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Original article

Contribution of the internal transcribed spacer regions to the detection and identification of human fungal pathogens

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A B S T R A C T

Fungi are morphologically and phylogenetically diverse. There identification is largely based on phenotypic methods. Thus, related species, phenotypic variants and rare species may be unidentified. So, molecular methods have been introduced for identification of pathogenic molds to overcome these problems. In this study, we report the contribution of molecular tools (PCR sequencing) to identify fungal pathogens in both clinical and environmental samples. A total of 82 mold isolates were used (50 clinical samples and 32 environmental samples). PCR and direct sequencing, targeting the internal transcribed spacer (ITS) regions, were performed. We employed comparative sequence analysis to identify molds by using the GenBank database. 89% of isolates were identified by phenotypic methods. PCR- sequencing allowed the fungal identification in all cases. The concordance between molecular and morphological identification was obtained for 33 cases (40.2%). In 36 cases (43.9%), the molecular study gave the exact species identification. PCR sequencing allowed as revising mycological identification for 13 fungi strains (15.9%). The concordance of identification at species level by phenotypic method and by sequence analysis was obtained for 28% of clinical samples and for 59% of environmental samples. The phylogenetic tree for the ITS sequences showed six different clusters that are composed of isolates belonging to the same genus or species.

PCR sequencing has been shown to be useful for the detection of the presence of fungal DNA in both environmental and clinical samples. It is rapid and more sensitive for the identification of medically important fungi.

1. Introduction

The frequency and the severity of fungal infections, particularly the invasive forms, are in increase in the last years. This is related to the growth in the number of fungi, the growing of the immunocompromised population and the use of highly immunosuppressive agents for treatment of a variety of diseases.

However, the diagnosis of these fungal infections, remain problematic because the classical methods such as culture, lack sensitivity and specificity, or take too long to yield a result to be clinically useful. In addition, species differentiation is difficult since many fungi have similar morphological features. So these methods are usually, not effective for early diagnosis, which, is critical to effective treatment [1].

Currently, molecular identification methods enable fast and sensitive identification at a species level. These methods includes the sequencing of internal transcribed spacer (ITS) region, and non-sequencing based tests like polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP), real-time PCR . . . (2).

The internal transcribed spacer (ITS) regions are the most commonly used as a target because of the following benefits: multiple copies of the ribosomal gene are present in all organisms, enabling sensitive detection by PCR, and the ITS regions contains both highly conserved and variable regions, and are therefore, the optimal target for developing fungi specific PCR primers that discriminate among closely related species [3,4]. Thus, taxonomy and identification of fungal pathogens are increasingly dependent on PCR sequencing methods.

In this study, we report the usefulness and the contribution of molecular tools (PCR sequencing) to identify fungal pathogens in both clinical and environmental samples.

2. Material and methods

2.1. Fungal isolates

A total of 82 isolates were used in the present study: 50 isolates were selected from clinical samples received in the Department of

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Parasitology - Mycology university hospital - Sfax, Tunisia, during a 15 year period (from January 2004 to December 2018).

These samples included (43) cultures and [7] tissues biopsy samples obtained from patients who had fungal infection in different sites: sinusal (38%), cutaneous (6%), auricular (34%), cerebral (4%), corneal (6%), palate specimen (2%), cavum specimen (2%), blood culture (2%), toe and hand nail (6%) (Table 1).

Isolate selection criteria were as follows: isolates most frequently recovered in our laboratory, medically important isolates recovered infrequently, or isolates unidentifiable by phenotypic methods.

32 environmental samples: 3 *Rhizopus* species isolated from hospital environmental samples (air, surfaces) and 29 *Aspergillus* species isolated from air and environmental samples of homes of asthmatic patients (Table 2).

Environmental samples, were collected by swabbing (bed, table, rug, carpet, floor, wall, covering, headset) and by using contact Petri. For air sampling, we used a bioimpactor (180 liters of air/minute) (SAS super 180).

2.2. Conventional identification

All specimens were cultured onto Sabouraud dextrose agar (SDA) for a maximum of 4 weeks, depending on the type of specimen. Fungal isolates were identified on the basis of macroscopic and microscopic morphological characteristics.

