infections. However, the taxonomy of both species has been revised recently and now comprises of seven species, with *C. neoformans sensu stricto* (formerly *C. neoformans* variety *grubii*, serotype A, genotype AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII) being the major cause of HIV-associated cryptococcal meningitis [4]. *Cryptococcus neoformans* variety *neoformans* was elevated to the species level as *C. deneoformans* (serotype D, genotype AFLP2/VNIV) [4]. The five *C. gattii* genotypes are now recognized as species, those frequently isolated from HIV-infected individuals include *C. bacillisporus* (genotype AFLP5/VGIII), *C. tetragattii* (genotype AFLP7/VGIV) and *C. decagattii* (genotype AFLP10/VGIV), while others often cause disease in otherwise healthy individuals namely *C. gattii sensu stricto* (genotype AFLP4/VGI) and *C. deuterogattii* (genotype AFLP6/VGII) [4].

Mortality as a result of cryptococcosis is highest in sub-Saharan Africa with an estimated annual case fatality of 504,000 [3]. This places cryptococcosis as the fourth highest cause of infectious disease deaths in sub-Saharan Africa ahead of tuberculosis. However, the mortality trends might have changed over the past few years because of the widespread use of HAART [3].

Given the high mortality, clinical differences, change in taxonomy and the extensive ecological niche of *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes, it is important for clinical laboratories in sub-Sahara African countries to adopt new affordable alternative diagnostic algorithms that rapidly identify and distinguish these species from other yeasts [4–6]. The conventional gold standard diagnostic method for distinguishing *C. gattii* and *C. neoformans* species complexes is based on biotyping with L-canavanine glycine bromothymol blue (CGB) media [7]. However, CGB media have been reported to give false positives and false negatives, despite its continued use in most laboratories [6,8–10]. Several biotyping methods have been developed for differentiating pathogenic *Cryptococcus* species and these exploit the fact that few strains of *Cryptococcus* are able to metabolise one or more amino acids as their only source of nitrogen. Members of the *Cryptococcus gattii* species complex can assimilate D-proline, D-tryptophan, L-malic acid and glycine as both carbon and nitrogen sources, and resist growth inhibition by cycloheximide and L-canavanine unlike *C. neoformans* species [4,11].

New alternative biotyping media such as yeast-carbon-base-D-proline-D-tryptophan (YCB-DP-DT) and creatinine dextrose bromothymol blue thymine (CDBT) have been developed to distinguish between the *C. neoformans* and *C. gattii* species complex [6,12]. However, no study has compared their diagnostic performance with CGB media. Despite the continued use of biotyping methods in research; there have proved to be tedious and require lengthy incubation periods [13].

Several genotyping assays have been developed to identify the different genotypes within the *C. gattii/C. neoformans* species complexes and to determine their epidemiology. These include restriction fragment length polymorphism (RFLP), multi-locus sequence typing (MLST), amplified fragment length polymorphism (AFLP) analysis and PCR fingerprinting [4,14–19]. With the advent of molecular techniques, it has become apparent that the epidemiology of *C. neoformans* and *C. gattii* species complexes is more diverse and complex than thought previously [4,16,17,20]. Genotyping of *Cryptococcus* species may be important in guiding clinicians on the management of their patients as well as the epidemiologists in determining the impact of these species on the population.

Our goal in this study was to determine the diagnostic performance of three biotyping media namely L-canavanine glycine bromothymol blue, yeast-carbon-base-D-proline-D-tryptophan and creatinine dextrose bromothymol blue thymine when compared to AFLP genotyping. The second goal was to estimate the prevalence of each species/genotype among the *Cryptococcus* isolates cultured from cerebrospinal fluid (CSF) obtained from a cohort of patients with cryptococcal meningitis.

Materials and Methods

Patients and isolates

Seventy-four *Cryptococcus* isolates which were isolated during a laboratory diagnostic process from a cohort of adults with HIV associated cryptococcal meningitis, were subjected to biotyping and genotyping. These isolates were collected from a group of patients with cryptococcal meningitis as identified with India ink, cryptococcal antigen testing and culture between June 2013 and September 2014. The Joint Research Ethics Committee of the Parirenyatwa Group of Hospitals and the College of Health Sciences (JREC), the Institutional Review Board of the Biomedical Research Training Institute (BRTI), the Medical Research Council of Zimbabwe (MRCZ) and Research Council of Zimbabwe (RCZ) approved the study.

