



Original article

Comparison of PCR protocols for detecting *Histoplasma capsulatum* DNA through a multicenter study

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ABSTRACT

Background: A multicenter study was conducted. A panel containing DNA from *Histoplasma capsulatum*, as well as negative and cross-reaction controls, was sent to five different laboratories, members of the MICOMOL network from CYTED Program.

Aims: The objective was to assess the accuracy of different PCR protocols to detect *H. capsulatum* DNA.

Methods: Seven different PCR protocols were tested. They were based on PCR techniques and used unicopy and multicopy targets.

Results: Most of these protocols (4/7) were able to detect the smallest amounts of fungal DNA (10^2 fg/ μ l). Overall sensitivity was 86% and specificity was 100%. The protocol based on a unicopy target (SCAR₂₂₀) presented lower sensitivity (43%) but 100% specificity. The real-time protocols tested were highly reproducible, sensitive, and specific. Neither false positives nor cross-reactions were detected in any protocol.

Conclusions: All laboratories were able to amplify *H. capsulatum* DNA, and real-time PCR seems to be a promising tool to efficiently detect this pathogen in clinical samples.

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Comparación de protocolos de la prueba de reacción en cadena de la polimerasa (PCR) para detección de ADN de *Histoplasma capsulatum* a través de un estudio multicéntrico

RESUMEN

Antecedentes: Se realizó un estudio multicéntrico en el que participaron cinco laboratorios miembros de la red MICOMOL a partir del programa CYTED. Los participantes recibieron un panel que contenía muestras de ADN de *Histoplasma capsulatum*, al igual que paneles con muestras de control negativas y de reacciones cruzadas.

Objetivos: el objetivo del presente estudio fue examinar la precisión de los diferentes protocolos de PCR para la detección de ADN de *H. capsulatum*.

Métodos: Se examinaron siete protocolos, todos ellos basados en técnicas de PCR, que empleaban como diana plásmidos unicopia o multicopia.

Resultados: la mayoría de estos protocolos (4/7) pudieron detectar las cantidades más pequeñas de ADN (10^2 fg/ μ l). La sensibilidad global fue del 86% y la especificidad del 100%. El protocolo basado en el plásmido unicopia SCAR₂₂₀ se asoció a la menor sensibilidad (43%) pero a una especificidad del 100%. Los protocolos de PCR en tiempo real fueron muy reproducibles, sensibles y específicos. En ningún caso se detectaron resultados falsos positivos ni reacciones cruzadas.

Palabras clave:

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Conclusiones: todos los laboratorios pudieron amplificar el ADN de *H. capsulatum* y las técnicas basadas en PCR en tiempo real parecen un instrumento prometedor para la detección eficiente de este patógeno en muestras clínicas.

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Histoplasmosis is an infection acquired by the inhalation of *Histoplasma capsulatum* conidia present in soils rich in organic matter, particularly guano of bats or birds.⁸ Although this fungus has a global distribution, the most endemic regions are found in the American continent: Mississippi, Ohio, and Missouri river valleys in United States of America, and Central and South America.^{6,7} Nowadays, it is the most common endemic mycoses in Europe, mainly due to immigration and travellers coming from these endemic areas.²

Microbiological classic diagnosis is based on isolating the organism in cultures, examining microscopically fluids and tissues, and doing serological techniques.¹² Nonetheless, all these methods have limitations. Cultures are slow, can take up to 3–4 weeks to be positive, and require level 3 facilities; the sensitivity and specificity of histopathological and serological techniques are quite different depending on the patient's clinical condition, thus the histopathology has a reduced sensitivity in the subacute or chronic forms of the pulmonary histoplasmosis, and serological tests are less sensitive in the progressive disseminated forms, probably because of underlying immunosuppression. In the acute forms, serology may be negative during the first 2 months of infection. Furthermore, a positive serology result can be misleading in patients from endemic regions because serologic test remain positive for several years.²¹ These limitations have led to the development of molecular techniques. In the last few years, PCR assays have been proposed for detection of *H. capsulatum*. Several conventional assays to detect *H. capsulatum* DNA, targeting single or multicopy genes, have been reported.^{3,7,10,18,20} Real-time PCR techniques showing a high sensitivity and specificity and yielding results in a few hours^{4,11,14} have also been published. All these molecular methods are faster than cultures and avoid the manipulation of the fungus, but although these techniques seem very promising, all of them are in-house methods with limited availability and without external validation of results.