2.3. DNA extraction

The genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN) as indicated by the manufacturer's instruction, and eluted with 50 μl of sterile water.

Table 1
Details of phenotypic and molecular identification of the 50 mold species isolated from clinical samples.

N°	Sample	Direct examination	Culture	PCR- sequencing result	Accession number
TN344D/11	Sinus biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH247233
TN345D/11	Sinus biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH247234
TN54D/11	Sinus biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH430185
TN290D/12	Sinus biopsy	hyphae	Not grow	*Curvilaria spicifera*	MH271090
TN299D/12	Sinus biopsy	hyphae	Not grow	*R.oryzae*	MH247235
TN403D/12	Nasal sinus polyp	hyphae	*A.sp + A.niger*	*A.awamori*	MH271091
TN422D/12	Cerebral abscess	hyphae	*A.sp + A.niger*	*A. oryzae*	MH271092
TN430D/12	Facial cellulitis	hyphae	*A.section flavi*	*A. oryzae*	MH271093
TN520D/12	Nasal biopsy	hyphae	*A.sp*	*A. flavus*	MH271094
TN533D/12	Cerebral biopsy	hyphae	*A.section flavi*	*A. flavus*	MH271095
TN539Hc/13	Blood culture	hyphae	*Fusarium solani*	*F.oxysporum*	MH271096
TN42COR/13	corneal	hyphae	*Bipolaris* sp	*Curvilaria spicifera*	MH271097
TN91D/13	sinus biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH271087
TN592D/13	Skin biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH271086
TN209AUR/14	auricular	hyphae	*A.parasiticus*	*A. oryzae*	MH271098
TN210AUR/14	auricular	hyphae	*A.parasiticus*	*A. oryzae*	MH271099
TN663D/14	Sinus biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH271088
TN902D/14	palate	hyphae	*Lichtheimia corymbifera*	*Lichtheimia ramosa*	MH685909
TN221D/15	Sinus biopsy	hyphae	Not grow	*Alternaria tenuissima*	MH329775
TN254AUR/15	auricular	hyphae	Undetermined Mucorale	*Saksenae vasiformis*	KU314816
TN1127D/15	nasal	hyphae	*R.oryzae*	*R.oryzae*	MH715404
TN252D/16	corneal	hyphae	*Fusarium sp*	*Fusarium solani*	MH329792
TN267D/16	Sinus biopsy	hyphae	*A.sp + R.oryzae*	*A.flavus*	MH681102
TN698D/16	Nasal sinus polyp	negative	negative	*Cladosporium cladosporoides*	MH329776
TN1093D/16	cavum	hyphae	Not grow	*R.oryzae*	MH271089
TN1142D/16	Sinus biopsy	hyphae	Not grow	*Cladosporium cladosporoides*	MH329777
TN85AUR/16	Auricular	hyphae	*A.sp*	*A. oryzae*	MH329778
TN594D/17	Sinus biopsy	hyphae	Undetermined fungi	*Alternaria alternanta*	MH329779
TN568AUR/17	auricular	hyphae	*A.sp*	*A.tamarii*	MH329780
TN573AUR/17	auricular	hyphae	*A.flavus*	*A.flavus*	MH329781
TN602AUR/17	auricular	hyphae	*A.sp*	*A. oryzae*	MH329782
TN611AUR/17	auricular	hyphae	*A.sp*	*A.oryzae*	MH329783
TN612AUR/17	auricular	hyphae	*A.sp*	*A.oryzae*	MH329784
TN551AUR/17	auricular	hyphae	*Emericella nidulans*	*Emericella.nidulans*	MH329785
TN619AUR/17	auricular	hyphae	*A.sp*	*A.oryzae*	MH329786
TN990D/17	Sinus biopsy	hyphae	*A.flavus*	*A.flavus*	MH329787
TN971D/17	Nasal biopsy	hyphae	*A.flavus*	*A.flavus*	MH329791
TN1231D/17	sinus	hyphae	*Not grow*	*R.oryzae*	MH665847
TN1242D/17	auricular	hyphae	*A.sp*	*A.flavus*	MH675522
TN1263D/17	Skin biopsy	hyphae	*R.oryzae*	*R.oryzae*	MH675533
TN1285OM/17	Hand nail	hyphae	*A.terreus*	*A.terreus*	MH329788
TN1345OM/17	Hand nail	hyphae	*Fusarium sp*	*Fusarium prolificans*	MH329789
TN2148OO/17	Toe nail	hyphae	*A.sp*	*Eurotium amstelodami*	MH329790
TN355D/18	Sinus	hyphae	*R.oryzae*	*R.oryzae*	MH660403
TN6OPHT/18	corneal	negative	*Alternaria sp*	*Alternaria alternata*	MH681051
TN44AUR/18	auricular	hyphae	*A.sp*	*A.oryzae*	MH675520
TN57AUR/18	auricular	–	*A.sp*	*A.flavus*	MH675519
TN75AUR/18	auricular	–	*A.flavus*	*A. tamarii*	MH675521
TN142AUR/18	auricular	–	*A.sp*	*A.oryzae*	MH681105
TN184AUR/18	auricular	hyphae	*A.sp*	*A.oryzae*	MH681104

