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ORIGINAL ARTICLE

Molecular Identification of two Isolates of Nannizia Fulva Causing Tinea Capitis in Iraq

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Abstract

Background: Tinea capitis is a clinical form of superficial fungal infection of the scalp and caused by filamentous fungi known as dermatophytes

Methods and Materials:Hairs and skin scrapings of scalp showing ringworm infection were collected from the affected regions and were subjected to direct microscopic examination and isolation on culture media.Identification of the causal agent was based on molecular analysis of ITS-rDNA region.

Results: The present study reported two cases of dermatophyte infection of tinea capitis (scalp ringworm) caused by Nannizia fulva.The causal agent was isolated in two occasions from ringworm lesions on scalp of 8 years old girl and 10 years boy from Zakho refuge camp school

Conclusion: Nannizia fulva is recorded for the first time as etiologic agent of tinea capitis in Iraq.

Keywords: Nannizzia fulva ,Tinia capitis, Dermatophytosis,ITS region,Iraq.

INTRODUCTION

Dermatophytes are keratinolytic filamentous fungi that infect the superficial epidermal layer of the skin as well as hairs and nails and caused dermatophytosis [1.] Dermatophytosis is a prevalent public health problem in the majority of the countries [2-4].Tinea capitis (TC) is a clinical form of superficial fungal infection of the scalp, eyebrows and eyelashes that is most common in school-aged children [5-7]. Tinea capitis is often caused by anthropophilic or zoophilic dermatophytes mostly assigned to the genera Trichophyton and Microsporum [8 -10] and rarely to the geophilic Nannizia species [11-13].

The incidence and distribution of tinea capitis as well as of its causative agents differs according to geographic regions [9,14-15]. In Iraq , tinea capitis infections have appeared as the second most common disease after tinea corporis as encounterd by several investigations on dermatophytosis [16-18]. During our survey on the incidence of dermatophytosis in Kurdistan region, North Iraq, we encountered two cases of tinea capitis on school children from a refuges camp at Zakho city. Isolation and identification of the causal agent ,revealed Nannizia fulva as confirmed by sequencing of ITS region of rDNA.

MATERIALS AND METHODS

Specimens collection: Samples were collected from primary school children at Zakho refuge camp,Kurdistan region of Iraq suspected to have been infected with ringworm during October 2017. Skin scrapings and hair fragments of scalp were collected from the affected lesions after proper sterilization with 70% alchohol (Figure 1 a and b) . Sufficient amount of specimens were taken from the edge of the lesion using a number 15 sterile surgical blade and were transported in folded paper envelope to the laboratory for direct microscopic examination and isolation on culture media [19].

Direct microscopic examination: Part of the specimen was observed through direct microscopic examination by mounting in 20% potassium hydroxide (KOH) solution (Merck, Germany). Then viewed under the light microscope (CX 40 Olympus USA) to look for fungal structures (Arthrospore or hyphae) [19] .

Primary isolation and culture on specific media: Second portion of the specimen was inoculated into Sabouraud Dextrose Agar (SDA) (CM41;Oxoid, ,UK) which supplemented with chloramphinicol (0.05%)(RS78;Oxoid,UK) to suppress any bacterial growth . Mycocel agar (Sigma-Aldrich, St. Louis, MO,USA) were used as well as, the medium was supplemented with both chloramphinicol (0.05%) and cycloheximide (0.05%) to inhibit the growth of environmental saprophytic fungi as appropriate [19] . Inoculated plates were incubated at 25°C for at least 3-4 weeks and examined periodically (twice a week) for existence of dermatophyte growth. Primary isolation and diagnosis of each species was based on phenotypic characteristics (Macroscopic and Microscopic feature of the colonies). Diagnosed species of dermatophyte isolates were subcultured in a new agar slant of SDA and maintained for further examination by molecular identification.

Molecular methods: Genomic DNA was extracted and purified by taking a proper amount of sample from a fresh colony (21 day-old, grown on SDA dishes). This performed by grinding in presence of liquid nitrogen for initial breaking up of mycelia. Total DNA isolation of fungi achieved using molecular biology kit; EZ-10 Spin Column Fungal Genomic DNA Mini-preps Kit(BIO BASIC INC., Canada) according to the instruction recommended by the manufacturer. The quantity, quality and the detection of genomic DNA were done by using gel electrophoresis and Nano drop UV spectrophotometer (Thermo Scientific,USA) at 260\280 nm . 6µL of the fungal DNA sample was mixed with 1µL of loading dye and loaded in the agarose gel (1.5%), run at 90 volts for 45 min. Then the gel was examined by gel image desktop to see the DNA bands and visualized under Ultraviolet Trans illumination (Sangabriel,UK) [20]. Amplification of Internal transcribed spacers (ITS) was performed using primers ITS1 (5'-

TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') according to [21]. The primers obtained from (MWG-Biotech AG ,Germany). The PCR mixture contained 10µl of ready to use prime Taq DNA polymerase master mix (2X) (Bioneer,Korea), 1-2µl of each primer (10pmol^{-1}) ,2-3µl of DNA template (50 ngl^{-1}), and deionized water was added to made up the total volume of the mixture to 25µl. Amplification was performed on a thermocycler (Bioneer,Korea), using the following conditions: An initial denaturation set up at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for 30 sec. , annealing at 58°C for 1 min. and extension at 72°C for 1 min. ,with a final extension step of 72°C for 10 min.

