Universidade Estadual de Londrina

AMANDA DE FÁVERI PITZ

DETECÇÃO MOLECULAR DE ESPÉCIES DE *CANDIDA* POR SEMI-NESTED PCR

Londrina
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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Patologia Experimental.

Orientador: Prof. Dr. Emerson José Venancio.

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Londrina, 28 de abril de 2017.

Dedico este trabalho a meus
pais, Roseli Fáveri Pitz e Jonas Villar Pitz
por serem as pessoas mais importantes em minha vida.

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RESUMO

O diagnóstico da candidemia é complexo, e a confirmação da infecção por meio de diagnósticos laboratoriais é fundamental. A técnica padrão para o diagnóstico é a hemocultura com o isolamento de fungos, entretanto, devido ao longo tempo requerido para a obtenção do resultado, confere-se a candidemia como uma infecção fúngica sistêmica com alta taxa de mortalidade. Visando agilizar o diagnóstico laboratorial, nos quesitos de tempo de obtenção do resultado, aumento da especificidade e da sensibilidade, bem como a monitorização epidemiológica das candidemias, o uso de testes baseados em PCR para detectar o DNA de *Candida* spp é atualmente considerado promissor na investigação em amostras clínicas, considerando baixas concentrações em pequenos volumes de sangue. Neste trabalho investigamos a utilização da técnica de *semi-nested* PCR para o diagnóstico da candidemia por meio de uma revisão sistemática com metanálise. Foram acessadas as bases: Medline, Web of Science, Scopus, LILACS, Scielo, Embase e Cochrane que identificou um total de 1906 estudos. Destes, 5 foram incluídos na revisão sistemática e analisados quanto ao risco de viés utilizando-se a ferramenta QUADAS-2. Ainda destes estudos incluídos, 3 preencheram os critérios para a metanálise, fazendo-se a comparação direta da *semi-nested* PCR com a cultura por meio de tabelas 2x2 e extração de resultados verdadeiro positivo (VP), falso positivo (FN), verdadeiro negativo (VN) e falso negativo (FN) em 50 pacientes com candidemia confirmada (controle positivo) e em 38 indivíduos saudáveis (controle negativo). A sensibilidade e especificidade nos estudos foram calculadas com um intervalo de confiança de 95% e foi encontrada especificidade de 100% e sensibilidade variável de 88 a 100%. Além disso, os estudos também testaram um total de 77 amostras de pacientes em suspeita de candidemia com hemoculturas negativas, e estes obtiveram uma positividade de 52%. Baseando-se nestes resultados podemos sugerir que a *semi-nested* PCR em amostras clínicas pode ser uma importante ferramenta no diagnóstico de candidemias na rotina laboratorial. Foi também padronizada uma *semi-nested* PCR em única etapa (OTsn-PCR) utilizando como alvo as sequências das regiões espassadoras internas – *Internal Transcriber Spacer* (ITS1 e ITS2), caracterizadas por ter regiões altamente conservadas e regiões altamente específicas para cinco espécies de importância clínica para o gênero *Candida*: *Candida albicans, Candida glabrata, Candida parapsilosis, Candida krusei* e *Candida tropicalis.* A reação apresentou especificidade ao ser testada com outras espécies fúngicas e ótimo limite de detecção, alcançando 0,25pg de DNA por amostra. Quando comparada com ensaios convencionais de *nested* ou *semi-nested* PCR, a OTsn-PCR mostra vantagens pois é mais rápida e apresenta menor risco de contaminação pela menor manipulação dos tubos, tendo um grande potencial para ser utilizada no âmbito do diagnóstico molecular de candidíases e candidemias.

Palavras-chave: Diagnóstico molecular. Ensaios por PCR. Semi-nested PCR. DNAr Candidíase. Candidemia.

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ABSTRACT

The candidemia diagnosis is complex and it is essential to confirm the infection through laboratory diagnoses. The gold standard technique for diagnosis is blood culture of the fungus, however, due to the long time required to obtain the result, candidemia is conferred as a systemic fungal infection with a high mortality rate. In order to speed up the laboratory diagnosis, in the questions of time to obtain the result, increase specificity and sensitivity, as well as the epidemiological monitoring of candidemia, the use of PCR-based tests to detect *Candida* spp DNA is currently considered promising in the investigation in *Candida* spp. Clinical samples, considering low concentrations in small volumes of blood. In this work we investigated the use of the semi-nested PCR technique for the diagnosis of candidemia by means of a systematic review with meta-analysis. The databases were accessed: Medline, Web of Science, Scopus, LILACS, Scielo, Embase and Cochrane which identified a total of 1906 studies. Of these, 5 were included in the systematic review and analyzed for bias risk using the QUADAS-2 tool. Of these included studies, 3 fulfilled the criteria for the meta-analysis, with the direct comparison of the semi-nested PCR with the culture by means of 2x2 tables and extraction of positive results (PV), false positive (FN), true negative (VN) and false negative (FN) in 50 patients with confirmed candidemia (positive control) and in 38 healthy subjects (negative control). Sensitivity and specificity in the studies were calculated with a 95% confidence interval and specificity of 100% and variable sensitivity of 88 to 100% were found. In addition, the studies also tested a total of 77 patient samples suspected of candidemia with negative blood cultures, and these obtained a positivity of 52%. Based on these results we can suggest that the semi-nested PCR in clinical samples can be an important tool in the diagnosis of candidemia in the laboratory routine. It was also standardized a semi-nested single-step PCR (OTsn-PCR) targeting the sequences of the internal Transcriber Spacer regions (ITS1 and ITS2), characterized by having highly conserved regions and highly specific regions for five species of importance clinic for the genus *Candida*: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and *Candida tropicalis*. The reaction showed specificity when tested with other fungal species and excellent detection limit, reaching 0.25pg of DNA per sample. When compared to conventional nested or semi-nested PCR assays, the OTsn-PCR shows advantages as it is faster and presents a lower risk of contamination due to the lower manipulation of the tubes, having a great potential to be used in the molecular diagnosis of candidiasis and Candidemias.

Keywords: Molecular diagnosis. PCR assays. Semi-nested PCR. DNAr Candidiasis. Candidemia.

LISTA DE ABREVIATURAS E SIGLAS

µl	microlitro
%	por cento
DNA	Ácido desoxirribonucléico
DNAr	DNA ribossômico
dNTPs	Desoxirribonucleotídeos
g	grama
L	litro
ITS	*Internal Transcribed Spacer*
M	Molaridade
mg	miligrama
min	minuto
Mm	milimolar
ng	nanograma
°C	Graus Celsius
pb	Pares de bases
pg	picograma
PCR	Reação em cadeia da polimerase

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1 INTRODUÇÃO

1.1 GÊNERO *CANDIDA* SPP

Candida spp. é um gênero pertencente a Divisão *Eumycota*, Subdivisão *Ascomycetes*, Classe *Debaryomycetaceae*, Família *Saccharomycetales*, no qual já foram descritas mais de duzentas espécies. Apenas 10% das espécies descritas são de interesse médico (EGGIMANN et al., 2003). O desenvolvimento de organismos desse gênero é pleomórfico predominantemente unicelular e normalmente a reprodução é por brotamento unilateral (blastoconídeo) (BERMAN; SUDBERY, 2002). Eles possuem parede celular bem definida, constituída por uma camada de manoproteína, uma camada com polímeros de glucanas com β-1,3-D-glucanas, β-1,6-D-glucanas, quitina e de uma pequena quantidade de proteínas e lipídios (AKPAN; MORGAN, 2002).

Algumas espécies de *Candida* spp. fazem parte da microbiota do trato gastrointestinal, entretanto, espécies desse gênero podem colonizar a pele, mucosas e cavidade oral (MIRANDA et al., 2009). Sabe-se que são poucas as espécies de *Candida* capazes de crescer a 37ºC e por isso é restrita a colonização em humanos. *Candida albicans* é a espécie mais comum em infecções fúngicas humanas, devido ao fato de apresentar uma grande variedade de fatores de virulência (DELAYOLE; CALANDRA, 2013). Porém, a infecção causada por *Candida* spp está mais relacionada ao estado imunológico do hospedeiro do que aos seus próprios fatores de virulência (MIRANDA et al., 2009). *C. albicans* possui a capacidade *switching*, que é habilidade de alterar seu fenótipo, adaptando-se ao microambiente (MORSCHHAUSER, 2010).

Entre as enzimas mais importantes para a patogenicidade de *Candida* estão as aspartilproteases secretadas (SAP), que atuam como queratinases e colagenases, e as fosfolipases, cuja função é degradar a membrana da célula hospedeira, levando a lise celular (NAGLIK ET AL., 2004; WILLIAMS; LEWIS, 2011).

Espécies patogênicas do gênero *Candida* spp expressam uma variedade de fatores de virulência, no qual podem aumentar a gravidade da infecção. Entre os principais fatores, estão a produção de proteinases, fosfolipases, adesinas, produção de D-arabinitol, formação de biofilme e de hifas (EGGIMANN et al., 2003).

1.2 ESPÉCIES DE INTERESSE MÉDICO

Espécies patogênicas do gênero *Candida* spp expressam uma variedade de fatores de virulência, no qual podem aumentar a gravidade da infecção. Entre os principais fatores, estão a produção de proteinases, fosfolipases, adesinas, produção de D-arabinitol, formação de biofilme e de hifas (EGGIMANN et al., 2003).

Entre o gênero *Candida* spp, a espécie *albicans* é a espécie oportunista mais importante, porém, as espécies de *Candida* não-*albicans* estão cada vez mais frequentes no âmbito clínico, principalmente as espécies *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei* e *Candida glabrata* (GIOLO; SVIDZINSK, 2010).

C. albicans apresenta forte aderência à mucosa e grande produção de enzimas, é responsável por muitos relatos de candidemia nosocomiais, (BECKER et al., 2010; WISSING et al., 2013) representando cerca de 40 a 50% dos casos e taxa de mortalidade variável de 40 a 60% dependendo de aspectos clínicos, fatores de risco e peculiaridades do paciente (GUINEA et al., 2014, FOONGLADDA et al., 2014).

C. glabrata também tem uma significativa relevância nos casos de candidemia em adultos, representando cerca de 5 a 14% dos episódios (COLOMBO et al., 2006; OESER et al.,2013). Entre os pacientes com maior risco de candidemia por *C. glabrata* estão os indivíduos submetidos a transplantes de órgãos, com insuficiência renal crônica, com tumores sólidos, submetidos à profilaxia antifúngica e procedimentos intensivos (MORAN et al., 2010). A candidemia por *C. glabrata* possui uma taxa de mortalidade entre as mais elevadas atribuídas às espécies de *Candida*, variando entre 40 a 70% (ALONSO-VALLE et al., 2003; MORAN et al., 2009).

Os principais fatores de virulência de *C. tropicalis* são a alta capacidade de aderência em mucosa, capacidade de formar biofilmes e grande capacidade de secreção de lipases e proteinases (WILLIAMS; LEWIS, 2011). Os principais fatores de riscos associados à infecção por *C. tropicalis* são admissão e o tempo de permanência em unidades intensivas de tratamento, pacientes em tratamento de câncer, utilização de cateter venoso central e antibioticoterapia de amplo espectro (NEGRI et al, 2012).

C. krusei possui resistência constitutiva ao fluconazol e relativa sensibilidade a anfotericina B (RICARDO et al., 2011). Tende a ser isolada de infecções em

pacientes neutropênicos, com câncer ou infectados pelo vírus da imunodeficiência (ALMIRANTE et al., 2005). Possui taxa de mortalidade em cerca de 40% (MÜH et al.,2006).

C. parapsilosis é a espécie mais comum de candidemias em recém-nascidos, entretanto, possui a menor taxa de mortalidade do gênero *Candida* spp (ORTEGA et al, 2011). Seus principais fatores de risco são nutrição parenteral e a utilização de cateter venoso central (MIRANDA et al., 2009).

