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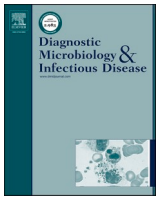

Original Article

Streamlining fungal diagnostics: Evaluation of a Direct-to-PCR extraction-free workflow for candida detection

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ABSTRACT

Fungal infections are an escalating health threat, and delays from conventional nucleic acid extraction hinder timely diagnosis and treatment. This study evaluated the diagnostic performance of a novel extraction-free technology, Direct-to-PCR (D2P; Scienetix, Tyler, TX, USA), for the detection of clinically significant *Candida* species (*C. albicans, C. glabrata, C. auris, C. parapsilosis*, and *C. tropicalis*). D2P was compared against conventional silica column-based (Qiagen) and magnetic bead-based (KingFisher) extraction methods, using microbial reference isolates, residual clinical specimens, and limit-of-detection analyses. Diagnostic sensitivity and specificity of D2P were comparable to conventional approaches, with specificity ranging from 96.77 % to 100 %. Concordance between methods was high, with Cohen's kappa coefficients (κ=0.93–1.00). Limit-of-detection analyses demonstrated strong analytical sensitivity, with excellent linearity (R^2=0.924–0.999) and low replicate variability (coefficient of variation 0.2–6.3 %). Statistical comparison of cycle threshold values revealed no significant differences between methods ($p > 0.05$), supporting equivalent nucleic acid recovery without the need for time-intensive extraction steps. Despite these strengths, the study has limitations. The relatively small number of residual clinical specimens ($n = 40$) may restrict the generalizability of findings, and further validation across broader patient populations, specimen types, and clinical settings is warranted. Nevertheless, D2P offers a streamlined, rapid diagnostic workflow that reduces turnaround times and enhances accessibility, particularly in resource-limited environments. Wider adoption of extraction-free PCR platforms such as D2P could facilitate earlier detection of invasive candidiasis, improve clinical outcomes, and mitigate healthcare-associated morbidity and mortality attributable to fungal infections.

1. Introduction

Fungal infections are an escalating global health threat, marked by rising incidence, antifungal resistance, and significant clinical burden. In 2022, the World Health Organization designated multidrug-resistant species, including Candida auris and Candida glabrata, as critical public health concerns [1]. These pathogens cause infections ranging from superficial mucosal disease to life-threatening invasive candidiasis, particularly in immunocompromised patients and those with prolonged antibiotic exposure [2,3]. Invasive candidiasis alone affects over 250,000 individuals annually, contributing to approximately 50,000 deaths worldwide [2].

Timely diagnosis of invasive Candida infections is essential for effective patient management [3]. However, conventional detection methods often fall short due to inherent limitations, including prolonged turnaround times, low sensitivity, and nonspecific clinical

Abbreviations: CFU, Colony Forming Unit; Ct, Cycle Threshold; D2P, Direct-to-PCR; DOR, Diagnostic Odds Ratio; FOR, False Omission Rate; FDR, False Discovery Rate; LR, Likelihood Ratio; LOD, Limit of Detection; MagB, Magnetic Bead-based Extraction; NA, Not Applicable; NPV, Negative Predictive Value; PCR, Polymerase Chain Reaction; PPV, Positive Predictive Value; qPCR, Quantitative Polymerase Chain Reaction; SD, Standard Deviation; TP, True Positive; TN, True Negative; FP, False Positive; FN, False Negative.

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manifestations. A major barrier to the advancement of molecular fungal diagnostics lies in the structurally complex and resilient Candida cell wall, which is composed predominantly of mannoproteins, β-glucans, and chitin, and poses significant challenges for efficient DNA extraction [4]. Traditional fungal DNA extraction protocols—such as mechanical disruption via bead-beating, enzymatic digestion (e.g., zymolyase treatment), or chemical lysis followed by silica-column or magnetic bead-based purification—are labor-intensive, prone to DNA degradation, and require specialized laboratory infrastructure [5,6]. These methodological constraints are particularly detrimental in resource-limited settings or when rapid clinical decision-making is critical [7,8].

Extraction-free nucleic acid amplification technologies represent a promising advancement over conventional diagnostic methods. Although their application to bacterial and viral pathogens has been demonstrated, their utility in fungal diagnostics, particularly for Candida species, remains insufficiently characterized. Current literature provides limited evidence regarding their diagnostic accuracy and clinical applicability in this context.

This study evaluates Direct-to-PCR (D2P) extraction-free technology (Scienetix, Tyler, TX, USA) in comparison to conventional silica column-based (Qiagen) and magnetic bead-based (KingFisher) extraction methods. The D2P method operates on the fundamental principle of achieving efficient microbial cell lysis to liberate nucleic acids suitable for direct amplification, bypassing conventional nucleic acid purification steps typically required for PCR-based diagnostics. Specifically, the D2P protocol employs a proprietary buffer system designed with carefully selected biochemical components, including protein-degrading enzymes, chelating agents, detergents, nucleic acid stabilizers, and biomimetic antimicrobial peptides. These components collectively disrupt pathogen cell membranes and walls while maintaining the integrity and accessibility of nucleic acids. This innovative approach facilitates rapid nucleic acid release into a PCR-compatible lysate, significantly streamlining the diagnostic workflow by reducing overall processing time, complexity, and cost. The principles underlying extraction-free PCR methodologies, including overcoming PCR inhibitors and maintaining nucleic acid integrity, align with established techniques described previously [9].

The primary objective is to assess whether the D2P workflow (Fig. 1) achieves comparable sensitivity, specificity, and reliability in detecting major Candida species, including C. glabrata, C. albicans, C. auris, C. parapsilosis, and C. tropicalis. Results from this investigation aim to inform the clinical utility of extraction-free PCR approaches and support their broader implementation in diagnostic mycology.

