IN VITRO AND *IN VIVO* CHARACTERISATION OF ISOLATES OF *CRYPTOCOCCUS*

NEOFORMANS CAUSING MENINGITIS IN HIV INFECTED AND UNINFECTED PATIENTS

IN VIETNAM

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Running title: Characterizing *Cryptococcus neoformans* from HIV infected and uninfected

patients

Keywords

Cryptococcal meningitis*, Cryptococcus neoformans,* MLST*,* immunocompetent*,* HIV-uninfected,

HIV, A/J mice, ST5, phenotype

Abstract

Most cryptococcal meningitis cases occur in immunocompromised individuals and are caused

by *Cryptococcus neoformans* var.*grubii.* We previously reported cryptococcal meningitis in

Vietnam arising atypically in HIV-uninfected, apparently immunocompetent patients, caused by

a single amplified fragment length polymorphism (AFLP) cluster of *C. neoformans* (VNI-γ). This

variant was less common in HIV-infected individuals. It is unclear why this lineage is associated

with HIV-uninfected, apparently immunocompetent patients. To better delineate the

phenotypic characteristics of *C. neoformans* VNI-γ in comparison with other *C. neoformans*

lineages we selected 30 representative isolates and compared their genotype-specific *in vitro*

pathogenic potential and *in vivo* virulence, and ability to induce inflammatory response in the

A/J mouse model. All isolates were genotyped using multi-locus sequence typing. All previously

AFLP-defined VNI-γ isolates were identified as sequence type (ST) 5. Compared with non-ST5

strains, ST5 isolates had significantly increased variability in cell and capsule size ($p<0.001$). ST5

and non-ST5 strains exhibited similar characteristics in relation to previously defined virulence

factors including melanization, growth at 37°C, and growth in cerebrospinal fluid. However, A/J

mice infected with ST5 isolates had significantly longer survival and lower fungal burdens at day

7 and death compared with non-ST5 isolates. ST5 isolates induced significantly greater initial

inflammatory responses than non-ST5 strains, measured by TNF-α concentrations ($p<0.001$).

Despite being generally less virulent in the mouse model, we hypothesize that the significant

within strain variation seen in ST5 isolates in the tested phenotypes may represent an

evolutionary advantage enabling adaptation to novel niches including apparently

immunocompetent human hosts.

Introduction

Cryptococcal meningitis (CM) is a life-threatening fungal infection. The largest burden of CM

occurs in immunocompromised individuals, including those with HIV infection, malignancy,

organ failure, autoimmune disease, and receiving immunomodulatory therapy (1, 2). Globally,

CM is estimated to result in 120,000 to 240,000 deaths per year (3).   Most cases of CM are

associated with two species: *Cryptococcus neoformans* and *Cryptococcus gattii* (2, 4). *C.*

neoformans is more commonly associated with CM in immunocompromised patients and

incorporates two varietal forms: *C. neoformans* var.*grubii* and *C. neoformans* var. *neoformans*.

C. neoformans var.*grubii* is isolated globally while *C. neoformans* var. *neoformans* is more

commonly isolated from patients in Europe (5, 6). The burden of CM is highest in sub-Saharan

Africa and South/Southeast Asia where there are more people living with HIV/AIDS. In these

tropical and subtropical regions, *C. gattii* CM is relatively uncommon in HIV-infected patients,

usually accounting for only 1-2% of cryptococcosis in HIV-infected individuals, although higher

rates have been reported from Botswana and Malawi (up to 30% of all CM cases) (7, 8). It is

widely acknowledged that *C. gattii* primarily causes disease in immunocompetent patients

while *C. neoformans* is associated with disease in immunocompromised patients(9, 10).

Where CM due to *C. neoformans* var.*grubii* occurs in HIV-uninfected individuals, reports most

often describe patients with an increased disease susceptibility due to some other underlying

immunosuppressive condition (11–13). However, in Australasia and the USA no clear underlying

immune deficit is identified in approximately 20% of CM cases in HIV-uninfected individuals

(14–19). In Vietnam, disease in HIV-uninfected patients accounts for approximately 10% of all

CM cases admitted to our referral hospital in Ho Chi Minh City (HCMC) (20). We have previously

reported that the majority of HIV-uninfected patients had no identified cause of

immunosuppression, and that >80% of HIV-uninfected CM patients were infected by yeasts

belonging to a single amplified fragment length polymorphism (AFLP)-defined cluster of *C.*

neoformans, which we had named VNI-γ (19, 20). Our clinical observation has been replicated

in China where over 70% of CM cases from apparently immunocompetent individuals were

infected with *C. neoformans* (17). The yeasts isolated from these immunocompetent patients in

China were closely related to each other, and belonged to a single multi-locus sequence type

(MLST), sequence type (ST)5 (17). Similarly, this ST accounted for more than 80% of non-HIV-

associated cases of CM in South Korea, although some patients from this group had other

underlying, potentially immunosuppressive conditions. (21). The association between a

particular ST/genotype and host immune phenotype could be explained by a lineage-specific

increase in pathogenic potential or microbial fitness, a currently unidentified host immune

deficit, or a combination of these factors.

In order to explore the hypothesis that VNI-γ strains have increased pathogenic potential, we

compared their virulence phenotypes in a range of in vitro experiments with non-VNI-γ strains.

Additionally, we exploited a mouse model of cryptococcosis to compare the relative *in vivo*

virulence and the differential immune responses of Vietnamese clinical isolates from CM

patients with and without HIV.