A: *Aspergillus*, R: *Rhizopus*.

Table 2
Molecular identification by PCR- sequencing of fungal strains isolated from environmental samples.

N°	Isolation source	Culture	ITS-Sequencing	Accession number
1ENV/18	Hospital environment	*R.oryzae*	*R.oryzae*	MH681100
2ENV/18	Hospital environment	*R.oryzae*	*R.oryzae*	MH681101
3ENV/18	Hospital environment	*R.oryzae*	*R.oryzae*	MH682260
TN-5	Air	*A. versicolor*	*A.unguis*	KX610751
TN-42	Air	*Aspergillus sp*	*A. sepultus*	KX610733
TN-44	Environmental	*A. section flavi*	*A. ochraceus*	KX610749
TN-10	Air	*A. section flavi*	*A. pallidofulvus*	KX610754
TN-7	Environmental	*Aspergillus sp*	*A. tamarii*	KX610719
TN-33	Environmental	*A. flavus*	*A. flavus*	KX610724
TN-49	Air	*Aspergillus sp*	*A. chevalieri*	KX610737
TN-1	Environmental	*A. candidus*	*A. candidus*	KX610753
TN-121	Environmental	*A. fumigatus*	*A. fumigatus*	KX610740
TN-27	Air	*A. flavus*	*A. flavus*	KX610722
TN-30	Environmental	*A. flavus*	*A. flavus*	KX610732
TN-57	Air	*A. flavus*	*A. flavus*	KX610728
TN-69	Air	*A.nidulans*	*A.nidulans*	KX610744
TN-80	Environmental	*Aspergillus sp*	*A. quadrilineatus*	KX610735
TN-85	Environmental	*A. nidulans*	*A. nidulans*	KX610745
TN-203	Environmental	*A. flavus*	*A. flavus*	KX610729
TN-205	Environmental	*Aspergillus sp*	*A. quadrilineatus*	KX610736
TN-24	Air	*A. versicolor*	*A.unguis*	KX610752
TN-25	Air	*A. oryzae*	*A. sepultus*	KX610734
TN-26	Air	*A. section flavi*	*A. ochraceus*	KX610750
TN-136	Environmental	*A.nidulans*	*A.nidulans*	KX610748
TN-139	Air	*A.nidulans*	*A.nidulans*	KX610747
TN-144	Environmental	*A.nidulans*	*A.nidulans*	KX610746
TN-263	Air	*A. section flavi*	*A. tamarii*	KX610720
TN-275	Environmental	*A. section flavi*	*A. tamarii*	KX610721
TN-230	Environmental	*A. flavus*	*A. flavus*	KX610730
TN-236	Environmental	*A. flavus*	*A. flavus*	KX610731
TN-235	Environmental	*A. flavus*	*A. flavus*	KX610727
TN-306	Environmental	*A. nidulans*	*A. nidulans*	KX610743

2.4. Amplification by ITS1-ITS4

For specimens received in our laboratory, amplification of the ITS1, 5.8S and ITS2 regions was performed using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al [5].

