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A multicentre external quality assessment: A first step to standardise PCR protocols for the diagnosis of histoplasmosis and coccidioidomycosis

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Abstract

Background: In-house real-time PCR (qPCR) is increasingly used to diagnose the so-called endemic mycoses as commercial assays are not widely available.

Objectives: To compare the performance of different molecular diagnostic assays for detecting *Histoplasma capsulatum* and *Coccidioides* spp. in five European reference laboratories.

Methods: Two blinded external quality assessment (EQA) panels were sent to each laboratory that performed the analysis with their in-house assays. Both panels included a range of concentrations of *H. capsulatum* ($n=7$) and *Coccidioides* spp. ($n=6$), negative control and DNA from other fungi. Four laboratories used specific qPCRs, and one laboratory a broad-range fungal conventional PCR (cPCR) and a specific cPCR for *H. capsulatum* with subsequent sequencing.

Results: qPCR assays were the most sensitive for the detection of *H. capsulatum* DNA. The lowest amount of *H. capsulatum* DNA detected was 1–4 fg, 0.1 pg and 10 pg for qPCRs, specific cPCR and broad-range cPCR, respectively. False positive results occurred with high concentrations of *Blastomyces dermatitidis* DNA in two laboratories and with *Emergomyces* spp. in one laboratory. For the *Coccidioides* panel, the lowest amount of DNA detected was 1–16 fg by qPCRs and 10 pg with the broad-range cPCR. One laboratory reported a false positive result by qPCR with high load of *Uncinocarpus* DNA.

Conclusion: All five laboratories were able to correctly detect *H. capsulatum* and *Coccidioides* spp. DNA and qPCRs had a better performance than specific cPCR and broad-range cPCR. EQAs may help standardise in-house molecular tests for the so-called endemic mycoses improving patient management.

KEYWORDS
Coccidioides, *Histoplasma*, kinetic polymerase chain reaction, multicentre trial, PCR, quality control

1 | INTRODUCTION

Histoplasmosis and coccidioidomycosis are caused by the thermally dimorphic fungi *Histoplasma capsulatum* (*H. capsulatum*), *Coccidioides immitis* (*C. immitis*) and *Coccidioides posadasii* (*C. posadasii*), respectively. *Histoplasma capsulatum* has a cosmopolitan distribution with some areas of high endemicity in Africa and North and South America, although the burden in Europe is very low. *Coccidioides* is present in arid and semiarid regions in the Americas. *Coccidioides immitis* is found mainly in central and southern California, but also in Washington State,[1] whereas infections by *C. posadasii* have been reported in parts of Latin America[2] and the United States from central and southern Arizona to western Texas and southern New Mexico.[3] Immigration and travel coming from these areas have caused an increasing number of histoplasmosis and coccidioidomycosis in Europe[4–6] (Staffolani, 2018 #1010). Diagnosis in European countries is challenging due to the lack of experience and available diagnostic methods. Although the gold standard methods are culture and microscopy,[7] their practical use is frequently restricted due to their limitations. Culture isolation of these biosafety level-3 (BSL-3) agents is time-consuming due to slow growth, delaying appropriate treatment and representing a potential health risk for laboratory workers.[8] On the other hand, histopathological observation of fungal structures in tissues requires skilled personnel, as several protozoa and fungi, especially other dimorphic fungi, may be challenging to differentiate from *Histoplasma* and *Coccidioides*.[8–12] In addition, the sensitivity of these conventional methods is moderate, ranging from 0 to around 75% depending on clinical manifestations and origin of the samples.[8] Besides these golden standards, specific antigen detection tests may be a good additional tool for the diagnosis of histoplasmosis and coccidioidomycosis; however, cross-reactions are described with other fungi, sensitivity is decreased in immunocompetent patients[13–20] and antigen detection tests are still not largely available.[21,22] As with other invasive fungal infections, the utility of antibody detection is limited, as the antibodies are produced only 4–8 weeks after exposure, may completely be absent in patients with impaired humoral immunity, and cross-reactions are possible with other fungal and granulomatous diseases[8,23–25] (Table S1).

Molecular diagnostic testing on clinical samples has shown its usefulness in diagnosing fungal infections in animals[26,27] and humans[12,28–35] and detecting environmental niches.[36–39] Besides existing broad-range fungal PCRs that rely upon amplicon identification by sequencing, multiple specific in-house assays were developed during the past years to detect *H. capsulatum* and *Coccidioides* spp. fast (Figure 1). They were applied on various sample types, such as formalin-fixed paraffin-embedded and fresh biopsies,[4,32,40,41] blood,[28,42–45] serum,[29,33,43,45–47] bronchoalveolar lavage fluid (BAL),[28,45,46,48] cerebrospinal fluid,[28,45,47,49] bone marrow,[28,43,48,49] pleural fluid[28,48] and urine.[50] They target multicopy ribosomal DNAs[28,29,33,36,39,43–45,47,49,51–59] (rDNAs) or species-specific genes,[27,41,42,46,48,60–73] mostly single copy, except for the region identified in the NCBI database as a 'copia-like retrotransposon'[1,37,38,74,75] with approximately 60 copies per *Coccidioides* genome.[74]

The specific PCRs targeting multicopy rDNAs, such as the internal transcribed spacer (ITS) region or the 5.8S, 28S and 18S rDNA, have the potential advantage of increased sensitivity, being able to detect less than one genome equivalent.

Only one specific real-time PCR (qPCR) kit, targeting the 'copia-like retrotransposon' of *Coccidioides* spp., is commercially available and has been FDA-approved for BAL and bronchial wash (class II)[74] (GeneSTAT.MDx Coccidioides test on the GeneSTAT System; DxNA LLC).

