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Cryptococcus gattii Infections

Sharon C.-A. Chen,[a,b,c,d] Wieland Meyer,[a,b,c,e] Tania C. Sorrell[a,b,c]

Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney, Sydney, NSW,[a] Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, Westmead, NSW,[b] Western Clinical School, Sydney Medical School, University of Sydney, Westmead, NSW,[c] Institute of Clinical Microbiology and Medical Research, Westmead, NSW,[d] and Molecular Mycology Research Laboratory, Department of Infectious Diseases, Westmead Hospital, Western Sydney Local Health District, Westmead, NSW,[e] Australia

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Address correspondence to Tania C. Sorrell, tania.sorrell@sydney.edu.au.

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SUMMARY

Understanding of the taxonomy and phylogeny of *Cryptococcus gattii* has been advanced by modern molecular techniques. *C. gattii* probably diverged from *Cryptococcus neoformans* between 16 million and 160 million years ago, depending on the dating methods applied, and maintains diversity by recombining in nature. South America is the likely source of the virulent *C. gattii* VGII molecular types that have emerged in North America. *C. gattii* shares major virulence determinants with *C. neoformans*, although genomic and transcriptomic studies revealed that despite similar genomes, the VGIIa and VGIIb subtypes employ very different transcriptional circuits and manifest differences in virulence phenotypes. Preliminary evidence suggests that *C. gattii* VGII causes severe lung disease and death without dissemination, whereas *C. neoformans* disseminates readily to the central nervous system (CNS) and causes death from meningoencephalitis. Overall, currently available data indicate that the *C. gattii* VGI, VGII, and VGIII molecular types more commonly affect nonimmunocompromised hosts, in contrast to VGIV. New, rapid, cheap diagnostic tests and imaging modalities are assisting early diagnosis and enabling better outcomes of cerebral cryptococcosis. Complications of CNS infection include increased intracranial pressure, severe neurological sequelae, and development of immune reconstitution syndrome, although the mortality rate is low. *C. gattii* VGII isolates may exhibit higher fluconazole MICs than other genotypes. Optimal therapeutic regimens are yet to be determined; in most cases, initial therapy with amphotericin B and 5-flucytosine is recommended.

INTRODUCTION

Cryptococcus gattii is a basidiomycetous yeast, which grows mainly as an asexual budding yeast in the environment and within human and animal hosts. Sexual reproduction can occur between cells of the opposite mating type or of the same mating type (1). During sexual development, yeast cells undergo a dimorphic transition to hyphal growth, generating a mycelium and forming basidiospores (2). It is not yet certain whether sexually produced blastospores, desiccated yeast cells, or sexually produced basidiospores are the infectious propagules.

C. gattii has long been recognized as an endemic pathogen in Australia. In the 1990s, it emerged and became established in British Columbia, Canada, and subsequently in the Pacific Northwest of the United States. In the last decade, major advances in molecular technology led to a revision of the taxonomy and phylogeny of this species and have enhanced our understanding of its ecology, epidemiology, and clinical associations. Furthermore, recognition of genotype-dependent differences within the species *C. gattii* has assisted studies of pathogenesis, which, while still limited, are revealing the complexity of transcriptional circuits, the phenotypic virulence composite, and host cellular responses. Significant advances in diagnosis, clinical epidemiology, and approaches to the management of human cryptococcosis have been

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made. This review places new information about *C. gattii* in a historical context.

TAXONOMY OF THE SPECIES *C. GATTII*

Historical Overview

The agents of cryptococcosis, *Filobasidiella neoformans* (teleomorph/sexual stage)-*Cryptococcus neoformans* (anamorph/asexual stage) and *F. bacillispora*-*C. gattii*, form a monophyletic group, which is placed taxonomically within the phylum Basidiomycota, subphylum Agaricomycotina, family *Tremellales*, and genus *Filobasidiella*, which contains only three additional, closely related sister taxa, *Filobasidiella depauperata*, *F. lutea*, and *F. amylolenta*. These species are distinct from the >80 species in the polyphyletic genus *Cryptococcus*, based on recent phylogenetic analysis of the D1/D2 region of the large-subunit (LSU) ribosomal DNA (rDNA) gene (3, 4).

Historically, several pathogens, *Saccharomyces neoformans* (5), *Cryptococcus hominis* (6), *Torula neoformans* (7), *Torula histolytica* (8), and *Debaryomyces hominis* (9), were identified by Benham as belonging to the same species, for which he proposed the name *Cryptococcus neoformans* (10, 11). Until 1949, *C. neoformans* was considered to be a homogeneous anamorph (asexual) species (12–14). In that year, three serotypes were described (serotypes A, B, and C) (15), and 20 years later, a fourth serotype (serotype D) was discovered (16). However, it was not until 1970 that Vanbreuseghem and Takashio described the production of distinct elliptical yeast cells by a cryptococcal strain isolated from an African patient with leukemia (RV20186, IHEM11796, CBS 6289, ATCC 32269, or MUCL 30449) and introduced the name *Cryptococcus neoformans* var. *gattii* (17). More recently, molecular analysis of a clinical isolate from France, reported by Curtis in 1896 as *Saccharomyces subcutaneus tumefaciens* (18), revealed that this was, in fact, the first reported case of human *C. gattii* infection (19).

Mating experiments conducted by Kwon-Chung in the early 1970s revealed two different teleomorph (sexual) species: *Filobasidiella neoformans*, which resulted from a cross between two serotype D strains of *C. neoformans* var. *neoformans*, B3501 (*MAT*α) and B3502 (*MAT***a**) (20, 21), and the morphologically distinct *Filobasidiella bacillispora*, characterized by smooth-walled, bacilliform-shaped basidiospores, which resulted from a cross between two *C. neoformans* var. *gattii* strains, a serotype C *MAT***a** strain (NIH 191) and a serotype B *MAT*α strain (NIH 444) (22). After the discovery of this new teleomorph species, *C. neoformans* var. *gattii* was raised to the species level and renamed *C. bacillisporus* (2). The teleomorph species *F. neoformans* corresponds to the two anamorphic (asexual) *C. neoformans* species (serotypes A and D), and *F. bacillispora* corresponds to *C. bacillisporus* (serotypes B and C). Further studies revealed that the *C. neoformans* var. *gattii* strain reported by Vanbreuseghem and Takashio and the *C. bacillisporus* strain (NIH 191, CBS 6955, or ATCC 32608) isolated from the cerebrospinal fluid (CSF) of a patient in California had the same biochemical, morphological, and serological characteristics (23).

Subsequently, it was revealed that strains of *F. neoformans* and *F. bacillispora* had intermediate DNA-DNA reassociation values compared to those of isolates of the same species (55 to 63% and 88 to 94%, respectively) (24). In addition, a *MAT*α strain (CBS 6289), type culture of *C. neoformans* var. *gattii* (serotype B), when crossed with the *MAT***a** strain (CBS 6901) of *C. neoformans* (serotype D), produced viable basidiospores, and the F1 generation showed a mixture of both *F. neoformans* and *F. bacillispora*. Based on these observations and additional biochemical, morphological, serological, and genetic data, the species nomenclature *C. bacillisporus* was again reduced to a variety of *C. neoformans*. The older nomenclature *C. neoformans* var. *gattii* was given precedence over *C. bacillisporus*, and *C. bacillisporus* was made a synonym of *C. neoformans* var. *gattii* (23).

After the 1970s, there was increasing evidence of differences in morphological, biochemical, molecular, ecological, pathobiological, and clinical features between the two varieties of *C. neoformans* (2, 20, 22, 23, 25–29). Phylogenetic relationships between the two varieties were scrutinized by using DNA sequences of multiple genes, including the *URA5*, *LAC1*, *CAP59*, and *CAP64* genes, as well as the intergenic spacer (IGS) and the internal transcribed spacer (ITS) regions of the rDNA gene cluster (4, 30–32). Two distinct monophyletic groups were identified, regardless of the genetic locus studied (Fig. 1). Population genetic analysis using either amplified fragment length polymorphism (AFLP) analysis or PCR fingerprinting approaches supported these two major groups (33–35). Since *C. neoformans* and *C. gattii* fulfilled the criteria for different species according to morphological, biological, and phylogenetic species concepts, they were designated as such in 2002. The name "*gattii*" was preserved, as it had been used preferentially over the previous 2 decades by the medical, veterinary, and scientific communities (19). Furthermore, genetic analysis of the F1 generation of a cross between strains of the two varieties showed no evidence of recombination. In addition, comparison of the entire genomes of B3501 (*C. neoformans* var. *neoformans*) and WM 276 (*C. neoformans* var. *gattii*) revealed only 87% identity between them (36).

The occurrence of outbreaks of cryptococcosis in new geographic locations over the last 2 decades (37–39) has stimulated detailed molecular studies of *C. gattii*.

Phylogenetic Studies and Molecular Subtyping

The application of PCR fingerprinting (34, 40), AFLP analysis (33), *PLB1* restriction fragment length polymorphism (RFLP) analysis (41), *URA5* RFLP analysis (40), and multilocus microsatellite type (MLMT) analysis (42, 43) revealed intraspecies genetic diversity within *C. gattii* and led to the discovery of interspecies hybrids of serotypes A and B and serotypes B and D (44, 45). *C. neoformans*-*C. gattii* is now considered a species complex, with the actual number of scientifically valid species being controversial (43).

Initial studies based on analyses of mitochondrial RNA (mtRNA), ITS1/2, and the *OMP* and *DOX* genes indicated that the two species separated 37 million years ago (32), with a subsequent analysis of the seven haploid molecular types, based on four genetic loci, *ACT1*, *IDE*, *PLB1*, and *URA5*, placing this separation at 49 million years ago (46). A Bayesian evolutionary analysis based on 10 genetic loci, *ACT1*, *IDE*, ITS1/2, IGS, *LAC1*, *PLB1*, *RPB1*, *RPB2*, *TEF1*, and *URA5* (C. K. M. Tsui and W. Meyer, unpublished data) and a subsequent whole-genome sequence-based study (47) estimated that the two species diverged either 70 million years ago or 80 million years ago (range, 16 million to 160 million years ago).

Four distinct genetic groups within *C. gattii* have been described, VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, and VGIV/

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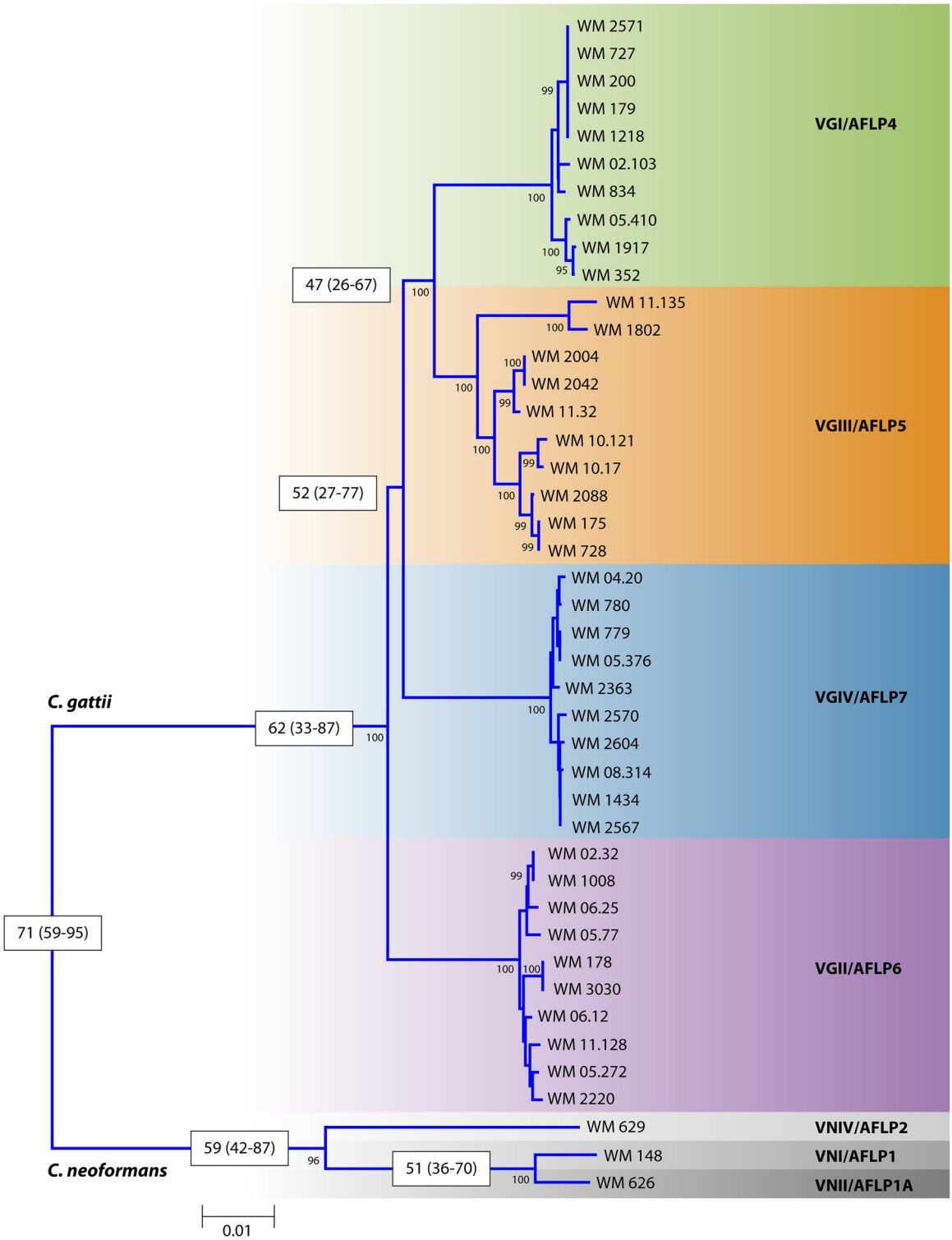

FIG 1 Rooted neighbor-joining tree inferred from the concatenated MLST typing scheme sequences (*CAP59*, *GPD1*, *LAC1*, *SOD1*, *URA5*, and *PLB1* genes and IGS) of 10 strains of each major *C. gattii* molecular type rooted with the standard strains of the three major *C. neoformans* molecular types. Numbers on branches are bootstrap support values obtained from 1,000 pseudoreplicates using the program MEGA version 5.05. Numbers in the white boxes indicate estimated diversion times based on the 10 genetic concatenated loci (*ACT1*, *LAC1*, *IDE1*, ITS1/2, IGS, *PLB1*, *RPB1*, *RPB2*, *TEF1*, and *URA5*), generated with the program BEAST.

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AFLP7 (Fig. 1), each of which contains serotype C and B strains in different proportions (33, 48, 49). The phylogenetic relationships between these group were investigated in two major studies. In the first study, analysis of the major AFLP types was conducted by using six concatenated genetic loci (*LAC1*, ITS1/2, IGS, *RPB1*, *RPB2*, and *TEF1*). VGII/AFLP6 was identified as the lineage that is basal to the *C. gattii* clade, followed by VGIV, which itself is basal to the sister groups VGI/AFLP4 and VGIII/AFLP5 (49). However, a phylogenetic analysis of two mitochondrial loci (mtlrRNA and *ATP6*) revealed evidence of recombination (50), unlike the one with six nuclear loci. In the second study, analysis of the major molecular types obtained by PCR fingerprinting was performed by using either individual genetic loci or a combined analysis of four concatenated genetic loci (*ACT1*, *IDE1*, *PLB1*, and *URA5*). This analysis identified four major clades corresponding to the four major molecular types of *C. gattii*. This study confirmed that the VGII/AFLP6 clade is the basal lineage of *C. gattii* (32, 46). Given that the major molecular types within *C. gattii* contain both serotype B and C isolates, further studies using four concatenated genetic loci (*ACT1*, *IDE1*, *PLB1*, and *URA5*) were undertaken and revealed that the major molecular types of *C. gattii* are younger than those of *C. neoformans*. The VGI/AFLP4, VGIII/AFLP5, and VGIV/AFLP7 clades were estimated to have diverged from the basal VGII/AFLP6 clade approximately 12.5 million years ago, the VGIV/AFLP7 clade was estimated to have diverged from the VGIII/AFLP5 and VGI/AFLP4 sister clades 11.7 million years ago, and the VGIII/AFLP5 and VGI/AFLP4 clades were estimated to have diverged from each other 8.5 million years ago (46). A recent combined study of all 10 loci (*ACT1*, *LAC1*, *IDE1*, ITS1/2, IGS, *PLB1*, *RPB1*, *RPB2*, *TEF1*, and *URA5*) confirmed the ancestral position of VGII/AFLP6 (48). The initial divergence of the VGII/AFLP6 basal clade and the remaining major clades was estimated to have occurred 62 million years ago (range, 33 million to 87 million years ago), that of the VGIV/AFLP7 clade and the VGIII/AFLP5/VGI/AFLP4 clade was estimated to have occurred 52 million years ago (range, 27 million to 77 million years ago), and that of the VGIII/AFLP5 clade and the VGI/AFLP4 clade was estimated to have occurred 47 million years ago (range, 26 million to 67 million years ago) (C. K. M. Tsui and W. Meyer, unpublished) (Fig. 1).

Notably, whole-genome sequence typing (WGST) was recently employed to investigate the spread of highly virulent *C. gattii* VGII strains from the Pacific Northwest. The highest genetic diversity was present among VGIIa strains, followed by VGIIc and VGIIb strains. The WGST data showed clearly that the VGIIc genotype responsible for the most recent infections in the Pacific Northwest is genetically distinct from both VGIIa and VGIIb Vancouver Island outbreak subtypes and suggest that all three subtypes are equally distinct from each other (52). Similar findings based on multilocus sequence typing (MLST) and MLMT analyses, which have been confirmed based on WGST, have been reported recently (53–55). All studies suggest that the three subtypes have arisen as a result of recombination events that took place prior to their introduction into the Pacific Northwest.

The genetic variation between the four major molecular types of *C. gattii* is similar to that observed between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* and suggests that they warrant at least varietal if not species status. Several lines of evidence support this contention. The four molecular types have been distinguished clearly from each other by using multiple molecular techniques, including PCR fingerprinting (35, 40) (Fig. 2A), AFLP analysis (33) (Fig. 2B), *PLB1* RFLP analysis (41) (Fig. 2C), *URA5* RFLP analysis (40) (Fig. 2D), hyperbranched rolling-circle amplification (RCA) (56) (Fig. 2E), and matrix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-TOF MS) (57, 58) (Fig. 2F) (see also Diagnostics, below). Furthermore, there is evidence for genotype-dependent differences in virulence in mouse and wax moth experimental models, for geographical localization of different molecular types, for differences in clinical epidemiology, and for reduced susceptibility to fluconazole among VGIII isolates. A detailed taxonomic description of the potential varieties and species within *C. gattii* is in preparation (T. Boekhout, K. J. Kwon-Chung, and W. Meyer, unpublished data).

Standardization of Molecular Typing Systems

Many typing techniques have been applied to individual strains of *C. gattii* to gain insights into the molecular epidemiology and population structure of this species. Random amplified polymorphic DNA (RAPD) analysis (59), PCR fingerprinting (35) (Fig. 2A), AFLP analysis (33) (Fig. 2B), MLMT analysis (42), and MLST analysis (43) are in common use. More recently, WGST has been implemented (52).

RAPD analysis, based on the random amplification of DNA fragments using short arbitrary primers, was used initially for cryptococcal strain typing (59, 60). Advantages included high discriminatory power, simplicity, and speed (61). This method separated *C. gattii* into three major molecular types, designated VGI, VGII, and VGIII (60). Use of the primers ERIC1 and ERIC2 revealed variation in the geographic distribution of these molecular types (59). RAPD analysis has been superseded by other methods, since this technique was never standardized and its interlaboratory reproducibility is poor.

PCR fingerprinting, based on the amplification of DNA sequences flanked by hypervariable DNA repeats, uses single primers specific to either minisatellites (the core sequence of wild-type phage M13, 5′-GAGGTGGCGGTTCT-3′) or microsatellites [$(GTG)_5$ and $(GACA)_4$] (34). This technique distinguished four major molecular types of *C. gattii*, designated VGI, VGII, VGIII, and VGIV (35, 62, 63) (Fig. 2A). Although more reproducible than RAPD, banding patterns can vary with the source of laboratory reagents and different experimental conditions, making it unsuitable for multicenter typing studies.

AFLP analysis is based on the digestion of genomic DNA by using frequent (EcoRI)- and rare (MseI)-cutting restriction enzymes, followed by ligation with an adaptor and subsequent PCR amplification of specifically selected fragments. Reproducibility is excellent, especially with the inclusion of an internal standard in each lane and direct fragment detection by using an automated DNA sequencer. Three major AFLP groups were separated within a global collection of *C. gattii*, named AFLP4, AFLP5, and AFLP6 (33), with a subsequent study revealing a fourth group, AFLP7 (64) (Fig. 2B). Serotype B strains were distributed among all three AFLP types, but serotype C strains in this particular study were restricted to type AFLP5 (VGIII); strains of serotype VGIV, which also contains serotype C isolates, were not tested in this study (33). A drawback of AFLP is the potential for artificial variation in banding patterns due to incomplete digestion; thus, samples must be run in duplicate.