In the present study, we performed an interlaboratory comparison of PCR protocols to detect *H. capsulatum* DNA. The objective was to evaluate the protocols routinely used in each laboratory using a common source of fungal DNA. The laboratories implicated were part of the MICOMOL-Network that emerged from the CYTED programme (Ciencia y Tecnología para el Desarrollo) which is an intergovernmental programme of multilateral cooperation in Science and Technology that aims at cooperation in research and innovation for the development of the Iberoamerican region.

Methods

Participants

Five laboratories were involved in this study. Four of them are located in endemic countries (Argentina, Brazil, Colombia, and México) and one in a non-endemic region (Spain); all are members of the MICOMOL-CYTED network. The centers have both clinical and scientific expertise in the field and use PCR techniques in routine laboratory tasks.^{15,20} They have been designated with the following numerical code: (Lab. 1) Unidad de Micología Médica y Experimental, Corporación para Investigaciones Biológicas, Medellín, Colombia; (Lab. 2) Departamento de Microbiología y Parasitología, UNAM, México D.F., Mexico; (Lab. 3)

Table 1

Content of the panel sent to each laboratory.

Tube number	Content (fg/μl)
1	10 ⁷ <i>H. capsulatum</i> DNA
2	10 ⁶ <i>H. capsulatum</i> DNA
3	Water
4	10 ⁵ <i>H. capsulatum</i> DNA
5	10 ⁴ <i>H. capsulatum</i> DNA
6	10 ³ <i>H. capsulatum</i> DNA
7	Water
8	10 ² <i>H. capsulatum</i> DNA
9	10 ⁴ <i>H. capsulatum</i> DNA
10	10 ⁶ <i>P. brasiliensis</i> DNA

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Two of these laboratories (Lab. 2 and Lab. 4) contributed with two different protocols and were designated as Lab. 2(1), Lab. 2(2), and Lab. 4(1), Lab. 4(2), respectively. The rest of the centers contributed with a single protocol.

Panel of DNAs

A blind panel with ten tubes containing different concentrations of fungal DNA was sent to each participant laboratory in May 2011 (see Table 1). Tubes with different concentrations of *H. capsulatum* DNA were included together with tubes containing water as negative controls and a tube with *Paracoccidioides brasiliensis* DNA. To avoid DNA degradation in freeze–thaw, aliquots containing samples were frozen and sent to the laboratories. Each laboratory thawed the panel samples only to perform the assays. To validate the panels, initial testing was performed by the organizing group before their distribution. The receiving centers were asked to confirm the panel reception, comment on its state and keep the tubes frozen at –20 °C until tested.

Strains

Clinical strains of *H. capsulatum* (CNM-CM-2721) and *P. brasiliensis* (CNM-CM-2908) belonging to the microorganism collection of the Spanish National Centre of Microbiology were used to extract DNA.

Each participant center used their own strains to include as positive controls in their respective PCR assays.

DNA extraction

DNA extraction from the strains was performed in biosafety level III facilities in compliance with Spanish law.¹ Briefly, the strains were grown statically at 30 °C in 10 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) for a week. Mycelia were harvested, dried and introduced in a Falcon tube with six glass beads of 4 mm diameter (Sigma–Aldrich Química, Madrid, Spain). The mycelia were subjected to two cycles of freezing in liquid nitrogen for 30 s and vortex for 30 s to obtain a fine powder. Then, the kit “DNeasy Plant Mini Kit” (Qiagen, Izasa, Madrid, Spain) was

Table 2
Assay details of PCR and equipment used by the five participant teams.