Though molecular diagnosis of histoplasmosis and coccidioidomycosis in Europe relies mainly on in-house assays (in-house in vitro diagnostic medical devices in the new EU regulation[76]), which need to be critically assessed, and if accreditation (DIN E. ISO/IEC 17043 or 15189 standards) is aimed, participation in external quality assessments (EQA) is mandatory. However, commercially available EQAs, evaluating techniques targeting *H. capsulatum* and *Coccidioides* spp., are currently lacking.

The objective of this work was to analyse the performance of different molecular diagnostic tests based on PCR to detect *H. capsulatum* and *Coccidioides* spp. DNA in a multicentre study that involved five reference laboratories in Europe.

2 | MATERIALS AND METHODS

2.1 | Participating centres

Five European laboratories, all located outside high-endemic countries (France, Germany, The Netherlands, Portugal and Spain), participated in this multicentre study. They have been designated with the following numerical code; Centre 1: FG16 Mycology,

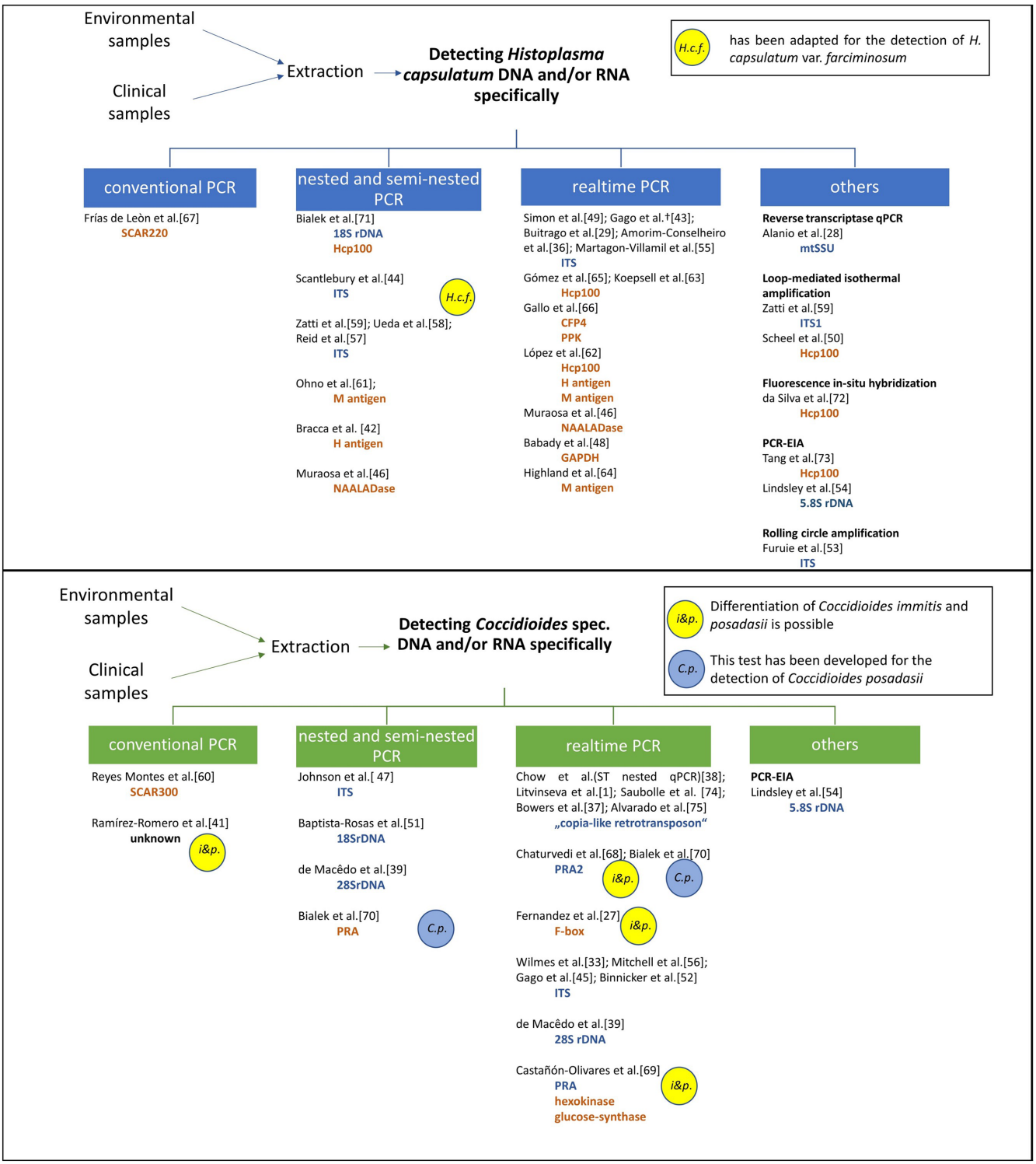

FIGURE 1 Schematic review of published PCR methods for specifically detecting *Histoplasma capsulatum* DNA (above) and *Coccidioides* spp. DNA (below). In orange, single copy targets and blue, multicopy targets. *CFP4*: culture filtrate protein 4 gene; FISH: Fluorescence in situ hybridisation; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase gene; *Hcp100*: 100-kDa-like protein gene; LAMP: loop-mediated isothermal amplification; *NAALADase*: N-acetylated α-linked acidic dipeptidase gene; PCR-EIA: PCR-enzyme immunoassay; *PPK*: predicted protein kinase gene; *PRA2*: proline-rich antigen gene; RT-qPCR: reverse transcriptase real-time PCR; SCAR: sequence characterised amplified region. †multiplex PCRs.

Robert Koch Institute (RKI), Berlin, Germany; Centre 2: Reference Mycology Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III (ISCIII), Madrid, Spain; Centre 3: Department of parasitology and Mycology, Hôpital Saint-Louis, APHP, Paris, France; Centre 4: Department of Medical Mycology, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; Centre 5: National

Reference Laboratory for Parasites and Fungal Infections, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisbon, Portugal.

