Most Cases of Cryptococcal Meningitis in HIV-Uninfected Patients in Vietnam Are Due to a Distinct Amplified Fragment Length Polymorphism-Defined Cluster of *Cryptococcus neoformans* var. *grubii* VN1

Jeremy N. Day,1,2* Thu N. Hoang,1,2 Anh V. Duong,1,2 Chau T. T. Hong,1,2,3 Pham T. Diep,1,2 James I. Campbell,1,2 Tran P. M. Sieu,3 Tran T. Hien,1,2 Tien Bui,4 Maciej F. Boni,1,2 David G. Lalloo,5 Dee Carter,4 Stephen Baker,1,2 and Jeremy J. Farrar1,2

Oxford University Clinical Research Unit, Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam1; Wellcome Trust Major Overseas Programme, Ho Chi Minh City, Vietnam2; Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam3; School of Molecular Bioscience, University of Sydney, Sydney, Australia4; and Liverpool School of Tropical Medicine, Liverpool, United Kingdom5

Received 1 October 2010/Returned for modification 17 November 2010/Accepted 3 December 2010

Cryptococcal disease most commonly occurs in patients with an underlying immune deficit, most commonly HIV infection, and is due to *Cryptococcus neoformans* var. *grubii*. Occasionally disease due to this variety occurs in apparently immunocompetent patients. The relationship between strains infecting immunosuppressed and immunocompetent patients is not clear. Amplified fragment length polymorphism (AFLP) analysis was used to characterize the relationship between strains infecting HIV-infected and uninfected patients. Isolates from 51 HIV-uninfected patients and 100 HIV-infected patients with cryptococcal meningitis were compared. *C. neoformans* var. *grubii* VNI was responsible for infections in 73% of HIV-uninfected and 100% of HIV-infected patients. AFLP analysis defined two distinct clusters, VNI1 and VNI6. The majority (84%) of isolates from HIV-uninfected patients were VNI1, compared with only 38% of isolates from HIV-infected patients (odds ratio, 8.30; 95% confidence interval [CI], 3.04 to 26.6; P < 0.0001). In HIV-uninfected patients, underlying disease was less frequent in those with VNI1 infections. Two clusters of *C. neoformans* var. *grubii* VNI are responsible for the majority of cases of cryptococcal meningitis in Vietnam. The distribution of these clusters differs according to the immune status of the host.

There are an estimated 1 million cases of cryptococcal meningitis, resulting in 625,000 deaths, every year (42). Most disease occurs in patients with underlying immunosuppressive conditions, including HIV infection, malignancy, organ failure, immunomodulating therapy, or autoimmune disease. Two species of *Cryptococcus* cause almost all human infections: *Cryptococcus neoformans* and *Cryptococcus gattii* (25, 28). Host and geographical factors influence which species is responsible for disease in different settings (8, 26). *C. neoformans* most commonly affects immunocompromised patients, and two varietal forms are known: *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*. *C. neoformans* var. *grubii* is distributed worldwide and is associated with pigeon guano (14). The vast majority of disease in HIV-infected patients is due to this variety, and while *C. neoformans* var. *neoformans* accounts for up to 25% of cases in Western Europe, it is rarely isolated outside this region (11, 12, 17–19, 22). *C. gattii* occurs mainly in the tropics and subtropics, is associated with eucalyptus and other tree species, and is most frequently described as causing disease in immunocompetent patients (9, 15, 16, 23, 27, 29–31, 38, 39, 49). An outbreak of disease due to *C. gattii* has been ongoing in British Columbia, Canada, since 1999, and there is evidence of autochthonous spread, suggesting that it is exploiting new environments and climatic zones (24, 51). Despite the high rates of HIV infection in the tropics, disease due to *C. gattii* is uncommon; in South Africa and Rwanda it is responsible for only 1 to 2% of cryptococcosis in HIV-infected patients, although the rate is higher (between 14 and 30%) in Botswana and Malawi (3, 34, 40, 50). Thus, due to the high prevalence of HIV infection, most cases of cryptococcal meningitis occur in the tropics and are caused by *C. neoformans* var. *grubii* (8, 22, 34, 37, 40, 42).

