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Abstract:

Laboratory diagnosis of histoplasmosis is based on various methods including microscopy, culture, antigen and DNA detection of *Histoplasma capsulatum* var. *capsulatum* (*Hcc*) or H. *capsulatum* var. *duboisii* (*Hcd*). To improve sensitivity of existing quantitative PCR assays, we developed a new reverse transcriptase qPCR (RTqPCR) assay allowing amplification of whole nucleic acids of *Histoplasma* spp.. and validated on suspected cases.

The limit of detection was 20 copies and the specificity against 114 fungal isolates/species was restricted to *Histoplasma* spp.. Whole nucleic acids of 1,319 prospectively collected consecutive samples from 907 patients suspected of histoplasmosis were tested routinely between May 2015 and May 2019 in parallel with standard diagnostic procedures performed in parallel. 44 had proven histoplasmosis due to Hcc (n=40) or Hcd (n=4) infections. RTqPCR was positive in 43/44 patients (97.7% sensitivity), in at least one specimen. Nine out of 863 cases (99% specificity) were RTqPCR positive and therefore classified as possible cases. RTqPCR was positive in 13/30 (43.3%) blood tested in proven cases. A positive RTqPCR in blood was significantly associated with *Hcc* progressive disseminated histoplasmosis with a positive RTqPCR in 92.3% of the immunocompromised patients with disseminated disease. This new *Histoplasma* RTqPCR assay enabling amplification of *hcc* and *hcd* is highly sensitive and allows the diagnosis of histoplasmosis advantageously from blood and BAL.

The fungus *Histoplasma* was first described in Panama by Samuel Taylor Darling in a lung sample from a patient from Martinique (French Caribbean islands)[1] by observation of free or intracellular small yeasts in a smear of respiratory specimen. Since then, two distinct varieties have been described in humans initially identified by morphological aspects in tissues and epidemiological criteria (i) *Histoplasma capsulatum* var. *capsulatum* (*Hcc,* New World human pathogen) associated with small ovoid (2-3 µm) yeasts, and (ii) *Histoplasma capsulatum* var. *duboisii* (*Hcd,* Old World human pathogen) associated with large citrus shaped yeasts (5-10 µm). A third variety was described in Africa, *Histoplasma capsulatum* var. *farciminosum* (Old World animal pathogen) associated with epizootic lymphangitis in horses. However, this classification has been revisited based on recent multilocus sequencing revealing several cryptic species, which are structured according to their geographical origin.[2] *Histoplasma capsulatum* is now composed of at least four phylogenetically different groups,[3] including a specific group for var. *duboisii*. *Histoplasma capsulatum* var. *farciminosum* appeared to be polyphyletic suggesting this variety does not correspond to a specific phylogenetic group.[3]

Recent analyses of multiple isolates by whole genome sequencing recognized the existence of four phylogenetic species corresponding to the geographical clades of North America (2 species), Latin America and Panama. The African variety (*Hcd*) is still not considered a different species mainly due to the small number of isolates studied so far.[4]

Nowadays, diagnosis of histoplasmosis still relies on microscopic direct examination and culture, together with antibody or antigen detection.[5] In addition, PCR methods based on the amplification of various DNA targets and using different methodologies have been reported[6–14] with a sensitivity ranging from 67 to 100% depending on the studies.[15] More recently, real-time quantitative PCR (qPCR) methods based on DNA detection[7,11,16–18] have been developed for human diagnosis to overcome the limits of the microbiological culture. Indeed, qPCR

assays are expected to be more sensitive,[16] more rapid than culture and prevent laboratory transmission of the mold form of *Histoplasma* spp. in the laboratory environment.[19,20]

A *Histoplasma* spp. RTqPCR assay was developed and validated based on a repeated gene target and the detection of whole nucleic acids (RNA plus DNA) to improve the specificity and the sensitivity of microscopy and culture and accelerate results. After validation, this RTqPCR was performed prospectively during four years and then evaluated its performance in all patients suspected of histoplasmosis in this French network.

Material and methods

Nucleic acids extraction

Extraction procedures were initially optimized. Preliminary experiments on a patient with a positive qPCR in blood (Supplemental Table 1) showed a 100-fold increased analytical sensitivity upon bead beating compared to without mechanical lysis (ΔCq=6.54). Therefore, all specimens were processed with bead beating in this study. Another preliminary experiment showed that whole blood was 100-fold increased analytical sensitivity as compared to plasma (ΔCq=6.7) (Supplemental Figure 1A and 1B) to detect circulating *Histoplasma* spp. nucleic acids. Therefore, 1.3 mL of whole blood was used with bead beating instead of serum or plasma in this study. Serum and plasma specimens were not considered further as relevant sample type.

Whole nucleic acids (WNAs) were extracted from non-fixed specimens using initial bead beating in a Precellys bead beater (Bertin Technologies, Montigny-le-Bretonneux, France). Then, the tubes were centrifuged 5 min at 10,000 g at 4°C. WNAs from the supernatant were extracted with addition of 10 µL/sample of 1:5 diluted internal control (DNA Virus Culture, DICD-CY-L100, Diagenode, Seraing, Belgium) using a Qiasymphony (Qiagen, Hilden, Germany) with the Virus Pathogen extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. WNAs were eluted in 85 µL volume. For the paraffin-embedded tissue specimens, extraction of three 10 µm-sections was performed using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations with a volume of elution of 110 µL in DNA grade water. All extracts and aliquots were stored at +4°C until use (less than 5 days). In a specific preliminary experiment comparing WNA and DNA amplification, the RTqPCR kit was used without or with the reverse transcriptase step (15 min at 50°C) for DNA and WNA amplification, respectively.

