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Evaluation of five conventional and molecular approaches for diagnosis of cryptococcal meningitis in non-HIV-infected patients

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Summary

Cryptococcal meningitis (CM) is a life-threatening mycosis primarily occurring in HIV-infected individuals. Recently, non-HIV-infected hosts were increasingly reported to form a considerable proportion. However, the majority of the reported studies on the diagnosis of CM patients were performed on HIV-infected patients. For evaluation of various diagnostic approaches for CM in non-HIV-infected patients, a range of conventional and molecular assays used for diagnosis of CM were verified on 85 clinical CSFs from non-HIV-infected CM patients, including India ink staining, culture, a newly developed loop-mediated isothermal amplification (LAMP), the lateral flow assay (LFA) of cryptococcal antigen detection and a qPCR assay. The LFA had the highest positive detection rate (97.6%; 95% CI, 91.8–99.7%) in non-HIV-infected CM patients, followed by the LAMP (87.1%; 95% CI, 78.0–93.4%), the qPCR (80.0%; 95% CI, 69.9–87.9%), India ink staining (70.6%; 95% CI, 59.7–80.0%) and culture (35.3%; 95% CI, 25.2–46.4%). All culture positive specimens were correctly identified by the LFA.

Key words: Cryptococcal meningitis, Diagnosis, non-HIV-infected host.

Introduction

Cryptococcal meningitis (CM) is a life-threatening mycosis caused by members of the *C. neoformans*/*C. gattii* species complex.[1] Seven new species have been recognised recently.[2] Globally, CM is primarily observed in HIV-infected patients with high mortality, ranging from approximately 20% (medically developed countries) to over 50% (in resource-limited regions such as sub-Saharan Africa),[3–5] causing over 625 000 deaths worldwide a year.[3–5] Recent studies revealed that non-HIV-infected hosts also form a considerable proportion of CM patients, particularly in Eastern Asia, such as China (approximately 17%), Taiwan (approximately 70%) and Japan (approximately 35%).[6–8] However, the diagnosis of CM has been reported to be more difficult in non-HIV-infected hosts than in HIV-infected hosts.[9] One potential reason was that physicians often easily overlook the possibility for CM in non-HIV-infected hosts.[1,4,5] Another reason was the potentially lower fungal burden in non-HIV-infected hosts than in HIV-infected CM patients. For example, the sensitivity of India ink staining for cryptococcosis is approximately 50% in non-HIV-infected patients,[10,11] whereas it is approximately 80% in HIV-infected patients.[12]

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The conventional diagnostic tests for CM, such as India ink staining or culture, have a low sensitivity, rely on experienced technicians and are time-consuming, particularly in clinical specimens with a low fungal burden. Detections of cryptococcal antigen (CrAg) using either latex agglutination (LA) or enzyme immunoassays (EIA) are highly sensitive and specific but they require a cold chain for specimen transport and technical expertise.[13,14] Recently, a lateral flow assay (LFA) for CrAg detection became widely recognised for the diagnosis of CM worldwide.[15–18] The reasons for its wide acclaim are not only because of the high specificity and sensitivity of the LFA for rapid diagnosis of CM in HIV-infected patients,[15–18] but also for its excellent usability which meets the ASSURED criteria,[19] i.e. affordable, sensitive, specific, user-friendly, robust and rapid. With this method, the results are usually available within 30 min, without requiring expensive equipment, and they are deliverable to the end user. In addition to the study by McMullan *et al.* [20], the majority of results of the LFA reported so far came exclusively from HIV-infected CM patients. The performance of the LFA and conventional diagnostic assays on CM is largely unknown in non-HIV-infected hosts.

Molecular techniques have potential added values for the diagnosis of CM when compared to routine methods.[9,21,22] Several molecular techniques, including various PCR-based assays,[9,23] rolling circle amplification (RCA) and Luminex *x*MAP,[24,25] have shown potential for rapid diagnosis of CM in clinics. In 2000, the LAMP technology was described by Notomi *et al.* [26], and it uses an isothermal step for accurate, cost-effective and rapid DNA amplification in one hour without the need for sophisticated equipment and the products can be assessed by the naked eye. Furthermore, the LAMP technology can amplify the target DNA from partially processed and/or non-processed specimens.[27] This technique also has been considered to be a potential molecular diagnosis platform that could meet the ASSURED criteria.[19,28] Here, we have developed a new LAMP assay targeting the intergenic spacer 1 (IGS1) region of the nuclear ribosomal rRNA gene that can simultaneously identify all members of the *C. neoformans*/*C. gattii* species complex at species complex level using a single set of primers. Notably, almost all conventional diagnostic approaches on CM are designed targeting the cryptococcal capsule, which may be a hidden challenge in the diagnosis of CM caused by capsule-deficient strains.