1.3 CANDIDEMIA

A candidemia é uma infecção da corrente sanguínea causada por leveduras do gênero *Candida*, comprovada por cultura sanguínea, considerando-se procedimentos estéreis. Sabe-se que a principal espécie envolvida é *C. albicans*, entretanto, tem-se observado que outras espécies do gênero *Candida* tem aparecido no contexto clínico (BRUDER-NASCIMENTO et al., 2010; PEMÁN et al. 2012). É possível observar que a frequência de candidemia por espécies não-*albicans* tem até ultrapassado a frequência por *C. albicans* em alguns estudos (GONZÁLEZ et al., 2008; LEE et al., 2007).

A candidemia é a infecção fúngica de corrente sanguínea mais frequente em ambientes hospitalares, especialmente em pacientes de alto risco como aqueles internados em unidades de tratamento intensivo (UTI), em tratamento com agentes imunossupressores, com câncer, vítimas de queimaduras, neonatos, pacientes com HIV, transplantados entre outros, alcançando até 50% de mortalidade nestes pacientes (AITTAKORPI et al., 2012; FORTUN et al., 2014).

Um estudo realizado sobre a ocorrência de candidemia em onze centros médicos brasileiros demonstrou que espécies não-*albicans* estão relacionadas a maioria dos casos (59%) e as principais espécies são: *C. tropicalis* (21%), *C. parapsilosis* (21%) e *C. glabrata* (5%) (COLOMBO et al., 2006). Outros estudos brasileiros demonstraram que *C. tropicalis* foi uma das principais espécies de *Candida* encontrada em pacientes com candidemia, tendo uma porcentagem de positividade de 26%, juntamente com *C. parapsilosis* (26%) e *C. albicans* (19%) (BRUDER-NASCIMENTO et al., 2010; PEREIRA el al., 2010; XAVIER et al., 2008).

1.4 FATORES DE RISCO

A maioria dos fatores de risco associados à candidemia está envolvida diretamente com aspectos clínicos do paciente, e especialmente à prematuridade na neonatalidade, estado imunológico do indivíduo entre outras condições (BERMAGASCO et al., 2012).

A candidemia está comumente relacionada à imunossupressão ligada a diminuição de atividade do sistema fagocitário do hospedeiro. Outros fatores como idade, cateterismo, queimaduras graves, sondagens, pacientes internados em unidades oncológicas e/ou hematológica, unidades de tratamento intensivo e também pacientes submetidos a antibioticoterapia de amplo espectro são fatores que podem favorecer o aparecimento das infecções por *Candida* spp. (ALANGADEN, 2011; DELALOYE; CALANDRA, 2013).

A candidemia apresenta manifestações clínicas variáveis, muitas vezes não específicas. Assim sendo, podem desenvolver sinais e sintomas cardíacos, digestivos, respiratórios, hepáticas, renais e nervosos. Por se tratarem de quadros graves requerem tratamento com antifúngicos sistêmicos (COLOMBO et al., 2013; PFALLER et al., 2012; HORN et al., 2009; OESER et al., 2013).

1.5 EPIDEMIOLOGIA DA CANDIDEMIA NO MUNDO E NO BRASIL

A importância do estudo da ocorrência de candidemia consiste pela necessidade de elucidar a prevalência, monitorar tendências de incidência, distribuição de espécies e perfil de sensibilidade a antifúngicos (COLOMBRO et al., 2006).

Um estudo realizado nos Estados Unidos da América entre os anos de 1979 e 2000 demonstrou um aumento das sepses fúngicas em 207% e enfatizou a importância do diagnóstico precoce nestes contextos (MARTIN et al., 2003). Ainda nos Estados Unidos relatou-se um aumento de 50% na incidência de candidemia no período de 2000 a 2005 (ZILBERBERG et al., 2008).

Uma pesquisa da Confederação Europeia de Micologia Médica sobre candidemia, ao analisar 2089 casos provenientes de 106 instituições de saúde de sete países da Europa, relatou que *C. albicans* foi a mais prevalente (56%), seguida de *C. glabrata* (13,6%), *C. parapsilosis* (13,3%) e *C. tropicalis* (7,2%), e a taxa de

mortalidade foi de 37,9% (TORTORANO et al., 2004). Em outro estudo realizado em hospitais da Espanha e Itália foram analisados 995 casos de candidemia distribuídos entre os anos de 2008 e 2010, as espécies mais isoladas foram *C. albicans* com (58,4%), seguida de *C. parapsilosis* (19,5%), *C. tropicalis* (9,3%) e *C. glabrata* (8,3%), e a taxa de óbito foi de (39,9%) (BASSETTI et al., 2013).

Na China, quando analisados 133 casos de candidemia entre os anos de 2009 e 2011, a espécie com maior ocorrência foi *C. tropicalis* com (28,6%), seguida por *C. albicans* (23,3%) e *C. parapsilosis* (19,5%) com uma taxa de mortalidade de (26%) sendo que, pacientes infectados por *C. tropicalis* apresentaram taxas mais elevadas (44,7%) (MA et al., 2013).

No Chile, um estudo realizado entre 2001 a 2003, relatou maior prevalência de *C. albicans* (44,4%) nas candidemias e as espécies *Candida* não-*albicans* representaram (55,6%), no qual, *C. tropicalis* foi a mais prevalente (27,8%) (AJENJO et al. 2011).

No Brasil, algumas pesquisas realizadas sobre a candidemia relataram alta prevalência da espécie *C. albicans,* nos quais as taxas que variaram entre 44% a 52% (CHANG et al. 2008; FRANÇA et al. 2008; MOTTA et al. 2010; XAVIER et al. 2008). Entretanto, foram encontrados dados divergentes em estudos feitos nos estados de Pernambuco e São Paulo, nos quais a principal espécie isolada foi *C. parapsilosis* (HINRICHSEN et al. 2008; MONDELLI et al. 2012) e no estado do Paraná, onde a espécie mais isolada foi *C. tropicalis* (FURLANETO et al., 2011).

Um estudo retrospectivo sobre as candidemias feito em um hospital universitário no sudeste do Brasil, entre os anos de 2006 a 2010, demonstrou que as espécies mais prevalentes foram *C. albicans* (44%), *C. tropicalis* (21,7%), *C. parapsilosis* (14,4%), *C. glabrata* (11,2%), e *C. krusei* (3,5%) (MORETTI et al., 2013).

Colombo e colaboradores (2006) realizaram um estudo epidemiológico acessando dados de pacientes de 9 grandes cidades brasileiras e *Candida* spp. foi o quarto microrganismo com maior frequência de isolamento em hemoculturas, precedido por Estafilococos coagulase-negativo, *Staphylococcus aureus* e *Klebsiella pneumoniae*, tendo o ranking de *C. albicans* com 40,9%, seguido de *C. tropicalis* com 20,9% e *C. parapsilosis* com 20,5%. A taxa de mortalidade para as candidemias neste estudo foi de 54%. Nesta mesma análise foi demonstrado que espécies não-*albicans* estão relacionadas a maioria dos casos de candidemia (59%) e as principais espécies são: *C. tropicalis* (21%), *C. parapsilosis* (21%) e *C. glabrata* (5%).

Um estudo realizado em 16 hospitais de várias regiões brasileiras, entre 2007 a 2010, descreveram *Candida* spp como o sexto microrganismo mais isolado nas Unidades de Terapia Intensivas. Quanto à distribuição das espécies, identificou-se que as espécies não-*albicans* foram responsáveis por mais de 50% cos casos de candidemia, sendo mais significativas as espécies *C. tropicalis* e *C. parapsilosis* (MARRA et al., 2011).

Outros estudos brasileiros demonstraram que *C. tropicalis* foi uma das principais espécies de *Candida* encontrada em pacientes com candidemia, tendo uma porcentagem de positividade de 26%, juntamente com *C. parapsilosis* (26%) e *C. albicans* (19%) (BRUDER-NASCIMENTO et al., 2010; PEREIRA el al., 2010; XAVIER et al., 2008).

1.6 TRATAMENTO

Os azólicos são os antifúngicos mais largamente utilizados no tratamento das candidemias. O fluconazol é um antifúngico triazólico de largo espectro, com excelente biodisponibilidade e um perfil farmacocinético favorável, utilizado no tratamento de várias infecções por *Candida* spp. e outros fungos. Entretanto, o surgimento de espécies resistentes a este fármaco tem restringido seu uso. O novo azólico voriconazol apresenta maior espectro de atividade, incluindo a espécies de *Candida* spp resistentes ao fluconazol (MAERTENS, 2004).

A atividade destes fármacos está baseada na interferência da síntese de ergosterol por meio da inibição da enzima lanosterol 14-α-demetilase, codificada pelo gene *ERG11*. A função da enzima é converter lanosterol a ergosterol e sua inibição leva à presença de precursores do ergosterol na membrana na célula fúngica, considerados tóxicos (CUENCA-ESTRELLA et al., 2001). Recentemente, uma nova geração de triazólicos, incluído posaconazol, ravuconazol, albaconazol e isavuconazol estão sendo desenvolvidos, e diversos trabalhos demonstram que estes medicamentos são altamente ativos contra isolados de *Candida* spp, incluindo isolados resistentes e sensíveis ao fluconazol (CALVO et al., 2003; CUENCA-ESTRELLA et al., 2006; GONZALEZ et al., 2008; GUINEA et al., 2008; PASQUALOTTO; DENNING, 2008; SEIFERT et al., 2007).

Os poliênicos como a anfotericina B e a nistatina pertencem à outra classe de antifúngicos que possuem como alvo, membranas contendo ergosterol. A

anfotericina B tem sido considerada um dos agentes antifúngicos mais eficientes para uso sistêmico; contudo, a toxicidade e a disfunção renal observada têm restringido sua utilização (COLOMBO et al., 2013). Estes problemas têm diminuído de acordo com que são desenvolvidas formulações destes medicamentos (CARRILLO-MUÑOZ et al., 2006).

Atualmente vários estudos preconizam o uso de uma das três equinocandinas (caspofungina, micafungina e anidulafungina) como a primeira linha de escolha para o início do tratamento dos episódios de candidemias, visto sua especificidade antifúngica e baixa toxicidade (COLOMBO et al., 2013; CORNELY et al., 2012; NUCCI et al., 2013). As equinocandinas constituem uma classe de antifúngicos disponibilizada para administração exclusivamente parenteral. Atuam como inibidores direcionados da biossíntese de um componente da parede celular dos fungos (KATIVAR et al., 2006).

A escolha da melhor terapêutica antifúngica para pacientes com candidemia deve considerar a presença de infecções em órgãos, gravidade do episódio, espécie de *Candida* envolvida, risco para toxicidade renal, exposição prévia a antifúngicos, presença de cateter venoso central e necessidade de cirurgia para remoção de focos infecciosos (COLOMBO et al., 2013).

A resposta à terapia antifúngica varia de acordo com a espécie fúngica causadora da infecção. *Candida albicans* e *C. parapsilosis* geralmente são sensíveis ao fluconazol, enquanto que a *C. glabrata* possui sensibilidade dose-dependente ou resistência ao mesmo. Já *C. krusei* é considerada resistente ao fluconazol (SHAH et al., 2011). Isolados clínicos da espécie *C. tropicalis* respondem bem ao tratamento com anfotericina B e azólicos, mas diversos estudos já demonstram o desenvolvimento de resistência ao fluconazol (BASSETTI et al., 2011; COLOMBO et al., 2013).

1.7 DIAGNÓSTICO

A sobrevivência dos pacientes com candidemia está diretamente associada à precocidade da identificação da espécie envolvida na infecção. Logo, a utilização de métodos rápidos e que resultem na identificação precisa da espécie e do padrão de sensibilidade é essencial para o tratamento clínico (MÍMICA et al., 2009).

O diagnóstico da candidemia é baseado nos achados clínicos combinados com os exames de laboratório. Entretanto, o diagnóstico clínico das fungemias é complexo, devido à inespecificidade dos sinais e sintomas.

O diagnóstico precoce e correto da candidemia é crucial para o aumento da sobrevida do paciente. Este se torna difícil principalmente pelo fato de que os sinais e sintomas da doença podem ser confundidos com vários outros processos infecciosos e não infecciosos (CORTÉS et al., 2011).

