The detection of cryptococcal antigen by latex agglutination tests (LATs), enzyme-linked immunoassays (ELISA), or lateral flow assay (LFA) is an important tool for diagnosis of a Cryptococcus infection. Cerebrospinal fluid and/or serum samples of 10 patients with cryptococcosis due to Cryptococcus gattii or a hybrid of Cryptococcus neoformans and C. gattii were examined by three LATs (the IMMY Latex-Crypto® test, the Pastorex™ Crypto Plus, and the Remel Cryptococcus Antigen Test Kit) and the LFA made by Immuno-Mycologics. LATs based on monoclonal antibodies (mAbs) like the Pastorex™ Crypto Plus or the Remel Cryptococcus Antigen Test Kit turned out to have an insufficient sensitivity to detect four out of 10 C. gattii infections, including one infection by a hybrid between C. gattii and C. neoformans. Reflecting the ongoing expansion of C. gattii in geographical zones outside of tropical and subtropical areas like Mediterranean countries, Vancouver Island (British Columbia, Canada) and the Pacific Northwest region (USA), these findings are alarming because of the risk of delayed diagnosis of infections caused by C. gattii. Therefore, the preliminary serological screening for cryptococcal antigen in the case of a suspected Cryptococcus infection should be performed by using an assay with a broad range specificity and sensitivity for C. neoformans and C. gattii, including their hybrids.

Key words: cryptococcosis, disseminated infection, antigen detection, false negative, Cryptococcus gattii, monoclonal antibodies, polyclonal antibodies.

Introduction
Screening for cryptococcal antigen in serum and/or cerebrospinal fluid (CSF) is of high value for laboratory diagnosis of cryptococcal infections because of the high sensitivity and specificity of the assays that are generally used [1–4]. Determination of the amount of antigen in serum and CSF provides clinically important information on the stage and course of infection, and may also indicate a relapse of cryptococcosis [5].

Available tests target the polysaccharide capsular antigen of infectious agents within the Cryptococcus gattii/Cryptococcus neoformans species complex.
Disseminated cryptococcosis without detectable cryptococcal antigen in sera and CSF has been reported rarely [6–11]. Several commercial assays are available for the serological diagnosis of cryptococcosis, namely, latex agglutination tests (LAT), enzyme immune assays (EIA), and, since 2009, the lateral flow immunoassay (LFA) developed by IMMY (Immuno-Mycologics, Norman, OK, USA). Motivated by a cerebral cryptococcosis highly suspicious to be caused by \( C. gattii \) without serological evidence when using a commercial assay, a retrospective analysis of the diagnosis of \( C. gattii \) infections in humans and animals was performed at a reference laboratory in a nonendemic area using commercially available serological tests. We conclude that the diagnosis of \( C. gattii \) infections may be impaired when some assays are used.

### Methods

#### Patients

Nine humans and one cat diagnosed with a proven \( C. gattii \) infection at the German reference laboratory for cryptococcosis between 1985 and 2014 whose sera were available for the comparison of commercially used assays were included in this study [12–15]. Five out of 10 serum samples were included in the present study. Four patients were under treatment at the time of sampling, and four patients had been treated before the start of antifungal therapy had been taken. The index patient (patient one in Table 1) was a 59-year-old immunocompetent Caucasian female originating from Germany who had developed cerebral cryptococcosis in 1985 while residing in Florida, United States, for an unknown number of years. Unfortunately, no details were available on the antifungal treatment that the patient received during the 4 months before hospital admission. The patient received the subsequent antifungal treatment at the Robert Koch Institute, Germany. The index patient was consulted for interpretation of the serological results because of the patient's history of cryptococcosis, but test results were negative. Because the patient had developed cerebral cryptococcosis before the serological screening for \( C. gattii \) infection, the serological screening was performed on the antifungal treatment that the patient received during the 4 months before hospital admission. The index patient was admitted to the hospital where serum and CSF samples were obtained on the antifungal treatment that the patient received during the 4 months before hospital admission. The patient was initially treated with fluconazole and consult a general practitioner because of progressive headaches. Because meningitis was suspected, she was admitted to the hospital where serum and CSF samples were obtained on the antifungal treatment that the patient received during the 4 months before hospital admission. The index patient was admitted to the hospital where serum and CSF samples were obtained on the antifungal treatment that the patient received during the 4 months before hospital admission. The patient was initially treated with fluconazole and had been taken before the start of antifungal treatment at the time of sampling.