2.2 | Panel preparation

The used strains belonged to the collection of the RKI and are presented in Tables 1 and 2. DNA was extracted from *C. posadasii*, *H. capsulatum* (yeast cells), *Paracoccidioides lutzii* and *Blastomyces dermatitidis* isolates in the BSL-3 facility, and DNA extraction from *Candida albicans*, *Aspergillus fumigatus*, *Trichophyton violaceum*, *Uncinocarpus reesii*, *Emergomyces europaeus* and *Emergomyces africanus* in the BSL-2 laboratory. For fungal DNA extraction, we used the MasterPure™ Yeast DNA purification kit (LGC Lucigen), including RNAse, with an additional bead beating step with 0.25 mm silicon-carbide sharps (BioSpec Products Inc.) in a FastPrep-24™ 5G machine (MP Biomedicals, LLC). Extracted DNA was eluted in 100 µL TE buffer. DNA quantities were measured fluorometrically (Qubit™ 2.0, Fluorometer, Invitrogen by life technologies with the Qubit™ dsDNA BR Assay Kit, Invitrogen by Life Technologies).

All DNA concentrations were standardised to 0.2 ng per microliter. The *H. capsulatum* and *C. posadasii* genomic DNAs were then serially 10-fold diluted with Triton 0.1%. Other fungal DNAs were diluted to 20 pg per microliter. Sterility was tested by cultivation.

These DNAs were used to put together the two blinded panels, including 14 samples for *H. capsulatum* and 10 samples for *Coccidioides* spp. (Tables 1 and 2). The tubes were immediately frozen after preparation and sent on dry ice. The receiving centres were requested to store the tubes at −20°C until further testing.

2.3 | Panel content

The first panel was sent in November 2021 to evaluate the *H. capsulatum* detecting PCR protocols. The *Coccidioides* spp. panel was sent in January 2022. Different concentrations of target fungal DNA and controls were included in each panel. The sensitivity and specificity were assessed by different concentrations of target fungal DNA and controls in each panel. Control fungal DNA consisted of frequent fungal pathogens and fungi closely related to *H. capsulatum* and *Coccidioides* spp. (Tables 1 and 2).

2.4 | PCR protocols

All centres used their current routine diagnostic techniques and equipment (Tables 3 and 4) and included their own positive and no-template controls. Centres 1 and 2 performed inhibition controls either in the same tubes (Centre 2) or in a separate reaction by qPCR[12] (Centre 1). All participating centres did their PCRs in duplicates, except for Centres 4 and 5, which performed their PCRs in a single replicate. A total of eight protocols were compared for the detection of *H. capsulatum* DNA and five for the detection of *Coccidioides* spp. DNA.

TABLE 1 Composition of the *Histoplasma capsulatum* panel.

Sample number	*Histoplasma* panel
1	Triton 0.1
2	*H. capsulatum* (RKI09-0599) 10 pg/5 µL
3	*C. albicans* (RKI11-0116) 100 pg/5 µL
4	*H. capsulatum* (RKI09-0599) 1 pg/5 µL
5	*Es. europaeus* (RKI17-1077) 100 pg/5 µL
6	*H. capsulatum* (RKI09-0599) 100 pg/5 µL
7	*H. capsulatum* (RKI09-0599) 0.01 pg/5 µL
8	*Es. africanus* (RKI17-1221) 100 pg/5 µL
9	*H. capsulatum* (RKI09-0599) 0.001 pg/5 µL
10	*H. capsulatum* (RKI09-0599) 0.1 pg/5 µL
11	*H. capsulatum* (RKI09-0599) 1000 pg/5 µL
12	*A. fumigatus* (RKI13-0959) 100 pg/5 µL
13	*B. dermatitidis* (RKI16-1033) 100 pg/5 µL
14	*P. lutzii* (RKI16-1032) 100 pg/5 µL

Abbreviations: A., *Aspergillus*; ATCC, American Type Culture Collection; B., *Blastomyces*; C., *Candida*; Es., *Emergomyces*; H., *Histoplasma*; P., *Paracoccidioides*; RKI, Robert Koch Institute.

2.4.1 | *Histoplasma capsulatum* DNA detecting protocols

Centre 1 and 2 performed different qPCRs targeting regions of the ITS1[12,49] and ITS2[43] of the *H. capsulatum* rDNA. Centre 3 used a RT-qPCR assays, targeting the mitochondrial ribosomal small subunit (mtSSU)[28] and Centre 4 three different qPCR assays targeting ITS2, ITS1[36] and the multicopy cytochrome C oxidase 2 (*COX2*) mitochondrial gene. Centre 5 performed a broad-range fungal conventional PCR (cPCR) targeting a part of ITS1 (primers ITS1 and ITS2[77]), and a specific cPCR[78] targeting a part of the ITS1 region with primers derived from Buitrago et al.[29] Both assays were followed by sequencing. Briefly, amplicons were purified using the ExoSAP-IT enzyme system (USB Corporation), according to the manufacturer's instructions. The sequencing of both strands was performed with the BigDye terminator v 1.1 cycle sequencing kit (Applied Biosystems) in the thermal cycler, using the same primers as were used in the PCR amplification. The methods are summarised in Table 3.

2.4.2 | *Coccidioides* spp. DNA detecting protocols

Centres 1 and 2 used for detecting *Coccidioides* spp. DNA qPCRs targeting parts of the ITS2 region of the rDNA genes.[33,45] Centre 3 performed a RT-qPCR amplifying total nucleic acids by performing a reverse transcription before the amplification of the mtSSU. Centre 4 used a duplex qPCR targeting the single copy proline-rich antigen 2 (*PRA2*) gene, able to differentiate *C. immitis* and *C. posadasii*.[68] Centre 5 performed a broad-range fungal cPCR with subsequent identification by sequencing.[78] The methods are summarised in Table 4.

