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Full Length Research Paper

Isolation of an emerging thermotolerant medically important Fungus, *Lichtheimia ramosa* from soil

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***Lichtheimia ramosa*, a ubiquitous clinically important mould was isolated during a screen for thermotolerant fungi obtained from soil on a freshly burnt field in Ikorodu, Lagos State. In the laboratory, as expected it grew more luxuriantly on Potato Dextrose Agar than on size limiting Rose Bengal Agar. The isolate had mycelia with a white cottony appearance on both media. It was then identified based on morphological appearance, microscopy and by fungal Internal Transcribed Spacer ITS-5.8S rDNA sequencing. This might be the first report of molecular identification of *L. ramosa* isolate from soil in Lagos, as previously documented information could not be obtained.**

Key words: Soil, thermotolerant, *Lichtheimia ramosa*, Internal Transcribed Spacer (ITS).

INTRODUCTION

Zygomycetes of the order Mucorales are thermotolerant moulds that are ubiquitous in nature (Nagao et al., 2005). The genus *Lichtheimia* (syn. Mycocladus, Absidia) belongs to the Zygomycete class and includes saprotrophic microorganisms that can be isolated from decomposing soil and plant material (Alastruey-Izquierdo et al., 2010a). The awareness of the thermotolerant genus *Lichtheimia* increased markedly since its separation from the mesophilic genus *Absidia* (Hoffmann et al., 2007) and its taxonomic revision (Alastruey-Izquierdo et al., 2010b). Members of the genus *Lichtheimia* are considered to constitute thermotolerant fungi, because they grow at a wide range of temperatures, from 20 to 53°C, with optimum temperature for growth being 37°C, where it exhibits rapid growth (André et al., 2014).

L. ramosa is abundant in soil, decaying plant debris and foodstuff and is one of the causative agents of mucormycosis in humans (Barret et al., 1999). Mucormycosis is an opportunistic invasive infection caused by *Lichtheimia*, *Mucor*, and *Rhizopus* of the order Mucorales. Soil serves as a habitat and spore reservoir for *Lichtheimia* species. Several cases are known where traumatic injuries contaminated with soil resulted in *Lichtheimia* infections in immunocompetent patients (Belfiori et al., 2007, Corti et al., 2009, Blazquez et al., 2010). Mucormycosis (both rhino-orbital-cerebral and pulmonary) are acquired by the inhalation of their spores. Despite their low virulence, *Lichtheimia* species are currently regarded as emerging pathogens among Mucoralean fungi (Kutlu et al., 2014). There has been an increase in reports of *L. ramosa* infections among

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immunocompromised patients (Schwartze and Jacobsen, 2014).

L. ramosa is a microbe of clinical importance. Reported cases include *L. ramosa* isolated from a young patient's infected wound after a road traffic accident (Neelaveni et al., 2017) and as a causal agent of primary cutaneous mucormycosis in a burn victim (Kaur et al., 2014). A fatal case of mucormycosis due to *L ramosa* affecting a 56-year-old male with diabetes mellitus and siderosis has also been reported (Mouronte-Roibás et al., 2016).

Genome sequence of *L. ramosa* and its close relative *L. corymbifera* have been published (Linde et al., 2014, Schwartze et al., 2014). Although *Lichtheimia* species tend to be morphologically and genetically distinct, they often share very similar antifungal drug susceptibilities.

The growth of the *L. ramosa* in media is rapid, with mycelia expanding to cover the entire plate in only a few (one to seven) days (Ziaee et al., 2016). Microscopically, *L. ramosa is* similar to *L. corymbifera* and differs in its ellipsoidal to cylindrical sporangiospores as compared to the subglobose to broadly ellipsoidal sporangiospores of *L. corymbifera* (Alastruey-Izquierdo et al., 2010a). In this study, a screen was undertaken to isolate and identify a thermotolerant fungus, *L. ramosa* from the environment by culture-based and molecular methods.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from a freshly burnt vegetation in Ikorodu Local Government, Lagos State with GPS coordinates 6°37'20.4"N 3°34'37.9"E. The farmland had been burnt following harvesting of farm produce. Samples were collected from the surface layer of the soil up to a depth of 7.5 to 10 cm.

Isolation of fungi

One gram of soil sample was placed in a test tube containing 9 ml of sterile distilled water and homogenized by shaking thoroughly. A ten-fold serial dilution scheme was made up to 10^{-5} dilutions. One milliliter aliquot of the homogenized solutions was taken from 10^{-3}, 10^{-4} and 10^{-5} dilutions and plated on Potato Dextrose Agar supplemented with chloramphenicol and Rose Bengal chloramphenicol agar using the pour plate technique. Plating was done in triplicates for both Potato Dextrose Agar and Rose Bengal Agar. The plates were incubated at 30°C and the growth observed for 7 days.

