Acta Veterinaria-Beograd 2024, 74 (3), 398-408
UDK: 582.282.123.4:547.963.3
DOI: 10.2478/acve-2024-0027

Research article

DEVELOPMENT OF A SIMPLE AND RAPID DNA EXTRACTION METHOD FOR *ASPERGILLUS FLAVUS*

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(Received 05 June, Accepted 05 August 2024)

Aspergillus species are known to be very important in human and domestic animal health. *Aspergillus* species commonly cause severe systemic and skin infections, as well as allergic lung diseases. With the development of PCR techniques, these methods are used to identify and diagnose fungi. DNA extraction from *Aspergillus* species is difficult because the fungal cell wall structure is very durable and complex. Fungal DNA extraction methods containing proteinase K and liquid nitrogen are widely used to break down the cell wall. However, these methods cause DNA loss during the extraction in *Aspergillus* species. In this study, on the contrary, the commonly used DNA extraction by means of ammonium hydroxide, which is generally used to break down chitin in DNA extraction of ticks and plants, is used. The efficiency of the cell wall lysis method from *A. flavus* with ammonium hydroxide was compared with methods containing proteinase K and liquid nitrogen. For this purpose, DNA extraction of *A. flavus* was tried using three different methods. As a result, the cell wall of *A. flavus* was lysed using ammonium hydroxide in this study. The obtained DNA's quality, concentration, and PCR performance were sufficient. This method has been evaluated as a faster, more straightforward, and more economical alternative.

Keywords: Aspergillosis; Ammonium hydroxide; Extraction; Proteinase K; Liquid nitrogen.

INTRODUCTION

Aspergillus is a fungus that is widespread in nature and can spread widely through airborne transmission [1]. Along with *A. fumigatus, A. flavus* is another dominant species involved in invasive aspergillosis [2]. Invasive aspergillosis plays an essential role in the morbidity and mortality of immunocompromised individuals, bone marrow transplant recipients, cancer patients, human immunodeficiency virus patients, and patients treated with immunomodulators worldwide [3,4]. *A. flavus* infections are common in various food and feed ingredients, especially in developing countries and regions [5]. Aflatoxins are secondary metabolites mainly produced by *A. flavus* and

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are highly toxic compounds. They pose a severe hazard to human and animal health due to their carcinogenicity, hepatotoxicity, teratogenicity, and immunotoxicity [6]. The *Aspergillus* ssp affects the human respiratory system, such as the lungs, pharynx, and trachea. Consumption of foods contaminated with aflatoxins can directly affect human health. Also, aflatoxins in feedstuffs ingested by dairy cows, goats or chickens can be transferred to milk or eggs and eventually be consumed by humans [7].

The antigen-based galactomannan (GM) test is the gold standard in diagnosing Aspergillus. However, because the performance of the GM test is still not very good and the result time is variable, some device tests, immunological marker tests, and tests related to iron gain and PCR are developed, and studies are continuing to optimize them by using them as a standard. The performance of this test can be variable, leading to the development and optimization of alternative diagnostic methods. These include device tests, immunological marker tests, tests related to iron metabolism, and PCR (Polymerase Chain Reaction) tests. The goal is to standardize and improve these methods to achieve more reliable and timely results. The most important issue in obtaining DNA from Aspergillus species is the most widely used genomic DNA extraction method, which is efficient in speed and sensitivity and breaks down the cell wall without damaging the genomic DNA. DNA extraction protocols are essential to obtaining high-quality quantity and purity in PCR applications [8].

Disruption of the fibrillar structure of the cell wall in filamentous fungi causes difficulties, and traditional DNA extraction protocols are insufficient [9–12]. There are two main steps in fungal genomic DNA extraction: first, the cell wall disruption and extraction, followed by purification of the genomic DNA. Liquid nitrogen is generally used to break down the cell wall [13,14]. Extraction of genomic DNA is usually performed by cetyl trimethyl ammonium bromide solution (CTAB) extraction and purification with phenol, chloroform, and precipitation with isopropanol or ethanol [15]. Liquid nitrogen, used to destroy the cell wall, has disadvantages such as being difficult to supply and adversely affecting the DNA extraction in the desired amount and quality due to its toxicity [10]. In the use of proteinase K, time is significant, and the DNA quality may be low due to its incorrect application [11,12]. Identifying a rapid fungal DNA extraction method will reduce the time required for fungal identification.