In this study, we aimed to evaluate various conventional and molecular approaches for diagnosis of CM in non-HIV-infected patients using spiked and clinical CSF specimens, including a newly developed loop-mediated isothermal amplification (LAMP), a newly reported qPCR for *Cryptococcus* based on the ITS of the rDNA,[23] India ink staining and culture.

Materials and methods

Patients and CSF specimens

Eighty-five specimens of clinical CSF were collected from 58 confirmed non-HIV-infected CM patients hospitalised in Shanghai Changzheng Hospital and Shanghai Huashan Hospital when they underwent laboratory examination on admission. A portion of each sample was immediately tested by India ink staining, culture and the LFA. The remaining CSF specimens were stored at −20 °C for molecular detection. With ethical approval (accession number: 2013SMMU-LL013), all patients involved understood and agreed to the use of these clinical specimens in this study. We also collected sterile human CSF from a non-infected patient with his consent in Shanghai Changzheng Hospital, who required CSF drainage due to high CSF pressure. The sterile CSF and ddH_2O were used as negative controls in the molecular detection of CM in clinical CSF specimens. *C. neoformans* strain H99 (CBS 8710), *C. gattii* strain CBS 10078 and a capsule-deficient strain (*cap*59Δ mutant H99 strain) were separately added into the sterile CSF for sensitivity testing in spiked specimens and as positive controls using the following concentrations of *Cryptococcus* cells 3×10^7 m^{-1}, 3×10^6 m^{-1}, 3×10^5 m^{-1}, 3×10^4 m^{-1}, 3×10^3 m^{-1}, 3×10^2 m^{-1}, 3×10^1 m^{-1}, 3 m^{-1} and 0 m^{-1} (Table S1).

The conventional diagnostic assays

For conventional diagnostic assays, the clinical CSF specimens were collected and centrifuged at $425 \times \boldsymbol{g}$ for 20 min. The precipitates were immediately tested using India ink staining and observed by microscopy for the presence of capsulated yeast cells. A small aliquot (500 μl) from each specimen was inoculated onto Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, Hampshire, U.K.) at 30 °C for 14 days. Positive yeast cultures were purified and confirmed as *C. neoformans* using multilocus sequence typing (unpublished data), following protocols described previously.[2,7]

The LFA

The 85 CSF specimens were tested using the IMMY LFA (ImmunoMycologics, Inc, Norman, OK, U.S.A)

following the instruction manual. Similar to the previous studies in HIV-infected CM patients,[15,16] reactivity at 1 : 2 dilution of CrAg was considered a positive result in our test.

DNA extraction

For molecular diagnosis, genomic DNA was extracted using benzyl chloride as previously described.[29] *Cryptococcus* DNA from the clinical and spiked specimens was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ Kit (ZYMO Research Co., Ltd, Los Angeles, CA, USA). The concentration and quality of DNA were evaluated using absorption ratios at 280 nm (OD_{280}) and 260 nm (OD_{260}) as assessed by fluorescence spectrophotometry (Colibri Microvolume Spectrometer, Berthold Titertek, Pforzheim, Germany). DNA with $OD_{260/280}$ absorbance ratios ranging from 1.8 to 2.0 was used by molecular assays (Table S2).[30]