2. Materials and Methods

This study employed a comparative framework to assess the diagnostic performance of an extraction-free nucleic acid processing method, Direct-to-PCR (D2P; Scienetix, Tyler, TX, USA), relative to conventional silica column-based (Qiagen) and magnetic bead-based (KingFisher) extraction technologies. Evaluation encompassed nucleic acid recovery from standardized microbial isolates, diagnostic performance in clinical specimens, and analytical sensitivity through limit-of-detection (LOD) analysis.

2.1. Nucleic Acid Extraction Methods

Three extraction methods were evaluated for their efficacy in lysing fungal cells and isolating DNA suitable for downstream quantitative PCR (qPCR) detection of *Candida* species.

Traditional silica column-based extraction was performed using the DNeasy Blood and Tissue Kit (Cat. No. 69504, Qiagen, Hilden, Germany). Samples underwent proteinase K digestion followed by enzymatic lysis with AL buffer, targeting disruption of the chitin-rich fungal cell wall [4]. Nucleic acids were subsequently bound to silica membranes in spin columns, washed with ethanol-based AW1 and AW2 buffers, and eluted in 50 μL of elution buffer. Although effective, this method required considerable processing time and multiple manual handling steps.

Magnetic bead-based extraction was conducted using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit with the KingFisher™ Flex Purification System (Cat. No. 5400610, Thermo Fisher Scientific, Waltham, USA). Fungal cells were lysed in a chaotropic buffer containing proteinase K to facilitate cell wall disruption [3]. DNA was captured by paramagnetic beads, washed to remove contaminants, and eluted in a low-salt buffer optimized for PCR. While this method offered automation and scalability, its efficacy remained dependent on efficient fungal lysis.

The D2P method (Cat. D2P-UN-192, Scienetix, Tyler, TX, USA) simplified sample preparation by eliminating conventional nucleic acid extraction steps. Clinical specimens (1 mL) were centrifuged at 10,000 × *g* for 10 minutes at 4°C, the supernatant was discarded, and the pellet was resuspended in 30 μL D2P-Universal Extraction Buffer. Samples were vortexed, incubated at 95°C for 10 minutes to achieve fungal cell wall disruption, stabilized with the addition of 30 μL D2P-Sample Dilution Buffer, and briefly centrifuged at 8,000 × *g* for 10 seconds. The resulting lysates were directly used for qPCR detection. This streamlined approach substantially reduced processing time and complexity while maintaining compatibility with molecular amplification workflows.

2.2. Application and Evaluation of Extraction Methods

Standardized microbial reference isolates of clinically significant Candida species, including C. glabrata, C. albicans, C. auris, C. parapsilosis, and C. tropicalis, were obtained from the American Type Culture Collection (ATCC) and BEI Resources (Manassas, VA, USA). To simulate clinically relevant conditions, these isolates were spiked into pooled urine matrices, creating biologically representative samples for evaluating nucleic acid extraction efficiency. Each spiked sample underwent parallel DNA isolation using the three evaluated methods—Qiagen silica column-based extraction, KingFisher magnetic bead-based extraction, and the extraction-free D2P workflow. Nucleic acid

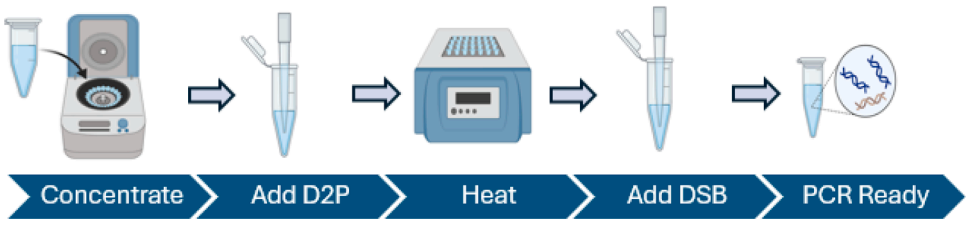

Fig. 1. Workflow of Direct-to-PCR (D2P) extraction-free sample processing. The D2P extraction-free protocol involves a streamlined five-step process for preparing fungal clinical specimens for PCR analysis. Samples are first concentrated by centrifugation, followed by the addition of D2P Universal Extraction Buffer. After heat-mediated fungal cell lysis, a sample dilution buffer (DSB) is added to stabilize the lysate. The resulting preparation is immediately PCR-ready, eliminating the need for conventional enzymatic, mechanical, or chemical DNA extraction steps.

yields and qPCR amplification performance were systematically compared across extraction methods to assess their relative efficiency and suitability for fungal DNA detection.

To assess clinical diagnostic utility, 40 de-identified residual clinical specimens from patients with confirmed or suspected Candida infections were obtained from Advanta Genetics (Tyler, TX, USA). These samples encompassed infections with C. glabrata, C. albicans, C. auris, C. parapsilosis, and C. tropicalis. Each specimen was processed using both the extraction-free D2P method and the KingFisher magnetic bead-based extraction system to enable direct comparison of fungal DNA isolation efficiency from clinical material. This evaluation aimed to determine whether D2P could serve as a rapid and reliable alternative within clinical laboratory workflows. Institutional ethical standards were upheld, and the study was classified as IRB-exempt due to the exclusive use of de-identified specimen samples [10].

Analytical sensitivity was further assessed by LOD analysis, directly comparing the D2P and KingFisher methods. Contrived urine specimens were spiked with known concentrations of C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, and C. auris, ranging from 10^4 to 10^1 CFU/mL through serial tenfold dilutions. Samples were processed in duplicate with each extraction method, followed by multiplex qPCR targeting species-specific genetic markers. Standard curves generated from positive controls allowed accurate quantification of fungal loads, and the LOD was defined as the lowest dilution consistently yielding reproducible amplification across replicates [11].