#### Table 1. Patients with cryptococcosis due to \( C. gattii \) and detection of cryptococcal antigen.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Human (H) / Animal</th>
<th>Age</th>
<th>Sex</th>
<th>Probable location where the infection was acquired</th>
<th>Underlying disease</th>
<th>Specimen</th>
<th>Strain / Serotype</th>
<th>AFLP/genotype</th>
<th>MLST</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H 59 F</td>
<td>USA</td>
<td>None</td>
<td>None</td>
<td>CSF</td>
<td>RKI 07–0123 (B)</td>
<td>AFLP9</td>
<td>ST058</td>
<td>Schaberg et al. 1988 [15]</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>H 45 M</td>
<td>Germany</td>
<td>None</td>
<td>None</td>
<td>Lung, cryptococcoma</td>
<td>RKI 85–0888 (B)</td>
<td>AFLP4/VGI</td>
<td>ST172</td>
<td>Dihgans et al. 1998 [12]</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>H 33 F</td>
<td>Brazil</td>
<td>None</td>
<td>None</td>
<td>CSF</td>
<td>RKI 96–0767 (B)</td>
<td>AFLP6/VGII</td>
<td>ST113</td>
<td>Grose et al. 2001 [14]</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>H 27 F</td>
<td>USA</td>
<td>None</td>
<td>None</td>
<td>Brain tissue</td>
<td>RKI 97–0310 (B)</td>
<td>AFLP5/VGIII</td>
<td>ST020</td>
<td>Georgi et al. 2009 [13]</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>H 53 F</td>
<td>Canada</td>
<td>None</td>
<td>None</td>
<td>CSF</td>
<td>RKI 06–0496 (B)</td>
<td>AFLP6/VGII</td>
<td>ST020</td>
<td>Georgi et al. 2009 [13]</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>H 54 M</td>
<td>Kenya</td>
<td>HIV positive</td>
<td>None</td>
<td>CSF</td>
<td>RKI 07–0288 (B)</td>
<td>AFLP8</td>
<td>n/a</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>H 61 M</td>
<td>Spain</td>
<td>None</td>
<td>None</td>
<td>Spinal tumour</td>
<td>RKI 09–0082 not cultured</td>
<td>AFLP4/VGII</td>
<td>n/a</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>H 44 M</td>
<td>Germany</td>
<td>None</td>
<td>None</td>
<td>CSF</td>
<td>RKI 09–0421 (B)</td>
<td>AFLP4/VGII</td>
<td>ST051</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>H 55 M</td>
<td>Germany</td>
<td>None</td>
<td>None</td>
<td>CSF</td>
<td>RKI 13–0681 (B)</td>
<td>AFLP4/VGII</td>
<td>ST156</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Animal 7 F</td>
<td>Germany</td>
<td>None</td>
<td>None</td>
<td>Pharyngeal tumour</td>
<td>RKI 14–0661 not cultured</td>
<td>AFLP6/VGII*</td>
<td>ST007</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

| Control 47 M | Myanmar | HIV positive| blood culture | CSF | RKI 08–0494 (A) | AFLP1/VNI | n/a | This study |
| Control 66 F | Germany | None| None | CSF | RKI 08–0436 (A) | AFLP1/VNI | n/a | This study |

Note: *Environmental isolate; n/a, not available in the legend.
Repeated analyses of CSF and serum using this test in our laboratory were likewise negative for cryptococcal antigen. CSF had also been taken for mycologic cultures, resulting in growth of one single colony of *Cryptococcus* species after two weeks of incubation on Niger seed agar at 26 °C. Of note, this isolate was phenotypically suspected to be *C. gattii* when the first subculture on l-canavanine glycine bromothymol blue (CGB) medium started to turn the medium blue and also the agglutination with antisera in the test kit Crypto Check® (Iatron Laboratories, Tokyo, Japan) revealed a single but clear agglutination with antiserum against *C. gattii* serotype B. After repeated subculturing the macroscopic morphology of the culture changed from mucoid to a drier phenotype; also the CGB agar remained negative and the agglutination with antiserum against serotype B was not reproducible. The internal transcribed spacer region was sequenced, which revealed that the isolate was *C. neoformans* variety *grubii*. However, subsequent genotyping by amplified fragment length polymorphism fingerprinting revealed that this isolate was an interspecies hybrid between *C. neoformans* variety *grubii* and *C. gattii*. The isolate clustered tightly with CBS10496, which is the representative strain for genotype AFLP9 (see Results) [16].

