UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

IDENTIFICAÇÃO E DETERMINAÇÃO DO PERFIL DE SENSIBILIDADE DE FORMA RÁPIDA DE *Candida* spp. ISOLADAS DE HEMOCULTURAS.

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RESUMO

Base Teórica: As infecções de corrente sanguínea causadas por espécies *Candida* têm aumentado e tornaram-se prevalentes com taxas de mortalidade próximas ou superiores a 50%. O início tardio da farmacoterapia adequada se correlaciona com um aumento da taxa de mortalidade e por esse motivo a rápida identificação das espécies e a determinação do seu perfil de sensibilidade são fundamentais para orientá-la, reduzir custos e tempo de internação. O MALDI-TOF é uma tecnologia que permite a identificação rápida e confiável de patógenos e pode auxiliar na agilidade desses resultados. **Objetivo:** Desenvolver um método rápido, prático e barato para a identificação e determinação do perfil de sensibilidade ao Fluconazol e Micafungina para *Candida* spp. isoladas de hemocultura. **Metodologia:** Identificação por MALDI-TOF e determinação do perfil de sensibilidade *Candida* spp. por microdiluição em caldo, diretamente do frasco de hemocultura positiva, após subcultivo e incubação por cerca de 5 horas, de amostras pacientes internados no Hospital de Clínicas de Porto Alegre entre 2019 e 2020. **Resultados:** Os testes diretamente do frasco permitiriam a obtenção dos resultados entre 24 a 48 horas antes do método padrão. Os resultados da identificação rápida obtiveram uma concordância categórica de 92,05% (220 em 239 amostras clínicas), comparado ao método padrão. Quando avaliado por espécie, *C. glabrata* e *C. krusei,* obtiveram 100% de concordância. Na microdiluição rápida para o Fluconazol a concordância foi de 97,06% ($p< 0,001$), o índice de concordância Kappa foi de aproximadamente 0,91 ($p< 0,001$), apresentou um erro menor (1,47%) e um erro maior (1,47%). Já na microdiluição rápida para a Micafungina não houve erros, obtendo concordância de 100% ($p< 0,001$) e o índice Kappa de 1,0 ($p< 0,001$). **Conclusão:** As metodologias rápidas para identificação e determinação do perfil de sensibilidade de *Candida* spp. são uma ótima alternativa aos métodos utilizados atualmente na rotina do laboratório de microbiologia. Além dessas metodologias serem eficazes, reprodutíveis, baratas e de fácil execução, são confiáveis e possibilitam a liberação do resultado de forma precoce impactando diretamente na eficácia do tratamento.

Palavras-chave: MALDI-TOF MS; *Candida* spp.; diagnóstico rápido; hemocultura; sensibilidade.

ABSTRACT

Background: Bloodstream infections caused by *Candida* species have increased and become prevalent with mortality rates close to 50%. The late start of adequate pharmacotherapy is correlated with an increase in the mortality rate and, for this reason, the rapid identification of the species and the determination of their susceptibility profile are essential to guide it, reduce costs and hospitalization time. MALDI-TOF is a technology that allows fast and reliable identification of pathogens and can help speed up these results. **Objective:** Develop a rapid, practical, and inexpensive method for identification and determination of the susceptibility profile to Fluconazole and Micafungin of *Candida* spp. isolated from blood culture. **Methods:** Identification through MALDI-TOF and determination of susceptible profile through broth microdilution, directly from the positive blood culture bottles after subculture and short-term incubation of *Candida* sp. samples from patients admitted to Hospital de Clínicas de Porto Alegre between 2019 and 2020. **Results:** Tests directly from the bottle would allow obtaining results 24 to 48 hours before the standard method. The rapid identification results obtained a categorical agreement of 92.05% (220 out of 239 clinical samples) compared to the standard method. Regarding species identification, *C.glabrata* and *C. krusei* obtained 100% agreement. In rapid broth microdilution for Fluconazole, the agreement was 97.06% ($p<0.001$), the Kappa agreement coefficient was approximately 0.91 ($p<0.001$), had a minor error (1.47%) and a major error (1.47%). In the rapid broth microdilution for Micafungin, there were no errors, obtaining 100% agreement ($p<0.001$) and the Kappa coefficient was 1.0 ($p<0.001$). **Conclusion:** Rapid methodology for identifying and determining the susceptibility profile of *Candida* spp. is an excellent alternative to the methods currently used in the routine of the microbiology laboratory. In addition to these methodologies are efficient, reproducible, inexpensive, and easy to perform, they are reliable and release of the result early, directly impacting effectiveness of the treatment.

Keywords: MALDI-TOF MS; *Candida* spp.; rapid diagnosis; blood culture; susceptible.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

CLSI	Clinical & Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

SUMÁRIO

1. INTRODUÇÃO

As infecções de corrente sanguínea causadas por espécies de *Candida* são cada vez mais frequentes em ambiente hospitalar atualmente, com incidência cinco vezes maior do que na última década (BASSETTI *et al*., 2013; LORTHOLARY *et al*., 2014).

Apesar de todos os avanços relacionados ao reconhecimento dos fatores de risco e desenvolvimento de novas ferramentas diagnósticas, tratamento de suporte e farmacoterapia para infecções fúngicas, as candidemias ainda estão entre as causas mais importantes de morbimortalidade em pacientes hospitalizados independentemente do estado imunológico, com taxas de mortalidade próximas ou superiores a 50% (BASSETTI *et al*., 2013; KULLBERG; ARENDRUP, 2015; MORENO-LOAIZA; MORENO-LOAIZA, 2017; DOI *et al.*, 2016; BRAGA *et al*., 2018; ALDARDEER *et al*., 2020; DE OLIVEIRA *et al*., 2021).

A epidemiologia da candidemia varia geograficamente, de modo geral, a *Candida albicans* é a espécie mais frequentemente isolada nas hemoculturas positivas para fungos (entre 50 a 60%) (GUINEA, 2014; LORTHOLARY *et al*., 2014; JABEEN *et al*., 2016; DA MATTA; SOUZA; COLOMBO, 2017) e possui alta sensibilidade ao fluconazol, antifúngico utilizado no Brasil e em outras partes do mundo como tratamento de primeira de escolha (NUCC*I et al.,* 2013; BONGOMIN *et al*., 2017). Porém há uma preocupação com o aumento da frequência ao longo dos anos de infecções causadas por espécies de *Candida* não *albicans* em diferentes partes do mundo, pois estas são mais propensas a serem resistentes às terapias empíricas utilizadas e apresentam potencial de causar surtos, o que possui um impacto clínico importante caracterizando um problema de saúde pública mundial (PFALLER *et al.,* 2011; FAROOQI *et al*., 2013; DOI *et al*., 2016; SCHELENZ *et al.,* 2016; DA MATTA; SOUZA; COLOMBO, 2017; DE OLIVEIRA *et al*, 2021; KOTEY *et al*., 2021; SOUZA *et al*., 2021).

A identificação rápida da espécie de *Candida* e a determinação do seu perfil de sensibilidade são fundamentais para direcionar a farmacoterapia, melhorando o manejo dos pacientes infectados, possibilitando a redução de custos e do tempo de internação, principalmente se a espécie isolada for resistente à droga empírica utilizada (PEREZ *et al.,* 2013, COLOMBO *et al.,* 2014, CORTÉS *et al.,* 2014). Um estudo de coorte retrospectivo realizado por Kollef e colaboradores (2012), durante

nove anos em um Hospital dos Estados Unidos com 1250 leitos, evidenciou que a taxa de mortalidade hospitalar para pacientes em choque séptico que tiveram a terapia antifúngica administrada dentro de 24 horas do início da candidemia foi de 52,8% (n = 142), comparada a uma taxa de mortalidade de 97,6% (n = 82) em pacientes que não tiveram essa administração. Esse estudo expõe o fato de que o início tardio da farmacoterapia adequada correlaciona-se com um aumento da taxa de mortalidade desses pacientes, fato demonstrado também em outros estudos mais recentes (CORTES *et al.*, 2014; LAUSCH *et al.*, 2018; KOTEY *et al*., 2021).

expondo o fato de que o início tardio da farmacoterapia adequada correlaciona-se com um aumento da taxa de mortalidade desses pacientes (CORTES *et al.*, 2014; LAUSCH *et al.*, 2018; KOTEY *et al*., 2021).

A tecnologia de MALDI-TOF (Matrix Assisted Laser Desorption/Ionization - Time Of Flight Mass Spectrometry) identifica com precisão e rapidez diversos microrganismos e representa uma alternativa atraente para métodos convencionais mais demorados (BUCHAN; LEDEBOER, 2013, HUANG *et al*., 2013). Com o uso desse equipamento e com a elaboração de novas metodologias e protocolos baseados na identificação e determinação do perfil de sensibilidade direta do frasco de hemocultura é possível ter de forma mais rápida os resultados e, assim, auxiliar na seleção de uma terapia antifúngica apropriada.

2. REVISÃO DE LITERATURA

2.1 Estratégias para localizar e selecionar as informações

Esta revisão da literatura está focada nos aspectos relacionados a definição, incidência, perfil de sensibilidade e diagnóstico de Candidemias. A estratégia de busca envolveu as bases de dados SciELO e PubMed no período de 1940 a 2020. Foram realizadas buscas através dos termos “Candida”, “Blood culture”, “MALDI-TOF”, “Susceptible Profile” e suas combinações apresentadas na Figura 1.

PubMed	Palavras-Chaves	SciElo
76.386	1. Candida	2.598
108.111	2. Blood culture	842
20.855	3. MALDI-TOF	128
34.883	4. Susceptible profile	195
1 + 2 = 2.125 1 + 3 = 458 1 + 4 = 858 1 + 2 + 3 = 51 1 + 3 + 4 = 45 1 + 2 + 3 + 4 = 0		1 + 2 = 47 1 + 3 = 7 1 + 4 = 15 1 + 2 + 3 = 0 1 + 3 + 4 = 2 1 + 2 + 3 + 4 = 7

Figura 1. Estratégias para localizar e selecionar as informações. Fonte: elaborado pela autora (2020).