Laboratory	Target	PCR type	Equipment	Polymerase
1	Hc100p	Nested ^a	PCT100 (MJ Research)	Taq (Invitrogen)
2(1) ^b	SCAR ₂₂₀	Conventional ^c	MX-BLC-7 (ESCO Technologies)	Taq (Fermentas Molecular Biology Tools)
2(2) ^b	Hc100p	Nested ^a		
3	Hc100p	Nested ^a	C100 (BioRad)	Taq (Invitrogen)
4(1) ^b	Hc100p	Nested ^a	iCycler (BioRad)	Taq (Invitrogen)
4(2) ^b	ITS1	Real Time	AB 7500 (Applied Biosystems)	PerfeCTa qPCR SuperMix (Quanta Bio Sciences, Inc)
5	ITS1	Real Time ^d	LC480 (Roche)	HotStart (Roche)

^a Bialek et al. (2002).

^b Two different protocols were tested in these laboratories.

^c Frías de León et al. (2012).

^d Buitrago et al. (2006).

employed to extract DNA from the obtained powder following the manufacturer's instructions. The quality and amount of DNA obtained was checked spectrophotometrically.

PCR protocols

The participants were told to use their current amplification systems. A total of seven protocols were tested. These protocols were based on conventional, nested and real time PCR and targeted different DNA regions. The enzymes, the target and the equipment used by each laboratory are described in Table 2. Reactions were performed in a final volume of 20 μ l, and 2 μ l of DNA from each tube in the panel were added to the reaction, with the final DNA concentration in the PCR tube being tenfold lower than in the original tube. Each laboratory included positive and negative controls.

Protocol based on SCAR marker

Lab. 2 tested the SCAR₂₂₀ marker (Sequence-Characterized Amplified Region) specific for *H. capsulatum*. In this protocol, a 220 bp fragment was amplified⁹ using the primers 1281-1283₂₂₀F (5'-CATTGTTGGAGGAACCTGCT-3') and 1281-1283₂₂₀R (5'-GAGCTGCAGGATGTTTGTG-3') in the following conditions: denaturation at 94 °C for 3 min and then 30 cycles of 94 °C for 30 s, 55 °C for 30 s; 72 °C for 2 min and a final extension step at 72 °C for 5 min. PCR products were subjected to electrophoresis in agarose gels following the protocols of Sambrook and Russel¹⁹ to confirm the PCR results. The amplicons were purified using the QIAquick purification Kit (Qiagen, Valencia, CA) and sequencing was performed using ABI equipment (Applied Biosystems Inc., Foster City, CA).

Protocols based on 100-kDa-like protein

Labs. 1, 2, 3 and 4 used the method described by Bialek et al.³ that targeted the 100-kDa-like protein unique in *H. capsulatum*. This protein has been described as essential for the survival of the pathogen.¹⁶ This protocol was a nested conventional PCR assay in which the first round amplified a 391 bp fragment and the second round a 210 bp fragment. Primers and PCR conditions were as described by Bialek et al.³ PCR results were verified by using electrophoresis in agarose gels.¹⁹

Protocols based on Real Time PCR (ITS1 region)

Lab. 4 used a real-time PCR assay that targeted the multi-copy ITS1 region of the ribosomal DNA and used the specific primers HcITS-54F (5'-ACCCTTGCTACCGACTGT-3') and HcITS-204R (5'-TTTTACTGGATTATTATCGCTCTCA-3') and the Taqman probe HcITS-155 (5' FAM-CGGTGAACGATTGGCGTCTGAGC-TAMRA 3'). These primers and probes were designed using the Program