Material and methods

C. neoformans organisms and culture conditions

The yeasts used in this study were clinical isolates from the cerebrospinal fluid (CSF) of patients

enrolled in a randomized controlled trial of antifungal therapy in HIV-infected patients, and a

prospective, descriptive study of HIV-uninfected patients with central nervous system (CNS)

infections (20, 22). All studies were approved by the Institutional Review Board of the Hospital

for Tropical Diseases, Ho Chi Minh City, and the Oxford Tropical Ethics Committee or Liverpool

School of Tropical Medicine UK. All yeasts were archived at -80°C in Microbank beads (Pro-Lab

Diagnostics, UK). We randomly selected 30 isolates for characterization (15 from HIV-infected

patients and 15 from HIV-uninfected patients), from which eight representative isolates were

randomly selected for assessment of *in vivo* virulence in A/J mice. For each isolate, a single

colony was selected from the initial archive and purified for all subsequent experiments. For

each experiment, a single purified *C. neoformans* colony was inoculated into 5ml of Yeast

Peptone Dextrose (YPD) broth and incubated overnight at 30°C with agitation. Cells were

harvested and washed three times with phosphate-buffered saline (PBS). Inoculum

quantification was performed using a Cellometer X2 (Nexelom Biosciences, USA). The

cryptococcal mating type was determined by polymerase chain reaction amplification of the

MAT locus as previously described (23). The characteristics of the selected yeasts and the

clinical data from the corresponding patients are shown in Table 1.

DNA extraction and Multi-locus Sequence Typing (MLST) determination

All isolates have been previously genotyped using amplified fragment length polymorphism

(AFLP) (19). Total genomic DNA was extracted using the Masterpure Yeast DNA kit (Epicentre

Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions after growth

on Sabouraud agar. Seven MLST loci (CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, and URA5) were

amplified and sequenced following the procedures of the International Society for Human and

Animal Mycology (ISHAM) MLST consensus typing scheme for *C. neoformans*

(http://mlst.mycologylab.org)(24). Sequencing was performed using BigDye v3.1 Chemistry

(Applied Biosystems, CA, USA) on an ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

Both forward and reverse amplicons of each locus were sequenced. Consensus sequences were

manually edited using ContigExpress and aligned in AlignX , implemented in VectorNTI Suite 7.0

(InforMax, USA) (25). A single Allele Type (AT) number was assigned to each of the seven loci

using the ISHAM database (http://mlst.mycologylab.org)(24), resulting in a seven-number allelic

profile for each isolate. The allelic profiles defined the corresponding STs.

Ex vivo CSF growth, melanin production, and temperature–dependent growth

We measured growth of the various *C. neoformans* strains at different temperatures and in *ex*

vivo human CSF following previously described procedures with modifications for quantitative

assessment (26). To assess fungal growth at selected temperatures, inocula were adjusted to

10^6 cells/ml, serially diluted, and inoculated in duplicate onto YPD agar plates in 5ul aliquots

and incubated at 30^oC or 37^oC for 48 hours. After 48 hours, colony forming units (CFU) were

counted. For the *ex vivo* CSF experiment, CSF supernatant from three random de-identified HIV-

infected patients enrolled into an antifungal therapy trial was pooled, filtered, and stored at -

80^oC until use (22). Growth was assessed by adding 10ul of 10^6 cells/ml into 90ul of the pooled

CSF and incubating at 37^oC in $5\%CO_2$; CSF was serially diluted and inoculated onto YPD agar at

days 1 and 3 post-inoculation. All experiments were repeated in triplicate. *C. neoformans* H99

was included as a control in all experiments; data were standardized by expressing the growth

results as a ratio of the CFU of the clinical yeast isolate to the CFU of H99. Melanin production

was assessed by plating a drop of cell suspension (10^6/mL$^)$ onto L-DOPA agar (1g/L L-asparagine

, 1g/L glucose, 3g/L KH_2PO_4, 250mg/L $MgSO_4.7H_2O$, 1mg/L Thiamine HCl, 5μg/L Biotin, 100mg/L

L-DOPA, 20g/L Bacto Agar)(27, 28). Plates were incubated in the dark at 30^oC or 37^oC for 3 days.

Differences in melanization were compared visually with a melanotic control strain.

Capsule and cell size measurement

To measure the capsule thickness of yeasts grown *in vitro*, all isolates were sub-cultured onto

DMEM media (Dulbeco Modified Eagle Medium powder with 4.5g/L glucose L-glutamine,

sodium pyruvate (without $NaHCO_3$),; $NaHCO_3$ 250mM (Merck); NaMOPS 1M; Neomycin

200mg/ml, Cefotaxime 100 mg/ml; (29) and incubated at 37^oC in 5% CO_2 until single colonies

were visible. Unless otherwise specified, all reagents were purchased from Sigma-Alrich. India-

ink-stained smears were prepared on a microscope slide and visualized at 100X magnification

using light microscopy (CX41, Olympus, Japan). Images were captured using a DP71 Camera

system with DP Controller software (Olympus, Japan) and processed using ImageJ

(rsb.info.nih.gov/ij/). Capsular thickness was calculated by subtracting the cell body diameter

(D_{CD}, no capsule) from whole cell diameter (D_{WC}, including capsule). At least 30 individual cells

were measured for each isolate.

Mouse infection model of cryptococcosis

All mouse infections were conducted as previously described (30) and were performed

according to Duke University's Institutional Animal Care and Use Committee guidelines and

approvals.

