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Dual one-step recombinase-aided PCR for rapid detection of *Candida* in blood

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Abstract

This study presents a novel dual one-step recombinase-aided PCR (DO-RAP) method, combined with recombinant human mannan-binding lectin protein (rhMBL; i.e., M1 protein)-conjugated magnetic bead (M1 bead) enrichment, for the early detection of *Candida krusei* and *Candida parapsilosis* bloodstream infections. Unlike previous studies that utilized the characteristic of docosane being solid at room temperature and melting above 44 °C as an impermeable barrier to separate two reaction steps, DO-RAP simplifies the process by eliminating this step. Specificity tests with the genomic DNAs from 11 bacterial strains and 3 fungi related to bloodstream infections (BSIs) confirmed no cross-reactivity, while sensitivity analysis demonstrated detection limits of 1 copy/μL for recombinant plasmids containing 26S ribosomal RNA gene fragment from *C. krusei* and NADH5 mitochondrial gene fragment from *C. parapsilosis* and 10^{-7} ng/μL for DNAs from standard strains of *C. krusei* and *C. parapsilosis*. In simulated infection samples enriched with M1 beads, DO-RAP achieved detection thresholds of 1 CFU/mL (colony-forming unit per milliliter) in simulated samples within 3.5 h, surpassing quantitative PCR (qPCR) performance, which has detection limits of 3–5 CFU/mL. Clinical validation showed strong agreement between DO-RAP and qPCR, with Kappa values of 0.936 for *C. krusei* and 0.904 for *C. parapsilosis* ($P < 0.05$). This integrated approach improves detection speed and sensitivity, eliminates the need for culturing, and offers a more efficient alternative to qPCR for diagnosing invasive *Candida* infections.

Key points

- *The DO-RAP method achieves a detection sensitivity of 1 CFU/mL, surpassing conventional qPCR.*
- *This approach eliminates the need for docosane, streamlining operations, and accelerating detection.*
- *M1 magnetic bead enrichment enhances pathogen capture, facilitating rapid Candida diagnosis.*

Keywords *Candida krusei* · *Candida parapsilosis* · Dual one-step recombinase-aided PCR detection · M1 magnetic bead

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Introduction

Bloodstream infections (BSIs) have been associated with significant morbidity and mortality, and early effective antimicrobial therapy has been demonstrated to improve patient outcomes (Peri et al. 2022). In recent years, opportunistic yeast infections in humans have gained prominence, posing significant diagnostic and therapeutic challenges due to the diversity and abundance of pathogenic organisms (García-Salazar et al. 2022). *Candida* species, the most prevalent fungal pathogens affecting humans, manifest as both superficial infections and life-threatening invasive infections, particularly in immunocompromised individuals (Cao et al. 2023). These infections, ranging from superficial to systemic, are predominantly caused by opportunistic yeasts with a broad infection spectrum and often lethal outcomes (Deurenberg et al. 2017). Among them, the mortality rate associated with various *Candida* species infections ranges from 35 to 80%, resulting in approximately 1.5 million deaths worldwide annually (Lass-Flörl and Steixner 2023). The primary risk factors for candidemia encompass central venous catheters (CVC) (84.38%), surgical history (56.88%), and invasive mechanical ventilation (42.50%). Among the identified species, *Candida albicans* accounts for 43.53% of cases, followed by *Candida parapsilosis* (31.76%), *Candida glabrata* (12.36%), *Candida krusei* (5.29%), *Candida tropicalis* (2.35%), and *Candida lusitaniae* (2.35%) (Mareković et al. 2021).

Current molecular detection methods for candidemia principally encompass PCR, T2 *Candida* panel (Zervou et al. 2017), and sequencing technologies (Fang et al. 2023). While these techniques have been shown to reduce detection time, they have not been adopted more broadly due to limitations in sensitivity or cost (Gudisa et al. 2024).

Our research group has previously developed a recombinase-aided amplification (RAA) combined with quantitative PCR (qPCR) method. In this method, the recombinase, supplied by the RAA kit (Amp-Future, Amp-Future Biotech Co., Ltd., Changzhou, Jiangsu, China), is RecA from *Escherichia coli* and plays a key role in DNA strand exchange and homologous recombination (Chen et al. 2018). The recombinase facilitates primer invasion and strand displacement, enabling efficient isothermal amplification and enhancing both amplification efficiency and sensitivity. The method is termed recombinase-aided polymerase chain reaction (RAP), as well as multiplex recombinase-aided PCR (M-RAP) for rapid and highly sensitive detection of various respiratory viruses (Fan et al. 2021, 2023). Furthermore, M1 protein (i.e., rhMBL; recombinant human mannan-binding lectin protein) is a recombinant protein that is expressed through genetic engineering. The carbohydrate recognition domain (CRD) of human mannose-binding lectin (MBL) facilitates binding to mannose and *N*-acetylglucosamine residues on the surface of various microorganisms, including *Candida* species. It has demonstrated robust in vitro binding capabilities towards multiple pathogens (Chen et al. 2020). The M1 method, a technique based on recombinant MBL magnetic bead enrichment, has been shown to enhance the detection efficiency of pathogens including bacteria and fungi in immunocompromised patients (Xiao-Ping et al. 2024).