Selection of target gene

The ribosomal small subunit RNA gene of *H. capsulatum* (GenBank accession number, GG663449.1) was used to design primers and probes using Primer3web v4.0.0 software. Outer primers (Hc_7F: GATGATGGCTCTGATTGAACG and Hc_361R: GATGATGGCTCTGATTGAACG) were first used to analyze the *Histoplasma* sequence in the region of interest of this gene for the presence of polymorphism. The amplicons obtained with two strains of *Hcc* (CNRMA16.205, GenBank MW317018 and CNRMA17.309, GenBank MW317017), one strain of *Hcd* (CNRMA16.638, GenBank MW317134) were sequenced resulting in a 100% (357/357bp) and 99.9% (356/357bp) similarity with the reference sequence (GenBank GG663449.1), respectively. The final assay was then designed in conserved regions excluding the single-nucleotide polymorphism observed in *Hcd*. Of note, no polymorphism at the primers and probe loci were observed upon sequencing of isolates from various lineages (GenBank MW317018, MW317017, MW317134)

Whole nucleic acid reverse transcriptase quantitative PCR assay (RT-qPCR)

WNA amplification was performed using the following conditions: 1× Invitrogen RT-qPCR buffer mix (Superscript III One step RT-PCR, Life Technologies Corporation, Carlsbad, CA, USA), 0.3 µM of each primer (Hc_24_55F: CGTACGACATCATATTAAAAATGA and Hc_22_128R: CTTTCTTTAAGGTAGCCAAAAT), 0.1 µM of probe (Hc_21_79P: FAM-TGTAGTGGTGTACAGGTGAGT-BHQ1), and 1 µM of Superscript III Platinum enzyme, in a total of 25 µL with 8 µL of WNA extract. The amplification consisted of one step of RT-PCR at 50°C for 15 min, followed by qPCR with one activation step at 95°C for 2 min and 50 cycles of denaturation at 95°C for 15 sec and annealing at 53°C for 30 sec. All the qPCR runs were

performed on a Light Cycler 480 thermocycler (LC480-II; Roche Diagnostics, Mannheim, Germany) with quantitative cycle (Cq) determination using the calculation of the second derivative of the amplification curve.[21]

Specificity

A total of 114 fungal strains of 99 species was tested in this study to assess the specificity of the qPCR assay. These strains are listed in Supplemental Table 2 and 3.

qPCR efficiency and limit of detection

A 129-base pair DNA amplicon containing the PCR target locus of the RTqPCR assay was synthetized at 12.57 fmol/ng (gBlock Gene Fragments, IDT, Coralville, Iowa, USA), diluted at different concentrations (100,000 to 1 copies/well) and tested on a LC480-IIThe standard curve allowing PCR efficiency calculation was obtained based on the result of two replicates of five 10-fold serial dilutions of the WNA of *Hcc* strain CNRMA16.205. Regression lines were constructed automatically by plotting the logarithm of the initial template concentration versus the corresponding Cq value by using Analysis package included in LightCycler 480 software v. 1.5 (Roche Diagnostics, Mannheim, Germany).[22]. In addition, 50, 20 and 10 copies dilutions were tested in ten replicates to obtain the limit of detection.

Comparison of three qPCR assays

The evaluated in-house RTqPCR targeting *mtSSU* was compared on the LC480-II with two previously published qPCR assays targeting Internal Transcribed Spacer (*ITS*) 1 regions of *H. capsulatum* following the published protocols.[11,16] Quantitative PCR reactions assay from Simon et al. publication (qPCR 1) was carried out as advised by the authors in a final volume

of 25 µL containing 1x LC480-II Probes Master, 0.9 µM of each primer, 0.25 µM of the probe, 7 µL of template DNA [16]. The qPCR assay from Buitrago et al. (qPCR 2) was carried out as advised by the authors in a final volume of 25 µL containing 2x LC480-II Probes Master, 0.5 µM of each primer, 0.2 µM of the probe, 7 µL of template DNA [11]. A total of 10 WNAs were tested including four WNA extracted from the two CNRMA strains of each *Hcc* (CNRMA17.309 and CNRMA16.205) and *Hcd* (CNRMA16.638 and CNRMA17.108) adjusted at 1 ng/µL, and positive WNA samples using the RTqPCR (n=6) from whole blood, one bone marrow aspiration, one lymph node, one digestive biopsy (4 patients infected with *Hcc*), and one bone biopsy and one brain abscess (2 patients infected with *Hcd*). The three assays were performed the same day on the same LC480-II and the dilutions aliquots realized extemporaneously. A standard curve allowing PCR efficiency calculation was obtained for each assay based on the result of PCR duplicates in six serial dilutions WNAs.