The virulence of each individual strain was assessed by measuring survival rate and fungal

burden in tissue from two independent experiments. Six week old female A/J mice were lightly

sedated with isoflurane and infected intranasally with the selected cryptococcal strain by

dropping 25ul of yeast suspension containing $5x10^4$ cells into the nares (31, 32). Animals were

monitored daily. Mice showing signs of distress or losing more than 15% body weight were

euthanized by CO_2 inhalation. Survival assays were performed using 10 mice per strain.

To measure fungal burden in the mice, five animals were infected with each clinical isolate and

euthanized by CO_2 inhalation either on day 7 or day 14 post-infection, or when the animal

showed signs of distress or had lost >15% of initial body weight. Fungal burden was assessed by

excising the left superior lobe of the lung and homogenizing the tissue by bead beating. Tissue

homogenates were serially diluted and plated onto YPD agar supplemented with 100mg/ml

ampicillin. The plates were incubated at 30^oC for 48 hours and the number of *C. neoformans*

colony forming units (CFU) recorded. Fungal burdens were expressed as CFU per gram of tissue

(CFU/g). At the same time points (7 and 14 days post-infection), the right superior lobe of the

lung from each mouse was excised and immersed in 10% formalin (replaced with 70% Ethanol

after 24 hours) for histopathology. Fixed specimens were stored at 4^oC until processing. After

paraffin embedding, sliced sections were stained using the Period Acid-Schiff method (PAS).

Histopathological examination was performed by an independent pathologist blinded to the

infecting strain. The resulting tissue damage was scored from 0 (no changes) to 10 (severe

changes), corresponding to the severity of pathology in 4 different categories: necrosis,

hemorrhage, edema and inflammation as per the Duke Veterinary Diagnostic Laboratory

(Division of Laboratory Animal Resources).

To assess the severity of the inflammatory responses the middle lobe from the right lung of

each infected mouse was excised and homogenized by bead beating in 1ml sterile PBS/Protease

inhibitor. 500ul of lung homogenate was used for cytokine profiling. Cytokines representing T-

helper type 1 (Th1) (IL-12p70, TNF-α, IFN-γ), T-helper type 17 (IL-17) and T-helper type 2 (Th2)

(IL-4, IL-5, IL-10) responses were measured using a customized Bio-Plex Pro™ Mouse Cytokine

Th1/Th2 Assay kit (Biorad, USA) with the BioPlex 200 platform according to the manufacturer's

guidelines. Data were retrieved using BioPlex Manager software. The upper and lower limits of

quantification (ULOQ and LLOQ) were based on a standard curve. All values falling below the

LLOQ were replaced with the midpoint between zero and the LLOQ. Data were standardized by

lung weight and presented as pg of cytokines per gram lung tissue (pg/g).

Statistical analysis

GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA;

www.graphpad.com) was used for data visualization and statistical analyses of fungal loads,

cytokine profiling, capsular/cell size, and survival proportions. Mann-Whitney U-test was used

for comparing fungal load and cytokine concentrations. Kaplan-Meier survival curves, and the

log-rank test were used for survival analysis. Capsule/cell size was analyzed in GraphPad Prism

5.04 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com) and

compared using Welch's t-test. The Fligner-Killeen test of variance homogeneity for analyzing

variation in capsule/cell size and Fisher's exact test were performed using R software, version

3.2.4 (http://www.r-project.org). One way ANOVA with a post hoc multiple comparison test

(Dunnett or Bonferroni) integrated in Graphpad was used to compare cytokine concentrations

between individual isolates.

The study was approved by the scientific committee of the Hospital for Tropical Diseases, Ho

Chi Minh City, Vietnam. All animal experiments were performed according to Duke University's

Institutional Animal Care and Use Committee guidelines and approvals.

Results

Multi-locus Sequence Typing of Vietnamese C. neoformans isolates

All 30 isolates selected for characterization in the study were confirmed to be *C. neoformans*

serotype A, mating type α (Aα). MLST of the 30 isolates identified four different STs: ST4

(12/30), ST5 (15/30), ST6 (2/30), and ST306 (1/30). All 15 of the previous described AFLP VNI-γ

isolates were ST5, while the 15 AFLP VNI-δ (non- VNI-γ) isolates comprised three different STs

(ST4, ST6, and ST306). None of the HIV-uninfected patients with an ST5 infection had any other

known or apparent underlying diseases. The two HIV-uninfected patients with an identified

underlying disease (lupus and gastric cancer) were infected with an ST4 and a ST306 *C.*

neoformans, respectively.

In vitro phenotyping of Vietnamese C. neoformans isolates

Upon microscopic examination we observed that the ST5 *C. neoformans* isolates developed

significantly thicker capsule during *in vitro* culture than non-ST5 isolates (mean 2.64 μm [95%CI:

2.49 - 2.79μm] versus mean 2.01μm [95%CI: 1.94 - 2.07μm], respectively, *p*<0.0001, Welch's t-

test; also see Table 2). Individual ST5 *C. neoformans* cells were also significantly larger in size

than cells from the non-ST5 strains (cell diameter excluding the capsule 11.38μm [95%CI: 10.96

- 11.79µm] versus mean 9.69µm [95%CI: 9.52 - 9.87µm], respectively, $p<0.0001$, Welch's t-test;

also see Table 2). In fact, a high degree of variation in capsule size and cell diameter was

detected in yeast isolates belonging to ST5 (Figure 1); a Fligner-Killeen test for homogeneity of

variance confirmed this variation to be significantly greater for ST5 than non-ST5 isolates

($p<0.0001$) for both capsule size and cell diameter). Strains of both lineages tended to appear

less melanized at 37°C, in comparison to the control strain, H99 (see Supplementary Figure S1).

