



Identification of some human pathogenic fungi using four DNA extraction methods

*¹SAMUEL, TO; ²EBABHI, AM; ¹ADEKUNLE, AA

¹Department of Botany, Faculty of Science, University of Lagos, Akoka-Yaba, Lagos, Nigeria

²Biology Unit, Department of Education Science, Distance Learning Institute, University of Lagos, Akoka, Lagos, Nigeria
temmitade@yahoo.com, ebabhi.meg@gmail.com, aaded63@yahoo.com

*Corresponding author: E-mail: temmitade@yahoo.com; tosamuel@yahoo.com

Tel: 2348036410449

ABSTRACT: Dermatophytes being animal and human pathogenic fungi infect some human at one point or the other in their lifetime. For effective control of dermatophytes, accurate identification of the specific species/strain involved must be known. Stocks from pathogenic fungi isolated from infected areas on different patients, around Lagos-Nigeria were analysed using molecular methods (DNA extraction, PCR-RFLP and DNA sequencing). Four DNA extraction protocols were employed in the identification of the fungal isolates. Sixteen different fungal isolates were identified, and based on the molecular data these were classified into six species of dermatophytes belonging to the genera *Microsporium*, *Trichophyton* and *Epidermaphyton*, two species of systemic mycoses fungi and eight opportunistic human pathogenic fungi. The results also revealed that CTAB protocol, Modified CTAB protocol and the GNOME kit used in this work were only able to extract non-dermatophytes DNA. Only the Zymo DNA kit was able to isolate dermatophytes DNA. DNA extraction which is the first step in all molecular studies showed that one DNA extraction method might not be able to extract all fungal DNA for proper identification, diagnosis and treatment of fungal infections. © JASEM

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Dermatomycoses is contagious and represents a significant health problem in Nigeria. In particular, Lagos metropolis has a higher incidence of dermatomycoses due to crowded living conditions (Adetosoye, 1977; Ogbonna *et al.*, 1985; Anosike *et al.*, 2005). Incidences of the prevalence is not only particular to Lagos-Nigeria but is observed in many countries and crowded places in the world. For instance, Lakshmanan *et al.* (2015) reported a high incidence of fungal infection in rural India while Teklebirhan and Bitew (2015) reported a prevalence of 79.4% in 242 sampled cases.

The current diagnosis of dermatomycoses in most mycological laboratories inclusive of Lagos state, Nigeria is based upon conventional methods which is not species-specific. This does not give the accurate diagnostic results as the etiological patterns of most pathogenic organisms are changing just as there is change in climate. Balajee *et al.* (2007) also reported that for the increasing number of isolates that are uncommon, atypical, or unusual, DNA-based identification methods are being increasingly employed in many clinical laboratories.

According to Havlickova *et al.* (2008), dermatomycoses accounts for 20-25% mycotic infection worldwide. An effective treatment and prevention of dermatomycoses depend on knowledge of the specific dermatophyte. This study therefore aims at isolating and identifying this group of human pathogenic fungi responsible for dermatomycoses using molecular methods.

MATERIALS AND METHODS

Samples collection: Stock cultures of sixteen different isolates of human pathogenic fungi were collected in

Mc-Cartney bottles from the Mycology unit of Department of Botany, University of Lagos. These were sub cultured on freshly prepared commercially produced Sabouraud dextrose agar (SDA). The Sabouraud dextrose agar (SDA) was prepared according to manufacturer specification (Oxoid, Basingstoke, England).

DNA extraction: Extraction of fungal DNA was done using 4 different protocols namely Cetyltrimethyl ammonium bromide (CTAB) protocol, Modified CTAB protocol, Zymo kit by Zymo Research Corp (Hatfield-Pretoria 0028, South Africa) and GNOME kit by Qbiogene Corp.