Patient classification

Patients included are consecutive patients admitted to hospitals from a French network from May 2015 to May 2019 as part of a routine procedure of diagnosis. All patients were at risk of histoplasmosis, as they all originated or travelled from endemic areas, and had compatible clinical symptoms. Routine diagnosis of histoplasmosis in metropolitan France does not include either detection of *Histoplasma* antigens because of the rarity of imported cases, nor detection of anti-*Histoplasma* antibodies for lack of accuracy to detect acute histoplasmosis in immunocompromised patients and even in immunocompetent patients from endemic areas.[5] Clinical data were recorded retrospectively for all PCR positive patients. Each histoplasmosis case was classified according to the EORTC/MSG criteria[23] and described according to the 2007 Infectious Diseases Society of America guidelines[24] and clinical textbook

213 on histoplasmosis.[25] For included each patient, the normal clinical work up for suspicions of
214 histoplasmosis (reference tests: histopathology and culture) have been performed according
215 to the clinical presentation and the technical method available locally. The results of these
216 tests have been collected retrospectively in all PCR-positive patients. In most cases, the results
217 of the reference tests were not available at the time of the PCR testing. PCR results were
218 delivered as a routine test to the prescriber. The probable case category defined as an isolated
219 positive antigen test was not possible as antigen testing is not routinely performed in France.
220 Disseminated cases were defined as those with ≥2 non-contiguous clinical locations associated
221 with mycological diagnosis on at least one lesion. Localized cases were those with lesions
222 observed only in one organ. Nevertheless, histoplasmosis cases where only qPCR was positive
223 without other mycological criteria (histopathology, direct microscopy, culture) in a compatible
224 epidemiological (living or having traveled in an endemic area for histoplasmosis) and clinical
225 context (including sepsis, respiratory, digestive, neurological hematological, dermatological
226 symptoms and/or lesions of deep organs) with a good outcome under adapted therapeutic
227 management were classified as possible cases. Galactomanan test (Biorad, Platelia) have been
228 performed as part of the routine procedure in histoplasmosis suspicion in centers were this
229 test was available.
230
231 **Samples**
232 The samples were taken from the patients prospectively at the time of the clinical suspicion
233 and sent to the Mycology-Parasitology laboratory, Saint-Louis Hospital, Paris, France for
234 routine testing. They consisted in various specimens including whole blood, tissue biopsies
235 (lungs, digestive tract, adrenal glands), liquids (bronchoalveolar lavage fluid, cerebrospinal
236 fluid, bone marrow aspirate, pleural, peritoneal, pericardial exudates) or skin/mucous

scrapings (skin or mucous ulcerations). Microscopic examination and culture and/or histology were performed in parallel to the RTqPCR. Paraffin-embedded tissue sections were also used in two cases requiring specific extraction procedures.

Polyphasic identification of clinical strains

Histoplasma isolates were sent to the National Reference Center for Invasive Mycoses & Antifungals, Institut Pasteur, Paris, France for identification which was handled in a Biosafety Level-3 (BSL-3) laboratory. Strains were sub-cultured on Sabouraud chloramphenicol agar slants and incubated at 27-30°C until sufficient sporulation was attained. The observed macroscopic features included white colonies with fine aerial mycelia or flat tan colonies diffusing a dark brown pigment with age. Microscopic preparations on lactic blue revealed the presence of thick-walled tuberculate macroconidia and smooth-walled microconidia. Molecular analyses were performed by sequencing the ITS1-5.8S-ITS2 (ITS) region of the ribosomal deoxyribonucleic acid (rDNA)[26] to confirm the identification of the genus. The small region (772 bp) of the gene *prp8*[27] allowed the differentiation of *Hcd* from the other geographical clades of *Histoplasma capsulatum* with a 97% degree of similarity between them. Of note all *hcd* cases had a typical positive direct examination showing the characteristic 5-10µM ovoid yeasts in specific specimens.

Statistical analysis

Intended sample size was not determined as the accuracy was unknown as a new assay and as the clinical characteristics of the patients was not targeted in this study. No indeterminate reference and PCR tests and no missing data on evaluated items were observed in this study. Median and interquartile range are given for specific descriptions in parameters with non-

normal distribution. Non-parametric paired t-test were performed to compare WNA and DNA Cq and for Cq comparison of the qPCR assays. Contingency tables and Fischer's exact test were performed to analyze the statistical link between clinical presentation, underlying disease, visited countries, positive galactomannan test and a positive qPCR test using Prism v9.0 (Graphpad, San Diego, CA, USA).

Ethics

All specimens have been tested as part of the routine diagnostic procedure in Saint-Louis Hospital, Paris, France (current care) and were not part of a clinical trial. No specific ethic validation was required. Patients' data have been recorded retrospectively and collected in an anonymized file. The data collection has been declared at the French Commission Nationale de l'Informatique et des Libertés (CNIL) under the number: YNb2747338P.

Results

Evaluation and validation of the *Histoplasma* RTqPCR

Interest of WNA over DNA – Limit of Detection – Efficiency.

The analytical performance of the RT-qPCR amplifying WNA was first compared to qPCRs amplifying only DNA. A comparison of the results on 11 WNA extracts from 3 patients showed that the sensitivity on WNA (11/11, 100%) was higher than on DNA (9/11, 81% specimens detected). In addition, WNA gave significantly lower Cq values (median 40.3 [29.5-41.2]) compared to DNA (median 40.2 [31.6-45]) (P=0.006, Figure 1). The limit of detection (LOD90) of the RTqPCR assay was 10 copies (Supplemental Table 4). The efficiency of the RTqPCR was 1.9 (95%) (Supplemental Table 4).