Heating with ammonium hydroxide is effectively used to break down the cell wall of cellulose and chitin in the DNA extraction of plants and parasites [16–18]. This method has the advantage of being a simple, fast, and inexpensive method whose suitability has been proven in ticks with the fibrillar structure of the cell wall equivalent to filamentous fungi [17]. However, the effectiveness of ammonium hydroxide in the breakdown of fungal cell walls has yet to be determined.

In this study, the ammonium hydroxide method will be used for the first time to break down the cell wall of fungi, and the two most widely used methods in the extraction of fungi, the liquid nitrogen method and proteinase K enzyme digestion methods, were compared in terms of time, DNA amount and quality. The study is also crucial in

developing a method that provides an effective and short-term diagnosis. In addition, this method is also vital in terms of its use in DNA extraction from other filamentous fungi. The effectiveness of ammonium hydroxide, a cost-effective chemical that does not require complex procedures, has been determined. This study aims to modify the aluminum hydroxide method, previously used for ticks, and make it usable in fungal infections. In addition, the study compares two widely used methods (Proteinaz K and liquid nitrogen) for DNA extraction in fungal infections.

MATERIAL AND METHODS

Preparation of *Aspergillus flavus* strain

In this study, the *A. flavus* strain ATCC® (16883) was obtained from the culture collection of Aksaray University, Faculty of Veterinary Medicine.

To reactivate the *A. flavus* strain in the culture collection, it was inoculated into Sabouraud-2 % Dextrose liquid agar (Merck, Germany; 105438) and incubated at 30°C for 72 hours. Fungal colonies were transferred into 5 ml 0.9% physiological saline, and spectrophotometrically analyzed A530 (McFarland standard 0.5) was measured at a concentration of 1-5 x 10^6 cfu/ml. It was transferred to a clean microcentrifuge tube, centrifuged at 4000 g for 5 minutes, and the pellet was prepared for use by pouring the supernatant (19).

Cell Wall Breakdown Methods

Proteinase K enzyme method

Fungal samples in TE buffer(10 mM Tris HCl [pH 7.6], 1Mm EDTA) 300 mg/ml proteinase K; 0.5% Tween-20; incubated 18 h at 60°C [20,21].

Liquid Nitrogen Method

After the fungal samples were kept in 300 µl of TE buffer at 200° C overnight (pre-sterilized by baking), 1 ml of liquid nitrogen was added to the microcentrifuge tubes and diluted until powdery in a mortar [20,22].

Ammonium Hydroxide Method

0.7 M 100 µl of ammonium hydroxide was added to the 10-20 mg fungal mass (20-40 mm^2 mycellial tissue) fungal samples and waited in the thermal block at 100°C for 15 minutes to expose the DNA. The lids of the microcentrifuge tubes were left open, and after cooling at room temperature, they were kept at 100°C for 15 minutes to evaporate the ammonium hydroxide [23].

Fungal Genomic DNA Extraction

Purification of Genomic DNA

600 µl of DNA extraction solution and 4.6 µl of RNAse at a final concentration of 15 mg/ml were added (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, %0.6 SDS) to the samples taken into a clean microcentrifuge tube for cell lysis. After mixing with the vortex for a short time, it was left in the thermal block at 65°C for 10 minutes [19,22].

For protein precipitation, 140 µL of protein precipitation solution was added (3 M sodium acetate, pH 5.3), slowly inverted, incubated at – 20°C for 5 minutes, and then centrifuged at 13,000 g for 5 minutes [19,22].

The supernatant was transferred to a clean microcentrifuge for DNA precipitation. The supernatant was slowly removed by adding 600 µl of isopropanol to the tube, slowly turned it upside down, waited for the DNA to become visible, and centrifuged at 13,000 g×2 min. To wash the DNA pellet, 650 µL of 75% ethanol was added and inverted 3-4 times. After the tubes were centrifuged at 16,000 g×2 minutes, the supernatant was removed with the help of a pipette, and the ethanol was evaporated on blotting paper at room temperature and kept at 65 °C for 1 hour. DNA was stored at – 20 °C until used in PCR analysis [20,22].