Gel electrophoresis: The PCR products were visualised by electrophoresing 10µl of amplicons in 1.5% (w/v) agarose gel in TBE buffer (90mM Tris,90mM boric acid ,2mM EDTA ,pH 8.3) , stained with ethidium bromide and supplied to the power at 75 volts for 60 min. The gel visualized under computerized UV trans-illuminator and photographed. A 100bp DNA Marker was used as a reference to determine the fragments size.

Sequencing, phylogenetic tree construction and identification of isolated fungi: The ITS region on rDNA gene was amplified using ITS 1 and ITS 4 primers which resulted in PCR product size ranked between (444 and 555) bp for Nannizzia fulva . The PCR productus were sequensed by Macrogen company,South korea.Sequence a alignment and checking of the obtained query sequences was conducted using BioEdit sequence alignment Editor 7.0.5.3 (http;//www.mbio.nesu.edu/Bio.edit/bioedit.html).

For final identification,the obtained consensus of query sequences was compared with ITS DNA database (https://blast.ncbi.nlm.nih.go/Blast.cgi.BLAST search (basic local alignment search tool) on the BLAST homepage,NCBI,Bethesda,USA to define the identity of the fungal strain and to find regions of local similarity between the sequences. The sequences of the current study were deposited in GenBank and accession numbers were recieved(MK112625 and MK112626). Sequencing of different amplicons were aligned using ClustalW MEGA7 software was used to determine genetic distance between the studied fungal isolates ,and a phylogenetic tree was reconstructed by the neighbor-joining method and the alignment were used to phylogened analysis using MEGA software version X.

RESULTS

Primary isolation and culture: Direct microscopic examination from the two scalp samples was positive showing hyaline hyphae typical for dermatophytes.Based on culture examination, isolates from the two samples were purified showing pinkish-buf fast growing colony at 25°C on SDA (Fig.2).

Sequencing and phylogenetic analysis: The ITS region on rDNA was amplified by using universal primers ITS1 and ITS4 which yielded in PCR product size ranged approximately between 444 and 555bp in N. fulva . Phylogenetic analyses of the ITS region of this study were compared with the dataset of different countries isolates originated from different sources .

Fig 1: (A) Symptoms of ringworm of scalp on 8-years old girl. (B) on 10-years old boy

Fig 2: Nannizia fulva culture showing pinkish-buff in colour

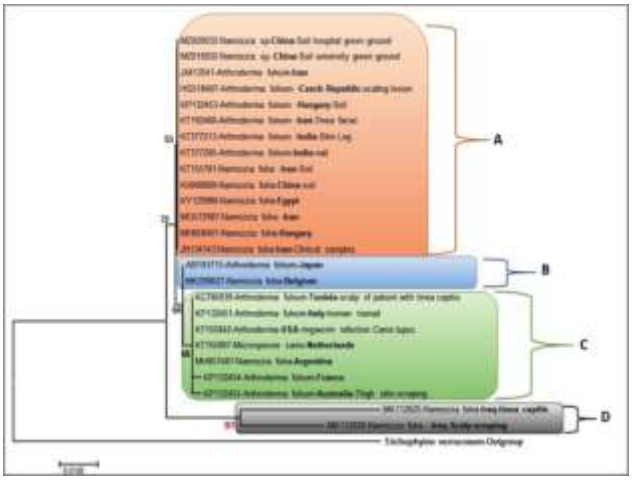

Figure 3: The represented phylogenetic tree of 25 ITS sequences using the Neighbor-Joining method . The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method by MEGA 7.

Table 1: The sequence similarity of this study starins with other countries and Isolated sources .