However, previous methods employed docosane, a white crystalline solid with a melting point of 44 °C, to separate the RAA and qPCR stages (Zhao et al. 2024). This substance functions as an impermeable barrier, melting above 44 °C and solidifying at room temperature, without affecting DNA amplification, but rendering the procedure relatively cumbersome. In the present study, we sought to eliminate the use of docosane by establishing a dual one-step recombinase-aided PCR (DO-RAP) method. This approach was then combined with M1 magnetic beads enrichment for the detection of *C. krusei* and *C. parapsilosis* in patients with bloodstream infections. Other members of our research group are conducting parallel studies on *C. albicans, C. tropicalis*, and *C. glabrata* using the same method as in this study. Preliminary results have shown sensitivities of 2, 2, and 1 CFU/mL for these species, demonstrating the feasibility of the proposed method. Therefore, this study focuses on *C. parapsilosis* and *C. krusei*, which are also closely related to bloodstream infections. The clinical performance of this method was evaluated by comparing the results with those obtained using qPCR.

Materials and methods

Sample collection and genomic DNA isolation

Standard strains of *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), and the M1 protein were provided by the Infectious Disease Institute, China CDC, Beijing, China. Plasma for simulated sample preparation was obtained from Hebei General Hospital. Whole blood from healthy volunteers was collected with informed consent, anticoagulated with heparin, and centrifuged to isolate plasma, which was stored at − 80 °C. The genomic DNA was extracted from the standard strains of *C. krusei* and *C. parapsilosis* using the FastPure® Microbiome DNA Isolation Kit (Vazyme, Nanjing Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China) and stored at − 80 °C. A total of 55 blood clinical specimens from Hebei General Hospital were used to validate the performance of the DO-RAP. The specimens included 10 *C.*

krusei-positive blood culture specimens, 15 *C. parapsilosis*-positive blood culture specimens, and 30 blood culture-negative specimens.

Design of DO-RAP primers, probes, and plasmid construction

Primers and probes targeting the 26S ribosomal RNA gene in *C. krusei* (U767347.1) and the NADH5 mitochondrial gene in *C. parapsilosis* (NC_005253.2) were designed based on published literature (Brinkman et al. 2003; Vahidnia et al. 2015), in which the species-specific detection assays for nearly all known ascomycetous yeast species were developed (Brinkman et al. 2003). The RAA primers for the DO-RAP assay were designed to be 30–35 base pair (bp) in length to optimize nucleic acid-protein complex formation between the recombinase and primers, avoiding shorter primers that could hinder this process or longer primers that may promote stable primer-dimer structures. The probe for the DO-RAP assay was identical to that used in the qPCR assay. Primer design was analyzed and optimized using AmplifX (v1.7.0, CNRS, Aix-Marseille University, France. Available at: https://inp.univ-amu.fr/en/amplifx) and Oligo7 (v7.56, Molecular Biology Insights, Inc., Cascade, CO, USA) software, considering factors such as GC content, primer dimers, hairpin structures, and tertiary formations. The primers and probes were analyzed in silico to ensure specificity and to confirm the absence of cross-reactivity. Full-length sequences of *C. krusei* and *C. parapsilosis* were retrieved from NCBI, and alignments were performed using Vector NTI (v11.5.1, Invitrogen, Carlsbad, CA, USA) to identify optimal locations for the DO-RAP primers and probes. All primers and probes were synthesized by Sangon Biotech (Shanghai, China) and the sequence details are shown in Table 1. A 290 bp fragment of the conserved 26S ribosomal RNA gene from *C. krusei* and a 334 bp fragment of the conserved NADH5 mitochondrial gene from *C. parapsilosis* were inserted into the pUC57 vector (TsingKe Biotech Corp., Beijing, China) to construct recombinant plasmids. These plasmids were synthesized and provided by TsingKe Biotech Corp. (Beijing, China). The quantity of plasmid DNA was determined using the Qubit dsDNA BR/HR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit 2.0 fluorometer (Life Technologies, Waltham, MA, USA). Plasmid copy numbers were calculated using the formula: DNA copy number (copies/μL) = {[plasmid concentration (ng/μL) × $6.02 \times 10^{23} \times 10^{-9}$]}/(DNA length × 660). The recombinant plasmids were diluted in tenfold serial concentrations ranging from 10^5 to 10^0 copies/μL in 1 × TE buffer and stored at − 80 °C until further use.

Establishment and optimization of the DO-RAP method

The optimized DO-RAP reaction system consisted of 10 μL of reaction buffer and RAA enzyme mixture (Amp-Future, Amp-Future Biotech Co., Ltd., Changzhou, Jiangsu, China), 1 μL (2.5 U) of Taq DNA polymerase (Nagene Diagnosis, Beijing, China), 0.4 μL each of forward and reverse primers (10 μM), 0.2 μL each of fluorescent probes (10 μM), 0.4 μL of single-stranded DNA-binding protein (Yugong Biotech, Yugong Biolabs Co., Ltd., Jiangsu, China) (500 μg/mL), 0.4 μL of betaine (Sigma-Aldrich, St. Louis, MO, USA) (5 M), 7.2 μL of nuclease-free water, 2 μL of 100 mM magnesium ions (added to the cap of the 8-strip tube, equivalent to 8 mM magnesium), and 2 μL of recombinant plasmids or extracted genomic DNA, making a total volume up to 25 μL. The subsequent amplification process was conducted within an Archimed X6 fluorescence qPCR instrument (manufacturer: Kunpeng Gene Technology Co., Ltd., Beijing, China). The reaction program was configured as follows: an initial cycle at 40 °C for 10 min, followed by 35 cycles of 95 °C for 15 s