**Cryptococcal Antigen Detection**

Antigen detection in serum and CSF was performed comparing the LATs of the IMMY Latex-Crypto® test (Immuno-Mycologics, Norman, OK, USA) to the Pastorex™ Crypto Plus (formerly Pastorex Cryptococcus® test; Bio-Rad, Marnes-la-Coquette, France). If available serum and CSF, the Remel *Cryptococcus* Antigen Test Kit (Remel, Lenexa, KS, USA) was also applied. Furthermore, the dipstick sandwich immunochromatographic assay Cryptococcus Antigen Lateral Flow Assay (CrAg® LFA; Immuno-Mycologics) was used in every sample. All these tests are qualitative and semiquantitative for the detection of capsular polysaccharide antigens of *C. neoformans* [17]. The LAT by IMMY uses polyclonal antibodies (pAbs) against the capsular polysaccharide of *Cryptococcus* bound to latex particles, while in the two other tests particles are sensitized with mAbs. For the CrAg® LFA a combination of mAbs was chosen. All assays were performed with undiluted samples according to the manufacturer’s instructions. To rule out false negative results due to a prozone effect, each sample was also diluted to 1:20 in a first step and screened for CSA. The titres for all LATs were consistently determined by two-fold serial dilutions to make data comparison possible. Serum samples from two patients with proven *C. neoformans* var. *grubii* infection were randomly selected and included as controls.

**Identification and Typing of the Isolates**

The *Cryptococcus* isolates were phenotypically examined according to their growth on Niger seed and on CGB agar, their ability to grow at 37 °C and to hydrolyze urea, as well as their assimilation of sugar in the API® ID 32C kit (BioMerieux, Marcy l’Etoile, France). Until 2007 serotyping of isolates was performed with polyclonal antibodies by using the Crypto Check® kit (Iatron, Tokyo, Japan), which has since been removed from the market. Molecular characterization was performed by amplified fragment length polymorphism (AFLP) genotyping [16], sequencing of the ITS and IGS regions of rDNA [18] and multilocus sequence typing (MLST) of the nuclear loci CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, and URAS5 [19].

In case a clinical isolate was not available and the diagnosis was histologically confirmed by the presence of encapsulated yeasts, fungal DNA was extracted and amplified from formalin fixed paraffin embedded (FFPE) tissue. The amplicons of the ITS2 region of rDNA and the IGS region were identified by hybridization and subsequently sequenced as described before [20]. Briefly, five tissue sections that were 5 μm thick were subjected to DNA extraction and purification with the FFPE Tissue LEV DNA Purification Kit designed for the Maxwell® 16 Instrument (Promega, Madison, WI, USA).

All sera, CSF and cryptococcal isolates were archived at −70 °C prior to further investigations.

**Results**

All clinical cryptococcal isolates cultured from patients 1 to 6, 8 and 9 (Table 1) grew well at 37 °C and on CGB agar, except for the subcultured isolate of patient 1 which showed a phenotypic switching on CGB agar (see case report in the method section). Identification by API® ID 32C resulted in an excellent identification of >99% as “*C. neoformans*”. Reidentification of the clinical isolates confirmed that six out of eight isolates were *C. gattii*, while the cryptococcal isolates from patient 1 and 6 turned out to be interspecies hybrids between *C. gattii* and *C. neoformans*. However, the isolate cultured from patient 1 was found to be closely related to CBS10496 (genotype AFLP9), the reference strain for the interspecies hybrid between *C. neoformans* var. *grubii* genotype AFLP1/VNI and *C. gattii* genotype AFLP4/VGI [16]. The isolate cultured from patient 6 clustered with reference strain CBS10488 (genotype AFLP8), representing the interspecies hybrid between *C. neoformans* variety *neoformans* genotype AFLP2/VNIIV and *C. gattii* genotype AFLP4/VGI [16].

Altogether 10 sera and one CSF sample from 10 patients suspected to have acquired a *C. gattii* infection, including...
the ones with an infection by an interspecies hybrid, and sera from two patients with a C. neoformans var. grubii infection as controls were tested with three or if possible four different assays (Table 2). Sera from the two patients with a proven C. neoformans infection and six of the 10 patients with a proven C. gattii infection (including the interspecies hybrids) had detectable cryptococcal antigen with each of the assays studied. Samples in four out of 10 patients with a C. gattii infection had a positive reaction using the IMMY assays, whereas there were no antigen detected using the LATs by Bio-Rad and Remel (Table 2).

Of all assays tested, those by IMMY gave the highest titers in cryptococcal antigen screening performed by the LATs based on mAbs, whereas there were no antigen detected using the LATs by Bio-Rad and Remel (Table 2). Of all assays tested, those by IMMY gave the highest titers in cryptococcal antigen screening performed by the LATs based on mAbs, whereas there were no antigen detected using the LATs by Bio-Rad and Remel (Table 2).