Determination of Absorbance of DNAs

Optical densities (OD) of DNA extracts at 260 nm and 280 nm wavelengths were determined using a spectrophotometer (Nanodrop2000, Thermo Scientific) to determine purity and quantity. DNA rehydration solution was used as a blank. Measurements of the 1µl sample were made at 260 nm (OD260) and 280 nm (OD280) wavelengths. Concentrations were obtained as ng/ml. The differences between the different cell wall lysis methods will be determined and compared, and the quality samples with values close to 1.8-2.0 were used in PCR by proportioning each other according to values of the OD260 and OD280. The averages of results were calculated in Microsoft Excel (Microsoft Office 2010).

Amplification and validation by PCR

PCR amplification and validation of fungal DNA with universal fungi primers

The highly conserved 18S rRNA region was targeted for PCR amplification via PCR of fungal DNA; for this purpose, universal primers (18S-Forward primer 5 – ACATCCAAGGAAGGCAGCAGG – 3 and 18S-Reverse primer 5 – GAGTTTCCCCGTGTTGAGTCAA – 3) were used, and a 783–800 bp band was considered as positive for fungal DNA. [24].

A total of 1.2 µl of primers, 4 µl of Master Mix, 14.8 µl of sterile ultrapure water, and 5 µl of DNA were prepared for a total of 25 µl for amplification in each PCR

tube content. This mixture was amplified in a heat cycler with a 35-cycle reaction, each cycle consisting of 15 s at 94 °C, 45 s at 58°C, and 30 s at 72°C after 5 min of pre-denaturation at 95°C. As a final extension, after the DNAs were kept for 5 min at 72 °C (24)A 1.5% agarose gel containing 5 µg/ml ethidium bromide was prepared and subjected to electrophoresis for imaging. Bands with a size of about 800 bp were evaluated as positive for NNA of fungal agents using a 100 bp DNA ladder as a marker.

PCR amplification and validation of *A. flavus* DNA with specific primers

Forward primer 5-CGACGTCTACAAGCCTTCTGGAAA-3 and reverse primer 5-CAGCAGACCGTCATTGTTCTTGTC-3 were used for PCR amplification of *A. flavus* DNA ~ a 200 bp band was considered as positive for *A. flavus* [25].

A total of 1.2 µl of primers, 4 µl of Master Mix, 14.8 µl of sterile ultrapure water, and 5 µl of DNA were prepared for a total of 25 µl for amplification in each PCR tube content. This mixture was amplified in a heat cycler with a reaction of 38 cycles, each cycle consisting of 1 min at 94 °C, 1 min at 59 °C, and 1 min at 72 °C, after 5 min of pre-denaturation at 94 °C. As a final extension, DNAs were staged for 5 min at 72 °C [19,25].

Statistical analysis

The General Linear Model (IBM SPSS Statistic 23 Programme) analyzed the mean quantities of bacterial DNA obtained from each isolate. Multivariate tests were used to determine the significance of the different results of cell wall breakdown methods. Such comparisons were considered statistically significant when P values were < 0.01.

RESULTS

Purity and quantification of DNA extracts

DNA extraction was performed after lysis of the cell wall with proteinase K, liquid nitrogen, and ammonium hydroxide. Optical density measurements of DNA extract concentrations were 37.8, respectively; 6.4; it was determined as 43.9 ng/µl. The purity of the extracted DNA varied as determined by the ratio of A260 to A280 (Table 1).

PCR amplification and validation of fungal DNA with universal fungi primers

Proteinase K targets the 18S rRNA region after PCR amplification of fungal DNA obtained after digestion with liquid nitrogen and ammonium hydroxide; a 783–800 bp band is observed (Figure 1). This image is positive for fungal DNA presence.

Table 1. Comparison of methods for *Aspergillus flavus* isolates

Method	Concentration of DNA ng/µl (mean ± sd)	Purity 260/280 nm (mean ± sd)
PK	37.8±0.834	1,79 ± 0,02
LN	6.4±1,06	1,91 ± 0,04
AH	43.9±0,744	1,91 ± 0,02

Mean values of various parameters from these methods
(**PK**: Proteinase K Method; **LN**: Liquid Nitrogen Method; **AH:** Ammonium Hydroxide Method)