Amplification reactions were performed in final volumes of 50 µl containing 400 ng of genomic DNA, 10 µl of 5X reaction buffer (pH 8.5), 25 mM of MgCl2, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (Promega), 25 pmol of each primer and 2.5 U of GOTaq® DNA polymerase (Promega). PCR was performed in a thermocycler (Eppendorf), it consisted of an initial denaturation at: 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C and a final extension at 72 °C for 10 min.

All PCR products were separated in 1.5% agarose gel, stained with ethidium bromide and visualized with a UV transilluminator and photographed.

2.5. Sequencing

PCR products were purified using PCR Clean-up system (Promega). Both strands of the PCR products were directly sequenced with a DNA Sequencing Kit BigDye® Terminator v3.1 cycle Sequencing Kits (Applied Biosystems) with ITS1-ITS4 primers and an automatic sequencer (ABI 3730 × 1 DNA Analyser), according to the manufacturer's instructions.

The sequences generated were used in BLAST searches (http://www.ncbi.nlm.nih.gov) to confirm the preliminary identification. The identity of each isolate was determined based on the sequence similarity of the ITS regions, specifically using those results with $\geq$ 99% similarity [6].

2.6. Phylogenetic analyses

The above mentioned sequences were aligned using the ClustalX V2.1 program [7] as implemented in BioEdit [8], sequence alignment Editor Version. 7.0.9.0 Software. The phylogenetic tree of ITS rDNA for available sequences of our strains was obtained using MEGA (Molecular Evolutionary Genetic analysis program) version 6 (Tamura K et al, 2013). Tree was created using UPGMA method. For bootstrap analysis, 1000 replicates were chosen for UPGMA tree. The evolutionary distance between organisms was indicated by the horizontal branch length, which reflects the number of nucleotide substitutions per site along that branch from the node to the endpoint. In the UPGMA tree, the percentage of bootstrap samplings that support the interior branches was noted.

3. Results

PCR of the ITS regions of the 82 clinical and environmental strains showed bands ranging from 570 to 735 bp. Direct sequencing allowed to identify fungi in all cases, with a sequence similarity $\geq$98%. Morphological methods allowed the identification of 89% of strains.

Phenotypic identification and sequencing results for all species are shown in Tables 1 and 2.

The concordance of identification at species level by microscopic examination of cultures and sequence analysis was obtained for 33 cases (40.2%). In 36 cases (43.9%), the molecular study gave the exact species identification. PCR sequencing allowed as revising mycological identification for 13 fungi strains (15.9%).

All of the obtained sequences were deposited in the GenBank database, the GenBank accession numbers are listed in Tables 1 and 2.

Table 3
Epidemiological and clinical characteristics for patients with fungal infections.

Epidemiological characteristics	
Age, years, median (range)	37.5 (1-66 years)
Gender, male (%)	54.0
Clinical characteristics	%
fungal sinusitis	22.4
rhino-orbito-cerebral mucormycosis	22.4
otomycosis	34.7
cutaneous mucormycosis	2
keratomycosis	6.2
cerebral abscess	4.1
onychomycosis	6.2
septicemia	2

3.1. Clinical samples

The general characteristics of the patients with fungal infections are shown in Table 3.

The concordance between morphological and molecular identification was obtained for 14 cases (28%). In 22 cases (44%), the molecular study gave the exact species identification. PCR sequencing allowed as revising mycological identification for **7** fungi strains (14%). In 7 cases (14%) with negative culture, the fungal species was directly identified by PCR sequencing from clinical specimen (Table 1).

In the cases TN403D/12 and TN422D/12, knowing that the two strains were isolated from the same patient, 2 different fungal species were isolated by culture. In this case, molecular study allowed us to identify the association of *A.awamori* and *A.oryzae* in sinus and cerebral samples.