No.	Accession No.	Location (country)	Isolation source	Identity
1.	MK112625	Iraq (This Study)	scalp scraping (tinea capitis)	100%
2.	MK112626	Iraq (This Study)	Scalp scraping	95
3.	MH858401	Hungary		96
4.	MG572987	Iran		96
5.	KY129988	Egypt		96
6.	KX668868	China	soil	96
7.	KT155781	Iran	soil	96
8.	KT377205	India	nail	96
9.	KT377213	India	Skin of leg	96
10.	KT192466	Iran	Tinea faciei	96
11.	KP132453	Hungary	soil	96
12.	HG518407	Prague, Czech Republic	scaling lesion	96
13.	JX413541	Iran		96
14.	MZ020032	China	Soil, hospital green ground	96
15.	MZ019932	China	Soil, university green ground	96
16.	JN134143	Iran	Clinical samples	95
17.	KP132454	France	Clinical and veterinary samples	95
18.	KP132455	Australia	Thigh skin scraping	95
19.	KC784939	Tunisia	scalp of patient with tinea capitis	95
20.	KP132451	Italy	human toe nail	95
21	KT155842	USA	ringworm infection; Canis lupus	95
22.	KT155887	Netherlands	Homo sapiens	95
23.	MH857487	Argentina		95
24.	AB193715	Japan		95
25.	MK298627	Belgium		95
26.	MK156725	outgroup		87

The isolates of these study are forming their own cluster in a group named by Clad D as type species Nannizzia fulva (MK112625 and MK112626), with the bootstrap support (97%) (Figure3). The sister cluster to this is Clade C which grouped different strains together including Arthroderma fulvum from following (Tunisia-scalp, Italy-human toenail, USA-ringworm infection Canis lupus, France and Australia-Thigh skin scraping), in addition to the Microsporum canis-Netherlands with the bootstrap support (66%). Clad A comprised only Nannizzia fulva – From different countries and different sources as show in (Figure 3 and Table1) with lower bootstrap (53%). The bootstrap -supported node (72%) is the basal to the Clad A, B and C. All branches between clades had a low bootstrap support values but no less than 50%, except for Clade D which high 97%. Trichophyton verrucosum used as an outgroup.

The identity matrices of the ITS sequences of this study compared with different geographical areas ITS sequences and are summarised in (Table1). Twenty four pairwise comparisons for ITS sequences were applied, BLAST search tool was used, to the sequence homology between the study isolates and others. Based on this Nannizzia fulva (MK112625, Iraq strain) showed that the obtained sequences shares 96% homology to Nannizzia fulva strains from: Hungary (MH858401 and KP132453), Iran (MG572987, KT192466, KT155781, JX413541 and JN134143), Egypt (KY129988), China(KX668868, MZ020032 and MZ019932), India (KT377205 and KT377205), Prague, Czech Republic (HG518407) (Table 1). While, the same sequence gives the similarity of (95%) with following; Iraqi isolate (MK_112626), Netherland (KT155887), Iranian (KT155887), France (KP132454), Australia (KP132455), Tunisia (KC784939), Italy (KP132451), USA (KT155842), Argentina (MH857487), Japan (AB193715) and Belgium (MK298627) (Table 1).

DISCUSSION

Dermatomycoses are very common infection of human and animals with wide spectrum of clinical manifestations [2,22] . Dermatophyte identification based on phenotypic and physiological methods is time-consuming and mostly has led to misdignosis due to the high degree of phenotypic similarities among relative species . Therefore, accurate identification of dermatophyte at the species level is essential due to therapeutic and epidemiological importance [23-25]. In current study, the two cases were found in patients aged ≤10 years,and this was in accordance with the results obtained by several studies [6-7,26-28]. Nevertheless, TC was recorded as the second most prevalent type of dermatophytosis in Iraq as revealed by several studies [16-18] .

The etiology and incidence of TC differs in various parts of the world [3,8,29] . Previous studies on diagnosis of tinea capitis in Iraq were mostly based on clinical features and phenotypic characteristics of the isolated causal pathogen. Our diagnosis of the two isolates was confirmed by the molecular analysis of ITS region of fungal ribosomal DNA. Studies on the etiology and incidence of TC in Iraq revealed different results. However, species of genus Trichophyton such as T.violaceum, T.mentogrophytes, T.verrucosum and T.tonsurans were the mostly reported as responsible for infections of the scalp [30-35] and to less extent T.audouinii [34,36]. Nannizia fulva formerly (Microsporum fulvum) is a geophilic dermatophyte of worldwide distribution, rarely encountered as etiologic agent of tinea infection in humans and animals [37] . The reason for the scanty of tinea reports on N.fulva (=M.fulvum) as etiologic agent of infections is probably due to misdiagnosis of M.fulvum with M.gypseum that mostly based on conventional methods. In recent investigation, molecular approach was employed to confirm the identification of the M. fulvum strains isolated from Iranian patient with tinea corporis and identified formerly as M.gypseum by culture and classical methods [38] . In previous studies in Iraq , N. fulva was not recorded as etiologic agent of tinea capitis in our country.

CONCLUSION

Nannizia fulva is newly recorded as causal agent of tinea capitis in Iraq. It seems that there is an epidemiologyical change regarding the etiological agents of tinia capitis in our region and currently N. fulva may represents important causal agent of TC particularly in Kurdistan Region, Iraq. Sequences of ITS rDNA as a general phylogenetic marker

succeeded to distinguish N.fulva from closely related species.

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