MLMT analysis is an even more discriminatory technique for strain typing. It is based on the identification of repeat variations within microsatellite sequences, short tandem repeats, or simple

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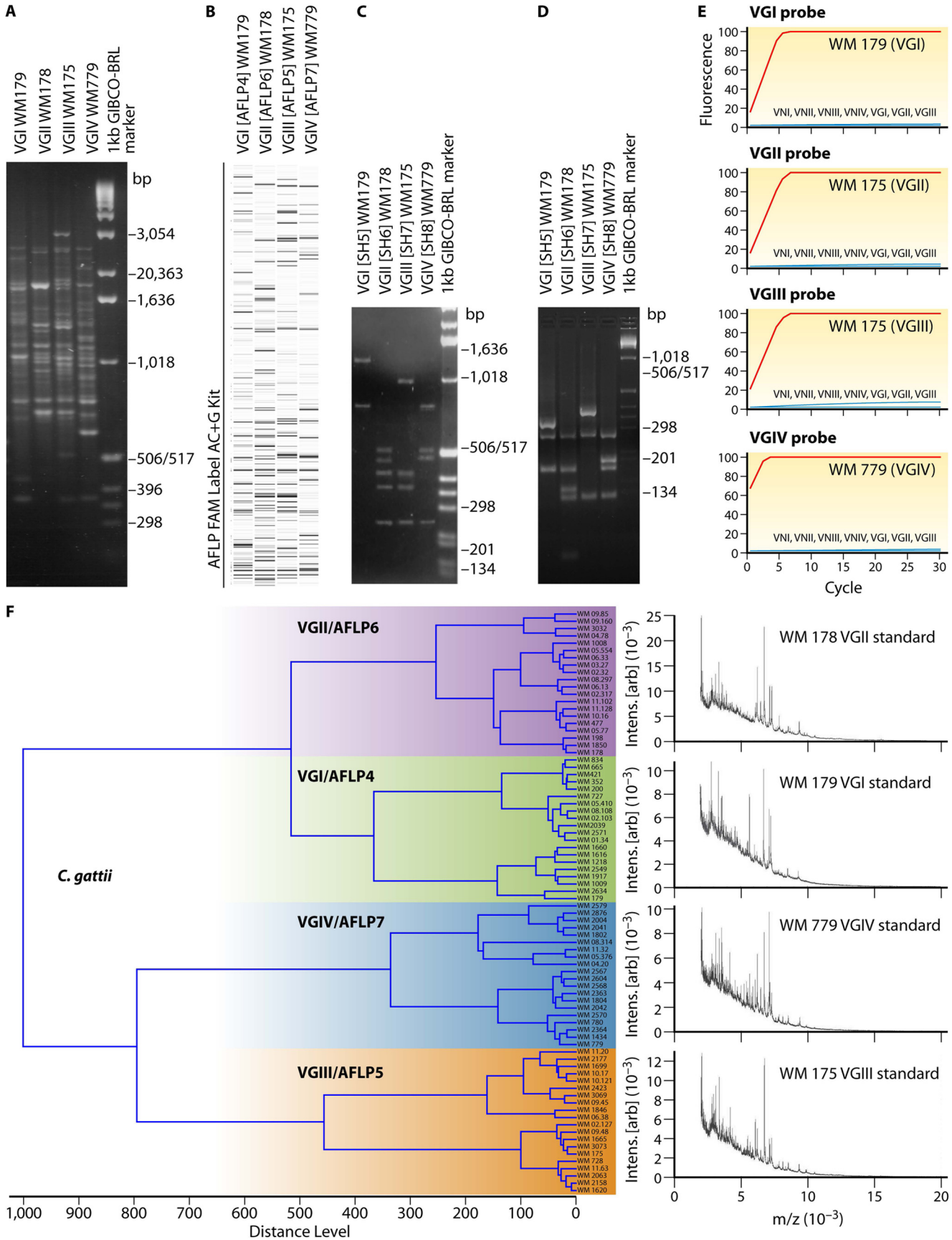

FIG 2 Identification of the major molecular types within *C. gattii*. (A) PCR fingerprinting generated with the primer M13; (B) AFLP profiles generated with the 6-carboxyfluorescein (FAM) label AC+G kit; (C) *PLB1* gene RFLP profiles identified via digestion with AvaI; (D) *URA5* gene RFLP profiles identified via double digestion with Sau96I and HhaI; (E) *PLB1* gene hyperbranched rolling-circle amplification; (F) MALDI-TOF MS profiles obtained from the reference strains of each major molecular type.

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TABLE 1 Concordance of different schemes used for molecular typing of *Cryptococcus gattii*

C. gattii serotype	PCR fingerprinting molecular type (40, 77)	AFLP genotype (33)	*URA5* RFLP type (40)	*PLB1* RFLP type (41)	ITS genotype (342)	IGS genotype (31, 350)	MLST type (43)
B/C	VGI	AFLP4A/AFLP4B	VGI (SH5)	A5	ITS3/ITS7	4	VGI
B/C	VGII	AFLP6	VGII (SH6)	A6	ITS4	3	VGII
B/C	VGIII	AFLP5A/AFLP5B/AFLP5C	VGIII (SH7)	A7	ITS5	5	VGIII
B/C	VGIV	AFLP7	VGIV (SH8)	A8	ITS6	6	VGIV

sequence repeats (SSRs) 1 to 6 bp in length following polyacrylamide gel electrophoresis (65). It is highly reproducible, and the primers used to amplify the microsatellites are species specific. This technique distinguishes all four major molecular types of *C. gattii* and has enabled the tracing of highly virulent strains (see below) (53, 66, 67).

MLST analysis is the most reproducible method and is readily standardized but expensive. Two main MLST schemes based on the amplification of highly variable partial sequences of 9 genetic loci (+22 additional loci) (1) or highly variable partial sequences of 7 genetic loci (43) within housekeeping genes have been developed. This method produces unambiguous, highly reproducible sequence data from which a sequence type is generated for each strain. Although both MLST schemes distinguish between the four major molecular types of *C. gattii* (1, 43, 68, 69), they do not yield comparable allele and sequence type numbers, thus precluding the possibility of combining them into a single global population genetic analysis.

WGST is the latest development in cryptococcal strain typing. It detects single nucleotide polymorphisms (SNPs) throughout the genome (52) and is the most accurate typing tool available. Currently, its use is limited by high costs and bioinformatic demands.

Despite the large number of typing tools available, understanding of the population structure of *C. gattii* is still fragmented. This is due mainly to the use of different nomenclatures and a lack of consensus between results obtained by using different systems. In 2007, a working group of the International Society for Human and Animal Mycology (ISHAM) was established to standardize genotyping of the *C. neoformans-C. gattii* species complex, thereby enabling the global tracking of highly virulent strains and, potentially, prediction of future outbreaks. Since the different typing methods yielded the same major cryptococcal molecular types (Table 1), it was agreed by this working group in 2007 that the VGI, VGII, VGIII, and VGIV nomenclature should be used to represent the global population structure of *C. gattii* (43). In addition, a set of standard strains was designated and deposited at major public culture collections, including the CBS Fungal Biodiversity Centre (CBS-KNAW), The Netherlands (http://www.cbs.knaw.nl/); the American Type Culture Collection (ATCC) (http://www.atcc.org/); the Fungal Genetic Stock Center (FGS) (http://www.fgsc.net/); the Westmead Hospital Collection (WM), Australia (http://www.mycologylab.org/); and Coleção de Micro-Organismos de Referência em Vigilância Sanitária (CMRVS), Brazil (http://cmrvs.fiocruz.br/). The respective culture collection strain numbers are listed in Table 2. Statistical analysis of all published MLST loci revealed that a minimum of seven loci is needed to type a given strain unambiguously. As a result, the ISHAM consensus MLST typing scheme for the *C. neoformans-C. gattii* species complex consists of the following seven variable unlinked genetic loci: *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and the IGS1 region (43). Three of the seven loci encode major cryptococcal virulence factors: the polysaccharide capsule (*CAP59*), melanin synthesis (*LAC1*), and cell invasion (*PLB1*). The MLST data, including allele and sequence types, are stored in a centralized database at the Molecular Mycology Research Laboratory at the University of Sydney and can be accessed at http://mlst.mycologylab.org/. All research groups using this MLST scheme are encouraged to deposit their data in this database. Allele and sequence types can be retrieved online.

Interspecies Hybrids

The application of PCR fingerprinting and AFLP typing revealed diploid or aneuploid hybrids in addition to the four major haploid molecular types in *C. gattii*. These hybrids may have originated from hybridization events between some of the major molecular types of *C. gattii* and its sister species *C. neoformans*. These hybrids include the VGI/VNIV (serotype B [VGI/AFLP4] and serotype D [VNIV/AFLP2]) hybrids (44), the VNI/VGI (serotype A [VNI/AFLP1] and serotype B [VGI/AFLP4]) hybrids (45, 70), and the VNI/VGII (serotype A [VNI/AFLP1] and serotype B [VGII/AFLP6]) hybrids (70). Virulence studies using the *Galleria mellonella* model revealed differences in the virulence of hybrids depending on the genome combinations present. The VNI/VGII (αAB**a**) hybrids were found to be as virulent as haploid strains H99 and CDC R265. The VNI/VGI (αAB**a**) and VNIII (αAD**a**) hybrids were virulent but less so than strain H99 (M. Aminnejad and W. Meyer, unpublished data).

EPIDEMIOLOGY, ORIGIN, AND EVOLUTION

Global Distribution and Movement of *C. gattii* Genotypes

With the recognition of the emergence of VGII and VGIII infections in North America, there is clear evidence that *C. gattii* has spread to new temperate climatic zones outside the tropical and subtropical areas identified by Kwon-Chung and Bennett (71). It has always been present in the southern temperate zone of Australia. The global distribution of the four major molecular types of *C. gattii* has been determined by analyses of clinical, animal, and environmental isolates from several studies (33, 38, 40, 72–81; W. Meyer and L. Trilles, unpublished data). Acknowledging potential sample biases, in contrast to previous reports, in which the most frequent molecular type was VGI (48, 80), VGII is now more common (comprising 47% of all isolates), followed by VGI (34%), VGIII (11%), and VGIV (8%) (Fig. 3, top). However, among clinical isolates, VGI and VGII are represented in equal proportions, followed by VGIII and VGIV. Most environmental isolates are of molecular type VGII, although this may reflect sampling bias associated with attempts to determine the origin of the Vancouver Island outbreak, followed by VGI and VGIII. VGIV has rarely been isolated from the environment (Fig. 3, top). The geographic variation in the distributions of VGI, VGII, VGIII, and VGIV is striking.

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TABLE 2 Standard/reference strains for *Cryptococcus gattii* strain typing[a]

C. gattii PCR fingerprinting molecular type (reference) and AFLP genotype (reference)	CBS strain designation	ATCC strain designation	FGS strain designation	WM strain designation	CMRVS strain designation	Other strain designation(s)	MAT type and serotype	Description	Reference(s)
VGI (40) and AFLP4 (33)	CBS 10078	ATCC MYA-4560	10419	WM 179	70298	Bryon, H33.1, MH56	αB	1993, Sydney, NSW, Australia; clinical, CSF, HIV negative; isolated by Sharon C.-A. Chen	40, 154
	CBS 6289	ATCC 32269		WM 01.124		MUCL 30449, RV 20186, CBS 8273	aB	1966, Kinshasa, Congo; clinical, CSF; isolated by E. Gatti and R. Eeckels; type strain of *C. neoformans* var. *gattii*	199
	CBS 10510			WM 276		TCS, SC1	αB	1993, Mt. Annan National Park, NSW, Australia; environmental, *Eucalyptus tereticornis* woody debris; isolated by Tania C. Sorrell and Sharon C.-A. Chen; genome sequence strain	40
VGII (40) and AFLP6 (22)	CBS 10082	ATCC MYA-4561	10420	WM 178	70302	49435, Colter, IFM 50894	αB	1991, Sydney, NSW Australia; clinical, CSF, HIV negative; isolated by Sharon C.-A. Chen	40, 154
	CBS 10514			WM 02.32		CDC R265	αB	2001, Duncan, Vancouver Island, BC, Canada; clinical, bronchial wash specimen; isolated by British Columbia CDC; highly virulent Vancouver Island outbreak strain of VGIIa; genome sequence strain	38
VGIII (40) and AFLP5 (22)	CBS 10081	ATCC MYA-4562	10421	WM 175	70299	WM 161, E698, 689, TP 0689, D1.13H	αB	1992, Blind Recreation Center/Park Boulevard UPAS Street, San Diego, CA, USA; environmental, *Eucalyptus* species woody debris; isolated by Tania Pfeiffer and David Ellis	40, 100
	CBS 6955	ATCC 32608	10424	WM 01.125		DBVPG 6225, WM 2220, MUCL 30454, NIH 191, CBS 6916	aC	Before 1970, San Fernando, CA, USA; clinical, CSF	2
VGIV (40) and AFLP7 (56)	CBS 10101	ATCC MYA-4563	10422	WM 779	70300	King Cheetah, IFM 50896	αC	1994, Johannesburg, South Africa; veterinary, cheetah; isolated by Valerie Davis	40, 389

[a] Adapted from reference 43.

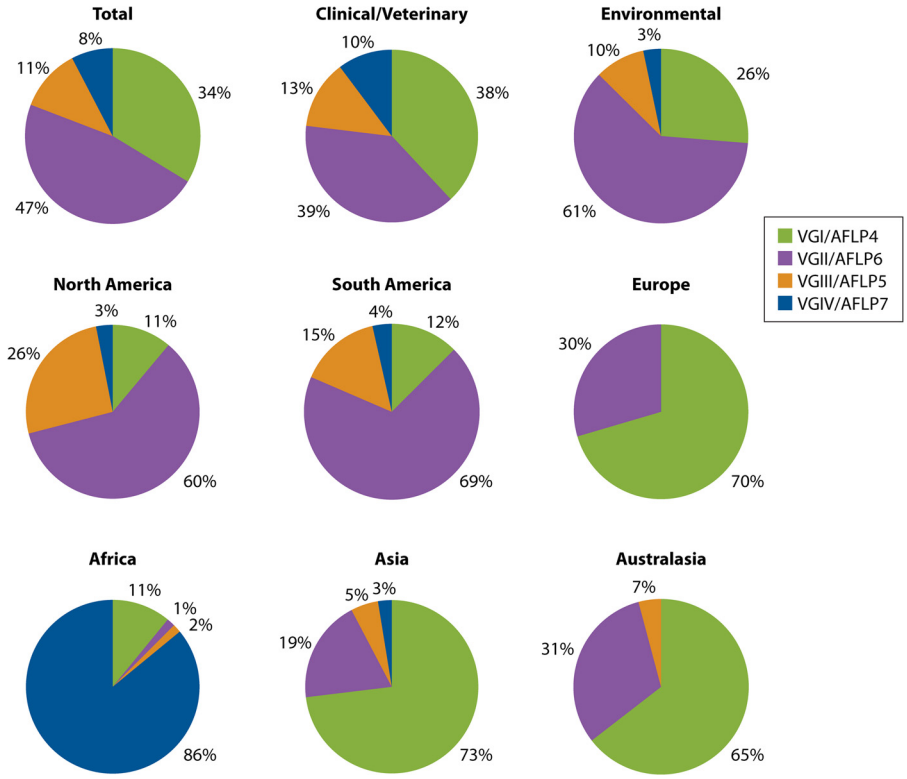

FIG 3 Distribution of the four major molecular types of *C. gattii* identified among a total of 980 clinical/veterinary and environmental global isolates. Shown is the distribution of the four major molecular types of *C. gattii*, combining clinical/veterinary ($n = 615$) and environmental ($n = 365$) isolates (top) from North America (including Mexico) ($n = 162$), South America ($n = 367$), and Europe ($n = 44$) (middle) and from Africa ($n = 64$), Asia ($n = 78$), and Australasia ($n = 265$) (bottom).

The Americas. In the Americas, VGII and VGIII predominate among clinical, veterinary, and environmental isolates (Fig. 3, middle, and 4), with most VGII isolates being obtained from South America (40). In North America, this distribution of molecular types is commensurate with the establishment of VGII infections in British Columbia and the Pacific Northwest (38, 39, 82, 83) and the emergence of VGIII in southern California and Mexico (39, 84; W. Meyer and C. Firacative, unpublished data). The Canadian and Pacific Northwest outbreak strains are all of mating type α and comprise two subtypes of the major molecular type VGII/AFLP6, namely, VGIIa/AFLP6A and VGIIb/AFLP6B (38). As well as in British Columbia, Canada, molecular type VGII has now been reported in the United States (68, 85, 86) (Fig. 4). AFLP analysis has identified the same VGII molecular subtypes in other temperate regions (1, 38, 87). Closely related strains of a new highly virulent subtype, VGIIc, have been identified in the state of Oregon and, subsequently, in other regions of the Pacific Northwest (68, 69, 86).

Based on MLST of global isolates, Fraser et al. initially proposed that the Vancouver Island VGII outbreak strains originated in Australia or South America (1). More recent studies support the proposal that *C. gattii* molecular type VGII originated in South America (46, 72) and that Australia and Thailand are possible "stepping stones" in the global spread of VGIIb from South to North America (88, 89). The introduction of VGIIb from its ancestral origin in South America, via different parts of the world, has recently been confirmed by using WGST (54). These findings agree with a recent comprehensive, global study of VGII strains, which traced the origin of both outbreak subgenotypes VGIIa and VGIIb back to an ancestral *C. gattii* strain, LMM645, from the Amazon rainforest. This study revealed the presence of the VGIIa and VGIIb genotypes and *MAT***a** and MATα strains in Brazil and showed clear evidence for recombination among the Brazilian *C. gattii* population (53).

Studies in South America, including Argentina, Brazil, Chile, Colombia, Mexico, Peru, Venezuela, and Guatemala, found that VGII/AFLP6 is common in this part of the world, with 64% of all *C. gattii* isolates from Brazil being of molecular type VGII (40, 78, 79). The greatest genetic diversity among VGII/AFLP6 strains was found in Brazil (42, 78) and Colombia (74), where both *MAT***a** and MATα strains are present. Environmental studies conducted in South America show that VGII is particularly well adapted to biotopes associated with decaying wood (73, 78, 90). The Brazilian epidemiological data suggest that there is also a north-to-south geographical trend, with *C. gattii* being endemic in the north and northeast Brazilian states of Amazonas, Bahia, Pernambuco, Piauí, and Roraima. Here primary cryptococcosis is caused predominantly by VGII and affects immunocompetent patients, unlike in the southern states (Mato Grosso do Sul, Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, and São Paulo) (78).

VGIII comprises 21% of Brazilian *C. gattii* isolates (78) and has been isolated from eucalypts in the Brazilian state of Rio Grande do Sul (72). A Mexican study using PCR fingerprinting with the primer M13 showed the presence of all four *C. gattii* molecular types, including VGIII (79). Molecular type VGIV is rarely found in the Americas (Fig. 3, middle, and 4).

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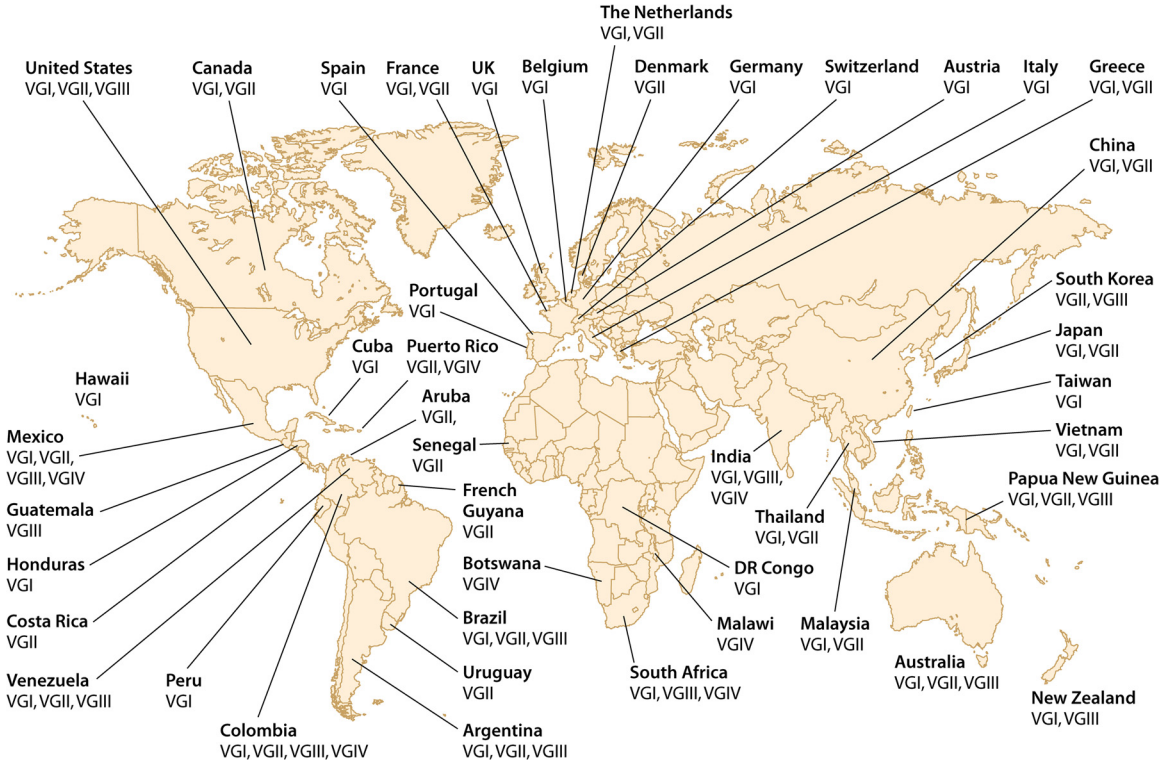

FIG 4 Global distribution of the four major molecular types of *C. gattii*, based on 980 isolates.

Europe. In Europe, *C. gattii* is isolated from patients and the environment only rarely, with the majority of human infections being imported and due to VGI (91). The occurrence of cases many years after travel to an area of endemicity is consistent with reactivation of dormant infection. In Spain, VGI caused an outbreak in goats (92). A small number of imported human cases of VGIIa/AFLP6A infection have occurred following travel to Vancouver Island (93–95).

Africa. Although little is known about *C. gattii* in Africa, its epidemiology appears to differ significantly from that in the rest of the world. Most VGIV strains come from the southern part of Africa, including Botswana, Malawi, and South Africa (96–98). VGI and VGII are mainly from central Africa, for example, the Democratic Republic of Congo (formerly Zaire), Senegal, and South Africa (33, 97, 98) (Fig. 3, bottom, and 4).

Asia and Australasia. In Asia and Australasia, the leading major molecular type is VGI, followed by VGII; VGIII has been isolated rarely (29, 60, 88) (Fig. 3, bottom, and 4). RAPD analysis of 62 clinical, 29 veterinary, and 45 environmental strains revealed a predominance of VGI among environmental and clinical isolates; two subgroups were identified within VGI and VGII (99, 100). RAPD analysis also identified environmental strains of molecular type VGII from the Northern Territory of Australia. Prior to that report, all *C. gattii* strains isolated from eucalypts in Australia were of molecular type VGI (29). A small number of VGI strains have been isolated from clinical and veterinary sources in China (75–77). VGIV was reported only from India; these isolates have been closely linked with VGIV isolates from South Africa, the major source of this molecular type. This association may represent an epidemiological link resulting from human migration between the two countries (81).