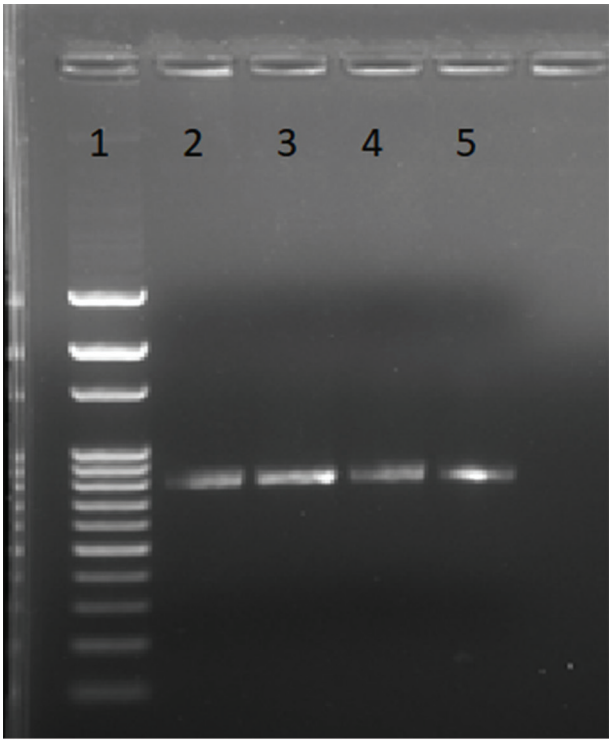

Figure 1. Fungal DNA 18S rRNA; 1.Ladner(100 bp) 2. Proteinase K 3. Liquid Nitrogen 4-5. Ammonium Hydroxide

PCR amplification and validation of *A. flavus* DNA with specific primers

A~200 bp band was observed after PCR amplification of *A. flavus* DNA obtained after degradation with proteinase K, liquid nitrogen, and ammonium hydroxide. (Figure 2).

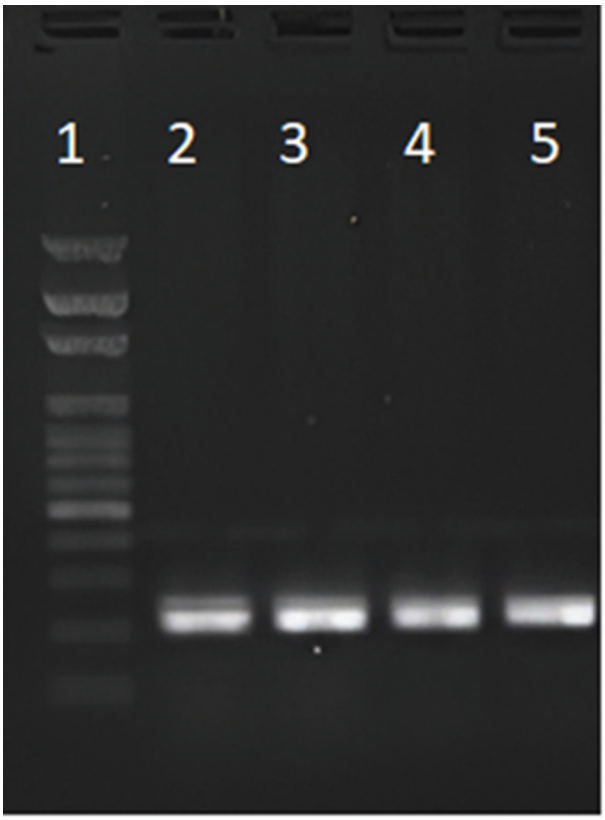

Figure 2. *Aspergillus Flavus* DNA; 1. Ladder (100 bp) 2. Proteinase K 3. Liquid Nitrogen 4-5. Ammonium Hydroxide

DISCUSSION

The importance of rapid identification of *A. flavus* in agriculture and animal products and animal feed increases the importance due to the ability of the organism to cause disease and produce aflatoxin. Therefore, classical PCR and gel electrophoresis methods are widely used to diagnose the agent [30].As molecular tools for diagnosis are increasingly used, DNA purification is a critical step in diagnosing infectious agents and is indispensable in daily laboratory life. Therefore, it is essential to be able to use affordable DNA purification methods to work on the samples with maximum efficiency, as only low amounts of target DNA may be present [26]. Aspergillosis is one of the most common fungal diseases and is accompanied by high morbidity and mortality [8]. Filamentous fungi cause significant problems in terms of human and animal health due to the mycotoxins they produce. People with weakened immune systems are susceptible to *Aspergillus* species. This gradually increases the importance of the disease. Current DNA extraction methods from filamentous fungi can take an hour to a day. These methods require tampons and tools to crush the fungal