O fato de possuir um difícil diagnóstico pela cultura ou hemocultura, faz com que a candidemia tenha uma elevada taxa de mortalidade em pacientes internados. Estudos mostraram que a cultura apresenta taxas menores que 50% para a detecção precoce destas infecções. Por esse motivo, métodos de diagnóstico não baseados em culturas têm sido desenvolvidos buscando-se agilidade e maior sensibilidade do diagnóstico e propiciando o início rápido de terapêutica (CERIKCIOGLU et al., 2004; GIOLO; SVIDZINSK, 2010).

1.7.1 Laboratoriais

O diagnóstico laboratorial é frequentemente realizado pelos métodos tradicionais de cultura de microrganismos, mas estes podem apresentar baixa sensibilidade e necessitar de vários dias para a detecção de positividade. Outros fatores ainda podem influenciar no resultado deste diagnóstico, como profilaxia ou tratamento empírico com antifúngicos (MONTAGNA et al., 2011).

O diagnóstico pode ser realizado por hemocultura, método baseado no isolamento das leveduras do gênero *Candida* que estejam presentes no sangue ou no soro (CAREY et al., 2008). O período de incubação da hemocultura está diretamente relacionado o prognóstico do paciente, pois estudos indicam que quanto maior a demora no resultado e consequente demora no início do tratamento, maior é o risco de morte do paciente. (TAUR et al., 2010).

As espécies de *Candida* spp podem ser isoladas na maioria dos meios de cultivo de uso rotineiro. Um dos mais utilizados é o Ágar *Sabouraud* dextrose, que permite a identificação de colônias de leveduras, de consistência cremosa e coloração branca a creme (ARAÚJO, 2012). Contudo, a tipagem de micro-organismos isolados de casos de infecção hospitalar por métodos moleculares tem

tido grande contribuição para o entendimento clínico-epidemiológico da candidemia (CANTÓN et al., 2001).

Embora a hemocultura seja considerada a técnica “padrão ouro” no diagnóstico de candidemias, muitas falhas podem aparecer nesta técnica, gerando falso-negativos em seu resultado apesar da disseminação de *Candida* spp em órgãos internos (GEHA; ROBERTS, 1994). A hemocultura pode apresentar uma especificidade de 100%, porém com apenas 50% de sensibilidade e tem a desvantagem de necessitar de um período mínimo para positividade de aproximadamente dois dias (CHARLES et al., 2012).

Os métodos de identificação fenotípica convencionais são utilizados para a identificação de espécies comumente encontradas na rotina, mas falham na identificação de espécies emergentes como *C. orthopsilosis, C. metapsilosis, C. nivariensis, C. bracarensis*, entre outros. Nestes casos a realização de métodos moleculares pode fornecer uma identificação rápida e precisa (CENDEJAS-BUENO et al., 2010).

Outra alternativa que vem sendo estudada para auxiliar no diagnóstico é a pesquisa do antígeno manana e de anticorpos anti-manana. A manana é um dos principais antígenos de *Candida* circulante durante a infecção. Este antígeno e seus anticorpos podem ser pesquisados no soro do paciente por testes de aglutinação em látex e ensaios imunoenzimáticos (MÍMICA et al., 2009).

1.7.2 Moleculares

A caracterização de métodos utilizando o DNA para a detecção de espécies de *Candida* spp revela-se potencialmente mais sensível e específica no diagnóstico de candidemia, no qual a identificação genômica pode ser específica para fungos e não para outros organismos celulares. A modalidade e tecnologia da Reação em Cadeia de Polimerase (PCR) tem sido desenvolvida e adaptada para amplificar DNA de *Candida* spp facilitando sua detecção em amostras de sangue total para o diagnóstico molecular de candidemias (AHMAD et al.,2004; BORST et al., 2001; CERIKCIOGLU et al., 2004; ELLEPOLA et al., 2003; KLAN; MUSTAFA, 2001).

Uma variedade de técnicas de tipagem molecular foram desenvolvidas e passaram a ser investigadas no uso para distinguir isolados de *Candida*, como Eletroforese de Enzimas Multilocus (MLEE), Análise por Enzimas de Restrição

(REA), DNA Polimórfico Amplificado Aleatoriamente (RAPD), Polimorfismo de Fragmentos de Amplificação (AFLP), Polimorfismo de Microssatélites (MLP) (SAGHROUNI et al., 2013), DNA "*fingerprinting*", eletroforese de campo pulsado (PFGE), Tipagem por Sequenciamento Multilocus (MLST) (HEO et al., 2011), Polimorfismo de Fragmentos de Restrição (RFLP) (VIJAYAKUMAR et al., 2012) e "*Southern blotting*" (MYOUNG et al., 2011).

Recentemente outra técnica que também vem sendo testada e investigada quanto ao uso no diagnóstico de infecções fúngicas é a espectrometria de massa (MALDI-TOF - *Matrix Assisted Lazer Desorption Ionization*), podendo discriminar diferentes espécies a partir de colônias, em períodos de tempo extremamente curtos. Muitos autores têm pesquisado no sentido de melhorar o diagnóstico, tornando-o mais rápido e melhorando o prognóstico dos pacientes de estado grave. Porém uma desvantagem desta metodologia é que os bancos de dados de depósito de informações para detecção dos microrganismos não são públicos, dificultando o acesso às informações de identificação. (POSTERARO et al., 2013).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar a utilização da técnica de *semi-nested* PCR para o diagnóstico molecular de candidemia e/ou candidíase.

2.2 OBJETIVOS ESPECÍFICOS

Realizar uma revisão sistemática da literatura sobre as técnicas de *semi-nested* PCR para a detecção molecular de espécies de *Candida* spp. em amostras clínicas de candidemia e candidíase;

Acessar a sensibilidade e especificidade das técnicas de *semi-nested* PCR em comparação à cultura;

Desenvolver uma metodologia de *semi-nested* PCR em única etapa para detecção molecular de *Candida albicans, C. tropicalis, C. krusei, C. parapsilosis* e *C. glabrata*.

3 RESULTADOS E DISCUSSÃO

3.1 **ARTIGO 1:** REVISÃO SISTEMÁTICA COM METANÁLISE SUBMETIDA PARA A REVISTA *J CLIN MICROBIOLOGY*

Is Semi-nested PCR effective in the diagnosis of Candidiasis and Candidemia? A systematic review with meta-analysis

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Running Title: Semi-nested PCR for Candidemia and Candidiasis

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Abstract

Recent studies indicate that rapid detection and identification of *Candida* sp. in clinical laboratories is extremely important for the management of high risk patients and a better prognosis. Candidemia and candidiasis diagnosis in clinical laboratories relies on culture-based methods however, these methods are time-consuming and sometimes species identification is not possible. Semi-nested PCR has been recognized as a rapid and efficient assay for this diagnosis but, very little is known regarding the accuracy of the assay. We performed a systematic review accessing: Medline (1950- April 2016), Web of Science, Scopus, LILACS-Latin American and Caribbean Health Science Literature Database, SCIELO - Scientific Eletronic Library Online, EMBASE, and Cochrane Controlled Trials Register Library. Two reviewers abstracted data independently. For meta-analysis three studies were included with 50 proven candidemia patient and 38 healthy subjects. Perfectly 100% specificity and 88% to 100% sensitivity was achieved in semi-nested PCR when compared with culture. When semi-nested PCR was performed to evaluate patients with suspected candidiases or candidemia the positivity was 52% versus 0% of culture. We conclude that direct semi-nested PCR using serum samples had good sensitivity and specificity for the diagnosis of candidemia and candidiasis and offers an attractive assay for earlier diagnosis of specific *Candida* species. Its effects on clinical outcomes and treatment after earlier diagnosis should be investigated.

Introduction

Candida albicans is a commensal fungus and major colonizer of human skin, mucosal surfaces, gastrointestinal and genitourinary tracts (1). Colonization is associated with patient condition such as immunological suppression caused by human immunodeficiency virus (HIV) infection, chemotherapy, and recently the use of corticosteroids or broad-spectrum antibiotics have been identified as major factors for development of candidiasis and candidemia. *Candida albicans* is the most pathogenic species of *Candida*, being the most prevalent etiological agent for human fungal infection (2, 3).

Candidiasis is characterized by an overgrowth of *Candida* species at restricted sites of the body, causing infectious diseases. The most common are mucocutaneous, which include oral and vulvovaginal candidiasis (4).

Oral candidiasis is manifested in the superficial epithelium of the oral mucosa and considered one of the most common clinical features of HIV patients, being observed in up to 90% of these patients. The most commonly isolated species from the oral cavity is *C. albicans* which is believed to be more virulent in humans, occurring in approximately 50% of cases (5, 6).

About three-quarters of women present at least one episode of vulvovaginal candidiasis during their reproductive age, and approximately half of these women present two or more episodes. The most common pathogen in this case is also *Candida albicans*, isolated in 85 to 90% of all cases (7, 8).

Candidemia or invasive candidiasis is defined by the presence of *Candida sp.* in a deep tissue biopsy, or a blood culture, both obtained by a sterile procedure (5). It is the most prevalent nosocomial fungal bloodstream. The incidence of candidemia is higher among cancer patients, burn victim, immunosuppressed individuals, neonates, HIV patients,

transplant recipients and patients in the intensive care unit (UCI), with mortality rates of 50% in these patients (9-12).

The most prevalent species causing candidemia include *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*, accounting for more than 92% of all diagnosed cases. However their relative frequency varies depending on the population involved, geographical region, previous anti-fungal exposure, and patient age (11, 13, 14).

The standard routine for the diagnosis of candidiasis or candidemia is by culture, which can take 24 to 48 hours to provide a result (15, 16). Currentlly, other tests performed include gram staining, germ tube assay, and other morphological and biochemical identification methods. These methods may present some disadvantages, such as being time-consuming, operator-dependent, high cost, and limitations in sensitivity and/or specificity. Problems related to diagnosis can directly affect the therapy choice and patient prognosis (10, 17). Consequently, delay in diagnosis may lead to increased mortality rate and affect the effectiveness and appropriateness of antifungal therapy (10, 17).

The time spent and reliable diagnosis are important clinical problems, considering that treatment is usually started without a diagnosis result, directly affecting patient prognosis. In this context, semi-nested PCR has been suggested as a rapid and efficient method for Candidiasis and Candidemia diagnosis, especially as it is a faster method and presents great sensitivity and specificity (18-26).

We performed a systematic review of studies assessing the diagnostic accuracy of direct semi-nested PCR for *Candida sp*. in human clinical samples. We attempted to define the sensitivity and specificity of the test through meta-analysis.

Materials and Methods:

Search Strategy. Systematic computerized searches were performed in the following databases until October 2016: Medline, Web of Science, Scopus, LILACS-Latin American and Caribbean Health Science Literature Database, Scielo - Scientific Electronic Library Online, EMBASE and Cochrane Controlled Trials Register Library. The search strategy was formulated carefully and included the following keywords both in isolation and combined:(patient OR client OR hospitalized adolescent adults OR institutionalized adolescent OR hospitalized child OR institutionalized child OR adult OR inpatient OR outpatient) AND (candidiasis OR invasive candidiasis OR candidemia OR moniliasis OR candidaemia) AND (Semi-nested PCR OR Polymerase Chain Reaction OR PCR OR Hemi-nested PCR).

Inclusion criteria. No age restrictions were applied, were included adults, pediatric group (children and adolescents), and elderly individuals. Studies evaluating at least 10 patients were considered and included when performed the semi-nested PCR method for Candidemia or Candidiasis diagnosis directly from human clinical samples from patients at risk or already diagnosed with Candidiasis or Candidemia. The index test included semi-nested PCR used for identification of *Candida spp.* to the genus or species level. All target genes and primers were accepted for semi-nested PCR analyzed in our study. It was defined that the studies included in this review should carefully follow the pattern of the established culture method criteria of the Revised Definitions of Invasive Fungal Definitions from the European Organization for Research and Treatment of Cancer/Invasive (15).

For inclusion in the current study, two authors screened the search results for potentially eligible studies. When titles and abstracts suggested that a study was potentially eligible for inclusion, a copy of the full text of the manuscript was obtained. Disagreements

between the two authors regarding the eligibility of a study were solved by discussion or, when necessary, by a third author. The reference lists from studies identified in all databases were checked and any duplicated study was excluded. Having selected all relevant studies for the review, both reviewers independently read the titles and abstracts of each reference identified by the searches in the databases. Disagreements between the two reviewers were solved through discussion and consensus with a third author.

