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Standardization And Comparison Of Four Different Dna Extraction Techniques Using Spiked Tissue Samples

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ABSTRACT

Background: The extraction of high-quality DNA is a critical prerequisite for molecular diagnostic techniques like PCR and gene sequencing, particularly for invasive fungal infections, where rigid cell walls complicate the process. This study aimed to standardise and compare four different DNA extraction protocols to identify the most effective method for clinical application.

Methods: Tissue samples were spiked with *Aspergillus flavus* conidia (10^8 CFU/ml). Four extraction techniques were evaluated: Phenol-Chloroform (Method A), CTAB (Method B), Potassium Acetate (Method C), and a Column-Based method (Method D). The quality and quantity of the extracted DNA were measured using a nanophotometer and confirmed via conventional PCR using pan-fungal (ITS1, ITS4) primers.

Results: Statistical analysis showed a significant difference between the methods (p=0.02). While Method A (Phenol-Chloroform) provided a high mean DNA yield (282.4 ng/µl), Method D (Column-Based) consistently produced the highest purity, with an ideal A260/280 ratio of 1.9. Methods B and C were found to be less effective in terms of the balance between yield and purity.

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INTRODUCTION:

The extraction and purification of DNA are foundational steps in molecular biology, serving as one of the diagnostic processes. The quality and the quantity of the purified DNA template acquired in the extraction is critical for conducting molecular experiments such as Polymerase Chain Reaction (PCR) and gene sequencing [1]. A successful DNA extraction involves three major steps: disruption of the tissue, denaturation of the protein complex and purification of the nucleic acid [2]. A single optimal extraction of DNA is challenging due to widely varying biological materials (3). For instance, extracting DNA from tissue with fungal infection is complicated due to their rigid cell walls, such as lipids, peptides and chitin [1].

Numerous extraction methods have been developed broadly and categorised into two methods: solution-based methods, such as the classic phenol-chloroform method, salting and cetyltrimethylammonium bromide (CTAB) method [1,4]. Solid-phase methods utilise materials such as silica, glass particles, and magnetic beads within spin columns, avoiding liquid-liquid separation steps, overcoming solution-based methods [1]. The constant evolution and diversity of available techniques necessitate continuous comparison to identify the most suitable protocol in terms of reproducibility, speed, cost-effectiveness, and efficacy for a given application. However, the clinical utility of these molecular methods has been limited due to a lack of standardisation. Establishing a standardised procedure is essential to

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ensure consistent results and integrating into diagnosing invasive fungal infections over gold standard methods, such as direct microscopy and culture. Hence, we aimed to compare and evaluate the quality and quantity of four different DNA extraction methods from spiked clinical samples.

MATERIAL & METHODS:

This study was conducted at Sri Ramachandra Institute of Higher Education and Research, India and approved by the Institutional Ethics Committee (Ref: IEC-NI/22/JUL/83/73). Tissue samples that were negative for 20% Potassium hydroxide (KOH) and culture were used for spiking in this study. *Aspergillus flavus* (ATCC 9643) were sub-cultured in Sabouraud's Dextrose Agar (SDA) and incubated at 37°C for 48 hours. The conidia were added to double-distilled autoclaved water (DDW) and counted using a Neubauer chamber, and adjusted to a 0.5 McFarland standard at a concentration of 10^8 colony-forming units (CFU) per ml. These conidia were added to each of ~50mg non-infected tissue for all the four extraction methods.

Nucleic acid extraction methods:

Extraction method A: Phenol-Chloroform method:

Based on the previously published work by **Vijayakumar et al. (2012)** minor adjustments were made in this method for standardisation [9]. To the sample, 500 µl of TESS buffer (100 mM Tris base, pH: 8.0; 10 mM EDTA, pH: 8.0; 100 mM NaCl, 3% SDS) and 20 µl of β-mercaptoethanol were added. All the tubes were incubated at 100°C for 15 min in a water bath, centrifuged at 10,000 rpm for 10 minutes. About 400 µl of the top-most layer was pipetted into a new set of 1.5 ml microcentrifuge tubes, then 500 µl of Phenol-Chloroform (1:1) was added to each tube, vortexed thoroughly and centrifuged at 10,000 rpm for 10 min. The top-most aqueous layer of about 400 µl was pipetted into a new 1.5ml microcentrifuge tube, then 500 µl of chloroform was added. The tubes were vortexed well and centrifuged at 10000rpm for 10min. The above step was repeated if more white precipitate was observed in the tube. About 300 µl of the topmost aqueous layer was pipetted into a new set of 1.5ml microcentrifuge tubes. An equal volume of absolute ice-cold Isopropyl alcohol (IPA) was added to the tube, and the contents were gently mixed by tilting. The tubes were centrifuged at 10,000 rpm for 10 min. The fluid was discarded without disturbing the pellet. 200µl of ice-cold 70% ethanol was added to the tube. The tubes were centrifuged at 10,000 rpm for 10 minutes. Then fluid was poured out, and tubes were air-dried inverted or in a dry bath at 60°C for 5-10 minutes. The DNA pellet was re-suspended in 100 µl of Nuclease-Free Water.