Table 1 The DO-RAP primer and probe sequences

Species	Type	Sequence	Source
C. krusei	CK-DO-RAP-F	ATCAAATAAGCGGAGGAAAAGAAACCAACA	This paper
	CK-DO-RAP-R	CAAGGGACTTGGACACCGCCTTCCACACAG	This paper
	CK-qPCR-F	GCTCAGATTTGAAATCGTGCTTT	Brinkman et al. (2003)
	CK-qPCR-R	GGGGCTCTCACCCTCCTG	Brinkman et al. (2003)
	CK-P[a]	FAM-CGGCACGAGTTGTAGATTGCACC-BHQ1	Brinkman et al. (2003)
C. parapsilosis	CP-DO-RAP-F	GCTAAAAGTGCTCAATTAGGTTTACATGCT	This paper
	CP-DO-RAP-R	ATCTAACTAACACATAAACTCCGGCACTTT	This paper
	CP-qPCR-F	GCTACTGCTAAAAGTGCTCAATT	Brinkman et al. (2003)
	CP-qPCR-R	ACATATACTCCAGCACATACCATAG	Vahidnia et al. (2015)
	CP-P[b]	VIC- TCCTACTCCTGTTAGTGCTTTATTACATGCT -BHQ1	Vahidnia et al. (2015)

[a]Probe modifications: *FAM* 6-carboxyfuorescein, [b]probe modifications: *VIC* 6-phosphoramidite, *BHQ*1 black hole quencher, *qPCR* quantitative polymerase chain reaction, *DO-RAP* dual one-step recombinase-aided polymerase chain reaction

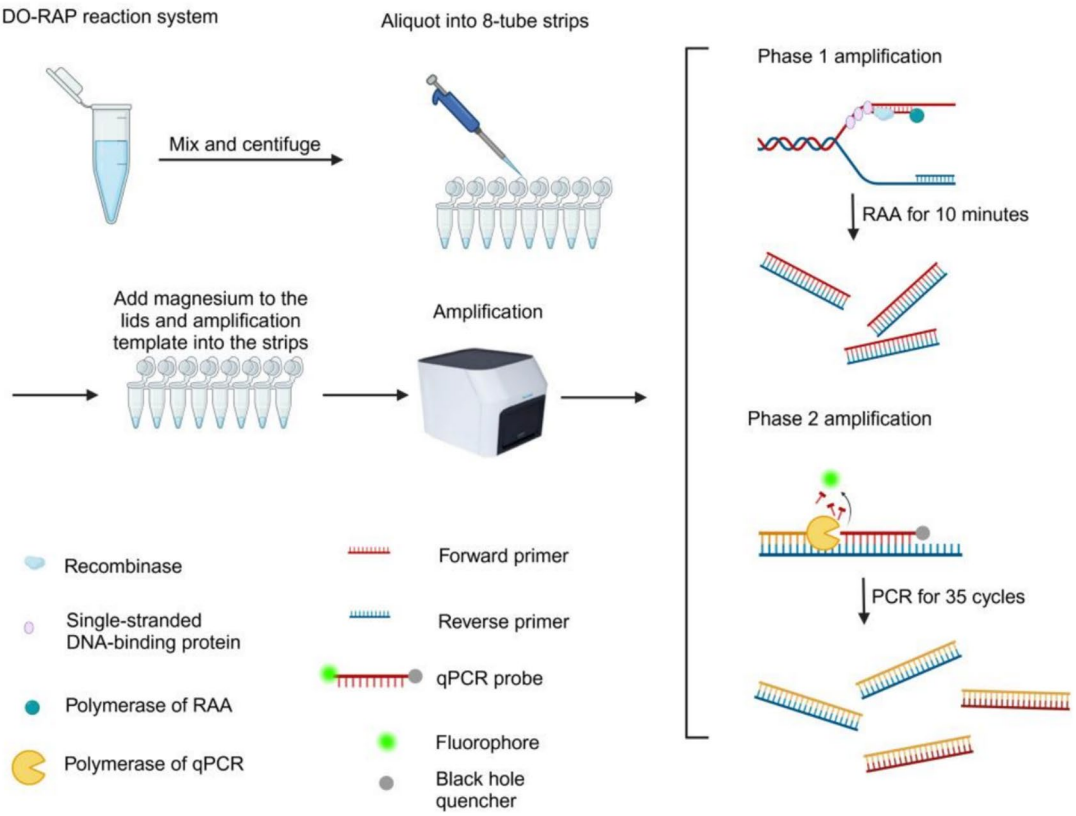

Fig. 1 The detection principle of DO-RAP

and 60 °C for 40 s with concurrent fluorescence acquisition. The principle of the reaction is shown in Fig. 1. To optimize this DO-RAP method, we evaluated various magnesium ion concentrations, qPCR annealing duration, and RAA reaction duration, using a recombinant plasmid template diluted to 10^2 copies/μL.

Sensitivity, reproducibility, and specificity of the DO-RAP method

The plasmids of *C. krusei* and *C. parapsilosis* were serially diluted in tenfold increments, ranging from 10^0 to 10^5 copies/μL. These diluted plasmids served as templates in the DO-RAP assay, with DEPC-treated water (DEPC, diethylpyrocarbonate, a reagent used to degrade RNases) employed as a negative control. Furthermore, genomic DNA from both *C. krusei* and *C. parapsilosis* was extracted in accordance with the guidelines provided by the FastPure® Microbiome DNA Isolation Kit (Vazyme, Nanjing Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China), the extracted DNA was subsequently diluted to concentrations ranging from 10^{-7} to 10^{-3} ng/μL and subjected to the aforementioned DO-RAP assay. To assess reproducibility, the DO-RAP sensitivity tests were repeated eight times at various intervals.

