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1           **Evaluation of a new *Histoplasma* spp. reverse transcriptase quantitative PCR assay**

2

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57

58

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61 of this study. AA, SB, ASL and FD are listed as inventors in a patent deposited on this assay.  
62 MGM, DGH, ASL, MB, FD, AMR have nothing to disclose.

63

64 **Abstract:**

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

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

82

83

84 The fungus *Histoplasma* was first described in Panama by Samuel Taylor Darling in a lung  
85 sample from a patient from Martinique (French Caribbean islands)<sup>1</sup> by observation of free or  
86 intracellular small yeasts in a smear of respiratory specimen. Since then, two distinct varieties  
87 have been described in humans initially identified by morphological aspects in tissues and  
88 epidemiological criteria (i) *Histoplasma capsulatum* var. *capsulatum* (*Hcc*, New World human  
89 pathogen) associated with small ovoid (2-3  $\mu$ m) yeasts, and (ii) *Histoplasma capsulatum* var.  
90 *duboisii* (*Hcd*, Old World human pathogen) associated with large citrus shaped yeasts (5-10  
91  $\mu$ m). A third variety was described in Africa, *Histoplasma capsulatum* var. *farciminosum* (Old  
92 World animal pathogen) associated with epizootic lymphangitis in horses. However, this  
93 classification has been revisited based on recent multilocus sequencing revealing several  
94 cryptic species, which are structured according to their geographical origin.<sup>2</sup> *Histoplasma*  
95 *capsulatum* is now composed of at least four phylogenetically different groups,<sup>3</sup> including a  
96 specific group for var. *duboisii*. *Histoplasma capsulatum* var. *farciminosum* appeared to be  
97 polyphyletic suggesting this variety does not correspond to a specific phylogenetic group.<sup>3</sup>  
98 Recent analyses of multiple isolates by whole genome sequencing recognized the existence of  
99 four phylogenetic species corresponding to the geographical clades of North America (2  
100 species), Latin America and Panama. The African variety (*Hcd*) is still not considered a different  
101 species mainly due to the small number of isolates studied so far.<sup>4</sup>  
102 Nowadays, diagnosis of histoplasmosis still relies on microscopic direct examination and  
103 culture, together with antibody or antigen detection.<sup>5</sup> In addition, PCR methods based on the  
104 amplification of various DNA targets and using different methodologies have been reported<sup>6-</sup>  
105 <sup>14</sup> with a sensitivity ranging from 67 to 100% depending on the studies.<sup>15</sup> More recently, real-  
106 time quantitative PCR (qPCR) methods based on DNA detection<sup>7,11,16-18</sup> have been developed  
107 for human diagnosis to overcome the limits of the microbiological culture. Indeed, qPCR

108 assays are expected to be more sensitive,<sup>16</sup> more rapid than culture and prevent laboratory  
109 transmission of the mold form of *Histoplasma* spp. in the laboratory environment.<sup>19,20</sup>  
110 A *Histoplasma* spp. RTqPCR assay was developed and validated based on a repeated gene  
111 target and the detection of whole nucleic acids (RNA plus DNA) to improve the specificity and  
112 the sensitivity of microscopy and culture and accelerate results. After validation, this RTqPCR  
113 was performed prospectively during four years and then evaluated its performance in all  
114 patients suspected of histoplasmosis in this French network.