Extraction method B: Cetyltrimethyl Ammonium Bromide (CTAB) method:

The genomic DNA was isolated as per **Inga *et al.* (2014**), using certain modifications [4]. To the sample, 500 µl TRISS lysis buffer (100mM Tris, PH:8.0, 20mM of EDTA, pH: 8.0, 2% CTAB) and 20µl of proteinase K were added to the tube, placed at incubated at 60°C for 30 min in a water bath. Around 140 µl of 0.5% CTAB (2M NaCl and 0.5% (w/v) CTAB) was added to the tube and incubated at room temperature for 10 min. 500 µl of chloroform: isoamyl alcohol (24:1) was added, vortexed well and centrifuged at 10,000rpm for 10 min. About 400µl of the topmost aqueous layer was pipetted into a new 1.5ml microcentrifuge tube. For precipitation, an equal volume of ice-cold isopropanol is mixed by inverting the tubes several times, then centrifuged at 10000rpm for 10 min, and the fluid is discarded. To the pellet, 300µl of ice-cold 70% ethanol was added and centrifuged at 10000 rpm for 10 min and then repeat the above step. Dry the tube in a biosafety cabinet by placing the tube in an inverse position for 1h, and the DNA was resuspended in 100µl of nuclease-free water.

Extraction method C: Potassium acetate method:

Standardisation of this method was done according to **De Armas et al. (2005) [10].** To the sample, 500 µl TRISS lysis buffer (50 mM Tris, pH:8.0, 10 mM EDTA, pH: 8.0, 200mM NaCl, 2% SDS) was added and incubated in a hot bath at 100°C for 15 min. Now add 200µl of potassium acetate, followed by mixing using vortex for 15sec, and incubate at room temperature for 10min. Around 500µl of chloroform: isoamyl alcohol (24:1) was added to the above mix and vortexed, followed by centrifugation at 10000rpm for 5min. Transfer supernatant to the new microcentrifuge tube, then add an equal volume of ice-cold isopropanol and mix by inverting the tube several times. Then, centrifuge at 10000rpm for 5 min and the fluid was discarded. To the pellet, add 300µl of ice-cold 70%ethanol and centrifuge at 10000rpm for 5 min. The ethanol step was repeated. Dry the tube in the biosafety cabinet by placing the tube inverse position for 1h. The DNA was resuspended in 100 µl of nuclease-free water.

Extraction method D: Column-Based Extraction method:

This method was done according to **Poh JJ *et. al.*,** (2014) with minor modification [7]. To the sample, 500 µl of TESS buffer (150 mM Tris base pH:8.0, 10 mM EDTA, 100 mM NaCl, 3% SDS) was added along with 20 µl of proteinase K**.** The tubes were incubated at 60°C for 30 min in a water bath, then

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centrifuged at 10,000 rpm for 10 min. About 200 µl of 5M potassium acetate was added to the tubes and incubated at room temperature for 10 min. Then, transfer 500µl of the fluid to the spin column and centrifuge at 10,000rpm for 1min. The fluid was discarded from the collecting tubes, and the above step was repeated for the remaining fluid. Ice-cold 70% ethanol was added to the spin column, followed by centrifugation at 10000rpm for 1min. Repeat this step. Then empty spin was done at 10,000rpm for 3min. The spin column was transferred into a new 1.5ml microcentrifuge tube, and 60µl of Nuclease-free water was added and incubated at room temperature for 2min. Then centrifuge the tubes at 10,000rpm for 1min and discard the spin column.

The comparison of the above four different DNA extraction methods is mentioned in Table 1

Table 1 – DNA extraction methods

Extraction method	Phenol-Chloroform	CTAB	Potassium acetate	Column-Based Extraction
Processing time (h: mm)	1:10min	0:50min	0:40min	0:45min
Reagents	Phenol, Chloroform	CTAB, Proteinase K	Potassium Acetate	Proteinase K, Spin column
Special Equipment	Dry bath, Centrifuge	Dry bath, Centrifuge	Centrifuge	Water bath, Centrifuge
Detection Limit	20ng/µl	50ng/µl	20ng/µl	40ng/µl

Spectrophotometric detection of DNA concentration

The concentration, purity and absorbance at 260-280 nm (A260/A280 ratio) were measured using a nanophotometer N60 (Implen, Germany) using 1 µl of each DNA template. NFW was used as a blank as well as in eluting of DNA during isolation.

Polymerase Chain Reaction:

Pan-fungal primers (ITS1, ITS4) were used for identification. Each reaction mixture contained 23µl of the PCR master mix plus 2µl of the extracted DNA. The amplifications were carried out for a total of 25µl reaction mixture. The conditions of the amplification were: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30sec, annealing at 56°C for 30sec, extension at 72°C for 30sec, and final extension at 72°C for 10 min.