ECOLOGY

Environmental Studies

The first studies of the environmental niche of *C. gattii* identified the red gum species of eucalypt, *Eucalyptus camaldulensis* and *E. tereticornis*, as the major environmental sources of *C. gattii* VGI (25, 101), and seasonal flowering was linked with airborne dispersal (26, 102, 103). *C. gattii* concentrations in air samples obtained during the Vancouver Island outbreak were significantly higher in the warm, dry summer months, although potentially infectious propagules (<3.3 μm in diameter) were present throughout the year (104). Decomposing wood in tree hollows has been identified as an environmental source (22, 105). Eucalypts are in fact only one of >50 different species of tree that can provide an ecological niche for *C. gattii* (106–110). Environmental isolates have been obtained from Africa (33, 110–112), Asia (76, 77, 108), Australia (26, 110, 113–118), Europe (110, 119, 120), North America (38, 110, 121), and South America (122). In addition, it has been shown that plant material promotes fertility (108, 123–125) and may render *C. gattii* strains more pathogenic (125). Molecular types VGI and VGII are those most commonly associated with a wide variety of tree species (74, 110, 126). VGIII has been isolated from eucalypts and almond trees in Colombia and other trees in the greater Los Angeles area of California (74, 126–128), and both mating types of VGIV were found in the same two species in Colombia (127) and Tunisia (112).

Notably, both *C. neoformans* and *C. gattii* were isolated concurrently from a hollow in a pink shower tree in Piauí in northeast Brazil (73, 107, 129), and *C. gattii* molecular types VGI and VGII coexisted in the hollow of an Australian *Eucalyptus tereticornis* tree (115).

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Initial studies of VGI isolates obtained from *E. camaldulensis* trees suggested that environmental populations of *C. gattii* are highly fragmented, have a limited ability to disperse, and are confined to individual tree hollows (114). AFLP analysis of clinical isolates revealed that they are geographically restricted, highly clonal, and of low fertility (53, 116, 117). Subsequent extensive testing of isolates from single tree hollows by AFLP analysis revealed that recombination is occurring in both *MAT***a** and *MAT*α or *MAT*α-only populations (118). Isolates of VGII from Australia are highly fertile and recombining (117), with a recent study also suggesting recombination within the *MAT*α-*MAT***a** population, especially in Western Australia (88). Among a global collection of *C. gattii* clinical, veterinary, and environmental strains, representing molecular types VGI, VGII, VGIII, and VGIV, the topology of the four clades was noncongruent, suggesting the occurrence of recent recombination events within *C. gattii* (46). These observations suggest that *C. gattii* populations are likely to be sustained in nature by recombination between the same or opposite mating types.

PATHOGENESIS

This section is intended to provide a historical summary and to highlight the complexity revealed by the use of genomic and other "omics"-related technological advances. Rather than providing a comprehensive review of the literature, we provide insights from key studies and, in addition, those that focus on *C. gattii*.

To establish invasive disease, inhaled cryptococci must penetrate lung tissue, reproduce, enter the bloodstream, and thence disseminate to other organs, predominantly the central nervous system (CNS). Experimental data on the pathogenesis of *C. gattii* infection are limited. In animal models, as with the much more intensively studied *C. neoformans*, the outcome of exposure is determined by a complex set of interacting pathogen-derived and host factors. Broadly, these factors include the extent of exposure to cryptococci (inoculum size), the cryptococcal strain/genotype and its virulence composite, and the host immune system. In human cryptococcosis due to *C. gattii*, genotype-associated clinical and host differences have been observed. For example, VGI infection typically presents with CNS disease, including cryptococcomas, often with concurrent lung lesions (130), whereas VGII infection more commonly presents with pulmonary disease (131, 132). Both of these molecular types of *C. gattii* have a predilection for immunocompetent hosts. In contrast, molecular type VGIII was overrepresented in a series of HIV-infected patients in southern California (84, 133), and VGIV has been identified in Africa in association with HIV/AIDS (see Clinical Epidemiology of Human Infection, below).

Cryptococcal Virulence Determinants

C. gattii expresses the same suite of major virulence determinants as *C. neoformans*. These determinants include the polysaccharide capsule, the ability to grow at 37°C, and laccase activity, which is responsible for the production of melanin, phospholipase B (Plb1), urease, superoxide dismutase (Sod1), and trehalose. Some gene products, such as the capsule (134) and Plb1 (135, 136), have been well characterized in *C. gattii*, but in few instances have deletion mutants or overexpressing strains been created and tested for virulence in animal models. An exception is the laccase gene *LAC1*, created in R265, the virulent VGIIa strain from the Vancouver Island outbreak, the overexpression of which was associated with increased virulence in a mouse inhalation model (137). In that same study, disruption of *CAS1*, which is associated with the construction of the polysaccharide capsule backbone in *C. neoformans* (138), did not affect capsule size but, when overexpressed, was associated with hypervirulence in mice. Disruption of the signal transduction pathway molecule MPK1, which causes defective melanin synthesis and cell wall integrity, was associated with reduced virulence (137). Additional genes, transcription factors, and signaling molecules that have been linked to virulence in *C. gattii* are summarized in Table 3. A recent comprehensive review of stress signaling pathways and the pathogenicity of *Cryptococcus* was provided by Bahn and Jung (139). Other virulence-related functions in *C. neoformans* are likely to be conserved in *C. gattii*. These functions include lipid signaling pathways, adaptation to hypoxia, determinants of proliferation and metabolism in the host, intracellular trafficking and pH sensing, iron and copper regulation and uptake (140), and zinc metabolism (141).

Function of major virulence determinants. The importance of tolerance to growth at physiological temperatures is self-evident. The polysaccharide capsule functions in evasion/suppression of the host immune response, laccase is responsible for the production of the oxidative stress protectant melanin, phospholipase B (Plb1) and urease promote invasion of host tissue, and superoxide dismutase (Sod1) and trehalose function as antioxidants (for a major review, see reference 142). In murine models of infection with *C. neoformans* strain H99 and deletion mutants, Plb1 and laccase are essential for the egress of cryptococci from the lung and dissemination via the blood to the CNS (143, 144). Plb1 and urease are required to establish cerebral cryptococcosis, urease promotes the migration of cryptococci across the blood-brain barrier (BBB) (143–146), and Plb1 is required for the extrusion of *C. neoformans* from macrophages (147). Urease has also been reported to promote an anti-inflammatory Th2 cellular response in the lung (148).

Virulence in experimental models. Virulence *per se* is most often assessed in survival studies by using mouse inhalational models (to mimic the route of natural infection), invertebrates or insects such as *Galleria mellonella* (wax moth) larvae, or rates of proliferation in the mouse macrophage-like cell line J774, expressed as the intracellular proliferation rate (IPR) (142). Inoculation of the tail vein in mice has also been commonly used, and since intravenous (i.v.) inoculation bypasses the respiratory route, it is especially useful in elucidating determinants of pulmonary versus CNS infection. The invertebrate/insect and macrophage models are cheap and convenient, allow higher throughput, and yield results similar to those in mice. Although none of these animals are natural hosts, their relevance has been validated by observations of human cryptococcosis. Thus, in the recent *C. gattii* VGII outbreak in British Columbia, ~90% of outbreak strains were of the VGIIa genotype, and ~10% were of the VGIIb genotype (suggesting that in humans, it is more virulent than the minor genotype VGIIb). This clinical observation is consistent with the much decreased survival and, hence, greater virulence of *C. gattii* VGIIa in mice and a substantially increased ability to replicate within J774 macrophages (1, 38, 84, 149). A more extensive study of VGIIa and VGIIb isolates from around the world confirmed these findings (150). More recently, zebrafish larvae have been proposed as a tractable model in which to study cryptococcal interactions with the innate immune system (151).

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TABLE 3 Comparison of cellular functions and virulence of *Cryptococcus gattii* and *Cryptococcus neoformans* by using deletion mutants[a]

Gene(s) of interest, product	Function	Phenotype	Required for virulence, model	Reference(s)
SOD1, superoxide dismutase	Cytoplasmic antioxidant	Required for production of virulence factors urease, PLB, and laccase in *C. gattii* but not *C. neoformans*	Yes, in BALB/c mouse intravenous inoculation model and in A/JCr mouse inhalational model of *C. neoformans*	390
SOD2, superoxide dismutase	Mitochondrial antioxidant	Required for growth at 37°C in 20% but not in 1.3% oxygen	Yes, for *C. gattii* BALB/c mouse inhalation and i.v. inoculation model; *C. neoformans* was not tested	391
TPS1 and *TPS2*, trehalose-6-phosphate synthase	Trehalose biosynthesis; trehalose functions as an antioxidant and stress protectant	Required for thermotolerance, capsule and melanin production, mating, and cell wall integrity in *C. gattii* and thermotolerance in *C. neoformans*	Yes, for *C. gattii Caenorhabditis elegans* (worm) and A/JCr mouse inhalational models; *TSP1* but not *TSP2* is required for virulence in *C. neoformans*	392, 393
PKA1, cAMP-activated protein kinase A	Signal transduction pathway regulator	Required for capsule production in *C. gattii* and mating and capsule and melanin production in *C. neoformans*	*C. gattii* was not tested; yes, for virulence of *C. neoformans* in BALB/c mouse inhalational model and immunosuppressed rabbit CSF inoculation model	394, 395
PKA2, cAMP-activated protein kinase A	Signal transduction pathway regulator	Required for mating and capsule and melanin production in *C. gattii* but not *C. neoformans*	Not tested	394, 395
PLC1	Signal transduction pathway regulator	Regulates growth at 37°C and melanin and PLB production in *C. neoformans* through the PKC/MAPK pathway (see below)	Yes, in *C. neoformans* BALB/c mouse inhalational model	396
MPK1	Signal transduction pathway regulator	Regulates melanin, capsule production, and cell wall integrity in *C. gattii* and thermotolerance and cell wall integrity at 37°C in *C. neoformans*	Yes, in *C. gattii* (BALB/c inhalational model) and in *C. neoformans* i.v. DBA/2 complement-deficient mouse model	397
*STE12*α	Transcription factor	Regulates melanin, mating, and ecological fitness in *C. gattii* and mating and capsule size in *C. neoformans*	Yes, in *C. gattii* but not *C. neoformans*	398, 399
GAT1	GATA transcription factor	Regulates nitrogen utilization	Yes, in *C. gattii* but not in *C. neoformans* BALB/c intrapharyngeal instillation model	400
CNA1, calcineurin catalytic subunit (A)	Subunit of the heterodimer calcineurin, a Ca^{2+} calmodulin-activated serine-threonine-specific protein phosphatase	In *C. gattii*, regulates thermotolerance (37°C) (strains differ) and is required for plasma membrane integrity, tolerance to fluconazole, and optimal growth in the presence of Ca^{2+} Li^{+}, with no role in melanin and a minor role in capsule production; in *C. neoformans*, lesser effect of Ca^{2+} and not required for fluconazole tolerance in	Yes, in *C. gattii* (molecular type-dependent) *G. mellonella* (wax moth) larva and A/JCr mouse inhalational models and also in *C. neoformans* rabbit intracisternal inoculation and BALB/c mouse i.v. inoculation models	156, 401, 402

[a] Note the functional divergence between *C. gattii* and *C. neoformans* for all genes analyzed, with an evolutionary switch of functions of PKA1 and -2. cAMP, cyclic AMP; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PLB, phospholipase B.

Correlations between the molecular type of *C. gattii* and virulence have been made. In general, VGIIa is more virulent than VGIIb in mice, and VGII overall is more virulent than VGI (36, 38). However, such global assessments of virulence may not yield consistent results, suggesting that molecular type may be a marker, but not a determinant, of virulence. For example, in mice, the clinical type strain of *C. neoformans*, H99, was less virulent than the *C. gattii* VGIIa type strain, R265, from the Vancouver Island outbreak (1, 150) but was equally virulent in another study (152). Indeed, threshold inocula required for disease expression and, in some cases, organ burdens are strain dependent (153), and individual isolates of the same molecular (sero)type of *C. gattii* may be more or less virulent than isolates of *C. neoformans* (154). Subtype-dependent differences in virulence have also been described for VGIII strains isolated from patients with AIDS. In particular, strains of VGIIIa were more virulent than those of VGIIIb (84). In this context, a recent report is of interest (155). Assessment of hypervirulence markers (IPR and tubularization of mitochondria) during mating of VGII and VGIII strains and within VGII strains revealed that these traits were infrequently transferred in outgroup crosses but readily transferred during ingroup (within VGII) crosses.

Phenotypic and genotypic virulence correlates. In early studies of *C. gattii* VGI, phospholipase production *per se* quantified *in vitro* was correlated with virulence in mice (154). Differences between the two closely related VGII Vancouver Island outbreak subtypes were noted; all VGIIa isolates were fertile, compared with only 17% of VGIIb isolates, and at 37°C, growth rates, melanin production, and capsule size were increased (150). In another study of Vancouver Island outbreak strains, capsule size; melanin, phospholipase, and proteinase production; and other enzyme activities were analyzed in 39 *C. gattii* strains of variable virulences and different molecular types. There was no correlation between virulence and individual virulence phenotypes. Despite this, expression of *PLB1*, *CRG1*, capsule-related genes, and genes on the mating-type locus correlated with the IPR in macrophages, suggesting that these genes contribute synergistically to virulence in the VGII (AFLP6) lineage (142). There is also evidence that the relative importance of particular virulence determinants varies between molecular types of *C. gattii*. For example, VGIIa was more tolerant to growth at 37°C in the presence of calcineurin inhibitors or in *CNA1* deletion mutants than isolates of VGI, VGIIb, VGIIc, VGIII, and VGIV. Calcineurin was required for virulence in the WM 276 and R265 backgrounds but much less so in the R272 background, whereas melanin production levels were similar in all three strains (156).

These observations confirm that virulence, as measured by disease outcome, is not simply the sum of individually expressed determinants but rather the outcome of a complex set of interacting factors. The term fungal virulence composite was coined by Cutler in 1991 (157) to reflect this in *Candida albicans*. More recently, the deterministic nature of cryptococcal virulence was elucidated in *G. mellonella* and *Acanthamoeba castellanii* models, which confirmed that the cryptococcal phenotype does not necessarily correlate with disease-causing potential, nor does the expression of individual virulence factors correlate directly with pathogenicity (158). The complexity of the fungal virulence composite is further exemplified by the study of genomic and transcriptomic profiles in cryptococcal species.

Genomic and Transcriptomic Profiles

The advent of the omics revolution has revealed a complex array of genes and their transcripts that contribute to the virulence composite in *Cryptococcus* spp. Sequencing of a *C. gattii* VGI environmental strain (WM 276) and the VGIIa Vancouver outbreak strain (R265) revealed that the genomes are colinear for most of the 14 chromosomes, with minor rearrangements, but comparison of different strains showed considerable variation in gene content and overall sequence identity. There was extensive variation between strains of *C. gattii* VGII, precluding simple explanations of differences in virulence based on genome content (36). *C. gattii* strains that are very closely related by genome analysis can have widely different transcriptional circuits and phenotypic differences in virulence in experimental models of infection (159).

Transcriptomes of *C. gattii* have been analyzed in an attempt to fully characterize the *C. gattii* virulence composite. A recent whole-genome expression study identified mitochondrial regulation as an important factor in the enhanced pathogenicity of genotype VGIIa (149); notably, mitochondria were shown to be essential for cryptococcal viability and for protection against hypoxia and oxidative stress. As mentioned above, expression of genes encoding known virulence factors, for example, *PLB1*, *CRG1*, capsule-related genes, and genes on the mating-type locus, correlated with the IPR in J774 macrophages, suggesting a synergistic contribution to virulence in the VGII lineage. Previously uncharacterized genes shown subsequently to influence melanin and capsule production and, thus, cryptococcal infectivity (for example, the COP9 signalosome complex and ubiquitin carboxyl-terminal hydrolase) were also upregulated in VGIIa strains (149).

Comparison of the transcriptomes of R265 (VGIIa) and R272 (VGIIb) revealed increased expression levels of genes encoding melanin, capsule, and growth at 37°C (*LAC1*, *LAC2*, *CAS3*, and *MPK1*). Levels of proteins involved in cell wall assembly, membrane components, carbohydrate and lipid metabolism, transport, the stress response, and lignin degradation were all increased in the VGIIa strain, whereas genes involved in the regulation of mitosis, ergosterol biosynthesis, and drug resistance were downregulated (137). Differences in several other genes, including cell wall assembly and mitotic regulatory genes, were identified. Unlike the study by Ma et al. (149), the expression of the mitochondrial genes was not different in R265 and R272. The differences in the results from the two studies can be explained by the similarity in mitochondrial genomes of strains R265 and R272, since creation of congenic **a** and α strains by backcrossing of R265 with a nonoutbreak VGII strain indicated that neither the mating type nor the mitochondrial genotype was a major virulence determinant (407).

Observations *In Vivo*

In both rat and mouse models, there is evidence that *C. gattii* VGII strains cause significant lung disease but disseminate to the CNS infrequently (153, 160). In a rat model of *C. gattii* VGII infection, the five strains studied included *MAT*α strains R265 (VGIIa) and R272 (VGIIb) from Vancouver Island, which caused progressive and ultimately fatal pneumonia, but consistent and substantial late dissemination to the CNS was noted only with a VGIIb strain of feline origin from Australia (153). In a rat intrapulmonary injection model, strain variation was again apparent, with an environmental isolate (VGI) and an Australian feline isolate (VGIIb)

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causing more severe pulmonary disease than 2 human clinical isolates of VGI. Increased virulence in rats was correlated directly with extensive infiltration of the lungs with budding cryptococci and inversely with the extent of the inflammatory response. Only the most virulent isolate disseminated to the brain; in these animals, microscopic examination revealed occasional single or budding yeasts in the meninges (153). Pulmonary responses in C57BL/6J mice infected with the H99 or R265 strain differed substantially, with proliferation of intra-alveolar yeast and reduced macrophage responses to infection with R265 and an intense nodular macrophage and multinucleated giant cell response to H99. Interestingly, mortality rates were similar in both groups (161, 162).

Another group found that mice infected by intrapharyngeal instillation of VGII strain R265 died from extensive pulmonary infection without significant brain involvement, whereas *C. neoformans* strain H99 disseminated readily to the brain and caused death from cerebral infection (160). Notably, strain R265 induced a relatively poor inflammatory response compared to that induced by strain H99, and both H99 and R265 crossed the blood-brain barrier to establish fatal brain infection upon intravenous inoculation. The predilection of R265 for the murine lung is consistent with the clinical observation that human infections with the *C. gattii* VGII genotype present more commonly with pulmonary than with neurological infection (131).

The influence of *C. gattii* on the host immune response is poorly understood, although studies with *C. neoformans* underscore the importance of the innate immune response in the pathogenesis of cryptococcosis. Cellular interactions between neutrophils, natural killer (NK) lymphocytes, monocytes, and macrophages have been investigated, predominantly *in vitro*, in order to understand the inflammatory/immune response *in vivo*. Cryptococcal genotype-, strain-, and species-dependent effects have been observed in different studies but have not necessarily correlated with the outcome of infection *in vivo*.

For example, a nuclear magnetic resonance (NMR) spectroscopic analysis of metabolites released by single strains of *C. gattii* and *C. neoformans* identified 23 strains with supernatant concentrations differing by at least 2-fold and two metabolites, acetoin and dihydroxyacetone, that were uniquely released by *C. gattii*. The effect of the supernatants from cultures of *C. neoformans* was generally more proinflammatory, causing increased neutrophil necrosis and phorbol myristate acetate (PMA)-induced neutrophil superoxide production and greater adhesion/migration of cryptococci through A549 lung epithelial cell monolayers. Despite these differences *in vitro*, these two strains produced similar neutrophil responses in the rat lung (162). In C57BL/6 mice infected with *C. gattii* VGII Vancouver Island outbreak strains R265 and R272, Australian environmental strain WM 276, and *C. neoformans* strain H99, early migration of neutrophils to sites of infection and production of protective cytokines were reduced in mice infected with the *C. gattii* strains, but strain R265 and *C. neoformans* strains were equally virulent in mice and more virulent than the other two *C. gattii* strains (152).

NK cells are the predominant lymphocytes involved in the innate immune response to cryptococcal infection. They exert a direct cytotoxic effect on cryptococcal cells via exocytosis of lytic granules containing the effector compound perforin (164). It was recently reported that perforin degranulation is increased in an acidic environment, at pH levels reported previously to be generated within cryptococcomas (163); this effect was observed with both *C. gattii* and *C. neoformans* (165).

Alveolar and pulmonary macrophages are a first line of defense against cryptococcal infection. Animal models reveal substantial numbers of cryptococci within pulmonary macrophages and an increase in the number of extracellular organisms during the progression of disease (for example, see reference 143). In *C. neoformans* models, cryptococci exit the lung and are transported in the blood in mononuclear phagocytes (143) and as free cells (146). The cryptococci cross the blood-brain barrier either via transendothelial cell transport of free cryptococci or by paracellular transport within the phagolysosome of mononuclear phagocytes (the so-called Trojan horse mechanism) (146, 166). Much is now known about phagocytosis, phagolysosomal inhibition, multiplication, and egress of *C. neoformans* from macrophages in the pathogenesis of disease (reviewed in reference 142), and the IPR of *C. gattii* VGIIa is enhanced in J774 murine macrophages and primary human monocyte-derived macrophages (149).

There are relatively few studies of host cell interactions with *C. gattii* that provide novel insights into pathogenesis, but a recent publication on the evasion of the adaptive immune response by *C. gattii* strains R265 and R272 is instructive, as similar evasion has not been demonstrated for *C. neoformans* (167). This group demonstrated that heat-killed *C. gattii* is efficiently bound to, and internalized by, human peripheral blood-derived dendritic cells (DCs), trafficked to late phagolysosomes, and killed. However, the DCs were not stimulated to mature into efficient antigen-presenting DCs, as measured by increased expression levels of major histocompatibility complex (MHC) class II and the costimulatory molecules CD86, CD80, CD83, and CCR7 (a chemokine associated with trafficking of DCs to lymph nodes) or decreased expression levels of CD11c and CD32. As a result, specific T cell responses were suboptimal. It was further determined that tumor necrosis factor alpha (TNF-α) levels were not increased, as would be expected following phagocytosis, but exogenous TNF-α or stimulation of its production restored DC maturation and normal T cell responses to cryptococci.