Additionally, studies were excluded when the semi-nested PCR was performed with samples from cultures or fungal isolates, review studies, meta-analysis, comments, letters and/or case reports. Furthermore duplicated studies published by the same author and those that only described the methodology or that showed unclear results were also excluded.

Data extraction. We compared the results of the semi-nested PCR method with the reference standard and performed the data analysis on a 2x2 table reporting data on false-positive (FP), true-positive (TP), false-negative (FN), and true-negative (TN) results of the diagnostic test or that permitted its calculation.

Assessment of risk of bias. Two authors independently assessed QUADAS-2 (27, 28) to analyze the quality and risk of bias of studies included in this review, following which. A figure showing this risk was constructed in Revman version 5.3 software (Figure 2).

Statistical analysis. The values of sensitivity and specificity were automatically computed in Review Manager 5.3 software. The diagnostic accuracy indexes and related 95% CIs were calculated when studies compared semi-nested PCR test and culture as the standard reference. The Kappa coefficient was calculated using SPSS 20 (Chicago, IL, USA) to assess the agreement among judges for risk of bias of studies.

Studies were performed constructing tables of TP, TN, FP, and FN results per study, crossing results of the semi-nested PCR and the reference standard (culture).

This systematic review followed the recommendations of the PRISMA Statement (29).

Results

The database search initially identified 2572 studies. After removing duplicates the total number was 1906. Preliminary screening identified 10 potentially relevant citations, published between 1994 and 2011, which were selected for further evaluation based on their titles and abstracts (Diagram 1); however five were subsequently excluded based on the selection eligibility criteria after complete study reading (Table 1). The remaining five studies were then analyzed regarding quality assessment and risk of bias, including cross sectional study with and without control group and retrospective cohort. The selection of samples were by convenience however all authors described inclusion criteria with details. Two studies (23, 24) assessed elderly individuals, adults, adolescents and children, and the other three did not state a specific patient group. Only one study (23) reported that sample specimens were obtained before initiating antifungal therapy.

Figure 2 shows the quality assessment results for the five selected individual studies. For all QUADAS-2 domains, the majority of studies presented low risk of bias and low concern regarding applicability. In the patient selection domain, all studies indicated a homogeneous and representative population of patients with suspected or confirmed candidiasis or candidemia; two were classified as high risk since the selection of the patients could have introduced bias and three studies were graded as having unclear risk of bias as the manner of patient selection was not stated.

In the index test domain, two studies were considered as low risk and three as unclear, while regarding applicability, three were graded as low risk and one as unclear. Only one

study was judged as high risk. In the reference standard domain, all studies were judged to have a low risk of bias as they stated that the reference standard results were interpreted without knowledge of the results of the index test. Applicability was also of low concern for all studies in the reference standard domain. In the flow and timing domain all studies were judged as having low risk of bias since all patients were included in the analysis, the reference standard was used, and information about the results was provided. The information was provided nearly complete in almost all studies.

For inclusion in the meta-analysis, the five pre-selected studies previously analyzed for risk of bias were analyzed and compared with respect to the culture and semi-nested PCR results, especially information on the tests result with the confirmed candidemic patient (blood cultures yielding *Candida* species on one or more occasion in a clinically suspected patient) and the negative controls (healthy subjects with no complaints of oral or vaginal *Candida* infection) to enable the calculation of TP, TN, FP, and FN results.

For this purpose, we tried to contact the authors from two studies (19, 30), as some additional information about their patient inclusion was necessary. We received an answer from the first author (19), who confirmed that the study only included 10 confirmed candidemic patients and did not include negative control patients. Attempts to contact author from the other study (30) by mail were made three times, to confirm information about the negative controls, however no response was received after 3 weeks. Considering the answer from the first (19) and missing of information from the second one (30) together with the use of outpatients and no negative controls subjects in this study, it was impossible to include these two studies in the meta-analyses.

Figure 3 presents analyses of the three remaining studies (23-25) performed using Revman software version 5.3. We constructed tables of TP, TN, FP, and FN results for each

study, crossing results of the semi-nested PCR and reference standard (culture). Sensitivity and specificity were calculated with 95% confidence intervals.

One study (24) verified 12 confirmed candidemic patients that were all positives in the culture and semi-nested PCR, and 12 negative controls patients that were negative both the culture and semi-nested PCR. The second (25) investigated 6 confirmed candidemic patients that were all positive in the culture and semi-nested PCR, and 10 negative controls that were negative in both the culture and semi-nested PCR. The third (23) analyzed 32 confirmed candidemic patients, of these 28 were positive in the semi-nested PCR and 4 were negative. Moreover 16 negative control patients were analyzed that were all negative in both culture and semi-nested PCR. A total of 50 proven candidemic and 38 healthy subject serum samples were involved in the three remaining studies selected for meta-analysis.

In the forest plot (Figure 3) it can be seen that all three studies presented 100% of specificity and sensitivity varied from 88% in one study (23) to 100%, in two studies (24, 25). All studies used the CTSF and CTSR universal primers, capable of amplifying the 3' end of 5.8S rDNA and 5' end of 28S rDNA, including the intervening space region (ITS). The semi-nested PCR sample-processing time ranged from 6 to 12 hours, allowing reporting of results within 1 working day.

It was also possible to extract information about the clinically suspected candidemia (fever that did not respond to 4 days of broad-spectrum antibiotic therapy and other risk factors: extended period of hospitalization, isolation of *Candida* species from one or more anatomic sites, presence of a catheter, recent history of a surgical procedure, and administration of immunosuppressive therapy) or candidiasis patient in these three studies. This information is shown in table 2. One study (24) demonstrated a positive response rate of 56.25% in the semi-nested PCR (9 of 16 samples). Other study (25) 40% (4 of 10 samples) and other (23) 53% (27 of 51 samples).

Of a total number of 77 clinically suspected patients in the three studies, all were negative in the culture method and 40 were positive in the semi-nested PCR, generating approximately 52% media positivity.

Discussion

Some advantages of the semi-nested PCR are the faster diagnosis when compared to the culture method and the possibility of monitoring the persistence or resolution of the candidemia or candidiasis infection. The patient prognosis can be better if the antifungal therapy is started with the correct *Candida* species identification (16, 18).

The studies assessing patients with candidemia versus healthy controls analyzed in this review showed that PCR of serum samples targeting the rDNA (3' end of 5.8S rDNA and the 5' end of 28S rDNA, including the intervening space region (ITS) genes) presented 100% specificity and sensitivity varied from 88% to 100%. Studies included in this meta-analysis established a specific and sensitive detection of four clinically important *Candida* species: *C. albicans*, *C. tropicalis*, *C. glabrata,* and *C. parapsilosis*.

The authors showed in some cases that semi-nested PCR was capable of detecting two *Candida* species in the same sample, demonstrating concomitant infection. This occurred in two samples from one study (24) and five samples from other one (23). In one sample from one study (23) semi-nested PCR identified three species in the same sample while the culture identified just one. This easily occurs since different *Candida* species have similar colonial morphological appearance and could miss cases of co-infecting species that yield only a few colonies, thus the result of the species with greater growth in the culture prevails.

One study (23) also reported in eight samples that the *Candida* species identifications were in disagreement, showing different results of species level in each test.

All the samples from healthy controls were semi-nested PCR negative, demonstrating the great specificity of the method. In addition, one study (24) also tested the semi-nested PCR on 10 colonized patients (samples from oral, urine, endotracheal aspirate, and vaginal swabs), with no suggestive clinical indications for systemic candidemia, the results being negative in both the culture and semi-nested PCR. This strongly confirms that healthy and colonized patients do not present detectable levels of *Candida* DNA in serum, reducing the risk of false positive results.

In clinically suspected candidemia or candidiasis patients, semi-nested PCR presented 52% positivity, being much higher than blood culture (0%). These results are understandable since molecular methods provide higher sensitivity than culture for the diagnosis of *Candida* infections, presenting approximately 96% sensitivity in this meta-analysis.

Due to poor reporting, it was impossible to examine the clinical conclusion of patient condition after diagnosis and treatment, or if complications or deaths occurred. This information is very important to verify the interference of diagnosis time and *Candida* species identification on better patient prognosis. Two studies (23, 24) related the underlying condition and only one (23) reported that their samples were obtained before initiating the antifungal therapy, this information about therapy is indispensable considering that is directly related to diagnosis, also affecting its results.

Other very important information from studies related to candidemia diagnosis is whether there was the use of antifungals chelants in culture in the case of patients who have already started prophylactic therapy, considering that the not use can directly affect culture results, generating false-negatives culture results.

The prophylaxis and effects of antifungal medicine or resistance were not stated by the groups. Thus, the studies currently available do not allow assessment of clinical outcomes on serum samples related to higher sensitivity and earlier diagnosis achieved with semi-nested

PCR. Thus, researchers in the area of mycological infection should perform comparisons of different diagnosis methods, especially culture and PCR methods, using the same sample at the same starting test time, and blinding the results, to enable analysis of the real impact on patient outcomes achieved from the medical actions in the face of faster diagnosis and earlier treatment.

Based on the results compiled in this review and meta-analysis, we can highlight that semi-nested PCR on serum samples might have an important diagnostic function and impact on improvement in high risk candidemic or candidiasis patient treatment considering the sensitivity and specificity achieved. We should also consider the technique for patients with aspergillosis since this fungal infection is also one of the most frequent in severe hospitalized patients (31).

In summary, this diagnostic accuracy review and meta-analysis proved that direct semi-nested PCR in serum samples has higher sensitivity for the diagnosis of candidemia and candidiasis than conventional identification by blood cultures, with a specificity of 100%, great results for laboratorial and clinical routines. Future studies should assess the clinical outcomes and effects of this diagnostic test following the recommendations of practicing different diagnosis methods with the same sample, comparing the results and effects on patient prognosis and clinical outcomes.

Figure 1. Diagram of the selection of eligible studies from all identified citations.