However, there were no genotype-specific differences in either growth at 30°C ($p=0.10$,

Welch's t-test; mean CFU/ml ratio to H99: 0.98[95%CI:0.74 to 1.22] for ST5 vs 1.01[95%CI:0.87

to 1.15] for non ST5) or 37°C ($p=0.23$, Welch's t-test; mean CFU/ml ratio to H99: 1.2[95%CI:0.98

to 1.42] for ST5 vs 1.11[95%CI:0.85 to 1.37] for non ST5) , or *ex vivo* survival in CSF after one day

exposure ($p=0.72$, Welch's t-test; mean CFU/ml ratio to H99: 11.72[95%CI:2.07 to 21.36] for ST5

vs 5.84[95%CI:1.37 to 10.31] for non ST5 at day 1; or *ex vivo* survival in CSF for three days

exposure: $p=0.77$, Welch's t-test; mean CFU/ml ratio to H99: 1.36[95%CI:0.67 to 2.06] for ST5

vs 3.23[95%CI:1.31 to 5.16] for non ST5 at day 3) (see Supplementary Figures S2 and S3).

Representative phenotyping data are shown in Supplementary Figures S4 and S5.

Mouse infection model of cryptococcosis with Vietnamese C. neoformans isolates

Animals were challenged individually with five randomly-chosen different ST5 strains (BK147,

BK44, BMD700, BMD1338 and BMD1646) and three non-ST5 strains (BMD1415, BK80 (both

ST4) and BMD1367 (ST306)). Infections with the ST5 strains were associated with significantly

longer survival time than infection with the` non-ST5 strains ($p<0.0001$, log-rank test, Figure 2).

However, there was significant variability in the effect of individual yeast strains of the same

lineage on survival time (Supplementary Figure S6). Notably, two ST5 isolates strains (BK147

and BMD1646) appeared to be substantially attenuated in comparison to the other ST5 yeast

strains with mice infected with these yeasts surviving up to day 40 post infection. However, the

differences in survival times between mice infected with ST5 and non-ST5 strains remained

significant when data from these two isolates were removed from the comparative analysis

(p=0.003, log-rank test, data not shown). Of the eight strains selected for infecting mice, the

two most virulent were BMD1415 (ST4), and BMD1338 (ST5); infection with these isolates

resulted in 100% mortality by days 17 and 18, respectively (median survival 15 and 17 days,

respectively). BMD1338 was significantly less virulent than BMD1415 (p=0.0015, log-rank test).

No significant difference in virulence was detected between BMD1338 and BMD1367, the

second most virulent non-ST5 strain. Although it was not as virulent as isolates BMD700 and

BMD1338, the ST5 isolate BK44 also resulted in 100% mortality by day 25.

All strains successfully established lung infection and disseminated to the brain of the mice as

early as day 7. Consistent with the survival data, the non-ST5 strains established a higher fungal

burden in the lung than the ST5 organisms at all time points (p=0.0002, p<0.0001, and p<0.0001

at day 7, day 14, and at the point of 15% weight loss, respectively, Mann-Whitney U-test)

(Figure 3). Moreover, these data were not driven by the two severely attenuated isolates

(BK147 and BMD1646) as the fungal loads associated with ST5 infections remained low even

when these two isolates were excluded from the analysis. The majority of mice infected with

BMD1646 (ST5) and BK147 (ST5) became sterile and therefore lung fungal burdens in these

animals were minimal up to day 14. On the other hand, despite these differences in fungal

burden in the lung, there were no ST5/non-ST5 specific differences in fungal burden in the brain

up to day 14. However, brain fungal burdens in non-ST5 infections were significantly higher

than the brain fungal burdens in ST5 infections at the point of 15% weight loss (mean logCFU.g^{-1}

(95%CI); 4.5 (3.9-4.7) and 2.9 (2.2-3.6) for non-ST5 and ST5 respectively, p=0.01, Mann-Whitney

U-test). Again, as seen with yeast cell size, significantly more intra-genotypic variation in mice

fungal burden was observed amongst the ST5 strains than amongst non-ST5 strains (p<0.001,

Fligner-Killeen test).

Inflammatory responses induced during a mouse infection model of cryptococcosis with C.

neoformans from HIV-uninfected patients

We next measured the concentration of cytokines from lung homogenates of A/J mice infected

with the various strains at days 7 and day 14 post-infection. Cytokine concentrations at these

time points are shown in Figure 4, according to the genotype of the infecting organism. At day

7, ST5 strain infections were associated with significantly higher concentrations of TNF-α in the

lung than non-ST5 strain infections (p=0.01, Mann-Whitney U-test; also see Table 4).

Furthermore, among ST5 strain infections at day 7, TNF-α concentrations were highest for the

two specific strains that appeared to be most attenuated in the mouse survival model (BK147

and BMD1646), and lowest in BMD1338, the most virulent ST5 isolate in the mouse model. By

day 14, mean TNF-α concentration associated with the ST5 strains had declined from 3933.71

pg/g to 2802.36 pg/g. Other Th-1 cytokines including IL-12, IL-17 and IFN-γ also decreased in

ST5-infected mice between day 7 and day 14 (p<0.001, p<0.01 and p=0.02, Mann-Whitney U-

test, respectively).