Statistical analysis:

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) software version 30.0. ANOVA test was used to compare the amount of fungal DNA of each extraction method, and $p<0.05$ was taken to be statistically significant.

RESULTS:

Four methods for the extraction of fungal DNA from spiked tissue samples were tried out. The main criteria for evaluation of the extracted DNA were purity and the amount of DNA acquired in all the methods, which were further amplified by conventional PCR and compared as mentioned in Table 2/Figure 1. Extraction method A showed an abundant amount of fungal DNA, whereas method D showed pure fungal DNA with R260/280 of 1.9. Extraction B and C did not show pure fungal DNA when compared to methods A and D. Statistical analysis using the ANOVA test showed a statistically significant difference with p value of 0.02 in terms of fungal DNA (ng/µl).

Table 2 – DNA concentration of different extraction methods

Extraction methods	**A**		**B**		**C**		**D**	
Samples weight/purity	**ng/µl**	**R260/280**	**ng/µl**	**R260/280**	**ng/µl**	**R260/280**	**ng/µl**	**R260/280**
1	617.15	1.8	89.7	1.9	486.3	2.2	1453.9	1.9
2	327.9	2.0	6.45	1.6	274.7	1.9	2722.2	1.9
3	293.90	1.9	12.8	2.2	150.7	1.9	1247.9	1.9
4	106.95	2.2	3.45	2.0	63.3	1.9	170.1	2.0
5	32.75	2.1	10	2.0	31.0	1.8	99.9	1.9
Mean	282.4	1.97	12.6	1.9	188.9	1.9	957.3	1.9

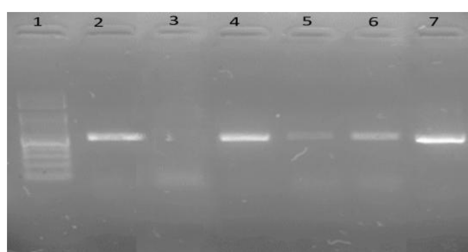

Figure 1 - Conventional PCR of four DNA extraction methods (Lane 1: DNA Marker, Lane 2: PC (*A.flaves* ATCC 9643), Lane 3: NC, Lane 4: Method A, Lane 5: Method B, Lane 6: Method C, Lane 7: Method D)

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DISCUSSION:

Extraction of DNA from fungi is challenging when compared to bacteria, virus or mammalian cells due to the complex fungal cell wall, which hinders the release of DNA. The inherent challenges in extracting high-quality DNA from fungal-infected tissues are characterised by complex and rigid cell walls composed of chitin, (1-3)-β-d-glucan and peptides. These structural components, particularly chitin microfibrils and melanin provides enormous tensile strength and chemical resistance, often hindering the efficient release of intracellular DNA [5]. This study highlights the comparative analysis of four different extraction techniques, which yield higher quantities of DNA.

In this study, extraction Method A (Phenol-Chloroform) demonstrated the highest mean DNA yield (282.4 ng/µL). This aligns with the established status of phenol-chloroform as a "gold standard" for maximizing yields from limited starting material [6]. The use of *β*-mercaptoethanol in this method likely assisted in denaturing proteins and inhibiting nucleases by reducing disulfide bonds [1]. In contrast, this study also demonstrated that Extraction Method D (Column-Based) yielded the highest purity with an average A260/280 ratio of 1.9, which falls within the ideal range (1.8–2.0) for downstream molecular applications. This method utilizes silica-gel membranes to adsorb DNA in the presence of high salt concentrations, effectively removing carbohydrates, proteins, and other tissue metabolites that can inhibit PCR. Though the yield of Method D was lower than that of Method A in our study, the safety, speed, and standardisation of the column-based method make it highly suitable for clinical diagnostic environments where high-throughput processing is required. Extraction Methods B (CTAB) and C (Potassium Acetate) did not provide the same balance of yield and purity as Methods A and D in this study. CTAB, effective for removing polysaccharides and polyphenols, showed limited success here, likely needing more intensive grinding to break the fungal cell wall [8]. Overall, our findings suggest the column-based method is preferable for downstream molecular analysis due to its higher purity. The purity is often more critical than absolute quantity for the success of sensitive techniques like conventional PCR or gene sequencing.

CONCLUSION:

This study successfully standardised and compared four DNA extraction methods from spiked tissue samples. Among all, the Extraction Method D (Column-Based) with high-purity DNA free from contaminants was found to be the most effective method, though it had less DNA quantity compared to Method A. Establishing the appropriate standardised procedure is a vital step towards integrating molecular diagnostics into the management of invasive fungal infections, ultimately providing results more quickly than traditional culture methods.

STUDY LIMITATION

This study was conducted from a single centre. There is a need of multicentric study for better understanding of different diagnostic molecular methods.

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