2.3. Quantitative PCR for Candida Detection

Quantitative PCR was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA) with preformulated multiplex reagents targeting species-specific Candida markers (Scienetix, Tyler, TX, USA). Each 10 μL reaction consisted of 2.5 μL of extracted DNA (Qiagen, KingFisher, or D2P) and 7.5 μL of master mix containing primers, probes, enzymes, and dNTPs. Thermocycling conditions included initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. A cycle threshold (Ct) value <35 was interpreted as positive for Candida detection; samples with Ct values ≥ 35 or no amplification were considered negative [12].

2.4. Statistical and Data Analysis

Statistical analyses compared extraction method performance across Ct values, diagnostic sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV). Mean Ct values and standard deviations were calculated to assess extraction consistency. Paired t-tests were used to evaluate differences in Ct values between methods, with statistical significance defined as $p < 0.05$. Additional diagnostic performance metrics, including false discovery rate (FDR), false omission rate (FOR), Cohen's kappa coefficient (κ), likelihood ratios (LR), and diagnostic odds ratios (DOR), were calculated to provide a comprehensive assessment of clinical reliability.

3. Results

The efficiency of the extraction-free D2P method was compared with conventional silica column-based (Qiagen) and magnetic bead-based (KingFisher) extraction techniques for the detection of Candida glabrata, Candida albicans, Candida auris, Candida parapsilosis, and Candida tropicalis from reference microbial isolates. Given the structural complexity of the fungal cell wall, this comparison assessed whether D2P could achieve nucleic acid recovery comparable to traditional extraction methods. Ct values served as the primary indicator of DNA yield, with lower Ct values reflecting higher nucleic acid recovery. Differences in Ct values were statistically analyzed to assess variations in DNA yield and diagnostic performance, and additional diagnostic parameters—including sensitivity, specificity, accuracy, PPV, and NPV—were computed to compare overall efficacy [13].

Comparison across extraction methods revealed notable differences in recovery efficiency. Qiagen consistently yielded the lowest Ct values across all species, indicating optimal DNA recovery. D2P achieved Ct values comparable to Qiagen for C. glabrata, suggesting effective fungal lysis without the need for conventional extraction. For C. albicans, D2P yielded slightly higher Ct values, indicating a modest reduction in DNA recovery. In contrast, D2P demonstrated superior performance in C. auris detection, achieving significantly lower Ct values than both Qiagen and KingFisher, consistent with enhanced lysis efficiency for this species (Table 1).

The KingFisher magnetic bead-based extraction method consistently exhibited higher Ct values across all Candida species tested, reflecting lower nucleic acid recovery efficiency despite the advantages of automation. Performance was weakest for C. auris, with a mean Ct value of 23.00 (SD 0.61), compared to 21.39 (SD 1.5) achieved with the D2P extraction-free method, further supporting the superior efficacy of D2P for the detection of this emerging multidrug-resistant pathogen.

D2P achieved DNA recovery comparable to Qiagen and superior to KingFisher, particularly for C. auris, where it demonstrated lower Ct values than either conventional method. Although Qiagen remained the gold standard for nucleic acid yield, D2P offered a faster and more streamlined alternative, particularly valuable in time-sensitive diagnostic settings. ΔCt analysis supported these findings, with D2P closely matching Qiagen for C. glabrata and achieving superior recovery for C. auris. In contrast, KingFisher consistently exhibited the highest Ct values across species, reflecting lower nucleic acid recovery efficiency despite the advantages of automation.

Key performance metrics, including sensitivity, specificity, accuracy, PPV, and NPV, confirmed the diagnostic reliability of each method. Both D2P and Qiagen achieved 100 % sensitivity for C. glabrata, indicating complete DNA recovery with no false negatives. For C. albicans, D2P showed slightly reduced sensitivity (88.89 %), consistent with higher Ct values and marginally lower DNA yield. For C. auris, D2P outperformed both conventional methods, achieving higher sensitivity and lower Ct values, suggesting more effective fungal lysis and DNA release for this emerging multidrug-resistant pathogen. Specificity remained 100 % for Qiagen and D2P across most species, whereas KingFisher exhibited minor reductions due to false positives, particularly in C. auris detection. Overall diagnostic accuracy mirrored these trends, with D2P performing comparably to Qiagen, while KingFisher demonstrated a modest decrease due to increased rates of false positives and false negatives.

These results confirm that D2P provides diagnostic accuracy equivalent to Qiagen while eliminating labor-intensive extraction steps, making it a practical alternative for high-throughput molecular

Table 1
Ct Value Comparison Across Extraction Methods for Candida Species.

Microorganism	Qiagen (Ct ± SD)	KingFisher (Ct ± SD)	D2P (Ct ± SD	ΔCt (KF-QI)	ΔCt (D2P-QI)	ΔCt (KF-D2P)
C. glabrata	18.19 ± 0.11	19.06 ± 1.76	19.40 ± 0.12	0.86	1.20	−0.34
C. albicans	23.34 ± 0.25	22.92 ± 0.14	25.20 ± 0.22	−0.42	1.85	−2.28
C. auris	24.73 ± 0.41	23.00 ± 0.61	21.39 ± 1.5	−1.73	−3.34	1.61
C. parapsilosis	24.63 ± 0.37	25.13 ± 1.10	25.13 ± 0.45	0.50	1.1	−0.55
C. tropicalis	24.26 ± 0.73	24.37 ± 0.88	24.58 ± 0.88	0.11	0.3	−0.21

Ct = cycle threshold; SD = standard deviation; D2P = Direct-to-PCR extraction-free method (Scienetix, Tyler, TX, USA); ΔCt = difference in mean Ct values between methods; KF = KingFisher magnetic bead-based extraction (Thermo Fisher Scientific, Waltham, USA); QI = Qiagen silica column-based extraction (Qiagen, Hilden, Germany).

diagnostics, particularly in resource-limited settings. Although KingFisher offers automation benefits, its reduced sensitivity and specificity suggest that D2P represents a superior option for clinical laboratories requiring rapid, cost-effective, and reliable fungal detection. Further validation with broader clinical specimen types remains necessary to fully establish the clinical applicability of the D2P workflow in direct patient sample testing.

