Molecular Identification of Trichosporon Asahii Used DNA Sequencing Methods Isolated from Patient with Diabetic Foot

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Abstract
The aim of this study was conduct and investigate to the genotyes identification of*T. asahii* isolates obtained from patients with diabetic foot in Al-Najaf province-Iraq , One pathogenic yeast, *T. asahii*, was used in this study isolated from patient with diabetic foot The first step they were activated all isolates on Brain heart infusion broth for 2 days at 25°C and examined for purity ,second step involved morphological and microscopic examination, to verify the isolation's identity by staining with lactophenol stain, DNA extraction and purification using Accu power PCR Pre Mix kit with both (ITS1-ITS4) primers ,The PCR product was then sequenced using the Sanger method by delivered to Macrogen Lab in the United States .The colonies were usually raised and have a waxy appearance, also developed furrows and irregular folds, on SDA after 3 days at 37°C, *T. asahi* that was discovered on chromagar, who appeared pale lavender with a white rim of *T. asahi* on chrome agar plate after 2 days of incubation at 37°C .The results of primer pair which was targeted ITS regions sample 30 refered to *T. asahi* with moleculare weight 736 pb .The strain L3_LF was found to be the nearest neighbor to *T. asahii* strain KTSMBNL-12 with identity 99.21% while the strain L3_LR was found to be the nearest neighbor to *T. asahii* strain AUMC 10759 with identity 98.97% .

1. Introduction

The clinical manifestations of T. asahii infected patients were non-specific, but varied with different infection sites and types of infections. The major types of infections were urinary tract infection, fungaemia and disseminated infection (Haitao, et al .2020).

Trichosporon species are distinguished microscopically by having yeast cells that germinate to produce hyaline hyphae that disarticulate at the septa, the hyphal compartments acting as arthroconidia (asexual propagules). No teleomorphic (sexual) states are known. Trichosporon sp. causes superficial and systemic nosocomial infections. These yeasts have been found to be the causative pathogens of white piedra, and opportunistic pathogens in invasive infections in immunocompromised patients in tertiary hospitals worldwide (Chagas Neto et al. , 2009 ; Adriana Araújo et al. , 2016).

Autopsy revealed hyphae with arthroconidia that were consistent with Trichosporon species widely dispersed throughout the lungs and heart , it is also found in the bone marrow, lymph nodes, adrenal gland, kidney (although not appearing extensive enough to account for renal failure), and thyroid gland, but they were not found in the liver, spleen, bancreas, or brain also present were micro nodular cirrhosis (probably secondary to earlier alcohol use as well as hepatitis C), diffuse and nodular glomerulosclerosis, acute pancreatitis, and an old infarct of the right parietal cortex. The bone marrow revealed all cell lines present, with only a modest decrease in megakaryocytes (Walsh , 1989 ; John et al. , 2001).

Trichosporon species, especially T. asahii, are emerging pathogens that have increasingly been reported in patients with cancer during the past 10–15 years. Although it is not unique, the case demonstrates that Trichosporon species also may cause life-threatening sepsis and widespread dissemination in patients who do not have either neutropenia or cancer, but who, nevertheless, are at risk for an opportunistic fungal infection due to chronic illness and compromised skin and mucous membranes. In addition, a case of uterine trichosporonosis, also in apatient with neither neutropenia nor cancer, was recently reported (Chan et al. , 2000; John et al. , 2001). The objective of this study was conduct and investigate to the genotyes identification ofT. asahii isolates obtained from patients with diabetic foot in Al-Najaf province

2. Materials and methods

A Study of Isolates

One pathogenic yeast, T. asahii, was used in this study isolated from patient with diabetic foot The first step they were activated all isolates on Brain heart infusion broth for 2 days at 25°C and examined for purity in the laboratory of Advanced Mycology at the Faculty of Science, University of Kufa. The second step involved morphological and microscopic investigation, which was used to verify the isolation's identity. A microscopic examination was carried out to determine the purity and morphology of T. asahii by adding one drop of water to a yeast colony,

doi.org/10.31838/hiv23.03.116 Received: 09.11.22, Revised: 10.12.22, Accepted: 18.01.23

staining it with lactophenol stain, and observing it under a microscope at a magnification power 100X.