115

116

## 117 **Material and methods**

### 118 **Nucleic acids extraction**

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

128 Whole nucleic acids (WNAs) were extracted from non-fixed specimens using initial bead  
129 beating in a Precellys bead beater (Bertin Technologies, Montigny-le-Bretonneux, France).  
130 Then, the tubes were centrifuged 5 min at 10,000 g at 4°C. WNAs from the supernatant were  
131 extracted with addition of 10  $\mu$ L/sample of 1:5 diluted internal control (DNA Virus Culture,  
132 DICD-CY-L100, Diagenode, Seraing, Belgium) using a Qiasymphony (Qiagen, Hilden, Germany)  
133 with the Virus Pathogen extraction Kit (Qiagen, Hilden, Germany) following the  
134 manufacturer's instructions. WNAs were eluted in 85  $\mu$ L volume. For the paraffin-embedded  
135 tissue specimens, extraction of three 10  $\mu$ m-sections was performed using the DNeasy Blood  
136 and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations with  
137 a volume of elution of 110  $\mu$ L in DNA grade water. All extracts and aliquots were stored at  
138 +4°C until use (less than 5 days). In a specific preliminary experiment comparing WNA and  
139 DNA amplification, the RTqPCR kit was used without or with the reverse transcriptase step (15  
140 min at 50°C) for DNA and WNA amplification, respectively.

141

142 **Selection of target gene**

143 The ribosomal small subunit RNA gene of *H. capsulatum* (GenBank accession number,  
144 GG663449.1) was used to design primers and probes using Primer3web v4.0.0 software. Outer  
145 primers (Hc\_7F: GATGATGGCTCTGATTGAACG and Hc\_361R: GATGATGGCTCTGATTGAACG)  
146 were first used to analyze the *Histoplasma* sequence in the region of interest of this gene for  
147 the presence of polymorphism. The amplicons obtained with two strains of *Hcc*  
148 (CNRMA16.205, GenBank MW317018 and CNRMA17.309, GenBank MW317017), one strain  
149 of *Hcd* (CNRMA16.638, GenBank MW317134) were sequenced resulting in a 100%  
150 (357/357bp) and 99.9% (356/357bp) similarity with the reference sequence (GenBank  
151 GG663449.1), respectively. The final assay was then designed in conserved regions excluding  
152 the single-nucleotide polymorphism observed in *Hcd*. Of note, no polymorphism at the  
153 primers and probe loci were observed upon sequencing of isolates from various lineages  
154 (GenBank MW317018, MW317017, MW317134)

155

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

157 WNA amplification was performed using the following conditions: 1× Invitrogen RT-qPCR  
158 buffer mix (Superscript III One step RT-PCR, Life Technologies Corporation, Carlsbad, CA, USA),  
159 0.3 μM of each primer (Hc\_24\_55F: CGTACGACATCATATTA AAAATGA and Hc\_22\_128R:  
160 CTTTCTTTAAGGTAGCCAAAAT), 0.1 μM of probe (Hc\_21\_79P: FAM-  
161 TGTAGTGGTGTACAGGTGAGT-BHQ1), and 1 μM of Superscript III Platinum enzyme, in a total  
162 of 25 μL with 8 μL of WNA extract. The amplification consisted of one step of RT-PCR at 50°C  
163 for 15 min, followed by qPCR with one activation step at 95°C for 2 min and 50 cycles of  
164 denaturation at 95°C for 15 sec and annealing at 53°C for 30 sec. All the qPCR runs were

165 performed on a Light Cycler 480 thermocycler (LC480-II; Roche Diagnostics, Mannheim,  
166 Germany) with quantitative cycle (C<sub>q</sub>) determination using the calculation of the second  
167 derivative of the amplification curve.<sup>21</sup>

168

### 169 **Specificity**

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

172

### 173 **qPCR efficiency and limit of detection**

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

183

### 184 **Comparison of three qPCR assays**

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

189 of 25  $\mu$ L containing 1x LC480-II Probes Master, 0.9  $\mu$ M of each primer, 0.25  $\mu$ M of the probe,  
190 7  $\mu$ L of template DNA <sup>16</sup>. The qPCR assay from Buitrago et al. (qPCR 2) was carried out as  
191 advised by the authors in a final volume of 25  $\mu$ L containing 2x LC480-II Probes Master, 0.5  
192  $\mu$ M of each primer, 0.2  $\mu$ M of the probe, 7  $\mu$ L of template DNA <sup>11</sup>. A total of 10 WNA were  
193 tested including four WNA extracted from the two CNRMA strains of each *Hcc* (CNRMA17.309  
194 and CNRMA16.205) and *Hcd* (CNRMA16.638 and CNRMA17.108) adjusted at 1 ng/ $\mu$ L, and  
195 positive WNA samples using the RTqPCR (n=6) from whole blood, one bone marrow  
196 aspiration, one lymph node, one digestive biopsy (4 patients infected with *Hcc*), and one bone  
197 biopsy and one brain abscess (2 patients infected with *Hcd*). The three assays were performed  
198 the same day on the same LC480-II and the dilutions aliquots realized extemporaneously. A  
199 standard curve allowing PCR efficiency calculation was obtained for each assay based on the  
200 result of PCR duplicates in six serial dilutions WNA.