2.2 Espécies de *Candida*

As leveduras do gênero *Candida* sp. são células fúngicas unicelulares, esféricas a elipsoides (3 a 15 µm), apresentam parede celular rígida que determina sua forma e as protegem do estresse osmótico e ambiental, podem ser encontradas em variados ecossistemas, como solo, alimentos, água e fazem parte da microbiota de homens e animais. Esses microrganismos possuem uma considerável capacidade adaptativa, podendo desenvolver-se na presença e na ausência de oxigênio e se reproduzem, predominantemente, de maneira assexuada, podendo também se multiplicar de maneira sexuada. São classificadas taxonomicamente no reino *Fungi*, Filo *Ascomycota*, Classe *Saccharomycetes*, Ordem *Saccharomycetales* e Família *Saccharomycetaceae* (GIOLO; SVIDZINSKI, 2010; BROOKS *et al.,* 2014).

As leveduras apresentam-se como comensais em humanos colonizando a pele e as superfícies de mucosas como a da boca, do trato gastrointestinal e urogenital. Esses microrganismos estabelecem uma relação de competição com as bactérias da microbiota exercendo uma pressão seletiva, o que resulta na eliminação dos microrganismos menos adaptados gerando um equilíbrio entre as populações microbianas nos tecidos colonizados (PAM *et al.,* 2012; BROOKS *et al.,* 2014). Porém, podem ser consideradas oportunistas, pois em caso de desequilíbrio de sua relação com o hospedeiro, tornam-se patogênicas causando infecções que podem variar entre

uma leve irritação das superfícies das mucosas até uma doença invasiva grave (BUCHAN; LEDEBOER, 2013).

2.2 Candidemia

O termo candidemia refere-se a presença de células leveduriformes da espécie *Candida* sp. na corrente sanguínea e é a forma mais comum de candidíase invasiva (KULLBERG; ARENDRUP, 2015). Está associada a altas taxas de mortalidade, mesmo com a introdução de novos agentes antifúngicos (BASSETTI *et al*., 2013, KULLBERG; ARENDRUP, 2015, MORENO-LOAIZA; MORENO-LOAIZA, 2017). Estima-se que o custo de cada internação hospitalar por um episódio de candidemia seja de aproximadamente US$ 40.000 (BASSETI *et al.,* 2013). São cada vez mais frequentes em ambientes hospitalares, com incidência cinco vezes maior do que na última década (BASSETTI *et al.,* 2013, LORTHOLARY *et al.,* 2014).

O aumento da incidência de candidemias têm sido atribuído ao grande número de pacientes imunocomprometidos existentes atualmente, como transplantados e portadores de imunodeficiências que são populações vulneráveis a esses patógenos oportunistas. Outros motivos que podem ser ressaltados para justificar o aumento nos casos de candidemia são o maior número de técnicas invasivas disponíveis e o avanço dos métodos de diagnóstico laboratorial (BUCHAN; LEDEBOER, 2013; KULLBERG; ARENDRUP, 2015; KAUFFMAN, 2017).

Os principais fatores de risco associados à candidemia são o uso de antimicrobianos de largo espectro, tempo prolongado de internação hospitalar, neutropenia, nutrição parental total, sonda vesical de demora, ventilação mecânica, cateter venoso central e colonização de vários sítios anatômicos por leveduras. Além desses fatores, também se destacam idades extremas (prematuros e idosos), imunossupressão, insuficiência renal, diabetes mellitus, quimioterapia, radioterapia, lesão de mucosas, hemodiálise, pancreatite, cirurgia prévia (principalmente procedimentos intra-abdominais maiores), corticoterapia e condições médicas mais imunocomprometedoras, como malignidades (KULLBERG; ARENDRUP, 2015; BONGOMIN *et al*., 2017)

Com o sistema imunológico debilitado ou com o rompimento da barreira física anatômica, ocorre um desequilíbrio entre os microrganismos que vivem comensalmente na microbiota tornando o meio propício para instalação da infecção.

Para iniciar um processo patológico, a levedura precisa atravessar as barreiras biológicas como pele, trato respiratório, trato gastrointestinal e trato geniturinário e, para isso, expressam alguns fatores de virulência como a adesão, formação de biofilme, alterações fenotípicas e morfológicas, como a filamentação e secreção de enzimas, que resultam no início do processo patológico (CORTÉS; A RUSSI, 2011; SEGHIR *et al*., 2014).

Existem cerca de 200 espécies distintas de *Candida*, porém mais de 90% das infecções de corrente sanguínea por células leveduriformes são causadas pela espécie *C. albicans* e por algumas espécies conhecidas como não-albicans (*C. parapsilosis, C. tropicalis, C. glabrata* e *C. krusei*) (GIOLO; SVIDZINSKI, 2010). A *Candida albicans* é a principal levedura patogênica oportunista isolada em humanos, entretanto, as infecções por espécies de *Candida* não-*albicans* têm apresentado aumento significativo, fato que é preocupante são mais propensas a serem resistentes aos antifúngicos e têm maior potencial de causar surtos, tornando-se uma preocupação em diferentes partes do mundo (PFALLER *et al.,* 2011; FAROOQI *et al*., 2013; DOI *et al*., 2016; SCHELENZ *et al.,* 2016; DA MATTA; SOUZA; COLOMBO, 2017; DE OLIVEIRA *et al*, 2021; KOTEY *et al*. 2021; RISUM *et al*., 2021).

Recentemente relatos documentaram surtos de candidemia *por C. auris* em centros médicos da Venezuela e da Colômbia (MATTA *et al*., 2017). O primeiro caso no Brasil foi confirmado em dezembro de 2020 em um hospital de Salvador, no estado da Bahia. Essa espécie tem facilidade em colonizar o ambiente hospitalar e os pacientes, apresenta alta transmissibilidade e pode causar surtos prolongados de difícil controle por sua difícil erradicação. A *C. auris* está associada à resistência a múltiplas drogas antifúngicas e infecções invasivas (DE ALMEIDA *et al*., 2021). Muitas vezes pode ser identificada erroneamente em laboratórios de rotina como *C. famata* e *C. haemulonii*, o que gera apreensão devido à sua evolução, perfil de resistência e propagação mundial (CHOWDHARY; VOSS; MEIS, 2016; MORALES-LÓPEZ *et al*., 2017).

2.3 Perfis de Sensibilidade

A introdução de drogas antifúngicas azólicas por via oral na década de 1980, particularmente o fluconazol, foi um desenvolvimento significativo que permitiu o tratamento de infecções fúngicas sistêmicas sem o efeito colateral da nefrotoxicidade

associada ao tratamento com anfotericina B. O fluconazol é um antifúngico triazólico bastante tolerante, possui alta biodisponibilidade e penetração tecidual (PERON *et al*., 2016). No entanto, períodos prolongados de tratamento podem induzir mutações que expressam a resistência a esse antifúngico causando falhas no tratamento, o que é preocupante, pois é um agente antifúngico comumente utilizado para profilaxia e tratamento de infecções por *Candida* spp. (BASSETTI *et al*., 2013, NUCCI *et al*., 2013).

O perfil de sensibilidade aos antifúngicos varia de acordo com as espécies de *Candida*. As espécies *C. albicans, C. dubliniensis* e *C. tropicalis* são normalmente sensíveis aos principais antifúngicos utilizados para o tratamento da candidemia, já a *C. glabrata* possui um perfil menos sensível, sendo naturalmente sensível aumentando a exposição ao fluconazol e com taxas crescentes de resistência às equinocandinas. *A C. parapsilosis* é menos sensível às equinocandinas, enquanto a *C. krusei* possui resistência ao fluconazol (PERON *et al*., 2016).

Durante a revisão de Matta e colaboradores (2017), foram encontradas taxas de resistência entre os isolados de *C. parapsilosis* variando entre 3,4% e 7,5% nos Estados Unidos e entre 0 e 6% na Europa, já a *C. tropicalis* apresentou taxas oscilando entre 2,4% e 11,6% nos Estados Unidos e de 1,7% a 22% na Europa. Na América Latina se ressaltou que ocorreu um ligeiro aumento nos isolados de *C. albicans*, *C. parapsilosis* e *C. tropicalis* exibindo um perfil não sensível ao fluconazol, com taxas de resistência que aumentaram de 0,4% para 1,2% entre os isolados de *C. albicans*, de 0,5% a 2,3% entre os de *C. tropicalis* e de 0 a 2,6% entre os de *C. parapsilosis*.

Como evidenciado anteriormente, estudos relataram uma incidência crescente de infecções causadas por leveduras que adquiriram resistência ou são intrinsecamente resistentes ao medicamento em uso, o que é preocupante, pois as opções de antifúngicos são limitadas às classes de azóis, equinocandinas, polienos e flucitosina, e o surgimento da resistência limita as opções farmoterapêuticas (PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017, LAMOTH *et al*., 2018).

Com a crescente resistência das leveduras, os testes de sensibilidade aos antifúngicos são fundamentais para que a farmacoterapia seja conduzida de maneira segura, correta e eficaz. Pelo antifungigrama é possível monitorar e detectar cepas de

Candida spp. resistentes, auxiliando o clínico na escolha da terapia antifúngica adequada (GIOLO; SVIDZINSKI, 2010).

2.4 Diagnóstico Laboratorial das Leveduras

Como em qualquer processo infeccioso, a identificação oportuna do organismo infectante e a determinação do seu perfil de sensibilidade aos antimicrobianos desempenham papéis-chave no manejo bem-sucedido do paciente. A hemocultura é o padrão ouro para o diagnóstico de infecções da corrente sanguínea e depende de técnicas convencionais subsequentes, incluindo coloração de Gram, subcultura seguida por testes bioquímicos e teste de sensibilidade aos antifúngicos (BUCHAN; LEDEBOER, 2013). Todo o procedimento leva em média de 48 a 72 horas para ser concluído e possui limitações, já que essas metodologias necessitam de processos metabólicos do microrganismo (CASPAR *et al*., 2016; MALDONADO; ROBLEDO; ROBLEDO, 2017; ZHOU *et al*., 2017).