PCR identification

The DNA mini-preps kit with an EZ-10 spin column (Favorgen Biotech Corp, Taiwan) was used to DNA extraction and purification Accu power PCR Pre Mix. Bioneer Corporation USA, (0.2ml) thin-wall 8-strip tubes with attached cup /96 tubes were used . The premier used in experiment, created via Bioneer company, Korea
ITS1-F 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4-R 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990).
Amplification of genes was carried out according to the experimental protocol of Accu power TLA PCR Premix tub under conditions of cycling as mentioned in cycling parameters .The PCR reaction mixture was prepared as 5µl of master mix, which is supplied to be ready for using in 100 µl PCR tube, followed by 5 µl template DNA extract, 2.5µl of 10 pmol/µl upstream (reveres) primer specific solution and 2.5 µl of 10 pmol/µl downstream (forward) primer specific solution and the volume was completed to 20 µl with deionized distilled water, the tube was mixed with vortex to dissolve the lyophilized blue pellet and briefly spin down.
The following protocol was used: initial denaturation for 4 minutes at 94°C, 30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C, 35 cycles; 7 minutes at 72°C. Electrophoresis on a 1.3 percent (w/v) agarose gel separated the PCR products, which were then stained with ethidium bromide.

DNA sequencing of PCR products

T.asahii PCR results were delivered to Macrogen Lab in the United States, where the sequence data for each isolates . Prior to the sequencing procedure, the PCR product was purified using a processor kit (Promega, Madison, USA) in accordance with industrialization company guidelines. The PCR product was then sequenced using the Sanger method. (Hawksworth et al., 2016).

3. Results

Macroscopic and Microscopic Characterizations of *T. asahii*

The colonies were usually raised and have a waxy appearance, also developed furrows and irregular folds, on SDA after 3 days at 37°C, or on SDA plate after 3 days of incubation at 27°C.
T. asahii characterized by light blue color, who discovered that colonies of the same species have the same traits *T. asahi* that was discovered on chromagar, who appeared pale lavender with a white rim of *T. asahi* on chrome agar plate after 2 days of incubation at 37°C .*T. assahi* after staining with lactophenol cotton blue appeared with pseudo hyphae, arthroconidia, and blastoconidia (Figure1 A,B ,C,D and E).
Microscopic examination is a preliminary test to diagnose the yeasts Each sample was stained of Lactophenol cotton blue stain and examined microscopically. Direct microscopic examination was used for identification the pseudo hyphae and one-celled blastoconidia of *T. asahii*,
chlamydoconidia, pseudo hyphae, and true hyphae , all species produce blastoconidia, which may be round or elongated. Most produce pseudo hyphae that are long, branched, or curved. In addition, true hyphae and chlamydospores are produced by some *T. asahii* strains.
Although members of the same genus, the various species present a degree of unique behavior with respect to their colony texture , where most species produce pseudo hyphae which may be long, branched or curved. True hyphae and chlamydospores are produced by strains of some *Candida* spp. , all diagnostic *T. asahii* exhibit a positive results when prepared with gram stain, these results were in agreement with Iehab, (2014); Zahra, (2016); Ali, (2018) ; Ataa, (2020). Figure (4.1). The genus *Trichosporon* is characterized by the ability to form arthroconidia, blasatoconidia, hyphae, and pseudo hyphae, these results were in agreement with Noha *et al.*, (2011).
This results were agreement with AL-Ameri (2021) , Al-Inizi (2022) and disagree with Dana, *et al* . (2010) which were found that *T. asahii* with White to cream-colored, folded colonies with low white aerial hyphae were recovered on SGA plates inoculated with the isolates from the six patients this is may be due to The difference in the isolation also , the conditions under which this isolation was studied were different