The LAMP assay

For testing the specificity of the LAMP, a total of 81 isolates covering 25 *Cryptococcus* species and species belonging to related genera (Table S3) were tested according to the previous phylogenetic analysis.[31] The LAMP primers were designed using Primer Explorer V4 software (http://primerexplorer.jp/e/) based on the available IGS1 sequence (GenBank EF211264) of type strain H99. The primers for LAMP were designed to specifically amplify the IGS1 region, a multiple copy region (Table 1). The reactions were carried out in duplicate in 25 μl reaction volume using a Loopamp Real-time turbidity LA-320C machine containing the following reagents: 1 μl FIP primer (40 μmol l^{-1}), 1 μl BIP primer (40 μmol l^{-1}), 1 μl F3 primer (5 μmol l^{-1}), 1 μl B3 primer (5 μmol l^{-1}), 12.5 μl of 2 × LAMP Reaction Mix (Eiken Chemical Co., Ohtawara, Tochigi, Japan), 1 μl Bst DNA Polymerase (8U), 4 μl template DNA and 3.5 μl ddH_2O. The reaction was performed at 63 °C for 90 min. Similarly, the sensitivity tests were repeated twice using template DNA extracted from reference strains, spiked specimens and clinical CSF respectively. Subsequently, the LAMP was performed on the 85 specimens of cryopreserved clinical CSF. Genomic DNA of strain H99 and DNA extracted from spiked specimens were used as positive control. Both DNA extracted from sterile CSF and ddH_2O were used as negative controls.

The qPCR assay

The qPCR reactions (20.0 μl final volume) were performed in a mixture containing 10 μl 2 × *Premix Ex Taq*™ (Probe qPCR, ROX plus) (Takara Bio Inc., Otsu, Shiga, Japan), 0.5 μl each primer for *Cryptococcus* (1 μmol l^{-1}), 1.0 μl probe (0.2 μmol l^{-1}),[23] 2.0 μl template DNA and 6.0 μl ddH_2O using a Rotor-Gene 3000 Advanced Real-Time DNA Detection System (Corbett Research Inc, Sydney, Australia). The PCR conditions were as follows: an initial step of 95 °C for 30 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s, with a cooling cycle at 40 °C for 30 s. The quantification cycle (*Cq*) value for the positive control, positive CSF specimens and negative control (DNA extracted from sterile CSF; ddH_2O) were at 17, 20–30 and >35 respectively.

Statistical analysis

The positive detection rates obtained from different diagnostic techniques and the 95% confidence intervals (95% CI) were calculated using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA). The 95% CI were used to test whether the positive detection rates were statistically significant. A P value of 0.05 was considered to be statistically significant.

Table 1 Sequence of the primers for the LAMP (FIP, BIP, F3 and B3) and qPCR assays (Primer F, primer B and probe) used in this study.

Primer	Sequence	Reference
FIP(F1c+F2)	5′-TCCGCAACCTACTACAGTGAATGGGTCTTCAGCTTGGGTCATTGA-3′	This study
BIP(B1c+B2)	5′-TGCAATTGAGACACTTACCAGGCCACGACCATAAAATCAGTAGCGT-3′	This study
F3	5′-GGACTTGTACACAGTCTCATCA-3′	This study
B3	5′-ATGCGACACAAGCACCAG-3′	This study
Primer F	5′-CCTGTTGGACTTGGATTTGG-3′	20
Primer R	5′-AGCAAGCCGAAGACTACC-3′	20
Probe	5′-Cyan 500-CGCGATCATTACGCCGGGCTGACAGGTAATCAGATCGCG-BHQ1-3′	20

Results

Patient characteristics

A total of 58 confirmed non-HIV-infected CM patients were included in this study, 35 of them were male and 23 were female. The mean age at onset was 43.9 ± 14.9 years and the most heavily represented age group was 51–60 years (31.0%, 18/58). Although these patients had no HIV-infection, they had other underlying conditions, including systemic lupus erythematosus (SLE) (3.4%, 2/58), type 2 diabetes mellitus (3.4%, 2/58), kidney transplant (3.4%, 2/58) and tuberculosis (1.7%, 1/58). Among these 58 patients, 15 (25.9%, 15/58) had been treated with antifungal drugs, such as amphotericin B before admission to our hospitals and before the CSF specimens were taken. Details of patient history are shown in Table 2 and Table S4.

The clinical performance of the LFA

The positive detection rate of the LFA for clinical CSF specimens was 97.6% (83/85) when the reactivity above the titres of 1 : 2 was considered a positive result. The limit of detection (LoD) of the LFA varies among the specimens, ranging from 3 to 3×10^2 *Cryptococcus* cells ml^{-1} when it was used for testing the spiked CSF specimens (Table 2; Table S1).