This contrasted with non-ST5 strain infections, in which mean TNF-α levels increased from

3256.25 pg/g to 6378.76 pg/g (p=0.0003 and p=0.0235, Mann-Whitney U-test, respectively;

also see Table 4) between days 7 and 14. However, the high concentration of TNF-α seen in

non-ST5 strain infections at day 14 was driven by a single isolate - BMD1415; TNF-α

concentrations in the lung in mice infected with this isolate were significantly higher than for

infections with any other ST5 or non-ST5 isolate (p<0.05, Mann-Whitney U-test). There were no

statistically significant differences in TNF-α concentration at day 14 when data from infections

due to this isolate were excluded from the analysis. The same BMD1415-driven pattern also

occurred when comparing IFN-γ and IL-17 concentrations at day 14. The concentrations of

these two cytokines in BMD1415-infected mice appeared significantly higher than in mice

infected with any other isolates (p<0.05, Mann-Whitney U-test; see Supplementary Figure S7).

However, IL-12 concentrations fell in non-ST5 strain infected mice, including BMD1415,

between day 7 and 14 (4 fold decrease, p<0.0001, Mann-Whitney U-test) (Figure 5).

We measured the TNF-α/IL-10 ratio as a proxy marker of the relationship between a Th-1 and a

Th-2 response (33). From day 7 to day 14 post-infection, the TNF-α: IL-10 ratio decreased

almost 1.28 fold in mice infected with ST5 isolates while those infected with non-ST5 strains

exhibited a TNF-α: IL-10 ratio increase of 1.25 fold. However, when BMD1415 (ST4) was

excluded from the analysis the TNF-α/IL-10 ratio in mice infected with non-ST5 strains

decreased by a factor of 0.67. By day 14 we detected elevated concentrations of IL-17 and IFN-γ

in non-ST5 infected mice (5-fold and 17-fold increments, respectively (Table 4), but not in ST5

infected mice. Again, this effect was largely associated with BMD1415, since the concentrations

of IL-17 and IFN-γ induced by this isolate at day 14 were significantly higher than for all others

($p<0.05$, one way ANOVA test; also see Supplementary Figure S7).

Histological examination of infected lung tissue revealed evidence of inflammation,

hemorrhage, edema and necrosis in most cases. These changes were generally greater by day

14 in comparison to day 7. There were no clear differences in histological scores between

sequence types, other than strain BMB1646 (ST5) which had only mild inflammation with no

necrosis or haemorrhage seen (Figure 6, also see Supplementary Figure S8). Though we could

not quantitatively assess and compare genotype-specific capsule size *in vivo,* ST5 yeasts

appeared extensively encapsulated in the lung sections, especially at day 14 (Figure 6). This

observation agreed with the previous finding that ST5 isolates have increased *in vitro* capsular

induction.

Discussion

Although *C. gattii* disease is well recognized in immunocompetent hosts worldwide [30], most

cases in apparently immunocompetent patients in Asia, including Vietnam, are due to *C.*

neoformans var.*grubii* [15,20,31,32]. We previously reported that 84% of *C. neoformans*

infections in HIV-uninfected patients in Vietnam are caused by a single AFLP-defined cluster

(VNI-γ); here we confirm these strains as MLST ST5, consistent with studies reported from

China, Japan and Korea [17,21,33,34]. It is unlikely that the high prevalence of ST5 infection

observed in HIV- uninfected patients is simply explained by significantly greater prevalence of

this lineage in the environment (and subsequent greater exposure) since ST5 accounts for only

35% of cases of disease in HIV- infected patients [19] within the same geographical area.

Furthermore, in the same locales ST5 is not more prevalent in any particular HIV- associated

sub-group such as intravenous drug users [19].

The high prevalence of ST5 strain infection in HIV-uninfected patients could be explained either

by the strains being intrinsically more virulent, or lineage–specific unidentified host immune

defects or exposures. In this study we investigated the first hypothesis by comparing previously

identified virulence-associated phenotypes (capsule size, *ex vivo* growth in CSF, cell wall

melanization and growth at human body temperature) as well as *in vivo* virulence and immune

responses in the mouse model by lineage [35–43]. Strains from all STs were able to grow in

human CSF and at 37°C - essential characteristics for human infections. However, we could not

identify ST-specific differences of growth under these conditions, suggesting that the ability to

establish disease in HIV-uninfected/immunocompetent patients is not driven by simple

adaptations to high temperature or growth in human CSF.

On the other hand, we found ST5 cells to be both significantly larger than non-ST5 cells and to

have thicker capsules in vitro, which might impact pathogenic potential [44–47]. The capsule of

C. neoformans plays important roles in pathogenesis, from interfering with phagocytosis by

host macrophages to modulating the host immune response [48–52]. Previously, it has been

shown that *in vitro*-induced capsular enlargement confers greater virulence in CBA/J mice [53–

55], and that variations in capsule size also correlate with pathogenic potential including

overcoming *in vivo* phagocytosis by host macrophages, leading to uncontrolled fungal growth

and host cell death [50,53,55]. *Ex vivo* capsule size has also been associated with higher

intracranial pressure, slower clearance of yeasts and paucity of inflammation in humans(34).

Our study demonstrates that while ST5 isolates have significantly higher capacity for *in vitro*

capsule enlargement, this phenotype did not translate into increased virulence in the mouse

model. Mice infected with ST5 isolates not only had better survival but also lower tissue fungal

burdens than those infected with non-ST5 strains, contrary to recent human data which

supports morphological changes being associated with more severe pathology in the human

host (e.g *ex vivo* capsule size and capsular shedding has been associated with increased

intracranial pressure in CM patients with HIV)(34).