Analytical specificity

A total of 114 strains from 99 different species were tested showing no cross-reactivity except for *Histoplasma* species (*Hcc* and *Hcd*) and 3 closely related species *Nannizziopsis* sp., *Emmonsia pasteuriana,* and *Emmonsia crescens* (Supplemental Table 2 and 3). The ability to detect 0.1 ng of DNA of various genetical clades of *Histoplasma capsulatum* including *Hcd,* LAm B, LAmE and RJ were similar (mean Cq = 22.51±0.82) whereas the 3 close relatives were not amplified in these conditions.

Comparison with qPCR assays

The RTqPCR assay was compared to two qPCR assays from the literature using 6 *Hcc* WNA and 4 *Hcd* WNA and gave significantly lower Cq values compared to qPCR 1 and qPCR 2 assays (P=0.004) (Figure 2A). Of 6 clinical specimens, one was not detected with qPCR 2. The RTqPCR

299 gave significantly lower mean Cq values (27.26 and 22.1) than qPCR 1 (31.0 and 25.3) and
300 qPCR 2 (31.6 and 28.8) assays targeting ITS for *Hcc* (P=0.008) or *Hcd* (P=0.042), respectively
301 (Figure 2B, Supplemental Table 5).
302
303 **Clinical evaluation**
304 Over 4 years, 1,319 samples from 907 patients suspected of histoplasmosis were tested
305 (Figure 3). A total of 44 (4.9%) patients had proven histoplasmosis based on a positive direct
306 examination and/or culture (Table 1, Table 2). In these 44 proven cases, RTqPCR was positive
307 in 43/44 (97.7% sensitivity) patients in at least one specimen (median: 2 RTqPCR-positive
308 specimens/patient) (Table 2). In addition to proven cases, 9 patients out of 863 (99%
309 specificity) with clinical presentation and exposure evocative of histoplasmosis were RTqPCR
310 positive. Positive and negative predictive value were 0.82 and 0.99, respectively, resulting in
311 a likelihood ratio of 93.7.
312 In proven patients, immunosuppression was reported in 25/44 (56.8%) patients, while 19/44
313 (43.2%) had no known major cause of immunosuppression and thereafter considered as
314 immunocompetent. The underlying disease, the clinical presentation, the country of origin or
315 travel history, and the species identified are summarized in Table 2.
316 In proven progressive disseminated histoplasmosis due to *Hcc* (23 patients), RTqPCR in
317 respiratory specimens was positive in 13/14 (92.3%) of the patients sampled (17/19, 88.9% of
318 the tested specimens) and in 7/7 patients with skin/mucosa sampled (8/9, 88.9% of tested
319 specimens). RTqPCR in bone marrow aspirates was positive in 8/8 (100%) patients tested
320 (9/10, 90% tested specimens) (Table 3). RTqPCR in blood was positive in 13/17 (76.5%)
321 patients tested (25/48, 52.1% of tested specimens). In the subgroup of disseminated *Hcc*
322 disease in immunocompromised patients (n=18) and HIV patients (n=12), the RTqPCR in blood

was positive in 24/44 (54.5%) specimens and 12/13 (92.3%) patients, and 20/38 (52.6%) and 10/11 (90.9%), respectively (Table 3). In proven acute pulmonary ± mediastinal lymphadenitis, RTqPCR was positive in 12/12 (100%) patients tested with 12/14 (85.7%) positive specimens tested (BAL, biopsies, lymph nodes) (Table 2). In CNS histoplasmosis, RTqPCR in CSF was positive in the 2/2 (100%) cases (4/7, 57.1% of tested specimens) (Table 2). Disseminated *Hcd* infections had various RTqPCR positive sample types, including skin, bone, brain, urinary and digestive tract specimens (Table 2).

A total of 169 specimens were recovered from these 44 proven cases including 97 PCR-positive specimens (positivity rate = 57.4%). In 94 specimens investigated in parallel to RTqPCR for microscopy and/or culture, PCR was positive in 59/64 (92.2%) specimens associated with a positive microscopy or culture vs. in 10/30 (33.3%) specimens associated with a negative microscopy or culture. In addition, 28/75 (37.3%) specimens not investigated in parallel with microscopy and/or culture were RTqPCR positive.

Most of the possible cases (RTqPCR positive / culture negative) had localized diseases (8/9, 88.9%) [lung (7/8), digestive tract (1/8)] and only 1/9 (11.1%) had a disseminated disease (Patient #16, immunocompetent, with a negative microscopy and negative culture in blood and BAL). RTqPCR positive specimens were respiratory specimens (n=8) and digestive tract (n=1) (Table 2). Parallel culture and/or direct examination/histopathology were negative in the seven patients for whom it was done in parallel to RTqPCR (three patients had no other investigation but the PCR to assess mycological diagnosis in a compatible epidemiological and clinical context). In one case under immunosuppressive therapy (#50), galactomannan detection was positive (index >0.5). Three out of 9 (33.3%) were immunocompromised and all (9/9, 100%) lived or travelled in endemic areas. For all patients, no other diagnosis explained