C. GATTII INFECTION IN ANIMALS

C. gattii is an emerging pathogen in a broad range of animals, including domestic cats, dogs, horses, sheep, cows, koalas, dolphins, gray squirrels, ferrets, birds, and marsupials. The epidemiology and clinical and pathological features of cryptococcosis, including that due to *C. gattii*, in various animals have been described in detail (168–171). This review focuses on more recent insights into *C. gattii* infection.

In North America, the prevalence of *C. gattii* infection relative to that of *C. neoformans* infection remains imprecise, since many veterinary isolates have not undergone identification to species level. Furthermore, the prevalence of each pathogen varies with the species of animal (168, 169). In two studies involving 82 cases of feline cryptococcosis in California (169) and western Canada (172), only 17 cryptococcal isolates were identified, 14 of which were *C. gattii* and 3 of which were *C. neoformans*. Much of our knowledge of species distribution comes from studies from Australia (99, 173). As companion dogs and cats are considered sentinel species, disease distribution may be expected to be similar to that in people (see Clinical Epidemiology of Human Infection, below).

In Australia, the majority of infections (and colonization) in

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native koalas and horses are due to *C. gattii* (174, 175), compared to about 33% in domestic cats and dogs (173). In Western Australia, *C. gattii* caused 5 of 9 feline and 11 of 22 canine infections (175). *C. gattii* causes disease in immunocompetent mammals and is considered a primary pathogen (168, 173). Different manifestations of cryptococcosis in different species may be due to a number of factors, including host anatomical differences, physiological differences (e.g., body temperature), ecological habitats, and species-specific differences in comorbidities, immunology, and behavior, which affect exposure and host reaction to the fungus. These aspects are not reviewed, but the salient features of *C. gattii* infection in various animals are presented.

Case reports and moderate-to-large case series of *C. gattii* in cats abound (37, 169, 171–173, 176–182). A review of the major studies where the etiologic cryptococcal species were reported indicates that there are no clinical features which reliably distinguish disease caused by *C. neoformans* from that due to *C. gattii*. As with *C. neoformans* infection, young to middle-aged cats (2 to 3 years of age) are typically affected (173), but the age range is wide. In Australia, Siamese, Birman, and Ragdoll breeds are overrepresented. Exposure to the outdoors is significantly associated with cryptococcosis, but indoor cats are also affected, presumably through exposure to aerosols, soil, or plant material (173, 178). Of note, one study found that cats in rural areas were significantly more likely to have *C. gattii* than *C. neoformans* infection (173). In the Vancouver Island outbreak, Duncan et al. reported that in a matched case-control study, cats that were active, had traveled on the island, or lived near a site of commercial environmental disturbance (including logging and soil disturbance) had a significantly increased risk of developing *C. gattii* infection (183). These findings pertained to the period between 2001 and 2003, and follow-up studies are worthwhile to determine if these activities still pose a risk 10 years later, since they have implications for pet owners. Interestingly, the majority of cats infected with the VGII genotype in two Australian studies (173) presented with more extensive disease than those infected with the VGI genotype, suggesting that VGII is more virulent than VGI. Studies of a larger numbers of cats with both types of infection, in other regions, are required to validate this hypothesis.

Cats present primarily with nasal and paranasal cavity disease. Other common sites of infection include the skin, lymph nodes, brain, meninges, and eyes. Typically, granulomatous lesions occur, especially in the head and neck regions, such as over the maxillary or frontal areas and the nares (173, 178, 179). Eye involvement, characterized by chorioretinitis and blindness, is not uncommon. Single or multiple cryptococcomas can occur in the brain and spinal cord (181). Unlike in koalas and horses (see below), lung manifestations, including pyothorax, are uncommon (180). However, sneezing, stertor, snuffling, and headshaking can occur (168, 169). In southwestern British Columbia, 27% of 78 feline cases had such respiratory symptoms (171). Nasal deformity with or without purulent, serous, or hemorrhagic nasal discharge, either unilateral or bilateral, is characteristic. Owners may report lethargy and poor appetite in their pets, but fever is uncommon (168, 169). Disseminated infection occurs in 8 to 16% of cases, and any organ may be affected, including the gastrointestinal and genitourinary tracts (169, 172, 173, 175, 178). In Canada, a multivariate survival analysis revealed that CNS infection but not respiratory disease or other medical history was a significant predictor of mortality (171). Retroviral infection in cats does not appear to predispose them to cryptococcosis (172). The prevalence of *C. gattii* infection in cats with feline immunodeficiency virus or feline leukemia virus infection is not known.

Collectively, young active dogs <6 years of age comprise the majority of cases, with no sex predilection. Large breeds (e.g., Border Collies, Boxers, Dalmatians, Doberman Pinschers, Great Danes, and German Shepherds) are overrepresented, presumably reflecting their active outdoor life-style (171, 172, 183). Outdoor walks through forested areas and accompanying humans on hiking activities were associated with *C. gattii* disease in the North American outbreaks (171, 183). In Australia, unlike with cats, a rural domicile was not associated with *C. gattii* infection (172, 173).

Dogs typically present with clinical signs involving more than one organ system (172, 184). In one study (173), although nasal cavity involvement was important, the canine cohort had a greater propensity to develop secondary CNS involvement and disseminated disease than feline cases. Neurological signs, including stumbling, paralysis, ataxia, hyperesthesia (often along the dorsal areas and neck), are most common, and seizures may occur. Blindness is not as common as in cats (168). Epistaxis, sneezing, and nasal discharge are present in ~50% of cases of cryptococcosis (173, 175, 184, 185). The skin, kidney, and gastrointestinal tract may also be involved. As for cats, there are no clinical features that distinguish cryptococcosis caused by *C. neoformans* from that caused by *C. gattii*.

Cryptococcosis was first reported in koalas in 1960 (186) and is almost always due to *C. gattii* (174, 187). The koala habitat in the wild is the same as that of the species of eucalypt that has been identified as the environmental niche of *C. gattii*. Animals become heavily colonized with the fungus: colonization rates of 94 to 100% were found in koalas residing in the Coffs Harbor Wildlife Park, New South Wales, Australia, and the Sydney Taronga Zoo (174, 188). Koalas are susceptible to invasive infection and may act as reservoir hosts for humans by amplifying the environmental burden of *C. gattii* (187, 189). Of interest, in a recent Japanese study of 15 imported Australian koalas in a zoo in Yokohama, only 1 of 10 (66.7%) animals colonized with *Cryptococcus* was colonized with *C. gattii* (190). Koalas with invasive cryptococcosis most often present with pneumonia and meningoencephalitis, although nasal infection also occurs (187). Identification of *C. gattii* in asymptomatic koalas (188–190) has spawned studies of subclinical cryptococcosis, of the mechanisms that trigger clinical infection, and of the need for effective cryptococcal eradication measures to prevent the spread of infection.

Sheep often develop infections of the nasal cavity or CNS, while horses and goats present primarily with lung disease (92, 168, 175). In horses, the sinuses (animals present with rhinitis) are most consistently involved, but both pneumonia and CNS disease are also common. Horses with *C. gattii* infection in Western Australia ($n = 9$) had primarily pneumonia; cases of bone, lymph node, and bowel involvement were described, but the causative cryptococcal species was not specified (175). In birds, the presentation is variable, but upper and lower respiratory infections are relatively common. Granulomatous skin nodules on the neck or body and retrobulbar lesions also occur (172, 191). At least five confirmed cases of cryptococcosis due to *C. gattii* in ferrets, including cases from Spain, have been reported, two of which were autochthonous cases (192, 193); lesions were present in the nasopharynx, lung, lymph nodes, CNS, and bone.

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In the United States and Canada, *C. gattii* infection in dolphins and porpoises has been reported (37). It is not clear how marine mammals become infected, but *C. gattii* can survive in freshwater and seawater for up to a year, suggesting that this may be a route of infection. Alternatively, these mammals may acquire infection through inhalation of aerosolized spores, as is the case for other mammals, since lung infections in dolphins and porpoises have been reported (37, 194). Norman et al. described a case of maternal-fetal transmission in a porpoise (195).

CLINICAL EPIDEMIOLOGY OF HUMAN INFECTION

In humans, *C. gattii* causes predominantly meningoencephalitis and other CNS and pulmonary diseases and is associated with substantial morbidity (27, 28, 130–132). It also poses a concern for veterinarians, and some animal infections, in particular those in feline and canine companion animals and Australian native koalas, represent sentinels for human exposure (37, 176, 188, 190). Shifts in the appreciation of the clinical epidemiology of *C. gattii* in the past decade include the recognition that it affects immunocompromised (including those with HIV/AIDS) as well as immunocompetent (130–133) hosts, the identification of multiple *C. gattii* molecular types (see Epidemiology, Origin, and Evolution, above) and their clinical implications, and the broadening of its geographic range beyond the tropical-subtropical locales of Northern Australia, Papua New Guinea (PNG), Southeast Asia, Africa, South America, and California (71) to the temperate regions of these and other countries (38, 69, 86, 91, 130, 132, 196–198). This review focuses on these changes and their possible associations with disease patterns in humans.

Epidemiology of Human Infection

The first clinical case of cryptococcosis in which mention of the name *C. gattii* was made was reported in 1970, involving a 7-year-old boy with leukemia from the Congo (formerly Zaire) who presented with meningitis (199), although in retrospect, the application of molecular methods revealed that an isolate recovered from a patient with infection due to *Saccharomyces subcutaneous tumefaciens* was, in fact, *C. gattii* (19). Since then, the literature has abounded with reports of this uncommon infection in single patients and small case series. The emergence of larger case clusters in 1999 in North America and reports of autochthonous cases outside known regions of endemicity has exponentially increased awareness of *C. gattii* as a pathogen. The major *C. gattii* reports (confirmed by culture- or molecular-based methods) in English are listed here or are referred to below (27–29, 38, 69, 86, 91, 94–96, 98, 106, 113, 130–133, 196–198, 200–253). Table 4 summarizes the pertinent epidemiological and clinical features in case series involving three or more patients. In addition, a number of reviews, editorials, and book chapters with substantive discussion on *C. gattii* clinical epidemiology and infection in humans have been published since 2000 (39, 85, 122, 254–265).

Geographic distribution and incidence. Knowledge regarding the global distribution of *C. gattii* cases assists in understanding its clinical epidemiology and, consequently, risk factors for infection (see below). However, this understanding is incomplete, in part because until recently, many laboratories did not distinguish between *C. gattii* and *C. neoformans* (247, 258). In Australia, where *C. gattii* infection is considered to be endemic, the two species have been distinguished routinely since the early 1990s by using canavanine-glycine-bromothymol blue (CGB) agar (266) (see Diagnostics, below). Earlier small (8 to 49 patients) case series of *C. gattii* infection were mainly from Australia (25, 27, 28, 99, 113, 204, 267), Papua New Guinea (208–213), and Brazil (206, 219). Incidence data from Brazil are imprecise, but compared to other parts of the world, children appear to be disproportionately affected by *C. gattii*. Prior to the emergence of AIDS in Papua New Guinea, *C. gattii* caused ~95% of cases of cryptococcal meningitis, with an estimated annual population incidence in the Central Province of 42.8 cases per million population (214). In Australia, the annual incidence in the late 1990s was 0.94 cases per million population; this incidence varied with jurisdiction, being highest in the Northern Territory (8.5 cases per million) (29). The incidence has remained unchanged over the succeeding decade (0.61 per million) (130). Population-based rates are significantly higher in the indigenous Aboriginal population than in nonindigenous persons (10.4 versus 0.7 cases per million) (29). In the tropical Arnhemland region of the Northern Territory, the relative risk for cryptococcosis in Aboriginal people compared with non-Aboriginals is 20.6 (204).

C. gattii has traditionally been considered a "tropical or subtropical fungus" despite the fact that even prior to the North American outbreak, a large proportion of disease in Australia occurred in its southern temperate region (27–29, 99, 268). Before 1999, *C. gattii* infections were uncommon in North America, with small numbers of cases being reported in California and Hawaii (83, 269) and Europe (reviewed in reference 122). The spread of *C. gattii* infection into new geographic regions was heralded by an unprecedented outbreak of infection on Vancouver Island, British Columbia, in 1999, with subsequent clusters in British Columbia and the Pacific Northwest of the United States.

From 1999 to 2007, 218 patients acquired *C. gattii* infection as part of the Vancouver Island outbreak (198, 270). Between 2002 and 2006, the annual average incidence in British Columbia was 5.8 cases per million population, and that on Vancouver Island was 27.9 per million (270). It now appears that an endemic focus of *C. gattii* cryptococcosis was established in 2004 in the Pacific Northwest. As of 2012, >96 infections have been reported, mainly from the states of Washington and Oregon (132, 197, 258), although incidence data have not yet been reported (271). Only after these outbreaks and the consequent interest in species distinction has the complexity of the epidemiology been appreciated.

In parallel with the emergence of case clusters, an explosion of *C. gattii* cases were reported, some after travel to areas of western North America where the disease is "newly endemic," including California, and others presenting as autochthonous cases (231, 238, 240, 247, 250). Single cases of infection have also been reported in the United States, including New England (85, 243, 247), New Mexico (272), and Florida (253); Europe (91, 95, 106, 245); Asia, including Japan and Singapore (227, 246, 249); and South America (223, 256). Most recently, Harris et al. reported 25 cases, inclusive of previously described patients (247, 253) diagnosed between 2009 and 2012, from Montana, Alabama, California, Hawaii, and Michigan; none of these patients had traveled to areas of *C. gattii* endemicity (251). In Australia, despite heightened awareness, no increase in the number cases has been observed (most large institutions care for 2 to 3 patients annually). Two reports (216, 217) highlighted the occurrence of immune reconstitution inflammatory syndrome (IRIS) in separate immunocompetent hosts with *C. gattii* meningitis (see Clinical Complications of Human Infection, below).

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TABLE 4 Epidemiology and clinical features of major case series of *Cryptococcus gattii* infection[c]

Reference	Location	No. of patients	Site(s) of infection	Host status(es)	Mortality rate (%)	Induction antifungal therapy	Complication(s) (no. of cases)	Sequela(e) (no. or % of survivors with sequela)
218	PNG	3 (all children)	Meninges	IC	33	cAMB (6 wk)	Abnormal mentation (2), papilledema (2), retinal hemorrhage (1)	None
213	PNG	7 (1 child)	Meninges	IC	43	cAMB + 5FC (mean, 7 wk)	Papilledema (3), blurred vision (3), deafness (1), seizures (1)	Not specified
208	PNG	82	Meninges	IC	Not stated	Not specified	Visual loss in 52.6% of survivors	Visual loss (52.6%)
209	PNG	88	Meninges	IC	34.1	Not specified	Not specified	Not specified
211	PNG	49	Meninges	IC	22.4	cAMB + 5FC; total dose of cAMB ranged from 890 mg to 2.4 g	Papilledema (26), blurred vision (25), abnormal mentation (12), cranial nerve palsy (10), seizures (7), raised ICP (3/6)	Visual loss (31%)
204	NT, Australia	18	CNS +/− lung	Primarily IC	Not specified for *C. gattii* but 9.1 overall	cAMB (range, 0.85 to 5 g)	Not specified	Not specified
27	Australia	26	CNS (26)	IC	15	cAMB + 5FC (median, 2.4 g cAMB)	Papilledema (13), abnormal mentation (8), focal signs (1), seizures (10), hydrocephalus (9)	Moderate-to-severe neurological sequelae (8 patients)
28	Australia	20	Meninges (17), brain (7), lungs (13)	IC	0	Not specified for *C. gattii*	Hydrocephalus (4), focal signs (5)	Neurological sequelae (7 patients)
29	Australia	47	Meninges (27), brain (10), lung (30), skin (3)	IC (41), IS (5)	Not specified		Hydrocephalus (5)	Not specified
215	NT, Australia	12	CNS +/− lung (8), lung only (4)	Not specified	33	cAMB + 5FC (avg, 43 days)	Hydrocephalus (at least 3)	Not specified
130	Australia	86	CNS +/− lung (73), lung only (10)	IC (62), IS (24)	13.6	AMB + 5FC (57 patients) (6 wk), FLU (9) (4 wk), AMB + 5FC (7) (2 wk), FLU (2) (not specified)	Papilledema (9), abnormal mentation (18), cerebellar deficit (10), limb weakness (6), seizures (5), cranial nerve palsy (13), raised ICP (31; 42%), hydrocephalus (22; 30%)	Neurological sequelae (20; 27%)[a]
98	South Africa	46	Mostly meninges	HIV (29), non-HIV/ unknown (18)	35.6	AMB (14), FLU (32), AMB + FLU (5)		
96	Botswana	29	Meninges	HIV (29)	17	AMB (14 days)		
131	Canada	3	Lung + CNS (1), lung only (2)	IC	0	AMB (1), FLU (2)	None	None
198	Canada	218	Lung only (167), CNS only (17), CNS + lung (22), other (2)	IC (148), IS (70)	8.7		Not specified	Not specified
230	Canada	38 in case-control study[b]	Meninges (10), lung (28)	IC (not stated), IS (not stated)				
132	USA (Pacific Northwest states)	96; 83 outbreak infections, 13 nonoutbreak infections	Meninges (29), Brain (6), lung (31)	IC (62), IS (34)	33	Not specified	Papilledema (3/49), blurred vision (9/49), seizures (4/49)	
248	USA (non-Pacific Northwest states)	25	CNS (19), lung (9), CNS only (12), lung only (12), blood (3), leg (1)	IC (20), IS (5)	24	AMB + 5FC (17 patients), AMB (1), FLU (1), unknown (2)	Papilloedema (2), blurred vision (8), seizures (1), cranial nerve palsy (5/13), hydrocephalus (4/18)	Not specified
239	Vietnam	10	Meninges	HIV negative	10	cAMB + 5FC (Vietnamese national guidelines)	Papilledema (5), abnormal mentation (1), blurred vision (4), focal signs (5)	Overall, blindness in 6 patients, deafness in 1 patient, and neurological deficits in 14 patients, but data were not stratified by infecting *Cryptococcus* species
206	Brazil	8	Unknown	IC (7), IS (HIV infection) (1)			Not stratified by cryptococcal species	Not stratified by cryptococcal species
220	Brazil	11	CNS alone (9), CNS and lung (2)	IC	18.2	AMB (7), AMB + 5FC (5)	Hydrocephalus (6)	Blindness (4 patients)

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222	Brazil	21	Meninges	HIV (2), non-HIV (19)			
221	Brazil	25	Meninges	HIV (4), non-HIV (21)	44		
256	Brazil	7 (previously unreported cases only)	Meninges	HIV (1), IC (6)	14.3	AMB only (5), AMB + FLU (2)	
251	Brazil	4	Meninges	HIV (2)	50		

[a] Cranial nerve lesions, epilepsy, memory impairment, and visual field loss.
[b] The case-control study identified oral steroid use, pneumonia, and other lung conditions to be associated with infection. In population comparisons, cases were more likely to be >50 years of age, to be current smokers, to have HIV infection, or to have a history of invasive cancer.
[c] Abbreviations: AMB, amphotericin B formulation; cAMB, conventional amphotericin B deoxycholate; CNS, central nervous system; 5FC, 5-flucytosine; IC, immunocompetent; ICP, intracranial pressure; IS, immunosuppressed; PNG, Papua New Guinea.

Central to the epidemiology of human infection is the appreciation of the distribution of *C. gattii* molecular types by geographic region (Table 1 and Fig. 4). Infection in Australia and PNG is caused largely by molecular type VGI (29, 99). VGII infections also occur but are, to date, geographically restricted to the Arnhemland region of the Northern Territory and the southwest of Western Australia (113, 117). In contrast, clonal VGII subtypes (referred to as outbreak strains) of *C. gattii* caused case clusters in British Columbia (subtypes VGIIa and VGIIb) and in the Pacific Northwest (subtypes VGIIa, VGIIb, and VGIIc) (38, 132, 198, 251); subtype VGIIc is a novel subtype not found outside the United States (68). Theories on how these VGII subtypes may have arisen in North America are discussed above (see Epidemiology, Origin, and Evolution, above). Infection outside the Pacific Northwest has been caused by VGI and VGIII (242, 243, 251, 272) as well as by subtype VGIIb and other, nonoutbreak subtypes of VGII (251).

The assignment of genotype is important not only for epidemiological purposes but also to track the origin of isolates. Pinto Junior et al. reported a child residing in Rio de Janeiro with *C. gattii* genotype VGII infection who had no history of travel outside the city (223). Given that VGII is the predominant molecular type (VGI is uncommon) in north/northeastern Brazil but not in the southern part of the country, those authors postulated that the ecological niche of this genotype may be expanding (223). Support for the notion that the niche of the VGII genotype is evolving is the observation of infection caused by molecular type VGIIa in a Japanese patient in Tokyo with no history of travel to an area of known endemicity; VGIIa genotype strains have not previously been documented in Japan (249). Further environmental surveillance is needed to determine new reservoirs of *C. gattii*. Obtaining a travel history from patients with suspected cryptococcosis in areas where *C. gattii* is not endemic should be routine. Hagen et al. described a Dutch patient who acquired infection due to molecular type VGIIb after visiting Vancouver Island (238). In a subsequent study, that same group of authors demonstrated that infections in Europe caused by molecular types VGIII and VGIV could be linked to exposure in regions of Africa (91).

Environmental exposure. The ecology of *C. gattii* and its environmental molecular epidemiology are described above (see Ecology, above).

Historically, the first hint of an environmental source of *C. gattii* was reported in the early 1990s in Australia, when the distribution of human disease was noted to match the geographic location of two species of eucalypt: *E. camaldulensis* (river red gum) and *E. tereticornis* (forest red gum) (26, 273). The density of *E. camaldulensis*, in particular along water courses in rural/semirural Australia, and the association of rural-dwelling Aborigines with these trees are thought to partly explain the relatively high prevalence of infection in indigenous Australians. This link was confirmed by subsequent epidemiological investigations (29, 99), as was the association between *C. gattii* and residence, or employment, in a rural/semirural environment (29, 130, 215). Whether a similar association is evident in British Columbia and the U.S. Pacific Northwest is unknown; in the setting of an outbreak, the epidemiology may be different. Additional risk factors, such as host genetic determinants of susceptibility, have not been well studied.