HIV-uninfected patients with cryptococcal meningitis due to *C. neoformans* usually have some other comorbidity present that is associated with immunosuppression (1, 8, 11, 13, 26, 39, 44, 47, 49). Occasionally, disease occurs in patients without any clear underlying immune deficit or predisposing condition. In Australia and New Zealand, up to 20% of all *C. neoformans* cryptococcosis has been reported to occur in immunocompetent individuals (8, 49). This contrasts with a recent report from China, where although disease is rare, most cases occur in patients without an obvious underlying immune deficit and are due to *C. neoformans* var. *grubii*. The strains found in China are closely related and of a single multilocus sequence type (MLST) (7).

The different species and varietal forms within the *C. neoformans* species complex can be distinguished by serological and molecular methods. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *URA5* gene reliably divides *C. neoformans* and *C. gattii* into eight molecular geno-
types (VNI to VNV and VGI to VGIV) (37). These genotypes are supported by PCR fingerprinting, amplified fragment length polymorphism (AFLP) analysis, and multigene sequencing studies (2, 5, 36, 41). More recently, AFLP analysis and MLST have successfully identified a novel genotype of C. neoformans var. grubii from Botswana that is closely related to the VNI genotype (35). While not all environmental strains of C. neoformans are able to cause disease, the pathological significance of differences in genotypes of clinical isolates at the subvarietal level has not been determined (33).

It is not clear why immunocompetent patients are occasionally infected with C. neoformans. The pathogen may exploit an unrecognized immune defect in the host or have some quality that increases its pathogenicity. In Vietnam, most HIV-uninfected patients with cryptococcal meningitis do not have obvious underlying conditions associated with immunosuppression.

We investigated the molecular epidemiological relationship between strains of C. neoformans from HIV-infected and uninfected patients in Vietnam using PCR-RFLP analysis of the URA5 gene and AFLP analysis in order to test the hypothesis that strains from HIV-uninfected patients are a closely related population.

**MATERIALS AND METHODS**

**Setting, participants, and strains.** The work was conducted at the Hospital for Tropical Diseases, Ho Chi Minh City, the tertiary referral center for infectious diseases in southern Vietnam. Strains were obtained from patients with cryptococcal meningitis enrolled into two clinical studies: a prospective descriptive study of cryptococcal meningitis in HIV-uninfected patients and a randomized controlled trial (RCT) of combination antifungal therapy in HIV-associated cryptococcal meningitis (http://www.controlled-trials.com/ISRCTN95123928/farrar). Cryptococcal meningitis was defined through the identification of C. neoformans or C. gattii from cerebrospinal fluid (CSF) by India ink staining, culture, or antigen testing or as a clinical syndrome consistent with cryptococcal meningitis and a positive blood cryptococcal antigen or culture. After giving informed consent, all patients underwent treatment and follow-up on the dedicated research ward. Ethical approval for the studies was obtained from the Hospital for Tropical Diseases, the Liverpool School of Tropical Medicine Research Ethics Committee, and the Oxford University Tropical Ethics Committee, United Kingdom. All patients were older than 15 years. Exclusion criteria for the randomized controlled trial were a previous episode of cryptococcal meningitis, liver or renal failure, and pregnancy.