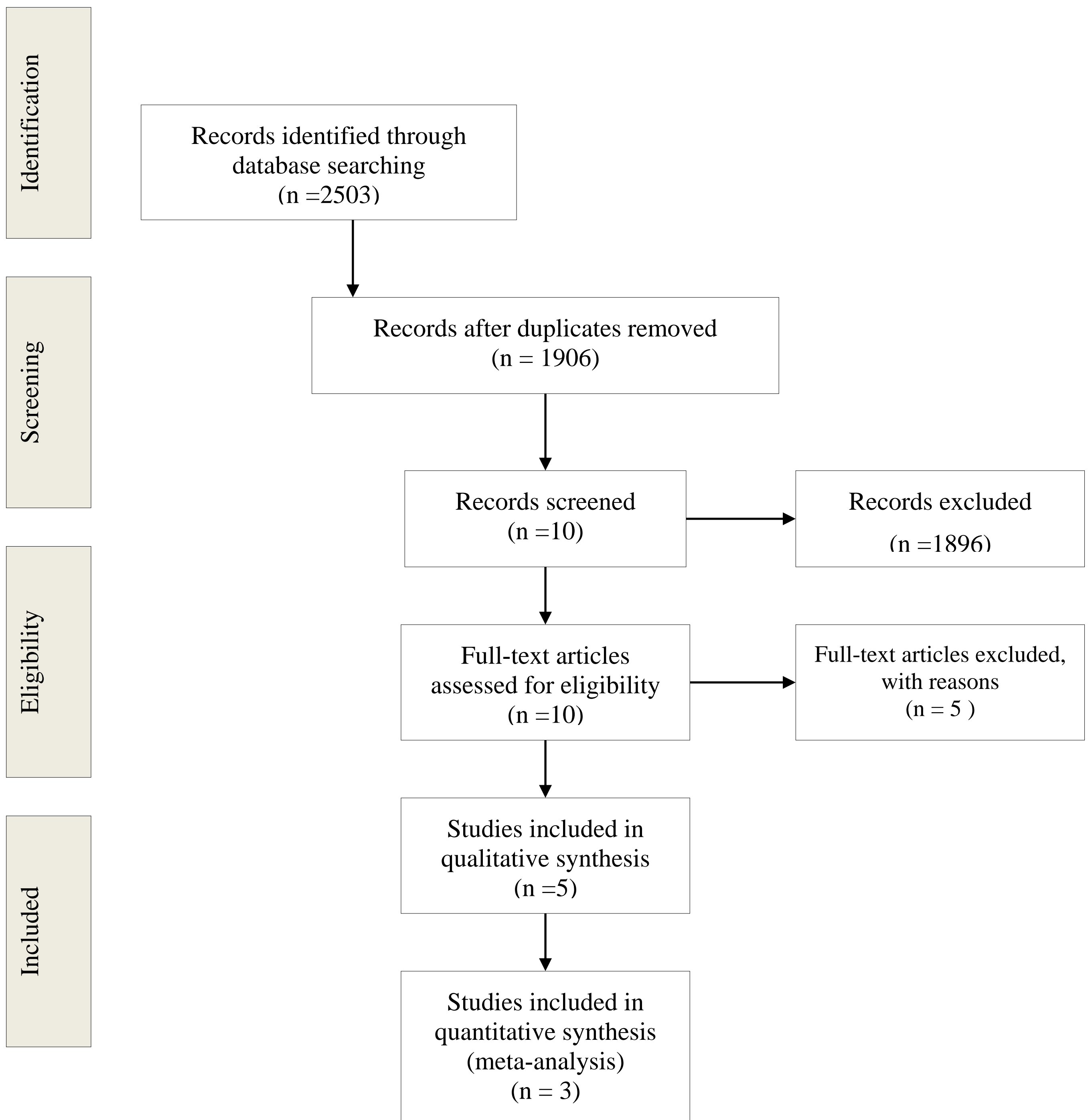

Table 1. Characteristics of excluded studies.

Authors	Reason for exclusion
Ahmad et al., 2005 Çerikçioglu et al., 2010	Identification of *Candida* species was not performed directly from clinical samples
Landlingler et al., 2009	Semi-nested PCR was used to confirm Luminex xMAP technology test
Ko et al., 2010	Semi-nested PCR was not compared with reference test (culture)
Nazzal, Yasin and Abu-Elteen, 2005	Full text was not found, we could only assess the abstract. Contact by mail with authors was performed, however without response. Furthermore the International Library comutation system (COMUT) also could not localize this manuscript.

Figure 2. Risk of bias and applicability concerns summary: review author´s judgment on each domain in all included studies.

	Risk of Bias				Applicability Concerns		
	Patient Selection	Index Test	Reference Standard	Flow and Timing	Patient Selection	Index Test	Reference Standard
Ahmad et al., 2002	−	?	+	+	+	−	+
Ahmad et al., 2004	?	+	+	+	+	+	+
Alam et al., 2007	?	?	+	+	+	+	+
Ospina et al., 2011	−	+	+	+	+	+	+
Rand et al., 1994	?	?	+	+	?	?	+

− High ? Unclear + Low

Figure 3. Forest plot of the semi-nested PCR – sensitivity and specificity of the method compared to culture (reference standard).

Study	**TP**	**FP**	**FN**	**TN**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
Ahmad et al. 2002	12	0	0	12	1.00 [0.74 - 1.00]	1.00 [0.74 - 1.00]
Ahmad et al. 2004	6	0	0	10	1.00 [0.54 - 1.00]	1.00 [0.69 - 1.00]
Alam et al. 2007	28	0	4	16	0.88 [0.71 - 0.96]	1.00 [0.79 - 1.00]

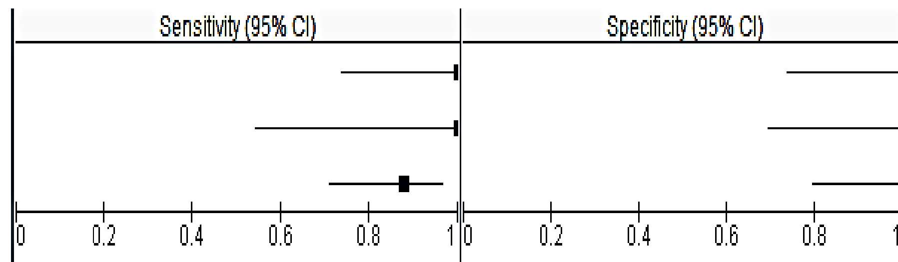

Table 2. Results of semi-nested PCR for clinically suspected candidemia or candidiasis patient.

Authors		Semi-nested PCR positive	Semi-nested PCR negative	Total
Ahmad et al., 2002, Ahmad et al., 2004 and Alam et al., 2007	Culture positive	0	0	0
	Culture negative	40	37	77
	Total	40	37	77

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3.2 **ARTIGO 2:** ARTIGO A SER SUBMETIDO À REVISTA DA SOCIEDADE BRASILEIRA DE MEDICINA TROPICAL

Title: One tube semi-nested PCR assay for *Candida* species detection.

Running title: *Candida* spp. detection by one tube semi-nested PCR

Título: Semi-nested PCR em única etapa para detecção de espécies de *Candida*.

Título corrente: Detecção de *Candida* spp. por semi-nested PCR em única etapa

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Conflict of interest

The authors declare that there is no conflict of interest.

ABSTRACT

Introduction: We report a one-tube semi-nested PCR (OTsn-PCR) for detection of *Candida* species. **Methods:** We developed the OTsn-PCR for five clinically medical important *Candida* species detection: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and *Candida tropicalis.* **Results:** OTsn-PCR presented specificity and detection limit of 0,25 pg. **Conclusions:** OT sn-PCR described in this paper presents high potential detection limit and presents perspective to be used on clinical routine laboratories for molecular detection of *Candida* spp. in Candidiasis and Candidemia patient. When compared with other conventional nested or semi-nested PCR assays, OTsn- PCR is faster with less prone to contamination risks.

Key words: *Candida* spp., molecular diagnosis, PCR assays.

RESUMO

Introdução: Descrevemos uma semi-nested PCR em única etapa (OTsn-PCR) para detecção de espécies de *Candida*. **Métodos:** Desenvolvemos uma OTsn-PCR para detecção de cinco espécies de *Candida* de importância médica: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* e *Candida tropicalis*. **Resultados:** OTsn-PCR apresentou especificidade e ótimo limite de detecção alcançando 0,25 pg. **Conclusões:** A OTsn-PCR descrita neste artigo apresenta alto potencial de limite de detecção e apresenta perspectiva para ser usada em rotinas clínicas de laboratório para a detecção molecular de *Candida* spp. em pacientes com candidíase e candidemia. Quando comparada com ensaios convencionais de nested ou semi-nested de PCR, a OTsn-PCR é mais rápida e com menores riscos de contaminação.

Palavras-chaves: Candidíse, candidemia, diagnóstico molecular.

Fungal infections have become an important problem in hospitals, especially among high risk patients with immunological suppression caused by human immunodeficiency virus (HIV) infection, cancer, burned victims, use of corticosteroids or broad-spectrum antibiotics, intravenous catheter, total parenteral nutrition and dialysis, including premature infants[1,2]. *Candida* spp. remains the most common cause of invasive fungal infections and five species account for approximately 92% of cases of candidemia, *C. albicans* (ATCC 90028)*, C. glabrata* (ATCC MYA 2950) *, C. tropicalis* (P-1011/12)*, C. parapsilosis* (ATCC 21019)*,* and *C. krusei* (ATCC 6258), considering that their distribution varies in different geographical areas. However *C. albicans* is the most frequent species worldwide, accounting 62% of cases[3,4].

Actually candidiasis or candidemia diagnosis is based on culture assays, which can take until 48 hours or more[5,6]. Culture presents several disadvantages, as time-consuming, high cost, limitations in sensitivity and sometimes making it impossible the specie level identification. Given the severity of cases associated with fungal infections, the antifungal treatment is often initiated prior to confirmation of laboratory fungal detection and these problems related to diagnosis can directly affect the therapy choice and patient prognosis[7,8]. Therefore, a rapid, sensible and specific diagnosis of *Candida sp.* is essential for the correct treatment of patients and will present a profound impact on the better and right treatment of the high risk patients[6].

Herein we propose a one-tube semi nested PCR (Otsn-PCR) for the five most prevalent *Candida* species: C. *albicans, C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei.* This assay is as efficient as classical nested and semi-nested PCR however with some advantages of being faster and decreasing assay contamination risks.

For this propose, we used the following microorganisms: *Aspergillus flavus* (ATCC 204304), *Aspergillus fumigates* (LIF 1621), *Aspergillus niger* (morphological identification),

Aspergillus terreus (morphological identification), *C. albicans* (ATCC 90028), *C. glabrata* (ATCC MYA 2950), *C. parapsilosis* (ATCC 21019), *Candida krusei* (ATCC 6258), *Candida tropicalis* (P-1011/12), *Cryptococcus gatti* (LIF 312), *Cryptococcus neoformans* (ATCC 90112), *Fusarium prolypheratum* (LIF 583), *Fusarium oxysporum* (LIF 428), *Paracoccidioides brasiliensis* (Pb18 e LDR1), *Sporothrix spp* and *Histoplasma capsulatum*.

DNA from the fungal cells were extracted by maceration in liquid nitrogen, followed by phenol-chloroform treatment and sodium acetate-ethanol precipitation as described by Koishi et al[5]. DNA concentration and purity were determined by spectrophotometry at 260/280nm.

The OTsn-PCR was developed to detect the internal transcribed spacer regions (ITS) of fungi. The first step was performed with primers ITS13B (5'-GTTTCCGTAGGTGAACCTGCGG-3'), a modified form of ITS1 primer, and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White et al.[9], and the second step was carried out with ITS13B and a species-specific primer. We tested the species-specific primers for *C. albicans* MJCaITS (5'-GCGGTAATAATCTGGTGTGACAAGTT -3'), *C. glabrata* MJCgITS (5'-AGAGATCCACGCACACTCCCAGGTC-3'), *C. parapsilosis* MJCpITS (5'-GGTTGAGTTTAATCTCTGGCAGGCC-3'), *C. krusei* MJCkITS (5'-AGGCATGCCCCCCGGAATGCCGA-3') and C. tropicalis MJCtITS (5'-CCAAACCGGGGGTTTGAGGG-3').

The reaction was performed with 1X Buffer (20 mM Tris--HCl (pH 8.4), 50 mM KCl), 2 mM of each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 1 μM ITS13B and species-specific primers, 0.1 μM ITS4 primer, 0.1 mg/ml of purified gelatin, 1 U Taq DNA polymerase, and ultrapure water, to a final volume of 25 μl. Ultrapure water and DNA from *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Cryptococcus gatti*, *Cryptococcus neoformans*, *Fusarium prolypheratum*, *Fusarium*

oxysporum, *Paracoccidioides brasiliensis*, *Sporothrix spp* and *Histoplasma capsulatum* were used as negative controls, and 5ng of purified DNA from each species of *Candida* were used as positive control. The PCR condition was 95°C for 2 min of initial denaturation, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min (first step), and still more 35 cycles of 95°C for 30 sec, 72°C for 30 sec, 72°C for 1 min (second step), and a final extension at 72°C for 5 min.

We tested the detection limit of the OTsn-PCR. For this, we used the five *Candida* sp. DNA with different concentrations by serial dilution (5ng, 2.5ng, 0.25ng, 25pg, 2.5pg and 0.25pg). Also was tested the specificity off the assay using all fungal DNA described above in this study. We repeated these tests at minimum three times to confirm sensitivity or detection limit results.

Considering the high mortality rate related to candidemia patients, it is essential to develop methods for an accurate identification of the causative agents, especially because problems related to diagnosis can directly affect the therapy choice and patient prognosis[7,8]. In this context, semi-nested PCR has been suggested as a rapid and efficient method for Candidiasis and Candidemia diagnosis, especially as it is a faster method and presents great sensitivity and specificity[10-17]. However common semi-nested PCR reactions are susceptible to accidental contamination during the transfer of aliquots form the first reaction mixture to new tubes used for the second reaction[18].