There are several possible explanations for the lower virulence of ST5 in mice we observed.

First, virulence in mice is variable depending on mouse breed and may not accurately reflect

the immunological heterogeneity of the human population [56]. Second, yeasts with different

pathogenic potentials, associated with their isolation from different sources (i.e. clinical versus

environmental, immunocompromised vs immunocompetent patients) may have the same or

paradoxical pathogenic potential in experimental animal models. For instance, *C. gattii* is often

associated with infection in immunocompetent patients and therefore is considered more

virulent (or fit) in the human host than *C. neoformans*. However, it has been shown that the *C.*

gattii strain R265, responsible for the on-going Vancouver outbreak, has similar virulence in

both C57BL/6 and A/J mice to the *C. neoformans* H99 strain, which is derived from a patient on

chemotherapy with Hodgkin's disease [57]. Furthermore, the same study reported it was

impossible to distinguish the virulence of strain R272, a Vancouver outbreak *C. gattii* VGIIb

strain, from WM276, an environmental isolate from Australia, with either murine model [57].

Third, the A/J mouse strain is not immunologically intact and thus may be an imperfect model

of infection for immunocompetent hosts [58,59]. Rather, it may be a better model of disease in

immunosuppressed patients, as the mice are highly susceptible to cryptococcal disease, and the

patterns of cytokine expression in mice with disseminated cryptococcosis are similar to those

seen in HIV-infected patients with CM [1,60]. Our results suggest that additional models to the

mouse may be needed to explain the complex interaction between *C. neoformans* and the

human host, particularly when the host is apparently immunocompetent.

In cryptococcal infection in mice, a Th1 type immune response, defined by the TNF-α/IL-10

ratio, is considered to be protective, while a Th2 response is associated with poor outcomes

[61,62]. We did not detect genotypic-driven changes in TNF-α/IL-10 ratios over time, although

higher TNF-α concentrations were induced by ST5 strains at day 7 suggesting this genotype

elicited a more intense initial inflammatory response. This finding could be explained by the

increased capsular size of ST5 organisms - previous studies have suggested that capsule

components, or cryptococcal cells themselves, have a dose-dependent ability to stimulate TNF-

α production by various immune effector cells [13,63–67]. We found that ST5 organisms

induced stronger inflammatory response in mice even though the ST5 fungal burden was

around 5 times lower than that of non-ST5 organisms 7 days post-infection, suggesting that

TNF-α production may be driven by capsular components rather than cell number. Previously, it

has been suggested that *C. gattii* is able to cause disease in apparently immunocompetent

patients because it induces a less severe inflammatory response than other cryptococci [57].

Data generated from our murine infection experiments do not support a less severe

inflammatory response being the mechanism underlying the ability of ST5 organisms to cause

disease in the immunocompetent.

In vivo controlled infection studies in mice commonly define pathogenicity as the microbe's

capability to cause disease in a susceptible host, whereas virulence corresponds to the severity

of the ensuing pathology [68]. Using the same infective dose for all strains we failed to

demonstrate that ST5 strains had greater virulence than non-ST5 organisms. The difference we

observe in prevalence of different lineages in immunocompetent and immunosuppressed

human patients may alternatively represent a specific difference in the ability of the organisms

to colonize the host and establish infection; we were not able to assess this with our

experimental systems. Spores are presumed to be the main infectious propagule for

Cryptococcus spp, although desiccated yeast cells have also been proposed [69]. Capsule

biosynthesis genes are required for both efficient sexual development and spore formation and

dispersal [70]. Given the higher rate of capsular enlargement and higher variation in capsule

size in ST5 strains, it is possible that capsule biosynthesis genes are more active in ST5 yeasts,

resulting in qualitative differences in their spores that confer higher infectivity when interacting

with immunocompetent hosts. Moreover, the higher rates of variability that we see in this and

other virulence phenotypes (for example, growth at 37C, see supplementary information)

within ST5 strains in this study hint that higher phenotypic variability in ST5 may be an adopted

strategy that facilitates the exploitation of novel niches. This strategy could have resulted in a

phenotype which allowed the successful colonization of one or more novel environmental

nich(es), and as a bystander-effect, allowed the exploitation of infrequent specific human

immune deficits. Thus, our cohort of ST5 yeast isolates displayed two notable phenotypes.

First, in general they appeared to be less virulent in a murine model than the other sequence

types, as demonstrated by reduced fungal burdens in tissue, induction of greater early

immunological responses and prolonged mouse survival. Second, ST5 strains had larger

capsules and cell sizes than the other genotypes, and notably greater variability between

individual strains for this phenotype.

Our data lead us to three main conclusions. First, clinical isolates, which have by their nature

already undergone selection within the human host, can possess wide variability in the

expression of virulence phenotypes within a single lineage. Secondly, the use of host risk

factors and immune phenotype to derive an understanding of the factors that drive the

pathogenicity of *Cryptococcus* may be more complex than anticipated. Associations may be

difficult to make due to the relevance of the particular *in vitro* phenotypes, animal models used,

within strain heterogeneity, and population substructure. Moreover, there may be significant

heterogeneity in the immune response of apparently immunocompetent patients which selects

particular sub-cohorts of isolates of the same lineage. Thirdly, the categorization of strains into

specific clades with limited genetic information such as MLST may lack precision to understand

the relative fitness of specific strains in the human host. It is likely that whole genome

sequencing will provide better mapping of the relationships between strains and virulence.

Lastly, we tested a relatively small number of strains, and may have lacked power to detect

relevant differences that are present at the wider population level.

