Abstract

Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are fungal pathogens found in the environment, which cause potentially life-threatening meningoencephalitis among human immunodeficiency virus (HIV) -infected and HIV-uninfected persons in South Africa. *Cryptococcus* is the most common cause of meningitis among South African adults. In South Africa, fluconazole is still used in combination with amphotericin B for induction treatment of HIV-associated cryptococcal meningitis and as monotherapy for the consolidation and maintenance treatment phases. Molecular characterisation using multilocus sequence typing (MLST) is a good discriminatory tool to genotype *Cryptococcus*. There is a high genetic diversity observed in strains isolated in southern Africa. Additionally, it was hypothesised that the molecular type VNB, that is endemic to southern Africa, may be more virulent among the *C. neoformans* molecular types. The overall aim of this study was to explore the genetic diversity, antifungal susceptibility and virulence of the two pathogenic *Cryptococcus* species complexes causing human disease in South Africa.

Materials and Methods

Multilocus sequence typing and fluconazole susceptibility testing was performed on a randomly selected sample of 251 *C. neoformans* and 146 *C. gattii* isolates collected from South African patients with cryptococcosis through laboratory-based surveillance from 2005 to 2009 and 2005 to 2013, respectively. We examined the association between clinical characteristics of patients and cryptococcal molecular type, and the effect of molecular type on in-hospital mortality. We

performed whole genome phylogenetic analysis of 15 *C. neoformans* isolates with the molecular type VNB and tested their virulence in a *Galleria mellonella* model. We also sequenced the genomes of 101 *C. gattii* isolates with the molecular type VGIV. We prospectively collected *C. neoformans* isolates from 1 January through 31 March 2017 from persons with a first episode of culture-confirmed cryptococcal disease. We determined fluconazole minimum inhibitory concentration (MIC) values (range: 0.125 μg/ml to 64 μg/ml) of 229 *C. neoformans* isolates using custom-made broth microdilution panels prepared, inoculated and read according to Clinical and Laboratory Standards Institute M27-A3 and M60 recommendations. We interpreted fluconazole MIC values using published epidemiological cutoff values (ECVs). The MIC values obtained in the 2017 survey were compared to MICs of 249 isolates from earlier surveillance (2007–2008).

Results

For *C. neoformans*, most isolates collected between 2005-2009 had the molecular type VNI (206/251, 82%), followed by VNII (25/251, 10%), VNB (15/251, 6%), and VNIV (5/251, 2%); with a total of 67 sequence types identified. There were no differences in fluconazole MIC values among molecular types and the majority of strains had low MIC values within the wild-type range (MIC_{50} of 1 μg/ml and MIC_{90} of 4 μg/ml). We did not find any associations between patients' clinical characteristics and molecular type and between molecular type and in-hospital mortality. Fifteen VNB strains were not highly virulent in a *G. mellonella* larval model. The majority of these VNB strains belonged to the VNBII clade and were very closely related by phylogenetic analysis.

In the 2017 survey, the fluconazole MIC_{50}, MIC_{90} and geometric mean MIC of 229 *C. neoformans* isolates was 4, 8 and 4.11 μg/ml compared to 1, 2 and 2.08 μg/ml in 2007–2008 (n = 249) respectively.

For *C. gattii*, most isolates had the molecular type VGIV (101/146, 70%), followed by VGI (27%, 40/146), VGII (2%, 3/146) and VGIII (1%, 2/146); with a total of 99 sequence types identified. The majority of *C. gattii* strains in our study had low fluconazole MIC values within the wild-type range (MIC_{50} of 4 μg/ml and MIC_{90} of 16 μg/ml). VGIV isolates had a lower fluconazole MIC_{50} value compared to non-VGIV isolates but still within one double-dilution. Compared to HIV-seronegative patients, HIV-seropositive patients were ten times more likely to be infected with a VGIV isolate on multivariable analysis, though with small numbers, the confidence interval (CI) was very wide (95% CI: 1.93-55.31, p=0.006). We did not find any association between molecular type and in-hospital mortality. Whole genome phylogenetic analysis of the VGIV strains in our study showed that the VGIV molecular type was highly diverse, with only some strains being closely related in two small clusters of ten and six strains, respectively.

Conclusion

Our studies showed that South African clinical *C. neoformans* and *C. gattii* isolates had a high genetic diversity with VNI and VGIV being the dominant molecular types among the two sibling species, respectively. Clinical *C. neoformans* and *C. gattii* strains collected between 2005 and

2013 mostly had low fluconazole MIC values, although the MIC_{50} and MIC_{90} values for *C. gattii* were two double-dilutions higher compared to those for *C. neoformans* and still within the wild-type range. We also observed that fluconazole MIC_{50} and MIC_{90} values were two-fold higher in clinical South African *C. neoformans* isolates from first episodes of meningitis collected in 2017 compared to 2007–2008. Our findings led to higher fluconazole dose recommendations for induction and consolidation phases in the 2019 Southern African guideline for HIV-associated cryptococcosis. The few South African VNB strains in our study were very closely related by whole genome phylogenetic analysis, suggesting a common environmental source. Whole genome phylogenetic analysis of 98 South African VGIV strains confirmed their high genetic diversity with most strains not being closely related. However, we observed two small clusters of closely related strains, one cluster from patients living in the Gauteng and Limpopo Provinces and the other cluster consisting of patients from the Mpumalanga Province of South Africa suggesting a similar environmental source.