A introdução do espectrômetro de massas MALDI-TOF (Matrix Assisted Laser Desorption/Ionization - Time Of Flight mass spectrometry) foi uma das maiores inovações tecnológicas dentro do setor de microbiologia. Em poucos anos deixou de ser uma novidade promissora, passando a ser uma tecnologia disponível e totalmente integrada na atividade clínica diária. Em 1975, Anhalt e Fenselau propuseram o uso da espectrometria de massas para a identificação de microrganismos e, 20 anos depois, foi publicado o primeiro estudo demonstrando a eficácia da MALDI-TOF MS na identificação de macromoléculas biológicas, o que rendeu o prêmio Nobel de Química de 2002 aos autores (MALDONADO; ROBLEDO; ROBLEDO, 2017; SILLER-RUIZ *et al*., 2017).

Ao final dos anos noventa, verificou-se que a aplicação de espectrometria de massas em células bacterianas produziu espectros proteicos característicos e reprodutíveis que poderiam ser usados para identificação a nível de gênero e espécie. A partir dessa descoberta, deu-se início ao desenvolvimento de bibliotecas espectrais baseadas em cepas de referência, integradas em programas de computador com algoritmos que permitiram a aplicação da espectrometria de massas na análise da composição proteica de microrganismos identificando-os de maneira rápida e precisa, uma vez que estas proteínas, principalmente de origem ribossômica na faixa de massa de 2-20 kDa, bem como suas variações, geram espectros de massa com picos que

podem ser específicos para o gênero e para espécie que são únicos, como uma impressão digital (MALDONADO; ROBLEDO; ROBLEDO, 2017).

Diversos estudos avaliaram o desempenho do equipamento MALDI-TOF na identificação de isolados de bactérias e leveduras a partir da cultura em meio sólido e todos obtiveram resultados com níveis de concordância superiores a 90% quando comparados a métodos moleculares. Quando a comparação foi feita entre o MALDI-TOF e os métodos bioquímicos e/ou o sistema Vitek, o MALDI-TOF obteve um melhor desempenho na identificação (CHERKAOUI *et al*., 2010; VAN VEEN; CLAAS; KUIJPER, 2010; HUANG *et al*., 2013; TEKIPPE; BURNHAM, 2014; GALÁN *et al*., 2015). Além do melhor desempenho, o equipamento necessita de menos insumos e dessa forma o volume de resíduos de risco biológico é reduzido em até 1/6 comparado aos produzidos usando métodos convencionais, sendo uma opção mais sustentável e com menor risco biológico (MALDONADO; ROBLEDO; ROBLEDO, 2017).

O equipamento MALDI-TOF tem a capacidade de analisar um grande número de amostras simultaneamente, quando usado em sua totalidade gasta um tempo médio de um minuto para identificar cada isolamento, o que diminui em aproximadamente 28,8 horas o tempo de identificação total do microrganismo quando comparado aos métodos bioquímicos tradicionais, o que acelera a liberação do resultado final (TAN *et al*., 2012; HUANG *et al*., 2013; PEREZ *et al*., 2013).

O aparelho possui um custo estimado de 22-32% do custo total dos métodos de identificação padrão, pois a preparação para análise de MALDI-TOF, incluindo a extração de proteínas, requer apenas reagentes mínimos e baratos que equivalem a aproximadamente US$ 0,50 por identificação e também é necessário um menor número de funcionários envolvidos, o que reduz a mão-de-obra. Embora o custo inicial de um instrumento MALDI-TOF possa ser significativo (US $ 180.000 a US $ 250.000) juntamente com a sua manutenção (até 10% do custo do equipamento anualmente), o baixo custo de reagente, a rapidez e a alta taxa de identificação reduzem significativamente o custo por isolado para identificação de microrganismos em comparação com os métodos convencionais (DHIMAN *et al*., 2011; BUCHAN; LEDEBOER, 2013; PEREZ *et al*., 2013). Um estudo realizado no laboratório de microbiologia do Hospital Johns Hopkins com 952 isolados clínicos determinou que o MALDI-TOF MS realizou a identificação de microrganismos 1,45 dias antes da metodologia padrão do laboratório e que a incorporação dessa tecnologia permitiu a

redução de 52% nos custos de mão-de-obra e reagentes investidos na identificação de microrganismos por ano (TAN *et al*., 2012).

A identificação precisa de microrganismos diretamente da hemocultura é uma das aplicações com maior impacto clínico em potencial da tecnologia MALDI-TOF, uma vez que contribui para o direcionamento correto da farmacoterapia de 24 a 48 horas antes da identificação baseada na subcultura do isolado. Diversos protocolos estão sendo validados, porém precisam superar alguns desafios como a interferência dos componentes do sangue (eritrócitos, leucócitos e proteínas séricas) que se apresentam como contaminantes espectrais, podendo interferir no espectro e na correspondência de pico, e a necessidade de uma alta concentração de microrganismos para obter um bom espectro de proteínas, uma vez que MALDI-TOF requer cerca de 10^5 unidades formadoras de colônias para obter um espectro confiável e específico. O uso dessas novas metodologias tem se mostrado muito benéfica ajudando a aumentar a sobrevida dos pacientes e reduzindo os custos derivados da assistência à saúde (BUCHAN; LEDEBOER, 2013; MALDONADO; ROBLEDO; ROBLEDO, 2017).

3. MARCO CONCEITUAL

As infecções de corrente sanguínea causadas por espécies de *Candida* tornaram-se prevalentes com altas taxas de mortalidade. O início tardio da farmacoterapia adequada se correlaciona com um aumento da taxa de mortalidade e por esse motivo a rápida identificação das espécies e a determinação do seu perfil de sensibilidade são fundamentais para orientá-la, reduzir custos e tempo de internação. Para isso é necessária a busca por metodologias rápidas que sejam práticas, confiáveis e baratas.

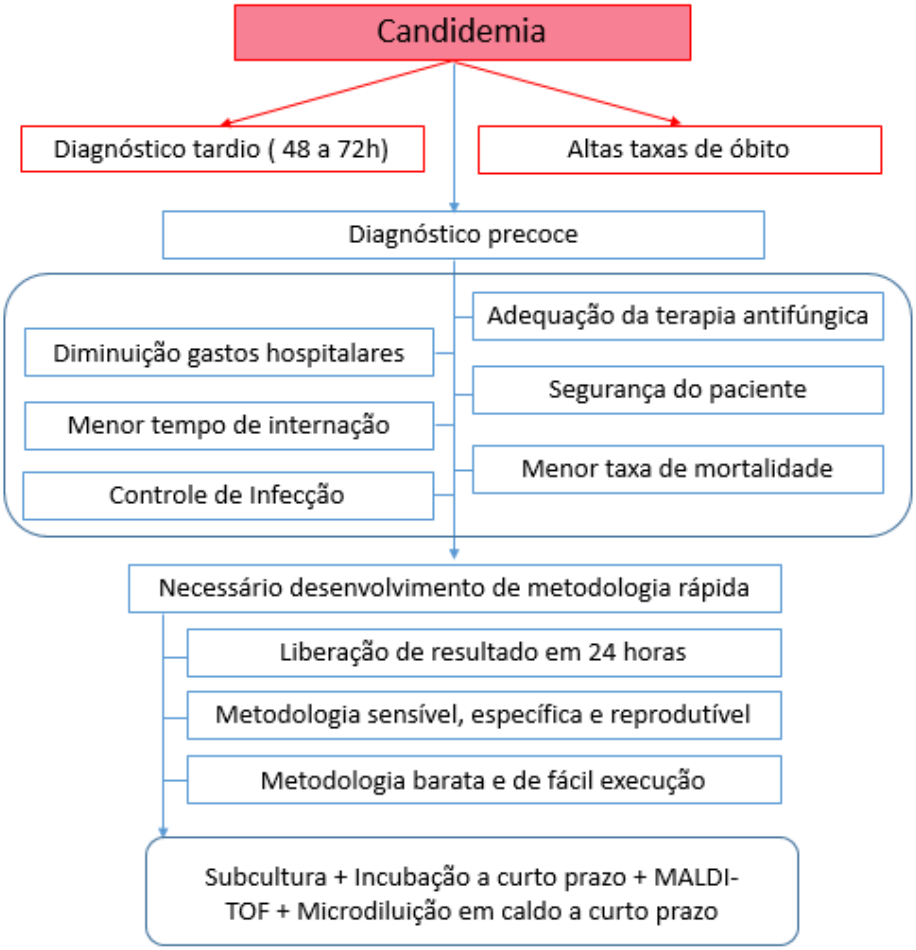

Figura 2. Marco conceitual do estudo. Fonte: elaborado pela autora (2021).

4. JUSTIFICATIVA

Com o aumento na incidência de infecções de corrente sanguínea nosocomiais causadas por diversas espécies de *Candida* com perfis de resistência variados, faz-se necessária uma rápida identificação e determinação do perfil de sensibilidade dessas leveduras para o correto direcionamento da farmacoterapia.

Uma das mais recentes aquisições do laboratório de microbiologia do Hospital de Clínicas de Porto Alegre foi o espectrômetro de massas MALDI TOF MS (Matrix Assisted Laser Desorption/Ionization - Time Of Flight Mass Spectrometry). Com o uso desse equipamento inovador e com a busca de novos protocolos, é possível diminuir ainda mais o tempo de identificação e realizar a determinação do perfil de sensibilidade das espécies causadoras de candidemias quando comparado ao método tradicional, o qual necessita do crescimento da colônia em meio sólido.

A escassez de estudos sobre novas metodologias de identificação, principalmente de origem brasileira, e a ausência de técnicas de determinação rápida do perfil de sensibilidade por microdiluição em caldo de *Candida* spp. isoladas em hemoculturas, culminou na necessidade do desenvolvimento e na validação de um método *in house* rápido e eficaz para a identificação e determinação do perfil de sensibilidade diretamente do frasco de hemocultura. Dessa maneira, os resultados poderão ser obtidos de forma mais breve e confiável, o que otimiza a rotina laboratorial na detecção de candidemias no Hospital de Clínicas de Porto Alegre acarretando na diminuição em mais de 24 horas no tempo de liberação dos resultados. Sendo assim, existe a possibilidade do direcionando mais rápido ao tratamento adequado podendo contribuir para o sucesso do manejo clínico, a melhoria na sobrevida dos pacientes e a redução dos custos hospitalares.