DNA extraction using CTAB protocol: Ten millilitres (10 ml) of sterilized distilled water was added to fresh pure culture of each isolate to be extracted, a suspension was made by gently probing the colony with the tip of a sterilised Pasteur pipette. The suspension was transferred into an eppendorf tube, and was spin for 30 seconds. The supernatant was decanted and 400 µl of CTAB buffer and 75 µl of 10 % SDS were added to the pellet. This was then incubated in water bath at the temperature of 65 °C for fifteen minutes. Five hundred microlitres (500 µl) of chloroform was added and this was mixed by rocking on an orbital shaker at 100 rpm for 5 minutes, which was then spinned at 10,000 rpm for 10 minutes. The supernatant was collected into fresh eppendorf tube and 500 µl of iso-propanol was added to the supernatant. This was kept at -20 °C for one hour after which it was spin at 14,000 rpm for ten minutes. The supernatant was decanted gently and the pellet (DNA) was washed with 70% ethanol then, the pellet was air dried for thirty minutes. The dried

*Corresponding author: E-mail: temmitade@yahoo.com; tosamuel@yahoo.com

pellet was then re-suspended in 200 µl of sterile distilled water.

DNA extraction using modified CTAB protocol: Ten millilitre (10 ml) of isolation buffer (10X CTAB) containing 80 µl of meta-mercaptoethanol in 50 ml blue cap tubes was pre-heated in 65 °C water bath. One gramme (1g) of freshly scrapped pure culture plates of each fungal isolates was added to the preheated isolation buffer in each tube. The mixture was then incubated at 65 °C for 15 minutes. Ten millilitre (10 ml) of SEVAG (24:1 chloroform: Isoamyl alcohol), then mixed gently but thoroughly. The cap of the tubes containing the mixture was then open to release gas, which was re-tightened then rocked the tubes using an orbital shaker (100 rpm) for 60 minutes. After rocking, the tubes were spinned at 4000 rpm at 25 °C for 20 minutes. The mixture gave a clear and colourless aqueous solution at the top (containing the DNA), the clear colourless aqueous was removed into another set of tubes with the aid of a plastic transfer pipette. Two-third volume of isopropanol was added to the aqueous solution. The mixture was then mixed gently and stored in freezer (-20°C) for 24 hours. This was to allow the DNA in it to precipitate. The mixture was later spinned in centrifuge at 3000 rpm for 5 minutes to collect the precipitate, and the liquid in the tube was discarded. Then 3 ml of 70% ethanol was added to dislodge the pellet in order to facilitate 'washing'. It was spinned again at 3000 rpm for 5 minutes, the liquid was discarded and the alcohol was allowed to evaporate by leaving the tubes open and on its side. The DNA was re-suspended in 1.5 ml of water and stored at -20°C prior shipments for PCR and Sequencing.

DNA extraction using GNOME kit: A fresh pure culture of each fungal isolates was scraped into a micro-centrifuge tube and suspended in 200 µl extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM Na₂EDTA). The suspension was centrifuged for 5 min at 13,500 rpm. The supernatant was transferred to a new micro-centrifuge tube, and the nucleic acids were precipitated by adding 1 ml absolute ethanol. Suspensions were mixed and centrifuged for 2 min (13,500 rpm). The pellet was re-suspended in 400 µl Tris-EDTA, pH 8.0, 3 µl RNase (10 mg/ml) and incubated for 5 min at 37° C. Then, 10 µl ammonium acetate (4 M) and 1 ml absolute ethanol were added and gently mixed. This mixture was centrifuged for 3 min at 13,500 rpm and the supernatant discarded. The DNA pellet was dried in airflow for 15 min and finally resuspended in 40 µl sterile distilled water.

DNA extraction using ZYMO kit: Ten grammes of freshly prepared pure fungal culture plate of each isolate was scrapped and suspended in 50 µl of water to the tubes of a ZR BashingBead™ Lysis Rack. Four