In our samples, *A.oryzae* species was identified only by PCR-sequencing. Other rare *Aspergillus* species, such as *E. nidulans*, *Erotium amstelodami* were also, isolated.

For Mucorales, *R. oryzae* was identified in majority of cases by morphological and molecular tools. However, for other Mucorale species, only sequencing allowed us to identify *Lichteimia ramosa* in palate sample and *Saksenae vasiformis* in auricular sample.

We have, also, identified by molecular tools dematiaceous fungi and *Fusarium* species in corneal (TN42COR/13, TN252D/16, TN6OHT/18), sinus (TN290D/12, TN221D/15, TN698D/16, TN1142D/16, TN594D/17), hand nail samples (TN1345OM/17) and blood culture (TN539Hc/13).

3.2. Environmental samples

PCR-sequencing of the 32 environmental strains allowed fungal identification of all cases (Table 2). Phenotypic and molecular identification were concordant in 59% of cases. PCR sequencing allowed as revising mycological identification in19% of cases and gave the exact species identification in 22% of cases.

In these environmental samples, our molecular study confirmed well the phenotypic identification of some species (*A. flavus*, *A. nidulans*, *R. oryzae*) and allowed us to identify rare and new *Aspergillus* species, such as *A. unguis*, *A. sepultus A. pallidofulvus* and *A. chevaleri.*

3.3. Phylogenetic analysis

The phylogenetic tree for the ITS sequences exhibits six different clusters that are composed of isolates belonging to the same genus. A higher-level sequence divergence was observed among the isolates and intraspecies diversity was observed for majority of fungi (Fig. 1).

In the *Aspergillus* clade, *A. flavus* was clustered in two different groups. One of the groups included the majority of clinical and environmental species of *A. flavus*, *A. oryzae* and *A. tamarii*.

Our study showed that the species *A. awamori* was, phylogenetically, closer to *A. fumigatus, A. candidus* and *A. terreus,* than to other *Aspergillus* species.

For the other rare isolated species, *A. chevaleri* was closely related to *E. amstelodami*. *A. unguis* species were related to the *A. nidulans* group. While, *A. sepultus* and *A. pallidofulvus* were clustered with *A. ochraceus* species.

Concerning, the Mucorales fungi, six clusters appeared for *R. oryzae* species. One of them included 2 environmental samples which were identical to those isolated from three sinus samples.

These *Rhizopus* species were more divergent from *L. ramosa* and more related to *S. vasiformis* species.

For dematiaceous fungi, *Alternaria* and *Curvilaria* genus were, phylogenetically closer, than the *Cladosporium* genus.

4. Discussion

The growth in the number of fungal pathogens and the poor sensitivity of culture has led investigators to develop and apply new methods for fungal identification that go beyond classical phenotypic methods.

So, there is an increased emphasis on the use of molecular methods as surrogates for culture in diagnosis of fungal diseases.

In our study, we employed a molecular method based on PCR sequencing of ITS regions for mold identification and differentiation of our isolates. Currently, the ITS regions are considered the most useful, molecular target to identify and characterize fungi to the genus and species level [2,9].

In fact, the ITS regions are non-coding sequences interspaced among highly conserved fungal rDNA and are characterized by a high heterogeneity among fungi. This heterogeneity has helped in the phylogenetic and taxonomic analyses of molds [2,10,11].

Sequence analysis of this highly variable ITS regions have been, successfully, used in several studies for detection and identification of both yeasts and filamentous fungi [11–16].

