TITLE: DEVELOPMENT OF A NEW ONE-TUBE MULTIPLEX PROBE-BASED qPCR ASSAY FOR DETECTING AND SPECIATION OF *PARACOCCIDIOIDES* SPECIES.

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ABSTRACT:
Paracoccidioidomycosis (PCM) is a neglected tropical disease caused by members of the *Paracoccidioides brasiliensis* complex (S1, PS2, PS3, and PS4) and *P. lutzii*. The disease is endemic to Latin America, where Brazil accounts for approximately 80% of cases. Its mortality rate justifies the need for rapid, accurate, and unambiguous diagnose. Laboratory testing is required to confirm the clinical diagnosis of PCM. Although the presence of pathognomonic forms of *Paracoccidioides* spp. provides valuable information, nucleic acid-based diagnostics are gradually replacing or supporting culture-based, biochemical, and immunological procedures in the routine microbiology laboratory. Here, we developed a one-tube multiplex probe-based qPCR assay to improve the molecular diagnosis of PCM. Polymorphisms among the ITS region (internal transcribed spacer) were exploited to speciate members of the *P. brasiliensis* complex and *P. lutzii*. We developed a genus-specific primer pair (Paracoco-F and Paracoco-R) and three hydrolysis probes with specificity to recognize the genus *Paracoccidioides* (Paracoco-VIC), members of the *P. brasiliensis* complex (Pbra-FAM), and *P. lutzii* (Plu-NED). A panel of 75 *Paracoccidioides* revealed 100% specificity (AUC=1.0) without cross-reacting with other medically relevant fungi (n=25), human, or murine DNA. Moreover, speciation via qPCR matched identification using DNA barcoding of the ITS1/2+5.8s region (*Kappa* value = 1.0; very good agreement), supporting its use as a reliable alternative to DNA sequencing. Remarkably, the lower detection limit for our qPCR assay was three copies of the target for all probes. To assess the value of our multiplex probe-based qPCR assay for environmental studies, soil samples were spiked with *Paracoccidioides*, and after DNA extraction, the sensitivity of the qPCR assay was established as 10 pg/µl of *Paracoccidioides* DNA for all probes. As a proof of concept, we demonstrated the accurate identification of members of the *P. brasiliensis* complex (n = 18) or *P. lutzii* (n = 2) from a broad range of formalin-fixed, paraffin-embedded (FFPE) tissues of PCM patient's organs. Positivity was indicated in 19 out of 23 FFPE samples, revealing a sensitivity of 82.6%. These pieces of information show that our multiplex probe-based qPCR assay is a valuable tool for detecting and diagnosing primary agents of PCM.

Keywords: paracoccidioidomycosis, molecular diagnosis, real-time PCR, qPCR
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