hundred microlitres (400 µl) of Lysis Solution was added to each tube. Tubes cap were tight to prevent leakage. The ZR BashingBead™ Lysis Rack was Centrifuge at 5,500 rpm for 5 minutes. Two hundred and fifty microlitres (250 µl) of the supernatant was transferred to each well of a Deep-Well Block. Seven hundred and fifty microlitres (750 µl) of Fungal DNA Binding Buffer was added to the supernatant in the Deep-Well Block from Step 3. Completely covered with cover foil then mixed thoroughly by vortexing block for 2 minutes. The Deep-Well Block was centrifuge at 5,000 rpm for 5 minutes. After removing cover foil, 500 µl of each of the supernatants from Step 5 was transferred to the wells of Silicon-A™ Plate on a collection plate. The assembly was centrifuge at 5,000 rpm for 5 minutes. The flow was discarding through from the collection plate and repeat Step 5. Two hundred microlitres (200 µl) of DNA Pre-Wash Buffer was added to the wells of the Silicon-A™ Plate on the emptied Collection Plate and centrifuge the assembly at 5,000 rpm for 5 minutes. Five hundred microlitres 500 µl Fungal DNA Wash Buffer was added to the wells of the Silicon-A™ Plate on the collection plate and centrifuge the assembly at 5,000 rpm for 5 minutes. The Silicon-A™ Plate was transferred to an Elution Plate and 100 µl DNA Elution Buffer was added directly to the matrices in the plate. The assembly was centrifuged at 5,000rpm for 5 minutes.

DNA verification on 1% agarose gel electrophoresis: One percent (1%) of agarose gel was prepared by mixing 1.5g agarose with 150 ml 1X TBE Buffer. The mixture was boiled in a microwave until all agarose has dissolved (usually, two minutes). This was cooled down under running cold tap water; after which, 6µl of Ethidium Bromide was added and swirled to mix (done in the fume-hood). The gel was then poured into the tray and allowed to stand for at least thirty minutes before removing the combs. The gel was then placed in an electrophoresis tank. Five microliters of loading dye was spotted for 10 µl of each DNA sample on a parafilm paper. Using a pipette, 15µl of each sample was loaded into each "well" of the prepared 1% agarose gel; also one-half microgram of 1kb ladder (Lambda DNA HindIII) was loaded into the "marker" well. It was run for 45minutes at 110Milli Amps. The set-up was then viewed under the ultraviolet trans-illuminator for DNA quality and yield assessments and the photograph was taken.

Polymerase Chain Reaction (PCR) and DNA sequence: The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is used to amplify the internal transcribed spacer (ITS) region of the rDNA. The PCR was performed using the universal primer pair ITS1-ITS4 (Jackson *et al.*, 1999). The thermal cycler was

programmed for 4 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 2 min at 58 °C and 1.5 min at 72 °C. The nucleotide sequences were determined automatically using the cycle sequencing protocol by the BigDye Terminator v3.1 Cycle Sequencing Kit in the ABI PRISM 377-DNA Sequencer. Further identification and blasting in order to confirm their identification was achieved by comparing nucleotide sequences data that was produced from the fungal DNA extracted to sequences database at the genbank and calculated the statistical significance of matches. BLAST (Basic Local Alignment Search Tool), is a program used to infer functional and evolutionary relationships between sequences as well as to help identify members of gene families.

RESULTS AND DISCUSSION

The extracted genomic DNA from each of the isolated fungus which was size separated on 1% Agarose gel are as shown in Plates 1a-d. Plate 1a shows the electrophorogram of fungal DNA extracted with the CTAB protocol, this protocol extracted DNA of all the fungal isolates and showed good bands on the electrophorogram expect for the dermatophytes isolates (first lane: well 4, 5, 6, & 7) (Table 1). Plate 1b shows the electrophorogram of fungal DNA extracted with the modified CTAB protocol. Here, it was also noted that this protocol was only able to extract few fungal while dermatophytes isolates were among those not extract. Same results was noted with extraction done with the GNOME kit (Plate 1c) where only the dermatophytes species DNA (first lane, well 2 and 3) did not show any band on the electrophorogram. This is not the same with extraction results done with the Zymo kit as shown in Plate 1d. Here, the entire extracted fungal DNA showed good band on the electrophorogram.

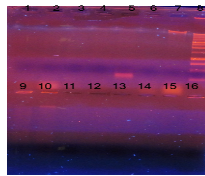


Plate 1a: Electrophorogram of extracted DNA samples using the CTAB protocol. The first lane contains the marker, C7, C8, B3, B4, B5, B6, D9. The second lane contains marker, D11, D12, D13, D14, D15, D16 and D10.

