Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.20, 2016

Molcular identification of *Aureobasidium pullulanus* isolated from pigeon dropping in Basra

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Abstract:

Aureobasidium pullulanus is black yeast like fungus, isolated from different environment and can cause various opportunistic human infections.

A local *Aureobasidium* isolate was collected from pigeon dropping in Basra. It was identified as *A. pullulanus* morphologically and confirmed on the basis of internal transcribed spacer (ITS) sequence similarity by using two primers, ITS-1 and ITS-4 which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene .

The availability of *A. pullulanus* was noted at the first time in stress habitat of pigeon dropping.

Key words: *Aureobasidium pullulanus***,** pigeon dropping, ITS sequence**.**

Introduction:

Aureobasidium pullulanus is a ubiquitous and widespread oligotrophic fungus that can be found in environments with fluctuation water activities, such as the phyllosphere, bathrooms, food and feeds. It can also be found in osmotically stressed, such as hypersaline water in salterns and rocks and mountains (Andrews *et.al.*1994; Urzi *et.al.*1999; Gunde-Cimerman *et.al.*2000; Samson *et.al.*2004). *A. pullulanus* was taxonomically characterized by de Hoog &Yurlova (1994) on the basis of its morphology and nutritional physiology. *A. pullulanus* is a causal agent of Phaeohyphomycosis occasionally found in men and animals.This mould is also responsible for arrange of opportunistic fungal infections, including fungemia (septicemia), systemic infections and abscesses in different viscera (Hawkes *et.al.* 2005 and Chan *et.al.* , 2011).

Various loci have been sequenced in the past to infer the taxonomy and phylogeny of the taxa in *Aureobasidium*, such as internal transcribed spacer (ITS) rDNA, intergenic spacer 1, translation elongation factor-1α, β- tubulin, and RNA polymerase II (Zalar,*et.al.*,2008; Manitchotpisit, *et.al.*, 2009 and Crous *et.al.*, 2011)

Material &methods

Isolation

Aureobasidium pullulans isolate was isolated from pigeon dropping sample which collected from Basra city and processed according to (Zarini *et.al.* 2010).

Identification:

After incubation period, all *Aureobasidium pullulans* like colonies were selected and subcultured on SDA to pure cultures. The fungus was then identified based on morphological observation and for more confirmation the isolate was prepared for molecular identification.

DNA extraction: The mycelium and spores of the activated isolate was grind and the DNA was isolated by using Bio basic Kit using the protocol described in the manufacturer's manual.

Amplification of isolated DNA using ITS: The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3' end of the 28S and the 5' end of the 28S gene were amplified using the two primers, ITS-1 and ITS-4 which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (Zhang *et.al.* 2000). The PCR-amplification reactions were performed in a 50µl mixture containing 25 µl Green Master Mix, 5 µl of each

primer (10 µm), 5 µl DNA template and the volume was completed to 50 µl by using of nuclease free water. The cycle parameters included an initial denaturation for 2 min. at 95°C, followed by 35 cycles of denaturation for 30 sec. at 95°C, primer annealing for 30 sec. at 55 ° C, primer extension for 1 min. at 72 °C, and a final extension for 10 min. at 72 ° C. amplified products were separated on 1% agarose gel in TBE buffer, pre-stained with ethidium promide (1µg/ml) and electrophoresis was carried out at 80 V for 75 min. in TBE buffer. One Kb ladder (Promega) was used as a marker. The gel was observed in transilluminator over ultraviolet light.

Purification of PCR product: The PCR product was purified by cutting desired band from the gel with minimum quantity of gel portion and purified using MEGA quick-spin total fragment DNA purification Kit (iNtRON Biotechnology). Using the protocol described in the manufacturer's manual.

DNA sequencing of the 28S rDNA fragment: A pair of universal ITS primers ITS-1 (forward) and ITS-4 (reverse) was used for sequencing of the amplified product and this step was carried out at the Macrogen Company (Koria) the details of the primers are mentioned in table (1).

Table 1. Gene sequence with universal primers.

Primer	sequence	Length	%GC
ITS1	TCCGTAGGTGAACCTGCGG	19	58
ITS4	TCCGTAGGTGAACCTGCGG	20	45

Sequence analysis: A comparison of 28S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) database was done using BLAST program (White *et.al.* 1990).