Using a serial dilution of purified DNA from the five species of *Candida* spp. tested, from 5ng to 0.25pg, the OTsn-PCR described in this study was able to detect 0.25pg from *C. albicans* (162-pb fragment) and *C. tropicalis* (349-pb fragment), 25pg from *C. glabrata* (244-pb fragment) and 2.5pg from *C. krusei* (308-pb fragment – data not shown) and *C. parapsilosis* (173-pb fragment). These results are shown in Table 1.

The found detection limits were comparable with that of the protocol developed by our group: Koishi et al[19] that standardized a semi-nested PCR for *P. brasiliensis* and Pitz et al[20] that tested the OTsn-PCR for *P. brasiliensis* clinical samples.

Specificity of OTsn-PCR was tested with DNA from *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Cryptococcus gatti*, *Cryptococcus neoformans*, *Fusarium prolypheratum*, *Fusarium oxysporum*, *Paracoccidioides brasiliensis*, *Sporothrix spp, Histoplasma capsulatum* and also amog *Candida* spp. and no cross-reactivity with other fungi was observed. The specificity results are shown in Table 2, the results did not show cross reactivity between the other 15 fungal DNA tested.

The OTsn-PCR we described here can be easily adapted for the detection of any pathogenic fungi simply changing the species-specific primer considering the peculiar characteristics of ITS region target in this reaction. Our group will future analyze some other important fungi as *Aspergillus fumigatus.*

We also suggest future studies also including clinical samples that make possible the observation of the OTsn-PCR efficiency in Candidemia detection on total blood or serum samples in laboratorial routine and compare it with the gold standard culture. Thus, the OTsn-PCR assay probably has a great potential for Candidemia diagnosis.

Table 1. Detection limit of the OTsn-PCR assay for identifying *C. albicans, C. tropicalis, C. krusei, C. parapsilosis* and *C. glabrata*. OTsn-PCR: one-tube semi-nested polymerase chain reaction.

	5 ng	2.5 ng	0.25 ng	25 pg	2.5 pg	0.25 pg
C. albicans (162-pb)	+	+	+	+	+	+
C. tropicalis (349-pb)	+	+	+	+	+	+
C. krusei (308-pb)	+	+	+	+	+	-
C. parapsilosis (173-pb)	+	+	+	+	+	-
C. glabrata (244-pb)	+	+	+	+	-	-

Table 2. Specificity of the OTsn-PCR for *C. albicans, C. tropicalis*, *C. krusei, C. parapsilosis* and *C. glabrata* tested with 15 different fungal DNA.

	C. alb	*C. tro*	*C. kru*	*C. par*	*C. gla*	*A. fum*	*A. fla*	*A. nig*	*A. ter*	*C. neo*	*C. gat*	*F. oxy*	*F. pro*	*P. bra*	*H. cap*	*S. sch*
MJCaITS	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MJCtITS	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MJCkITS	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
MJCpITS	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
MJCgITS	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-

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4 CONCLUSÕES

Com o presente trabalho e com os resultados obtidos, foi possível desenvolver uma revisão sistemática da literatura com meta-análise, acessando as reações de semi-*nested* PCR utilizadas para detecção molecular de espécies de *Candida*.

Foi possível comparar os resultados das reações de semi-*nested* PCR com a cultura, padrão-ouro para o diagnóstico das candidemias. A especificidade alcançada foi de 100% e a sensibilidade variou de 88 a 100% nos três estudos analisados.

Também foi possível desenvolver e padronizar uma reação de semi-*nested* PCR em única etapa para: *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* e *Candida krusei*.

O limite de detecção das reações foi de 0,25 pg DNA de *C. albicans* e *C. tropicalis,* 2,5 pg de *C. parapsilosis* e *C. krusei*, e 25 pg de *C. glabrata.*

Não foram observadas reações cruzadas entre o gênero *Candida*, assim como com outras espécies fúngicas testadas: *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Cryptococcus gatti*, *Cryptococcus neoformans*, *Fusarium prolypheratum*, *Fusarium oxysporum*, *Paracoccidioides brasiliensis*, *Sporothrix schenkii* and *Histoplasma capsulatum*

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ANEXOS

ANEXO 1: QUADAS 2 TOOL - BACKGROUND DOCUMENT

QUADAS-2: Background Document

QUADAS-2

QUADAS-2 is designed to assess the quality of primary diagnostic accuracy studies; it is not designed to replace the data extraction process of the review and should be applied in addition to extracting primary data (e.g. study design, results etc) for use in the review. It consists of four key domains covering patient selection, index test, reference standard, and flow of patients through the study and timing of the index test(s) and reference standard ("flow and timing") (Table 1). The tool is completed in four phases: 1) state the review question; 2) develop review specific guidance; 3) review the published flow diagram for the primary study or construct a flow diagram if none is reported; 4) judgement of bias and applicability. Each domain is assessed in terms of the *risk of bias* and the first three are also assessed in terms of *concerns regarding applicability*. To help reach a judgement on the risk of bias, *signalling questions* are included. These flag aspects of study design related to the potential for bias and aim to help reviewers make risk of bias judgements.

Phase 1: Review Question

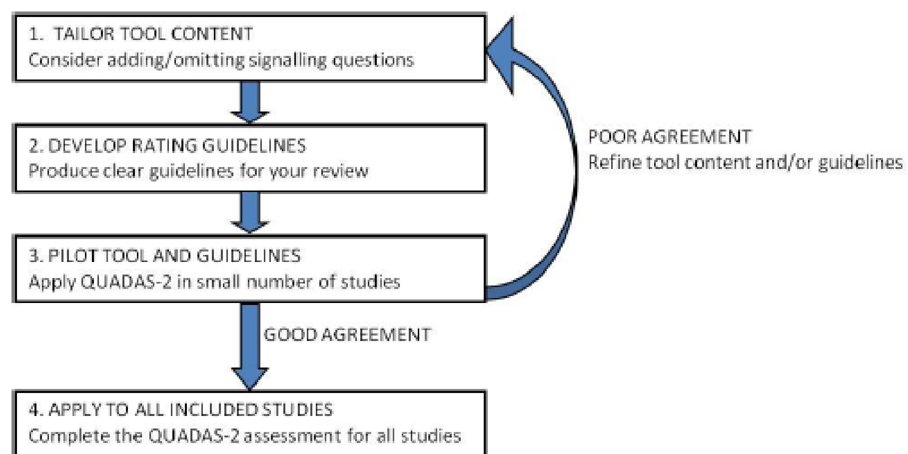
Review authors are first asked to report their systematic review question in terms of patients, index test(s), and reference standard and target condition. As the accuracy of a test may depend on where in the diagnostic pathway it will be used, review authors are asked to describe patients in terms of setting, intended use of the index test, patient presentation and prior testing.(1;2)

Phase 2: Review Specific Tailoring (Figure 1)

It is essential to tailor QUADAS-2 to each review by adding or omitting signalling questions and developing review-specific guidance on how to assess each signalling question and use this information to judge the risk of bias. The first step is to consider whether any signalling question does not apply to the review or whether any specific issues for the review are not adequately covered by the core signalling questions. For example, for a review of an objective index test it may be appropriate to omit the signalling question relating to blinding of the test interpreter to results of the reference standard. Review authors should avoid

complicating the tool by adding too many signalling questions. Once tool content has been agreed, review-specific rating guidance should be developed. The tool should be piloted independently by at least two people. If agreement is good, the tool can be used to rate all included studies. If agreement is poor, further refinement may be needed.

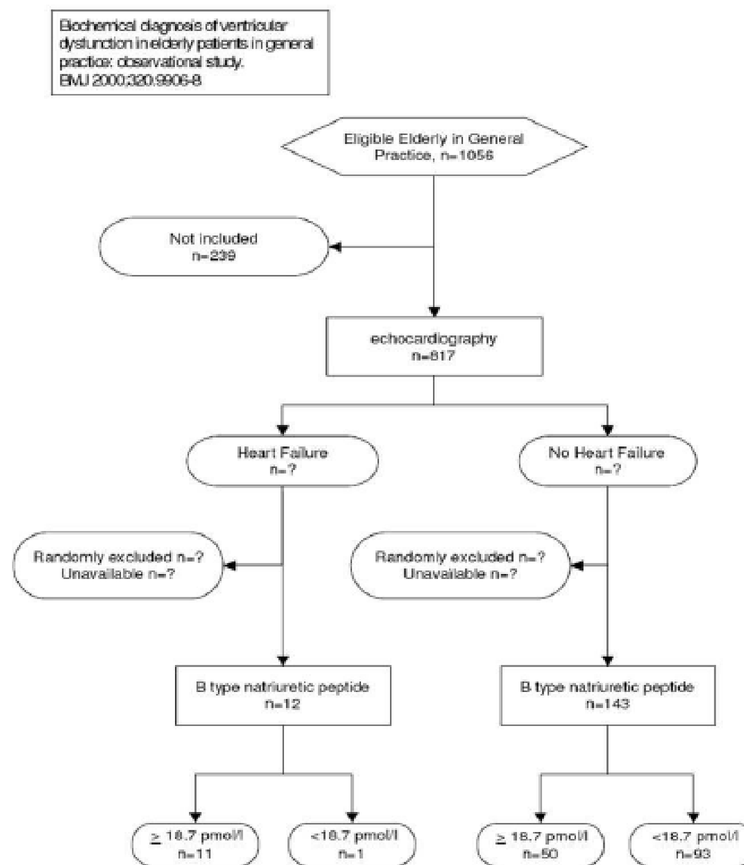
Figure 1: Process for tailoring QUADAS-2 to your systematic review



Phase 3: Flow Diagram

The next stage is to review the published flow diagram for the primary study or to draw one if none is reported or the published diagram is not adequate. The flow diagram will facilitate judgments of risk of bias, and should provide information about the method of recruitment of patients (e.g. based on a consecutive series of patients with specific symptoms suspected of having the target condition, or of cases and controls), the order of test execution, and the number of patients undergoing the index test and the reference standard. A hand drawn diagram is sufficient as this step does not need to be reported as part of the QUADAS-2 assessment. Figure 2 shows an example based on a primary study of B type natriuretic peptide for the diagnosis of heart failure.

Figure 2: Flowchart based on diagnostic cohort study of BNP for diagnosing heart failure



Phase 4: Judgments on bias and applicability

Risk of bias

The first part of each domain concerns bias and comprises three sections: 1) information used to support the risk of bias judgment, 2) signalling questions, and 3) judgment of risk of bias. By recording the information used to reach the judgment ("support for judgment"), we aim to make the rating transparent and facilitate discussion between review authors completing assessments independently.(3) The additional signalling questions are included

to assist judgments. They are answered as "yes", "no", or "unclear", and are phrased such that "yes" indicates low risk of bias.

Risk of bias is judged as "low", "high", or "unclear". If all signalling questions for a domain are answered "yes" then risk of bias can be judged "low". If any signalling question is answered "no" this flags the potential for bias. Review authors then need to use the guidelines developed in phase 2 to judge risk of bias. The "unclear" category should be used only when insufficient data are reported to permit a judgment.

Applicability

Applicability sections are structured in a similar way to the bias sections, but do not include signalling questions. Review authors are asked to record the information on which the judgment of applicability is made and then to rate their concern that the study does not match the review question. Concerns regarding applicability are rated as "low", "high" or "unclear". Applicability judgments should refer to the first phase, where the review question was recorded. Again, the "unclear" category should only be used when insufficient data are reported.

The following sections provide brief explanations of the signalling questions and risk of bias/concerns regarding applicability questions for each domain.

DOMAIN 1: PATIENT SELECTION

Risk of bias: Could the selection of patients have introduced bias?

Signalling question 1: Was a consecutive or random sample of patients enrolled?

Signalling question 2: Was a case-control design avoided?

Signalling question 3: Did the study avoid inappropriate exclusions?