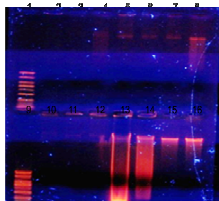


Plate 1b: Electrophorogram of extracted DNA samples using the modified CTAB protocol. The first lane contains B1, B2, B3, B4, C8, C7, D9 and the marker. The second lane contains D10, D11, D12, B5, B6, D14, D16 and D13.

The sub culture of the fungal isolates revealed that six of the isolates are likely to be dermatophytes due to their slow growing nature. These isolates showed colonies on the SDA plates after day 12 of sub culturing (Table 1). Other fungal isolates showed growth colonies within 48 hours of incubation.

The genera *Microsporium*, *Trichophyton* and *Epidermaphyton* were the group of dermatophytes identified (Table 3). These included: *Epidermaphyton floccosum*, *Microsporium audouinii*, *Microsporium nanum*, *Trichophyton concentricum* *Trichophyton mentagrophytes var. quinckeanun*, and *Trichophyton violaceum*. On the basis of their primary habitat associations, the above isolates are of three groups. These are the anthropophilic, zoophilic, and geophilic as depicted in Table 2. Two of the other identified species of the isolated fungi belong to the systemic mycoses group otherwise known as the “deep mycoses” and these are *Blastomyces dermatitidis* and *Exophiala dermatitidis*. The other eight species of the isolated fungi belong to the opportunistic mycoses group, and these include: *Absidia corymbifera*, *Aspergillus terreus*, *Emericlla nidulans*, *Mucor racemosus*, *Pencillium aschersonia*, *Pencillium citrinum*, *Aspergillus flavus*, *Aspergillus fumigatus*.

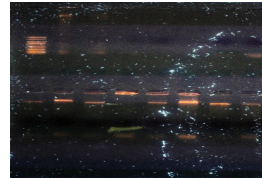


Plate 1c: Electrophorogram of extracted DNA samples using the Gnome kits. The first lane contains the marker, B3, B4, C7, C8, D9, D10, D11. The second lane contains the marker, C8, D9, D12, D13, D14, D15 and D16.

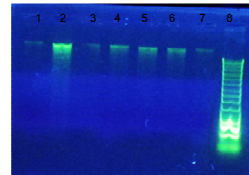


Plate 1d Electrophorogram of extracted DNA samples using the Zymo kits. The lanes labelled 1,2,3,4,5,6,7,8 contained sample number B1, B3, C7, B2, B4, B5, B6 and the Marker respectively.

The isolation and adequate identification of human pathogenic fungi is significant to clinical diagnosis and appropriate treatment of fungal infection in medical mycology. Over the years, it is noted that fungal isolates have been identified based on observations of colonial and microscopic morphology, along with tables, keys and textbook descriptions. Although, according to Balajee *et al.* (2007) this approach still has value for the identification of many fungal organisms, but requires expertise and can be problematic in determining a

species identification that is timely and useful in the management of high-risk patients.

Using four distinct DNA extraction protocols, six species from the three genera of dermatophytes, two species of systemic mycoses and eight species of opportunistic mycoses fungi were isolated and identified. According to Fredricks *et al.*, (2005) no single extraction method amongst those currently available is optimal for the molecular analysis of fungi. From the result of this present study, no single protocol was able to isolate all DNA from the samples. This agrees with the work of Knudsen *et al.* (2016) who had to use eight commonly used DNA extraction kits and sixteen protocols to extract quality DNA from human faeces, pig faeces and hospital sewages. They further concluded that DNA concentration, purity, and stability depend on the type of specimen and DNA isolation method. The quality of DNA extracted from the samples varied with the type of extraction protocol used. Of the four protocols used in the present study, quality DNA for molecular analysis was only extracted using Zymo kit. This could have been due to the ability to lyse the fungal cell wall. Zhang *et al.* (1996) and Muller *et al.* (1998) remarked that the major challenge for isolation of DNA of good quality and quantity from fungi lies in breaking the rigid cell walls, due to fungal nucleases and high polysaccharide contents of the cell wall. Melo *et al.*, (2006) agreed with Zhang *et al.*, (1996) and Muller *et al.* (1998) by concluding that DNA isolation from some fungal species is difficult. This is