As in Australia, *C. gattii* has been recovered from eucalypts in other countries with small numbers of human cases, including

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southern California, Italy, and Brazil (273, 274). However, infection also occurs in regions where eucalypts do not occur naturally, in particular Malaysia; South America; the Arnhemland region of the Northern Territory, Australia; and, most notably, Vancouver Island (38, 113, 274). In Arnhemland, the local reservoir of *C. gattii* remains uncertain; it is of interest that the predominant *C. gattii* molecular type in this location is VGII and not the VGI genotype that has been linked with eucalypts (99). While eucalypts are not indigenous to PNG, extensive sampling of imported trees in central PNG proved negative (212, 214). On Vancouver Island, in addition to imported eucalypts, trees native to the island have been implicated as being a niche for *C. gattii* and include Douglas fir, coastal western hemlock, alder, Garry oak, grand fir, and cedar (38, 258); *C. gattii* has also been found in soil.

Host factors. A better understanding of the global distribution of *C. gattii* has been accompanied by an appreciation of evolving host risk factors. Unlike *C. neoformans* cryptococcosis, where HIV/AIDS, for example, is a major risk factor, risks for *C. gattii* infection are less well defined.

Cryptococcosis *per se* has occurred more frequently in males than in females, independent of the disproportionate representation of males with AIDS in data sets from Western countries. In one Australian study, male gender was a significant risk factor for cryptococcosis but only in patients with *C. gattii* infection (male-to-female ratio of 3.3:1) (29). Similar observations were reported in PNG and were attributed to an increased exposure of males to environmental sources of *C. gattii* (209). On Vancouver Island, however, only 55% of the outbreak cases affected males, and in the Pacific Northwest, 54% affected males (132, 198), suggesting that exposure is the predominant factor under these circumstances. Interestingly, 85% (21 of 25) of sporadic cases in the United States affected men (251). Population-based studies using uniform case definitions are needed to clarify whether there is a gender predisposition to *C. gattii* infection and, if so, whether there are additional genotype-dependent associations.

Data on host genetic factors as risks for *C. gattii* cryptococcosis are sparse. Indigenous Australians are consistently overrepresented in reports of *C. gattii* from rural Australia (29, 130, 204, 215), presumably due mainly to increased environmental exposure. However, the incidence of *C. neoformans* infection is also higher in Aborigines, suggesting that genetic and/or socioeconomic factors may be important. Likewise, exposure to *C. gattii*, even though the ecological niche has not been identified, has been linked to cryptococcosis in PNG, where the indigenous population is Melanesian. In another PNG study, neither leukocyte antigen class I nor class II phenotypes were associated with *C. gattii* infection, although there was a trend toward increased susceptibility in patients carrying the human leukocyte antigen B*5601 (275). Recently, high levels of certain anticytokine antibodies (e.g., anti-granulocyte-macrophage colony-stimulating factor [GM-CSF] antibodies) were associated with cryptococcosis in HIV-negative patients (276). These antibodies predispose individuals to opportunistic infections by impairing innate immunity and may be especially relevant among HIV-negative patients in Asia (277, 278). Saijo et al. most recently reported the presence of anti-GM-CSF autoantibodies in the plasma of seven apparently immunocompetent patients with *C. gattii* meningoencephalitis but, interestingly, not in the plasma of patients with *C. neoformans* disease (279). Their results, which suggest that anti-GM-CSF autoantibodies predispose otherwise immunocompetent individuals to meningoencephalitis caused by *C. gattii*, but not necessarily that caused by *C. neoformans*, are worthy of further study.

Distinct from *C. neoformans*, which typically causes disease in individuals with cell-mediated immune deficiencies (255, 260), historically, our understanding is that *C. gattii* causes disease predominantly in persons with apparently normal immune systems (72 to 100%) (27–29, 206, 211, 213, 215, 219). However, following the North American outbreaks and, more recently, outbreaks in Australia, new risk groups have been recognized. These groups include patients with underlying HIV infection, cancer, solid organ transplantation, and other immunodeficiencies (96, 98, 130, 132, 198, 230) (Table 4). In an Australian study of 86 patients, HIV was rare (1 of 79 patients tested for antibodies to HIV), but 7% of patients had underlying idiopathic CD4 lymphopenia, 3% had previously received kidney transplants, 8.1% had a malignancy, and 14% had received corticosteroids or immunosuppressive drugs (130). Notably, host immunocompromise was associated with increased mortality (29% versus 5%; $P < 0.05$). Overall, 72% of patients were immunocompetent, compared to 91.5% of patients in a previous study (29).

In British Columbia, only 62% of patients were considered previously healthy (198); a case-control analysis identified oral corticosteroid use, smoking, older age, HIV infection, and malignancy as risk factors (230). The proportion of patients who had predisposing conditions or who were taking immunosuppressive medications was even higher (76%) in U.S. cases ($n = 76$ patients) infected with "outbreak" strains. Here 4 of 59 patients were HIV positive, 50% of patients were immunocompromised, and 20% and 23% had had a solid organ transplant and cancer, respectively. These outbreak strains were defined as belonging to molecular types VGIIa, VGIIb, and VGIIc (132). There is increasing evidence of an association between the *C. gattii* molecular type and host status, although the pathogenic link between the two is not yet understood. Several studies have confirmed that molecular types VGI and VGII of *C. gattii* have a predilection for immunocompetent hosts, as discussed above (Fig. 5). In contrast, molecular type VGIII was overrepresented in a series of HIV-positive patients in southern California (84, 133), and VGIV has been identified in Africa in association with HIV/AIDS (Fig. 5) (29, 99). There are also data indicating an association between molecular type and clinical presentation (see Clinical Manifestations of Human Infection, below).

Patients with *C. gattii* infection may have subtle defects in immune function (276, 279). Marr et al. reported IgG2 antibody deficiency in one such patient (280). It is possible that selective antibody deficiencies are risk factors for *C. gattii* infection, rendering the host unable to mount a protective IgG2 antibody response to cryptococcal capsular polysaccharide. Idiopathic CD4 lymphopenia is a risk factor for *C. neoformans* infection (281), but its association with cryptococcosis due to *C. gattii* is new (130). Taken together with the identification of anticytokine antibodies in some patients with *C. neoformans* and *C. gattii* cryptococcosis (see above), these results suggest that immunological assessments, at least performing HIV antibody and T cell subset measurements, should be undertaken for patients with *C. gattii* infection.

Other comorbidities may also pose a risk for cryptococcosis caused by *C. gattii*. Almost 30% of patients in the Pacific Northwest outbreak had chronic lung disease (132). Concurrent corticosteroid use in such patients and/or changes in lung function associated with cigarette smoking may contribute to this risk. On

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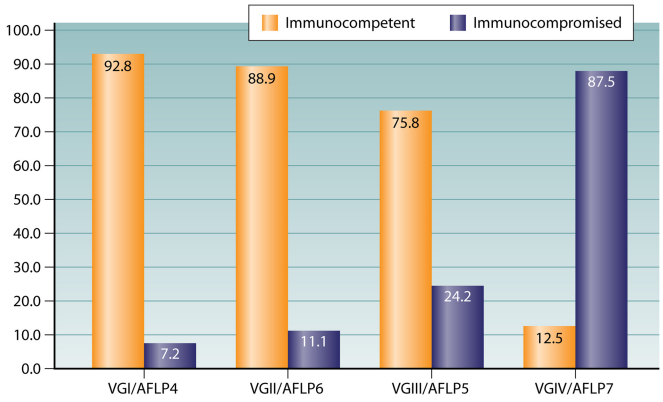

FIG 5 Percentages of 256 clinical isolates obtained from immunocompetent and immunocompromised patients (HIV positive) and from patients with other risk factors per major molecular type, identified by *URA5* RFLP analysis, PCR fingerprinting, or AFLP analysis, for which clinical data were available. Other risk factors are alcoholism, corticosteroid use, disorder T immunity, diabetes, leukemia, systemic lupus erythematosus, transplant, and tumor.

Vancouver Island, case-control studies identified an association between underlying lung conditions, including chronic obstructive pulmonary disease (COPD), and *C. gattii* infection. However, in population analyses, only current smokers ($P < 0.001$), not those with a history of COPD or asthma, were more likely to develop infection (231). Smoking was also an independent risk factor, occurring in 36% of cases in a recent Australian study (130). Other cases of *C. gattii* cryptococcosis have been reported in smokers (231, 272). Pregnancy has been proposed to be a risk factor, and in two studies, 2 of 26 and 1 of 49 patients were pregnant (29, 130); however, the proportion of pregnant patients in a recent Australian study of *C. gattii* infections was not higher than that in the general female population of reproductive age. An association between diabetes mellitus and *C. gattii* has not been established. In one study, 6% of patients had diabetes, no higher than in the general population (29), but in another study, 20% were diabetic (132). Notably, *C. gattii* is overrepresented in Australian Aborigines (29), in whom diabetes is common. Diabetes was not a risk factor for *C. gattii* in British Columbia (230).

The possible clinical associations of *C. gattii* molecular types are further discussed below (see Clinical Manifestations of Human Infections).

Infection in children. Cryptococcosis is uncommon in children, including those with HIV/AIDS (232, 282), and the same appears to be the case for *C. gattii* infection (213, 218, 232, 268), with the possible exception of children in Brazil. An earlier study from PNG showed that exposure to the fungus, as demonstrated by levels of serum IgG antibody directed against *C. gattii*, was significantly greater in adults, and especially adult males, than in children (209), suggesting that exposure increases with age and that it occurs away from the home environment. Antibodies to *C. neoformans* (including *C. gattii*) are uncommon in children <2 to 5 years old (268, 283).

In contrast, in the north and northeast regions of Brazil, particularly in the states of Para, Roriama, Piaui, Pernambuco, and Bahia, which include the Brazilian Amazon forest and savannah areas, a relatively high proportion of HIV-negative, otherwise healthy children present with cryptococcal meningitis (age range, 5 to 14 years; average age, 8.4 years) and are infected with *C. gattii* (219, 256, 284). Although sample sizes were small and only selected isolates were identified, Correa et al. reported that at least 47.3% of children with cryptococcal meningitis had *C. gattii* infection, while for 22 patients reviewed by Severo et al., *C. gattii* caused 33.3% of cases (219, 256). In that same study, 16 of 53 cases (30%) in adults and children were due to *C. gattii* (256); underlying conditions were not stratified by cryptococcal species, but at least 14 children were previously healthy. Small epidemiological studies suggest that *C. gattii* molecular genotype VGII predominates in infected children in north/northeast Brazil and that VGI is uncommon (221, 222). Infection in immunocompetent children in southern and southeastern Brazil has also been reported (223, 224, 256).

Among 16,192 episodes of cryptococcosis (due to both *C. neoformans* and *C. gattii*) in predominantly HIV-infected persons in South Africa from 2005 to 2007 (232), a bimodal distribution of infection was noted, with a peak in children <1 year old and a second peak in those aged 5 to 10 years. Although most pediatric infections were due to *C. neoformans*, children were significantly more likely than adults to be infected with *C. gattii* (9% versus 3%; $P < 0.001$).

CLINICAL MANIFESTATIONS OF HUMAN INFECTION

Incubation Period

The incubation period of *C. gattii* infection has been deduced from instances of exposure in persons visiting areas of endemicity or in an outbreak setting. Studies of travelers to Vancouver Island revealed a median time to clinical presentation of 6 to 7 months (range, 2 to 11 months) (229). However, in one instance, the incubation period was as short as 6 weeks (94), while other patients developed infection 13 to 35 months after visiting the island (95, 238, 250). Tsunemi et al. reported a Japanese national who developed infection 2 to 4 weeks after returning from Australia, where he had had close contact with koalas (246). Since the incubation period of *C. gattii* infection may be prolonged (>2 years), diagnosis may be delayed in an area where the disease is not endemic unless an appropriate travel history is obtained.

This estimated incubation period compares with 110 months for *C. neoformans* (serotype A) infection (285). The relatively long latent period of *C. neoformans* infection suggests that latency is established following primary infection and that reactivation of dormant infection can result from subsequent immunosuppression (286); the extent to which this happens in *C. gattii* infection is uncertain. Using MLST analysis, Hagen et al. described 24 *C. gattii* cases that were linked to exposure in regions where *C. gattii* is endemic in the United States, Brazil, Vancouver Island, and Africa; although the time to infection following exposure was not specified, the findings provide support for reactivation of dormant infection after many years (91).

Sites of Infection

CNS and lung infection. *C. gattii* cryptococcosis most commonly affects the CNS and lung, although it can involve any body site. Importantly, there appear to be differences in clinical presentation and site(s) of infection between disease that occurs sporadically and that seen in the reported case clusters. Studies from Papua New Guinea indicate that CNS infection, particularly meningitis, is typical (208, 211, 213, 218). In Australia and New Zealand, meningoencephalitis and/or cerebral involvement occurred in 74% of patients with culture-confirmed *C. gattii* infection in the

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1990s (29), while in a contemporary study, in which most patients underwent cerebral computerized tomography (CT) and magnetic resonance imaging (MRI), CNS infection was diagnosed in 85% of 86 patients; overall, 76% of patients had meningitis and 52% had brain infection, including cerebral cryptococcomas (130). Up to 97% of cases of *C. gattii* infection in patients with HIV/AIDS present as meningitis (96). Neurological disease was also common in non-outbreak-related infections in the United States, Brazil, and Europe (106, 223, 238, 242, 243, 248, 258).

In contrast, lung disease predominated in the North American outbreaks (131, 132, 252), with only 20 to 40% of patients having CNS infection. Among 218 patients in British Columbia, 189 (87%) presented with a respiratory illness, 22 of whom (10%) had concurrent CNS disease (198). In the U.S. outbreak, 31 of 57 (54%) patients developed pneumonia, and 20 of 61 (33%) patients had lung cryptococcomas (132). Patients considered to have acquired their infection in the northwest of North America also presented primarily with lung disease (231, 237, 240, 250). While lung disease is common in Australia, affecting 63 to 67% of cases in three population-based studies, it occurs mostly in association with CNS disease (81% of cases) (28, 29, 130). Notably, the pattern of disease in the Northern Territory differs from that in Australia as a whole, with isolated lung disease accounting for two-thirds of cases (215). Thus, there are regional differences in clinical presentation between, and within, countries.

Other sites of infection. Other than pulmonary and neurological (including eye) disease, *C. gattii* infections may involve skin, soft tissues, bone, joint, larynx, bone marrow, and lymph nodes (29, 131, 206, 234, 287). Intra-abdominal infection is uncommon but is important, as it may be mistaken for underlying cancer (233). Primary cutaneous *C. gattii* infection has been reported for only a few patients, including patients from Australia and Brazil. It is typically preceded by traumatic inoculation and affects the scalp and limbs of immunocompetent patients (228, 235, 256, 287–290). Lesions may be solitary or present as extensive ulceration or cellulitis. Primary skin involvement is rare in Australia and British Columbia (130, 198). Skin lesions may herald disseminated disease, with papules, nodules, ulcers, or cellulitis occurring typically on the face and neck; these patients may be immunocompromised (236, 256, 290). Musculoskeletal infection has been reported. Byrnes et al. described a patient with a cryptococcoma in the thigh, which progressed to disseminated infection despite surgical resection and antifungal therapy (68). Another patient from Florida had disease in the femur together with lung and CNS infection (253). Finally, *C. gattii* fungemia was detected in 10 patients in one series (130), but its prevalence either alone or in the setting of CNS or other infection is unknown, since blood cultures are not taken routinely.

***C. gattii* molecular types, host risk factors, and disease patterns.** The distinctive features of the North American outbreaks have raised the possibility of an association between clinical presentation and *C. gattii* molecular type (see Clinical Epidemiology of Human Infection, above). Among patients in the Canadian outbreak, the VGIIa molecular type was responsible for 86.3% of cases, with VGIIb and (nonoutbreak strains of) VGI accounting for 7.3% and 6.5% of cases, respectively (198). In the U.S. Pacific Northwest, the distribution of molecular types was as follows: 50% VGIIa, 32% VGIIc, 10% VGIIb, 5% VGI, and 3% VGIII (86). It is possible that the VGII genotype has a propensity to cause respiratory disease (see above) and that it is associated with host immunocompromise or predisposing medical conditions (40 to 73% of patients) (86, 132, 230). Although features of infection differ between "outbreak" and "nonoutbreak" disease, the reason for the difference has not been determined. It is possible that there are genotype-dependent differences in the host response to the organism (see Pathogenesis, above) or an effect of concomitant illnesses. There is also debate as to whether these hypotheses are generalizable to the nonoutbreak setting. In Australia, the epidemiology has changed over the last 10 to 15 years, with an increase in the proportion of HIV-negative immunocompromised patients (130), although most infections were still caused by molecular type VGI (S.C.-A. Chen and T. C. Sorrell, unpublished data). As with VGI infections, the majority of infections due to the VGII molecular type occurred in nonimmunosuppressed hosts (Fig. 5) (29, 99). VGII genotype strains were isolated from otherwise healthy Brazilian patients and a single patient each from Japan, Holland, and Florida; all had CNS disease (222, 238, 249, 256).

Clinical Findings

Clinical findings depend on the site of infection. Systemic features such as chills, fever, and weight loss were reported in 17 to 47% of cases in the Pacific Northwest (132, 252). Fever was present in 54% of patients with HIV infection in South Africa (98) but was uncommon (10%) in an Australian survey (130); in the latter study, the median time from the onset of CNS symptoms to diagnosis was 45 days (interquartile range [IQR], 21 to 120 days), compared with 56.8 days (IQR, 1 to 180 days) in those with isolated lung infection. In a U.S. study outside the outbreak setting, the median time from symptom onset to diagnosis was 32 days (range, 2 to 263 days) (251).

Neurological disease. Headache, sometimes with vomiting, and neck stiffness are common neurological presentations (130, 198, 211, 252). Abnormal neurological signs or deficits may be present or can evolve during illness. These include cranial nerve deficits, visual and hearing impairment or loss, cerebellar abnormalities, local limb weakness, seizures, and abnormal mentation (e.g., confusion, personality change, and coma) (27, 208, 211). Chen et al. reported that 73 patients had CNS infection, 43 had abnormal neurological manifestations at presentation, 18 had impaired consciousness (Glasgow coma scale range, 6 to 14) and 13 had cranial nerve abnormalities (1 patient with VII nerve palsy, 7 with VI nerve palsies, and 5 with optic atrophy) (130) (Table 4). In a recent U.S. series of cases outside the Pacific Northwest, headache occurred in 67% and blurred vision occurred in 62% of patients (251). Contemporary studies have reported seizures in 5.8 to 8% of cases (130, 132).

Importantly, papilledema was present in 15 to 53% of patients in earlier studies (29, 211, 213) but in only 6% of cases in the U.S. outbreak and in 12.4% of cases in a recent Australian study (130). Optic disc swelling is typically present, with loss of definition of the disc margins. Hemorrhages are found around the disc in severe cases and are accompanied by a loss of visual acuity.

Pulmonary disease. Cough, dyspnea, chest pain, and hemoptysis are the most common symptoms of lung involvement, but asymptomatic pulmonary nodules or mass lesions (cryptococcomas) observed on chest X ray or CT scan (see Diagnostics, below) are not uncommon (130, 198, 240, 252). Very large lesions are typical of disease due to *C. gattii* (215, 231) and have been associated with Pancoast syndrome with respiratory stridor (205). Johannson et al. reported a patient with lung infection accompanied

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by systemic symptoms with an unproductive cough and dyspnea (250).

Clinical Manifestations in Children

Most children with *C. gattii* infection present with CNS involvement. In Brazil, symptoms of headache, fever nuchal rigidity, nausea, and vomiting were common (219, 221, 256). Cranial CT scans of 11 children with CNS disease revealed hypodense nodules in all patients, cerebral atrophy in 9, hydrocephalus in 6, and hydrocephalus with cerebral atrophy in 5 (220). Meningoencephalitis is also characteristic of most pediatric cases in PNG (218). Laboratory and treatment variables for outcome were cryptococcal species independent, but hospital stays were longer for children than for adults. Data from case reports and small case series in Brazil estimated the mortality rate to vary between 37.5 and 42.8% (256).

CLINICAL COMPLICATIONS OF HUMAN INFECTION

Neurological Complications and Sequelae

Compared with *C. neoformans* infection, neurological complications, including cranial nerve palsies, loss of visual acuity, blindness, seizures, ataxia, and focal long tract signs, have been frequently reported for disease due to *C. gattii* (27, 28, 211); significant disability can result. In one earlier report, 7 of 18 (39%) patients with *C. gattii* disease developed sequelae, including cranial nerve lesions, epilepsy, memory impairment, and visual field loss (28), while another report identified 8 of 26 (30.8%) patients with moderate-to-severe sequelae (27) (Table 4). In another study, 43 of 73 patients had a range of neurological complications at diagnosis, and of 63 survivors, 20 (31.7%) had persistent sequelae, including visual impairment ($n = 8$ patients; 3 became blind), deafness ($n = 3$), limb weakness ($n = 2$), and dysphasia ($n = 2$). The proportions of patients with concomitant CNS and lung infection or CNS infection with neurological sequelae were similar (24% versus 29%) (130). Cranial nerve palsies developed in 5 of 13 (38%) cases from the United States that were not associated with the outbreaks (251), but long-term sequelae (if any) from the *C. gattii* case clusters have not yet been reported. In a Vietnamese study, a large proportion of patients with cryptococcal meningitis had neurological sequelae (14 of 20; 70%); however, the frequency of sequelae was not specified for those with *C. gattii* infection (239). Whether there are species-dependent effects on neurological outcomes or whether these differences reflect differences between patients in seeking medical attention is uncertain.