201

## 202 **Patient classification**

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

213 on histoplasmosis.<sup>25</sup> 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  $\geq 2$  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 where 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

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

240

#### 241 **Polyphasic identification of clinical strains**

242 *Histoplasma* isolates were sent to the National Reference Center for Invasive Mycoses &  
243 Antifungals, Institut Pasteur, Paris, France for identification which was handled in a Biosafety  
244 Level-3 (BSL-3) laboratory. Strains were sub-cultured on Sabouraud chloramphenicol agar  
245 slants and incubated at 27-30°C until sufficient sporulation was attained. The observed  
246 macroscopic features included white colonies with fine aerial mycelia or flat tan colonies  
247 diffusing a dark brown pigment with age. Microscopic preparations on lactic blue revealed the  
248 presence of thick-walled tuberculate macroconidia and smooth-walled microconidia.  
249 Molecular analyses were performed by sequencing the ITS1-5.8S-ITS2 (ITS) region of the  
250 ribosomal deoxyribonucleic acid (rDNA)<sup>26</sup> to confirm the identification of the genus. The small  
251 region (772 bp) of the gene *prp8*<sup>27</sup> allowed the differentiation of *Hcd* from the other  
252 geographical clades of *Histoplasma capsulatum* with a 97% degree of similarity between  
253 them. Of note all *hcd* cases had a typical positive direct examination showing the characteristic  
254 5-10µM ovoid yeasts in specific specimens.

255

#### 256 **Statistical analysis**

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

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

266

## 267 **Ethics**

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

273

274

275 **Results**

276

277 **Evaluation and validation of the *Histoplasma* RTqPCR**

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

279 The analytical performance of the RT-qPCR amplifying WNA was first compared to qPCRs  
280 amplifying only DNA. A comparison of the results on 11 WNA extracts from 3 patients showed  
281 that the sensitivity on WNA (11/11, 100%) was higher than on DNA (9/11, 81% specimens  
282 detected). In addition, WNA gave significantly lower Cq values (median 40.3 [29.5-41.2])  
283 compared to DNA (median 40.2 [31.6-45]) (P=0.006, Figure 1). The limit of detection (LOD90)  
284 of the RTqPCR assay was 10 copies (Supplemental Table 4). The efficiency of the RTqPCR was  
285 1.9 (95%) (Supplemental Table 4).

286

287 *Analytical specificity*

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

294

295 *Comparison with qPCR assays*

296 The RTqPCR assay was compared to two qPCR assays from the literature using 6 *Hcc* WNA and  
297 4 *Hcd* WNA and gave significantly lower Cq values compared to qPCR 1 and qPCR 2 assays  
298 (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

323 was positive in 24/44 (54.5%) specimens and 12/13 (92.3%) patients, and 20/38 (52.6%) and  
324 10/11 (90.9%), respectively (Table 3). In proven acute pulmonary ± mediastinal lymphadenitis,  
325 RTqPCR was positive in 12/12 (100%) patients tested with 12/14 (85.7%) positive specimens  
326 tested (BAL, biopsies, lymph nodes) (Table 2). In CNS histoplasmosis, RTqPCR in CSF was  
327 positive in the 2/2 (100%) cases (4/7, 57.1% of tested specimens) (Table 2). Disseminated *Hcd*  
328 infections had various RTqPCR positive sample types, including skin, bone, brain, urinary and  
329 digestive tract specimens (Table 2).