Primer Express[®] Software version 3.0 (Applied Biosystems). Each reaction mixture contained 0.5 μ M of each primer and 0.2 μ M of probe. The cycling conditions included the next steps: 2 min at 50 °C, 10 min at 95 °C and then 50 cycles as follows: 15 s at 95 °C and 1 min at 60 °C. Lab. 5 used a previously described real-time PCR assay^{4,5} that had been validated in clinical samples of patients with imported histoplasmosis.⁵ This protocol used specific FRET probes that targeted the ITS1 region of ribosomal DNA and included an internal control. For both assays, standard curves were constructed with PCR results from five repetitions of different dilutions of genomic DNA of *H. capsulatum*. Dilutions ranged between 10⁷ and 1 fg DNA/ μ l. A line ($y = mx + b$) was constructed by plotting the standard curve of log quantity versus its corresponding C_T value, a cycle in which fluorescence becomes detectable from the background. An average value and a 99% confidence interval for crossing-point values obtained for each DNA concentration were calculated.

Results

Participants submitted results at the end of September 2011. All centers were able to amplify *H. capsulatum* DNA and 100% of protocols detected the highest DNA concentrations (Tubes 1, 2, and 4). Results are summarized in Table 3. Sensitivity and specificity values for each protocol are shown in Table 4.

PCR results for SCAR marker protocol

The protocol based on SCAR marker [Lab. 2(1)] presented the lowest sensitivity for the panel sent (43%) and was not able to detect DNA concentrations of less than 10⁴ fg/ μ l. However, this protocol did not produce false positives or cross-reactions.

PCR results for 100-kDa-like protein protocols

The limit of detection was different for the four laboratories that performed this protocol. In Lab. 3 and Lab. 4 the protocols based on the 100-kDa-like protein were able to detect the different *H. capsulatum* DNA concentrations used, reaching the lowest concentration (10 fg/ μ l). In Lab. 1 and Lab. 2(2), fungal DNA concentrations in the PCR reaction tube of less than 10² and 10³ fg/ μ l, respectively, were not detected. The overall sensitivity for the panel using this protocol was 89.5%. The detection limit was established between 10³ and 10 fg/ μ l. False positives were not detected in any case.

PCR results for protocols based on real-time PCR

Both protocols based on real-time PCR were able to detect the lowest concentrations of DNA (10 fg/ μ l).

Table 3
PCR results for the seven PCR protocols tested.

Tube	Final DNA amount ^a (fg/μl)	PCR results								
		Lab. 1	Lab. 2 (1)	Lab. 2 (2)	Lab. 3	Lab. 4 (1)	Lab. 4(2)		Lab. 5	
							Ct	DNA [#] (fg/μl)	Ct	DNA [#] (fg/μl)
1	10 ⁶	Positive	Positive	Positive	Positive	Positive	15.00	3.2 × 10 ⁵	15.71	1 × 10 ⁶
2	10 ⁵	Positive	Positive	Positive	Positive	Positive	20.28	3.5 × 10 ⁴	19.46	10 ⁵
3	Water	Negative	Negative	Negative	Negative	Negative	–	–	–	–
4	10 ⁴	Positive	Positive	Positive	Positive	Positive	24.34	1.9 × 10 ³	23.23	8.6 × 10 ³
5	10 ³	Positive	Negative	Positive	Positive	Positive	27.70	2.7 × 10 ²	26.71	7.9 × 10 ²
6	10 ²	Positive	Negative	Negative	Positive	Positive	30.48	2.3 × 10 ¹	30.19	7 × 10 ¹
7	Water	Negative	Negative	Negative	Negative	Negative	–	–	–	–
8	10 ¹	Negative	Negative	Negative	Positive	Positive	37.00	0.21	33.96	5.4
9	10 ³	Positive	Negative	Positive	Positive	Positive	26.96	2.9 × 10 ²	26.50	9.1 × 10 ²
10	<i>P. brasiliensis</i>	Negative	Negative	Negative	Negative	Negative	–	–	–	–

Ct: threshold cycle; #: amount of DNA estimated from the standard curve in the reaction tube.

^a The amount of DNA refers to the fg per μl in the reaction tube (a tenfold dilution of panel tubes).

Table 4
Sensitivity and specificity of protocols used in the multicentre study for the panel sent.