Biotyping

Two-day-old *Cryptococcus* isolates grown on Sabouraud dextrose agar and chloramphenicol (SAB+C) were inoculated on quad plates containing L-canavanine glycine bromothymol blue (CGB), yeast-carbon-base-D-proline-D-tryptophan (YCB-DP-DT) and creatinine dextrose bromothymol blue thymine (CDBT) media (all Sigma-Aldrich, St Louis, MO, U.S.A.). The 3 biotyping media were prepared according to previously described protocols (6,7,12). Quad plates were inoculated with a 2-day old culture and incubated at 25°C for 5 to 10 days. *Candida albicans* ATCC 10231, *Candida* parapsilosis ATCC 22019 and *Cryptococcus neoformans* ATCC 204092 were used as quality control strains.

Interpretation criteria positive results on CGB medium—On CGB a positive result was indicated by growth and colour change of medium to deep blue indicating *C. gattii sensu lato* and minimal growth when the medium becomes yellow or green interpreted as *C. neoformans sensu lato*.

Interpretation criteria positive results on CDBT medium—As for CDBT, plates inoculated with *C. gattii sensu lato* develop a blue green colour, the intensity of which does not change by day 7. *Cryptococcus neoformans sensu stricto* (serotype A), grows but no colour change occurs on the media.

Interpretation criteria positive results on YCB-DP-DT agar—*Cryptococcus gattii sensu lato* is able to grow on this media and produces brown colonies and a pigment of varying intensity. Absence of growth on YCB-DP-DT indicated presence of *C. neoformans sensu lato* species that are unable to grow on this medium.

Molecular characterization of the isolates

Genomic DNA of *Cryptococcus* isolates was extracted from 2-day-old SAB+C cultures. A loop-full of *Cryptococcus* cells was suspended in 400 µl bacterial lysis buffer followed by bead-beating in Green Beads tubes. After 15 min incubation at 100°C, 200 µl suspension was transferred to a 96-deepwell plate followed by automatic DNA extraction on a MagNA Pure 96 platform by using the Pathogen 200 protocol with a final elution volume of 100 µl (all Roche Diagnostics, Almere, The Netherlands).

Amplified fragment length polymorphism (AFLP) genotyping was performed according to previously described protocols [21]. Reference strains 125.91, H99 (both *C. neoformans* AFLP1/VNI), Bt1 (*C. neoformans* AFLP1A/VNB/VNII), WM626 (*C. neoformans* AFLP1B/VNII), JEC20, JEC21 (both *C. deneoformans* AFLP2/VNIV), CBS132 and WM629 (both *C. neoformans* × *C. deneoformans* AFLP3/VNIII), WM179 (*C. gattii sensu stricto* AFLP4/VGI), WM161 (*C. bacillisporus* AFLP5/VGIII), WM178 (*C. deuterogattii* AFLP6/VGII), WM779 (*C. tetragattii* AFLP7/VGIV) and IHEM14941 (*C. decagattii* AFLP10/VGIV) were included [16,18,19,22].

Statistical analysis

Categorical data was presented as frequencies and proportions. The diagnostic performance of the different biotyping media was assessed by their sensitivity, specificity, negative predictive value and positive predictive values using AFLP genotyping as the reference method. The prevalence of the different *Cryptococcus* species was calculated as a proportion of the total isolates that grew on culture and were positive by AFLP genotyping. All statistical analyses were performed using the Stata software, version 13 (StataCorp, College Station, TX, U.S.A.).

Results

Comparison of CGB and YCB-DP-DT culture media

Of the 74 isolates, 60 (81.1%) of the cultured isolates were *C. neoformans sensu lato* based on CGB and YCB-DP-DT whilst 14 (18.9%) were identified as *C. gattii sensu lato*. Table 1 demonstrate that YCB-DP-DT was 100% accurate in differentiating *C. gattii* and *C. neoformans* species complexes when compared with the gold standard CGB.