346 the symptoms. Blood was tested negative in 7 cases out of 7 tested. No treatment and follow
347 up were available for these patients.
348 One proven case of histoplasmosis has been reported out of the 855 (0.12%) RTqPCR-negative
349 patients. This case had a negative RTqPCR test in BAL and blood but the diagnosis has been
350 done on the microscopic visualization of yeasts evocative of *Hcc* in a lung biopsy (not tested
351 by RTqPCR) before lung transplantation.
352
353 *Blood RTqPCR for dissemination and follow-up analysis*
354 A median of 1 blood specimen [range 1-9] per patient was tested (Table 1). RTqPCR in blood
355 obtained within the first 7 days after diagnosis was positive only in 13 proven cases out of 30
356 cases with blood tested before treatment (43.3%). RTqPCR positivity in blood and not in all
357 specimens was significantly associated with the underlying disease and the clinical
358 presentation (<0.01, Table 4). Indeed, a positive RTqPCR in blood was significantly associated
359 with proven progressive disseminated histoplasmosis due to *Hcc* with an increased rate of
360 positive RTqPCR in disseminated (13/17, 76.5%) vs. localized (0/13, 0%) cases (P<0.0001). In
361 acute pulmonary, CNS and localized histoplasmosis, the RTqPCR in blood was never positive
362 (Table 3). The same feature was observed considering specimens (chi-square, P=0.001), with
363 25/58 (43.1%) positive RTqPCR in blood specimens from disseminated vs. 0/24 (0%) in blood
364 specimens from localized cases (P<0.0001) (Table 4).
365 RTqPCR detection in blood was significantly associated with the underlying disease (P=0.005),
366 with RTqPCR positivity rate significantly increased in 10/18 (55.5%) immunocompromised
367 proven cases vs. 1/10 (10%) in immunocompetent proven cases (P=0.041). The association
368 was also significant if blood samples were considered with RTqPCR positivity observed in
369 24/60 (40.0%) specimens from immunocompromised vs. 1/22 (4.5%) in specimens from

immunocompetent cases (P=0.003 (Table 4). Of note, blood was obtained from 3/4 immunocompetent patients with *Hcd* disseminated infections and RTqPCR was negative in all 3 cases (100%). Thirty-seven blood samples from 9 patients with at least one RTqPCR positive and one follow-up blood sample were studied for a median follow-up duration of 25 days [13.5-82] and with a median number of blood sample per patient of 3 (Table 5). All patients were treated with liposomal amphotericin B at D0 (date of the first positive blood specimen). The kinetics of the fungal load is plotted in Figure 4A. The rate of negativation was dependent on the initial fungal load (Figure 4B) with a R^2 at 0.58 and a slope at -1.04 ± 0.44. This gain of 1 Cq per day corresponded approximately to a 2-fold decrease of the fungal load per day of treatment. Of note, for 3 of the 9 patients (Patients # 14, 38, 45), RTqPCR remained positive as the patient was lost (Table 5). In Patient#14 (HIV positive and inobservant), RTqPCR was still positive 334 and 611 days after the first positive result in blood with no intermediate testing.

Discussion

The present evaluation of this reverse transcriptase qPCR (RTqPCR) assay demonstrates an increased sensitivity of the Histoplasma detection using WNA instead of DNA and *mtSSU* gene instead of *ITS* as target gene. Thus, we were able to detect WNA in 97.7% (43/44) of microscopy and/or culture proven cases in contrast with recent publications comparing *ITS* Loop-mediated Isothermal Amplification (LAMP) PCR and *HSP100* nested PCR for which a positive PCR was observed in only in 54% and 64% of the culture positive specimens, respectively.[10] The high sensitivity of the present assay also allowed detection of Hcc disseminated diseases in the blood of 92.3% of immunocompromised patients and the detection of 9 patients including seven patients for whom the conventional mycology investigations were tested negative. Moreover, the high sensitivity of the test allows a rapid follow-up of treatment efficacy with the observation of a 2-fold decrease of the fungal load per day during optimal treatment.

To obtain a high sensitivity in proven cases not only confirms the diagnosis before the culture and without manipulating it and the hazard of laboratory-associated infection, but also allows a better delineation of dissemination in the patients with proven histoplasmosis. Indeed, the RTqPCR was able to detect 10 more specimens than with conventional diagnosis in proven cases. Unsurprisingly, a positive RTqPCR in blood was found significantly associated with immunocompromised patients and with disseminated infection, as already observed.[28] Indeed, more than 50% of HIV-positive patients with histoplasmosis had positive blood culture.[29] In this study, from the 30 patients with proven histoplasmosis and blood tested, 15 were HIV-positive patients and 10/15 (66.6%) had a positive RTqPCR in blood. No blood culture was positive in these patients. Of note, a positive RTqPCR in blood was also detected in a solid organ transplant recipient (#27), a patient with hematological malignancy (#04) and

in an apparently immunocompetent patient (#10) (Table 5). All of them had a progressive disseminated disease. No positive RTqPCR in blood was observed in patients with *Hcd* and non-disseminated *Hcc* histoplasmosis (CNS, lung, localized mucocutaneous, localized osteoarticular and digestive histoplasmosis). This suggests that either the fungal load of circulating yeasts in blood is low resulting in a negative RTqPCR, or that dissemination occurred previously and was not present at diagnosis. The later hypothesis is more likely, since the patients presented at the hospital with very chronic disease without acute symptoms and since the volume of the lesions observed in tissues were high.