TABLE 2 Composition of the *Coccidioides* spp. panel.

Sample number	*Coccidioides* panel
1	*C. posadasii* (RKI06-0090) 10 pg/5 μL
2	Triton 0.1
3	*C. posadasii* (RKI06-0090) 0.01 pg/5 μL
4	*C. posadasii* (RKI06-0090) 1 pg/5 μL
5	*T. violaceum* (RKI16-0839) 100 pg/5 μL
6	*C. posadasii* (RKI06-0090) 0.001 pg/5 μL
7	*Candida albicans* (RKI11-0116) 100 pg/5 μL
8	*U. reesii* (RKI19-0061) 100 pg/5 μL
9	*C. posadasii* (RKI06-0090) 0.1 pg/5 μL
10	*C. posadasii* (RKI06-0090) 100 pg/5 μL

Abbreviations: C., *Coccidioides*; RKI, Robert Koch Institute; T., *Trichophyton*; U., *Uncinocarpus*.

2.5 | Interpretation of the results

For the sensitivities, we compared the percentage of true positive PCR signals detected in each panel, the lowest detected concentration and absolute amount of DNA, and we transformed the latter into genome equivalents (GE). For the calculation of the absolute amount of DNA, and the GEs, the volume of sample per reaction was considered (Tables 3 and 4). GE in each well was calculated by the following formula:

$$\text{Number of copies} = \frac{c^{\text{sample}} \times \text{target} \times 6.022 \times 10^{23}}{\text{length} \times 650 \times 10^{9}}$$

'c^{sample}' stands for sample concentration and 'target' for target volume per well. Concerning the 'length', the length of the whole genome sequence of RKI 09-0599 (42 884 016 bp) was used for *H. capsulatum* and for *Coccidioides* spp., the mean size of the 13 currently (date 19.05.2022) in NCBI available *C. posadasii* WGSs (27 456 599 bp; range: 25.45–28.62 Mb).

We compared the percentage of false positive results detected in each panel for the specificities.

For the graphical presentation, we used the GraphPad Prism 9.1.0 software.

3 | RESULTS

The raw results of the participating laboratories can be found in the Supporting information (Tables S2 and S3). No PCR inhibition was detected by Centres 1 and 2, which performed internal amplification controls.

3.1 | *Histoplasma capsulatum* panel

Results are summarised in Figure 2 and Tables S4 and S5. The median sensitivity of all protocols was 100% (range: 42.5%–100%, 3 to 7 positive results out of 7 tested *H. capsulatum* DNAs). The median sensitivity of the specific assays was 100% (range: 71.4%–100%, 5 to 7 positive results out of 7) and that of the broad-range fungal cPCR 42.8% (3 positive *H. capsulatum* sequencing results out of 7). The median sensitivity of the specific qPCRs was 100% (range: 85.7%–100%, 6 to 7 positive results out of 7), and the sensitivity of the specific cPCR 71.4% (5 positive *H. capsulatum* sequencing results out of 7). The lowest detected concentration of the latter was 2 pg/1 μL. For centres 1, 3 and 4, using all qPCRs (Table 1), the lowest detected concentration of *H. capsulatum* DNA was 0.2 fg/1 μL. For Centre 2, using a qPCR targeting ITS1, the lowest detected concentration was 2 fg/1 μL (Figure 2). Centre 5 amplified *H. capsulatum* DNA concentrations until 20 fg/1 μL with the specific cPCR.

The resulting lowest detected absolute quantities of DNA were 1 fg (0.02 GE) for Centre 1; 1.6 fg (0.03 GE) for Centres 3 and 4 (all three tests); 4 fg (0.08 GE) for Centre 2; 100 fg (2.13 GE) for Centre 5 in the specific cPCR and 10 pg (213 GE) in the broad-range fungal cPCR (Figure 3 and Table S5).

Results regarding specificity are summarised in Table S4. The median specificity of all assays was 100%, (range: 57.1%–100%, means 4 to 7 non-*H. capsulatum* DNAs were tested negative for *H. capsulatum*). The specificity of the broad-range fungal and specific cPCR was 100% and the qPCR's median specificity 100% (range: 57.1%–100%, 4 to 7 non-*H. capsulatum* DNAs were tested negative for *H. capsulatum*). Samples containing high concentrations (20 pg/1 μL) of *B. dermatitidis* (Cq = 25.74/25.85), *Es. europaeus* (Cq = 35.59/35.6) and *Es. africanus* (Cq = 35.53/35.81) DNA tested positive in Centre 3 with the RT-qPCR targeting mtSSU. The sample with *B. dermatitidis* DNA also tested positive in Centre 4 (Cq = 32.61) with the qPCR targeting ITS2 (assay 1). In Centre 5, *A. fumigatus* DNA (20 pg/1 μL) tested positive in the specific *Histoplasma* cPCR, targeting ITS1, but subsequent sequencing demonstrated that it was *Aspergillus* DNA. The broad-range fungal cPCR in Centre 5, the qPCRs targeting ITS1 in Centres 1, 2 and 3 (test 2) and the qPCR targeting *COX2* in Centre 3 (assay 3) had a specificity of 100%.