5. OBJETIVOS

5.1 Objetivo geral.

Desenvolver e validar método rápido para identificação e determinação do perfil de sensibilidade de espécies de *Candida* isoladas a partir de hemoculturas de pacientes atendidos no Hospital de Clínicas de Porto Alegre.

5.2 Objetivos específicos

- Avaliar a performance do sistema MALDI-TOF VITEK®MS System (bioMérieux, France) para a identificação direta de espécies de Candida dos frascos de hemocultura positivas e comparar com a metodologia padrão utilizada na rotina laboratorial;
- Avaliar a performance do sistema MALDI-TOF VITEK®MS System (bioMérieux, France) para a identificação de espécies de Candida dos frascos de hemocultura positivas a partir de uma cultura de crescimento rápido (4-6h) em meio sólido e comparar com a metodologia padrão utilizada na rotina laboratorial;
- Determinar o perfil de sensibilidade ao fluconazol pela técnica de disco difusão diretamente do frasco de hemocultura e determinar a acurácia dos resultados em comparação com a técnica padrão de disco difusão;

- Determinar o perfil de sensibilidade aos antifúngicos micafungina e fluconazol de isolados clínicos de *Candida* spp. pela técnica de microdiluição em caldo, a partir de uma cultura de 4-6h em meio sólido e determinar a acurácia dos resultados em comparação com o método padrão;

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7. ARTIGOS

7.1 Artigo 1

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Research Article

Evaluation of Identification and Susceptibility for *Candida* Spp. Isolated Directly from Positive Blood Culture Bottles

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Determination of the susceptibility profile of isolates of *Candida* from blood culture bottles is extremely important for correctly guiding patient pharmacotherapy. The aim of this study was to compare the results of analysis of *Candida* isolated directly from blood culture bottles by the VITEK MS MALDI-TOF identification system and the fluconazole disk diffusion assay with those of standard identification methods. Testing directly from the bottle allowed results 24 to 48 hours quicker than the standard method. There was a categorical agreement of 51.64% (47 of 91 samples) between the results of analysis directly from the bottle and analysis by the standard method. Regarding species identification, there was 96.15% agreement for *Candida parapsilosis* (25 of 26 samples). Categorical agreement between the rapid and standard disk diffusion methods was 95%, and the agreement between the rapid disk diffusion method and the broth microdilution method was 97%. Only minor errors in the rapid method were observed: 3 (5%) in the standard disk diffusion method and 2 (3%) in the broth microdilution method. Our study concluded that the rapid disk diffusion method for fluconazole is a fast, easy, reproducible, and consistent method. Its timely implementation for testing antifungal agents in the clinical microbiology laboratory can help reduce profile release times, thus helping to determine the most appropriate antifungal treatment.

1. Introduction

Bloodstream infections caused by *Candida* species are becoming increasingly common in hospitals, with the incidence being fivefold higher than that in the last decade, and are associated with high morbidity and mortality rates [1, 2]. Fluconazole is a well-tolerated triazole antifungal with high bioavailability and tissue penetration ability [3]. However, prolonged treatment could induce resistant mutations in *Candida* that lead to therapeutic failure, which is a critical concern since fluconazole is the most commonly used antifungal agent for the prophylaxis and treatment of *Candida* infections in many parts of the world [1].

In a 9-year retrospective cohort study at a 1250-bed US Hospital, Kollef et al. found that the hospital mortality rate for septic shock patients who received antifungal therapy within 24 hours of candidemia onset was 52.8% ($n = 142$), compared to 97.6% ($n = 82$) in those who did not receive antifungal therapy [4]. Other studies have found that the 30-day survival rate of candidemia patients who receive appropriate pharmacotherapy was better than that of patients who received delayed or no treatment. These studies show that late initiation of adequate pharmacotherapy in infected patients correlates with an increased mortality rate [5–7]. Thus, determining the species responsible for the infection and the susceptibility profile of *Candida* spp. is important not only for guiding pharmacotherapy but also for

monitoring the treatment efficacy and the emergence of resistance.

This study aimed to use a rapid method to identify species of *Candida* and assess fluconazole susceptibility directly from positive blood cultures.

2. Methods

The study was approved by the local ethics committee.

2.1. Yeast Strains. Isolates of *Candida* were obtained from blood cultures of patients admitted to a tertiary care hospital in Southern Brazil. For the rapid identification method, we included all the samples of blood cultures from patients with *Candida* species isolated between September 2018 and June 2019. For the rapid disk diffusion method, we included only one sample per patient between September 2018 and September 2019.

Blood samples were inoculated in aerobic bottles and incubated in the BacT/ALERT® 3D system (bioMérieux, France) for microorganism growth monitoring. We excluded from the study samples from which more than one microbial species was isolated. *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 750, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 90018 were included as quality control strains.

2.2. Standard Method. Following microorganism growth identification by the BacT/ALERT® 3D system, blood culture media were collected from each bottle and subjected to Gram staining. Then, samples were subcultured on solid growth media, including blood agar (bioMérieux) and Sabouraud agar (Merck, Germany), and incubated at 35°C for 18–24 hours. To estimate the cell numbers in the bottles, 5 positive blood culture bottles were randomly selected. Then, 1 mL sample was aspirated from each of these bottles, serially 10-fold diluted with sterile saline, and 50 μL of suspensions was plotted on the Sabouraud agar plate, and colonies were counted after 24 h of incubation (ranged from 7×10^5 to 5×10^7 CFU/mL). The rapid disc diffusion method was performed according to the RAST methodology standardized by the European Committee on Antimicrobial Susceptibility Testing. Following incubation, isolated colonies were subjected to analysis by the MALDI-TOF VITEK MS® 3.0 system (bioMérieux, France) according to the manufacturer's instructions. Fluconazole susceptibility was assessed using a disk diffusion method according to the CLSI M44-A2 guidelines and a broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing guidelines [8, 9].

2.3. Rapid Identification Method. The rapid identification method was performed according to the protocol proposed by Spanu et al. [10]. Each test was conducted in duplicate. An 8 ml aliquot from the blood culture bottle was centrifuged at 10,000 rpm for 2 minutes at room temperature. The supernatant was discarded, and the pellet was washed twice with 1 ml of pure water and recentrifuged. It was suspended in 1 ml of 0.1% Tween 80, incubated for 2 minutes, recentrifuged, washed twice with 1 ml of pure water, recentrifuged, suspended in 300 μl of pure water plus 900 μl of absolute ethanol, and recentrifuged. Then, 30 μl of 70% formic acid plus 30 μl of pure acetonitrile was added to the pellet, and it was thoroughly vortexed and centrifuged at 14,000 rpm for 2 minutes. A 1 μl aliquot of the supernatant was collected and applied to a steel MALDI target plate. Finally, the sample was subjected to analysis by the MALDI-TOF VITEK MS® 3.0 system (bioMérieux, France).

2.4. Rapid Disk Diffusion Method. The rapid disk diffusion method was performed according to Jabeen et al. [11]. A 100 μL aliquot from the blood culture bottles was used to make lawns on Mueller–Hinton agar supplemented with 2% dextrose and 0.5 μg/ml methylene blue dye. Two discs with 25 μg of fluconazole were placed on the plates, and the plates were incubated.

2.5. Statistical Analysis. The kappa coefficients and categorical agreement of the data were determined using software PASW v.18 (IBM, USA). The acceptable rate agreement was ≤90% (10). Errors were classified into very major errors, major errors, and minor errors, and the acceptable rates were ≤1.5%, ≤3%, and ≤10%, respectively [12].

3. Results

A total of 91 blood culture samples from 46 patients were tested by rapid identification method tests. The overall agreement of *Candida* species identification between the rapid and standard methods was 51.64%. *Candida parapsilosis* had the highest agreement (96.15%) of the tested samples (Table 1). No sample containing *Candida orthopsilosis* or *Candida pelliculosa* was identified, and the agreement for other species varied from 30 to 67% (Table 1).

A total of 62 samples were used to assess fluconazole susceptibility by the standard disk diffusion method, the rapid disk diffusion method, and the broth microdilution method. Table 2 presents the susceptibility profiles of the *Candida* species isolated in the study obtained by the gold standard method (broth microdilution). The minimum inhibitory concentration (MIC) found for *Candida* spp. ranged from 0.125 to 32.0 μg/mL, and MIC 50 and MIC 90 were 0.5 and 4.0 μg/mL, respectively. For *Candida albicans*, the MIC range was 0.125 to 1.0 μg/mL, and MIC 50 and MIC 90 were 0.5 μg/mL and 1.0 μg/mL, respectively. For the *Candida parapsilosis* complex, the MIC range was 0.25 to 4.0 μg/mL, and MIC 50 and MIC 90 were 1.0 and 2.0 μg/mL, respectively. Approximately 87% of *Candida* spp. samples were sensitive to fluconazole (all *C. albicans* and 80% of non-*albicans Candida* isolates).

The categorical agreement between the rapid disk diffusion method and the standard disk diffusion method was approximately 95% and involved 3 minor errors (5%) (Figure 1). The kappa coefficient ($K = 0.77$; $p < 0.001$) showed strong agreement between these two methods. The

Table 1: *Candida* species identified directly from 91 blood culture samples from 46 patients.

Standard identification	Number of isolates		% agreement
	Total tested	Identification matching	
Candida albicans	28	14	50.00
Candida glabrata	3	2	66.67
Candida krusei	5	3	60.00
Candida orthopsilosis	16	0	0
Candida parapsilosis	26	25	96.15
Candida pelliculosa	3	0	0
Candida tropicalis	10	3	30.00
Total	91	47	51.64

Table 2: Distribution of *Candida* spp. and their fluconazole susceptibility profiles according to the gold standard (broth microdilution). The minimum inhibitory concentration (MIC) found for *Candida* spp. ranged from 0.125 to 32.0 μg/mL, and MIC 50 and MIC 90 were 0.5 and 4.0 μg/mL, respectively.