A study should ideally enrol all consecutive, or a random sample of, eligible patients with suspected disease – otherwise there is potential for bias. Studies that make inappropriate exclusions, e.g. excluding "difficult to diagnose" patients, may result in overoptimistic estimates of diagnostic accuracy. In a review of anti-CCP antibodies for the diagnosis of rheumatoid arthritis, we found that some studies enrolled consecutive patients who had confirmed diagnoses. These studies showed greater sensitivity of the anti-CCP test than

studies that included patients with suspected disease but in whom the diagnosis had not been confirmed – "difficult to diagnose" patients.(4) Similarly, studies enrolling patients with known disease and a control group without the condition may exaggerate diagnostic accuracy.(5;6) Exclusion of patients with "red flags" for the target condition, who may be easier to diagnose, may lead to underestimation of diagnostic accuracy.

Applicability: Are there concerns that the included patients and setting do not match the review question?

There may be concerns regarding applicability if patients included in the study differ, compared to those targeted by the review question, in terms of severity of the target condition, demographic features, presence of differential diagnosis or co-morbidity, setting of the study and previous testing protocols. For example, larger tumours are more easily seen with imaging tests than smaller ones, and larger myocardial infarctions lead to higher levels of cardiac enzymes than small infarctions making them easier to detect and so increasing estimates of sensitivity.(7)

DOMAIN 2: INDEX TEST

Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?

Signalling question 1: Were the index test results interpreted without knowledge of the results of the reference standard?

This item is similar to "blinding" in intervention studies. Interpretation of index test results may be influenced by knowledge of the reference standard.(6) The potential for bias is related to the subjectivity of index test interpretation and the order of testing. If the index test is always conducted and interpreted prior to the reference standard,this item can be rated "yes".

Signalling question 2: If a threshold was used, was it pre-specified?

Selecting the test threshold to optimise sensitivity and/or specificity may lead to overoptimistic estimates of test performance, which is likely to be poorer in an independent sample of patients in whom the same threshold is used.(8)

Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?

Variations in test technology, execution, or interpretation may affect estimates of its diagnostic accuracy. If index tests methods vary from those specified in the review question there may be concerns regarding applicability. For example, a higher ultrasound transducer frequency has been shown to improve sensitivity for the evaluation of patients with abdominal trauma.(9)

DOMAIN 3: REFERENCE STANDARD

Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?

Signalling question 1: Is the reference standard likely to correctly classify the target condition?

Estimates of test accuracy are based on the assumption that the reference standard is 100% sensitive and specific disagreements between the reference standard and index test are assumed to result from incorrect classification by the index test.(10;11)

Signalling question 2: Were the reference standard results interpreted without knowledge of the results of the index test?

This item is similar to the signalling question related to interpretation of the index test. Potential for bias is related to the potential influence of prior knowledge on the interpretation of the reference standard.(6)

Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?

The reference standard may be free of bias but the target condition that it defines may differ from the target condition specified in the review question. For example, when defining urinary tract infection the reference standard is generally based on specimen culture but the threshold above which a result is considered positive may vary.(12)

DOMAIN 4: FLOW AND TIMING

Risk of Bias: Could the patient flow have introduced bias?

Signalling question 1: Was there an appropriate interval between index test and reference standard?

Ideally results of the index test and reference standard are collected on the same patients at the same time. If there is a delay or if treatment is started between index test and reference standard, misclassification may occur due to recovery or deterioration of the condition. The length of interval leading to a high risk of bias will vary between conditions. A delay of a few days may not be a problem for chronic conditions, while for acute infectious diseases a short delay may be important. Conversely, when the reference standard involves follow-up a minimum follow-up period may be required to assess the presence or absence of the target condition. For example, for the evaluation of magnetic resonance imaging for the early diagnosis of multiple sclerosis, a minimum follow-up period of around 10 years is required to be confident that all patients who will go on to fulfil diagnostic criteria for multiple sclerosis will have done so.(13)

Signalling question 2: Did all patients receive the same reference standard?

Verification bias occurs when not all of the study group receive confirmation of the diagnosis by the same reference standard. If the results of the index test influence the decision on whether to perform the reference standard or which reference standard is used, estimated diagnostic accuracy may be biased.(5;14) For example, a study evaluating the accuracy of the D-dimer test for the diagnosis of pulmonary embolism carried out ventilation perfusion scans (reference standard 1) in those testing positive and used clinical follow-up to determine whether or not those testing negative had a pulmonary embolism (reference standard 2). This may result in misclassifying some of the false negatives as true negatives as some patients who had a pulmonary embolism but were index test negative may be missed by clinical follow-up and so be classified as not having a pulmonary embolism. This misclassification will overestimate sensitivity and specificity.

Signalling question 3: Were all patients included in the analysis?

All patients who were recruited into the study should be included in the analysis.(15) There is a potential for bias if the number of patients enrolled differs from the number of patients included in the 2x2 table of results, for example because patients lost to follow-up differ systematically from those who remain.

Incorporating QUADAS-2 assessments in diagnostic accuracy reviews

We emphasise that QUADAS-2 should not be used to generate a summary "quality score", because of the well-known problems associated with such scores.(16;17) If a study is judged as "low" on all domains relating to bias or applicability then it is appropriate to have an overall judgment of "low risk of bias" or "low concern regarding applicability" for that study. If a study is judged "high" or "unclear" on one or more domains then it may be judged "at risk of bias" or as having "concerns regarding applicability".

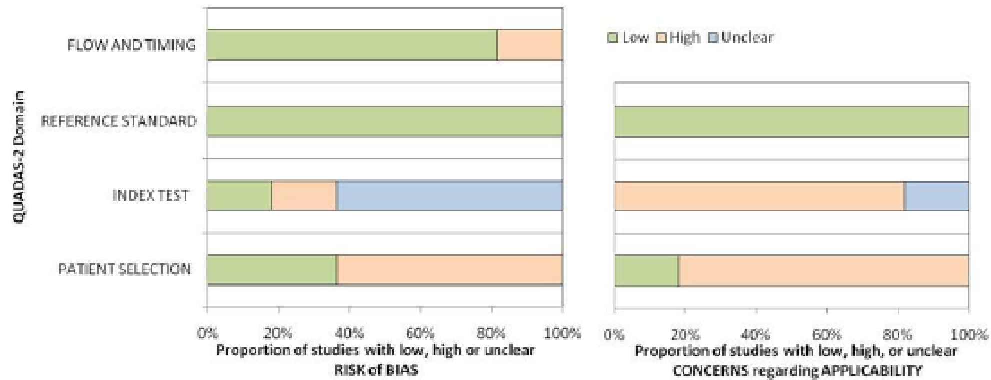
At minimum, reviews should present a summary of the results of the QUADAS-2 assessment for all included studies. This could include summarising the number of studies that found low, high or unclear risk of bias/concerns regarding applicability for each domain. If studies are found to consistently rate well or poorly on particular signalling questions then reviewers may choose to highlight these. Tabular (Table) and graphical (Figure 3) displays are helpful to summarise QUADAS-2 assessments.

Table: Suggested tabular presentation for QUADAS-2 results

Study	RISK OF BIAS				APPLICABILITY CONCERNS		
	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD
Study 1	☺	☺	☺	☺	☹	☺	☺
Study 2	☺	☺	☺	☺	☹	☺	☺
Study 3	☹	☹	☺	☺	☹	☺	☺
Study 4	☹	☹	☺	☺	☹	☺	☺
Study 5	☹	?	☺	☺	☹	☺	☺
Study 6	☹	?	☺	☺	☹	?	☺
Study 7	☹	?	☺	☺	☹	☺	☺
Study 8	☹	?	☺	☺	☹	?	☺
Study 9	☹	?	☺	☺	☹	☺	☺
Study 10	☹	?	☺	☹	☹	☺	☺
Study 11	☺	?	☺	☹	☺	☺	☺

☺ Low Risk ☹ High Risk ? Unclear Risk

Figure 3: Suggested Graphical Display for QUADAS-2 results



Review authors may choose to restrict the primary analysis so that only studies at low risk of bias and/or low concern regarding applicability for all or specified domains are included. It may be appropriate to restrict inclusion to the review based on similar criteria, but it is often preferable to review all relevant evidence and then investigate possible reasons for heterogeneity.(13;18) Subgroup and or sensitivity analysis can be conducted by investigating how estimates of accuracy of the index test vary between studies rated as high, low, or unclear on all or selected domains. Domains or signalling questions can be included as items in meta-regression analyses, to investigate their association with estimated accuracy.

Website

The QUADAS website (www.quadas.org) contains QUADAS-2, information on training, a bank of additional signalling questions, more detailed guidance for each domain, examples of completed QUADAS-2 assessments, and downloadable resources including a Microsoft Access™ database for data extraction, an Excel™ spreadsheet to produce graphical displays of results, and templates for Word™ tables to summarise results.

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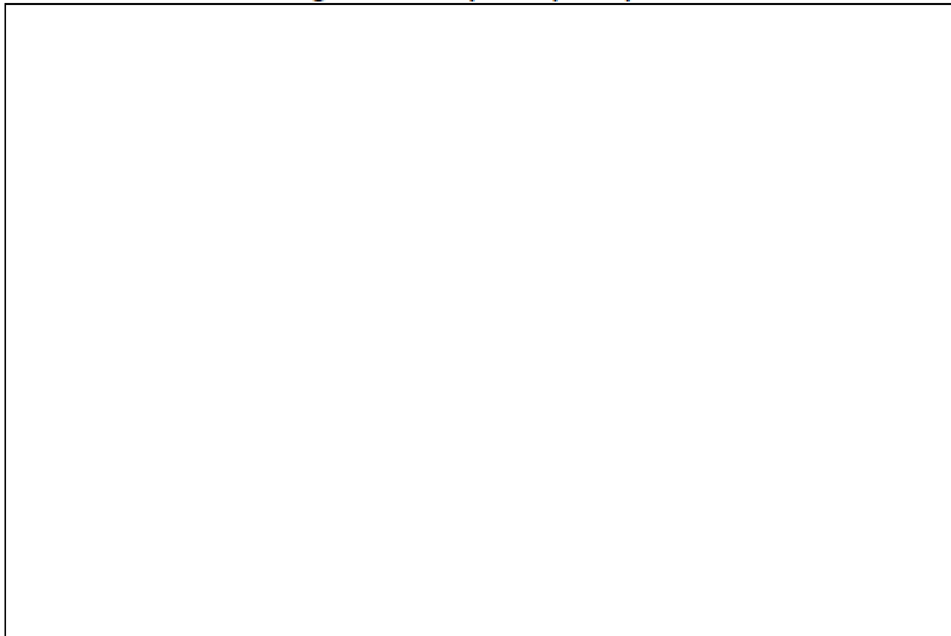
ANEXO 2: QUADAS 2

QUADAS-2

Phase 1: State the review question:

Patients (setting, intended use of index test, presentation, prior testing):
Index test(s):
Reference standard and target condition:

Phase 2: Draw a flow diagram for the primary study



Phase 3: Risk of bias and applicability judgments

QUADAS-2 is structured so that 4 key domains are each rated in terms of the risk of bias and the concern regarding applicability to the research question (as defined above). Each key domain has a set of signalling questions to help reach the judgments regarding bias and applicability.

DOMAIN 1: PATIENT SELECTION

A. Risk of Bias

Describe methods of patient selection:

- Was a consecutive or random sample of patients enrolled? Yes/No/Unclear
- Was a case-control design avoided? Yes/No/Unclear
- Did the study avoid inappropriate exclusions? Yes/No/Unclear

Could the selection of patients have introduced bias? RISK: LOW/HIGH/UNCLEAR

B. Concerns regarding applicability

Describe included patients (prior testing, presentation, intended use of index test and setting):

Is there concern that the included patients do not match the review question? CONCERN: LOW/HIGH/UNCLEAR

DOMAIN 2: INDEX TEST(S)

If more than one index test was used, please complete for each test.