3.1. Comparative Analysis of D2P and KingFisher in Residual Clinical Isolates

The diagnostic performance of the extraction-free D2P method was compared with the KingFisher magnetic bead-based extraction system for the detection of clinically relevant Candida species, including C. glabrata, C. albicans, C. auris, C. parapsilosis, and C. tropicalis, in residual clinical specimens. These pathogens represent major causes of invasive candidiasis, where rapid and accurate identification is critical due to the clinical challenges posed by the thick fungal cell wall. The evaluation aimed to determine whether D2P could match or exceed the diagnostic performance of KingFisher while eliminating the need for labor-intensive DNA extraction steps. Sensitivity, specificity, accuracy, PPV, and NPV were calculated to assess clinical effectiveness, and results are summarized in Table 2.

D2P achieved 100 % sensitivity for C. glabrata, C. tropicalis, and C. auris, with no false negatives observed, indicating high DNA recovery efficiency for these species. Sensitivity for C. albicans was slightly lower at 88.89 %, consistent with higher Ct values and suggesting a modest reduction in nucleic acid yield compared with conventional extraction methods. Specificity remained 100 % across all species except C. glabrata (96.77 %), where a single false positive was recorded. D2P achieved 100 % accuracy for C. albicans, C. auris, C. parapsilosis, and C. tropicalis, with PPV and NPV consistently high, further supporting its diagnostic reliability.

Both D2P and KingFisher demonstrated 100 % sensitivity for C. tropicalis. However, D2P demonstrated superior sensitivity and lower Ct values compared with KingFisher for C. glabrata, C. albicans, and particularly C. auris, a clinically important multidrug-resistant pathogen. KingFisher exhibited minor reductions in sensitivity for C. glabrata and C. albicans, primarily driven by isolated false-negative results, and showed a slightly higher false positive rate for C. glabrata. Overall diagnostic accuracy followed similar patterns, with D2P performing comparably or better than KingFisher, particularly in the detection of C. auris, where lower Ct values and improved sensitivity were observed.

Taken together, these results indicate that D2P provides diagnostic performance equivalent to, or exceeding, KingFisher for the detection of clinically significant Candida species while offering the advantages of a streamlined, extraction-free workflow. Although KingFisher offers benefits in automation, its reduced sensitivity and specificity for certain species highlight potential limitations in specific clinical applications. Given D2P's strong diagnostic performance, elimination of labor-intensive extraction steps, and cost-effectiveness, it represents a practical and efficient alternative for high-throughput and resource-limited settings in fungal diagnostics.

3.2. Limit of Detection (LOD) Analysis of Candida Species

The LOD analysis systematically evaluated the analytical sensitivity of the extraction-free D2P method compared to the conventional magnetic bead-based KingFisher extraction protocol across five clinically significant Candida species. Contrived urine specimens were spiked with C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, and C. auris at defined concentrations ranging from 1×10^5 CFU/mL to 1×10^1 CFU/mL, and serial tenfold dilutions were analyzed by qPCR to determine the lowest dilution at which consistent and reproducible amplification was observed, thereby establishing the detection threshold for each method.

Comparative results between D2P and magnetic bead-based extraction methods are illustrated in Figs. 2A–E. For C. glabrata (Fig. 2A), both methods demonstrated near-perfect linearity, with correlation coefficients (R^2) of 0.998 for D2P and 1.000 for magnetic bead-based, indicating equivalent analytical performance across the full dilution series. Analysis of C. tropicalis (Fig. 2B) also showed strong correlation ($R^2 = 0.9244$ for D2P; $R^2 = 0.9944$ for magnetic bead-based), although minor variability was observed for D2P at lower fungal concentrations, possibly reflecting slight differences in cell lysis efficiency. In the case of C. auris (Fig. 2C), excellent linearity was confirmed for both methods ($R^2 = 0.995$ for D2P; $R^2 = 0.9995$ for magnetic bead-based), supporting the sensitivity of the extraction-free workflow even at low fungal burdens. For C. albicans (Fig. 2D), D2P and magnetic bead-based yielded near-identical amplification performance ($R^2 = 0.9999$ and $R^2 = 0.998$, respectively). Similarly, for C. parapsilosis (Fig. 2E), robust extraction and amplification efficiency were demonstrated by both methods ($R^2 = 0.9575$ for D2P; $R^2 = 0.9985$ for magnetic bead-based).

3.3. Complementary Diagnostic Metrics Supporting D2P Clinical Accuracy

Additional diagnostic analyses were conducted to further evaluate the clinical reliability of the D2P extraction-free method compared with KingFisher. Complementary measures—including false discovery rate (FDR), false omission rate (FOR), Cohen's kappa coefficient (κ), likelihood ratios (LRs), and diagnostic odds ratio (DOR)—provided a more comprehensive assessment of diagnostic agreement, error rates, and clinical utility.