Species	Sensitive isolates	Dose-dependent isolates	Resistant isolates	Total isolates
Candida albicans	22	0	0	22
Candida dubliniensis	1	0	0	1
Candida glabrata	0	0	2	2
Candida krusei	0	0	2	2
Candida orthopsilosis	9	2	0	11
Candida parapsilosis	15	1	0	16
Candida pelliculosa	1	0	0	1
Candida tropicalis	6	1	0	7
Total	54	4	4	62

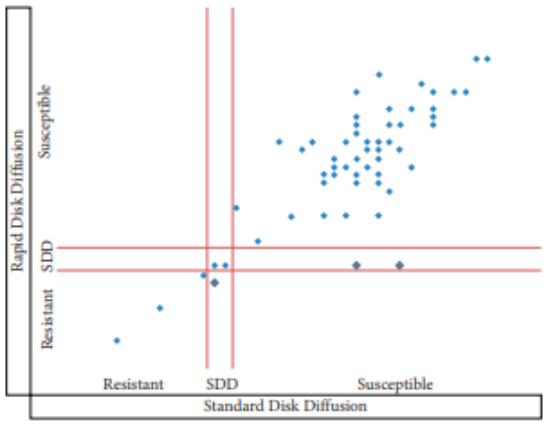


Figure 1: Distribution of susceptible profiles of *Candida* spp. according to the standard and rapid disk diffusion methods. SDD: fluconazole susceptible-dose dependent.

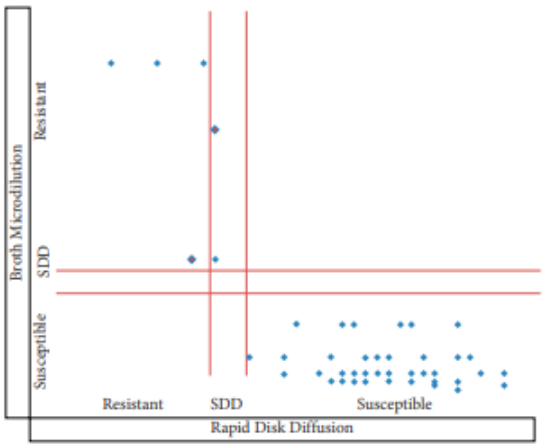
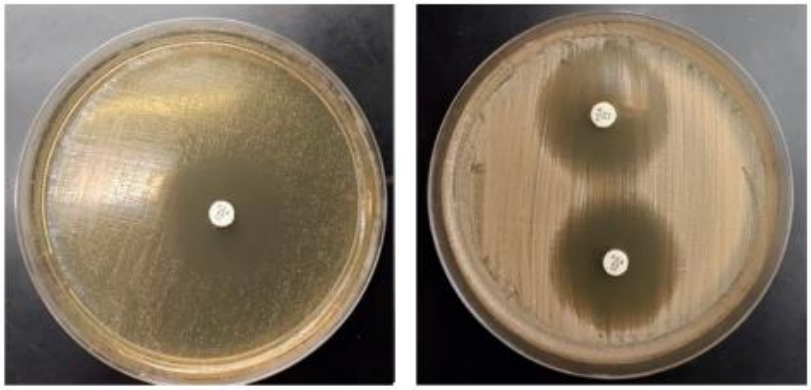


Figure 2: Distribution of susceptible profiles of *Candida* spp. according to the broth microdilution method and the rapid disk diffusion method. SDD: fluconazole susceptible-dose dependent.

categorical agreement between the rapid disk diffusion method and the broth microdilution method was 97% and involved 2 minor errors (3%) (Figure 2). The kappa coefficient ($K = 0.86$; $p < 0.001$) showed almost perfect agreement between these methods. A comparison of the results of the rapid disk diffusion method, the standard disk diffusion method, and the broth microdilution method (gold standard) is shown in Figures 1 and 2.

4. Discussion

The classical diagnostic workflow takes up to several days due to the slow growth of yeasts. The overall performance of our standard identification (Bruker Biotyper and VITEK MS) was in accordance with published data, with 70.7% of yeast correctly identified to the species, genus, or complex



(a) (b)

FIGURE 3: Comparison between standard disk diffusion (a) and rapid disk diffusion (b) susceptibility testing of *Candida* spp. for fluconazole.

level [12]. Unlike in the study conducted by Lévesque et al., the overall identification rate for our rapid method was lower than expected (52%) [13]. These authors used the Bruker Biotyper MALDI-TOF system (Daltonik GmbH, Leipzig, Germany) and obtained identification rates of 95.9% for *C. albicans* and 86.5% for non-*albicans Candida* species.

The ability to rapidly identify *Candida* species may be useful to promptly streamline the development of antifungal therapy based on empirical evidence [14, 15]. However, the emergence and spread of fluconazole-resistant *Candida* have introduced a pressing need for rapid antifungal susceptibility tests [16]. Our rapid disk diffusion method was reproducible, yielding concordant results and few errors compared to standard disk diffusion and broth microdilution methods. For our method, there were three minor errors compared to the standard disk diffusion method for the *C. parapsilosis* complex, two minor errors compared to broth microdilution for *C. glabrata* and *C. parapsilosis*, no errors for *C. albicans* isolates (which have the highest incidence in the hospital, 35%), and no errors for *C. krusei* and *C. tropicalis*. Moreover, in this study, the rapid disk diffusion method was more reliable for broth microdilution, which is the gold standard, than for standard disk diffusion, exhibiting a smaller number of errors, a higher kappa, and a higher categorical agreement rate.

The results indicate that the rapid disk diffusion test is promising for testing additional antifungal agents in microbiology laboratories, given that it can shorten the time needed for the identification of *Candida* spp. susceptibility profiles by up to two days [17]. This direct method saved on average 21.5 h for identification and 12.1 h for susceptibility testing compared to standard methods. The test is practical, easy to use, inexpensive, and rapid. It eliminates process steps, and interpreting halos is clearer and safer. Therefore, with this method, the halo is better delimited, which prevents conflicting results and interoperator error, as shown in Figure 3. By releasing susceptibility profile results more quickly, harm from inappropriate and sometimes ineffective pharmacotherapy can be reduced, aiding in patient recovery and reducing mortality and the length of stay, thus contributing to better patient safety. Further studies of susceptibility testing for other antifungal agents including echinocandins are necessary.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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7.2 Artigo 2

Rapid identification, fluconazole and micafungin susceptibility testing of *Candida species* from blood culture by a short incubation method.

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Abstract

Bloodstream infections caused by *Candida species* have increased and become prevalent with mortality rates close to 50%. The late start of adequate pharmacotherapy is correlated with an increase in the mortality rate and, for this reason, the rapid identification of the species and the determination of their susceptibility profile are essential to guide it, reduce costs and hospitalization time. MALDI-TOF is a technology that allows rapid and reliable identification of pathogens and can help speed up these results. This study aimed to develop a rapid, practical, and inexpensive method for identification through MALDI-TOF and determination of the susceptibility profile to Fluconazole and Micafungin of *Candida* spp. through broth microdilution, directly from the positive blood culture bottles after subculture and short-term incubation of patients admitted to Hospital de Clínicas de Porto Alegre between 2019 and 2020. Tests directly from the bottle would allow obtaining results 24 to 48 hours before the standard method. The rapid identification results obtained a categorical agreement of 92.05% (220 out of 239 clinical samples) compared to the standard method. Regarding species identification, *C. glabrata* and *C. krusei* obtained 100% agreement. In rapid broth microdilution for Fluconazole, the agreement was 97.06% ($p<0.001$), the Kappa agreement coefficient was approximately 0.91 ($p<0.001$), and had a minor error (1.47%), and a major error (1.47%). In the rapid broth microdilution for Micafungin, there were no errors, obtaining 100% agreement ($p<0.001$) and the Kappa coefficient was 1.0 ($p<0.001$). It is possible to conclude that the rapid method for identifying and determining the susceptibility profile of *Candida* spp. is an excellent alternative to the methods currently used in the routine of the microbiology laboratory. These methodologies are effective, reproducible, cheap, and easy to perform, in addition they are reliable and enable the release of the result early, directly impacting effectiveness of the treatment.

Keywords: MALDI-TOF MS; *Candida*; rapid diagnosis; blood culture; microdilution.

Introduction

Bloodstream infections caused by *Candida species* have increased significantly in the last decade, representing 6 to 13% of all nosocomial infections (1–6), making this fungus one of the most prevalent infectious agents in sepsis and, consequently, a major problem of world public health (7–9). In Latin America, candidemia incidence rates range from 0.74 to 6.0 per 1,000 hospital admissions (10).

Despite all the advances related to the recognition of risk factors and the development of new diagnostic tools, supportive treatment and pharmacotherapy for fungal infections, candidemia are still among the most important causes of morbidity and mortality in hospitalized patients regardless of immunological status, with rates of mortality close to or above 50% (1,7,10–13).

The epidemiology of candidemia varies geographically and, although *Candida albicans* remains the most frequent causal agent in this type of infection, there is concern about the increasing frequency over the years of infections caused by *non-albicans Candida species* in different parts of the world, as they are more likely to be resistant to the empirical therapies used, which has an important clinical impact (1,7,10,14–25). Studies demonstrated that the late start of adequate pharmacotherapy is correlated with an increase in the mortality rate and, for this reason, the rapid identification of species and the determination of their susceptibility profile are essential for the patient outcome (14,17,26–29).

The introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has been a major technological innovation, revolutionizing clinical microbiology due to its short response time (about 24 hours) (30–33). MALDI-TOF is an easy, fast and economical technology that allows the reliable identification of the pathogen in a shorter time than traditional methods (32,34,35). A survey by Clerc *et al*. (36) at Lausanne University Hospital showed that pharmacotherapy was adjusted in 35.1% of the analyzed bacteremia cases and that hospitalization time was reduced by approximately two days when MALDI-TOF MS was implemented in blood culture processing.

The search for rapid protocols to identify and determine the susceptibility profiles of *Candida* spp. is a major objective of clinical microbiology, as it directly contributes to patient outcome. Faster diagnostic methods are essential to not only guide pharmacotherapy, improve patient management, and reduce costs and length of stay, especially if the isolated species is resistant to the prescribed drug but also to

define institutional empirical therapy, control rates of nosocomial infection in different locations and during an investigation of outbreaks (10,37–40). This approach becomes even more important with the emergence of *Candida auris*, as this species has high transmissibility, high resistance rates and can cause prolonged outbreaks that are difficult to control (41).

This study aimed to develop a rapid, practical and inexpensive method to identify *Candida* spp. and determine its susceptibility profile to Fluconazole and Micafungin, drugs used worldwide in empirical therapy, from the positive blood culture bottle by a short incubation method.