Results:

The *A. pullulans* isolate was collected from sample which taken from Silo Street region. The isolate grown on SDA at 25°C and 37°C with and without cyclohexemide. The colonies growing rapidly cream –tan colored with slimy exudates, later was become blackish. Conidia produced in dense group from small denticles and adhering in slimy heads with measured 6-9*3.5-6 µ (fig. 1).

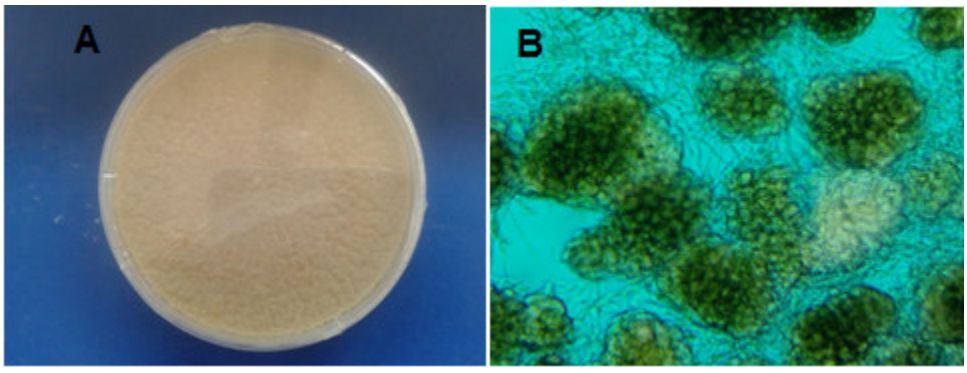

Figure 1: (A) *Aureobasidium pullulans* colony on SDA at 25°C, (B) Slimy heads conidia of *A. pullulans*.

The genomic DNA was extracted from isolated fungal isolate an *A. pullulans* and universal ITS-1 (19F) and ITS-4 (20R) primers were used for the amplification and sequencing of the 28S rRNA gene fragment. A total of 656 bp of the 28S rRNA gene was sequenced (Fig. 2).

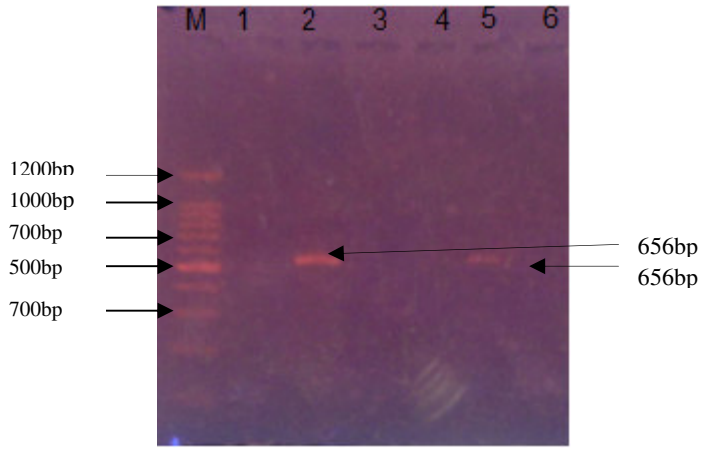

Figure 2 : 2% agarose gel electrophoresis analysis for PCR assay with two primer set ITS1, ITS4.

M: DNA marker 1200bp; Lanes 2 and 5 28S rRNA gene for *Aureobasidium pullulans*

Comparison of test strain against known sequences of SSU and LSU rRNA databases showed that the gene *ansl*sequence of this isolate 100% sequence similarity with 28S rRNA gene sequence of *Aureobasidium pullu* (GenBank LN899831.1) (Fig.3&4)