Ocular complications of *C. gattii* infection are notable and include papilledema, especially in the presence of raised intracranial pressure (ICP) (see below). Although papilledema is the most common manifestation, blurred vision, visual field defects, extraocular muscle paresis, impaired pupillary/accommodative function, choroiditis, retinal hemorrhage, endophthalmitis, and optic atrophy have all been reported (27, 130, 208, 211) (Table 4). Visual loss is particularly disabling; in *C. neoformans* infection, it is thought to be due to direct invasion of the optic nerve by cryptococci or to the effects of raised ICP or from local arachnoiditis leading to choroidal infiltration with fungus (291, 292). The mechanism of eye involvement is likely to be similar in *C. gattii* infection. Visual loss has been reported in 1 to 9% of patients with *C. neoformans* infection (291, 293), much lower than the 18 to >50% of patients with blindness noted with *C. gattii* infection in PNG and Brazil, including children (208, 211, 219). These high rates have not been observed recently in Australia (28, 130). Chen et al. reported blindness in 3 of 73 patients, with significant visual impairment in a further 5 patients (130). Rates of visual sequelae in the North American case clusters have not been reported, but Harris et al. described papilledema in 9 of 49 patients with infection due to outbreak strains (132). In a later study of cases outside the Pacific Northwest, 8 of 25 patients reported blurred vision (251) (Table 4).

Elevated ICP (CSF pressure of ≥20 cm water) is common in *C. gattii* meningitis (50 to 60% of cases), as it is with *C. neoformans* (30 to 75%) (130, 210, 211, 282, 294). Early raised ICP was a poor prognostic indicator in PNG patients (208, 211). In a contemporary Australian study, although it was not a risk factor for mortality, elevated ICP predicted neurological sequelae and/or death at 12 months ($P < 0.05$) (130). Clinical manifestations, including severe headache, papilledema, loss of vision or hearing, other neurological deficits, and altered mental consciousness, are common, and death may result, presumably from cerebral ischemia (211, 291, 295). Cryptococcomas due to *C. gattii* have caused raised ICP or obstructive hydrocephalus via a mass effect (27, 296) but are not necessarily associated with measurably raised ICP (130). Patients with elevated pressures are more likely to present with abnormal neurological signs ($P < 0.05$) (130). Rapid diagnosis is the key to achieving good outcomes, and routine measurement of CSF pressures is essential whenever a lumbar puncture is performed (see Diagnostics, below).

Clinicians should also be vigilant regarding the presence or development of hydrocephalus, which is usually obstructive. Identification of dilated ventricles by cerebral imaging (CT or MRI) is required to establish diagnosis. Symptomatic patients with hydrocephalus present with ataxia and deteriorating mental status due to meningoencephalitis. Although the number of cases studied is small, the proportion of patients with hydrocephalus has ranged from 10.6 to 34.6% in Australia. In the largest series of 86 patients, hydrocephalus was present in 22 (30%) patients, typically at diagnosis, although in a small minority, it may not be detected for 2 to 12 months (130). Six of 11 children in Brazil with *C. gattii* CNS infection were diagnosed with hydrocephalus by cerebral imaging, while in one U.S. study, Harris et al. reported this complication in 4 of 18 patients (251). Notably, 25% of patients presenting with hydrocephalus had "silent" or unexpected findings of meningitis upon CSF examination (27). In *C. neoformans* infection, most patients with hydrocephalus have indolent meningitis (296). Factors that predispose patients with *C. gattii* meningitis to developing hydrocephalus remain unclear. As for *C. neoformans*, delays in diagnosis and treatment may lead to serious, permanent loss of cognitive function (282, 297). The management of raised ICP and hydrocephalus is discussed below (see Antifungal Therapy and Management, below).

Immune Reconstitution Inflammatory Syndrome

Immune reconstitution inflammatory syndrome (IRIS) is best studied in HIV-infected individuals with infections due to *C. neoformans*. In these patients, the development of IRIS is associated with immune reconstitution and typically presents with worsening of disease or recurrence in the same or new anatomical sites despite mycological evidence of effective antifungal therapy (reviewed in reference 298). IRIS has been described infrequently in association with *C. gattii* infection: occasional cases were reported

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in apparently healthy hosts in association with pregnancy and in immunocompromised individuals (216, 217, 241, 299). No cases were reported for 10 HIV-negative Vietnamese patients with *C. gattii* meningitis (239). However, in an Australian study, IRIS occurred in 9.4% of patients, two-thirds of whom had no underlying immunocompromise, although one patient was postpartum (299). In that study, IRIS was diagnosed when symptoms or radiological features consistent with inflammation worsened or appeared following a clinical and/or microbiological response to anticryptococcal therapy, and cultures were negative.

The pathogenesis of IRIS is incompletely understood. Clinical observations in patients with HIV/AIDS or organ transplantation indicate that it develops at the time of immune reconstitution or reduction in immunosuppression, respectively. Risk factors reported for HIV-infected patients include a poor baseline inflammatory response (indicated by low CSF white blood cell counts and protein levels and associated failure to sterilize the CSF rapidly) (300), rapid immune reconstitution from a low base, and a high organism or antigen burden at presentation (301) or at the time of initiation of antiretroviral therapy (ART) (302). The original hypothesis that IRIS results from dysregulation of the early Th2 (anti-inflammatory) and subsequent Th1 (proinflammatory) cytokine responses appears to be too simplistic, but dysregulation within the mononuclear phagocyte lineage is universal (303). Recent evidence from cases of HIV-associated IRIS suggests that IRIS results from the failure of a dysfunctional immune system to clear antigen during the early phase of immune recovery. This is followed by a supranormal proinflammatory response as immune reconstitution occurs during ART, which is not accompanied by appropriate effector responses (302, 303). Additional factors, such as cryptococcal genotype, may influence the development of IRIS (304). In healthy hosts, it is proposed that cryptococcal capsular polysaccharide induces an initial Th2 cytokine response, which is then replaced by a Th1 response during successful antifungal therapy, although the stimulus for an excessive response sufficient to cause IRIS in a small percentage of patients is unknown.

Risk factors for IRIS in *C. gattii* infection include female gender and initial presentation with brain involvement or brain, meningeal, and lung involvement. Notably, in that study, median CD4 counts were higher than those in patients who did not develop IRIS (299). Unlike in HIV-positive patients, neither initial CSF cryptococcal antigen (CRAG) titers nor CSF leukocyte counts and protein levels differed from those in patients who did not develop IRIS (299, 300). However, since patients with *C. gattii* who subsequently developed IRIS all presented with cryptococcomas in the brain, CSF parameters likely did not reflect the fungal load (or immune response) in this site.

In patients with *C. gattii* infection, IRIS develops following an initial clinical and microbiological response, 4 weeks to as long as 12 months after initiation of antifungal drugs (299). The clinical presentation ranges from headache or drowsiness to new or recurrent neurological manifestations, including blindness, ataxia, dysarthria, deafness, hemianesthesia, seizures, and falls. Neuroimaging studies reveal new or worsening inflammatory mass lesions, meningeal enhancement, hydrocephalus, or increased CSF inflammation. In the largest study to date, all patients presented with enlarging or new brain lesions, typically with surrounding edema; occasionally, patients also developed pulmonary lesions with or without subcarinal lymphadenopathy (299).

The recognition of IRIS is important, as it can be misdiagnosed as clinical failure and can lead to unnecessary reinduction antifungal therapy. In a series by Chen et al. (299), three of eight patients also received gamma interferon (IFN) for presumed failure of antifungal therapy. The use of gamma interferon in this setting carries a theoretical risk of exacerbating the severity of IRIS due its proinflammatory effect. Treatment of IRIS is discussed below (see Antifungal Therapy and Management, below).

Mortality from *C. gattii* CNS Cryptococcosis and Prognostic Determinants

Mortality data are summarized in Table 4. The mortality rate was high in early studies of *C. gattii* meningitis in PNG (22.4 to 43%) (210, 211, 213) and remains so, especially if diagnosis is delayed (218). In Brazil, the mortality rate has ranged from 18.2 to 50% between studies (219, 221, 248, 256), and in the Northern Territory of Australia, it has been as high as 33% (215). Lower death rates of 0 to 15% were reported in three other Australian studies conducted between the late 1980s and 2007 (27, 28, 299). In a recent Vietnamese study, 1 of 10 *C. gattii*-infected patients died (239). Mortality is likely to be influenced not only by patient factors but also by medical awareness and access to health resources, which vary between countries. The mortality rate in predominantly HIV/AIDS patients in Africa ranged from 17 to 35.6% (96, 98). In the outbreak setting, mortality has likewise varied substantially: in British Columbia, 8.7% of patients died, compared to 20 to 33% in the Pacific Northwest (132, 198, 252).

Determinants of mortality and patient outcomes, although well described in immunocompromised patients infected with *C. neoformans*, are less well defined in *C. gattii* infection. Seaton et al. studied 88 patients in PNG with *C. gattii* meningitis and found that the mortality rate was higher in men ($P = 0.025$), older patients ($P = 0.39$), those with altered consciousness ($P < 0.001$), and those with a history of convulsions prior to commencement of therapy ($P = 0.002$) (210). In a more recent study of 86 patients, immune compromise was associated with an increased risk of death by univariate analysis. Initial CSF CRAG titers of ≥256 independently predicted death and/or neurological sequelae (130); unlike *C. neoformans* infection in HIV-infected hosts (305), a high cryptococcal load (serum CRAG titer, ≥512) and abnormal neurological findings at presentation were not associated with increased mortality (130).

DIAGNOSTICS

Imaging

Imaging of the lungs, brain, and other clinically appropriate sites is essential for *C. gattii* infections because the type and duration of induction and total antifungal therapy are site dependent. Furthermore, surgical resection of selected mass lesions or CSF shunting may be required.

Lung infection. Imaging of the chest may provide the first clue to the presence of cryptococcal disease, which most commonly manifests as one or more circumscribed cryptococcomas (Fig. 6). Pulmonary alveolar or interstitial infiltrates caused by *C. gattii* genotype VGI are uncommon in areas of endemicity such as Australia (130) and accounted for 14 to 17% of imaging abnormalities

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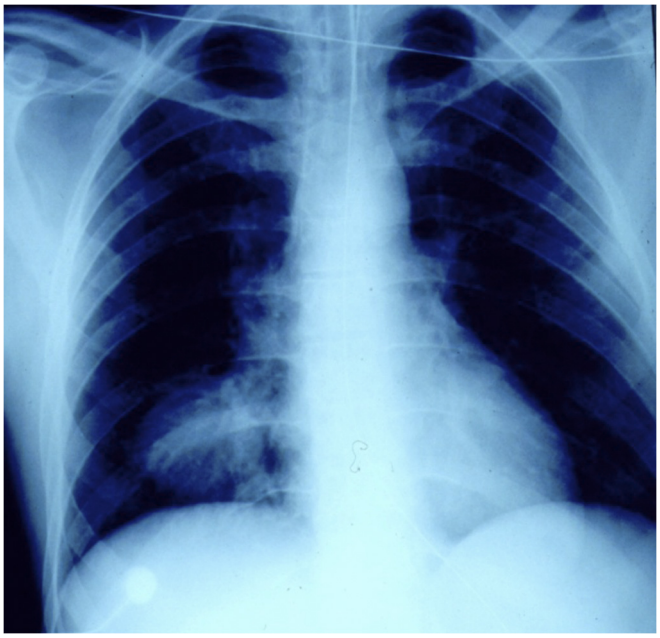

FIG 6 Chest X ray of a patient with a large mass lesion in the right lower lobe. Bronchoscopy and collection of bronchoalveolar lavage fluid showed multiple encapsulated yeasts upon cytological examination, and a culture grew *Cryptococcus gattii*. (Reprinted from reference 388 with permission of the publisher. Copyright 2013 UpToDate, Inc. [www.uptodate.com].)

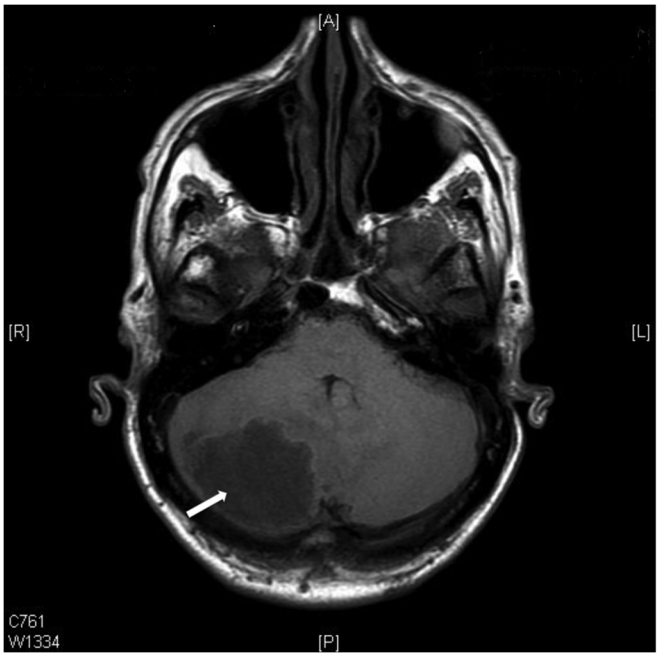

FIG 7 Computerized tomographic scan of brain showing a large mass lesion in the left cerebellar hemisphere (indicated by arrow). A provisional diagnosis of a cerebral neoplasm was made. Excision biopsy of the lesion showed encapsulated yeasts, and *Cryptococcus gattii* was isolated in culture.

in the British Columbian VGII outbreak (198). In contrast, in patients immunosuppressed by HIV or other causes, alveolar and interstitial pulmonary infiltrates account for ≈70% of lung lesions.

Lung cryptococcomas are more often due to *C. gattii* than to *C. neoformans*, both in patients with isolated lung infection (odds ratio [OR], 12.2; 95% confidence interval [CI], 3.3 to 46; $P < 0.001$) and in those with combined lung and CNS disease (29, 130, 215). In British Columbia, 75% of patients had pulmonary nodules (198) of various sizes, compared with 33% in the Pacific Northwest (132). In a smaller U.S. study, cryptococcomas were present in 14/23 (61%) patients (251). Cryptococcomas can appear in any region of the lung and have ranged from 1 to 8 cm in diameter in a population-based survey (our unpublished data), although larger lesions (up to 10 cm in diameter) have been reported (231, 243). Uncommonly, pleural effusions, abscess, cavitation, and lymphadenopathy are observed, and one report described a patient with Pancoast syndrome associated with opacification of the entire upper lobe (205, 251). Both cryptococcomas and lung infiltrates can be present in the same patient (29, 132).

Neurological infection. (i) Computerized tomography. Cerebral imaging is required to exclude brain involvement, which may manifest as cryptococcomas, hydrocephalus, or abnormal foci of reduced attenuation, contrast enhancement, and edema. Mass lesions often resembling "abscesses" have been reported, most commonly in the basal ganglia, thalamus, and cerebellum, in cranial CT scans in 33 to 58% of cases of *C. gattii* disease (27–29, 130, 251) (Fig. 7). In one study, cerebral cryptococcomas were more common in patients with *C. gattii* infection than in those with *C. neoformans* infection but not when the analysis was confined to immunocompetent hosts (29). In contrast, concurrent pulmonary and cerebral cryptococcomas and hydrocephalus were more frequently associated with *C. gattii* independent of host status (27, 29). Correa et al. reported hypodense nodules on cerebral CT scans, often in the basal ganglia, in all 11 immunocompetent children studied (220). Obstructive hydrocephalus has been described in 17 to 50% of *C. gattii* infections (27–29, 130, 251).

(ii) Magnetic resonance imaging. MRI technology is more sensitive than CT scans, especially for detection of small lesions. Cryptococcal infection spreads from the basilar cisterns, causing dilatation of the perivascular (Virchow-Robin) spaces as a result of accumulation of cryptococci and associated capsular material along the "walls" of these spaces. This abnormality is readily seen on MRI scans, most commonly near the basal ganglia. It is present in almost 50% of cases of HIV-associated cryptococcosis and is a diagnostic clue in such patients (306, 307). MRI scans are useful for delineating basilar meningeal enhancement after injection of gadolinium.

(iii) Newer techniques. Nuclear magnetic resonance (NMR) spectroscopy is a technology that can be linked to MRI to identify the location of cryptococcomas 2 cm in diameter or more and their metabolic "fingerprint" in a single examination. This approach is already established for noninvasive diagnosis of brain tumors and pyogenic abscesses (308). By generating data through computerized pattern recognition methods, based on the chemical composition and metabolite profiles of various human and/or microbial cells, NMR spectra provide information on the content of a specific abnormality or lesion. Experimental studies showed that uniquely among pathogenic fungi, cryptococci cultured *in vitro* are characterized by an abundance of the disaccharide α,α-trehalose, which can therefore be used as a marker that contributes to their identification *in vivo* (309–311). *In vivo*, it has been dem-

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onstrated that identification of cerebral cryptococcomas and differentiation from cerebral tumor are feasible, although the acquisition of spectra from more cases is required to achieve an unambiguous diagnosis (308).

Microbiological Diagnosis

Conventional and antigen-antibody-based tests. Almost all cases of cryptococcosis are readily diagnosed by visualization of the organism and its recovery in culture from appropriate clinical specimens and/or a positive serum or cerebrospinal fluid (CSF) cryptococcal antigen (CRAG) titer. Culture remains essential for definitive diagnosis since CRAG detection does not distinguish between *C. neoformans* and *C. gattii*.

C. gattii can be cultured from any body site. To diagnose lung infection, bronchoalveolar lavage (BAL) fluid, fine-needle aspirates, or lung biopsy sections are the preferred specimens, but sputum cultures can also be useful. Positive sputum cultures are indicative of invasive disease with few exceptions. As CNS disease is common, CSF should always be examined for cryptococci unless lumbar puncture cannot be performed; biopsy of other affected tissues may also be indicated. Positive blood cultures indicate disseminated disease, but as discussed above (see Clinical Manifestations of Human Infection, above), the frequency of recovery of *C. gattii* from blood is not known. Clinical specimens should be sent for both culture and histopathological examination.

(i) Histopathological stains. Hematoxylin-and-eosin stains will detect cryptococci in tissue sections, although Gomori methenamine silver and calcofluor white are specific for the fungal cell wall. Mucicarmine stains the cryptococcal polysaccharide capsule in tissue samples, revealing organisms as bright carmine red structures with a spiny, scalloped appearance; the capsule can also be stained by using alcian blue and periodic acid-Schiff (PAS) stain. Cryptococci contain melanin in their cell wall (see Pathogenesis, above), which, upon staining with the Fontana-Masson reagent, appears brown to black. The use of more than one stain improves the sensitivity of detection of cryptococci in tissue samples. Either mucicarmine or alcian blue combined with Fontana-Masson stain is superior to any of these stains alone; the combination has the advantages of highlighting both the cell wall (Fontana-Masson stain) and mucin-positive capsule (mucicarmine/alcian blue) and of identifying rare capsule-deficient strains (summarized in reference 312).

(ii) Other stains. India ink staining is a cheap and simple diagnostic test for CSF samples, becoming positive in <5 min. Cryptococci appear as encapsulated 5- to 7-μm-diameter structures with a halo, against a black background. The presence of budding cells distinguishes cryptococci from leukocytes. CSF India ink tests were positive in 70 to 95% of patients with *C. gattii* meningitis in three studies (29, 130, 211). It is a sensitive test in patients with *C. gattii* meningitis, in contrast to *C. neoformans* cryptococcosis, where the sensitivity is lower in HIV-negative patients (30 to 72%) than in those with AIDS (80%) (312, 313).

(iii) Culture. Cryptococci grow readily on bacteriological agar (for example, blood agar) and standard mycological culture media such as Sabouraud's dextrose agar (SDA), Mycosel agar (BBL, Becton Dickinson, USA), and brain heart infusion agar. Enriched media, for example, brain heart infusion agar, may improve recovery. Cryptococci grow selectively on bird seed agar, which differentiates *C. neoformans-C. gattii* (brown colonies are formed due to melanin production) from other *Cryptococcus* species but which is time-consuming to prepare (266). Although two other cryptococcal species form brown colonies on bird seed agar (*Cryptococcus podzolicus* and *Cryptotrichosporon anacardii*), neither are human pathogens (314, 315). CGB agar reliably distinguishes between *C. gattii* (colonies produce a blue pigment) and *C. neoformans* (no change in colony color); the blue color results from the utilization of glycine, which releases ammonia, causing alkalinization and, hence, the blue color and susceptibility to canavanine (23, 266). True false-negative tests for *C. gattii* (the medium does not turn blue even after several days) are uncommon. Very recently, *Candida famata* was noted to give a "false-positive" blue color on CGB agar (316). Although it is unlikely that *Candida* strains would be mistaken for *C. gattii* or vice versa, laboratory scientists should be vigilant to carefully inspect and test all colonies that are suspected to be *C. gattii*.

(iv) Cryptococcal antigen. CRAG has long been detected by incubating specific rabbit anti-*C. neoformans* antiserum with CSF and/or serum specimens. The test can be quantified (expressed as a titer) and is rapid (2 to 3 h). It is a sensitive, specific, and accurate diagnostic test for cryptococcosis in experienced hands.

There are two common commercial CRAG test formats: the latex agglutination test (LAT) and the enzyme-linked immunoassay (EIA). In general, agreement between the LAT and EIA results is excellent (92 to 98%) (summarized in reference 312). As the antibody used in these tests was developed from a strain of *C. neoformans*, in theory, occasional false-negative results may result from a lower affinity of the antibody for *C. gattii*. In clinical practice, the sensitivity of the test for *C. gattii* infection remains high, including for HIV-positive patients—90% for lung disease, with 87 to 100% sensitivity for CSF CRAG tests (29, 98, 130, 239). The level of detection of most LAT kits is at least 10 ng of polysaccharide/ml. On balance, the EIA is slightly more sensitive than the LAT (312), but it requires a plate reader and is more expensive.

Based primarily on studies of *C. neoformans* infection, false-negative results of the LAT are uncommon. They may result from prozone effects (where the amount of antigen in the sample is in excess of the amount of antibody in the assay mixture) or, occasionally, from a low organism burden or infection due to rare, capsule-deficient strains (131, 266, 317). In clinical practice, false-positive results are also uncommon but may be caused by *Trichosporon beigelii*, *Stomatococcus mucilaginosus*, *Klebsiella* spp., and *Capnocytophaga canimorsus* and by the presence of rheumatoid factor, collagen vascular disease, malignancy, and agar syneresis fluid (266, 312, 318). To minimize false-positive results, pretreatment of specimens with heat and proteolytic enzymes (pronase) is recommended, as is inclusion of a control reagent containing latex beads coated with normal rabbit globulin (319). Inclusion of IgM monoclonal antibody (MAb) effectively eliminates reactions with rheumatoid factor. CRAG testing by EIA does not require enzyme pretreatment of samples, nor is it associated with false-positive results in sera containing rheumatoid factor (320).