3.2 | *Coccidioides* panel

Results are summarised in Table S6. The median sensitivity of all PCRs was 83.3% (range: 33.3%–100%, 2 to 6 positive results out of 6 tested *C. posadasii* DNAs). The sensitivity of specific qPCR assays ranged from 75% to 100% and that of the broad-range cPCR was 33.3%. For Centres 1 (one replicate out of two) and 4, targeting ITS2 and *PRA2*, respectively, the lowest detected concentration of *C. posadasii* DNA was 0.2 fg/1 μL. For Centres 2 (one replicate out of two) and 3, targeting ITS1 and mtSSU, respectively, 2 fg/1 μL was the lowest detected concentration (Figure 4). For Centre 5, the lowest detected concentration using the broad-range fungal cPCR was 2 pg/1 μL. Only Centres 4 and 5 could identify the *Coccidioides* species as *C. posadasii*. The resulting minimal detected DNA amounts were 1 fg for Centre 1 (0.03 GE); 1.6 fg for Centre 4 (0.05 GE); 4 fg

TABLE 3 Methods used in the different participating laboratories to detect *Histoplasma* DNA.

Centre	Type of PCR (target)	Amplicon length (bp)	Volume of mastermix and sample (μL)	Number of cycles	Number of tested replicates	Polymerase	PCR cycler
1	*Histoplasma* qPCR (ITS1)[12,49]	63	20 + 5	45	2	TaqMan MM2 (Invitrogen)	7500 Real-Time PCR System (Applied Biosystem)
2	*Histoplasma* qPCR (ITS1)[43]	182	18 + 2	50	2	Sensimix Probe no ROX (Bioline)	Light Cycler 480 II thermocycler (Roche)
3	*Histoplasma* RTqPCR (mtSSU)[28]	74	17 + 8	50	2	Superscript III one step RT-PCR (Life Technologies Cooperation)	Light Cycler 480 thermocycler (Roche)
4	Assay 1: *Histoplasma* qPCR (ITS2)[a]	112	12 + 8	45	1	SensiFast no-ROX qPCR mastermix (Bioline)	Light Cycler 480 II thermocycler (Roche)
	Assay 2: *Histoplasma* qPCR (ITS1)[36a]	154					
	Assay 3: *Histoplasma* qPCR (COX2)	163					
5	Assay 1: *Histoplasma*-specific cPCR (ITS1)[b]	182	20 + 5	45	1	Illustra PuReTaq Ready-to-Go (Cytiva)	MyCycler (Biorad)
	Assay 2: panfungal cPCR (ITS)[77,78]	289	20 + 5	45	1	Illustra PuReTaq Ready-to-Go (Cytiva)	MyCycler (Biorad)

Abbreviations: COX2, cytochrome C oxidase 2; cPCR, conventional PCR; ITS, internal transcribed spacer; mtSSU, mitochondrial ribosomal small subunit RNA; qPCR, real-time PCR; RT-qPCR, reverse transcriptase real-time PCR.

[a]10 μL SensiFast no-ROX qPCR mastermix (Bioline), 2 μL primers/probe mix (1.76 μL 1× TE pH 8, 0.1 μL of forward and reversed primer [100 pmol/μL] and 0.04 μL TaqMan-probe [100 pmol/μL]).

[b]Histoplasma-specific cPCR was done using the Primer HC ITS1-1 (10 μmol) 5′-CCACCCTTGTCTACCG-3′ and Primer HC ITS1–2 (10 μmol) 5′-GGAACCAAGAGATCCGT-3′, Amplifications were performed in a 25 μL volume reaction of PCR beads (Illustra PuReTaq Read-to-Go; GE Healthcare), containing 15 pmol of each primer and 20–50 ng of genomic DNA.

TABLE 4 Methods used in the different participating laboratories to detect *Coccidioides* species DNA.

Centre	Type of PCR (target)	Amplicon length (bp)	Volume of mastermix and sample (μL)	Number of cycles	Number of tested replicates	Polymerase	PCR cycler
1	*Coccidioides* qPCR (ITS2)[33,52]	98	20+5	45	2	TaqMan MM2 (Invitrogen)	Biorad CFX Maestro cycler
2	*Coccidioides* qPCR (ITS1)[45]	266	18+2	50	2	SensiMix Probe no ROX(Bioline)	Light Cycler 480 II thermocycler (Roche)
3	*Coccidioides* RTqPCR (mtSSU)[a]	51	17+8	50	2	Superscript III One step RT-PCR (Life Technologies Cooperation)	Light Cycler 480 thermocycler (Roche)
4	*Coccidioides* duplex qPCR (PRA2)[68]	*Coccidioides posadasii*: 67 *Coccidioides immitis*: 130	12+8	45	1	SensiFast no-ROX qPCR mastermix (Bioline)	Light Cycler 480 II thermocycler (Roche)
5	panfungal cPCR (ITS)[77,78]	289	20+5	45	1	Illustra PuReTaq Ready-to-Go (Cytiva)	MyCycler (Biorad)

Abbreviations: cPCR, conventional PCR; ITS, internal transcribed spacer; mtSSU, mitochondrial ribosomal small subunit RNA; PRA2, proline-rich antigen 2; qPCR, real-time PCR; RT-qPCR, reverse transcriptase real-time PCR.

[a]The conditions for the *Coccidioides* spp. RT-qPCT were: 1× Invitrogen RT-qPCR buffer mix, 0.3 mM of each primer (Ci_27_129F: 5′-CCTTTCTTTAAGGTAGTTAAAATATTT-3′ and Ci_24_52R: 5′-AATCG AACGTCATTATCTATATAAG-3′), 0.1 mM of probe (Ci_21_101P: 5′-FAM- ACTCACCTGTACACCACTTCA-BHQ1-3′), and 1 μM of polymerase, in a total of 25 μL with 8 μL of extracted DNA. 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 s and annealing at 53°C for 30 s.