The strains used were the initial isolates identified at diagnosis. Strains from HIV-uninfected patients were from patients sequentially enrolled into the study between April 1996 and June 2009. Strains from HIV-infected patients were from patients sequentially enrolled into the RCT between April 2004 and June 2009. All strains were confirmed as C. neoformans or C. gattii from cerebrospinal fluid (CSF) by India ink staining, culture, or antigen testing or as a clinical syndrome consistent with cryptococcal meningitis and a positive blood cryptococcal antigen or culture. After giving informed consent, all patients underwent treatment and follow-up on the dedicated research ward. Ethical approval for the studies was obtained from the Hospital for Tropical Diseases, the Liverpool School of Tropical Medicine Research Ethics Committee, and the Oxford University Tropical Ethics Committee, United Kingdom. All patients were older than 15 years. Exclusion criteria for the randomized controlled trial were a previous episode of cryptococcal meningitis, liver or renal failure, and pregnancy.

The strains used were the initial isolates identified at diagnosis. Strains from HIV-uninfected patients were from patients sequentially enrolled into the study between April 1996 and June 2009. Strains from HIV-infected patients were from patients sequentially enrolled into the RCT between April 2004 and June 2009. All strains were confirmed as C. neoformans or C. gattii from cerebrospinal fluid (CSF) by India ink staining, culture, or antigen testing or as a clinical syndrome consistent with cryptococcal meningitis and a positive blood cryptococcal antigen or culture. After giving informed consent, all patients underwent treatment and follow-up on the dedicated research ward. Ethical approval for the studies was obtained from the Hospital for Tropical Diseases, the Liverpool School of Tropical Medicine Research Ethics Committee, and the Oxford University Tropical Ethics Committee, United Kingdom. All patients were older than 15 years. Exclusion criteria for the randomized controlled trial were a previous episode of cryptococcal meningitis, liver or renal failure, and pregnancy.

The strains used were the initial isolates identified at diagnosis. Strains from HIV-uninfected patients were from patients sequentially enrolled into the study between April 1996 and June 2009. Strains from HIV-infected patients were from patients sequentially enrolled into the RCT between April 2004 and June 2009. All strains were confirmed as C. neoformans or C. gattii from cerebrospinal fluid (CSF) by India ink staining, culture, or antigen testing or as a clinical syndrome consistent with cryptococcal meningitis and a positive blood cryptococcal antigen or culture. After giving informed consent, all patients underwent treatment and follow-up on the dedicated research ward. Ethical approval for the studies was obtained from the Hospital for Tropical Diseases, the Liverpool School of Tropical Medicine Research Ethics Committee, and the Oxford University Tropical Ethics Committee, United Kingdom. All patients were older than 15 years. Exclusion criteria for the randomized controlled trial were a previous episode of cryptococcal meningitis, liver or renal failure, and pregnancy.

The strains used were the initial isolates identified at diagnosis. Strains from HIV-uninfected patients were from patients sequentially enrolled into the study between April 1996 and June 2009. Strains from HIV-infected patients were from patients sequentially enrolled into the RCT between April 2004 and June 2009. All strains were confirmed as C. neoformans or C. gattii from cerebrospinal fluid (CSF) by India ink staining, culture, or antigen testing or as a clinical syndrome consistent with cryptococcal meningitis and a positive blood cryptococcal antigen or culture. After giving informed consent, all patients underwent treatment and follow-up on the dedicated research ward. Ethical approval for the studies was obtained from the Hospital for Tropical Diseases, the Liverpool School of Tropical Medicine Research Ethics Committee, and the Oxford University Tropical Ethics Committee, United Kingdom. All patients were older than 15 years. Exclusion criteria for the randomized controlled trial were a previous episode of cryptococcal meningitis, liver or renal failure, and pregnancy.
are shown in Table 1. Thirty-seven isolates were *Cryptococcus neoformans* var. *grubii* molecular genotype VNI, and 14 were *Cryptococcus gattii* (13 *C. gattii* molecular genotype VGI and one *C. gattii* molecular genotype VGII). A total of 238 HIV-infected patients were enrolled into the randomized controlled trial between April 2004 and June 2009. The median age of the patients was 26 years (range, 15 to 62 years), and the median CD4 count was 15 cells/μL. A total of 211 baseline CSF isolates were available from these patients. All were *C. neoformans* var. *grubii* URA5 molecular genotype VNI. Representative strains and the characteristic banding patterns produced by URA5 PCR-RFLP analysis are shown in Fig. 1.