Furthermore, to evaluate the specificity of the method, DNA of various microorganisms closely associated with BSIs, including *C. albicans*, *C. tropicalis*, *C. glabrata*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*, were then evaluated in parallel with the DO-RAP assay, which was designed to target *C. krusei* and *C. parapsilosis*. These strains are listed in Supplementary Table 1. This comprehensive approach enables a thorough assessment of the method's specificity.

Sensitivity of quantitative polymerase chain reaction (qPCR)

In order to assess and compare the sensitivity of the DO-RAP assay with that of standalone qPCR, we conducted qPCR analysis on recombinant plasmids derived from *C. krusei* and *C. parapsilosis*. The qPCR procedure was executed in strict adherence to the manufacturer's instructions for the qPCR probe set (Entrans qPCR Probe Set V2, ABclonal, Wuhan, China). The amplification protocol encompassed an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min.

Preparation of simulated samples and M1 magnetic beads

The standard strains of *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC22019) were cultivated on Sabouraud agar plates (Hopebio, Qingdao Hopebio Biotechnology Co., Ltd., Qingdao, Shandong, China) and subsequently incubated in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for a period of 48 h. A single colony was isolated and propagated in yeast extract peptone dextrose medium (Hopebio, Qingdao Hopebio Biotechnology Co., Ltd., Qingdao, Shandong, China) by overnight shaking at 220 rpm in a shaking incubator (THZ-032, Shanghai Boqi Biological Technology Co., Ltd., Shanghai, China) at 37 °C. Subsequently, 1 mL of the culture was subjected to centrifugation, the resultant pellet was resuspended in 1 mL of phosphate-buffered saline (PBS) (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), and the pellet was then discarded. This PBS washing procedure was repeated twice. The concentration of the bacterial suspension was determined using a hemocytometer.

Simulated plasma samples were generated by introducing < 2, 4, 6, 8, 10, 50, 100, 300, 500, 700, and 900 colony-forming units (CFU) of the bacterial suspension into 1 mL of sterilized human plasma (final concentrations: < 2, 4, 6, 8, 10, 50, 100, 300, 500, 700, and 900 CFU/mL). In a similar manner, simulated PBS samples were prepared by mixing the bacterial suspension with 1 mL of PBS. These samples were then utilized to enumerate colonies on Sabouraud agar plates.

The preparation of M1 magnetic beads entailed the conjugation of 1 mg of protein A magnetic beads (Biocanal, GenScript Biotech Corporation, Nanjing, Jiangsu, China) with 1 mL of PBS. Protein A, a bacterial cell wall protein derived from *S. aureus*, specifically binds to the Fc region of immunoglobulin G (IgG), thereby enabling the immobilization of M1 protein on the magnetic beads. The mixture was then subjected to vortexing and subsequently separated on a magnetic stand for a period of 3 min. This was followed by the removal of the upper layer of the mixture. This process was repeated twice. The beads were then detached from the magnetic stand and resuspended in 1 mL of PBS. Subsequently, 232 μg of M1 protein was added, and the mixture was agitated for 30 min using a suspension mixer. Finally, the mixture was separated on a magnetic stand for 3 min, the upper layer was discarded, and the beads were resuspended in 100 μL of PBS. The prepared M1 magnetic beads can be stored at 4 °C for a maximum of two weeks.

Capture of Candida and genomic DNA extraction

Simulated plasma samples containing 2, 4, 6, 8, and 10 CFU were aliquoted into 14-mL round-bottomed tubes, each supplemented with 1 mg of M1 magnetic beads. The volume was adjusted to 10 mL with PBS, followed by the addition of $CaCl_2$ (Yuanye, Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) to a final concentration of 4 mMol/L. After thorough mixing for 1 h, magnetic separation was performed using a magnetic stand. The supernatant was discarded, and the system was washed three times with PBS to remove unbound plasma components and pathogens. The sample was then resuspended in 400 μL of PBS. Similarly, simulated PBS samples were enriched with 2, 4, 6, 8, and 10 CFU using the same method, and colony counts were obtained after overnight incubation on plates. DNA extraction from both unenriched simulated plasma samples and M1-enriched samples was performed using a DNA extraction kit (Vazyme Biotech, Nanjing Vazyme Biotech Co., Ltd., Nanjing, China). The extracted DNA was used as a template for DO-RAP and qPCR.

Evaluation of DO-RAP combined with M1 magnetic bead enrichment using simulated samples

In this experiment, genomic DNA was extracted from varying concentrations of M1-enriched and non-enriched simulated plasma samples of *C. krusei* and *C. parapsilosis*. Extracted DNA was then analyzed using DO-RAP and qPCR. This experiment was replicated three times to ensure the reliability of the results.