For the diagnosis of histoplasmosis due to *Hcd*, the RTqPCR gained in the detection of *Hcd* over the other classical tests,[11,16] which is of utmost importance because this infection is frequently underdiagnosed in Africa.[30] The clinical picture of histoplasmosis due to *Hcd* is clearly different than that of *Hcc* patients in patients without immunosuppression, but the clinical picture could present as a disseminated infection in HIV-positive patients.[27] In this cohort, *Hcd* infections was diagnosed in four patients considered immunocompetent but with multiple organ infections and in 3/3 patients tested, RTqPCR in blood was negative.

The overall positivity rate in the 169 specimens of the proven cases seems not so high (57.4%) but this overall analysis contains specimens taken from patients with no evidence of Histoplasma by culture of direct examination. Indeed, when focusing on culture/microcopy positive specimens, the rate of positivity was 92.2%. In addition, the RTqPCR was also positive in 10/30 (33.3%) culture/microscopy negative which were not useful for the diagnosis of the patients using culture/microscopy. RTqPCR positivity rate in blood was positive in 92.3% of the immunocompromised patients with dissemination.

The higher sensitivity of a PCR test over conventional methods raises the issue of the meaning of an isolated positive PCR result. Indeed, here, the RTqPCR detected seven patients

for whom conventional diagnosis failed to detect *Histoplasma* microscopically or by culture. These patients can be classified as possible cases. Six of these cases were immunocompetent and were not treated with antifungal. Indeed, five of them had mild acute pulmonary histoplasmosis and did not required treatment.[24] These cases are indeed debatable since no other diagnostic mean was positive for *Histoplasma*. Anti-*Histoplasma* antibody or *Histoplasma* antigen were not tested in these patients since no reproductible test are implemented in our lab or in our reference center due to lack or reproducibility of the immunodiffusion test and poor performances in immunocompromised patients.[24] However, antigen detection seems more adapted to diagnose histoplasmosis in immunocompromised patients as parallelized from what is known in patients with invasive aspergillosis.[23] Nevertheless, systematic review of the detection of antigen in patients with histoplasmosis failed to find studies dealing with large cohort of patients and homogenous group of patients.[31]

A highly sensitive assay on blood can help following the patients after treatment. These results demonstrated a decrease of the fungal load overtime under liposomal amphotericin B therapy with a rate of 2-fold decreased every day of treatment. Thus, a follow up using the present RTqPCR allows the determination of the fungal load in blood to help managing the patient. This follow-up has been already advocated several years ago based on blood culture in patients treated with liposomal amphotericin B or itraconazole.[32] The present RTqPCR provides a more suitable means to achieve this goal. Of note, in one patient, RTqPCR was persistently positive over a 2 years period in a context of very poor HIV replication control highlighting that antifungal treatment without improvement of the immune status is potentially insufficient, as described with leishmaniasis.[33]

Because of the number of patients (n=907), It was not possible to get clinical data (country of origin, travel, antifungal treatment, outcome along with other microbiological investigations) for every patient but for the 54 patients with proven histoplasmosis (53 RTqPCR positive and 1 RTqPCR negative). Thus, clinical data from 854 patients (1135 samples of which 510 blood) with a negative RTqPCR result are lacking. Data from these patients has been collected only on case that had been diagnosed as a localized pulmonary histoplasmosis. As a reference center with an implemented management stewardship of invasive fungal infection in France, suspicion or histoplasmosis cases are referred by clinician or mycologist to us (two referents and three referent mycologists) at the reference center. There is little chance that such unusual case in France would have been not referred for the purpose of diagnosis or clinical management, but it cannot be excluded that it could be the cases for some patients included in this study.

Consequently, to fully evaluate the RTqPCR and evaluate fully this diagnostic tool, a prospective study including the evaluation of conventional diagnosis, but also *Histoplasma* antigen testing in serum and/or urine should be implemented. Additionally, this tool will be used to investigate the rate of decrease of the fungal load under different therapeutic regimens aiming to show that rapid decrease will be associated with better outcome in patients.

In non-endemic regions receiving patients originated for endemic regions, or travelers to endemic region, the rarity of histoplasmosis justify the use of a very sensitive test to assess the diagnosis more widely, more rapidly, and more safely than pathology and mycological culture. The present RTqPCR can confirm the diagnosis in respiratory specimens in 92.8 % of all disseminated cases and 100% of acute pulmonary cases and in blood in 92.3% of the immunocompromised patients with disseminated proven cases without waiting for a

confirmation on culture saving several days or weeks. Moreover, *Histoplasma* spp. are Biosafety level 3 (BSL3) organisms with the recommendation not to handle the positive cultures when histoplasmosis is suspected unless a BSL3 facility is available.[20] Based on these results, this RTqPCR should be considered as a very useful diagnostic test both for the diagnosis and the management of histoplasmosis.

