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Cryptococcosis due to *Cryptococcus gattii* in Germany from 2004–2013

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ABSTRACT

The fungal pathogen *Cryptococcus gattii* was considered to be restricted to tropic and sub- tropic regions. A recent outbreak in North America due to isolates belonging to molecular type VG II, affecting mostly non-immunocompromised hosts, documented the potential public health impact of this fungal pathogen also in temperate regions. Surveillance of these infections in Germany is challenging, as cryptococcosis is not notifiable and often *C. gattii* is diagnostically not distinguished from the more prevalent *Cryptococcus neoformans*. We used hospital discharge data and identified cryptococcal isolates received by the German cryptococcosis reference laboratory at the species level to gain insights into the epidemiology of *C. gattii*-infections in Germany between 2004 and 2013. Between 49 and 60 (Median 57) hospitalizations for cryptococcosis are documented per year. Between 5 and 28 (Median 14) isolates were received at the reference laboratory per year. Among 155 single patient isolates, four *C. gattii* (3%) of the molecular types VGI and VG III were identified from patients with meningoencephalitis, including one interspecies hybrid. Patient histories and molecular typing suggest that half of the infections were acquired abroad. Only one patient survived the infection. *C. gattii* remains rarely identified as agent of cryptococcosis in Germany but underestimation is likely. Definition of environmental niches occupied by *C. gattii* in Germany may help to assess the associated risk of infection and prevent this deadly fungal infection.

1. Introduction

Cryptococcosis is the most common fungal infection of the central nervous system worldwide, leading to an estimated 600,000 deaths annually. The majority of these infections is due to *Cryptococcus neoformans* and diagnosed in AIDS patients from developing countries (Park et al., 2009). The sister species *Cryptococcus gattii* was initially isolated in tropical and subtropical regions from non-immunocompromised patients (Harris et al., 2012). However, starting in 1999, an unprecedented outbreak of *C. gattii*-infections in temperate regions of North America, centered on Vancouver Island (British Columbia, Canada) has been documented. Between 1999 and 2007, 218 human cases of *C. gattii* infections have been reported, representing an average annual incidence of 5.8 cases per million inhabitants. The majority of affected persons (62%) was not immunocompromised and sought treatment for a respiratory syndrome (77%). Infections were associated with mortality in 8.7% (Galanis et al., 2010). The predominant outbreak strains, including the major molecular type (VGIIa) have been isolated from trees and soil in British Columbia (Bartlett et al., 2011). Phylogenetic studies suggest that these strains may have been introduced into the region from South America (Hagen et al., 2013). This highlights the potential public health impact of cryptococcosis and the need for surveillance including *C. gattii* infections in temperate regions such as northwestern Europe. In Germany, surveillance of cryptococcosis is challenging, as the infection is not notifiable. Furthermore, identification of cryptococci to the species level is not performed in many laboratories. We used hospital administrative data, identified clinical cryptococcal isolates received by the German reference laboratory to the species level and typed *C. gattii* isolates in order to gain insights into the current epidemiology of *C. gattii* infections in Germany.

2. Methods

Single patient isolates from cryptococcosis-patients diagnosed in Germany between 2004 and 2013 and submitted to the German reference laboratory for cryptococcosis at the Robert

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Table 1
PCR assays, primer-sequences and amplification conditions used to characterize *Cryptococcus gattii* isolates.

Locus	Reference	Primer	Pcr conditions
CAP59	Meyer et al. (2009)	*CAP59*f: CTCTACGTCGAGCAAGTCAAG *CAP59*r: TCCGCTGCACAAGTGATACCC	94°3 min, (94°30 s, 57°30 s, 72°60 s) × 35, 72°10 min
GPD1	Litvintseva et al. (2006)	*GPD*f: ATG GTC GTC AAG GTT GGA AT *GPD*r: GTA TTC GGC ACC AGC CTC A	94°3 min (94°45 s, 55°60 s, 72°60 s) × 35, 72° 10 min
LAC1	Litvintseva et al. (2006)	*LAC*f: GGC GAT ACT ATT ATC GTA *LAC*r: TTC TGG AGT GGC TAG AGC	94°3 min (94°30 s, 54°30 s,72° 60 s) × 35, 72° 10 min
PLB1	Meyer et al. (2009)	*PLBf*: CTTCAGGCGGAGAGAGGTTT *PLB*r: GATTTGGCGTTGGTTTCAGT	94°3 min (94°45 s, 61°45 s, 72° 60 s) × 35, 72° 10 min
SOD1	Meyer et al. (2009)	*SODf*: GATCCTCACGCCATTACG *SOD*r: GAATGATGCGCTTAGTTGGA	94°3 min (94°30 s, 52°30 s, 72°90 s) × 35, 72° 10 min
URA5	Meyer et al. (2009)	*URA5f*: ATGTCCTCCCAAGCCCTCGAC *URA5*r: TTAAGACCTCTGAACACCGTACTC	94°3 min (94°30 s, 52°30 s, 72°90 s) × 35, 72° 10 min
IGS1	Meyer et al. (2009)	*IGS1*f: ATCCTTTGCAGACGACTTGA *IGS1*r: GTGATCAGTGCATTGCATGA	94°3 min (94°30 s, 58°30 s, 72°60 s) × 35, 72° 10 min
STR1	Feng et al. (2013a)	STR1f: GAGATTCGGCAGGAAGAAGC *STR1r*: CGTAAGGGATGACGAAAAGGTA	94°5 min (94°60 s, 58°60 s, 72°120s) × 35, 72°7 min
Mat a	Bovers et al. (2006)	*STE12*a-F537: GTTCTTTGGAATGGCTTATTTCATAT *STE12*a-R1299: GMCTTGCGTGGATCATATCTA	
Mat alfa	Bovers et al. (2006)	*STE12*a-F809: TTGACCTTTTRTTCCGCAATG *STE12a*-R1607: TTTCTTCTCCCCTGTTTATAGGC	94°5 min (94°60 s, 58°60 s, 72°120 s) × 35, 72°7 min
Genotype	Feng et al. (2013b)	*VAC*f: AGCCCACGGCAAAATAGTG *VAC*r: CACGGTCCAAAACTTGATTGTT *IGS*f: CCGAGGCAGGACACACATAC *IGS*r: GGCGGAATACAAATACTACTTACCT	94°5 min (94°30 s, 56°30 s, 72°20 s) × 35, 72°6 min