FDR and FOR were calculated to quantify the reliability of positive and negative test results, respectively. FDR, defined as the proportion of false positives among positive results, remained at or near zero across most Candida species, indicating high test precision. C. glabrata exhibited a slightly elevated FDR (10 %), attributed to a single false positive, whereas C. albicans, C. auris, C. parapsilosis, and C. tropicalis showed FDR values of 0 %. Similarly, FOR values were extremely low, particularly for C. glabrata (0.0 %), C. auris (0.0 %), C. parapsilosis (0.0 %), and C. tropicalis (0.0 %), demonstrating minimal risk of false negatives. C. albicans exhibited a slightly elevated FOR (3.1 %), reflecting a single false-negative result. These findings support the high specificity and reliability of the D2P workflow across clinically relevant fungal pathogens.

Agreement between D2P and KingFisher relative to conventional methods was assessed using Cohen's kappa coefficient (κ). Values of κ were 0.93 for C. glabrata and C. albicans, indicating near-perfect

Table 2
Diagnostic performance of D2P versus KingFisher extraction for clinical detection of Candida species.

Microorganism	TP (D2P)	TN (D2P)	FP (D2P)	FN (D2P)	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV	NPV
C. glabrata	9	30	1	0	100.00 %	96.77 %	96.77 %	0.90	1.00
C. albicans	8	31	0	1	88.89 %	100.00 %	100.00 %	1.00	0.97
C. auris	0	40	0	0	NA	100.00 %	100.00 %	NA	1.00
C. parapsilosis	0	40	0	0	NA	100.00 %	100.00 %	NA	1.00
C. tropicalis	6	34	0	0	100.00 %	100.00 %	100.00 %	1.00	1.00

TP = true positive; TN = true negative; FP = false positive; FN = false negative; PPV = positive predictive value; NPV = negative predictive value; NA = not applicable.

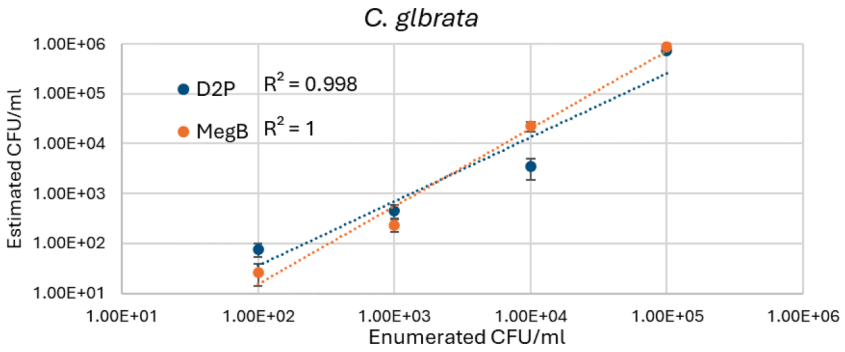

Fig. 2A. Limit-of-detection analysis comparing D2P and magnetic bead-based extraction methods for Candida glabrata detection across serial tenfold dilutions.

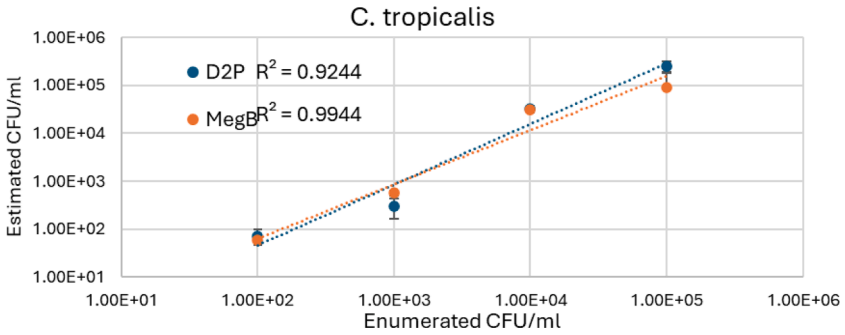

Fig. 2B. Limit-of-detection analysis comparing D2P and magnetic bead-based extraction methods for Candida tropicalis detection across serial tenfold dilutions.

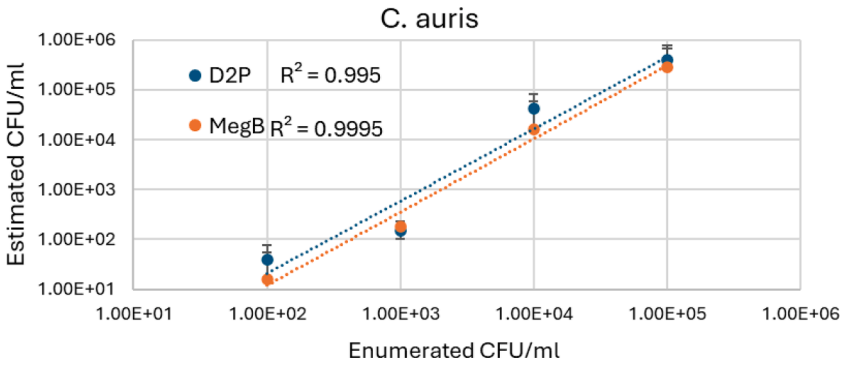

Fig. 2C. Limit-of-detection analysis comparing D2P and magnetic bead-based extraction methods for Candida auris detection across serial tenfold dilutions.