Materials and methods

Isolates of *Candida* were obtained from blood cultures of patients admitted to the Hospital de Clínicas de Porto Alegre, Brazil. For the rapid identification method, we included all the samples of blood cultures from patients with *Candida* species isolated between March 2019 and November 2020. For rapid broth microdilution method, we included only one sample per patient between September 2019 and November 2020. Blood samples were inoculated in aerobic bottle and incubated in the BacT/ALERT® 3D system (bioMérieux, France) for microorganism growth monitoring. Yeasts belonging to the *Candida parapsilosis* complex (*C. orthopsilosis*, *C. parapsilosis* and *C. metapsilosis*) were considered concordant when identified as one of the Candida species belonging to the complex. Polymicrobial blood cultures that were not identified by the standard method were excluded from the study. The study included only blood cultures from the routine laboratory of microbiology, artificially inoculated bottles were not evaluated in this study.

Identification

Standard Method

The positive-flagged blood culture by the BacT/ALERT® 3D system was collected an aliquot from each bottle and subjected to Gram staining to confirm the presence of yeast cells. Then, samples were subcultured onto solid growth media including blood agar (bioMérieux, France) and Sabouraud agar (Merck, Germany) and incubated at 35°C for 18-24 hours. Following incubation, isolated colonies were

subjected to the MALDI-TOF Vitek MS® 3.0 system (bioMérieux, France) according to the manufacturer instructions.

Rapid Method

After the positive blood culture is detected by the BacT/ALERT® 3D system, a Gram is performed to confirm the presence of yeast cells. Then, an aliquot of the blood culture is removed, with the aid of a syringe, and about 3 to 5 drops are placed on top of each other in Sabouraud agar to concentrate the growth. After this procedure, this agar is incubated at 35 °C, normal atmosphere, for 5 hours (+/- 1h) and the grown colonies identified by the Vitek MS® 3.0 system (bioMérieux, France). Identifications with percentages equal to or greater than 80% were accepted as valid.

Susceptibility Profile

Standard Method

Broth microdilution for Fluconazole (Sigma-Aldrich, St Louis, MO, USA) and Micafungin (Sigma-Aldrich, St Louis, MO, USA) was performed according to the guidelines of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) – E.DEF. 7.3.2., 2020 (42) and interpreted according to the breakpoint tables of the same committee version 10.0, 2020 (43). The following concentration ranges were tested: 0.125–64 µg /mL for Fluconazole and 0.03–16 µg / mL for Micafungin. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls.

Rapid Method

From the short incubation culture (5 hours), a suspension in saline with turbidity of 0.5 was prepared using the McFarland Scale. The determination of the susceptibility profile for Fluconazole and Micafungin is performed according to the technique recommended by EUCAST (42). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls.

Statistical analysis

Data were analyzed by a chi-square test using PASW v.18 software (IBM, USA). The total acceptable agreement rate was ≤ 90% (44). Errors were classified into very

major errors, major errors and minor errors, and the acceptable rates were considered ≤ 1.5%, ≤ 3% and ≤ 10%, respectively (45).

Results

Rapid identification

A total of 239 blood culture samples were analyzed by the rapid identification method. Of these samples 36.6% were *Candida albicans*, 35.3% *Candida parapsilosis* Complex, 16% *Candida tropicalis*, 7.1% *Candida glabrata*, 2.5% *Candida krusei* and 2.5% other species less frequent (graph 1).

The overall agreement of the standard method with the rapid method was 92.05% (Table 1). The mean of the confidence value of identification released by the MALDI-TOF equipment was 98.6%. Among the 19 samples (7.95%) not identified, 7 were *C. albicans*, 6 *C. orthopsilosis*, 2 *C. tropicalis* and 4 were infrequent *Candidas* (2 *Candida dubliniesis*, 1 *Candida helenica* and 1 *Candida rugosa*).

Rapid Susceptibility Profile

To assess the agreement of species that have standardized susceptible, the Susceptibility ranges Sensitive, Sensitive increasing exposure and Resistant were used according to EUCAST 2020 (43) and for species without standardization it was used the value of the Minimum Inhibitory Concentration (MIC), considering a variation of ± 1 dilution as acceptable.

Fluconazole

A total of 68 blood culture samples were analyzed by the rapid susceptibility profile method for Fluconazole. Of these samples 36.2% were *C. albicans*, 15.9% *C. tropicalis*, 21.7% *C. parapsilosis* complex, 13% *C. glabrata,* 4.3% *C. krusei* and 8.7% other less frequent species.

The general agreement of the standard method with rapid method was 97.06% ($p<0.001$), the Kappa agreement coefficient (46) was approximately 0.91 ($p<0.001$) and presented two errors, one minor error (1 .47%) and a major error (1.47%).

The minimum inhibitory concentration found for *Candida* spp. by the standard method it ranged from 0.125 to 32.0 µg / mL, and the MIC 50 and MIC 90 obtained were 0.5 and 8.0 µg / mL, respectively, in the rapid method the ranged was from 0.125

to 64.0 µg / mL and the MIC 50 and MIC 90 obtained were 0.5 and 16.0 µg / mL, respectively. For *Candida albicans*, the MIC range in the standard method was from 0.125 to 8.0 µg / mL, in the rapid method the range was from 0.125 to 4.0 µg / mL. The MIC 50 and MIC 90 were 0.25 µg / mL and 1.0 µg / mL, respectively, in the two methods (Table 2). For the *Candida parapsilosis* complex, the MIC range in the two methods was 0.125 to 1.0 µg / mL, and the MIC 50 and MIC 90 in the standard method were 0.5 µg/ mL, whereas in the rapid method they were 0, 25 µg/ mL and 1.0 µg/ mL, respectively. Approximately 81% of *Candida* spp. were sensitive to Fluconazole (Table 2).

Micafungin

A total of 62 blood culture samples were analyzed by the rapid susceptibility profile method for Micafungin. Of these samples 37.1% were *C. albicans*, 12.9% *C. tropicalis*, 22.6% *C. parapsilosis* complex, 14.5% *C. glabrata*, 4.8% *C. krusei* and 8.1% of others less frequent species.

The general agreement of the standard method with the rapid method was 100% ($p<0.001$) and the Kappa agreement coefficient (46) was 1.0 ($p<0.001$), therefore the method did not show errors.

The minimum inhibitory concentration found for *Candida* spp. ranged from 0.008 to 0.06 µg / mL, and the MIC 50 and MIC 90 obtained were 0.008 and 0.016 µg / mL, respectively, in the two methods. For *Candida albicans*, the MIC range was 0.008 to 0.016 µg/mL, and the MIC 50 and MIC 90 were 0.008 µg/mL, respectively, similar values in the two methods. For *Candida parapsilosis* complex, the MIC range in the two methods was 0.008 to 0.06 µg/ mL, and the MIC 50 and MIC 90 in the standard method were 0.008 µg/ mL and 0.016 µg/ mL, respectively, and in the rapid method were 0.008 µg / mL and 0.03 µg/ mL, respectively (Table 2). All samples of *Candida* spp. interpretables were sensitive to Micafungin.

Discussion

The rapid identification method proved to be very promising with a high overall agreement (92.05%), good confidence values and no incorrect identifications, which demonstrates that the technique is reproducible, reliable and does not lead to incorrect diagnosis and pharmacotherapy targeting. The method for determining the rapid

susceptibility profile had significant categorical agreement and a high kappa agreement coefficient (0.91 ($p<0.001$) for Fluconazole and 1.0 ($p<0.001$) for Micafungin), demonstrating an almost perfect agreement with the standard method (46).

The method with Micafungin showed no errors, whereas with Fluconazole there were two errors (in red in Figure 1). A minor error (1.47%) in which one *C. glabrata* was Sensitive, increasing the exposure (MIC = 16.0µg/mL) in the Standard method and Resistant (MIC = 32.0µg/mL) in the Rapid method. A major error (1.47%) in which one *C. albicans* was Resistant (MIC = 8.0µg/mL) in the Standard Method and Sensitive, increasing exposure (MIC = 4.0 µg/mL) in the Rapid method, which is in accordance with the recommended limits (larger errors ≤ 3% and smaller errors ≤ 10%) (45), respectively.

In the study previously published by our research group (47) a lysis-centrifugation methodology was used, which obtained an identification rate of approximately 52%, in which it was not possible to identify any *C. orthopsilosis*. In the present study, the methodology used was a short incubation culture, this change in methodology led to high identification rates and managed to identify *C. orthopsilosis* within its complex, which substantially improved the test performance. Furthermore, the present methodology is easy to perform and less operator-dependent, as it does not need several centrifugations and washings like the lysis- centrifugation methodology. It is also important to emphasize that the identification by short-incubation culture methodology does not need extra toxic reagents or a powerful centrifuge as in the previous study, making it cheaper, as it does not have any extra material compared to the standard technique. Another technical advantage of this new methodology is the lesser handling of the sample by the operator and the small volume of blood used (less than 0.5mL) compared to the previous one (8mL), which reduces the operator's exposure to biological hazards.

In Table 1, it is possible to observe that the rapid identification method obtained 100% agreement for *C. glabrata* and *C. krusei*, species that have high rates of resistance to Fluconazole and their early identification may have a greater impact on the direction of pharmacotherapy compared to other species (48). In addition, the performance of this method is not suitable for less frequent Candida species (those that are not *C. albicans*, *C. tropicalis*, *C. parapsilosis* complex, *C. glabrata* and *C. krusei*) with only 33.33% agreement. Of the 19 samples that were not identified, 4

belong to this group (2 *C. dubliniesis*, 1 *C. helenica* and 1 *C. rugosa*). Species in this group have a low worldwide prevalence, between 3 to 10%(13,20,49), which makes the database of these species smaller, which may justify the difficulty in identifying these yeasts, as reported in previous studies (47,50,51).

Two samples with *C. albicans* had little sample volume, which led to late positivity and a possible reduction in yeast load, impairing the rapid method. According to Bouza *et al*. (52), the volume of cultured blood is a variable that influences the blood culture yield regardless of the methodology used.