Evaluation and comparison of DO-RAP and qPCR assays for clinical specimen

A total of 25 clinical specimens with positive blood cultures were collected from Hebei General Hospital, including 10 specimens of *C. krusei* and 15 specimens of *C. parapsilosis.* Additionally, 30 control specimens with negative blood cultures were included. Following the extraction of genomic DNA, both the DO-RAP assay and qPCR were performed on the specimens. The consistency between the two detection methods was then compared to assess the clinical performance of the DO-RAP assay.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (v25.0, IBM Corp., Armonk, NY, USA). Kappa analysis was utilized to evaluate the consistency between the two methods, with statistical significance set at $P < 0.05$.

Results

Optimization of magnesium ion concentration

The concentration of magnesium ions in the RAA reaction mixture is different from that in the qPCR reaction mixture. Consequently, it became imperative to ascertain the optimum magnesium ion concentration for the DO-RAP reaction, ensuring the efficient progression of both reactions. The experimental results (Supplementary Fig. 1) indicated that a magnesium ions concentration of 8 mM was determined to be optimal for the complete optimization of both RAA and qPCR reactions of the DO-RAP reaction.

Optimization of PCR annealing time

The duration of qPCR annealing is a critical determinant of the effectiveness of the qPCR stage in the DO-RAP reaction. Experimental data (Supplementary Fig. 2) suggested that annealing times of 40 and 50 s exhibited superiority over a 30-s annealing period in terms of efficiency. Given the comparable amplification outcomes observed at 40 and 50 s, a 40-s annealing time was selected for the qPCR stage of the DO-RAP, balancing detection requirements with the minimization of overall reaction time.

Optimization of RAA reaction time

Extended RAA reaction times have been demonstrated to compromise detection sensitivity (Fan et al. 2021). To address this, the RAA reaction time was fine-tuned to a range of 8 to 14 min, aiming to identify the most efficient duration for the initial RAA stage. An analysis of the experimental outcomes (Supplementary Fig. 3) revealed that a reaction time of 10 min was not only more efficient but also contributed to a reduction in the overall reaction duration.

Evaluation of the sensitivity and reproducibility of DO-RAP

In order to evaluate the sensitivity and repeatability of the DO-RAP methods, recombinant plasmids of *C. krusei* and *C. parapsilosis* with concentrations ranging from 10^0 to 10^3 copies/μL and the diluted genomic DNA of the standard strain with concentrations ranging from 10^{-7} to 10^{-3} ng/μL were utilized to assess the sensitivity of the DO-RAP method. DEPC water was utilized as the negative control for each test. As illustrated in Fig. 2, the results showed that when utilizing recombinant plasmids as templates, the sensitivity was both 10^0 copy/μL (Fig. 2a, 2b). When genomic DNA was employed as the template, both exhibited a sensitivity of 10^{-7} ng/μL (Fig. 2c, 2d).

To assess the reproducibility of the DO-RAP, sensitivity tests were performed eight times at various time points

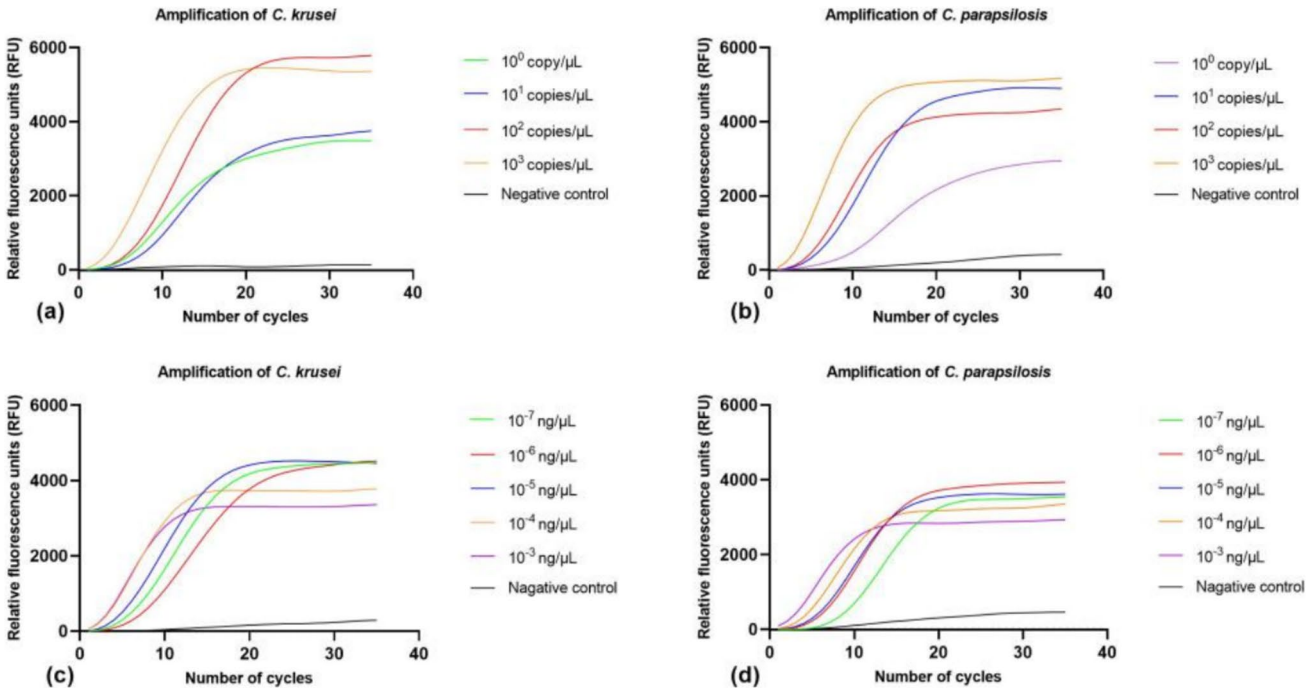

Fig. 2 DO-RAP sensitivity evaluation. The sensitivity of detecting C. krusei and C. parapsilosis was evaluated using two different templates: plasmid in (**a**) and (**b**) (10 0 copy/μL for both species), and genomic DNA of standard strains in (**c**) and (**d**) (10 − ^{7} ng/μL for both species)

using serial tenfold dilutions of recombinant plasmids, and the results were meticulously recorded. The results indicated that the DO-RAP method could consistently detect 10^0 copy/μL (Supplementary Table 2).