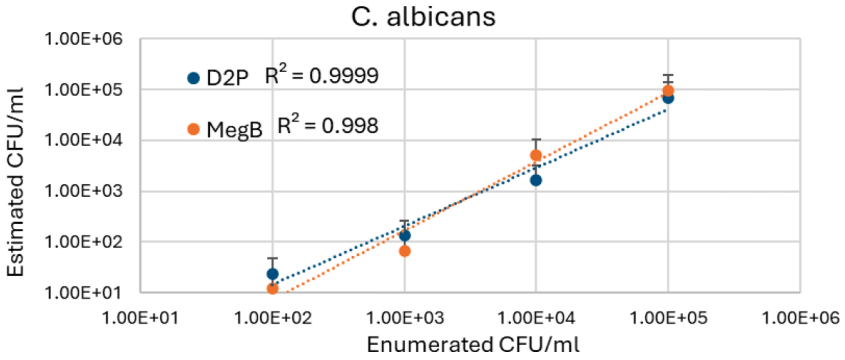

Fig. 2D. Limit-of-detection analysis comparing D2P and magnetic bead-based extraction methods for Candida albicans detection across serial tenfold dilutions.

agreement, and 1.0 for C. tropicalis, confirming complete concordance. For C. auris and C. parapsilosis, κ could not be computed due to the absence of false positives or false negatives, consistent with 100 % diagnostic accuracy for these species.

LR and DOR were also calculated to evaluate the ability of D2P to confirm or exclude infection. Positive likelihood ratios (LR+) were high

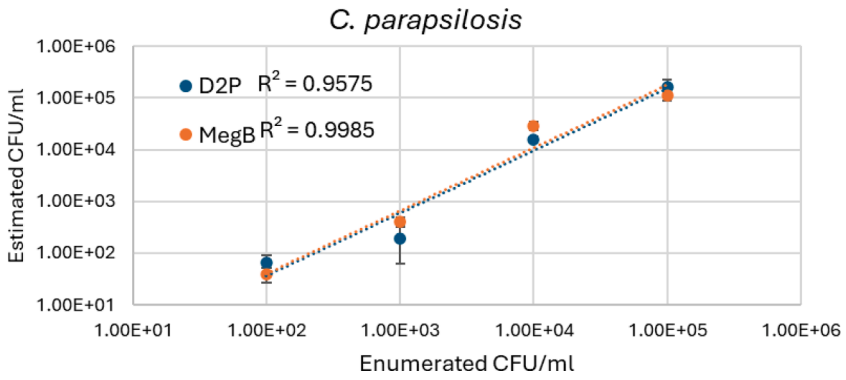

Fig. 2E. Limit-of-detection analysis comparing D2P and magnetic bead-based extraction methods for Candida parapsilosis detection across serial tenfold dilutions.

across most species, indicating strong positive predictive performance. Notably, C. glabrata exhibited an LR+ of 31.0, while C. albicans and C. tropicalis yielded LR+ values of infinity (∞), reflecting perfect classification without false positives. Negative likelihood ratios (LR−) were near zero, with C. glabrata and C. tropicalis both achieving LR− values of 0.0, and C. albicans demonstrating an LR− of 0.11, indicating strong negative predictive performance.

The DOR, summarizing overall discriminatory power, was also calculated. D2P achieved infinite DOR values for C. glabrata, C. albicans, and C. tropicalis, reflecting perfect distinction between infected and non-infected cases. For C. auris and C. parapsilosis, DOR values were undefined due to the absence of classification errors, reinforcing the precision and clinical reliability of the D2P method in detecting invasive fungal pathogens.

3.4. Operational Advantages of Extraction-Free Direct-to-PCR Diagnostics

In addition to diagnostic accuracy, workflow efficiency is critical when selecting optimal methods for clinical microbiology laboratories. The D2P extraction-free approach eliminates the need for labor-intensive nucleic acid extraction steps, substantially reducing sample processing time while maintaining high diagnostic performance. Although KingFisher offers automation benefits, it still requires additional workflow steps, contributing to longer turnaround times [14]. These findings support D2P as not only a diagnostically reliable alternative but also an efficient and scalable molecular tool capable of improving fungal detection workflows. The combination of high accuracy and reduced hands-on time positions D2P as a practical diagnostic solution for mycology laboratories, particularly in high-throughput or resource-constrained settings.

3.5. Statistical Evaluation of Nucleic Acid Recovery Across Extraction Methods

The relative efficiency of D2P, Qiagen, and KingFisher in nucleic acid recovery was assessed by comparing Ct values, with lower Ct values indicative of higher DNA yield. Given Qiagen's status as the benchmark for fungal DNA extraction, direct statistical comparisons were made to evaluate the viability of D2P as an extraction-free alternative. This analysis was restricted to C. glabrata, C. albicans, and C. auris, the only species tested across all three methods in both microbial isolates and clinical specimens; thus, findings are specific to these species.

Table 3 presents the t-statistics and p-values for pairwise comparisons. No statistically significant differences ($p > 0.05$) were observed between any of the methods. Although Qiagen demonstrated slightly lower Ct values than KingFisher ($t = -2.09$, $p = 0.17$) and D2P ($t = -1.14$, $p = 0.37$), the differences were not significant, suggesting comparable nucleic acid recovery across methods. Similarly, the KingFisher versus D2P comparison ($t = 0.75$, $p = 0.53$) reinforced the equivalence of DNA yield, supporting the analytical efficiency of the D2P extraction-free workflow.

Table 3
Statistical Comparison of Ct Values Across Qiagen, KingFisher, and D2P Extraction Methods.