Conclusions

In this study, we demonstrated genotype-specific differences in *in vitro* and *in vivo* virulence

phenotypes between *C. neoformans* var.*grubii* strains isolated from host with different immune

status. There was also significant variation among strains isolated from apparently

immunocompetent patients in specific *in vitro* and *in vivo* phenotypes tested. This higher rate

of phenotypic variation may represent an evolutionary strategy for *C. neoformans* var.*grubii* to

take advantage of novel niches and contribute to their ability to infect apparently

immunocompetent hosts, despite generally being less virulent in a mammalian animal model.

Furthering the understanding of the pathogenesis of cryptococcal meningitis will require

investigation of large numbers of strains with associated robust clinical information, and the

development of high throughput laboratory phenotypic studies that have clinical relevance in

humans.

Acknowledgements

JND is a Wellcome Trust Intermediate Fellow (WTWT097147MA). SB is a Sir Henry Dale Fellow,

jointly funded by the Wellcome Trust and the Royal Society (100087/Z/12/Z). Multiplex

cytokine profiling was performed in the Immunology Unit of the Duke Regional Biocontainment

Laboratory, which received partial support for construction from the US National Institutes of

Health, National Institute of Allergy and Infectious Diseases (UC6-AI058607).

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FIGURE 1: *In vitro* **induced capsule thickness and cell diameter of individual** *Cryptococcus neoformans* **strains from Vietnam:** Cells were grown on DMEM medium/5% CO_2 and visually assessed by India ink staining. Images were taken for single cells measurement using ImageJ software. Capsule thickness is obtained by subtracting cell body diameter from total cell diameter. AFLP-VNI-γ/MLST-ST5 strains expressed higher degree of variation in both capsule size and cell diameter *in vitro*. Scattered plot represents single cells from an individual strain. Data for individual strains are presented as mean with error bars denoting standard deviation.

FIGURE 2: Kaplan-Meier survival curves for mice infected with either VNI-γ or VNI-δ *Cryptococcus neoformans* strains. 10 mice were infected per strain **ST5** N = 5, **non-ST5** N = 3). Mice were monitored daily until the point of more than 15% weight loss or visible suffering and were then sacrificed by CO_2 inhalation. Mice infected with ST5 strains had statistically significantly longer survival times than those infected with non-ST5 strains (P<0.0001, Mantel-Cox log rank test).

FIGURE 3: Fungal burden in lung tissue at day 14 (A), lung fungal burden at point of impending death (B) and brain fungal burden at point of impending death (C) according to infecting genotype. 5 mice were infected per strain **(ST5** N = 5, **non-ST5** N = 3). Mice were monitored daily until there was > 15% weight loss or visible suffering and were then sacrificed by CO_2 inhalation. Non-ST5 fungal burden appeared higher than that of ST5 in both lung and brain tissue at all time points (Mann-Whitney test, **: $P<0.0001$, *: $P<0.05$). The horizontal line within the box indicates the median; boundaries of the box indicate the 25th and 75th percentile and the whiskers indicate the highest and lowest values of the results; outliers are denoted as black dots.

FIGURE 4: Genotype-specific cytokines concentrations from lung homogenate of A/J mice at 7 and 14 days post infection with 5x10^4 *C. neoformans* cells/mouse. Box and whisker plots compare levels of each cytokine between each genotype at day 7 and day 14 post-infection. The horizontal line within the box indicates the median; boundaries of the box indicate the 25th and 75th percentile and the whiskers indicate the highest and lowest values of the results; outliers are denoted as black dots. Data are standardized as picogram of cytokine per gram lung tissue. Asterisks show statistical significance by Mann-Whitney test.

FIGURE 5: Genotype-specific changes of cytokine concentration from lung homogenate of A/J mice infected with 5x10^4 *C. neoformans* cells/mouse between day 7 and day 14 post-infection. Box and whisker plots compare levels of each cytokine between day 7 and day 14 for each genotype. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentile and the whiskers indicate the highest and lowest values of the results; outliers are denoted as black dots. Data are standardized as picogram of cytokine per gram lung tissue. Asterisks show statistical significance by Mann-Whitney test.

Day 7

Day 14

FIGURE 6: Periodic Acid Schiff (PAS) staining of pulmonary tissue from mice infected with BMD1338 and BMD1415 on days 7 ad 14. The two strains represent ST5 and non-ST5, respectively. A/J mice were inoculated intranasally with 5×10^4 yeast cells. Lung specimens were harvested at days 7 and 14 for histopathological examination. Photomicrographs were obtained at 200X magnification; the scale bar represents 215 µm). (A-B): lung sections from mice infected with BMD1338 (VNI-γ/ST5) at day 7 and day 14, respectively. (C-D): lung sections from mice infected with BMD1415 (VNI-δ/ST4) at day 7 and day 14, respectively. Perivascular infiltration (red arrows) and necrosis are more marked by day 14 for both strains. Encapsulated yeasts (yellow arrows), notable for the larger cell size and capsule thickness of BMD 1338 compared with BMD1415.