A. Risk of Bias

Describe the index test and how it was conducted and interpreted:

- Were the index test results interpreted without knowledge of the results of the reference standard? Yes/No/Unclear
- If a threshold was used, was it pre-specified? Yes/No/Unclear

Could the conduct or interpretation of the index test have introduced bias? RISK: LOW /HIGH/UNCLEAR

B. Concerns regarding applicability

Is there concern that the index test, its conduct, or interpretation differ from the review question? CONCERN: LOW /HIGH/UNCLEAR

DOMAIN 3: REFERENCE STANDARD

A. Risk of Bias

Describe the reference standard and how it was conducted and interpreted:

- Is the reference standard likely to correctly classify the target condition? Yes/No/Unclear
- Were the reference standard results interpreted without knowledge of the results of the index test? Yes/No/Unclear

Could the reference standard, its conduct, or its interpretation have introduced bias? **RISK: LOW /HIGH/UNCLEAR**

B. Concerns regarding applicability

Is there concern that the target condition as defined by the reference standard does not match the review question? **CONCERN: LOW /HIGH/UNCLEAR**

DOMAIN 4: FLOW AND TIMING

A. Risk of Bias

Describe any patients who did not receive the index test(s) and/or reference standard or who were excluded from the 2x2 table (refer to flow diagram):

Describe the time interval and any interventions between index test(s) and reference standard:

- Was there an appropriate interval between index test(s) and reference standard? Yes/No/Unclear
- Did all patients receive a reference standard? Yes/No/Unclear
- Did patients receive the same reference standard? Yes/No/Unclear
- Were all patients included in the analysis? Yes/No/Unclear

Could the patient flow have introduced bias? **RISK: LOW /HIGH/UNCLEAR**

ANEXO 3: SHORT COMMUNICATION: AN OPTIMIZED ONE-TUBE, SEMI-NESTED PCR ASSAY FOR *PARACOCCIDIOIDES BRASILIENSIS* DETECTION

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Short Communication

An optimized one-tube, semi-nested PCR assay for *Paracoccidioides brasiliensis* detection

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ABSTRACT

Introduction: Herein, we report a one-tube, semi-nested-polymerase chain reaction (OTsn-PCR) assay for the detection of *Paracoccidioides brasiliensis*. **Methods:** We developed the OTsn-PCR assay for the detection of *P. brasiliensis* in clinical specimens and compared it with other PCR methods. **Results:** The OTsn-PCR assay was positive for all clinical samples, and the detection limit was better or equivalent to the other nested or semi-nested PCR methods for *P. brasiliensis* detection. **Conclusions:** The OTsn-PCR assay described in this paper has a detection limit similar to other reactions for the molecular detection of *P. brasiliensis*, but this approach is faster and less prone to contamination than other conventional nested or semi-nested PCR assays.

Keywords: Mycosis. Molecular diagnosis. Polymerase chain reaction assays.

Paracoccidioidomycosis (PCM) is an endemic infection in Latin America and is caused by *Paracoccidioides brasiliensis*, a thermal-dimorphic fungus[1]. The distribution of infections is restricted to Central and South America and is observed between Mexico (23°N) and Argentina (34°S)[1]. Brazil is the country with the largest number of cases of the disease, with an estimated incidence ranging between 10-30 cases/million inhabitants and a mortality rate of 1.4 deaths/million inhabitants[2]. PCM diagnosis is based on clinical symptoms, direct microscopic examination of clinical specimens, isolation of fungus in culture and the detection of specific antibodies by serological techniques[3]. However, these methods are dependent on the skills of laboratory staff, the capacity for the organism to be cultured and the cross-reactivity with antigens of other fungi[3]. Thus, several new molecular methods have been developed for the detection of *P. brasiliensis*, including polymerase chain reaction (PCR) assays[3,4].

In this study, we have proposed a one-tube, semi-nested PCR (OTsn-PCR) approach for the detection of *P. brasiliensis*, which is as efficient as classical nested and semi-nested PCR assays and can be adapted for the detection of other pathogenic fungi.

For this study, we used the following microorganisms: *P. brasiliensis* (LDR 1 and Pb 18 strains), *Cryptococcus* sp., *Sporothrix* spp., *Histoplasma capsulatum*, *Candida albicans* (strain CR15) and *Trichophyton rubrum*. In addition, positive sputum samples from 14 patients with Paracoccidioidomycosis, diagnosed by direct observation of fungal cells and clinical signs (including a productive cough and weight loss), were obtained from the University Hospital of Western Paraná State University (UNIOESTE, Paraná, Brazil). This study was approved by the Western Paraná State University Ethics Committee for Human Beings (UNIOESTE - N° 27373/2009).

Deoxyribonucleic acid (DNA) from yeast-phase fungal cells and clinical samples were extracted by cell lysis via a liquid nitrogen freeze-thaw, followed by phenol-chloroform treatment and sodium acetate-ethanol precipitation as described by Koishi et al.[5] The DNA concentration and purity were determined by spectrophotometry measurement at 260/280nm. Due to the possible presence of inhibitors, the quality of DNA extracted from the clinical samples was evaluated by PCR using two primers specific to the human CXCR4 gene; SDF1 (5'-CAGTCAACCTGGGCAAAGCC-3') and SDF2 (5'-CCTGAGAGTCCTTTTGCGGG-3')[6].

The OTsn-PCR approach was developed to detect the internal transcribed spacer regions (ITS) of fungi. The first step was performed with primers termed ITS13B (5'-GTTTCCGTAGGTGAACCTGCGG-3'), a modified form of an ITS1 primer, and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'), as described by White et al.[7] The second step of the OTsn-PCR assay was performed using the ITS13B primer and a species-specific primer. We tested a species-specific primer for *P. brasiliensis*, termed MJ034B (5'-ATAGGTCTCAGACGTCAAAGCTCC-3'), a modified form of the MJ03 primer described by Koishi et al.[5], and a species-specific primer for *H. capsulatum*, which we termed HC013B (5'-TCATGCTCAGACGCCAATCGTTC-3').

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The reaction was performed with 1X Buffer (20mM Tris-HCl [pH 8.4] and 50mM KCl), 2mM of each deoxynucleotide triphosphate (dNTP), 2mM $MgCl_2$, 1µM ITS13B primer and a species-specific primer, 0.1µM ITS4 primer, 0.1mg/ml of purified gelatin, 1U Taq DNA polymerase and ultrapure water to a final volume of 25µl. DNA from *Cryptococcus* sp., *Sporothrix* spp., *C. albicans* (strain CR15) and *T. rubrum* and ultrapure water were used as negative controls. Additionally, 2.5ng of purified DNA from *P. brasiliensis* or *H. capsulatum* were used as positive controls. The PCR conditions were as follows: 95°C for 2min (for the initial denaturation of the DNA); 35 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 1min (initial amplification phase); 35 cycles of 95°C for 30sec, 72°C for 30sec, and 72°C for 1min (secondary amplification phase); and a final extension at 72°C for 5min.

This OTsn-PCR approach was compared with the nested and semi-nested PCR methods described by Imai et al.[8], Theodoro et al.[9] and Koishi et al.[5] Similar to our OTsn-PCR assay, these reactions use sequences of ribosomal DNA as targets, especially from the ITS, and are specific for the detection of *P. brasiliensis*. For the sake of comparison, we used five different serially diluted *P. brasiliensis* DNA concentrations (2.5ng, 250pg, 25pg, 2.5pg and 0.25pg). All of the protocols were repeated at least three times, as originally described. Furthermore, to compare cycle number effects on the limit of detection among the methods, the methods developed by Imai et al.[8] and Theodoro et al.[9] were also performed using 35 cycles.

Considering the high incidence of fungal infections and the expanding spectrum of fungal pathogens, it is essential to develop methods for the early and accurate identification of the causative agents of fungal infections. Recently, several semi-nested and nested PCR assays have been developed for fungal detection[10]. However, these reactions are time consuming and susceptible to accidental contamination during the transfer of aliquots from the first reaction mixture to new tubes used for the second reaction[11-13].

Using a serial dilution of purified DNA from *P. brasiliensis* (from 2.5ng to 0.25pg), the OTsn-PCR assay described in this study was able to detect up to 2.5pg of *P. brasiliensis* DNA **(Figure 1A)** without cross-reactivity with other fungi (data not shown). The limit of detection for the OTsn-PCR assay was comparable with that of the protocol developed by Koishi et al.[5] **(Figure 1B)** and was more sensitive than the protocols in the Imai et al.[8] and Theodoro et al.[9] studies **(Figures 1C-1D)**. Furthermore, when using DNA extracted from the clinical samples, the OTsn-PCR assay and the protocol developed by Koishi et al.[5] were able to detect *P. brasiliensis* DNA in all of the samples (100%) **(Table 1)**, while the protocols of Imai et al.[8] and Theodoro et al.[9] only detected *P. brasiliensis* DNA in four (28.5%) and eight (57.2%) of the clinical samples, respectively **(Table 1)**. A possible cause for this difference may be the lower numbers of cycles used in these reactions. For this reason, all of samples were retested following the Imai et al.[8] and Theodoro et al.[9] PCR methods using 35 cycles. The numbers of positive samples increased from four to six (42.8%) and from eight to 10 (71.4%) using the PCR methods of Imai et al.[8] and Theodoro et al.[9], respectively **(Table 1)**. However, the methods developed by Imai et al.[8] and Theodoro et al.[9] failed to detect *P. brasiliensis* DNA in some of the *P. brasiliensis*-positive clinical specimens.

In general, nested or semi-nested PCR assays performed in a single tube are less sensitive when compared with assays that take place in two stages and in separate tubes[11-13]. However, in our study, we achieved the same limit of detection for the OTsn-PCR assay as the semi-nested PCR approach described by Koishi et al.[5], which was only possible due to the use of gelatin in the reaction. When the OTsn-PCR assay was performed

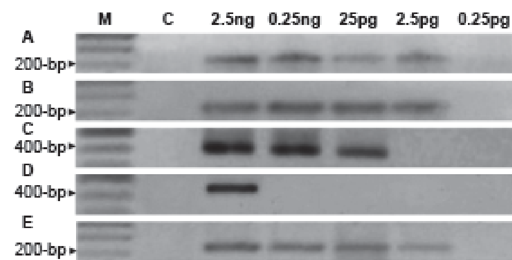

FIGURE 1 - Sensitivity of the OTsn-PCR assay for identifying *Paracoccidioides brasiliensis* (A) when compared with the nested or semi-nested PCR reactions developed by Koishi et al. (B), Theodoro et al. (C) and Imai et al. (D). Sensitivity of the OTsn-PCR assay for the identification of *Histoplasma capsulatum* (E). OTsn-PCR: one-tube semi-nested-polymerase chain reaction; M: 100-bp DNA Ladder (Invitrogen, Brazil); C: negative control. The agarose gel was stained with SYBR SAFE (Invitrogen, Brazil).

TABLE 1 - Detection of *Paracoccidioides brasiliensis* in clinical (sputum) samples from 14 patients. Comparison of the OTsn-PCR assay with the nested or semi-nested PCR assays developed by Koishi et al.[5], Theodoro et al.[9] and Imai et al[8].

	Positive		Negative	
Protocols	n	%	n	%
Imai et al.	4 (6)*	28.5 (42.8)	10 (8)	71.5 (57.2)
Theodoro et al.	8 (10)	57.2 (71.4)	6 (4)	42.8 (28.6)
Koishi et al.	14	100.0	0	0
OTsn-PCR	14	100.0	0	0

OTsn-PCR: one-tube semi-nested-polymerase chain reaction. *Methods of Imai et al. and Theodoro et al. using 35 cycles.

without gelatin, the limit of DNA detection was 0.25ng (data not shown). Despite the use of gelatin in some studies, its role in these types of PCR reactions remains unknown[11].

To assess the use of the OTsn-PCR assay for the detection of other fungi, we modified the assay to specifically detect *H. capsulatum* DNA by changing only the species-specific primer used in the reaction. The detection limit of this assay was as low as 2.5pg **(Figure 1E)**, which was similar to that observed when the *P. brasiliensis* species-specific primer was used. No cross-reactivity with the DNA of other fungi was observed.

In conclusion, our results demonstrate that the OTsn-PCR assay described in this study has a limit of detection identical to or better than the conventional nested and semi-nested PCR assays for *P. brasiliensis* detection and can easily be adapted for the detection of any pathogenic fungi simply by changing the species-specific primer. This approach will be a focus in our future studies. Furthermore, this reaction is faster and less prone to contamination than the conventional nested or semi-nested PCR assays. Thus, the OTsn-PCR assay has great potential for laboratory PCM diagnoses.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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