Extraction Method Comparison	t-Statistic	p-Value	Statistical Significance ($p < 0.05$)
Qiagen vs. KingFisher	−2.09	0.1717	Not Significant
Qiagen vs. D2P	−1.14	0.3724	Not Significant
KingFisher vs. D2P	0.75	0.5297	Not Significant

Table 4 presents the 95 % confidence intervals (CIs) for sensitivity and specificity across all extraction methods, providing further evaluation of diagnostic reliability. D2P achieved 100 % sensitivity for C. glabrata and C. tropicalis, with high specificity for C. glabrata (96.77 %), indicating minimal false positives. For C. albicans, sensitivity was slightly lower at 88.89 %, although specificity remained 100 %, supporting D2P's ability to accurately identify true negatives. The confidence intervals reinforce the precision and consistency of D2P performance across clinically relevant Candida species.

4. Discussion

This study demonstrated that extraction-free D2P technology achieves diagnostic sensitivity, specificity, and overall accuracy comparable—and in key instances superior—to conventional silica column-based (Qiagen) and magnetic bead-based (KingFisher) extraction methods for the detection of clinically significant Candida species. Notably, D2P achieved equivalent or enhanced DNA recovery for C. auris and C. glabrata, both pathogens of critical clinical concern due to their intrinsic antifungal resistance and frequent implication in healthcare-associated infections.

LOD analysis confirmed the analytical robustness of D2P, with sensitivity consistently matching that of the gold-standard KingFisher method across all evaluated species. Strong linearity, reproducibility, and low replicate variability (coefficient of variation 0.2–6.3 %) were observed. Although minor variability was detected at lower fungal concentrations, particularly for C. tropicalis, this did not materially impact diagnostic performance. The ability of D2P to maintain analytical sensitivity without reliance on traditional nucleic acid extraction underscores its potential to overcome longstanding technical barriers imposed by the resilient cell walls of fungal pathogens.

These findings are particularly significant given the critical importance of rapid, accurate diagnosis of invasive candidiasis for optimizing patient management and healthcare resource utilization. Conventional extraction workflows, while effective, are limited by prolonged turnaround times, specialized infrastructure requirements, and operational costs. By eliminating labor-intensive extraction steps without

Table 4
Diagnostic Sensitivity and Specificity with 95 % Confidence Intervals for Candida Detection, Stratified by Extraction Method.

Extraction Method	Candida Species	Sensitivity (%)	Sensitivity 95 % CI	Specificity (%)	Specificity 95 % CI
KingFisher	*C. glabrata*	90.00	(59.58 % – 98.21 %)	96.67	(83.33 % – 99.41 %)
	C. albicans	88.89	(56.50 % – 98.01 %)	100.00	(89.28 % – 100.00 %)
	C. auris	100.00	(100.00 % – 100.00 %)	100.00	(91.40 % – 100.00 %)
	C. parapsilosis	100.00	(100.00 % – 100.00 %)	100.00	(91.40 % – 100.00 %)
	C. tropicalis	100.00	(61.02 % – 100.00 %)	100.00	(89.42 % – 100.00 %)
D2P	*C. glabrata*	100.00	(70.09 % – 100.00 %)	96.77	(83.81 % – 99.43 %)
	C. albicans	88.89	(56.50 % – 98.01 %)	100.00	(89.28 % – 100.00 %)
	C. auris	100.00	(100.00 % – 100.00 %)	100.00	(91.40 % – 100.00 %)
	C. parapsilosis	100.00	(100.00 % – 100.00 %)	100.00	(91.40 % – 100.00 %)
	C. tropicalis	100.00	(61.02 % – 100.00 %)	100.00	(89.42 % – 100.00 %)

compromising analytical performance, D2P substantially streamlines diagnostic workflows and enhances the feasibility of fungal detection in both high-throughput and resource-limited laboratory environments.

Clinically, D2P positions itself as a scalable, cost-effective platform for the early detection of fungal infections, particularly in critical care and immunocompromised populations where diagnostic timeliness is directly linked to therapeutic outcomes. The technology enables accelerated clinical decision-making by preserving assay sensitivity while removing the bottleneck of nucleic acid extraction. Broader adoption of extraction-free PCR workflows, exemplified by D2P, has the potential to transform fungal diagnostics, facilitating earlier identification of invasive candidiasis, improving patient outcomes, and reducing healthcare-associated morbidity and mortality.

4.1. Strengths and Limitations

A major strength of this study is its comparative framework, directly evaluating the performance of the extraction-free D2P method against widely used conventional extraction platforms (Qiagen and KingFisher). The inclusion of both reference microbial isolates and residual clinical specimens enhances the translational relevance of the findings. Moreover, the use of quantitative PCR-based performance metrics—including sensitivity, specificity, accuracy, PPV, and NPV—enabled a robust statistical assessment of nucleic acid recovery efficiency and diagnostic performance [15].

Several limitations warrant consideration. The relatively small sample size of clinical specimens ($n = 40$), while adequate for initial proof-of-concept validation, may limit the generalizability of the findings [16]. Larger, multi-center studies involving diverse patient populations and clinical settings will be necessary to confirm the broader applicability [17] of the D2P method. In addition, although analytical sensitivity was rigorously assessed using contrived urine matrices, further validation across other biological matrices, including blood, cerebrospinal fluid, and bronchoalveolar lavage fluid, is needed to fully characterize performance under varied clinical conditions and lower pathogen concentrations [18].

The potential impact of PCR inhibitors, a well-recognized challenge for extraction-free methodologies [19], was not extensively evaluated. Biological matrices such as blood and urine commonly contain substances such as hemoglobin and urea that may inhibit amplification [20]. Although the proprietary buffer formulation used in D2P may mitigate some inhibitory effects, future studies should systematically investigate the influence of common PCR inhibitors and explore optimization strategies, including the use of inhibitor-resistant polymerases and pre-treatment approaches such as enzymatic digestion, dilution, or bovine serum albumin supplementation. Addressing these factors will be critical to further enhance the reproducibility, robustness, and clinical reliability of extraction-free fungal diagnostics [21].