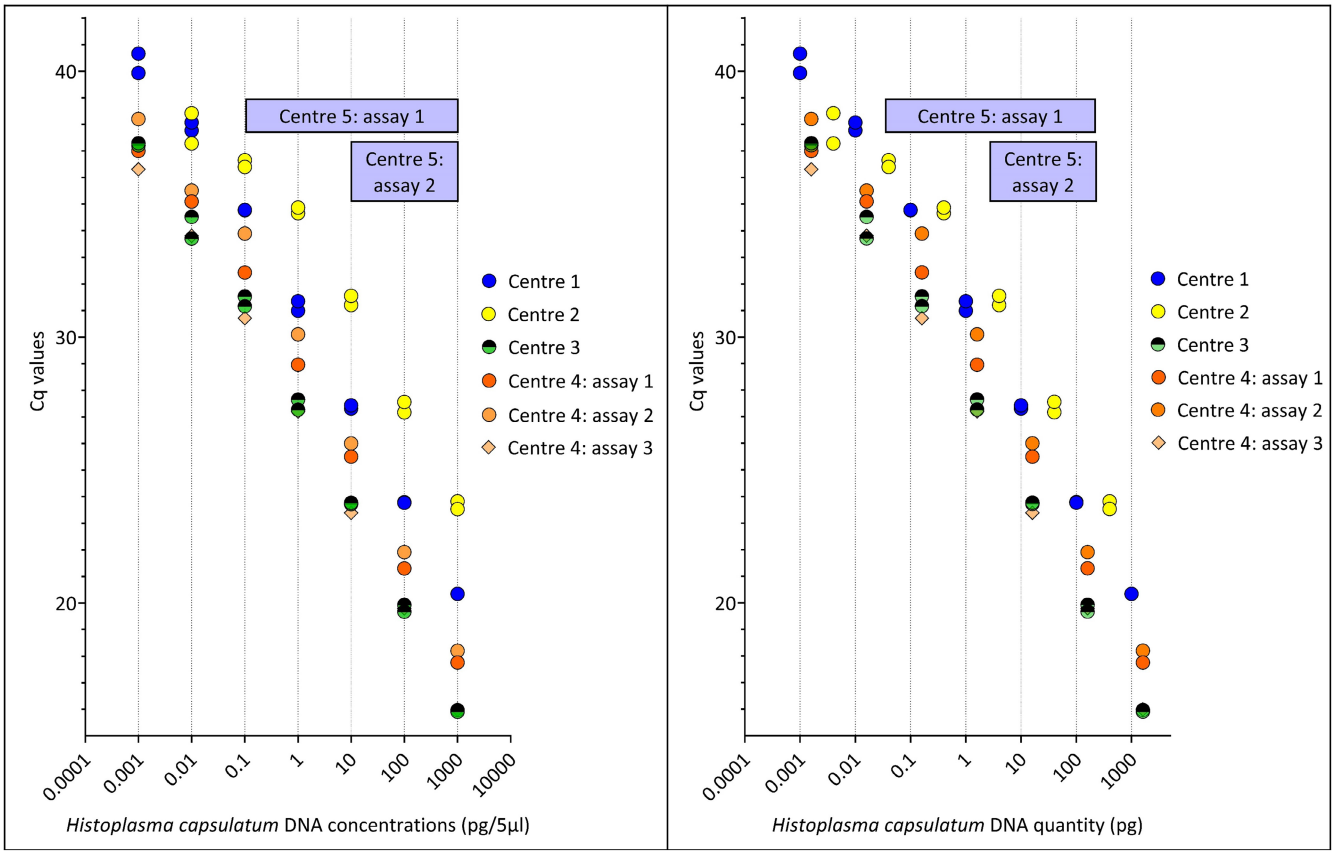

FIGURE 2 Results of the different assays for detecting *Histoplasma capsulatum* DNA. The Cq results of the real-time PCRs are represented in relation to the DNA concentration of the samples (left) or the applied quantity of DNA per reaction. The range of positive results of Centre 5, which performed a panfungal conventional PCR (cPCR) (test 2) and a *Histoplasma*-specific cPCR (test 1), are indicated with boxes. Centres 1, 2 and 3 examined the samples in two replicates, and Centres 4 and 5 tested their samples in one replicate. PCRs targeting multicopy ribosomal DNAs are indicated with circles, the PCR targeting a monocopy genus-specific DNA (COX-2) with a diamond.

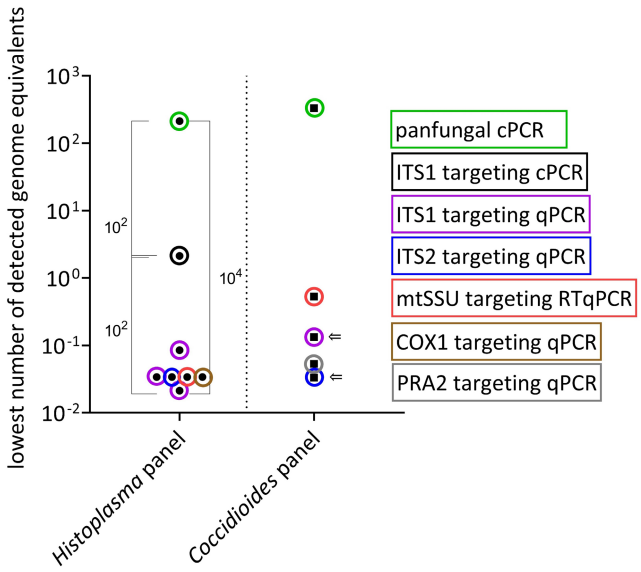

FIGURE 3 Lowest detected number of genome equivalents (GE) of *Histoplasma capsulatum* and *Coccidioides posadasii* in the two corresponding panels. The results found in only one of two replicates are indicated with an arrow. The lowest number detected of GE in the panfungal conventional PCR (cPCR) was 10^4-fold higher than in the real-time PCR (qPCR).

for Centre 2 (0.1 GE); 16 fg for Centre 3 (0.5 GE) and 10 pg in the broad-range fungal cPCR (332 GE) (Figure 3 and Tables S5 and S6).