Isolation of pure cultures and microscopy

Pure cultures of the white mycelial fungus were selected from the PDA plates and sub-cultured onto fresh PDA plates. The strain was stored on PDA slants at -20ºC for future use. For microscopic identification, a mycelial mat of the fungus was placed on a grease-free microscope glass slide and a drop of lactophenol cotton blue was added to the mycelia mat. A coverslip was placed on it with the aid of sterile forceps and the microscope slide was viewed under the microscope. Micrographs were thereafter obtained.

Molecular identification of the isolate

Genomic DNA was extracted from a five-day-old fungi culture grown on PDA using ZR Quick-DNA Fungal/Bacterial Miniprep™ extraction kit (Zymo Research, CA, USA) according to the manufacturer's instructions. To check the DNA quality, it was run on a 1% ethidium bromide agarose gel (Figure 2A). Polymerase Chain Reaction (PCR) of the extracted genomic DNA was done in a GeneAmp PCR system 9700 PCR thermal cycler. ITS5F (GGAAGTAAAAGTCGTAACAAGG) and ITS4R (TCCTCCGCTTATTGATATG) primers were used for amplification. The PCR reaction mix (25 µl) contained 2.5 µl of 10x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of forward and reverse primers, 1 µl of DMSO, 2 µl of 2.5 mM dNTPs, 0.1 µl of 5µg/ µl Taq DNA polymerase, 3 µl of 10ng/µl DNA and 13.4 µl Nuclease free water. The thermal cycler program used was as follows: Initial denaturation at 94˚C for 5 min, followed by 36 cycles of denaturation at 94˚C for 30 s, annealing at 54˚C for 30 s, elongation at 72˚C for 45 s, a final elongation step at 72˚C for 7 min and hold temperature at 10˚C. The amplicons were electrophoresed on 1.5% agarose gels. The gel was prepared and run at 75 volts for 90 min and visualized with a UV Transilluminator (Figure 2B). The PCR product was Sanger sequenced using the BigDye 3.1 reaction protocol on 3130XL genetic analyzer (Applied Biosystems) at the Bioscience Center, IITA, Ibadan, Oyo. The sequences were checked for quality and assembled using BioEdit (version 7.2.5) Sequence Alignment Editor (Hall, 1999). The consensus sequence was compared to the GenBank nucleotide data library using the Basic Local Alignment Search Tool, BLAST software (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI(http://www.ncbi.nlm.nih.gov). The sequences were submitted to the GenBank database and an accession number was assigned to the isolate.

RESULTS

Isolation of thermotolerant fungus from the soil sample

After 7 days of incubation, mixed culture PDA plates of few fungi were obtained (Figure 1A). From the 10^{-3} dilution plate, a dominant whitish fungus that appeared on all the other serial dilution plates was selected and sub-cultured. This selected strain (IYN69) had a more luxuriant growth on PDA plates than on Rose Bengal agar plate when grown in pure culture (Figures 1B and C). The strains grew at room temperature up to 37°C. The micrograph showed sporangiospores ellipsoidal to cylindrical in shape (Figure 1D).

Molecular identification of Isolate IYN69

In order to confirm the identity of the isolate IYN69 by molecular techniques, DNA product was visualized on 1% agarose gel stained with ethidium bromide (Figure 2A) and then the PCR product amplified by the pair of ITS4-R and ITS5-F primers were confirmed on a 1.5% agarose gel (Figure 2B).

The sequencing results from ITS5-ITS4 amplification of the ITS regions of isolate IYN69 was edited using BioEdit (version 7.2.5) Sequence Alignment Editor (Hall, 1999)

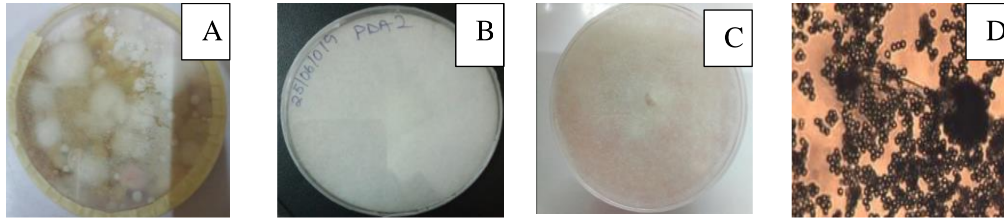

Figure 1. A. Mixed culture of 10^{-3} dilution PDA plate. Growth of strain (IYN69 on laboratory media **B.** PDA + Chloramphenicol. **C.** Rose Bengal Chloramphenicol agar. **D.** Micrograph of sporangiospores of the new isolate.