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600

601 Table 1: Distribution of the specimens in the 53 cases of histoplasmosis

	ALL (n=53)		PROVEN (n=44)		POSSIBLE (n=9)	
	Median	Range	Median	Range	Media n	Range
nb specimens/patient	3	[1-12]	3	[1-12]	2	[1-3]
nb RTqPCR positive specimens/patient	1	[1-7]	2	[1-7]	1	[1-2]
nb blood/patient	1	[0-9]	1	[0-9]	1	[0-2]
nb RTqPCR positive blood/patient	0	[0-4]	0	[0-4]	0	[0-0]

602

603 Table 2: Clinical characteristics of the 53 cases of histoplasmosis

	ALL (n=53)		PROVEN (n=44)		POSSIBLE (n=9)	
	Median	Range	Median	Range	Media n	Range
nb specimens/patient	3	[1-12]	3	[1-12]	2	[1-3]
nb RTqPCR positive specimens/patient	1	[1-7]	2	[1-7]	1	[1-2]
nb blood/patient	1	[0-9]	1	[0-9]	1	[0-2]
nb RTqPCR positive blood/patient	0	[0-4]	0	[0-4]	0	[0-0]
	n	%	n	%	n	%
Underlying disease						
Immunocompetent	25	47.2	19	43.2	6	66.7
HIV	16	30.2	16	36.4	0	0.0
Hematological malignancies	3	5.7	2	4.5	1	11.1
Immunosuppressive therapy	6	11.3	4	9.1	2	22.2
SOT	2	3.8	2	4.5	0	0.0
Solid cancer	1	1.9	1	2.3	0	0.0
Clinical presentation						
Progressive disseminated histoplasmosis	28	52.8	27	61.4	1	11.1
Acute pulmonary histoplasmosis ± mediastinal lymphadenitis	19	35.8	12	27.3	7	77.8
CNS Histoplasmosis	2	3.8	2	4.5	0	0.0
Localized muco-cutaneous	1	1.9	1	6.8	0	0.0
Localized adrenal Gland	1	1.9	1	4.5	0	0.0
Localized osteoarticular	1	1.9	1	2.3	0	0.0
Localized digestive tract	1	1.9	0	0.0	1	11.1
Country of origin or travel history						
Sub-Saharan Africa	17	32.1	17	38.6	0	0.0
Central and south America	18	34.0	11	25.0	7	77.8
Carribean islands	10	18.9	9	20.5	1	11.1

Asia	4	7.5	4	9.1	0	0.0
North America	3	5.7	3	6.8	0	0.0
French Polynesia	1	1.9	0	0.0	1	11.1
Species identified						
Hcc	/	/	40	90.9	/	/
Hcd	/	/	4	9.1	/	/
Galactomannan index						
≥0.5	12	22.6	11	25.0	1	11.1
<0.5	22	41.5	22	50.0	0	0.0
nd	19	35.8	11	25.0	8	88.9
RTqPCR positive in blood						
Positive	13	24.5	13	29.5	0	0.0
Negative	21	39.6	17	38.6	4	44.4
nd	19	35.8	14	31.8	5	55.6

nd: not done

607 Table 3: Proportion of RTqPCR positive specimens in patients belonging to the four most
608 frequent clinical presentations
609

Clinical presentation	Species	Patients (n)	Specimen	Nb of specimen positive (%)	Positive patients/sampled patients (%)
Progressive disseminated histoplasmosis	*Hcc*	23	All Pulmonary specimens (BAL, Biopsy)	17/19 (88.9)	13/14 (92.8)
			Skin/mucosa	8/9 (88.9)	7/7 (100)
			Blood	25/48 (52.1)	13/17 (76.5)
			Bone marrow aspirate	9/10 (90.0)	8/8 (100)
			Digestive tract biospy	3/5 (60.0)	3/4 (75.0)
			Adrenal Gland biospy	1/1 (100)	1/1 (100)
			Osteo-articular	1/1 (100)	1/1 (100)
			CSF	0/3 (0)	0/3 (0)
Progressive disseminated histoplasmosis and immunocompromised	*Hcc*	18	All Pulmonary specimens (BAL, Biopsy)	10/12 (83.3)	8/9 (88.9)
			Blood	24/44 (54.5)	12/13 (92.3)
Progressive disseminated histoplasmosis and HIV	*Hcc*	12	All Pulmonary specimens (BAL, Biopsy)	8/9 (88.9)	5/5 (100)
			Blood	20/38 (52.6)	10/11 (90.9)
Acute pulmonary histoplasmosis ± mediastinal lymphadenitis	*Hcc*	12	BAL	2/3 (66.7)	2/3 (66.7)
			Lung biopsy	5/6 (83.3)	5/6 (83.3)
			Mediastinal lymph node	5/5 (100)	5/5 (100)
			All pulm specimens	12/14 (85.7)	12/12 (100)

			Blood	0/12 (0)	0/9 (0)
CNS histoplasmosis	*Hcc*	2	CSF	4/7 (57.1)	2/2 (100)
			Blood	0/5 (0)	
Disseminated *duboisii* histoplasmosis	*Hcd*	4	Bone biopsy	2/2 (100)	2/2 (100)
			Skin	6/6 (100)	2/2 (100)
			Brain biopsy	4/4 (100)	1/1 (100)
			Urinary tract	1/4 (25)	1/3 (33)
			Digestive biopsy	1/1 (100)	1/1 (100)
			Sputum	0/1 (0)	0/1 (0)
			Blood	0/8 (0)	0/3 (0)
			Bone Marrow	0/1 (0)	0/1 (0)