Assessment of the specificity of DO-RAP

The DO-RAP methodology developed in this study exhibits a high degree of specificity for the concurrent detection of *C. krusei* and *C. parapsilosis*, without any cross-reactivity with the genomic DNA of other microbial pathogens as depicted in Supplementary Fig. 4. The associated strains utilized in this study are documented in Supplementary Table 1.

Sensitivity evaluation of qPCR detection

A serial tenfold dilution series of plasmids derived from *C. krusei* and *C. parapsilosis*, with concentrations ranging from 10^0 to 10^5 copies/μL, was subjected to qPCR analysis to determine sensitivity. As illustrated in Supplementary Fig. 5a and 5b, the sensitivity was both 10 copies/μL. Furthermore, genomic DNA extracted from these two *Candida* species was diluted to concentrations ranging from 10^{-7} to 10^{-3} ng/μL and subjected to qPCR analysis, with the results presented in Supplementary Fig. 5c and 5d, the sensitivity of *C. krusei* and *C. parapsilosis* was 10^{-5} ng/μL and 10^{-4} ng/μL, respectively.

Comparative analysis of DO-RAP and qPCR before and after simulated sample enrichment

Genomic DNA extraction was performed on simulated samples of both *Candida* species, with concentrations ranging from 100 to 900 CFU/mL, both before and after enrichment with M1 magnetic beads. Subsequent analysis of these samples was conducted using both dual qPCR and DO-RAP. The results are depicted in Fig. 3. Prior to enrichment, the limit of detection (LOD) for DO-RAP was determined to be 100 CFU/mL for *C. krusei* and 50–100 CFU/mL for *C. parapsilosis*. Conversely, the LOD for qPCR was 300 CFU/mL for *C. krusei* and 500 CFU/mL for *C. parapsilosis*.

For simulated samples with concentrations below 10 CFU/mL, after enrichment, the LOD for DO-RAP was improved to 1 CFU/mL for both *C. krusei* and *C. parapsilosis,* while the LOD for qPCR was 3 CFU/mL for *C. krusei* and 5 CFU/mL for *C. parapsilosis* (shown in Fig. 4). The corresponding colony counts from plate cultures are provided in Supplementary Fig. 6.

Comparative evaluation of DO-RAP and qPCR assays for clinical samples

Genomic DNA extracted from clinical specimens was analyzed using both DO-RAP and qPCR, and the results were compared (Table 2). The kappa values obtained were 0.936