because they have cell walls or capsules that are relatively unsusceptible to lysis. Although dermatophyte infections are treatable, there is a high rate of reinfection as a result of poor analysis of the causal organism. Of the four DNA extraction protocols employed in the study, the CTAB protocol, Modified CTAB protocol and the GNOME kit were able to extract DNA from the non-dermatophytic group. Only the Zymo DNA kit was able to extract DNA from the dermatophytic group. Growth of the non-dermatophytes group was noted in the fourth day of incubation which could lead to ineffective diagnoses if the observation was to stop there because the dermatophytic group started growing between day 12 - 14 of incubation. The result from this present study is in agreement with previous works (Fredricks *et al.*, 2005; Leite *et al.*, 2014) as it shows that no single DNA extraction protocol can isolate fungal DNA from the three groups of fungi encountered.

Conclusion: The results from this study revealed that DNA extraction which is the first step in all molecular studies showed that one DNA extraction method might not be able to extract all fungal DNA. The purity and yield of different fungal DNA extracts leads to adequate and effective sequence data for accurate identification, proper diagnosis and better understanding of the etiology of this group of pathogenic fungi in the prevention, treatment and eradication of infectious diseases.

Table 1: Fungal isolates showing

S/N	Fungi	Code	Day growth was observed	Growth rate (mm)
1	<i>Microsporum nanum</i>	B1	14	5.0
2	<i>Microsporum audouinii</i>	B2	14	5.0
3	<i>Trichophyton concentricum</i>	B3	14	5.0
4	<i>Epidermaphyton floccosum</i>	B4	13	5.0
5	<i>Trichophyton mentagrophytes</i>	B5	14	5.0
6	<i>Trichophyton violaceum</i>	B6	15	5.0
7	<i>Exophiala dermatitidis</i>	C7	6	5.0
8	<i>Blastomyces dermatitidis</i>	C8	6	5.0
9	<i>Emericella nidulans</i>	D9	4	5.0
10	<i>Aspergillus terreus</i>	D10	4	5.0
11	<i>Absidia corymbifera</i>	D11	4	5.0
12	<i>Penicillium aschersonia</i>	D12	4	5.0
13	<i>Penicillium citrinum</i>	D13	4	5.0
14	<i>Mucor racemosus</i>	D14	4	5.0
15	<i>Aspergillus flavus</i>	D15	4	5.0
16	<i>Aspergillus fumigatus</i>	D16	4	5.0

Table 2: The three groups of isolated dermatophytes based on their primary habitat

Primary habitat	Isolates			
Anthropophilic	<i>Epidermaphyton floccosum</i>	<i>Microsporum audouinii</i>	<i>Trichophyton violaceum</i>	<i>Trichophyton concentricum</i>
Zoophilic	<i>Trichophyton mentagrophytes var. quinckeanum</i>			
Geophilic	<i>Microsporum nanum</i>			

Table 3: Generated sequence data from the isolated fungi and the percentage of their significance of matches with the sequences database at the Genbank