### Discussion

Although the number of patients with a C. gattii infection in the current study was limited, the results strikingly document that the detection of CSA depends upon the design of the serological test. C. gattii infections in four out of five patients with only moderate CSA titres in the IMMY Latex-Crypto® test would have been overlooked using the Pastorex™ Crypto Plus or the Remel Cryptococcus Antigen Test Kit. The latter tests are based on mAbs, whereas the IMMY Latex-Crypto® test uses polyclonal antibodies (pAbs). If an assay is constructed from mAbs specific for epitopes shared across serotypes or is constructed with a cocktail of mAbs that are collectively reactive across serotypes, the test will have a broad spectrum of reactivity. A combination of two mAbs in the recently launched CrAg® LFA reduced serotype bias and increased the sensitivity for C. neoformans as well as for C. gattii [21], findings, which have been confirmed by our data. Likewise, a higher sensitivity of the CrAg® LFA compared to the LATs has been reported in two comparative studies [17,22].

In general, detection of cryptococcal serum antigen (CSA) can be next to impossible in patients with only localised pulmonary cryptococcosis [23–25] and in primary cutaneous manifestations [26,27]. Failure of CSA detection has been reported rarely in patients with disseminated cryptococcosis [6,7,9,28,29]. Lack of diagnosis by CSA may be accounted for by acapsular or minimally encapsulated isolates [10,11]. In view of the clearly positive antigen detection in the LAT and the LFA produced by IMMY, this explanation can be ruled out in four of our patients (1, 2, 4, and 7) with discrepant serologic results. Neither does it seem to have any effect on the results whether the patients were under antifungal therapy or not (Table 2).

Among isolates with different serotypes a high variability of antigenic structure has been observed [30]. Few data are available on the analytical cut-off of assays to detect cryptococcal antigen that address this problem. The Pastorex™ Crypto Plus is described to have an analytical cut-off of 5 ng/ml of antigen for serotype A, whereas cut-offs are considerably higher for serotypes D, B, and C with values...
of 100, 500, and 1000 ng/ml, respectively [31]. Although deemed to be sensitive and specific, one could speculate that the reference study for the Pastorex™ test did not include enough sera from patients infected by C. gattii, which had been considered a variety of C. neoformans leading to a gap in diagnosis.

Of note, comparative studies on cryptococcal antigen assays often ignore the influence of the genotype or even the species of the fungal pathogen [2,5,22,32–33]. Furthermore, case reports on patients with disseminated cryptococcosis in which CSA has not been detected failed to impart enough information about CSA screening tests used, the etiological agents identified, and, if cultivated, their in-depth molecular characterisation [8,9].

Our study did not include samples from patients infected by C. gattii serotype C, which has been described as causing serodiagnostics problems in commercially available assays [21]. Nor did our study have enough serotype B isolates to attempt a correlation between certain genotypes and detection limits for cryptococcal antigen. The serological results in the one animal included in the present study are concordant with published data on the high diagnostic value of CSA detection in cats with cryptococcosis [36].

The possibility of delayed or missed diagnosis of a C. gattii infection using the Pastorex™ Crypto Plus or the Remel Cryptococcus Antigen Test Kit is alarming considering the frequent use of these LATs in routine laboratories, especially in Europe. In our laboratory the IMMY Latex-Crypto™ test is used for routine serological diagnosis. We were thus able to uncover a positive CSA in patient 2 [15] not detected by one of the LATs based on mAbs. Because this patient denied any travel history outside of Europe, a C. gattii infection was not taken into account and the isolate was identified phenotypically as C. neoformans in the late 1980s. That CSA in patient 1 was not detected with the Pastorex™ Crypto Plus is because the test was not sensitive for the causative infectious agent later isolated and identified as a hybrid species between C. gattii and C. neoformans var. grubii. Similarly, a clinical isolate from the late 1980s, previously identified as being a C. gattii [37], was identified to be an interspecies hybrid two decades later by using molecular techniques [38]. Due to increasing numbers of endemic zones for C. gattii, for example, by anthropogenic dispersal and distribution of contaminated tree by-products as well as frequent travelling to and migration from endemic areas, we need to be ever more aware of C. gattii infections [19,39,40].

C. gattii infections may be underdiagnosed or diagnosed with considerable delay, especially in nonendemic areas, if only the LATs by Bio-Rad and Remel are utilized. If cryptococcosis is suspected, serological screening for cryptococcal antigen should be performed using a test not only able to detect C. neoformans, but one that includes a broader range of specificity and sensitivity for C. gattii and interspecies hybrids. Reliable diagnosis is of utmost importance to determine optimal treatment durations.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**References**