If the aforementioned cases (rare species and samples with low volume) are disregarded, it is possible to obtain an agreement of 94.4%. With this analysis, it is possible to realize that this method is limited by the collection of an inadequate volume of blood and the presence of less frequent species. Even with the limitation of the technique, it obtained excellent agreement rates, as well as high average identification rates.

When the MIC 50 and 90 are analyzed together with the values of the minimum inhibitory concentrations described in Table 2, it is possible to see that when there is a difference in values, it is only one dilution, which is acceptable due to the complexity involved in the microdilution technique in broth, not characterizing an error (42). Furthermore, in the case of *C. glabrata*, antifungal therapy with Fluconazole is discouraged due to its high levels of resistance (40,48), so this disagreement would not affect the patient. When analyzing Table 2, it is possible to observe that the distribution of MIC for Micafungin is restricted to very low values, which could be characterized as a limitation of the technique.

The rapid methodologies of this study are easy to perform, little operator-dependent and low cost, as they do not need any additional material, reagents or equipment and, in the case of identification, the Sabouraud agar used is a classic, inexpensive and easily accessible medium, different from the chromogenic media used in other studies (50,51,53–55). Another difference between this study and the other authors analyzed is that this is the only one in which the MALDI-TOF Vitek MS® 3.0 system (bioMérieux, France) was used and the only one in a large number of *C. orthopsilosis* samples were analyzed and even with its low worldwide prevalence (56,57). This methodology was able to identify this species within the *C. parapsilosis* complex with high concordance rates.

The methodology developed in this study obtained higher identification rates than others previously published by other authors, such as Bellanger and collaborators (53) which obtained 60%, Wang and collaborators 75% (54) and Bidart and collaborators 88.8% (55), these using previously contaminated samples. Idelevich and collaborators 62.5% (58), Vecchione and collaborators 86.3% (51), and Spanu and collaborators 91.82% (50), these using clinical samples.

This study gains strength in relation to other previously published studies, not only for the better performance in fungal identification and for the pioneering in the broth microdilution directly from the blood culture bottles, but also for the low cost of the test, ease of execution, variety of tested species and mainly due to the use of a large number of samples from hospitalized patients and not previously contaminated samples. The performance of tests performed under controlled conditions tend to be different from those performed in routine care in which uncontrolled conditions. In the samples contaminated, the fungal inoculum can be measured, making it a possible bias that does not occur when clinical samples are used.

The methodologies for rapid identifying and determining the susceptibility profile of this study obtained a high agreement, which makes its use promising in the routine of the clinical microbiology laboratory. With this methodology it is possible to obtain the identification result in about 5 hours and the susceptibility profile in 24 hours, processes that can normally last between 24 and 72 hours (35,59).

These methodologies should be replicated in other centers with different candidemia epidemiology to assess their performance in different scenarios. The next big step is to obtain certification from microbiology societies so that the methodology can integrate international guidelines such as EUCAST/CLSI and, thus, make it possible to carry out faster diagnostic tests in candidemia worldwide.

Conclusion

It is possible to conclude that the rapid methodology for identifying and determining the susceptibility profile of *Candida* spp. could be an excellent alternative to the methods currently used in the routine of the microbiology laboratory. In addition to these methodologies are efficient, reproducible, inexpensive, and easy to perform, they are reliable and release of the result early, directly impacting effectiveness of the treatment. With the quick release of the result, the patient receives pharmacotherapy

adjustment earlier, which directly affects a favorable outcome, and there may be a reduction in mortality and length of stay. The work aims to encourage a more active posture of clinical laboratories in the search for methodologies for the most opportune release of results, thus improving early diagnosis and patient management.

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Attachment

Table 1: Performance of *Candida* spp. identification directly from the blood culture bottle.

Standart Identification	**Number of Isolated**			**%Agreement**
	Total Tested	Concordant Identification	Confidence value of identification (%)	
Candida albicans	88	81	98,5	92,01
Candida glabrata	17	17	99,6	100,00
Candida krusei	6	6	99,2	100,00
C. parapsilosis complex	84	78	97,9	92,86
Candida tropicalis	38	36	99,4	94,74
Other less frequent species	6	2	99,9	33,33
Total	239	220	98,6	92,05

Table 2: Distribution of Minimum Inhibitory Concentrations by *Candida* spp. isolate from blood cultures.

Specie/MIC(µg/ml)	Drug	Method	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0
Candida albicans	Fluconazole	Standard					10	4	7	2	1		1			
		Rapid					2	11	5	5	1	1				
	Micafungin	Standard	22	1												
		Rapid	22	1												
Candida glabrata	Fluconazole	Standard								1		1	4	2	1	
		Rapid								1		2		4	1	1
	Micafungin	Standard	8	1												
		Rapid	8	1												
Candida krusei	Fluconazole	Standard											1	2		
		Rapid												1	2	
	Micafungin	Standard	2	1												
		Rapid	1	2												
Candida parapsilosis complex	Fluconazole	Standard					4	1	9	1						
		Rapid					2	7	2	4						
	Micafungin	Standard	11	2		1										
		Rapid	8	4	1	1										
Candida tropicalis	Fluconazole	Standard					2	2	2	3	2					
		Rapid						5		4	2					
	Micafungin	Standard	8													
		Rapid	7	1												
Other less frequent species	Fluconazole	Standard					2	4								
		Rapid					1	3	1							
	Micafungin	Standard	5													
		Rapid	5													

Graph 1: Distribution of the number of *Candida* spp. isolates in blood culture samples per species submitted to the rapid identification test

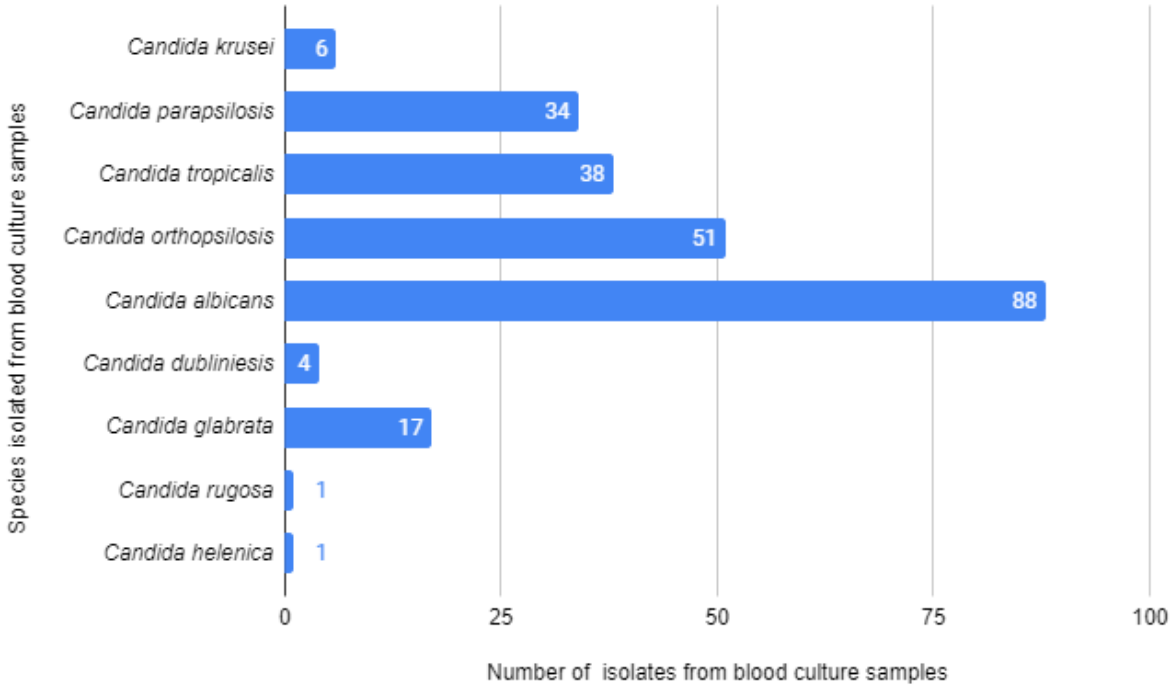
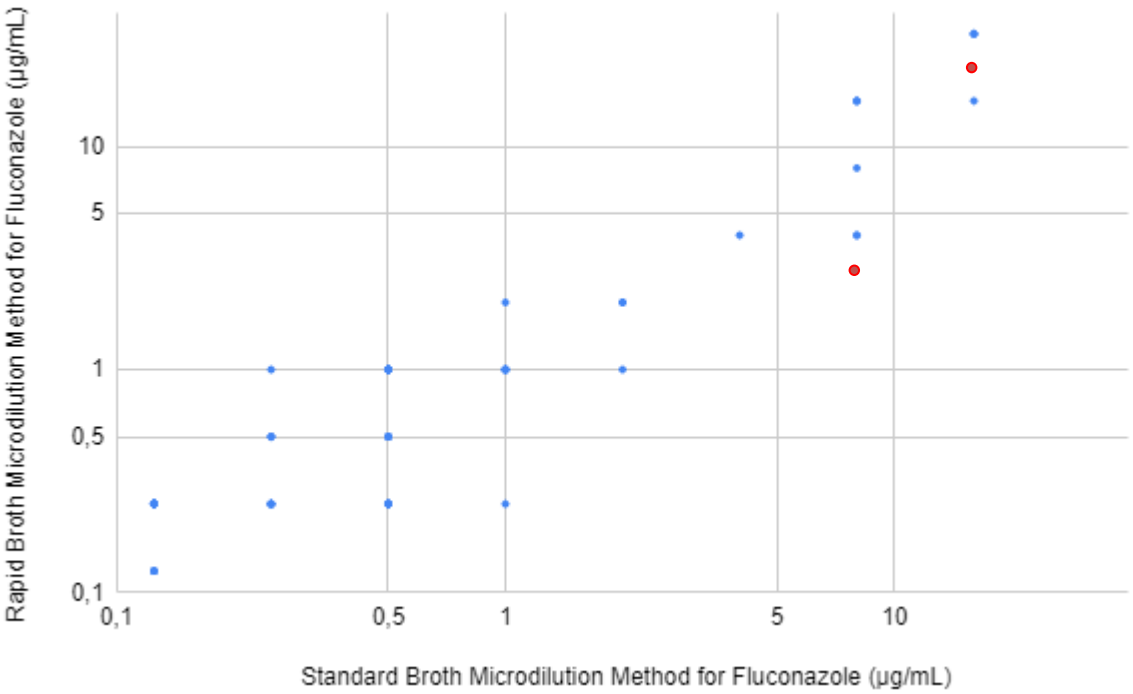


Figure 1: Distribution of susceptibility profiles to Fluconazole according to Standard (colony) and Rapid (bottle) Broth microdilution.