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

336 Most of the possible cases (RTqPCR positive / culture negative) had localized diseases (8/9,  
337 88.9%) [lung (7/8), digestive tract (1/8)] and only 1/9 (11.1%) had a disseminated disease  
338 (Patient #16, immunocompetent, with a negative microscopy and negative culture in blood  
339 and BAL). RTqPCR positive specimens were respiratory specimens (n=8) and digestive tract  
340 (n=1) (Table 2). Parallel culture and/or direct examination/histopathology were negative in  
341 the seven patients for whom it was done in parallel to RTqPCR (three patients had no other  
342 investigation but the PCR to assess mycological diagnosis in a compatible epidemiological and  
343 clinical context). In one case under immunosuppressive therapy (#50), galactomannan  
344 detection was positive (index >0.5). Three out of 9 (33.3%) were immunocompromised and all  
345 (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

370 immunocompetent cases ( $P=0.003$  (Table 4). Of note, blood was obtained from 3/4  
371 immunocompetent patients with *Hcd* disseminated infections and RTqPCR was negative in all  
372 3 cases (100%). Thirty-seven blood samples from 9 patients with at least one RTqPCR positive  
373 and one follow-up blood sample were studied for a median follow-up duration of 25 days  
374 [13.5-82] and with a median number of blood sample per patient of 3 (Table 5). All patients  
375 were treated with liposomal amphotericin B at D0 (date of the first positive blood specimen).  
376 The kinetics of the fungal load is plotted in Figure 4A. The rate of negativation was dependent  
377 on the initial fungal load (Figure 4B) with a  $R^2$  at 0.58 and a slope at  $-1.04 \pm 0.44$ . This gain of  
378 1 Cq per day corresponded approximately to a 2-fold decrease of the fungal load per day of  
379 treatment. Of note, for 3 of the 9 patients (Patients # 14, 38, 45), RTqPCR remained positive  
380 as the patient was lost (Table 5). In Patient#14 (HIV positive and inobservant), RTqPCR was  
381 still positive 334 and 611 days after the first positive result in blood with no intermediate  
382 testing.  
383

384 **Discussion**

385         The present evaluation of this reverse transcriptase qPCR (RTqPCR) assay  
386 demonstrates an increased sensitivity of the Histoplasma detection using WNA instead of DNA  
387 and *mtSSU* gene instead of *ITS* as target gene. Thus, we were able to detect WNA in 97.7%  
388 (43/44) of microscopy and/or culture proven cases in contrast with recent publications  
389 comparing *ITS* Loop-mediated Isothermal Amplification (LAMP) PCR and *HSP100* nested PCR  
390 for which a positive PCR was observed in only in 54% and 64% of the culture positive  
391 specimens, respectively.<sup>10</sup> The high sensitivity of the present assay also allowed detection of  
392 Hcc disseminated diseases in the blood of 92.3% of immunocompromised patients and the  
393 detection of 9 patients including seven patients for whom the conventional mycology  
394 investigations were tested negative. Moreover, the high sensitivity of the test allows a rapid  
395 follow-up of treatment efficacy with the observation of a 2-fold decrease of the fungal load  
396 per day during optimal treatment.

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

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

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

423 The overall positivity rate in the 169 specimens of the proven cases seems not so high  
424 (57.4%) but this overall analysis contains specimens taken from patients with no evidence of  
425 *Histoplasma* by culture of direct examination. Indeed, when focusing on culture/microcopy  
426 positive specimens, the rate of positivity was 92.2%. In addition, the RTqPCR was also positive  
427 in 10/30 (33.3%) culture/microscopy negative which were not useful for the diagnosis of the  
428 patients using culture/microscopy. RTqPCR positivity rate in blood was positive in 92.3% of  
429 the immunocompromised patients with dissemination.