**AFLP analysis of 144 representative *Cryptococcus* isolates from HIV-infected and uninfected patients.** AFLP analysis was performed with 144 of the isolates described above. These included all 37 *C. neoformans* var. *grubii* isolates from HIV-uninfected patients, 100 randomly selected *C. neoformans* var. *grubii* isolates from HIV-infected patients, the four *C. neoformans* control strains (*C. neoformans* var. *grubii* VNI and VNII, *C. neoformans* var. *neoformans* VNIV, and an AD hybrid VNIII), and three *C. gattii* strains. The median similarities of fingerprint patterns from duplicate experiments on identical isolates were 95.1% (interquartile range [IQR], 94.2 to 98.1%) for the GT/GT primer set and 92.5% (IQR, 88.85 to 99.4%) for the AC/G primer set. Both primer sets reliably distinguished strains by species and RFLP molecular group. The neighboring joining trees generated with each primer pair for the experimental set are shown in Fig. 2a and b.

The GT/GT primer set divided the VNI population into two clusters designated VNIγ and VNIδ (Fig. 2a). The cophenetic correlation value at the separating node was 0.91, demonstrating robust clustering. Cluster VNIγ consisted of 69 isolates, and cluster VNIδ consisted of 68 isolates. The HIV serostatus of the source patient was related to isolate cluster (Table 2). Thirty-one isolates (84%) from HIV-uninfected patients were VNIγ, compared with 38 (38%) of isolates from HIV-infected patients (odds ratio, 8.30; 95% confidence interval [CI], 3.04 to 26.6; *P* < 0.0001). Additionally, in HIV-uninfected patients, other underlying diseases were more common in those with VNIδ infections than in those with VNIγ infections (4 of 6 [67%] versus 7 of 31 [23%]; odds ratio, 6.43; 95% CI, 0.75 to 85.1; *P* = 0.05). In HIV patients the clusters did not segregate by HIV risk group; 36% of intravenous drug users had VNIγ infections, compared with 41% of those believed to have had a sexual route of acquisition of HIV infection (*P* = 0.67). While a larger proportion of HIV-infected patients were male (87%), there was no difference in cluster distribution by patient gender (38% versus 45% VNIγ for males and females, respectively; *P* = 0.75). HIV-infected patients were more likely to be from Ho Chi Minh City than the provinces (64% versus 29%; *P* = 0.007). However, within groups (HIV infected or uninfected), the patient address (Ho Chi Minh City versus province) had no impact on the distribution of isolate cluster; for HIV-associated isolates, 25 of 63 (40%) of isolates from patients with Ho Chi Minh City addresses were VNIγ, compared with 13 of 36 (36%) of isolates from province dwellers (*P* = 0.83); for HIV-uninfected patients, 9 of 10 (90%) of Ho Chi Minh City residents had VNIγ infections versus 20 of 25 (80%) of those from the provinces (*P* = 0.65). Isolates from HIV-negative patients were collected over a period of 13 years. There was no change in the relative proportion of VNI clusters over the study period (*P* = 1.0).

The AC/G primer set defined a monophyletic cluster that included the same 31 HIV-negative isolates assigned to the
VNIγ genotype cluster by the GT/GT primer set (Fig. 2b). The cophenetic correlation value of the node defining this cluster was 0.96, again supporting robust clustering.

The results of AFLP typing were not perfectly consistent between primer sets: five strains from HIV-infected patients identified in cluster VNIγ by the GT/GT primer set were not preserved in the monophyletic cluster using the AC/G primer set, and three HIV-associated strains that the AC/G primer set identified within the monophyletic cluster were assigned to cluster VNIδ by the GT/GT primer set. The AC/G-derived dendrogram suggested more variability within this population than that derived from the GT/GT primer set. All the major nodes along the tree were well supported, with cophenetic correlation values of greater than 0.9.