Comparison of CGB/YCB-DP-DT culture media versus AFLP

Sixty-six pathogenic *Cryptococcus* isolates were used to assess the diagnostic performance of CGB with amplified fragment length polymorphism. The CGB or YCB-DP-DT media was able to identify 62 (94%) of the *Cryptococcus* isolates as either *C. neoformans sensu lato* or *C. gattii sensu lato*. Two isolates were incorrectly identified as *C. gattii sensu lato* on CGB and YCB-DP-DT and later turned out to be *C. neoformans sensu lato* with AFLP genotyping. Hence, false positives and false negatives were present in equal percentages of 3% (*n*=2/66) for both CGB and YCB-DP-DT. Both CGB and YCB-DP-DT had a sensitivity of 96.8% and specificity of 50% when compared to AFLP genotyping. Based on AFLP genotyping, *C. gattii sensu lato* was isolated at a prevalence of 16.7%. This is summarised in table 2.

Comparison of CDBT culture media versus AFLP

Sixty-two (94.0%) isolates were accurately identified to species level based on the CDBT media when it was compared to the *Cryptococcus* species genotyping gold standard AFLP. Creatinine dextrose bromothymol blue thymine had a sensitivity of 100% and a low specificity of 33.3%. However, it also gave 4 (6%) false positives when distinguishing *C. neoformans sensu lato* from *C. gattii sensu lato*. Table 3 summarizes the findings.

Discussion

Since cryptococcal meningitis (CM) is a severe fungal infection associated with neurological complications and high mortality, successful treatment of CM depends on rapid and accurate identification of the *Cryptococcus* species [5,23]. This study is the first study to our knowledge to compare the diagnostic performance of three-biotyping media (yeast-carbon-base-D-proline-D-tryptophan [YCB-DP-DT], creatinine dextrose bromothymol blue thymine [CDBT] and L-canavanine glycine bromothymol blue [CGB]) with amplified fragment length polymorphism (AFLP) genotyping as the gold standard for characterizing pathogenic *Cryptococcus* species.

Yeast-carbon-base-D-proline-D-tryptophan was able to distinguish pathogenic *C. gattii sensu lato* from *C. neoformans sensu stricto* species isolated from cultures of patient CSF samples with 100% accuracy when comparing with the biotyping gold standard CGB. However, both CGB and YCB-DP-DT gave a 94% (*n*=62/66) agreement with AFLP genotyping. Despite their high diagnostic accuracy, CGB and YCB-DP-DT gave low false positives (3%; *n*=2) and false negatives (3%; *n*=2) at equal percentages. Here, we also report false negatives and false positives produced on CGB, a phenomenon that has been reported before [9,24]. This is the first report of false positive and false negative results on YCB-DP-DT agar.

Creatinine dextrose bromothymol blue thymine (CDBT) medium was also able to differentiate *C. gattii sensu lato* from *C. neoformans sensu stricto* and its results were comparable to those obtained with YCB-DP-DT and CGB. The CDBT medium had a 94% (*n*=62/66) agreement with AFLP genotyping. However, 6% (*n*=4/66) of the isolates were false positive for *C. gattii sensu lato*. This demonstrates that CDBT agar can be used as an

alternative for AFLP genotyping in resource limited areas. Nevertheless, all isolates that are identified, as *C. gattii sensu lato* need to be confirmed by using molecular characterisation.

L-Canavanine glycine bromothymol blue remains a user-friendly medium to prepare as compared to YCB-DP-DT or CDBT media, which are both complex in preparation and require experienced skills. Creatinine dextrose bromothymol blue thymine medium is highly pH sensitive, and does not support growth of *Cryptococcus* isolates if the pH is below 5.65 or if the pH is above 5.75. In contrast to CDBT agar, YCB-DP-DT requires filtration of the amino acids D-proline and D-tryptophan before mixing with the agar base. However, despite all these challenges CDBT or YCB-DP-DT are good alternatives that can complement CGB in speciating and determining the epidemiology of pathogenic *Cryptococcus* species in resource-limited areas.

This study demonstrates the accuracy and reliability of biotyping methods in differentiating the different pathogenic *Cryptococcus* species. However, biotyping media cannot replace molecular typing as gold standard in accurately distinguishing *C. neoformans sensu stricto* and *C. gattii sensu lato*.

Genotyping by AFLP identified all pathogenic *Cryptococcus* isolates to species level. Amplified fragment length polymorphism, although expensive, is rapid and reliable for the identification of *Cryptococcus* species. It also gives more epidemiological information as compared to the conventional biotyping methods [4,8].