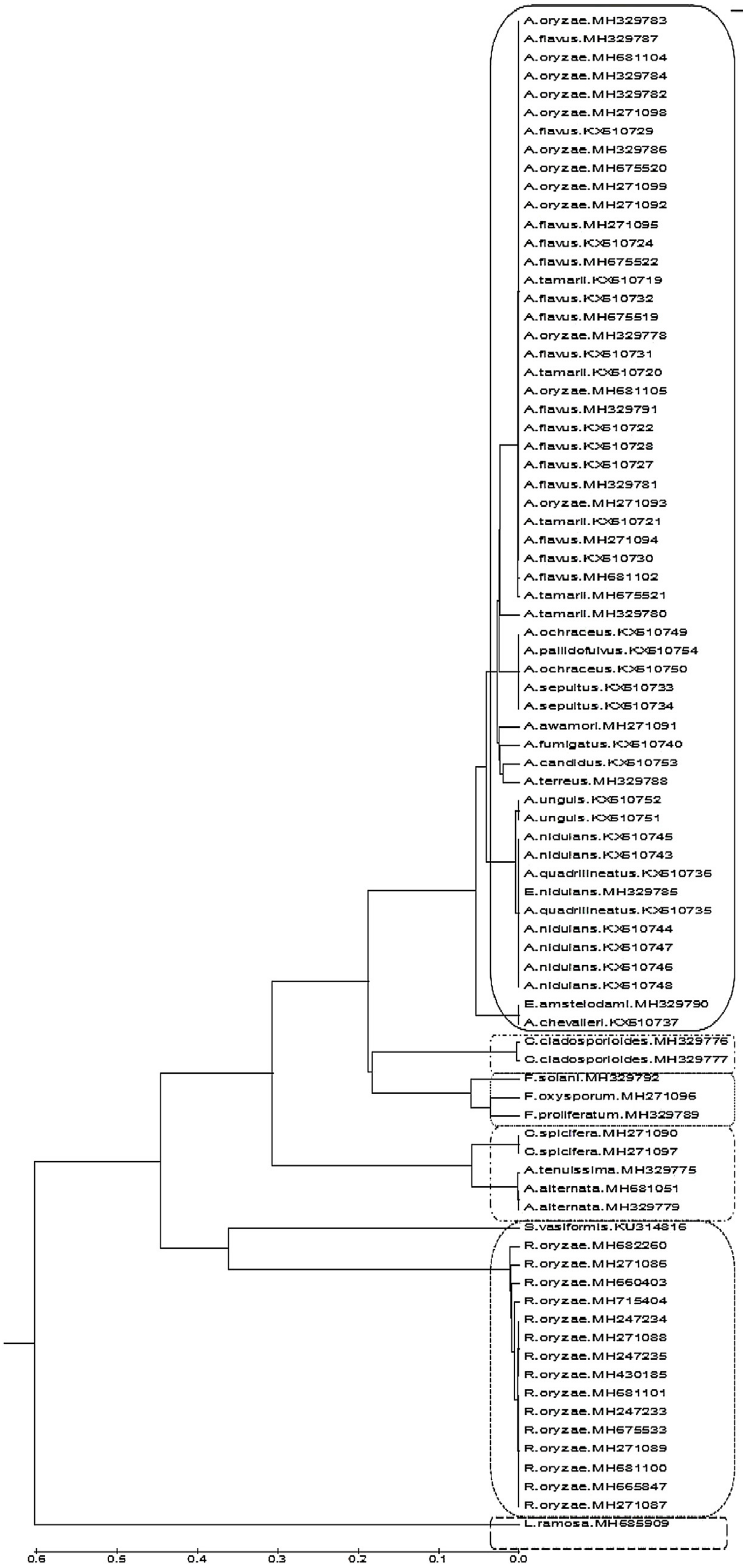
In our isolates, this method gave the exact species identification in 43.9% of cases and allowed as revising mycological identification for 15.9% of fungi strains.

In the study of Dannaoui et al, in which they compared between morphological and molecular identification of 27 Zygomycetes clinical isolates, mycological identification proved erroneous in more than 20% of cases [17].

In the clinical isolates, PCR sequencing allowed fungal species identification in 14% of cases directly from specimen. Hendolin et al., using a PCR- ITS based method, were able to detect fungal pathogens directly from fresh tissue. They showed that the ITS-PCR was a rapid method for detection and identification of fungal pathogens in tissue and it is a more sensitive technique than microscopy and culture [18].