The median specificity of the herein-used PCRs was 100% (range: 87.5%–100%). The specificity of the broad-range fungal cPCR and the median specificity of the qPCRs were both 100% (range: 87.5%–100%). In Centre 5, high concentrations (20 pg/1 µL) of *U. reesii* tested positive (Cq = 41.96) in one replicate out of 2. The specificity for the rest of the qPCRs was 100%.

4 | DISCUSSION

In the present multicentre study, we evaluated 13 different in-house PCR protocols for detecting *H. capsulatum* and *Coccidioides* spp. Techniques based on PCR are essential to achieve a rapid diagnosis of imported[12,34,35,40,79,80] and autochthonous[26] endemic mycosis cases.

Until today, only one inter-laboratory study compared seven different *H. capsulatum* PCR protocols in four laboratories from Latin America and one in Spain[81] showing that protocols based on qPCR were the most sensitive and reproducible. False positive results or cross-reactions were not seen; however, only the DNA of

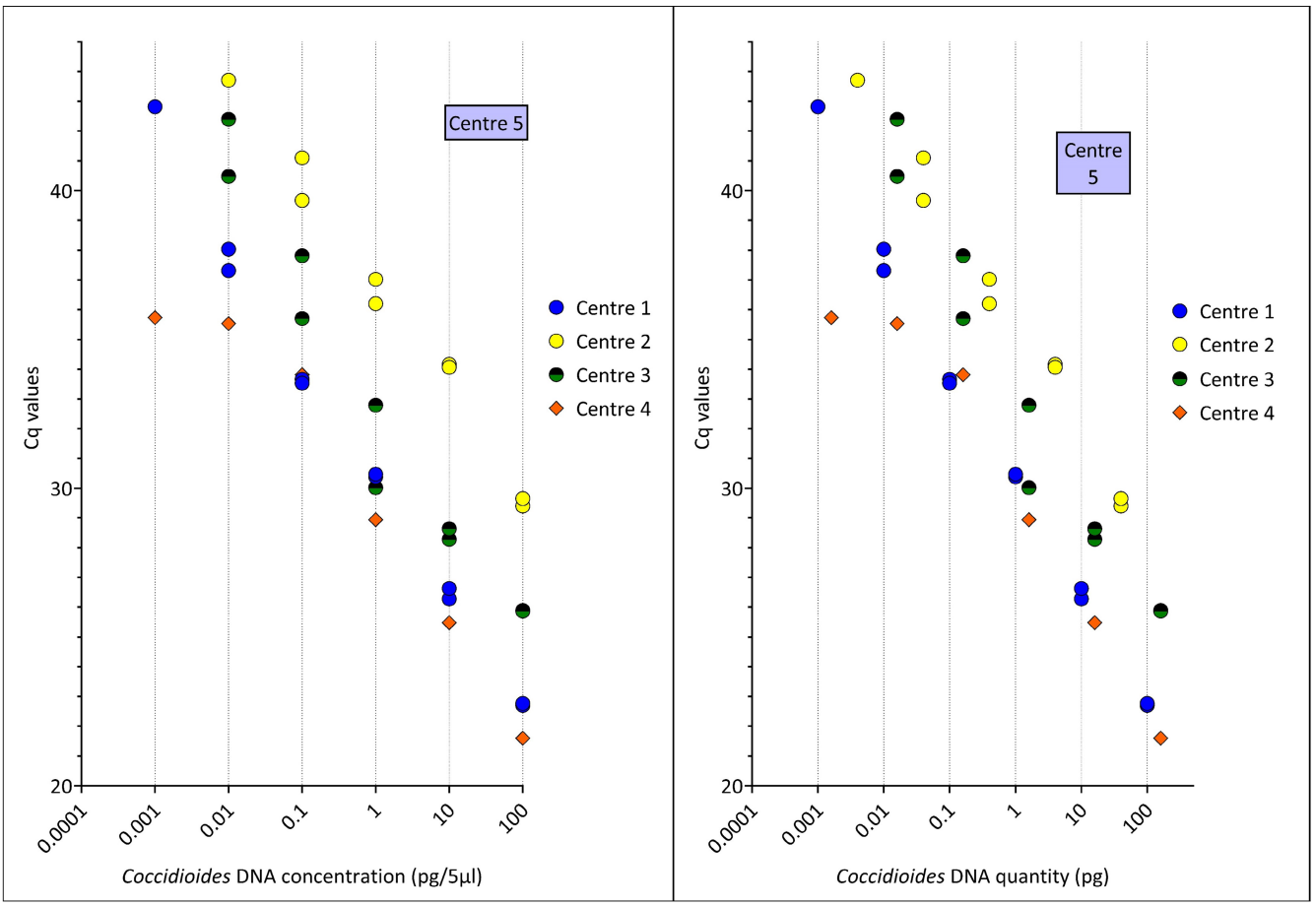

FIGURE 4 Results of the different assays for detecting *Coccidioides* spp. DNA. The Cq results of the real-time PCRs (qPCR) are represented in relation to the DNA concentration of the samples (left) or the applied quantity of DNA per reaction. The range of positive results of Centre 5, which performed a panfungal conventional PCR, is indicated with a box. Centres 1, 2 and 3 examined the samples in two replicates, and Centres 4 and 5 tested their samples in one replicate. PCRs targeting multicopy ribosomal DNAs are indicated with circles, and the PCR targeting a monocopy species-specific DNA (PRA) with a diamond.