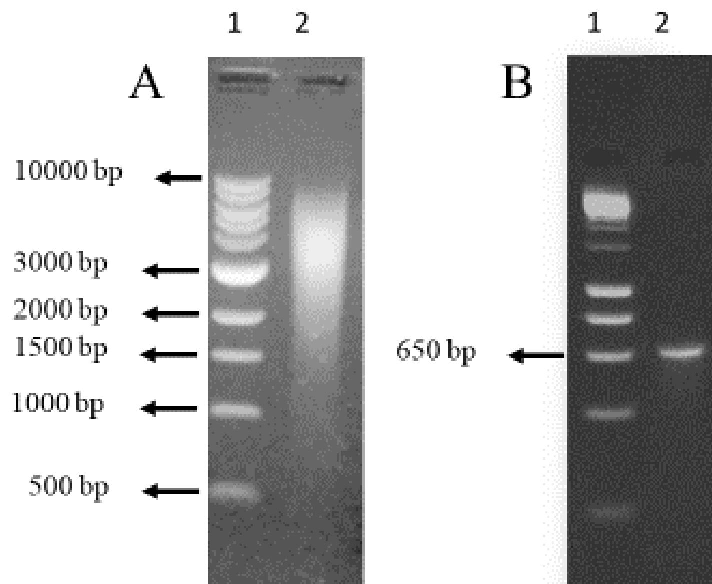

Figure 2. Agarose gel electrophoresis. **A.** 1% gel of genomic DNA from Isolate IYN69. Lane 1: 1 kb NEB ladder; lane 2: 5 µl of genomic DNA. **B.** 1.5% gel of the expected PCR amplicon size of 650 bp. Lane 1- Bioline Hyperladder 1 kb; lane 2- Isolate IYN69.

and compared with the GenBank database using NCBI Basic Local Alignment Search Tool (BLASTn). The mould was identified as *Lichtheimia ramosa* with 100% identity to *Lichtheimia ramosa* isolate PUR 3 internal transcribed spacer 1 on the NCBI platform.

Nucleotide sequence

The partial sequence of the internal transcribed spacer (ITS) region of this isolate has been submitted to GenBank and can be found under accession number MT373684.

DISCUSSION

L. ramosa was the dominant fungus among others from a screen of thermotolerant fungi from recently burnt vegetation. Its isolation under such conditions is an indication that it can withstand high temperatures. Its thermotolerance has been linked to its virulence (Schwartze et al., 2012). Morphological and microscopic characteristics and phylogenetic identification were sufficient to confirm that the isolate was *L. ramosa* strain. Its features are similar to *L. ramosa* strain H71D and mycelia were identical to the type species of *L. ramosa* described by Hoffmann (2010) and Alastruey-Izquierdo et al. (2010a).

It is likely that this report might be the first documenting soil isolation of *L. ramosa* from Lagos and its molecular identification as no existing records were found. The BLAST hits showed many *L. ramosa* species that had significant sequence alignments with the query sequence. Based on the available molecular data, *L. ramose* isolate IYN69 most closely matched another

isolate PUR 3 (GenBank Accession Number: MF033505) associated with a study on soil fungi and *L. ramosa* clone 7 (Alastruey-Izquierdo et al., 2010a). *L. ramosa* isolate IYN69 ITS 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence can be found in GenBank under accession number MT373684.

Incidences of *L. ramosa* infection in patients have been linked to road traffic accidents (RTAs) suggesting contamination by this soil fungus (Neelaveni et al., 2017; Bibashi et al., 2012). This is of great significance because RTAs are a major public health concern and third leading cause of death in Nigeria (Dixie, 1999; Onyemachi and Ofoma, 2016). The next steps would be to do whole genome sequencing and compare its genome with the existing reference genome (Linde et al., 2014) especially regions responsible for sensitivity to antifungals. This strain would be tested for its sensitivity to existing antifungal drugs (Amphotericin B, posaconazole, itraconazole) that are known to be effective against *Lichtheimia* species. Although, there are not many cases of *L. ramosa* infections worldwide (Mouronte-Roibás et al., 2016); for an emerging pathogen, the information from such studies would help to put in place good treatment options should burn, accident victims or diabetics get infected with this strain from this locality. Since *L. ramosa* is also known for the production of enzymes like xylanase (Alvarez-Zuniga et al., 2017), and mannase (Xie et al., 2019), an assay for these and other thermophilic enzymes with biotechnological applications will be conducted on this new isolate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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