The clinical performance of the LAMP

After DNA extraction, the positive detection rate of the LAMP for clinical CSF specimens was 81.0% (47/58). No cross-reaction was observed in other species after 90 min of LAMP reaction, including using DNA of a closely related sibling species, *Cryptococcus amylolentus* (CBS6039) (Fig. 1; Table S3). The LoD of the LAMP for *Cryptococcus* genomic DNA was 20 fg (approximately 30 genomic copies) per run (Fig. 1), but the corrected LoD of the LAMP was 3×10^2 *Cryptococcus* cells/mL when it was further evaluated with spiked specimens (Table S1). Approximately, 17.4% (19/109) of DNA specimens extracted from clinical and spiked specimens can be considered as good quality in our study (Table S2).

Comparison of conventional and molecular approaches for diagnosis of CM in non-HIV-infected patients

Comparison of the positive detection rates among the five assays showed that the LFA provided the highest scores (97.6%, 83/85; 95% CI, 91.8–99.7%), followed by the LAMP assay (87.1%, 74/85; 95% CI, 78.0–93.4%), qPCR (80.0%, 68/85; 95% CI, 69.9–87.9%), India ink staining (70.6%, 60/85; 95% CI, 59.7–80.0%) and culture (35.3%, 30/85; 95% CI, 25.2–46.4%) (Table 2). The LFA showed a significantly higher positive detection rate than the qPCR assay, India ink staining and culture ($P < 0.05$), whereas it was comparable to the LAMP assay ($P > 0.05$) (Table S5). The LAMP (70.8%) and the qPCR (75.0%) assays showed a higher positive detection rate than the culture and ink staining methods when using the clinical CSF with low LFA titres as the standard (≤1 : 80) (Table 3).

Discussion

Cryptococcal meningitis is primarily observed in HIV-infected patients worldwide. However, the infection also occurs in non-HIV-infected hosts, not only in Eastern Asia, but also in Australia and New Zealand (57%),[32] France (23%),[33] Germany (43%),[34] The Netherlands (50%)[35] and Mexico (18%).[36] However, the clinical performance of the LFA and other diagnostic assays is largely unknown in non-HIV-infected CM patients. Meanwhile, the information on molecular diagnosis of CM in clinical specimens is limited except for a recent study that used a normal PCR assay for CM in Brazil.[37] Therefore, we compared the conventional and molecular diagnostic assays for the diagnosis of CM using clinical CSF obtained from non-HIV-infected patients.

High sensitivity is important for microbial diagnostic techniques to be used in clinical settings. In this study, the positive detection rate for the diagnosis of CM in non-HIV-infected patients from clinical CSF is significantly higher with the LFA than that obtained for culturing and India ink staining. Our results are similar to previous results on the diagnosis of CM in HIV-infected patients.[15–18] Furthermore, our results showed that only the LFA had 100% sensitivity when compared with positive culture, the current gold standard for diagnosing CM from CSF. The conventional diagnostic assays such as India ink staining and culture for CM successfully detected *Cryptococcus* in 70.6% and 35.3% of the clinical specimens in our study respectively. Our results are lower than those previously reported from France (88.6% positive culture for clinical CSF).[33] One potential reason for the low culture success rate could be antifungal drug therapy for some of the patients analysed in our study. Specifically, 21.2% of the CM patients in our study were transferred from other hospitals due to poor

Table 2 Clinical CSF samples were detected using different diagnosis techniques.