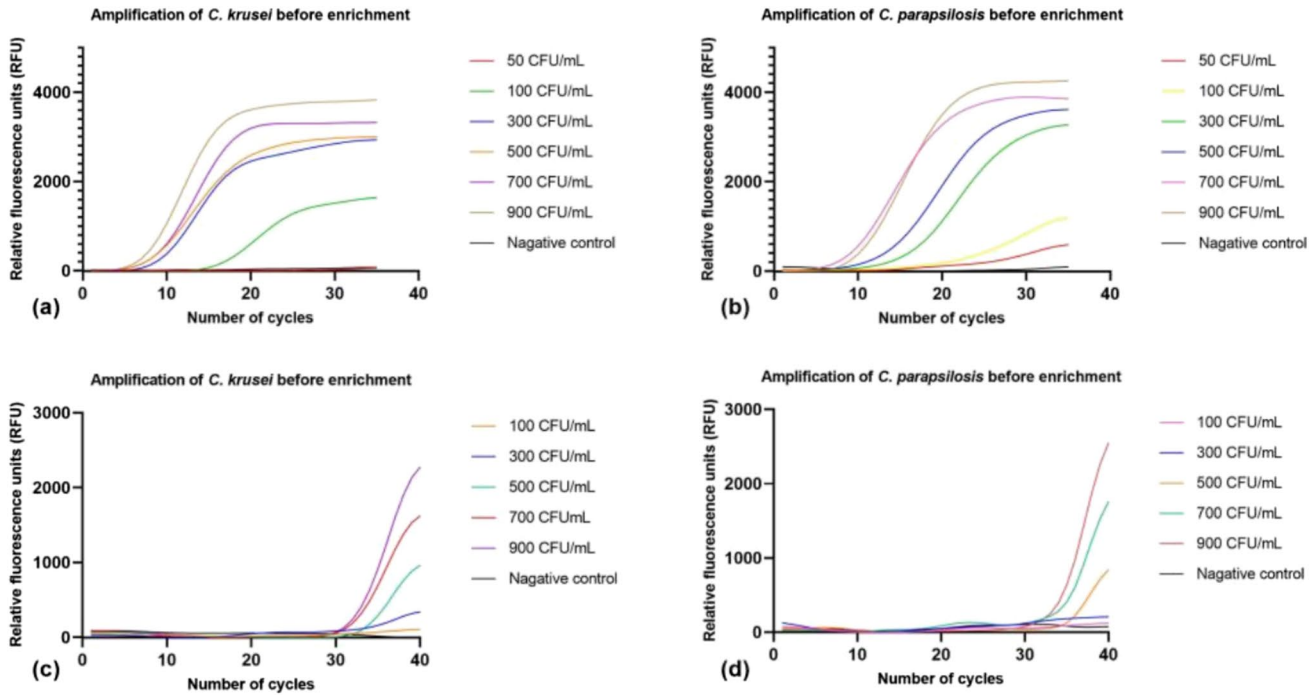

Fig. 3 Detection limits of the two methods before M1 magnetic-bead enrichment *C. krusei* using DO-RAP, with a LOD of 100 CFU/mL (**a**) and qPCR with a LOD of 300 CFU/mL (**b**) before enrichment; *C. parapsilosis* using DO-RAP, with a LOD of 50–100 CFU/mL (**c**) and qPCR with a LOD of 500 CFU/mL (**d**) before enrichment

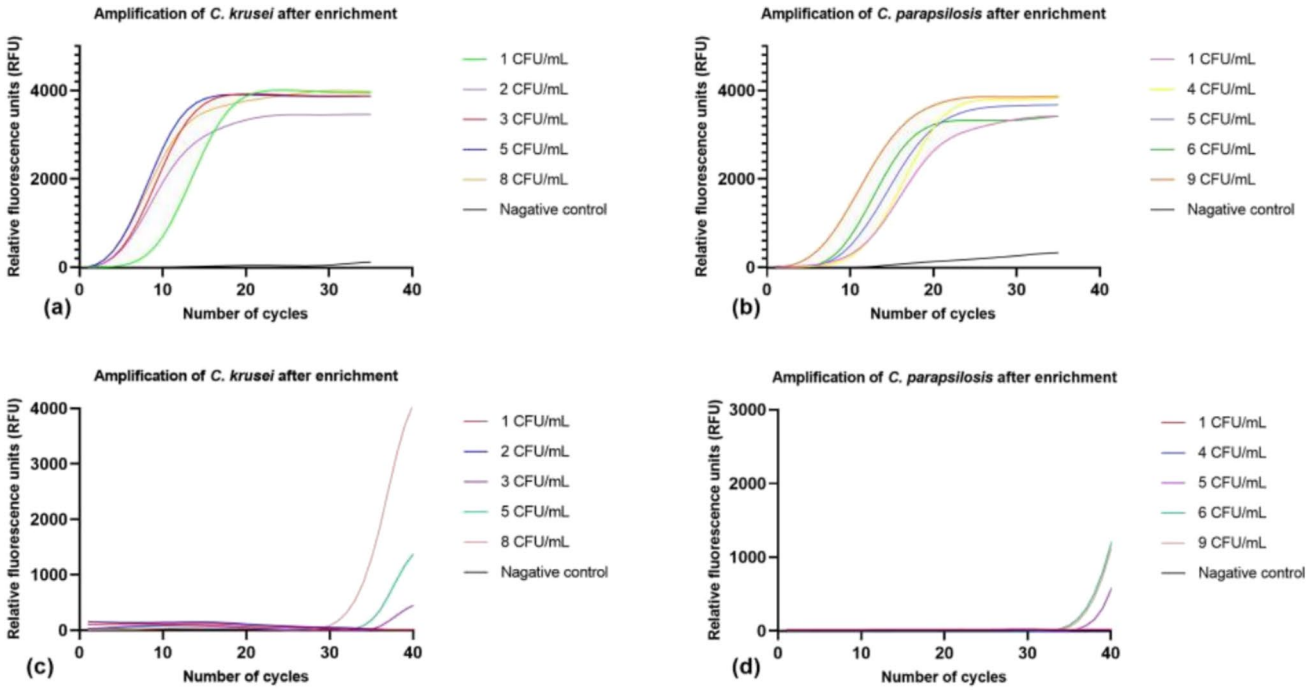

Fig. 4 Detection limits of the two methods after M1 magnetic-bead enrichment *C. krusei* using DO-RAP, with a LOD of 1 CFU/mL (**a**) and qPCR with a LOD of 3 CFU/mL (**b**) after enrichment;*C. parapsilosis* using DO-RAP, with a LOD of 1 CFU/mL (**c**) and qPCR with a LOD of 5 CFU/mL (**d**) after enrichment

Table 2 Detection of *C. krusei* and *C. parapsilosis* in clinical samples

Species	DO-RAP			qPCR			Kappa value
	Positive	Negative	Positive accuracy rate (%)	Positive	Negative	Positive accuracy rate (%)	
C. krusei	10	45	100	9	46	90	0.936
C. parapsilosis	15	40	100	13	42	86.7	0.904

and 0.904, respectively. $P < 0.05$ indicates a strong concordance between the detection results obtained by the two methods.

Discussion

In recent years, a notable surge has been observed in the incidence and mortality rates associated with infections caused by pathogenic *Candida* species, particularly among immunocompromised individuals, organ transplant recipients, and those with human immunodeficiency virus (HIV) (Ismadi et al. 2023). This increase can be attributed to several major risk factors such as immunosuppression, neutropenia, the administration of broad-spectrum antibiotics, corticosteroids, disruptions to mechanical or mucosal barriers, underlying conditions like diabetes, and global pandemics (e.g., COVID-19) (Cafardi et al. 2021). Consequently, *Candida* infections present a significant and pressing challenge in contemporary medical practice.