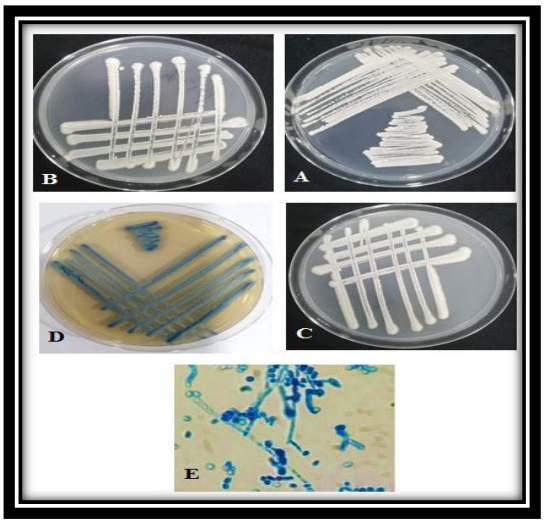


Figure (1) :Macroscopic and Microscopic of T. asahii isolate in different laboratory conditions A and B : on SDA plate after 3 days of incubation at 37°C. C: on SDA plate after 3 days of incubation at 27°C. D: on chrome agar plate after 2 days of incubation at 37°C. E: under a microscope with lactophenol cotton blue stain, magnification X 100

Molecular identification of *T. asahii*

PCR assay

The results showed that our different molecular sizes of ITS region of Yeast species. In addition, it offered PCR products of these isolates in Figure (2) The primer pair was used for the same sample is (ITS1-ITS4) was targeted ITS regions sample 30 refered to

T. asahi with moleculare weight 736 pb .The identification of yeast species was followed according of Bellemain *et al.*, (2010) key. This results are compatible with what it says Adriana *et al*, (2016), AL-Ameri(2021) and Al-Inizi (2022).

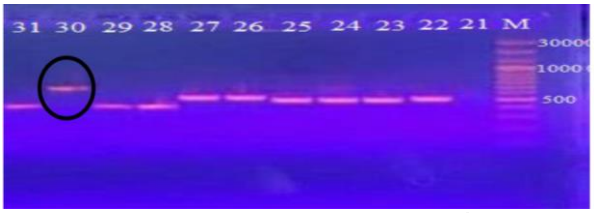


Figure (2): Agarose gel electrophoresis of ITS regions PCR product by pair primers (ITS1-ITS4) of different yeast spp.(1.3g agarose gel, 80 volts for 1 hour) (M: DNA ladder; lane 21-31: yeast spp. , lane 30 T.asahii).

Sequences analysis

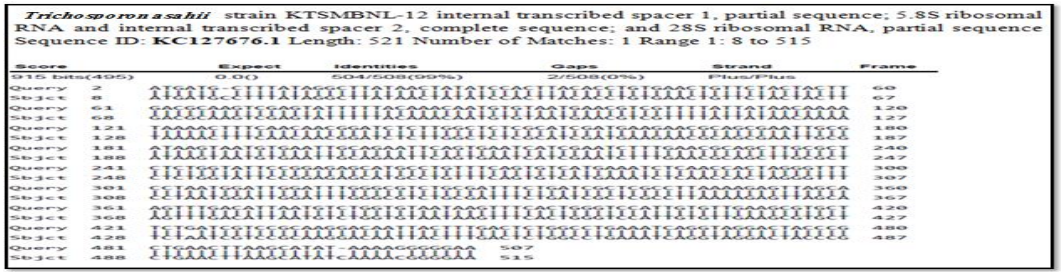
The PCR products were delivered to Macrogen Lab in the USA. There, 3 samples of amplified PCR-products (forward and reverse strand) were examined, and our sequences were compared with reference worldwide sequences in the NCBI Gene Bank.

