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

2

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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)[1] 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 µm) yeasts, and (ii) *Histoplasma capsulatum* var.
90 *duboisii* (*Hcd,* Old World human pathogen) associated with large citrus shaped yeasts (5-10
91 µm). 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.[2] *Histoplasma*
95 *capsulatum* is now composed of at least four phylogenetically different groups,[3] 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.[3]
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.[4]
102 Nowadays, diagnosis of histoplasmosis still relies on microscopic direct examination and
103 culture, together with antibody or antigen detection.[5] In addition, PCR methods based on the
104 amplification of various DNA targets and using different methodologies have been reported[6–
105 14] with a sensitivity ranging from 67 to 100% depending on the studies.[15] More recently, real-
106 time quantitative PCR (qPCR) methods based on DNA detection[7,11,16–18] 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,[16] more rapid than culture and prevent laboratory
109 transmission of the mold form of *Histoplasma* spp. in the laboratory environment.[19,20]
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 (ΔCq=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 (ΔCq=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 µL/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 µL volume. For the paraffin-embedded
135 tissue specimens, extraction of three 10 µm-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 µL 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: CGTACGACATCATATTAAAAATGA 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 (Cq) determination using the calculation of the second
167 derivative of the amplification curve.[21]
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 synthetized 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-IIThe 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 Cq value by using Analysis package included in LightCycler 480
181 software v. 1.5 (Roche Diagnostics, Mannheim, Germany).[22]. 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.[11,16] 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 µL containing 1x LC480-II Probes Master, 0.9 µM of each primer, 0.25 µM of the probe,
190 7 µL of template DNA [16]. The qPCR assay from Buitrago et al. (qPCR 2) was carried out as
191 advised by the authors in a final volume of 25 µL containing 2x LC480-II Probes Master, 0.5
192 µM of each primer, 0.2 µM of the probe, 7 µL of template DNA [11]. A total of 10 WNAs 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/µL, 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 WNAs.
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.[5] Clinical data were recorded retrospectively for all PCR positive patients. Each
211 histoplasmosis case was classified according to the EORTC/MSG criteria[23] and described
212 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

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)[26] to confirm the identification of the genus. The small
251 region (772 bp) of the gene *prp8*[27] 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 ± 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.[10] 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.[28] 403 Indeed, more than 50% of HIV-positive patients with histoplasmosis had positive blood 404 culture.[29] 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,[11,16] which is of utmost importance because this infection is
418 frequently underdiagnosed in Africa.[30] 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.[27] 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.[24] 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 reproductible 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.[24] 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.[23]
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.[31]

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.[32] 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.[33]

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.[20] 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**

493


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599

600

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 | Median | 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] |

Table 2: Clinical characteristics of the 53 cases of histoplasmosis

| | ALL (n=53) | | PROVEN (n=44) | | POSSIBLE (n=9) | |
|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | Median | 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 |

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 | *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 biopsy | 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) |

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) | | 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

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 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 |

619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652

653 Cq Quantification cycles ; Cq at 45 represents a negative result
654
655
656

657

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). $R^2$, 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.