**DISCUSSION**

Our findings demonstrate that the vast majority of cases of cryptococcal meningitis in Vietnamese patients, irrespective of HIV infection status, are due to *C. neoformans* var. *grubii* genotype VNI. Most HIV-uninfected patients in Vietnam (76% of all patients and 68% of patients with *C. neoformans* var. *grubii* infection) have no obvious underlying immunosuppressive disease. In this tropical location, it might have been predicted that *C. gattii* would account for more infections in HIV-negative patients, yet we found it to account for only 25% of cases. This is higher than is reported in locations in the Far East, albeit with more temperate climates (7, 10).

AFLP analysis with the GT/GT primer set defined two clusters of VNI in the *C. neoformans* var. *grubii* population, which we have termed VNIγ and VNIδ. The majority of strains from HIV-uninfected patients clustered within the VNIγ cluster. The rate of underlying disease was lower in HIV-uninfected patients infected with strains from this cluster. Typing using the AC/G primer set confirmed this observation, with the strains from HIV-uninfected patients being closely related to each other and forming a monophyletic cluster. In contrast, the VNIγ cluster caused only 38% of infections in HIV-infected patients. A small number of strains were allocated to different clusters according to the primer set used. Whether these are genuine differences or the result of experimental variability is not clear. A sequence-based typing method, such as MLST, might resolve these differences.

The difference in cluster distributions by HIV serostatus could be explained by a number of factors. Differences in cluster presumably relate to some difference in environmental niche adaptation. Different behaviors of different patient groups could lead to different risks of exposures to each cluster. For example, a large proportion of HIV-infected patients in Vietnam are intravenous drug users (59% in this cohort). However, we found no difference in the prevalence of cluster by HIV risk factor. Moreover, the HIV-uninfected patients are demographically diverse, implying that shared risk factors are unlikely, yet they are predominantly infected with a single cluster.

The geographical location of patients might explain different exposures. A sophisticated geospatial analysis is not possible with our data, and uncertainty over the incubation period with cryptococcal disease in any case makes this difficult, but a simple analysis suggests that there is no difference in cluster spread according to residency in Ho Chi Minh City or the provinces. The HIV-infected and HIV-uninfected groups contained patients from all provinces in southern Vietnam, as would be expected in our hospital, which is the tertiary referral center for all infectious diseases in the region.

Another possibility is that the clusters we identified are artificial and a consequence of error somewhere in the experimental process. AFLP analysis is a multistage procedure, and error could enter through different efficiencies of digestion, ligation, PCR amplification, or fragment detection (45). We performed multiple experiments on single strains to determine the rate of experimental variability, and we randomized the selection of strains for experimentation from DNA extraction through to final amplification. Fragment analysis was performed simultaneously in all experiments and in duplicate on all isolates from HIV-uninfected patients to minimize the effect of sequencer-induced noise. While the dendrograms were slightly different for each primer set, the positions of strains within the trees are similar, with the same HIV-negative isolates found in a monophyletic cluster in both, suggesting that the grouping of a majority of HIV-negative isolates in cluster VNIγ is genuine.

A further possibility is that the VNIγ cluster may represent strains with increased pathogenicity. If this is the case, an explanation is needed as to why it is not the dominant genotype causing disease in HIV-infected patients. This is explainable if VNIγ occurs less frequently than VNIδ in the environment. Differences in environmental prevalence correlating with differences in pathogenicity are biologically plausible. *C. neoformans* is a facultative pathogen, and human-to-human transmission is not important in the disease cycle. Differences in pathogenicity are likely bystander effects of adaptation to particular environmental niches. It is already known that not all environmental strains are pathogenic (32, 33, 55). The prevalence and site of a given niche in the environment, along with dispersal from the niche as an infectious propagule, will deter-
mine the risk of a potentially infectious contact between the pathogen and susceptible hosts. Thus, a strain with increased pathogenicity may be infrequent in the environment because its niche is rare, explaining a low overall disease prevalence. Alternatively, the low prevalence may reflect an additional need for an as-yet-unidentified host immune defect for successful infection in HIV-uninfected patients.