tissues. Commercial extraction kits are known to reduce the time spent preparing and extracting genomic DNA from fungal samples, but they are expensive methods [27]. Like filamentous fungi, ticks also have a chitin layer, making DNA extraction from ticks difficult. Protocols include freezing, phenol-chloroform, and proteinase K steps in nucleic acid extraction [28]. These multi-stage protocols waste time, complicate the procedure, and increase the cost. For this purpose, a more straightforward method, extraction by heating with ammonium hydroxide [16] is applied and reported in studies on ticks [18,29]. This method has the advantage of being a simple, fast, and inexpensive method that is proven to be suitable for the extraction used in ticks and for the isolation of DNA from plants [17,26]. Similar to our study, these studies were conducted on ticks [17,26]. Among the methods used, aluminum hydroxide was especially applied to fungi for the first time. In addition, it has been reported that there is no risk of environmental contamination in the DNA isolation method used [26].

In this study, overnight incubation was carried out using proteinase K and liquid nitrogen methods to break down the cell wall during the DNA isolation phase from *A. flavus*. In contrast, DNA isolation was successfully carried out by breaking the cell wall in 15 minutes with the ammonium hydroxide method. This situation also shows that it performs in breaking down the cell wall. According to the quantitative measurement results of the obtained DNA purity, it was determined that the concentration of proteinase K was higher than for liquid nitrogen, and the applicability of the method was high. In addition, the storage of ammonium hydroxide and the fact that it is very easy to obtain compared to liquid nitrogen makes the method advantageous. Compared to the liquid nitrogen method, it has been highly applicable because it has significant advantages regarding environmental contamination.

CONCLUSION

In conclusion, this study determined that the ammonium hydroxide method performed well in breaking down the cell wall, and DNA was obtained in a shorter time than the other two classical methods. However, studies with more clinical samples may be needed to validate this method as a new DNA isolation test from fungi. This study is presented as a new candidate for use in invasive aspergillosis diagnostic techniques.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

As stated on the author guidelines page, the authors attest to their adherence to the journal's ethical norms. Given that the research presented in this publication involved microorganisms, ethical approval was not necessary.

Authors' contributions

GSG, KA contributed to the study's design and conception, while KA, GSG were involved in data curation and visualization. All authors participated in writing and revising the manuscript, which they then read and approved as final.

Declaration of interest

There is no conflict of interest between the authors.

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RAZVOJ JEDNOSTAVNE I BRZE METODE EKSTRAKCIJE DNK IZ *ASPERGILLUS FLAVUS*-A

Gökçenur SANİOĞLU GÖLEN, Kadir AKAR

Poznato je da su vrste *Aspergillus* veoma važne za zdravlje ljudi i domaćih životinja. Vrste *Aspergillus* obično izazivaju teške sistemske i kožne infekcije, kao i alergijske bolesti pluća. Sa razvojem PCR tehnika, ove metode se koriste za identifikaciju i dijagnostiku gljivica. Ekstrakcija DNK iz vrste *Aspergillus* je teška jer je struktura ćelijskog zida gljivica veoma izdržljiva i složena. Metode ekstrakcije DNK gljivica koje sadrže proteinazu K i tečni azot se široko koriste za razbijanje ćelijskog zida. Međutim, ove metode izazivaju gubitak DNK tokom ekstrakcije kod vrsta *Aspergillus*. U ovoj studiji, naprotiv, koristi se najčešće korišćena ekstrakcija DNK pomoću amonijum hidroksida, koja se uglavnom koristi za razgradnju hitina u ekstrakciji DNK krpelja i biljaka. Efikasnost metode lize ćelijskog zida iz *A. flavus* sa amonijum hidroksidom je upoređena sa metodama koje sadrže proteinazu K i tečni azot. U tu svrhu je pokušana ekstrakcija DNK *A. flavus* pomoću tri različite metode. Kao rezultat toga, ćelijski zid *A. flavus* je liziran korišćenjem amonijum hidroksida u ovoj studiji. Kvalitet dobijene DNK, koncentracija i PCR performanse su bili dovoljni. Ova metoda je ocenjena kao brža, jednostavnija i ekonomičnija alternativa.