4.2. Comparison with Previous Studies

Although extraction-free PCR is generally established for certain DNA/RNA diagnostics [22–25], its application in fungal detection remains comparatively underexplored. Fungal species—particularly Candida—present distinct challenges due to their thick, multilayered cell walls enriched with β-glucans and chitin. The present findings align with previous research emphasizing the critical role of efficient cell lysis and PCR optimization in achieving robust fungal DNA amplification [26].

Compared with traditional silica column- and magnetic bead-based extraction methods, which rely on enzymatic digestion and chemical lysis, the D2P workflow streamlines sample processing by eliminating the need for complex extraction steps while preserving nucleic acid integrity. This operational simplification offers a substantial advantage for high-throughput and resource-limited diagnostic settings, where the cost, time, and technical burden of DNA extraction represent significant barriers to rapid fungal detection. However, the slightly elevated Ct values observed for C. albicans suggest that further optimization of the D2P lysis conditions, such as buffer composition or thermal treatment protocols, may be necessary to maximize DNA yield for specific fungal species.

4.3. Potential Applications in PCR Laboratories

The findings of this study demonstrate that D2P extraction-free technology can substantially amend fungal diagnostics in PCR laboratories by eliminating the need for labor-intensive nucleic acid extraction, thereby reducing processing times, consumable costs, and operational complexity. This advancement positions D2P as an efficient and scalable solution for a range of clinical and diagnostic applications. In resource-limited and low-infrastructure settings, the capacity for rapid fungal detection without reliance on specialized extraction equipment can significantly enhance diagnostic accessibility. Similarly, high-throughput laboratories requiring rapid, reliable screening for invasive candidiasis could integrate D2P into molecular workflows to improve sample processing efficiency and reduce turnaround times. In emergency and critical care environments, where prompt initiation of antifungal therapy is essential, the ability of D2P to deliver rapid and accurate diagnostic results could directly influence early treatment decisions and improve clinical outcomes.

Beyond workflow efficiency, D2P holds particular promise for advancing fungal detection in high-risk patient populations, including individuals in oncology wards, transplant units, and intensive care settings, where invasive candidiasis represents a substantial clinical threat. The early and accurate identification of C. auris, a multidrug-resistant pathogen associated with nosocomial outbreaks and high antifungal resistance rates, is critical for implementing infection control measures, limiting transmission, and improving patient prognosis. By offering a cost-effective, rapid, and clinically reliable alternative to conventional fungal diagnostics, D2P has the potential to transform molecular mycology workflows. Moreover, it has potential to enhance patient care across both routine and high-acuity clinical settings, particularly in high-complexity laboratory developed tests [27].

4.4. Future Directions

To establish D2P extraction-free technology as a viable alternative in fungal diagnostics, future research will require large-scale, multicenter clinical validation studies. Expanded evaluations across diverse clinical settings and patient populations—including immunocompromised individuals undergoing chemotherapy, organ transplant recipients, and critically ill patients—will be critical to comprehensively assess diagnostic accuracy, clinical reliability, and generalizability. In addition, systematic validation of D2P performance across a broader range of clinical specimen types, including whole blood, cerebrospinal fluid, bronchoalveolar lavage fluid, and tissue biopsies, will be essential to confirm analytical robustness within complex, inhibitor-rich biological matrices.

Targeted investigations into low fungal-burden samples [28], common in early-stage infections and among patients receiving antifungal prophylaxis, are particularly important to define the lower diagnostic thresholds and sensitivity limits of D2P in clinically challenging scenarios. Future studies should also incorporate direct benchmarking against emerging molecular diagnostic platforms, including digital PCR and metagenomic sequencing [29], to position extraction-free PCR methodologies within the evolving landscape of mycological diagnostics. Addressing these research priorities through comprehensive, multicenter investigations will generate the clinical evidence necessary to support broader adoption, regulatory approval, and ultimately, the integration of extraction-free PCR technologies into routine clinical practice, advancing the early detection and management of invasive fungal infections.

5. Conclusions

This study demonstrates that extraction-free D2P technology achieves diagnostic accuracy comparable to conventional extraction-based PCR methods for the detection of clinically significant Candida species. By eliminating labor-intensive nucleic acid extraction steps without compromising sensitivity or specificity, D2P substantially reduces turnaround times, streamlines molecular workflows, and offers a scalable, cost-effective solution for fungal diagnostics. Broader clinical implementation of extraction-free PCR methods, exemplified by D2P, has the potential to enhance the early detection of invasive candidiasis, accelerate clinical decision-making, and improve patient outcomes. Adoption of such streamlined technologies is expected to reduce healthcare-associated morbidity and mortality associated with invasive fungal infections, advancing the efficiency and impact of molecular mycology in both routine and critical care settings.

CRediT authorship contribution statement

Sadia Almas: Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Rob E. Carpenter:** Writing – review & editing, Writing – original draft, Resources. **Vaibhav K. Tamrakar:** Writing – original draft, Methodology, Formal analysis, Data curation. **Aditya Sharma:** Resources, Data curation. **Kamalpreet Suri:** Investigation, Data curation. **Salima Karki:** Data curation. **Katelyn Kyser:** Formal analysis, Data curation. **Randy Sronce:** Formal analysis, Data curation. **Rahul Sharma:** Writing – original draft, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

REC, RS, and SA report financial interests in Scienetix, LLC. These interests have been disclosed in accordance with journal policy, and all efforts have been made to ensure the objectivity and integrity of the research presented in this study. All other authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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This study was conducted in accordance with institutional ethical guidelines. Ethical review and approval were waived as it involved the use of de-identified residual clinical specimens.

Informed Consent Statement

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Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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