Patients	Samples	Time	Conventional assays		Detection of CrAg LFA assay	Molecular detection	
			Ink staining	Culture		LAMP	qPCR (*Cq* value)
Patient 01	CSF 01*	05/01/2010	+	+	1 : 5120	+	+ (27.90)
Patient 02	CSF 02*	02/03/2010	+	+	1 : 1280	+	+ (27.11)
Patient 03	CSF 03*	12/03/2010	+	–	1 : 1280	+	+ (27.2)
	CSF 04	18/05/2010	+	+	1:320	+	+ (29.63)
Patient 04	CSF 04*	22/04/2010	+	–	1 : 320	+	+ (27.23)
	CSF 06	20/05/2010	+	+	1 : 160	+	+ (26.81)
	CSF 07	08/06/2010	+	–	1 : 160	+	+ (28.45)
Patient 05	CSF 05*	03/06/2011	+	+	1 : 2560	–	+ (27.11)
	CSF 09	15/06/2011	+	+	1 : 2560	+	+ (27.43)
	CSF 10	06/07/2011	+	+	1 : 1280	+	+ (27.36)
	CSF 11	21/07/2011	+	+	1 : 640	+	+ (27.57)
	CSF 12	09/08/2011	+	+	1 : 320	+	+ (27.79)
	CSF 13	03/09/2011	+	–	1 : 320	+	+ (27.92)
	CSF 14	10/10/2011	+	–	1 : 160	+	+ (27.53)
Patient 06	CSF 6*	03/06/2011	+	+	1 : 1280	+	– (36.63)
Patient 07	CSF 7*	08/07/2011	–	–	1 : 160	+	– (36.25)
	CSF 17	22/07/2011	+	–	1 : 160	+	+ (27.01)
	CSF 18	03/08/2011	+	–	1 : 160	+	+ (27.41)
Patient 08	CSF 8*	04/07/2011	+	–	1 : 5	+	+ (26.65)
	CSF 20	10/08/2011	+	–	1 : 5	+	+ (27.98)
	CSF 21	27/09/2011	+	–	1 : 5	+	+ (27.92)
Patient 09	CSF 09*	11/10/2011	+	–	1 : 2560	+	– (38.85)
Patient 10**	CSF 10*	28/09/2011	+	–	1 : 320	+	– (37.45)
	CSF 24	18/10/2011	+	–	1 : 80	+	+ (27.94)
	CSF 25	07/11/2011	+	–	1 : 80	+	– (36.63)
Patient 11	CSF 11*	03/11/2011	–	+	1 : 640	+	+ (28.65)
Patient 12**	CSF 12*	19/12/2011	+	–	1 : 80	+	+ (27.47)
	CSF 28	06/01/2012	+	–	1 : 80	+	+ (27.46)
Patient 13**	CSF 13*	15/02/2012	–	–	1 : 5	+	+ (27.94)
Patient 14	CSF 14*	21/03/2012	+	–	1 : 320	+	+ (26.63)
	CSF 31	05/04/2012	+	–	1 : 320	+	+ (27.47)
	CSF 32	21/04/2012	+	–	1 : 320	+	+ (27.46)
Patient 15	CSF 15*	09/05/2012	+	–	1 : 320	+	+ (27.94)
	CSF 34	16/05/2012	+	–	1 : 160	+	+ (27.38)
	CSF 35	22/05/2012	+	–	1 : 160	+	+ (27.49)
Patient 16	CSF 16*	08/08/2012	–	–	1 : 5	+	+ (28.82)
Patient 17	CSF 17*	10/10/2012	+	+	1 : 2560	+	+ (25.66)
Patient 18	CSF 18*	24/09/2012	–	–	–	+	+ (25.83)
	CSF 39	27/09/2012	+	–	1 : 5	+	+ (27.07)
	CSF 40	15/10/2012	+	–	1 : 20	+	+ (27.39)
Patient 19	CSF 19*	27/08/2012	+	+	1 : 640	+	+ (27.46)
	CSF 42	11/09/2012	+	+	1 : 320	+	+ (27.94)
	CSF 43	15/10/2012	+	+	1 : 640	+	+ (27.38)
	CSF 44	27/10/2012	+	–	1 : 320	+	+ (27.49)
Patient 20	CSF 20*	13/12/2012	+	–	1 : 2560	–	– (36.67)
	CSF 46	14/01/2013	+	+	1 : 1280	+	+ (25.66)
	CSF 47	04/02/2013	+	+	1 : 1280	+	+ (25.83)
Patient 21	CSF 48*	08/03/2013	+	–	1 : 1280	+	+ (27.07)
Patient 22	CSF 49*	09/07/2013	+	–	1 : 640	+	+ (27.39)
Patient 23	CSF 50*	11/04/2013	+	+	1 : 2560	+	+ (27.01)
Patient 24	CSF 51*	25/11/2013	+	+	1 : 5120	+	– (38.21)
Patient 25	CSF 52*	21/11/2013	+	–	1 : 2560	+	+ (27.01)
Patient 26	CSF 53*	25/02/2014	–	+	1 : 5120	+	+ (27.4)
Patient 27	CSF 54*	17/02/2014	+	–	1 : 5120	+	+ (27.47)
Patient 28	CSF 55*	12/08/2014	+	+	1 : 5120	+	– (36.65)

(continued)

Table 2 (continued)