In the cases TN403D/12 and TN422D/12, molecular study, allowed us to identify *A. oryzae* and *A. awamori*. This later species is included in the *Aspergillus* section Nigri, and is morphologically indistinguishable from *A. niger* species [19]. Recently, clinical isolates thought to belong to *A. niger* were re-classified by genetic tools as *A. awamori* [20,21]. Consequently, we diagnosed a rare case of fungal sinusitis complicated with cerebral abscess and caused by an association of *A. awamori* and *A. oryzae*.

In our samples, we could not identify *A. oryzae* by the phenotypic methods. It was identified only by PCR-sequencing. In fact, this species is frequently confused with other *Aspergillus* species belonging to *Aspergillus* section Flavi, such as, *A. parasiticus*. This group now comprises six important species that are very closely related morphologically and phylogenetically: *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae* and *A. tamarii* [22].



	ID	sample	Accession number
A.oryzae.MH329783	TN611AUR/17	auricular	MH329783
A.flavus.MH329787	TN990D/17	Sinus biopsy	MH329787
A.oryzae.MH681104	TN184AUR/18	auricular	MH681104
A.oryzae.MH329784	TN612AUR/17	auricular	MH329784
A.oryzae.MH329782	TN602AUR/17	auricular	MH329782
A.oryzae.MH271098	TN209AUR/14	auricular	MH271098
A.flavus.KX610729	TN-203	environnemental	KX610729
A.oryzae.MH329786	TN619AUR/17	auricular	MH329786
A.oryzae.MH675520	TN44AUR/18	auricular	MH675520
A.oryzae.MH271099	TN210AUR/14	auricular	MH271099
A.oryzae.MH271092	TN422D/12	Cerebral abscess	MH271092
A.flavus.MH271095	TN533D/12	Cerebral biopsy	MH271095
A.flavus.KX610724	TN-33	environnemental	KX610724
A.flavus.MH675522	TN1242D/17	auricular	MH675522
A.tamarii.KX610719	TN-7	environnemental	KX610719
A.flavus.KX610732	TN-30	environnemental	KX610732
A.flavus.MH675519	TN57AUR/18	auricular	MH675519
A.oryzae.MH329778	TN85AUR/16	Auricular	MH329778
A.flavus.KX610731	TN-236	environnemental	KX610731
A.tamarii.KX610720	TN-263	Air	KX610720
A.oryzae.MH681105	TN142AUR/18	auricular	MH681105
A.flavus.MH329791	TN971D/17	Nasal biopsy	MH329791
A.flavus.KX610722	TN-27	Air	KX610722
A.flavus.KX610728	TN-57	Air	KX610728
A.flavus.KX610727	TN-235	environnemental	KX610727
A.flavus.MH329781	TN573AUR/17	auricular	MH329781
A.oryzae.MH271093	TN430D/12	Facial cellulitis	MH271093
A.tamarii.KX610721	TN-275	environnemental	KX610721
A.flavus.MH271094	TN520D/12	Nasal biopsy	MH271094
A.flavus.KX610730	TN-230	environnemental	KX610730
A.flavus.MH681102	TN267D/16	Sinus biopsy	MH681102
A.tamarii.MH675521	TN75AUR/18	auricular	MH675521
A.tamarii.MH329780	TN568AUR/17	auricular	MH329780
A.ochraceus.KX610749	TN-44	environnemental	KX610749
A.pallidofulvus.KX610754	TN-10	Air	KX610754
A.ochraceus.KX610750	TN-26	Air	KX610750
A.sepultus.KX610733	TN-42	Air	KX610733
A.sepultus.KX610734	TN-25	Air	KX610734
A.awamori.MH271091	TN403D/12	Nasal sinus polyp	MH271091
A.fumigatus.KX610740	TN-121	environnemental	KX610740
A.candidus.KX610753	TN-1	environnemental	KX610753
A.terreus.MH329788	TN1285OM/17	Hand nail	MH329788
A.unguis.KX610752	TN-24	Air	KX610752
A.unguis.KX610751	TN-5	Air	KX610751
A.nidulans.KX610745	TN-85	environnemental	KX610745
A.nidulans.KX610743	TN-306	environnemental	KX610743
A.quadrilineatus.KX610736	TN-205	environnemental	KX610736
E.nidulans.MH329785	TN551AUR/17	auricular	MH329785
A.quadrilineatus.KX610735	TN-80	environnemental	KX610735
A.nidulans.KX610744	TN-69	Air	KX610744
A.nidulans.KX610747	TN-139	Air	KX610747
A.nidulans.KX610746	TN-144	environnemental	KX610746
A.nidulans.KX610748	TN-136	environnemental	KX610748
E.amstelodami.MH329790	TN2148OO/17	Toe nail	MH329790
A.chevalieri.KX610737	TN-49	Air	KX610737
C.cladosporioides.MH329776	TN698D/16	Nasal sinus polyp	MH329776
C.cladosporioides.MH329777	TN1142D/16	Sinus biopsy	MH329777
F.solani.MH329792	TN252D/16	corneal	MH329792
F.oxysporum.MH271096	TN539Hc/13	Blood culture	MH271096
F.proliferatum.MH329789	TN1345OM/17	Hand nail	MH329789
C.spicifera.MH271090	TN290D/12	Sinus biopsy	MH271090
C.spicifera.MH271097	TN42COR/13	corneal	MH271097
A.tenuissima.MH329775	TN221D/15	Sinus biopsy	MH329775
A.alternata.MH681051	TN6OPHT/18	corneal	MH681051
A.alternata.MH329779	TN594D/17	Sinus biopsy	MH329779
S.vasiformis.KU314816	TN254AUR/15	auricular	KU314816
R.oryzae.MH682260	TN3ENV/18	Environnemental hospital	MH682260
R.oryzae.MH271086	TN592D/13	Skin biopsy	MH271086
R.oryzae.MH660403	TN355D/18	Sinus	MH660403
R.oryzae.MH715404	TN1127D/15	nasal	MH715404
R.oryzae.MH247234	TN345D/11	Sinus biopsy	MH247234
R.oryzae.MH271088	TN663D/14	Sinus biopsy	MH271088
R.oryzae.MH247235	TN299D/12	Sinus biopsy	MH247235
R.oryzae.MH430185	TN54D/11	Sinus biopsy	MH430185
R.oryzae.MH681101	TN2ENV/18	Environnemental hospital	MH681101
R.oryzae.MH247233	TN344D/11	Sinus biopsy	MH247233
R.oryzae.MH675533	TN1263D/17	Skin biopsy	MH675533
R.oryzae.MH271089	TN1093D/16	cavum	MH271089
R.oryzae.MH681100	TN1ENV/18	Environnemental hospital	MH681100
R.oryzae.MH665847	TN1231D/17	sinus	MH665847
R.oryzae.MH271087	TN91D/13	sinus biopsy	MH271087
L.ramosa.MH685909	TN902D/14	palate	MH685909