	Laboratory							
	Lab. 1	Lab. 2 (1)	Lab. 2 (2)	Lab. 3	Lab. 4 (1)	Lab. 4 (2)	Lab. 5	Overall
Sensitivity	86%	43%	72%	100%	100%	100%	100%	86%
Specificity	100%	100%	100%	100%	100%	100%	100%	100%

When DNA concentration was estimated based on the calibration curves, the average of relative errors was 77% for Lab. 4 and 18.5% for Lab. 5 (Table 3).

Neither false positives nor cross-reactions were detected with these protocols.

Discussion

This is the first multicenter study addressed to evaluate molecular protocols used to detect *H. capsulatum* DNA. A blind panel with ten tubes was sent to five different laboratories with the main purpose of assessing the analytical sensitivity of seven PCR protocols to detect *H. capsulatum* DNA. The results presented in this study show that all laboratories were able to amplify *H. capsulatum* DNA. All the protocols were specific and no false positive results were detected although some of the PCR schemes used were based on nested PCR which is more prone to produce false positive results. The overall sensitivity and specificity for the panel was 86% and 100%, respectively. Regarding sensitivity, the protocol based on SCAR marker⁹ presented a sensitivity of 43%, while the nested PCR protocols based on amplification of the gene coding for the 100-kDa-like protein³ presented a high sensitivity. However, Lab. 1 was unable to detect the lowest concentration (10 fg/μl) and Lab. 2 only detected up to 10³ fg/μl of fungal DNA; the other two laboratories (3 and 4) detected all tested fungal DNA concentrations. Consequently, the overall sensitivity for these protocols was 89.5%. The differences in the limit of detection obtained between laboratories that used the same protocol (100-kDa-like protein) may be due to the different PCR equipment, the polymerase or other technical aspects. Further studies will be conducted. Finally, protocols based on real-time PCR detected the lower concentrations and presented 100% sensitivity and specificity.

As we expected, there was a correlation between sensitivity and the PCR target. The unicopy target as SCAR marker exhibited a lower sensitivity than protocols based on multicopy target (ITS1) or nested PCR assays. Nested PCR has the problem of high risk of contamination,²³ although in this study no laboratory produced false positive results, which reflects appropriate precautions to avoid DNA-contamination. Real-time protocols were the most accurate and results were generated in a short time. In addition, no further post-PCR analysis such as electrophoresis or

sequencing was required. Regarding the quantification of DNA in real-time protocols, there were important differences between Lab. 4 and Lab. 5, suggesting differences in the quantification of the DNA that was used as template to construct the standard curves; a further study is needed to improve this pitfall. In recent years, there have been several multicenter studies with the aim of reaching a consensus on PCR techniques, especially for the detection of fungal genera such as *Aspergillus* or *Candida*.^{17,22,24} In these works, spiked samples with known quantities of a microorganism were distributed among the different laboratories. The methodologies and platforms were used according to the requirements of each laboratory. Some of these works are the result of the recently created working group “European *Aspergillus* PCR Initiative” (EAPCRI) which aims to standardize molecular methods to detect *Aspergillus fumigatus* in blood and serum. Different reports with the results of multicenter collaborative studies have been published.^{13,25} Recommendations for DNA extraction, sample volumes and PCR protocols have been forwarded based on the results obtained from these multicenter assays.

In this collaborative work, we can conclude that the protocols based on multicopy targets are more efficient in the detection of the smallest amounts of DNA, making them more suitable for their use in diagnosis. Moreover, real time PCR protocols were highly sensitive, and specific and they seem to be a promising tool to efficiently detect this pathogen in clinical samples. This work is the first joint effort to achieve a consensus on the PCR methods for the detection of *H. capsulatum* DNA. Although clinical samples were not used, this study allowed assessing the molecular techniques used by laboratories that diagnose histoplasmosis routinely. Future collaboration will be established to determine the best PCR protocol and the clinical samples to be used for the molecular diagnosis of histoplasmosis.

Conflict of interest

The authors declare that there is no conflict of interest.

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