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

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

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

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

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

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

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

484

485

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492 **Reference**

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- 599
- 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
Carribbean 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						
<i>Hcc</i>	/	/	40	90.9	/	/
<i>Hcd</i>	/	/	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

604 nd: not done

605

606

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	<i>Hcc</i>	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 biopsy	3/5 (60.0)	3/4 (75.0)			
			Adrenal Gland biopsy	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	<i>Hcc</i>	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	<i>Hcc</i>	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	<i>Hcc</i>	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	<i>Hcc</i>	2	CSF	4/7 (57.1)	2/2 (100)
			Blood	0/5 (0)	
Disseminated <i>duboisii</i> histoplasmosis	<i>Hcd</i>	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)

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610 CNS, Central nervous system  
611

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)	p	Total (n=82)	Negative PCR (n=57)	Positive PCR (n=25)	p
	Total	n (%)	n (%)		Total	n (%)	n (%)	
<b>Underlying disease</b>								
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)	
<b>Hematological malignancies</b>								
Immunosuppressive therapy	11	4 (36.4)	7 (63.6)	0.36	5	2 (40)	3 (60)	0.005
SOT	14	7 (50)	7 (50)		6	6 (100)	0 (0)	
Solid cancer	7	1 (14.3)	6 (85.7)	1	0 (0)	1 (100)		
	2	1 (50)	1 (50)	1	1 (100)	0 (0)		
<b>Clinical presentation</b>								
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)		
<b>Country of origin or travel history</b>								
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)	
Carribbean 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)		
<b>Species identified</b>								
<i>Hcc</i>	157	64 (40.8)	93 (59.2)	0.53	74	49 (66.2)	25 (33.8)	0.10
<i>Hcd</i>	27	13 (48.1)	14 (51.9)		8	8 (100)	0 (0)	
<b>Galactomannan index</b>								
≥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

616 Table 5: RTqPCR results in the blood of patients with follow up and a initial blood positive  
 617 RTqPCR test.  
 618

	Sample date	Delay from first positive sample (days)	RTqPCR result (min Cq)	
Patient 22	17/03/2017	0	24.2	619
	18/03/2017	1	24.59	620
	24/03/2017	7	33.23	621
	29/03/2017	12	36.02	622
	10/04/2017	24	45	623
	17/04/2017	31	45	624
	24/04/2017	38	45	625
	02/05/2017	46	45	626
	03/07/2017	108	45	627
Patient 38	06/12/2018	0	26.61	628
	07/12/2018	1	28.28	629
Patient 03	01/02/2016	0	41	630
	04/02/2016	3	45	631
	12/02/2016	11	45	632
Patient 45	19/04/2019	0	27.77	633
	29/04/2019	10	33.55	634
	09/05/2019	20	38.89	635
	17/05/2019	28	45	636
Patient 34	26/11/2018	0	29.66	637
	30/11/2018	4	34.64	638
	12/12/2018	16	38.22	639
Patient 08	15/09/2016	0	32.5	640
	03/10/2016	18	45	641
	10/10/2016	25	45	642
Patient 10	05/08/2016	0	36	643
	16/08/2016	11	45	644
	22/08/2016	17	45	645
Patient 23	09/05/2017	0	39.69	646
	29/05/2017	20	45	647
	06/06/2017	28	45	648
	13/06/2017	35	45	649
	20/06/2017	42	45	650
	27/06/2017	49	45	651
	04/07/2017	56	45	652
Patient 14	24/09/2014	0	40.61	
	24/08/2015	334	40	
	27/05/2016	611	37.3	

653 Cq Quantification cycles ; Cq at 45 represents a negative result

654

655

656



658 **Legend to Figures:**

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

662

663 Figure 2: Comparison of 3 quantitative PCR assay including the evaluated RTqPCR assay using  
664 6 clinical specimens and 4 strain WNA including all specimens (A) or specifically for *Hcc*  
665 (*Histoplasma capsulatum* var. *capsulatum*) or *Hcd* (*Histoplasma capsulatum* var. *duboisii*)  
666 specimens (B). \*\* P<0.01, \*P<0.05.

667

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

669

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

673

674 **Supplement material:**

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

678 Supplemental Table 1-5: see specific file.