TABLE 1 Strain source and typing (n=30)

Isolate data				Patient data	
Order	Strain name	AFLP	MLST	Underlying Disease	Sex
1	BK14	VNI-δ	4	HIV	M
2	BK163	VNI-δ	4	HIV	M
4	BK225	VNI-δ	4	HIV	M
6	BK48	VNI-δ	4	HIV	M
7	BK59	VNI-δ	4	HIV	F
8	BK69	VNI-δ	4	HIV	M
9	BK74	VNI-δ	4	HIV	M
10	BK80*	VNI-δ	4	HIV	M
11	BK87	VNI-δ	4	HIV	M
12	BK88	VNI-δ	4	HIV	M
13	BK89	VNI-δ	4	HIV	M
15	BMD1415*	VNI-δ	4	Lupus	M
3	BK218	VNI-δ	6	HIV	M
5	BK234	VNI-δ	6	HIV	F
14	BMD1367*	VNI-δ	306	Gastric cancer	F
16	BK147*	VNI-γ	5	HIV	M
17	BK44*	VNI-γ	5	HIV	M
18	BMD101	VNI-γ	5	None known	M
19	BMD1228	VNI-γ	5	None known	F
20	BMD1291	VNI-γ	5	None known	F
21	BMD1338*	VNI-γ	5	None known	M
22	BMD1353	VNI-γ	5	None known	M
23	BMD1452	VNI-γ	5	None known	F
24	BMD1646	VNI-γ	5	None known	M
25	BMD1716	VNI-γ	5	None known	F
26	BMD367	VNI-γ	5	None known	M
27	BMD673	VNI-γ	5	None known	F
28	BMD700*	VNI-γ	5	None known	M
29	BMD854	VNI-γ	5	None known	F
30	BMD899	VNI-γ	5	None known	F

AFLP = amplified fragment length polymorphism
MLST = multi locus sequence typing
M = Male, F = Female
* indicates isolates selected for mouse experiment

TABLE 2 Variability in *in vitro* capsule thickness and cell diameter between ST5 and non-ST5
Cryptococcus neoformans strains from Vietnam

	MLST Group	
Variable	**ST5**	**Non-ST5**
Capsule Thickness (µm)		
Mean*	2.64	2.01
95% CI	2.49 - 2.79	1.94 - 2.07
Range	0.01 - 9.56	0.02 - 6.16
Coefficient of Variation$ (%)	0.63	0.37
Cell diameter (µm)		
Mean*	11.38	9.69
95% CI of Mean	10.96 - 11.79	9.52 - 9.87
Range	4.74 - 27.22	5.25 - 19.89
Coefficient of Variation$ (%)	0.41	0.20

* P<0.0001, t-test with Welch's correction.

$ *P< 0.0001, Fligner-Killeen test of homogeneity of variance.*

TABLE 3 Tissue fungal burden in mice at days 7, 14 and death by infecting Sequence Type, log10Colony Forming Units of yeast per gram of tissue

Fungal burden

(Mean $\log_{10}$ colony forming units per gram of tissue)

[95%CI]

Tissue	ST5	Non-ST5	P Value
Lung (Day 7)	4.96	7.07	<0.01
	[3.86 - 6.05]	[6.85 - 7.29]*	
Brain (Day 7)	0.81	1.59	0.05
	[0.38 - 1.24]	[0.87 - 2.30]	
Lung (Day 14)	5.48	8.00	<0.0001
	[4.34 - 6.62]	[7.77 - 8.23]*	
Brain (Day 14)	1.56	1.91	0.36
	[0.81 - 2.31]	[1.12 - 2.69]	
Lung (Death)	3.81	6.53	<0.0001
	[3.05 - 4.57]	[6.30 - 6.76]*	
Brain (Death)	2.90	4.29	0.01
	[2.23 - 3.56]	[3.90 - 4.68]*	

Mann-Whitney test.

TABLE 4: Cytokine concentrations from lung homogenate of infected mice at day 7 and day 14 post-infection according to infecting *Cryptococcus neoformans* AFLP group

Cytokine		Mean cytokine concentration (pg per gram of lung tissue) [95%CI]		*P Value (ST5 vs non-ST5)
		ST5	Non-ST5	
Day 7				
Th-1	IL-12	853.12 [750.50 - 955.80]	822.87 [608.90 - 1037.00]	0.30
	IFN-γ	136.02 [115.00 - 157.00]	150.59 [105.80 - 195.30]	0.97
	TNF-α	3933.71 [3486.00 - 4381.00]	3256.25 [2630.00 – 3882.00]	**0.01**
Th-2	IL-4	230.79 [162.80 - 298.80]	253.24 [11.60 - 349.90]	0.92
	IL-5	561.05 [369.00 - 753.10]	459.85 [344.40 - 575.40]	0.91
	IL-10	145.01 [128.20 - 161.80]	139.99 [119.30 - 160.70]	0.68
Th-17	IL-17	268.48 [155.90 – 381.00]	243.41 [165.60 - 321.20]	0.24
TNF-α/IL-10 ratio		27.13	23.26	N/A
Day 14				
Th-1	IL-12	547.84 [412.40 - 683.30]	213.72 [120.50 - 306.90]	**<0.01**
	IFN-γ	91.2 [57.25 - 125.20]	1760.67 [166.50 - 3355.00]	0.09
	TNF-α	2802.36 [2385.00 - 3220.00]	6378.76 [3374.00 - 9384.00]	**<0.01**
Th-2	IL-4	1154.72 [673.50 – 636.00]	800.35 [604.70 – 996.00]	0.60
	IL-5	294.6 [170.40 - 418.80]	347.55 [279.20 - 415.90]	0.15
	IL-10	132.76 [102.10 - 163.40]	220.15 [172.40 - 267.90]	**0.01**
Th-17	IL-17	175.75 [65.54 – 286.00]	1012.49 [459.20 – 1566.00]	**0.01**
TNF-α/IL-10 ratio		21.11	28.97	N/A

* Mann-Whitney test.