Sequence data	Organism and % of significant match
110719-03_A07_TOC-ITS1 AACATGCGTGAGATTGTAAGTCTAGCTTTCCCCCTGTCC ATCACTTCCCCTCTATTTGGATAATGTTGCCCACTTAAG CGTCCGCTTCA	This sequences data was 99% significant matched with <i>Trichophyton mentagrophytes var. quinckeanum</i>
110719-09_I05_TOD-ITS4 GGACGAGGGCCCAACACACAAGCCGGGCTTGAGGGCA GCAATGACGCTCGACAGGCATGCCCCCGGAATACCA GGGGGCG	This sequences data was 99% significant matched with <i>Aspergillus terreus</i>
110719-03_K05_TOE-ITS1 TGGCCACTTGAACAACTTGTGGACCACGCTTACAT GCATGTGGGTTCATTTTTCGAACCGACGCCGCTTCTA ACTC	This sequences data was 99% significant matched with <i>Blastomyces dermatitidis</i>
110719-03_E07_TOG-ITS4 CACTGAACCTCATGCCTGAGAGTGATGCAGTCTGAGCC TGAATACAAATCAGTCAAACTTTCAACAATGGATCTCT TGGTTCGGCA	This sequences data was 97% significant matched with <i>Exophiala dermatitidis</i>
110719-09_A07_TOH-ITS CGGCTGGCGCCGGCCGCTAATCGAGCGGGTGAC AAAAGCCCCATACGCTCGAGGACCGGACACGGTGCCGCC GCTGCCTTTC	This sequences data was 98% significant matched with <i>Microsporium audouinii</i>
110719-03_G07_TOI-ITS1 ACCACTGAACCTCATGCCTGAGAGTGATGCAGTCTGAG CCTGAATACAAATCAGTCAAACTTTCAACAATGGATCT CTTGGTTC	This sequences data was 97% significant matched with <i>Penicillium citrinum</i>
110719-03_M07_TOM-ITS1 CTCCGTCAGGCTTCTTCTCCAGTTTTTGACAGGGATT CTTGTCGTTATACCTATTTCCCTACAATTACCTCTGCC TTCGATTGCTGG	This sequences data was 99% significant matched with <i>Penicillium aschersonia</i>
110719-09_K07_TOP-ITS4 GGCCCTAGTCGAGCGGGTGACAAAGCCCCATACGCTCG AGGACCGGACACGGTGCCCGGCTGCCTTTCGGGCCCC TCC	This sequences data was 99% significant matched with <i>Epidermaphyton flucosum</i>
110719-03_A09_TOQ-ITS1 ACTTCATGCCTGAGAGTGATGCAGGCTGGCTCTGAATC TAAAGCAAACTTCTTCTGAACTTTTGCTTCCC GCC TCCA	This sequences data was 98% significant matched with <i>Mucor racemosus</i>
110719-03_C09_TOR-ITS1 AACATGCGTGAGATTGTAAGTCTAGCTTTCCCCCTGTCC ATCACTTCCCCTCTATTTGTGGTAGATTGCGACGTGCG GCGAAAACA	This sequences data was 95% significant matched with <i>Trichophyton concentricum</i>
110719-09_O07_TOS-ITS4 AACCATGTGGATGGGCAACGAACATGTTACCGGCCCTG ATAATTTGCTTAAGCCTGGTATCTATTA AAACTCTAC CTTCAGAT	This sequences data was 96% significant matched with <i>Absidia corymbifera</i>
110719-09_K05_TOU-ITS4 ATGCGTCGGCGGGCGCCGGCCGGGCTACGAGCGGA AGACGAAGCCCCATACGCTCGAGGACCGGACGCGGTG CCGCCGC	This sequences data was 98% significant matched with <i>Microsporium nanum</i>
110719-09_I05_TOW-ITS4 AACATGCGTGAGATTGTAAGTCTAGCTTTCCCCCTGTCC ATCACTTCCCCTCTATTTGCCCGGGAGCCGGGGGAC GAGGGCCCA	This sequences data was 99% significant matched with <i>Trichophyton violaceum</i>
110719-03_E05_TOY-ITS1 ACGGATCTCTGGTTCGGCATCGATGAAGAACGCAGC GAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGA ATCATCG	This sequences data was 99% significant matched with <i>Emericella nidulans</i>
110719-09_I05_TOB-ITS4 AAACAGTTGGGCATGAAATGCACTCTAAACCTTACACA CAGTGTCTATACAACTCTTGCTGGTTTGGCCTAGGGTA GGTTG	This sequences data was 97% significant matched with <i>Aspergillus flavus</i>
110719-03_E05_TOV-ITS1 ACAAATTAATATCTCATGATAATCTTCAAACTCAACAA CGGATCTCTGGTCTCTATCGATGAAAACCGAAAT GATAAGTAATA	This sequences data was 97% significant matched with <i>Aspergillus fumigatus</i>

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