Patients	Samples	Time	Conventional assays		Detection of CrAg LFA assay	Molecular detection	
			Ink staining	Culture		LAMP	qPCR (*Cq* value)
Patient 29	CSF 56*	12/09/2014	+	+	1 : 5120	+	+ (23.08)
Patient 30	CSF 57*	25/10/2012	+	+	1 : 1280	+	+ (22.46)
Patient 31	CSF 58*	04/11/2012	–	–	1 : 160	+	+ (28.82)
Patient 32**	CSF 59*	08/12/2012	–	–	1 : 40	–	+ (28.85)
Patient 33**	CSF 60*	08/03/2013	+	–	1 : 160	+	+ (27.98)
Patient 34**	CSF 61*	09/10/2013	–	–	1 : 40	+	– (39.02)
Patient 35	CSF 62*	13/12/2013	+	+	1 : 640	+	+ (26.63)
Patient 36	CSF 63*	03/07/2013	–	+	1 : 640	+	– (38.21)
Patient 37	CSF 64*	15/01/2013	+	–	1 : 640	+	– (39.46)
Patient 38	CSF 65*	27/09/2012	+	+	1 : 1280	+	+ (28.65)
Patient 39	CSF 66*	19/09/2012	+	–	1 : 640	–	– (35.74)
Patient 40	CSF 67*	02/08/2013	–	+	1 : 640	–	– (36.46)
Patient 41**	CSF 68*	28/07/2014	–	–	1 : 80	–	– (36.65)
Patient 42**	CSF 69*	28/07/2014	–	–	1 : 40	+	+ (26.56)
Patient 43	CSF 70*	28/08/2014	+	+	1 : 1280	+	+ (27.98)
Patient 44	CSF 71*	28/08/2014	–	–	1 : 40	+	+ (28.85)
Patient 45	CSF 72*	29/08/2014	+	–	1 : 640	+	+ (28.45)
Patient 46**	CSF 73*	05/09/2014	+	–	1 : 160	+	+ (27.94)
Patient 47**	CSF 74*	05/09/2014	–	–	1 : 80	–	+ (29.63)
Patient 48**	CSF 75*	15/09/2014	–	–	1 : 40	–	– (35.74)
Patient 49	CSF 76*	18/08/2014	–	+	1 : 40	+	+ (26.63)
Patient 50**	CSF 77*	12/09/2014	–	–	1 : 160	+	+ (23.7)
Patient 51	CSF 78*	18/09/2014	–	–	1 : 320	+	+ (22.78)
Patient 52	CSF 79*	30/09/2014	–	+	1 : 1280	+	+ (21.80)
Patient 53	CSF 80*	30/09/2014	–	–	1 : 160	+	+ (28.65)
Patient 54**	CSF 81*	08/10/2014	–	–	1 : 40	+	+ (26.56)
Patient 55**	CSF 82*	13/10/2014	–	–	1 : 1	–	– (36.46)
Patient 56**	CSF 83*	06/10/2014	–	–	1 : 80	–	– (37.78)
Patient 57	CSF 84*	04/08/2014	–	–	1 : 20	+	+ (28.82)
Patient 58	CSF 85*	09/09/2014	+	–	1 : 320	–	+ (21.20)

ND, no data.

*CSF samples were collected from the patients when they underwent laboratory examination on admission.

**Patients were transferred from other hospitals who had been treated with antifungal drugs before admission in our hospitals.

therapeutic effects and/or other reasons. Almost all of these CM patients had been treated with antifungal drugs prior to admission to the study hospitals. Such treatments could have killed the cells without affecting the antigen, contributing to the relatively low culture positive rate in this investigation as compared to the study by Dromer *et al.* [33] In addition, the detection limit of the LFA and the LAMP assay were both approximately 300 *Cryptococcus* genomic copies when tested using spiked CSF specimens. This is similar to values obtained by the Luminex *x*MAP assay (10^1–10^3 cells per run),[25] and superior to results obtained by India ink staining (10^3–10^4 cells per run).[38]