The sequencing for the (L3) strains was obtained online, aligned to the NCBI database using the blast program, multiple aligned to one another using the Bio Edit program, and then sent in fasta format to the NCBI through the sequin program. Only one of the 3 samples amplified by the yeast primers (ITS1-ITS4) was particularly intriguing (L3). Each of the 3 resultant sequences was compared to the NCBI database using the NCBI blast program.

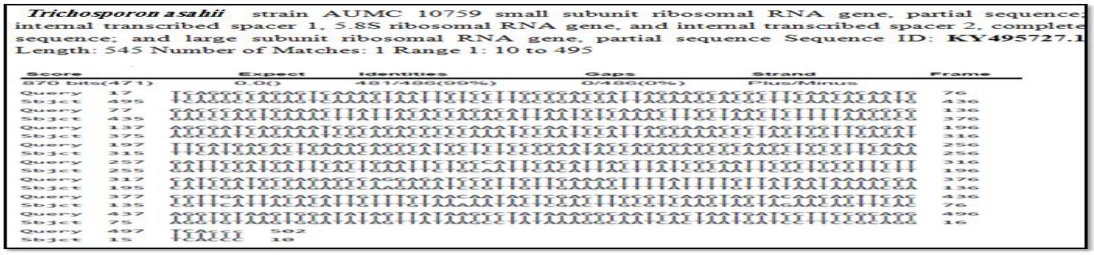
The strain L3_LF was found to be the nearest neighbor to T. asahii strain KTSMBNL-12 with identity 99.21% Table (1) and Figure (3), the strain L3_LR was found to be the nearest neighbor to T. asahii strain AUMC 10759 with identity 98.97% Table (2) and Figure (4). This results are in consistent with AL-Ameri (2021)

Table(1):DNA sequences alignments of isolated L3_LF (*T. asahii*) in comparing with database obtained from NCBI website

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Trichosporon asahii strain KTSMBNL-12 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Trichosporon a...	915	915	98%	0.0	99.21%	521
Trichosporon asahii isolate CDCF2158 internal transcribed spacer 1, partial sequence; 5.8S riboso...	Trichosporon a...	911	911	98%	0.0	99.21%	519
Trichosporon asahii strain AMC_TA005 internal transcribed spacer 1, partial sequence; 5.8S ribos...	Trichosporon a...	909	909	98%	0.0	99.21%	546
Trichosporon asahii isolate CDCF2160 internal transcribed spacer 1, partial sequence; 5.8S riboso...	Trichosporon a...	907	907	98%	0.0	99.02%	528
Trichosporon asahii strain ITS1; BMU internal transcribed spacer 1, partial sequence; 5.8S riboso...	Trichosporon a...	907	907	97%	0.0	99.60%	517



Figure(3):Pairwise alignment of ITS region of L3_LF (T. asahii)strain KTSMBNL-12 using NCBI online blast



Figure(4):Pairwise alignment of ITS region of L3_LR (T.asahii)strain AUMC 10759 using NCBI online blast

Table(2):DNA sequences alignments of isolated L3_LR (*T. asahii*) in comparing with database obtained from NCBI website

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Trichosporon asahii strain AUMC 10759 small subunit ribosomal RNA gene, partial sequence; inte...	Trichosporon a...	870	870	94%	0.0	98.97%	545
Trichosporon asahii partial 18S rRNA gene, strain AH16	Trichosporon a...	869	869	96%	0.0	98.19%	1539
Trichosporon sp. strain HBUM07182 small subunit ribosomal RNA gene, partial sequence; interna...	Trichosporon sp.	865	865	94%	0.0	98.77%	546
Trichosporon asahii strain AUMC 11242 small subunit ribosomal RNA gene, partial sequence; inte...	Trichosporon a...	865	865	94%	0.0	98.77%	545
Trichosporon asahii strain AMC_TA002 18S ribosomal RNA gene, partial sequence; internal trans...	Trichosporon a...	865	865	94%	0.0	98.77%	545

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