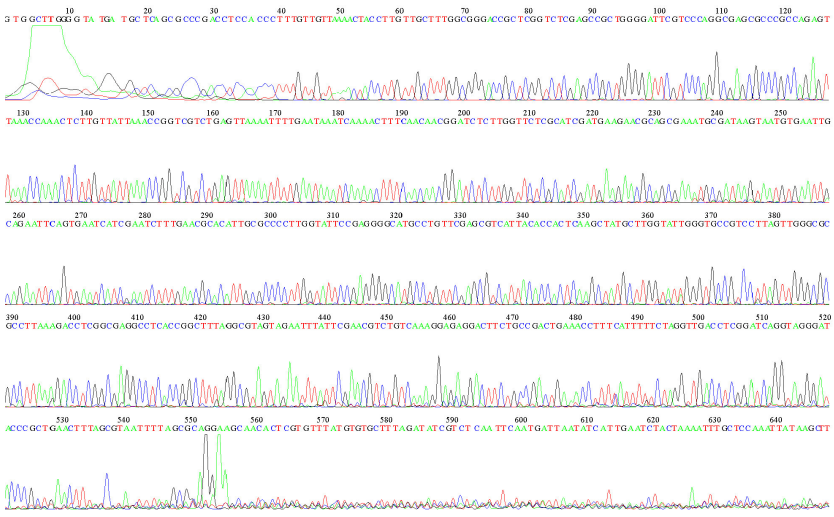

Figure 3: Alignment of sequencing of regions ITS1/4 of gene 28S rRNA *A. pullulans*, corresponds to studied .(strain, shows 100% similarity when compared to environmental strain (GenBank acces number LN899831.1

1- *Aureobasidium pullulans* isolate DFFSCS014 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene-656bp-100%

TGCTCAGCGCCCGACCTCCACCCTTTGTTGTTAAAACTACCTTGTTGCTTTGGCGGGACCGCTCGGTC
TCGAGCCGCTGGGGATTCGTCCCAGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTAAA
CCGGTCGTCTGAGTTAAAATTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT
GAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATTACACCACTCAAG
CTATGCTTGGTATTGGGTGCCGTCCTTAGTTGGGCGCGCCTTAAAGACCTCGGCGAGGCCTCACCGG
CTTTAGGCGTAGTAGAATTTATTCGAACGTCTGTCAAAGGAGAGGACTTCTGCCGACTGAAACCTTTT
ATTTTTCTAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCGTAATTTTAGCGCAGG
AAGCAACACTCGTGTTTATGTGTGCTTTAGATATCGTCTCAATTCAATGATTAATATCATTGAATCTAC
TAAAATTTGCTCCAAATTATAAGCTTGGAATTCATACTTATTATCAGTATAATTATTA

Figure 4: 28S rRNA gene sequence of *Aureobasidium pullulans*

Discussion:

A. pullulans belongs to the phylum Ascomycota, class Euascomycetes, order Dothideales, family Dothioraceae. Teleomorph of *A. pullulans* is *Discosphaerina fulvida* (Kirk *et.al.* , 2008) and it has been recovered from diverse habitats. Which characterized by its morphological variability (Salkin *et.al*., 1986; Perez *et.al.*, 1997 and Zalar *et.al.*, 2008). This yeast like fungus has many importance in biotechnological applications and also medical and agriculture importance (Kazemi *et.al.* , 2005).

In our study shape and size of conidia of *A. pullulans* were very variable, but generally similar to those described by de Hoog (2000) and Gniewosz (2003), hyphae frequently rare, while darkly pigmented conidia were accumulate as a slimy heads.

Up to date, none of the *A. pullulans* stored in gene bank originated from pigeon dropping. This fungus commonly found in environment (Salkin *et.al*., 1986; Sun *et.al*., 2008), but there is no reference refers to its isolation from like this stress habitat (dropping). Gostincare *et.al.* (2014) was concluded in his study that *A. pullulans* was considered a species of exceptional adaptability; this was reflected in its great phenotypic plasticity polyextrem tolerance and the ability to survive across a wide variety of habitats. Thus, the genome sequence generated here to allow the correct identification of *A. pullulans*.

Species within the genus *Aureobasidium* have most often been distinguished based on multilocus analysis of different regions of the rDNA gene clusters. The ITS region has been most commonly used (Zalar *et.al.*, 2008; Manitchotpisit *et. al.*, 2009; Crous *et.al.*, 2011; Peterson *et.al.*, 2013) it is recognized as insufficient in certain genera (Schoch *et.al.*; 2012). Based on the ITS rDNA, the distinction of *A. pullulans* was possible (Zalar *et.al.* 2008).

Acknowledgement

We are thankful to Dr. Abd Al- Abbas, M.J. University of Basra, College of Science (Iraq) for providing DNA sequencing data analysis.

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