CNS, Central nervous system

612 Table 4: Description of the RTqPCR results in the 184 specimens tested in the 53 patients
613

	Number of specimens tested				Number of Whole blood tested			
	Total (n=184)	Negative PCR (n=77)	Positive PCR (n=107)		Total (n=82)	Negative PCR (n=57)	Positive PCR (n=25)	
	Total	n (%)	n (%)	p	Total	n (%)	n (%)	p
Underlying disease								
Immunocompetent	72	32 (44.4)	40 (55.6)	0.80	23	22 (95.7)	1 (4.3)	0.003
HIV	78	32 (41)	46 (59)		46	26 (56.5)	20 (43.5)	
Hematological malignancies	11	4 (36.4)	7 (63.6)		5	2 (40)	3 (60)	
Immunosuppressive therapy	14	7 (50)	7 (50)		6	6 (100)	0 (0)	
SOT	7	1 (14.3)	6 (85.7)		1	0 (0)	1 (100)	
Solid cancer	2	1 (50)	1 (50)		1	1 (100)	0 (0)	
Clinical presentation								
Progressive Disseminated Histoplasmosis	127	48 (37.8)	79 (62.2)	0.36	58	33 (56.9)	25 (43.1)	0.005
Acute pulmonary histoplasmosis ± mediastinal lymphadenitis	38	18 (47.4)	20 (52.6)		16	16 (100)	0 (0)	
CNS Histoplasmosis	12	8 (66.7)	4 (33.3)		5	5 (100)	0 (0)	
Localized muco-cutaneous	2	1 (50)	1 (50.0)		1	1 (100)	0 (0)	
Localized adrenal Gland	1	0 (0)	1 (100)		0	0 (0)	0 (0)	
Localized osteoarticular	3	2 (66.7)	1 (33.3)		2	2 (100)	0 (0)	
Localized digestive tract	1	0 (0)	1 (100)		0	0 (0)	0 (0)	
Country of origin or travel history								
Sub-Saharan African	70	29 (41.4)	41 (58.6)	0.94	27	20 (74.1)	7 (25.9)	0.74
Central and south America	48	19 (39.6)	29 (60.4)		21	15 (71.4)	6 (28.6)	
Carribean islands	31	12 (38.7)	19 (61.3)		14	9 (64.3)	5 (35.7)	
Asia	26	12 (46.2)	14 (53.8)		17	10 (58.8)	7 (41.2)	
North America	7	4 (57.1)	3 (42.9)		2	2 (100)	0 (0)	
French polynesia	2	1 (50)	1 (50)		1	1 (100)	0 (0)	
Species identified								
Hcc	157	64 (40.8)	93 (59.2)	0.53	74	49 (66.2)	25 (33.8)	0.10
Hcd	27	13 (48.1)	14 (51.9)		8	8 (100)	0 (0)	
Galactomannan index								
≥0.5	53	18 (34)	35 (66)	0.37	27	13 (48.1)	14 (51.9)	0.11
<0.5	83	35 (42.2)	48 (57.8)		32	23 (71.9)	9 (28.1)	

614 CNS, Central nervous system
615

Table 5: RTqPCR results in the blood of patients with follow up and a initial blood positive RTqPCR test.

	Sample date	Delay from first positive sample (days)	RTqPCR results (min Cq)
Patient 22	17/03/2017	0	24.2
	18/03/2017	1	24.59
	24/03/2017	7	33.23
	29/03/2017	12	36.02
	10/04/2017	24	45
	17/04/2017	31	45
	24/04/2017	38	45
	02/05/2017	46	45
	03/07/2017	108	45
Patient 38	06/12/2018	0	26.61
	07/12/2018	1	28.28
Patient 03	01/02/2016	0	41
	04/02/2016	3	45
	12/02/2016	11	45
Patient 45	19/04/2019	0	27.77
	29/04/2019	10	33.55
	09/05/2019	20	38.89
	17/05/2019	28	45
Patient 34	26/11/2018	0	29.66
	30/11/2018	4	34.64
	12/12/2018	16	38.22
Patient 08	15/09/2016	0	32.5
	03/10/2016	18	45
	10/10/2016	25	45
Patient 10	05/08/2016	0	36
	16/08/2016	11	45
	22/08/2016	17	45
Patient 23	09/05/2017	0	39.69
	29/05/2017	20	45
	06/06/2017	28	45
	13/06/2017	35	45
	20/06/2017	42	45
	27/06/2017	49	45
	04/07/2017	56	45
Patient 14	24/09/2014	0	40.61
	24/08/2015	334	40
	27/05/2016	611	37.3

Cq Quantification cycles ; Cq at 45 represents a negative result

657

Legend to Figures:

Figure 1: Comparison of the detection of Histoplasma spp. DNA by qPCR and WNA by RTqPCR on 11 clinical specimens from 3 RTqPCR positive patients. WNA, whole nucleic acid; ** P<0.01

Figure 2: Comparison of 3 quantitative PCR assay including the evaluated RTqPCR assay using 6 clinical specimens and 4 strain WNA including all specimens (A) or specifically for *Hcc* (*Histoplasma capsulatum* var. *capsulatum*) or *Hcd* (*Histoplasma capsulatum* var. *duboisii*) specimens (B). ** P<0.01, *P<0.05.

Figure 3: Flow-chart of the design of the study

Figure 4: Evolution of the fungal load under treatment in 8 patients with an initial positive RTqPCR in blood and follow up (A). The time to negativation was dependent of the initial fungal load (B). R^2, correlation coefficient.

Supplement material:

Supplemental Figure 1: Comparison of the Cq values obtained from whole blood and serum/plasma in six patients with positive blood RTqPCR as dotplots (A) or linked plots (B). *P<0.05

Supplemental Table 1-5: see specific file.