8. CONSIDERAÇÕES FINAIS

Com o desenvolvimento desse trabalho foi possível concluir que a metodologia rápida para identificação e determinação do perfil de sensibilidade de *Candida* spp. é uma ótima alternativa aos métodos utilizados atualmente na rotina do laboratório de microbiologia. Além dessas metodologias serem eficientes, reprodutíveis, baratas e de fácil execução, possibilitam a liberação do resultado de forma precoce impactando diretamente na segurança do paciente. Com a agilidade na liberação do resultado, o paciente recebe adequação da farmacoterapia com maior antecedência, o que impacta diretamente em um desfecho favorável, podendo haver redução da mortalidade e do tempo de internação. O trabalho visa encorajar a postura mais ativa dos laboratórios clínicos na busca de metodologias para a liberação mais oportuna de resultados, melhorando assim o diagnóstico precoce e o manejo do paciente.

9. PERSPECTIVAS FUTURAS

As metodologias desenvolvidas no presente estudo necessitam ser reproduzidas em outros centros com epidemiologias de candidemia diferentes para ampliar a variabilidade na distribuição de concentrações inibitórias mínimas e espécies identificadas. O próximo grande passo é a obtenção da certificação junto às sociedades de microbiologia para que as metodologias possam integrar diretrizes nacionais como EUCAST e CLSI e, assim, seja possível viabilizar testes diagnósticos mais rápidos em candidemia mundialmente.

10. ANEXO

MICRO framework - A checklist of items that should be addressed in reports of studies involving human clinical microbiology data

[Core “must include” items are indicated by an asterisk]

Section	Item No	Recommendation	Completed Yes / No / NA	Page No
Methods				
Study design	1*	**Specimen types**: Describe the types of specimen included, i.e. clinical (e.g. blood cultures) or non-diagnostic surveillance (e.g. admission and other screening swabs to diagnose carriage). If specimens were obtained for diagnostic reasons, clinical syndromes should be described where possible, and specimens / isolates stratified by clinical syndrome.	**Yes**	**32**
	2*	**Sampling period:** State the collection timeframe for specimens yielding isolates for which data is reported, e.g. from MM/YY to MM/YY to be able to identify variability between seasons.	**Yes**	**32**
	3*	**Sampling strategy:** Describe the strategy for specimen collection, e.g. asymptomatic screening, sampling of all febrile patients, sampling at clinician discretion, sampling of specific patient groups, convenience sampling (e.g. use of isolates from an existing sample repository). Specify whether sampling followed routine clinical practice or was protocol driven. Classify specimens as from community-acquired (CAI) or hospital-acquired (HAI) infections. The definition of HAI used (e.g. HAI defined by specimen collection >48h after hospital admission) should be provided and should use ideally an international standard (e.g. US-Centers for Disease Control (1, 2)).	**Yes**	**32**
	4	**Target organisms:** Explicitly state which organisms / organism groups were included in the report. Nomenclature should follow international standards (i.e. using approved genus / species names as summarised in the International Journal of Systematic and Evolutionary Microbiology). Lists of approved bacterial names can be downloaded from Prokaryotic Nomenclature Up-to-Date and the List of Prokaryotic Names with Standing in Nomenclature. Organisms considered contaminants should be listed, if appropriate (e.g. coagulase negative staphylococci or *Corynebacterium* spp. (3, 4)).	**Yes**	**32**
Setting	5*	**Geographical setting:** Describe the geographical distribution of specimens / patients from which isolates were obtained; at least to a country level, but preferably to a sub-national level or a geoposition.	**Yes**	**32**
	6*	**Clinical setting:** Describe the type and level of the healthcare facilities (e.g. primary, secondary, tertiary) from which specimens were obtained. If stating a microbiology laboratory, the centres served by the laboratory should be specified.	**Yes**	**32**

Laboratory work	7	**Specimen processing:** If applicable, describe specimen collection and handling, processing and sub-culture methods for all types of specimen included. For example, if reporting AST results for blood culture and cerebrospinal fluid culture isolates, the processing of these specimens by the laboratory should be briefly explained, including how specimens are sub-cultured, the media used, incubation conditions and duration. A summary of specimen processing steps (e.g. pre-processing steps, nucleic acid extraction method (if applicable), amplification platform, contamination avoidance strategy) should be provided for molecular-only workflows (e.g. to detect *Mycobacterium tuberculosis* and rifampicin resistance using the Cepheid Xpert MTB / RIF system).	**Yes**	**32-33**
	8*	**Target organism identification:** Details of identification methodology should be reported briefly. Where identification databases were used (e.g. bioMerieux API / bioMerieux VITEK-MS / Bruker Biotyper), the version should be specified. In general, all pathogens should be identified to species level. In the case of *Salmonella* species, organisms should be identified to at least the *S.* Typhi, *S.* Paratyphi, or non-typhoidal salmonella (NTS) level. Strain subtyping methods should be reported according to STROME-ID (5).	**Yes**	**32-33**
	9*	**Antimicrobial susceptibility testing:** Describe the antimicrobial susceptibility testing methods used, internal quality control processes, and their interpretation, with reference to a recognised international standard – e.g. CLSI, EUCAST. Where an international standard was followed, the specific edition(s) of guidelines used should be referenced. Deviations from standard methodology should be described, along with evidence of validation. Handling of any changes to interpretative criteria during the sampling period should be documented. State whether the raw AST data (zone diameters and / or minimum inhibitory concentrations) were re-categorised with updated breakpoints or left as-is.	**Yes**	**32-33**
	10	**Additional tests performed to identify resistance mechanisms:** Describe the testing methods used for adjunctive / confirmatory antimicrobial susceptibility tests, such as enzymatic / molecular assays (e.g. Xpert MTB / RIF, mecA PCR) and inducible resistance assays, with reference to a recognised international standard, where available. Where an international standard was followed, the specific edition of guidelines used should be referenced. Deviations from standard methodology should be described, along with evidence of validation.	**NA**	
	11*	**Antimicrobial resistance definitions:** Define resistance for each antimicrobial class (i.e. are isolates in the "intermediate" category included within "susceptible" or "resistant" or analysed as a distinct category). If using the term, define MDR (e.g. ≥1 agent in ≥3 classes tested). For each organism type, an MDR test panel must be defined, consisting of the minimum panel of individual antimicrobial agents / classes against which an isolate must be tested for that isolate to be considered tested for MDR status. Antimicrobials to which an organism is intrinsically resistant	**Yes**	**33**

		cannot be part of the test panel or contribute to MDR status (6, 7).		
Quality assurance	12*	**External quality assurance:** State whether the microbiology laboratory participates in an external quality control programme and, if so, provide scheme details. Examples include the UK National External Quality Assurance Scheme and the American College of Pathologists External Quality Assurance / Proficiency Testing Program.	**Yes**	**33**
	13	**Accreditation:** State whether the laboratory is accredited through a national or international body (e.g. the International Standards Organisation, ISO) and specify which assays are covered in the accreditation.	**NA**	
Bias	14*	**Duplicate and sequential isolates:** The strategy for accounting for duplicate and sequential isolates from the same patient should be clearly detailed. Duplicate isolates are multiple isolates of the same phenotypic organism (i.e. same species and same resistance profile) from the same patient on the same date cultured either from the same clinical specimen, or from two separate clinical specimens, such as blood and CSF. Sequential isolates are isolates of the same phenotypic organism from the same patient at different dates, such as blood cultures taken on different dates. Various strategies for the handling of duplicate and sequential isolates exist (8), and the strategy used should be transparent as it will bias pooled resistance results. For example, inclusion of all isolates (the 'all isolate strategy'), has been shown to shift pooled resistance proportions toward greater resistance, whilst inclusion of only the first isolate per patient (the 'first isolate strategy') or only the first isolate per infection episode (the 'episode-based strategy') will shift pooled results towards susceptibility.	**Yes**	**32**
Results				
	15*	**Population:** Describe the demographics of the population from which clinical specimens and subsequent isolates have been obtained, disaggregating age and gender data.	**Yes**	**32**
	16*	**Denominators:** Patient and isolate denominators should be used appropriately to ensure clarity regarding the numbers included in each analysis. Of particular importance is the reporting of resistance where first- and second-line AST panels were used (i.e. not all isolates of a particular species were tested against all agents). For drugs where only a subset of isolates were tested, reporting of a percentage without the numbers of isolates tested / resistant may be highly misleading.	**Yes**	**34-35**
	17	**Site / place of acquisition**: AST data from CAI and HAI should be reported and analysed separately.	**NA**	
	18*	**Reporting resistance proportions for single agent and class resistance:** Proportions of resistant isolates should be reported as number of isolates susceptible or resistant to a given antimicrobial agent / class out of actual number of isolates tested for susceptibility to that agent / class.	**Yes**	**34-35**
	19	**Reporting multidrug resistance proportions:** If defined, the proportion of MDR isolates should be expressed as the number of MDR isolates out of the number of isolates tested (i.e. the number undergoing the MDR test panel	**NA**	

		specific to that organism). Single agent / class resistance should be always be reported, regardless of MDR reporting.		
Discussion				
Limitations	20	Discuss any reasons why bias may have been introduced into the reported data, due to patient / specimen selection, isolation of organisms, or otherwise. Consider factors which may have either introduced bias into the types of organisms isolated or the antimicrobial susceptibility profiles, e.g. receipt of antimicrobials prior to specimen collection will reduce the yield of certain species and also select for more resistant organisms.	Yes	36-38

Note

It is recommended that this checklist is used in conjunction with the original article (9), available on the Web site of BMC Medicine at https://bmcmedicine.biomedcentral.com/articles/10.1186/s12916-019-1301-1 (DOI: https://doi.org/10.1186/s12916-019-1301-1). The checklist is reproduced from this article under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0; http://creativecommons.org/licenses/by/4.0/).

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