Host and geographical factors are known to influence the species of Cryptococcus that cause meningitis in particular groups (8, 29, 46, 52). However, a subgroup of C. neoformans var. grubii that is associated with HIV-uninfected individuals has been described only once before, despite extensive study using numerous typing methods (4, 6, 21, 32, 37, 52). This may be explained by the relatively small number of isolates from HIV-uninfected patients, particularly from those without underlying disease. Only Varma et al. found a segregation of genotype by HIV status (52). In a study of 45 clinical isolates (23 from HIV-uninfected patients and 25 from HIV-infected patients), one of nine genotypes was found to be more frequent in HIV-uninfected patients, but no association was found for isolates from HIV-infected patients. It is not clear whether all isolates in this study were of the same variety.

The fact that we were able to demonstrate an association between a subgroup of C. neoformans var. grubii and the HIV infection status of the isolate source, in contrast to the case in most of the studies described above, may reflect the fact that all of our isolates were from a small, well-defined geographical area, reducing the “noise” that might be found in a more diverse population. More recently, it has been reported that in 91 immunocompetent patients with cryptococcal meningitis in China, disease was due to infection with a highly homogeneous group of C. neoformans var. grubii VNI strains, a finding consistent with our work (7). However, no comparison was made with strains from HIV-infected patients. This same genotype is also reported from Korea, although most affected patients have serious underlying disease (10). The strains described had variable virulence in a mouse infection model despite the genetic similarity, suggesting that they are not a single hypervirulent clone. Assessing the virulence of the Vietnamese isolates

### Table 2. Distribution of C. neoformans var. grubii isolates between clusters by HIV status of host

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HIV infected</th>
<th>HIV uninfected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>VNIιγ</td>
<td>38</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>VNIιδ</td>
<td>62</td>
<td>6</td>
<td>68</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>37</td>
<td>137</td>
</tr>
</tbody>
</table>

*Odds ratio, 8.30; 95% CI, 3.04 to 26.6; P < 0.0001 (Fisher’s exact test).*

FIG. 2. (a) AFLP-derived neighbor-joining tree obtained using the GT/GT primer set for Vietnamese C. neoformans var. grubii clinical isolates. Gray bars, isolates from HIV-uninfected patients; white bars, C. neoformans controls; black bars, C. gattii controls. Circles represent well-supported clusters; the numbers are the cophenetic correlation values (%). (b) AFLP-derived neighbor-joining tree obtained using the AC/G primer set for Vietnamese C. neoformans var. grubii clinical isolates Gray bars, isolates from HIV-uninfected patients; white bars, C. neoformans controls; black bars, C. gattii controls. Circles represent well-supported clusters, the numbers are the cophenetic correlation values (%).
from cluster VN1y, and determining their relationship to the Chinese isolates from HIV-uninfected patients will be interest- ing areas for further study.

Conclusions. We have identified a cluster of C. neoformans var. grubii strains that is responsible for the majority of disease in HIV-infected patients in Vietnam, most of whom have no comorbidities. This genotype accounts for only one-third of cases in HIV-infected patients. Whether this difference is due to host or pathogen factors is not yet clear.

ACKNOWLEDGMENTS

Wieland Meyer (Molecular Mycology Research Laboratory, Centre for Infectious Disease and Microbiology, University of Sydney at the Westmead Millennium Institute, Westmead, NSW, Australia) kindly provided Cryptococcus neoformans and Cryptococcus gattii control strains.

This work was funded by the Wellcome Trust UK. J. N. Day was in part supported by a British Infection Society Fellowship.

The authors have no conflicts of interest.

REFERENCES