The detection rate of the LAMP or the qPCR was significantly higher ($P < 0.05$) than those obtained by India ink staining or culture, and they were comparable to one another ($P > 0.05$). Although the positive detection rate of LAMP (87.1%) is higher than the qPCR (80.0%), there is no statistical significance ($P > 0.05$) between these two molecular assays, which could be related to the different working principle and different targeting genome regions of these two molecular assays. It may be difficult for physicians to make an accurate diagnosis of CM when the titres of CrAg are low, and results of the conventional diagnostic assays are negative, particularly as is the case during early stages of CM. In our laboratory, we normally consider CrAg ranging from 1 : 2 to 1 : 80 as low LFA CrAg titres, and recording low titres is not unusual in the study hospitals. In this study, the results of the molecular assays showed a relatively high positive detection rate (approximately 75%) for clinical CSF specimens with low LFA CrAg titres. The characteristics demonstrate

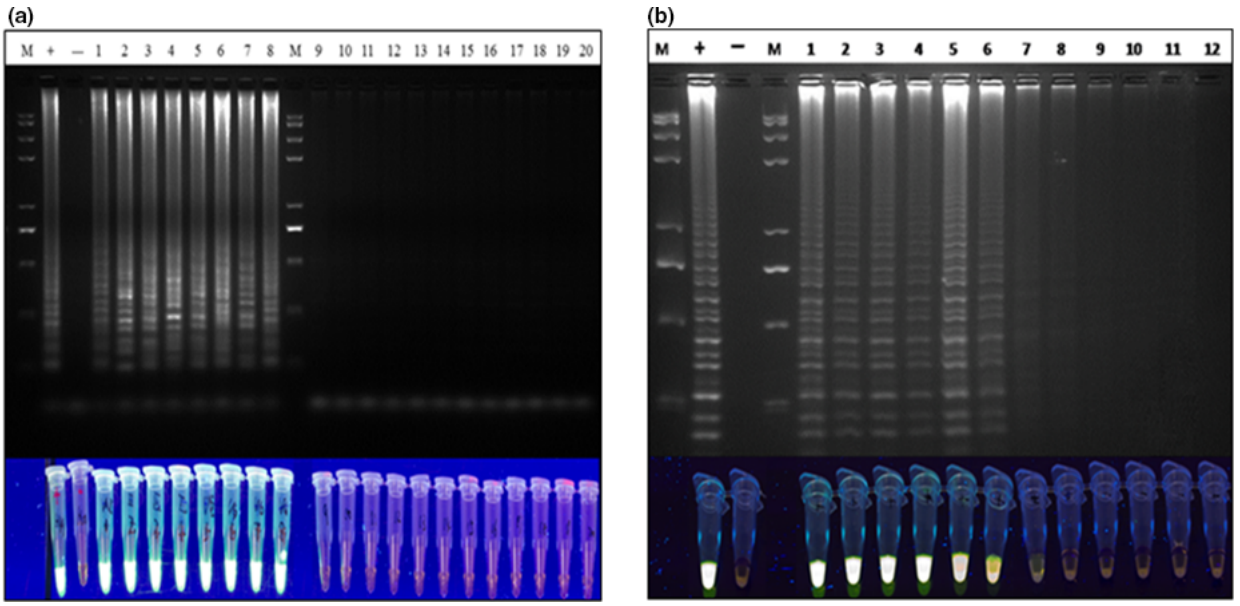

Figure 1 Gel and fluorescence representation of the specificity(a) and sensitivity (b) of the *C. neoformans*/*C. gattii* species complex using the LAMP primers. a, Lane M: DL2000 plus II DNA ladder (0.1–5 kb); lanes 1–8, eight genotypes of the species complex [CBS10085 (VNI), CBS10083 (VNII), CBS10080 (VNIII), CBS10079 (VNIV), CBS10078 (VGI), CBS10082 (VGII), CBS10081 (VGIII), CBS10101 (VGIV)]; lanes 9–20, other related species (CBS6039, *Cryptococcus amylolentus*; CBS7140, *Cryptococcus laurentii*; CBS2994, *Cryptococcus uniguttulatus*; CBS6819, *Cryptococcus podzolicus*; CBS5029, *Cryptococcus skinneri*; CBS570, *Cryptococcus curvatus*; CBS9964, *Cryptococcus fagi*; CBS922, *Cryptococcus albidus*; CBS2206, *Cryptococcus macerans*; ATCC10671, *Cryptococcus luteolus*; CBS7748, *Trichosporon asahii*; CBS8758, *Candida albicans*). b, Left to right: Lane M: DL2000 plus II DNA ladder (0.1–5 kb); lane+, positive control, 20 ng DNA from the strain H99; lane-, negative control, ddH_2O; lanes 1–12: 3.0×10^6, 3.0×10^5, 3.0×10^4, 3.0×10^3, 3.0×10^2, 3.0×10^1, 3.0, 3.0×10^{-1}, 3.0×10^{-2}, 3.0×10^{-3}, 3.0×10^{-4}, 3.0×10^{-5} copies of DNA respectively. Sensitivity testing showed that LAMP yields positive signals in a wide range of genomic DNA, as low as 3.0×10^1 copies of DNA.