Low titers of CRAG may be found in the urine of patients with disseminated cryptococcosis and in bronchoalveolar lavage fluid and lung aspirates from those with pulmonary infection. These observations were based on small studies mainly in HIV positive patients (and, by inference, with *C. neoformans* infection) (321–323). The utility of CRAG testing of BAL fluid and urine in *C. gattii* infection is untested.

Recently, a lateral flow immunoassay (LFA) for CRAG (Immuno-

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Mycologics [IMMY], Norman, OK) was developed as a simple, rapid (10- to 15-min), inexpensive, point-of-care test that requires no sample preparation. Kits can be stored at room temperature. Each test contains an immunochromatographic dipstick-like strip impregnated with MAbs directed against epitopes on glucuronoxylomannan (GXM) of the four major serotypes of *C. neoformans* and *C. gattii* (324). Experience using the LFA to diagnose *C. gattii* infection is limited; in one study, CSF and serum specimens from four patients with *C. gattii* infection returned positive tests by the LFA, with no false-negative results (325). Testing of CSF/sera from subsequent *C. gattii* patients in the same laboratory has demonstrated no false-negative results (S.C.-A. Chen, unpublished data). The LFA is likely to perform similarly in *C. gattii* infection and *C. neoformans* infection (see below), but further evaluation of its utility in the diagnosis of *C. gattii* infections is needed, especially in patients with extra-CNS or lung infections.

The LFA has been extensively evaluated in *C. neoformans* infections, with excellent results. In two evaluations of its performance against culture and EIA, in HIV-positive patients with and without *C. neoformans* meningitis, LFA sensitivities when performed on serum were 90 to 99.8% (sensitivities of 70 to 92% for urine and 96 to 100% for CSF). Agreement between CRAG EIA values and LFA results was high (>93%), and dipstick titers correlated strongly with EIA results (326, 327). In another study of predominantly HIV-negative patients ($n = 26$) with CNS and lung cryptococcosis, the sensitivity of the LFA using both serum and CSF was 100%, with no false-negative results (325), but the correlation between LFA and LAT titers was poor ($r = 0.85$). Some LAT-negative samples had low LFA titers, and similar results were obtained in another study (324). Data from HIV-infected Ugandan patients showed that the LFA sensitivity for CSF specimens is higher than that of the LAT; likewise, Kabanda et al. and Rajasingham et al. reported both a sensitivity and specificity of 100% in their cohorts of HIV/AIDS patients (328, 329).

Until more data are available, titers obtained by the LFA and LAT should not be directly compared, and comparison of titers in the same patient should always be made by using the same test.

In contrast to their diagnostic value, serum or CSF CRAG titers are not helpful in monitoring the response to therapy, as the correlation between changes in titer and clinical progression or mycological responses is poor. Titers remain elevated for prolonged periods (months to >1 year) (130). Hence, CRAG results should always be interpreted in conjunction with clinical assessment. In one Australian study, CSF CRAG titers of ≥256 predicted death and/or neurological sequelae at 12 months after initiation of antifungal therapy (130). More data are required to support (or refute) this finding, which contrasts with observations of *C. neoformans* meningitis, where both high pretreatment LAT titers (≥512) and, more recently, LFA titers (≥1,280) were predictive of adverse outcomes (328, 330).

The place of the LFA in cryptococcosis diagnosis is yet to be clarified. Currently, it is recommended for use on serum, plasma, or CSF for diagnosis of cryptococcosis in symptomatic patients with established infection. Further evaluation of its utility in the diagnosis of *C. gattii* infections is needed, especially in patients with infection outside the CNS or lung. It is likely that the LFA will occupy an important role given its simplicity and accuracy thus far.

Globally, screening of asymptomatic patients for cryptococcosis in populations with a high prevalence of cryptococcal antigenemia has become a priority with endorsement by the World Health Organization. In countries with a high prevalence of HIV infection, the strategy of screening for antigenemia by the LFA and the use of targeted preemptive therapy is cost-effective; this may also be so in resource-rich countries (329, 331). Costs, including labor, shipping, and overheads, range from US$2.50 to US$5.50 per test in resource-poor settings to US$10 in higher-income countries (331).

Nucleic acid-based tests. In clinical practice, molecular methods are seldom required to confirm the diagnosis of suspected cryptococcosis. Nevertheless, several assays have been evaluated for the rapid and sensitive detection of *Cryptococcus* in clinical specimens and to distinguish between *C. neoformans* and *C. gattii*. These assays may be useful when the organism does not grow, for example, where the fungus is visualized in tissue biopsy sections but is not cultured, or if a patient has had substantial anticryptococcal antifungal therapy. In the uncommon situation where phenotypic methods provide an ambiguous result, DNA sequencing of isolates for species assignment is helpful, but multiplex PCR and other molecular methods can also be used. Inclusion of primers specific for *Cryptococcus* in multiplex assays based on syndromic clusters (for example, "septicemia" and "CSF infection") or for analysis of blood cultures or biopsy samples where the differential diagnosis includes fungal disease has been useful in some laboratories (C. Halliday and S.C.-A. Chen, unpublished data).

(i) Detection of *C. gattii* directly from clinical specimens. PCR-based and non-PCR-based molecular techniques have demonstrated good sensitivity in identifying *C. gattii* in clinical specimens. Methods include single or multiplexed PCR assays, which may be nested or real time in format (332). Most approaches have exploited nucleotide polymorphisms in the ITS region or the large ribosomal subunit of the fungal rDNA complex, although other gene targets (e.g., *URA5*, *CAP59*, and M13) have also been used (see Taxonomy of the Species *C. gattii*, above). Positive broad-range PCR assays can also be followed by DNA sequencing of the amplicon.

(ii) Panfungal PCR. Many panfungal PCR assays target the multicopy fungal rRNA gene, for example, the ITS region, to identify fungi. Such assays have detected DNA from members of the *C. neoformans* complex directly from clinical specimens, including CSF (333), tissue specimens (334), and blood (335). For laboratories choosing this approach, the entire ITS region should be amplified and sequenced to allow identification of *C. gattii* to the species level; in addition, querying the unknown sequence should be performed against a database which distinguishes *C. gattii* from *C. neoformans* (see below).

(iii) Nested PCR. Nested PCR assays have successfully detected cryptococcal DNA in clinical specimens and have been validated for *C. neoformans*, but their utility in detecting *C. gattii* is largely untested. Rappelli et al. developed a nested ITS-targeted PCR protocol for detecting *C. neoformans* as well as *C. gattii* (serotype B) from CSF samples from patients with neurocryptococcosis (336). All 21 CSF samples from HIV/AIDS patients with confirmed cryptococcal meningitis yielded positive results. The analytical sensitivity of the assay was 10 cells/μl, and the specificity was 100%. Nested PCR has also been employed to detect *C. neoformans* in brain tissue from infected mice (337) and in the diagnosis of patients with primary lymphonodular abdominal cryptococcosis (338).

(iv) Real-time PCR. Real-time PCR is attractive since it is rapid

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and provides much higher assay sensitivity without compromising specificity. Bialek et al. showed that real-time PCR was faster than nested PCR for the detection of *C. neoformans* in the brains of experimental mice (337). Amplification of the 18S and 28S rRNA genes using real-time PCR has been successfully employed to detect *C. neoformans* directly from pericardial fluid (339). Using a TaqMan real-time assay, other investigators showed that seven clinical samples that were culture positive for *C. neoformans* and *C. gattii* were confirmed to be positive by the real-time assay, with no false-negative, or false-positive, results (340).

The utility of panfungal, nested, and real-time assays to distinguish *C. neoformans* from *C. gattii* is uncertain, and where this is not possible, the organism should be identified as "*C. neoformans* complex."

(v) DNA sequencing as an identification method. As for other fungi, DNA sequencing has been considered to be the gold standard for accurate species identification. Apart from its high level of reproducibility and objectivity, data generated by sequencing methods are deposited in public central repositories and are readily accessible. It should, however, be noted that there are errors within unrefereed public gene databases, collectively comprising the International Nucleotide Sequence Database (INSD), containing GenBank (http://www.ncbi.nlm.nih.gov/); the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/); and the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/). Error rates for fungal sequences within GenBank, for example, can be as high as 14 to 20%. The commercial MicroSeq D2 DNA sequencing kit system (Applied Biosystems, Rotkreuz, Switzerland) is unable to distinguish *C. neoformans* from *C. gattii*. However, the two species can be identified by using curated databases containing ITS sequences for pathogenic fungi, available through the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (which also contains a proportion of GenBank data associated with CBS strain numbers) (www.cbs.knaw.nl/collections/) and at the University of Sydney, Australia (http://its.mycologylab.org/).

In addition to the ITS gene region, other cryptococcal genes may have utility for the identification of *C. gattii* (discussed in detail in Taxonomy of the Species *C. gattii*, above). While acknowledging the limitations of ITS sequence analysis, the ITS region remains one of the more informative regions for species identification. Sequencing of both the ITS1 and ITS2 (including the 5.8S rRNA gene) regions is required for species identification of *C. gattii*. Using this approach, Katsu et al. accurately differentiated members of the *C. neoformans* complex (341). ITS sequences delineated genetic polymorphisms that allowed the distinction of *C. neoformans* from *C. gattii*; furthermore, species-specific ITS "sequence types" correlated with PCR fingerprinting/random amplification of polymorphic DNA (RAPD) molecular types, and ITS sequencing was able to assign subtypes within *C. gattii*. In contrast, sequencing of the ITS1, ITS2, or D1/D2 amplicons alone identified *C. neoformans* complex isolates only as "*C. neoformans*" (334, 342). ITS sequence analysis is especially useful when combined with sequencing of other genes, for example, in MLST (see Taxonomy of the Species *C. gattii*, above). Even with automation of sequencing, proofreading of the sequences still constitutes a significant task in a busy, high-throughput laboratory.

(vi) Multiplex PCR. Multiplex PCR has the advantage of simultaneously detecting a number of gene targets and can be performed quickly with a small amount of DNA compared to conventional PCR. It allows rapid identification to the species level and can distinguish between serotypes by using different combinations of primers. Assays can be coupled with real-time PCR (343). Multiplex PCR has also been used to determine mating-type profiles through the amplification of *STE* (sterile) gene sequences of *C. neoformans*, although there are no similar reports for *C. gattii* (344, 345). Leal et al. described a multiplex PCR protocol based on amplification of the ITS region with species-specific primers. The results allowed rapid differentiation between isolates of *C. neoformans* and *C. gattii*, and the PCR assay showed specificity and sensitivity comparable to those of the Crypto Check serotyping kit (Iatron Laboratories; no longer available commercially) and CGB agar (343). Recently, Feng et al. developed a uniplex assay for the rapid identification of *C. neoformans* and *C. gattii*. The assay targeted the putative sugar transporter (*STR1*) gene; amplified PCR fragments sizes for *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* were 274 bp and 224 bp, respectively, while that for *C. gattii* was 170 bp (346). The identity of all amplicons was confirmed by DNA sequencing of representative strains. By PCR analysis, 253 of 255 (99.2%) isolates of the *C. neoformans-C. gattii* complex were correctly assigned to the respective species, including serotype AB, AD, and BD hybrid strains. Subsequently, those same authors developed a duplex PCR assay to differentiate isolates within the species *C. gattii* (346). Whether this technique detects *C. gattii* from clinical samples has not yet been tested.

(vii) Isothermal amplification method. Isothermal amplification approaches, including hyperbranched rolling-circle amplification (RCA), targeting single nucleotide polymorphisms (SNPs) in the ITS region, have also successfully identified *C. gattii* (347). This technology uses circularizing padlock probes of approximately a 50- to 100-mer length that contain target complementary sequences by a linker. Specialized DNA polymerases are employed under isothermal conditions to replicate short single-stranded DNA circles. This technique is simple, robust, and highly specific, detecting differences in target nucleic sequences down to the single-nucleotide level (348). It can be performed within 2 h by using a water bath, heating block, or thermocycler. By using real-time PCR (RT-PCR) systems, an RCA signal is detected within 15 min of initiation of the RCA reaction. Studies examining fungal identification with this method have reported good results with cost-effectiveness in comparison with DNA sequencing (349). Based on this approach, Kaocharoen et al. (347) designed four padlock probes targeting cryptococcal species-specific SNPs. The assay was validated with 99 *C. neoformans* complex isolates; it clearly distinguished between *C. neoformans* and *C. gattii*. However, false-positive "*C. neoformans* var. *grubii*" signals were obtained when an RCA3-ITS cryptococcal probe was challenged with certain *Candida* species, including *Candida albicans*. Hence, careful design of padlock probes is essential for specificity. Further evaluation of RCA as a technique for identification of *C. gattii* is required.

(viii) Probe-based microarrays and the Luminex bead suspension array. The position of probe-based microarrays in fungal identification is currently limited by the high cost of microarray technology, limited platform flexibility, slow hybridization kinetics, and the need for strict optimization of test parameters to achieve reproducibility (for methodological details, see reference 312). Nonetheless, a variation of this assay format, the high-throughput Luminex x-map technology system (Luminex Corp., Austin, TX), has been evaluated for its ability to identify both *C.*

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neoformans and *C. gattii*; the assay is based on detecting gene polymorphisms within the intergenic spacer (IGS) regions. This system employs flow cytometry principles to detect and identify DNA targets. It detects 5′-biotin-labeled PCR amplicons hybridized to specific probes of the target species and can utilize a direct DNA hybridization or solution-based enzymatic reaction capture format. As its name suggests, specificity is achieved by the use of uniquely color-coded beads or microspheres to capture "species-specific" combinations with red/infrared fluorescence, and it is capable of detecting up to 500 different analytes (representing up to 100 species) in a reaction tube/well. By using direct hybridization in the Luminex system, Diaz et al. described an 8-plex hybridization array for the simultaneous detection of species, varieties, and genotypes within the *C. neoformans* complex, which allowed discrimination of 1-bp mismatches with no apparent cross-reactivity (350). This assay was subsequently validated with 58 clinical and environmental isolates, and isolates were correctly identified to the species and genotype levels, including hybrids (serotypes AD and BD). Furthermore, the assay identified *C. neoformans* directly from stored CSF samples from eight patients with cryptococcal meningitis, although none of the samples evaluated contained *C. gattii* (351). Because of their potential for automation, bead-based technologies show promise for DNA-based diagnostics. Their current high acquisition costs restrict their wider use.

Proteomic approaches. In parallel with advances in proteomic approaches in diagnostic microbiology, methods such as matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) have been evaluated for the identification of *Cryptococcus* spp. (57, 58, 352). In the largest evaluation to date, 81 clinical and environmental *C. gattii* isolates representing each of the four major molecular types (VGI, VGII, VGIII, and VGIV) were tested. Protein extracts were obtained by a standard formic acid extraction method (summarized in reference 353). MALDI-TOF MS using the Bruker Microflex system (Bruker Daltonik GmbH) and MALDI Biotyper software (version 3.0) correctly identified 100% of *C. gattii* isolates and correctly grouped each isolate according to its molecular type; furthermore, the technique was able to identify hybrids of *C. neoformans* and *C. gattii*. It is important to note that those authors first established reference spectra of all four molecular types by using well-characterized reference strains and subsequently added all spectra generated to an "in-house" supplementary MALDI Biotyper library (2011), thus extending a validated spectral library (57). Head-to-head comparisons of MALDI-TOF MS with traditional genotyping methods are essential in positioning MALDI-TOF MS as a typing tool.

In another study, McTaggart et al. compared MALDI-TOF MS analysis with DNA sequencing to identify *Cryptococcus* species. They studied 27 strains of *C. gattii* (23 clinical and 4 reference strains) and compared spectra against the MALDI Biotyper version 2.0.1 database (Bruker Daltonik). When the Bruker library was supplemented with in-house-generated spectral entries, all 27 strains were correctly identified to the species level (score, ≥2.0). However, when queried against the existing Bruker library, a substantially smaller proportion of isolates was identified. If *C. neoformans* plus *C. gattii* isolates were taken into consideration, only 58.4% of isolates were correctly assigned (352). One other evaluation, which also complemented the Bruker library (version 3.0) with in-house spectra, yielded correct species identification for all 10 strains studied (58). Very few studies have tested the ability of the current Bruker library alone to identify *C. gattii*. In a study in which only three isolates of *C. gattii* were encountered, two isolates yielded scores of ≥2.0, and one yielded scores of ≥1.7 to 1.99 (354). Ongoing construction of libraries containing reliable spectra from known isolates is essential. Until these extended libraries are more widely available, identification of *C. gattii* should not be reliant only on MALDI-TOF MS.

There is ongoing debate about the requirement for extraction of yeast protein for MALDI-TOF MS. A review of published studies (beyond the scope of this article) indicates that the use of the extraction procedure recommended by the manufacturer yields optimal and reproducible spectra (353). With respect to *C. gattii*, Firacative et al. reported that no spectra were obtained from intact yeast cells (57). The number of colonies used and the time of incubation did not affect results. Whether MALDI-TOF MS can be successfully applied to detect cryptococci in clinical specimens such as CSF is uncertain. In a small study, 11 CSF samples were inoculated in blood culture bottles, cultured, and then analyzed by MALDI-TOF MS for bacteria and yeasts, but none of the patients had cryptococcal meningitis (355).

ANTIFUNGAL SUSCEPTIBILITY

On casual reading, there appears to be little value in routinely determining the susceptibility of *C. gattii* to antifungal agents, given that firm therapeutic recommendations based on MICs cannot be made due to the lack of clinical breakpoints (CBPs). Only *C. neoformans* has been included in Clinical and Laboratory Standards Institute (CLSI) guidelines for testing of yeasts (356). There are as yet no data to indicate that adverse patient outcomes are correlated with raised MICs and, if so, at what level.

However, antifungal susceptibilities have been used to guide antifungal treatment, especially in patients failing to respond to antifungal therapy. MICs, mainly of amphotericin B, 5-flucytosine, and fluconazole, which have been the cornerstone of anticryptococcal treatment for decades and determined by CLSI broth microdilution methods, have been reported for both *C. neoformans* and *C. gattii* (see below; see also Antifungal Therapy and Management, below). There are also *in vitro* data for the newer azoles voriconazole, posaconazole, and isavuconazole.

Epidemiological Cutoff Values for *C. gattii*

Prior to the recent *C. gattii* outbreaks, there was evidence that MICs of antifungal drugs varied with the geographic origin of the isolates, although many of the isolates tested were derived from individual institutional culture collections. Subsequently, MICs of fluconazole were noted to be increased among isolates from *C. gattii* VGII case clusters (see discussion below). This prompted an ongoing large-scale study of MIC CBPs and of epidemiological MIC cutoff values (ECVs) based on MIC distributions of wild-type strains (357–359). ECVs for fluconazole were determined in an international consensus study of a large number of clinical isolates from Europe, the United States, Australia, Brazil, Canada, India, and South Africa, *viz.*, 8 mg/liter for *C. gattii* molecular types VGI, VGIIa, and VGIII and nontyped strains; 16 mg/liter for molecular type VGIV; and 32 mg/liter for molecular type VGII (except for VGIIa). Although varying by genotype, the ECVs are within 1 to 2 dilutions of each other. Similar molecular type-specific ECVs have been reported for itraconazole, voriconazole, and posaconazole (358). Espinel-Ingroff et al. proposed that ECVs of 0.5 mg/liter for amphotericin B and 4 mg/liter for 5-flucytosine

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would encompass >99% and >95.7% of *C. gattii* strains, respectively (357). Lockhart et al. (359) also established azole ECVs for *C. gattii* based on MIC distribution, proposing an ECV of 32 mg/liter for fluconazole, but acknowledged that >50% of their isolates were from the Pacific Northwest (these isolates were predominantly of molecular type VGII [see below]). ECVs are useful adjuncts for identifying isolates with acquired or intrinsic resistance or those that need further study. Whether they can be universally accepted as surrogates for CBPs to define "susceptibility" or "resistance" is less clear (360, 361).

Antifungal Susceptibility

A number of studies have documented comparatively low MICs of standard antifungals against *C. gattii* (and in comparison with *C. neoformans*), which have not increased over time (29, 362–364); resistance to amphotericin B and 5-flucytosine was rare. Based on the fluconazole CBPs for *Candida* spp. reported in CLSI document M27-A3 (356), fluconazole "resistance" (MIC, ≥64 mg/liter) among *Cryptococcus* spp. was uncommon in Australia (29, 362). There are no data to suggest whether the new CBPs for *Candida* spp. (resistance defined by an MIC of ≥8 mg/liter [361]) are applicable to *Cryptococcus*. The apparently low frequency of fluconazole resistance appears to be the case in other regions, including Malaysia, India, Spain, and Taiwan (Table 5) (29, 365–368). In a Malaysian study, 2 of 48 (4.2%) isolates had fluconazole MICs of >64 mg/liter. In addition, there were no significant differences in MICs between *Cryptococcus* species ($P > 0.05$), indicating that *C. gattii* was as susceptible as *C. neoformans* var. *grubii*. Furthermore, no significant difference in the MICs for *C. gattii* isolates collected from 1980 to 1990 and 2002 to 2004 were observed (365). In a Brazilian study, eight *C. gattii* strains (fluconazole MIC range, 8 to 32 mg/liter) were susceptible to most drugs tested, and susceptibility profiles of *C. gattii* were similar to those of *C. neoformans* (369). Table 5 summarizes fluconazole susceptibility data from major clinical studies.

Nonetheless, there remains concern over whether *C. gattii* may be less susceptible to some antifungal agents. In a study from Taiwan, *C. gattii* was less susceptible to 5-flucytosine and amphotericin B (75), but the geometric means (GMs) of the MICs were still within the range considered "susceptible." A group in Brazil reported significantly higher GMs of MICs for fluconazole, voriconazole, amphotericin B, and 5-flucytosine against *C. gattii* than against *C. neoformans* (370); likewise, Torres-Rodriguez et al. (368) reported fluconazole, voriconazole, and posaconazole MICs to be significantly higher for *C. gattii*, although the overall prevalence of strains with fluconazole resistance was still low (range, 0.25 to 2 mg/liter for *C. gattii*) (Table 5). Similarly, Chowdhary et al. noted that *C. gattii* strains from India were less susceptible than *C. neoformans* var. *grubii* to fluconazole, itraconazole, and voriconazole ($P < 0.05$) but not amphotericin B and 5-flucytosine (367). Elevated MICs of amphotericin B and 5-flucytosine were rare in the United States (371).