The prevalence of *C. gattii sensu lato* as identified by genotyping was 16.7% (11/66) and *C. neoformans sensu stricto* was responsible for infection in 83.3% (55/66) in our cohort of patients with HIV associated cryptococcal meningitis. Eight isolates did not grow after they were transferred to another laboratory for molecular analysis; hence, these isolates were not genotyped. *Cryptococcus gattii sensu lato* has been isolated before in Zimbabwe, however this is the first study that has reported such a high prevalence [23,25]. Other studies conducted in Africa have reported a prevalence of *C. gattii sensu lato* that ranged between 1.6% – 30% [25–31]. This is also the first report with the highest prevalence of *C. gattii sensu lato* in an African-based study, as the *C. gattii sensu lato* isolates from a study conducted in Botswana were not confirmed by molecular typing [26].

Our study presents the latest evidence on the prevalence of *Cryptococcus* species causing HIV associated cryptococcal meningitis in Zimbabwe and further supports the information gathered from other studies around the world. *Cryptococcus neoformans sensu stricto* is still the most prevalent species causing HIV associated cryptococcal meningitis in Zimbabwe; however our findings suggests that *C. gattii sensu lato* is also becoming more common in this region.

Acknowledgements

This work was supported by Grant number 2U2RTW007367 from the Fogarty International Centre, National Institutes of Health (NIH, USA) through the International Clinical, Operational and Health Services and Training Award (ICOHRTA) and Grant number “HIVRT15-065” from the HIV Research Trust. The authors would also like to thank the CryptoZim group for their technical assistance. Finally yet importantly, the authors would like to

extend their gratitude to the personnel of the departments of Chemical Pathology and Medical Microbiology, College of Health Sciences, University of Zimbabwe for all the support throughout the whole project.

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Table 1

Biotyping of pathogenic *Cryptococcus* species by CGB and YCB-DP-DT media

***Cryptococcus* species**	**Biotyping method**		**Statistical analysis**
	CGB (gold standard)	**YCB-DP-DT (Test)**	Sensitivity = 100%; C.I. = 94–100% PPV = 100% ; C.I. = 94–100% Specificity = 100%; C.I. = 73–100% NPV = 100%; C.I. = 73–100%
C. neoformans sensu lato	60 (81.1%)	60 (81.1%)	
C. gattii sensu lato	14 (18.9%)	14 (18.9%)	
Total	74 (100.0%)	74 (100.0%)	

C.I. = confidence interval, NPV =Negative predictive value, PPV = Positive predictive value.

Table 2

Comparison of *Cryptococcus* typing based on AFLP genotyping and CGB/YCB-DP-DT media

***Cryptococcus* species**	**Typing methods**		**Diagnostic accuracy**
	CGB/YCB-DP-DT (test)	**AFLP genotyping (gold standard)**	
C. neoformans sensu lato	53 (80.3%)	55 (83.3%)	Sensitivity = 96.8%; C.I. (88.1–99.4%) PPV= 96.8%; C.I. (88.1–99.4%) Specificity = 50.0% C.I. (9.1–90.8%) NPV = 50.0%; C.I. (9.1–90.8%)
C. gattii sensu lato	13 (19.7%)	11 (16.7%)	
Total isolates	66 (100%)	66 (100%)	

C.I. = confidence interval, NPV =Negative predictive value, PPV = Positive predictive value.

Table 3

Comparison of *Cryptococcus* typing based on AFLP genotyping and CDBT media

***Cryptococcus* species**	**Typing methods**		**Diagnostic accuracy**
	CDBT (test)	**AFLP (gold standard)**	
C. neoformans sensu lato	51 (77.3%)	55 (83.3%)	Sensitivity = 100%; C.I. (92.7–100%) PPV= 93.9%; C.I. (84.4 – 98.0%) Specificity = 33.3%; C.I. (6.0–75.9%) NPV = 100%; C.I. (19.8 – 100%)
C. gattii sensu lato	15 (22.7%)	11 (16.7%)	
Total isolates	66 (100%)	66 (100%)	

C.I. = confidence interval, NPV =Negative predictive value, PPV = Positive predictive value.