Koch-Institute, were included. Data on the organ involvement of infections, travel history and clinical outcomes of patients were provided by physicians referring the isolates. Isolates were confirmed as *C. neoformans* or *C. gattii* by a typical brown color effect on Niger-seed agar (Staib et al., 1987) and production of Urease using Christensen's agar (Roberts et al., 1978). Production of a blue color effect on CGB medium was assessed to identify *C. gattii* (Kwon-Chung et al., 1982). All media were made in house. Correct performance was assessed using appropriate control strains. In addition, identification of all *C. neoformans* isolates from 2004 to 2010 was confirmed by specific PCR assays as reported previously (Sanchini et al., 2013). The serotype of isolates received from infections diagnosed between 2011 and 2013 and all *C. gattii* isolates was identified by a PCR assay targeting the *STR1* gene (Feng et al., 2013a). The geno-, and mating-types of *C. gattii* were determined by PCR assays including appropriate controls (Bovers et al., 2006; Feng et al., 2013b). Isolates were typed using a consensus scheme with 7 loci (*CAP59, GPD1, LAC1, PLB1, SOD1, URA5, IGS1*) (Meyer et al., 2009). All PCR reactions were performed in a thermocycler (Mastercycler, Eppendorf, Germany) using a final volume of 25 μl of mastermix consisting of 1×PCR buffer, 2 mM MgCl2, 0.2 mM dNTP-mixed solution, 1 pmol/ml of each primer and 0.05 U/ml of BioTherm™ DNA-Polymerase (Rapidozym, Berlin, Germany) using PCR primer and conditions summarized in Table 1. The PCR products were purified using the ExoSAP-IT® kit (Affymetrix UK Ltd, High Wycombe, UK) in a thermocycler for 15 min at 37 °C followed by 15 min at 80 °C. Forward and reverse strands of the PCR products were sequenced using the primers used for amplification in an Applied Biosystems 3500xl Genetic Analyser (Life Technologies GmbH, Darmstadt, Germany). Forward and reverse sequences were assembled using Geneious version 7 (created by Biomatters, available from http://www.geneious.com/).

Reference strains representing the four *C. gattii* genetic groups VG I (CBS 6289.85), VG II (CBS 10514), VG III (CBS 10081) and VG IV (CBS 10101) were included. Sequences from the clinical isolates were submitted to the Cryptococcus MLST database (http://mlst.mycologylab.org/). The concatenated MLST sequences were aligned with sequences from selected strains from previous reports representing clinical or environmental isolates from defined geographic regions in order to obtain information on the origin of the infections using MEGA version 5.2 (http://www.megasoftware.net/) with manual corrections using the default settings (Chowdhary et al., 2012; Hagen et al., 2012). A Maximum likelihood tree was constructed using MEGA with the Kimura-2 parameter model according to a published guideline (Hall, 2013). The percentage of replicate trees in which the associated taxa clustered together was estimated by bootstrap test inferred from 500 replicates.

Annual hospital discharges due to cryptococcosis from German hospitals were extracted from publicly available data using an online query tool (www.gbe-bund.de).

3. Results

Administrative data document a total of 491 discharges from German hospitals due to cryptococcosis in the observation period, between 49 and 60 (Median 57) per year (Fig. 1). A total of 155 single patient isolates were included in this analysis. The annual number of isolates received at the reference laboratory ranged between 5 and 28 (Median 14) (Fig. 1). Isolates were identified as *C. neoformans var. grubii* ($n = 110$), *C. neoformans var. neoformans* ($n = 31$), *C. neoformans* intraspecies hybrids ($n = 10$) or *C. gattii* ($n = 4$). The identification of *C. gattii* appeared to be more likely in years in which more than 20 strains have been received by the reference laboratory (2/2 vs. 1/7; $p = 0.067$; Fisher's exact test).

Patients with *C. gattii*-infection were males aging between 43 and 55 years of age (Table 2). Underlying immunocompromising conditions were documented in three patients, including AIDS ($n = 2$) and idiopathic CD4-lymphopenia ($n = 1$). Travel history included visits to the United States ($n = 1$) and Kenia ($n = 1$) while travel outside Germany was denied in two patients. *C. gattii* was isolated from the cerebrospinal fluid in all patients. In two patients,