Of note, there are data to indicate that fluconazole susceptibility, and, to some extent, those of the other azoles, varies with the molecular type of *C. gattii*. Elevated MICs against fluconazole (and voriconazole) have been reported from the Pacific Northwest, with genotypes VGIIa and VGIIc having the highest MICs (359, 371–374). In one study of 43 clinical isolates from the Pacific Northwest, molecular type VGIIc had the highest fluconazole GM MIC (12.7 mg/liter), while types VGI and VGII had the lowest GM MICs (2.38 mg/liter). Among the azoles, fluconazole had the least activity (GM MIC 16- to 63-fold lower for all isolates) (371). Lockhart et al. also noted that molecular type VGII strains had the highest fluconazole GM MICs (8.6 mg/liter), with molecular type VGI isolates exhibiting the lowest GM MICs (1.7 μg/ml) (359). In a study of 350 clinical (human and veterinary) and environmental isolates, Datta and coworkers reported that clinical VGII isolates had significantly higher GM MICs for flucytosine and fluconazole than did clinical VGI isolates; isolates from veterinary sources were the least susceptible to the azoles (374). In another large study of clinical, veterinary, and environmental isolates (375) (Table 5), most of which originated from Australia and Canada, *C. gattii* genotype VGII isolates also had significantly higher MICs for all azole antifungals, particularly fluconazole, than did isolates of other genotypes. Notably, the inclusion of large numbers of environmental isolates may skew results, since there are data to suggest that these isolates may be less susceptible to antifungal agents (367). In addition, comparison of MIC results between studies is complicated by the fact that many studies were done by using different MIC testing methods. Although the implications of higher fluconazole MICs for therapeutic dosing and clinical outcomes have not been determined, there is major interest in this issue since *C. gattii* genotype VGII is the genotype associated with the ongoing *C. gattii* outbreak in North America.

Several studies have documented that voriconazole, posaconazole, and isavuconazole are more active *in vitro* against *C. gattii* than fluconazole (359, 366, 371–374), and hence, they may represent valuable treatment alternatives to fluconazole. In a study of 90 clinical and environmental isolates of *C. gattii* from the Pacific Northwest of the United States, fluconazole MICs against *C. gattii* VGII clinical and environmental isolates were higher than those of isavuconazole, voriconazole, and itraconazole (374). U.S. and Spanish data revealed GM MICs of voriconazole of 0.1 mg/liter and 0.03 mg/liter, respectively (371, 376). In another study that tested 298 strains, the modal MICs were 0.12 mg/liter for voriconazole, 0.5 mg/liter for itraconazole, and 0.5 mg/liter for posaconazole, compared with a GM fluconazole MIC of 8 mg/liter (359). Thompson et al. tested 35 *C. gattii* strains by using the Etest (AB Biodisk, Solna, Sweden) and CLSI broth microdilution (overall agreement, 97.8%). GM MICs of isavuconazole were 0.027 mg/liter by broth microdilution and 0.03 mg/liter by Etest; the corresponding MIC_{90} values (the concentration of an antimicrobial agent at which 90% of the organisms are inhibited) were 0.06 and 0.125 mg/liter, respectively, indicating a high level of susceptibility. That same group of investigators also showed that isavuconazole had the lowest MIC_{90} and GM MIC values, with the next most active azole being posaconazole, followed by voriconazole and then itraconazole (363).

In summary, despite the fact that there are no data on the relationship between MICs, drug dosing, and clinical outcome, susceptibility testing may be helpful for patients failing to respond to apparently appropriate therapy, when a high MIC result may favor substituting an alternative drug. Susceptibility testing is recommended to monitor epidemiological trends.

Mechanisms of Azole Resistance in *C. gattii*

The emergence of *C. gattii* strains with elevated MICs of fluconazole has sparked interest in the mechanisms of azole resistance in this organism. A few studies have examined the roles of the cryptococcal *ERG11* gene and of the ATP-binding cassette transporters

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TABLE 5 Major clinical studies reporting antifungal susceptibility testing of *Cryptococcus gattii* against fluconazole[c]

Reference	Origin(s) of isolates	Total no. of isolates	Source (no.) of isolates	Modal MIC (mg/liter)[a]	MIC_{90} (mg/liter)	GM MIC (mg/liter)	MIC range (mg/liter)	% of isolates with MIC ≥ 32 mg/liter	Description
29	Australia	18	C	4	16	NS	0.5–64	11	Primarily VGI isolates
75	Taiwan	21	C	8	16	NS	0.125–16	0	*C. gattii* was less susceptible to AMB and 5FC than *C. neoformans*; all *C. gattii* isolates were FLU susceptible
403	South America	11	C	NS	NS	NS	8–16	0	High degree of susceptibility to AMB, 5FC, FLU, ITC
370	Spain	57	C (52)	NS	32	9.54	1–64	NS	*C. gattii* was less susceptible than *C. neoformans* to FLU but not AMB and 5FC
			E (5)						
368	Spain	30	C (mainly)	NS	NS	0.7–1	0.25–2	0	Overall low-level resistance; MICs for FLU were higher for *C. gattii* than for *C. neoformans* ($P = 0.007$)
404	Brazil	23	C	NS	25.6	15.52	4–>64	NS	All strains had low MICs of AMB (0.03–0.25 mg/liter); among azoles, POS had greatest activity, followed by VOR, ITC, and FLU
366	USA	35	C (NS)	NS	0.064	0.03	0.03–0.06	0	Highly susceptible to isavuconazole
			E (NS)		0.06	0.027	0.015–0.25	0	
363	Worldwide	42	C (NS)	NS	4	2.36	0.25–64		Few differences in susceptibility between *C. gattii* and *C. neoformans*
			E (NS)						
371	USA	43	C	4	16	6.38	0.5–32	0.05	VGII molecular type strains, especially those of VGIIc, had highest FLU MICs
375	Australia, USA, Canada	45	C (NS) and E (NS); study I	NS	8	3.54	1–8	0	*C. gattii* less susceptible than *C. neoformans*; strains of the VGII molecular type had significantly higher MICs for 5FC and all azoles, especially FLU
		103	Extended study	NS	8	4.96	1–32	NS	
373	Worldwide	350	C (215)	NS	8	3.573	0.25–64	NS	Veterinary isolates were least susceptible to ITC, POS, VRC, and ISA; MICs varied with molecular type for FLU and 5FC
367	India	62	C (2)		1.9	1.55	1–16	0	VGI; *C. gattii* less susceptible than *C. neoformans* to FLU, ITC, and VRC but not AMB and 5FC; E was less susceptible than C and more resistant than C
			E (60)	NS	8	8		0	
405	Brazil	13	C	NS	NS	NS	16–>64	15	VGII molecular type studied
372	Brazil	49	C (43) and E (6); VGII		16	6.08	0.25–>64	NS	VGII molecular type strains less susceptible to FLU, VRC, and 5FC than VGI strains
			VGI		4	1.55	1–8	0	
359	Global	298	C and E (NS)	8	16	5.51	0.5–32	20	Molecular type VGII strains had the highest FLU GM MICs, and molecular type VGI had the lowest (8.6 vs 1.7 mg/liter)
406	Brazil	54	C (50)	NS	16	NS	1–64	NS	Mainly molecular type VGII strains
			E (4)						
369	Brazil	8	C	NS	32	13.45	8–32	0	Susceptible to most drugs; no differences in susceptibility between *C. neoformans* and *C. gattii*; ECV = 32 mg/liter
374	Canada, USA	90	C (75)	4	8	4.5	2–64	NS	Molecular type VGII studied, of which types IIb and IIc were less susceptible
			E (15)						
376[b]	Brazil	24	NS	≤1	4	NS	<0.12–32	3	3 isolates with MIC of 32 mg/liter; no molecular type stated

[a] In some instances, calculated by the authors.
[b] Measured by flow cytometry.
[c] Abbreviations: AMB, amphotericin B; C, clinical strains; E, environmental; 5FC, 5-flucytosine; FLU, fluconazole; GM, geometric mean; ITC, itraconazole; ISA, isavuconazole; POS, posaconazole; NS, not specified; VRC, voriconazole.

in resistance in *C. neoformans* (377–380); whether these observations are applicable to *C. gattii* are uncertain. Mutations in, or overexpression of, the *ERG11* gene, encoding the azole target lanosterol 14-α demethylase, and overexpression of one or more plasma membrane proteins that pump azoles out of the cell are well recognized mechanisms by which fungi acquire resistance to azoles (379).

Rodero et al. studied five sequential *C. neoformans* isolates recovered from a patient with recurrent cryptococcal meningitis; four were fluconazole susceptible (MICs, 1.5 to 2 mg/liter), while the fifth isolate, associated with clinical relapse, was considered resistant (MIC, 32 mg/liter). The latter isolate contained a point mutation responsible for the amino acid substitution G484S (377). However, the possibility of another mechanism of resistance was not excluded. Sionov et al. identified a single missense mutation in a fluconazole-resistant strain of *C. neoformans* resulting in the replacement of tyrosine by phenylalanine at amino acid position 145 (Y145F); this conferred resistance to fluconazole and voriconazole but not itraconazole and posaconazole (378). Most recently, the role of *ERG11* mutations in conferring azole resistance in *C. gattii* was studied in 25 U.S. strains with relatively high MICs of fluconazole (GM MIC of 20 mg/liter and MIC_{90} of 64 mg/liter) and 34 isolates with lower MICs (GM MIC of 9.9 mg/liter and MIC_{90} of 32 mg/liter). Amino acid substitutions in the deduced Erg11p sequences of strains with higher fluconazole MICs were not associated with differences in the susceptibilities of these proteins to inhibition by the azole (381). This conclusion was supported by experiments determining azole MICs for conditional *Saccharomyces cerevisiae erg11* mutants expressing variant gene sequences. In comparison to the "wild-type" isolates, azole MICs were all within 1 to 2 dilutions of each other. Gast et al. also compared *ERG11* mRNA levels in the *C. gattii* strains; *ERG11*/*ACT1* mRNA ratios did not correlate with fluconazole MICs. They concluded that neither *ERG11* overexpression nor variation in *ERG11* coding sequences was responsible for the high fluconazole MICs observed in their study (381).

Enhanced activity of plasma membrane azole efflux pumps, which has been associated with azole resistance of *C. neoformans*, has not yet been studied in *C. gattii* (380, 382). Upregulation of the ATP-binding cassette transporter-encoding gene *AFR1* in *C. neoformans* was associated with resistance to fluconazole *in vitro* and in mice with systemic cryptococcosis (380, 383).

An additional phenomenon of heteroresistance among *C. neoformans* strains in AIDS patients undergoing fluconazole maintenance therapy has been reported (384, 385). This is a form of intrinsic and adaptive resistance that has been associated with clinical failure. Researchers in the United States have established that *C. neoformans* strains are innately heteroresistant to fluconazole, with each strain producing a subpopulation that can survive concentrations of fluconazole well above the MICs (385). Sionov and colleagues have subsequently shown that, *in vitro*, strains adapt to high fluconazole concentrations by undergoing disomy (where the cryptococcal cell has 2 members of a pair of homologous chromosomes), first in chromosome 1 and then in multiple other chromosomes. The duplication of chromosome 1 parallels the duplication of the *ERG11* and *AFR1* genes and is reversed by removing the drug from the cultures. Whether a similar mechanism of heteroresistance involving chromosome duplication occurs in *C. gattii* exposed to azoles is uncertain but is an important area of future study.

ANTIFUNGAL THERAPY AND MANAGEMENT

Antifungal Therapy

Current recommendations for the management of infections caused by *C. gattii* are based largely on extrapolation from clinical trials in patients with infection caused by *C. neoformans* and on individual case reports, case series, and expert opinion (282). This review focuses only on the most recently reported data, as a comprehensive review and an article detailing practical management guidelines were published in 2013 (262, 386).

Infectious Diseases Society of America (IDSA) guidelines outline therapeutic approaches based on host status, site of infection, complications of cryptococcosis, and limitations placed on therapeutic options in resource-limited settings. In general, these guidelines recommend similar antifungal treatment strategies for *C. gattii* and *C. neoformans* (282). There is some evidence to support this approach from, for example, a subanalysis of a retrospective study of *C. gattii* and *C. neoformans* meningitis in immunocompetent hosts (27) and reports from Africa of outcomes of cryptococcal meningitis in patients with AIDS (96–98). Notably, the genotype of HIV-associated *C gattii* infections was not stated, and it is possible that disease caused by *C. gattii* VGIV, which has been isolated from HIV-associated infections in Africa (see Epidemiology, Origin, and Evolution, above), resembles that due to *C. neoformans*. Diagnostic distinction between *C. gattii* and *C. neoformans* by the simple laboratory test of growth on CGB agar has long been implemented in Australia due to the endemicity of *C. gattii* (now typed as VGI), its association with substantial pulmonary and cerebral cryptococcomas (29), and the attendant differences in the duration of induction and consolidation/maintenance therapy.

More recent and larger studies of *C. gattii* infection in Australia and of the outbreaks in Canada and the United States suggest that therapeutic recommendations may be refined by taking into account the genotype of the causative *C. gattii* strain and possibly its antimicrobial susceptibility (130, 131, 258, 299), although the latter is yet to be confirmed *in vivo*. Recently reported data indicate that for *C. gattii* VGI infection involving the lung and/or the CNS, induction therapy with amphotericin B plus 5-flucytosine is appropriate (~6 weeks for cerebral disease and 2 weeks for isolated lung disease) (299). Shorter induction courses may be effective in patients with CNS disease and minimal brain involvement, but current data are insufficient to confirm this proposition. As recommended by IDSA guidelines, for lung infection not considered "serious" (282), single small cryptococcomas due to *C. gattii* can be treated initially with fluconazole (386). Consolidation, and then maintenance, therapy with fluconazole is recommended universally. In patients with *C. gattii* cryptococcosis, there is a compelling argument to use the term "eradication therapy" rather than "consolidation" and "maintenance" therapy, as infection is curable with adequate antifungal and ancillary therapies. The minimum duration required to achieve cure in an immunocompetent patient has not been defined, but durations of 6 to 12 months for isolated and circumscribed lung infection and 12 to 18 months for cerebral disease are commonly used (262, 282, 299, 386).

Surgery

There are few studies that address indications for surgery in cryptococcosis. Single large lesions, especially when encroaching on

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local structures in the lung or with mass effect and surrounding edema upon cerebral imaging, require resection if they are surgically accessible (262). Insertion of a lumbar drain or an early CSF shunt procedure may be required for raised ICP not controlled by repeated lumbar punctures, and shunting is necessary in the presence of symptomatic hydrocephalus (see "Management of Complications," below).

Management of Complications

Raised intracranial pressure. Control of raised ICP is a critical determinant of the outcome of CNS cryptococcosis. Repeated lumbar punctures or, if necessary, insertion of a lumbar drain or a CSF shunt is indicated to reduce the ICP to acceptable levels. Symptomatic hydrocephalus visible upon cerebral imaging likewise requires the insertion of a shunt to relieve the high ICP, regardless of the cryptococcal species responsible (262, 282, 299).

Immune reconstitution inflammatory syndrome. In all cases, consolidation/maintenance antifungal therapy (in the case of *C. gattii* which can be cured, this is actually eradication therapy) should be continued. Patients with minor manifestations of IRIS can be managed with supportive care. Corticosteroids have been used successfully to control symptoms and reduce the size of inflammatory lesions (299). An early retrospective observational study in PNG suggested that corticosteroid use improved visual outcomes in patients with *C. gattii* meningitis (387). After initiation, a slow reduction in corticosteroid doses is needed to prevent a relapse of inflammation. Most cases of IRIS will resolve within days to weeks. In a small series of 8 patients with *C. gattii* neurological infection and IRIS, none died, but 4 had persistent neurological sequelae at 12 months (3 had received gamma IFN). Cerebral imaging abnormalities returned to pre-IRIS appearances within 4 to 6 months (299).

CONCLUSIONS

In conclusion, based on morphological, biological, and phylogenetic species concepts, *C. gattii* is recognized as a valid species in the *C. neoformans-C. gattii* species complex. This is congruent with its ecology, epidemiology, and clinical associations, which differ substantially from those of *C. neoformans*. Modern genetic techniques have determined the population genetic structure of *C. gattii* and include recombination studies, which have given us insight into how *C. gattii* is sustained in nature. There are four main genetic groups of *C. gattii* (VGI, VGII, VGIII, and VGIV, which are equivalent to AFLP4 through to AFLP7), with MLST typing superseding earlier methods of strain typing. The ISHAM consensus method is based on MLST using a minimum of seven predetermined genetic loci; this approach is recommended to enable strain pattern comparisons. Whole-genome sequencing, which is the most discriminatory strain typing method, is likely to become more common with time.

Clinically apparent cryptococcosis represents the outcome of a complex set of interactions that include the size of the inhaled inoculum, the individual *C. gattii* virulence composite (which is not simply the sum of individual virulence phenotypes), and the host status/response. Through the application of genomics and other omics technologies, it is increasingly apparent that the outcome of the host-cryptococcus interaction is regulated by complex sets of transcriptional circuits in both the fungus and host cells and that differences between human disease caused by *C. gattii* and that caused by *C. neoformans* can be elucidated in relevant animal models.

The ecology and epidemiology of infection vary with geographic region. Both humans and animals are susceptible to *C. gattii*, and in some circumstances, animals may be considered sentinels of human disease. Despite the emergence of *C. gattii* infections in new environments where 40 to 73% of patients have predisposing or immunosuppressive medical conditions, its propensity to cause disease in healthy hosts remains strong. Many infections, including those in new geographic locations, have occurred in apparently immunocompetent patients. However, the recent focus on identifying at least subgroups of patients with a genetic predisposition or immune dysfunction, for example, an association with anti-GM-CSF antibodies, may alter this thinking.

CNS and lung infections are the most common manifestations, although any body site may be affected. CNS infection is frequently encountered in the nonoutbreak setting; hence, routine lumbar puncture of patients with suspected cryptococcosis is recommended. CNS complications are common, particularly raised intracranial pressure, hydrocephalus, and severe neurological sequelae. New, rapid diagnostic tests and imaging modalities are assisting early diagnosis. Differentiation of *C. gattii* from *C. neoformans* is recommended as a routine and is achievable in most cases by simple phenotypic methods. Molecular techniques are definitive and provide subtype data. Despite the fact that there are no data on the relationship between MICs, drug dosing, and clinical outcome, antifungal susceptibility testing of azole drugs may be helpful for patients failing to respond to apparently appropriate therapy, and regular MIC surveillance in reference laboratories is recommended to monitor trends.

There are no antifungal drug trials to guide antifungal treatment of *C. gattii* infections. However, data from contemporary studies suggest that the site(s) of infection is the main influence on the choice of the antifungal drug regimen. For CNS infection and for severe pulmonary disease, intensive induction therapy with amphotericin B plus 5-flucytosine is necessary to optimize outcomes. The duration of induction therapy in patients with CNS disease is recommended to be at least 6 weeks, with a total duration of therapy of 18 to 24 months, although further evaluation of shorter induction courses is warranted. For isolated lung disease, induction therapy should be offered for 2 weeks, with a total duration of therapy of approximately 12 months. Fluconazole remains the preferred azole for use after induction therapy. Aggressive management of hydrocephalus and raised intracranial pressure is essential and may require early surgical drainage of CSF. Surgery may also be indicated for relief of anatomical complications caused by mass lesions. IRIS is an occasional complication that must be distinguished from failure of antifungal treatment.

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Sharon C. A. Chen is the Director of the Centre for Infectious Diseases and Microbiology Laboratory Services, Westmead Hospital, Sydney, Australia, incorporating a reference laboratory in Mycology, and is an Associate Professor at the University of Sydney. She is also an Infectious Diseases physician with a research interest in medical mycology. She is also the Medical Mycologist for the Molecular Mycology Research Laboratory at Westmead. She is the immediate past scientific chair of the Australia and New Zealand Mycoses Interest Group (ANZMIG), a member of the ASID Clinical Trials Network Steering Committee, a member of National Antimicrobial Committee of Australia, and a member of the National Scientific Advisory Committee for the Australian Society for Microbiology. Her research interests include clinical and molecular epidemiology of fungal infections; infections in immunocompromised hosts, critically ill persons, and those with chronic lung disease; novel diagnostic methods for fungal and bacterial infections; and resistance to antifungal agents.

Wieland Meyer is the Head of the Molecular Mycology Research Laboratory at the Center for Infectious Diseases and Microbiology and Professor for Molecular Medical Mycology, Sydney Medical School-Westmead Hospital, Marie Bashir Institute for Infectious Diseases and Biosecurity, the University of Sydney, Westmead Millennium Institute, Sydney, Australia. He obtained his Ph.D. in 1992 from the Humboldt University of Berlin. His research focuses on the evolution, phylogeny, identification of species, population genetics, molecular epidemiology, and typing of human-pathogenic fungi; the development of fast, simple, and reliable molecular identification techniques for human/animal fungal pathogens; and the understanding of fungal pathogenesis on a molecular level. He has published more than 130 peer-reviewed research papers. He is Vice President of the International Society of Human and Animal Mycology and an associate editor of *Persoonia—Molecular Phylogeny and Evolution of Fungi*, *Australasian Mycologist*, *Microbiology Australia*, *IMA Fungus*, *Japanese Medical Mycology Journal*, and *DNA Barcoding*.

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Tania C. Sorrell is the Director of the Marie Bashir Institute for Infectious Diseases and Biosecurity at the University of Sydney and a Senior Physician in Infectious Diseases at Westmead Hospital. She graduated in Medicine and obtained her research M.D. from the University of Adelaide, South Australia. She has longstanding interests in prevention, diagnosis, and treatment of infectious diseases, especially in immunocompromised hosts, and in the emergence of resistant microorganisms. Her research on the serious fungal infection cryptococcosis has provided new insights into host-microbe interactions and resulted in a new antifungal drug development program. She has developed new diagnostics for infectious diseases and led national studies on the epidemiology of invasive fungal infections. She is a previous President of the Australasian Society for Infectious Diseases and has served/serves on national advisory committees on Infectious Diseases and the Research and Human Ethics Committees of the National Health and Medical Research Council of Australia.

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