Table 3 Comparison of LAMP, qPCR, Indian ink staining and culture for diagnosing CM in non-HIV-infected patients with different titres of CrAg.

	Indian ink staining			Culture			LAMP			qPCR		
Titres of CrAg	+	–	Positive rate (%)	+	–	Positive rate (%)	+	–	Positive rate (%)	+	–	Positive rate (%)
1 : 5120 (n = 6)	6	0	100%	5	1	83.3%	6	0	100%	4	2	66.7%
1 : 2560 (n = 7)	6	1	85.7%	4	3	57.1%	5	2	71.4%	5	2	71.4%
1 : 1280 (n = 11)	10	1	90.9%	8	3	72.7%	11	0	100%	10	1	90.9%
1 : 640 (n = 11)	8	3	72.7%	7	4	63.6%	9	2	81.8%	7	4	63.6%
1 : 320 (n = 12)	11	1	91.7%	2	10	16.7%	11	1	91.7%	11	1	91.7%
1 : 160 (n = 8)	4	4	50.0%	0	8	0%	8	0	100%	7	1	87.5%
≤1 : 80 (n = 24)	9	15	37.5%	1	23	4.2%	17	7	70.8%	18	6	75.0%

+, positive; –, negative.

that the molecular assays have the potential to be an alternative to the LFA for the diagnosis of CM in the clinic. Certainly, realising this potential will rely on the development of improved protocols for the extraction of cryptococcal DNA from clinical CSF specimens in the future.

The World Health Organization (WHO) has compiled the ASSURED criteria for evaluating diagnostic tests suitable for use in developing countries.[19] The LFA has been widely recognised as meeting the ASSURED criteria for diagnosing CM, even in resource-limited regions, such as those in sub-Saharan Africa and Southeast Asia.[3,4] Our results demonstrate that the LFA should also be used for diagnosing non-HIV-infected CM patients because most of them also come from resource-limited regions.

The limitation of this study was that it was a retrospective study applied to specimens known to be associated with CM. Therefore, we cannot calculate the parameters of these diagnostic techniques such as specificity and sensitivity in broad specimens. Previous studies have shown high specificity and sensitivity of the LFA for diagnosing CM in HIV-infected patients.[15–18] The present results suggested that the LFA for diagnosing CM should have a similar performance in non-HIV-infected patients to that reported in HIV-infected patients. Certainly, a prospective diagnostic study on CM in a non-HIV-infected population is needed in the future.

In summary, our study suggests that the LFA should be recommended for diagnosing CM in a non-HIV-infected population, and it has a high level of agreement with the current gold standard, positive culture. Molecular diagnostic techniques, such as the LAMP or qPCR assays, have the potential to be used for the diagnosis of CM in the clinic in the future. However, more efficient protocols for the extraction of *Cryptococcus* DNA from clinical CSF specimens are needed in order to improve the sensitivity of the molecular methods.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MC participated in molecular test and drafted the manuscript. JZ, JL and ML carried out the traditional and molecular diagnostic studies. JS and WJF performed the statistical analysis. TB, JPX and AMS participated in designing this study and revising the manuscript. WQL and WHP conceived and designed the study. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Detection of the *C. neoformans*/*C. gattii* species complex in spiked CSF specimens using the LAMP assay.

Table S2. The evaluation of DNA extracted from clinical CSF specimens and spiked CSF specimens using absorption ratios at $OD_{260/280}$ assessed by fluorescence in this study.

Table S3. Fungal isolates ($n = 81$) tested in the LAMP assay for special detection of the *C. neoformans*/*C. gattii* species complex in this study.

Table S4. The characteristics of CM patients included in this study; *Patients were from Shanghai Huashan hospital.

Table S5. Pairwise comparison of diagnostic assays for CM in non-HIV-infected patients in this study.