In the present study, we developed the DO-RAP detection method, which seamlessly integrates the rapid amplification capabilities of RAA with the widespread applicability of qPCR. This methodology eliminates the need for docosane to separate the two reaction stages, thereby streamlining and accelerating the process without compromising sensitivity. Additionally, the DO-RAP method requires only a single pair of RAA primers per target, which can also function as qPCR primers. This reduction in the number of primers necessary is instrumental in facilitating multiplex detection. The utilization of qPCR probes eliminates the necessity for the more complex design of RAA-specific probes. Furthermore, this technique is compatible with standard qPCR instruments, thus bypassing the need for specialized isothermal equipment.

M1 magnetic beads have been demonstrated to be effective in enriching common pathogens implicated in bloodstream infections (Zheng et al. 2023). Given that *Candida* concentrations in patient blood are often low, traditional molecular biology techniques exhibit limited sensitivity and may fail to provide definitive identification (Pfeiffer et al. 2011). This study employed M1 magnetic beads to capture *C. krusei* and *C. parapsilosis* from samples, thereby enhancing the relative concentration of pathogenic fungi while effectively removing plasma-derived interferents. This approach augments amplification efficiency and detection sensitivity (Zhang et al. 2023).

A variety of molecular diagnostic methods are currently available for the detection of *Candida*. Among these methods, real-time PCR is widely utilized, with a sensitivity range of 500 to 10^3 CFU/mL (García-Salazar et al. 2022; Mulet Bayona et al. 2021). Digital droplet PCR has been shown to achieve a detection sensitivity of 10 CFU/mL for *Candida*, while microarray technology has been demonstrated to detect *Candida* at a sensitivity of 100 CFU/mL (Tjandra et al. 2022). Furthermore, recombinase polymerase amplification coupled with lateral flow strip (RPA-LFS) has a detection sensitivity of 5.85×10^3 copies/reaction (Zhu et al. 2023). In contrast, the DO-RAP method developed in this study, in combination with M1 magnetic bead enrichment, achieved a minimum detection limit as low as 1 CFU/mL for both *C. krusei* and *C. parapsilosis*.

The DO-RAP method, when used in conjunction with M1 magnetic bead enrichment, has been shown to significantly enhance detection sensitivity by concentrating target pathogens. This, in turn, enables the direct detection of bloodstream infections with fewer than 10 CFUs without the necessity for extensive pre-culturing. This method is capable of producing reliable results within 3.5 h, thus presenting a novel opportunity for the rapid and precise clinical detection of *C. krusei* and *C. parapsilosis*.

However, the DO-RAP methodology is subject to certain constraints. Prior investigations conducted by our research group imply that these limitations could stem from by-products generated during the initial RAA phase, which subsequently impede the amplification efficiency of the qPCR (Fan et al. 2021). Given the high amplification efficacy of RAA, it is possible that certain samples may attain the qPCR detection threshold during the RAA stage itself, leading to the absence of cycle threshold (Ct) values in the subsequent qPCR phase. This phenomenon is particularly pronounced in samples with elevated fungal concentrations. Mitigation strategies may include the optimization of RAA reaction conditions, the incorporation of enzymatic digestion or purification steps to remove interfering by-products, and the fine-tuning of qPCR parameters to enhance overall amplification efficiency. Additionally, the specific enzyme composition and concentrations in the commercial RAA kit are undisclosed, further optimization in this aspect remains a potential avenue for improvement. Despite its limitations, this study has demonstrated that DO-RAP technology enables the effective detection of *C. krusei* and *C. parapsilosis*. The adaptability of the technology further underscores its potential for broad clinical and research applications, as it allows for integration with other techniques for rapid detection.

In summary, the DO-RAP method, when used in conjunction with M1 magnetic beads enrichment, enables the direct detection of *C. krusei* and *C. parapsilosis* in specimens. This approach eliminates the necessity for culture-based methods and significantly reduces the detection timeframe. This method introduces a novel approach for the early diagnosis and therapeutic intervention of invasive *Candida* infections, thereby possessing substantial clinical relevance.

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Author contribution X.S. and X.M. conceptualized and designed the study. X.L. conducted the experiments, curated and analyzed the data, and drafted the manuscript. X.L., Z.H., and H.L. repeated and validated the experimental methods. X.C., Y.W., and S.G. provided new reagents and data analytical tools. N.K., Y.G., Y.T., and J.W. provided clinical specimens. Z.F. and X.M. reviewed and edited the manuscript for critical intellectual content. All authors have read and approved the manuscript.

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Data availability The datasets used and/or analyzed in the current study can be provided on reasonable request. All requests should be made to the corresponding author.

Declarations

Ethics approval Ethical clearance for this study was granted by the Hebei General Hospital Ethics Committee (Research Ethics Committee approval, reference 2024-LW-0240). The study was conducted in accordance with the ethical guidelines and regulations. The samples used in this study were residual clinical specimens collected from previously approved projects after the completion of diagnostic testing. According to the guidelines established by the ethics committee, the requirement for additional informed consent was deemed unnecessary on the grounds that all patient identifiers had been meticulously removed, and the study did not impose any additional risks to the participants.

Conflict of interest The authors declare no competing interests.

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