P. brasiliensis was used to study the specificity of the amplification protocols. In this study, we included the detection of *Coccidioides* spp., lower target DNA concentrations and more potentially cross-reacting fungal DNAs.[82]

As expected, all laboratories were able to detect *C. posadasii* and *H. capsulatum* DNA. However, sensitivity and specificity depended on the method. The broad-range fungal cPCR was around 2.5×10^3–10^4 times less sensitive than the specific qPCRs in detecting *H. capsulatum* DNA and 6.2×10^2–10^4 times less sensitive than the specific qPCRs in detecting *Coccidioides* DNA. *H. capsulatum* specific qPCRs were superior compared to cPCR which was 25–100 times less sensitive. The lowest amount of *H. capsulatum* DNA was detected by Centres 1, 3 and 4 and by Centres 1 (one replicate out of two) and 4 for *Coccidioides* spp. Centre 2 used less sample volume (2 µL) and multiplexed their qPCRs. This may be a reason that it could not detect DNA in the samples with the lowest DNA concentrations (0.2 fg/1 µL) of both panels. No decrease in sensitivity was observed for the assay using single copy target *PRA2* in comparison to multicopy targets amplifying protocols. However, it cannot be excluded that further dilutions would have shown differences. The herein used qPCR assays were the most sensitive and rapid approaches, indicating that they may be the most promising technique towards an improvement of the diagnosis.

The overall specificity was 91.1% and 97.5% for the *H. capsulatum* and *Coccidioides* spp. panels, respectively. Cross-reactions occurred mainly in the presence of high concentrations of DNA from closely related fungi. In Centre 3, *B. dermatitidis*, *Es. africanus* and *Es. europaeus* DNA were positive with the RT-qPCR targeting mtSSU in the *H. capsulatum* panel, and *B. dermatitidis* DNA alone with a qPCR assay 1 (targeting ITS2) in Centre 4. Both Centres used 8 µL of sample for their qPCRs, which could explain partly the loss of specificity. Additionally, for Centre 3, the primers target conserved regions on mtSSU. This loss of specificity could turn as an advantage as this molecular assay is able to detect simultaneously *Histoplasma*, *Blastomyces* and *Emergomyces*. Centre 2 detected *U. reesii* with their ITS1 targeting qPCR in the *Coccidioides* spp. panel in one duplicate and was reported by the laboratory as negative. Centre 5 obtained a positive result in the sample containing high concentrations of *A. fumigatus* DNA, but sequencing confirmed the presence of *A. fumigatus*. As the fungal burden in clinical samples is usually much lower than the herein used ones, future comparisons should include DNA

concentrations which may be found in clinical samples. However, these protocols should be reviewed by Centres 3 and 4.

Although the broad-range fungal assay was less sensitive in this study, it has well-known limitations (turnaround time, contamination risks); broad-range cPCRs are helpful when there is no clear suspicion of a specific fungal infection.[12,82]

A limitation of this research was that panels contained neither *C. immitis* DNA nor DNAs from other *H. capsulatum* phylogenetic clades, and it cannot be ruled out, that sensitivities of the PCRs for the detection of these other species aren't decreased. Especially in the case of the duplex qPCR from Centre 4, which uses two species-specific primer pairs for the detection of *C. immitis* and *posadasii*. More complete panels should be tested and include more laboratories, also from highly endemic regions. Even if several laboratories from these areas[21,22] lacked equipment for performing PCR assays, the global severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has allowed the implementation of conventional and qPCR technologies worldwide, including laboratories with less resources, which implies that more laboratories could participate in future inter-laboratory studies.

Additionally, the inclusion of specimen matrices should be performed to compare the efficiency of different nucleic acid extraction methods and assay performances.

A consensus about issues such as best target, extraction method, sample preparation, etc., should be reached to include the PCR for the diagnosis of the so-called endemic mycoses in forthcoming diagnostic guidelines. In this context, a European Confederation of Medical Mycology working group has been founded to perform multicentre studies aiming to improve diagnosis and providing better information on the epidemiology of these infections (https://www.ecmm.info/working-groups/working-group-on-the-diagnosis-and-the-epidemiology-of-endemic-mycoses), which are included in the WHO fungal priority pathogens list.[83]

AUTHOR CONTRIBUTIONS

Dunja Wilmes: Investigation; writing – original draft; writing – review and editing; visualization; project administration; formal analysis; data curation; methodology; conceptualization; supervision; validation. **Ferry Hagen:** Investigation; writing – review and editing. **Cristina Verissimo:** Investigation; writing – review and editing. **Alexandre Alanio:** Investigation; writing – review and editing. **Volker Rickerts:** Conceptualization; writing – review and editing. **Maria José Buitrago:** Conceptualization; investigation; methodology; project administration; writing – review and editing; supervision; validation.

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CONFLICT OF INTEREST STATEMENT

Dunja Wilmes, Volker Rickerts and Cristina Verissimo declare no conflict of interest. Ferry Hagen has received grants from Health~Holland and the European Society for Clinical Microbiology & Infectious Diseases (ESCMID). He is Vice-President of the International Society for Human and Animal Mycology (ISHAM), Treasurer of the Netherlands Society for Human and Animal Mycology (NVMy) and Chair of the Division of Microbial Genomics of the Royal Netherlands Society for Microbiology (KNVM-DMG). He has been involved in the validation of molecular detection kits from Bruker MDx and Pathofinder/Pathonostics. Alexandre Alanio has received a travel grant from Gilead and Pfizer, is supported by grants from the French National Research Agency (ANR 20CE35000701) and has received consulting fees from Gilead Sciences. He has received payments from Gilead Sciences and PR edition. Maria José Buitrago is a founding partner and holds shares of Micologia Molecular S.L. She has received grant support from the Instituto de Salud Carlos III and has been paid for talks on behalf of United Medical LTDA and Research Foundation from Hospital 12 de Octubre.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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