Fig. 1. Phylogenetic tree based on ITS region rDNA sequences of mold species using UPGMA method. The percentages of bootstrap samplings, derived from 1000 samples which were supporting the interior branches, are noted.

Also, our molecular study allowed us to identify other *Aspergillus* species, such as *E. nidulans* and *Erotium amstelodami*. These species were rarely reported as agents of invasive and superficial fungal infections.

For Mucorales, *R. oryzae* is considered as a major zygomycete genus responsible of rhino-orbito-cerebral and cutaneous infections. It was easily, identified in majority of our cases by morphological and molecular tools. However, PCR-sequencing allowed us to identify other rare and emergent agents of human mucormycosis. We diagnosed an invasive fungal sinusitis case caused by *L. ramosa,* which is morphologically identified as *L. corymbifera.* In fact, several studies, showed that a significant number of isolates identified as *L. corymbifera* were, really, *L. ramosa* [23,24]. We have also, diagnosed a rare case of malignant *S. vasiformis* otomycosis. This case is the first reported in Tunisia and the second in the literature.

PCR-sequencing has rectified the diagnosis of disseminated *Fusarium oxysporum* infection with endocarditis and as identified *F. solani* morphologically.

Also, we isolated Dematiaceous fungi, such as (*C. spicifera*, *C. cladosporoides*, *A. alternata*, *A. teniusima*) in keratomycosis and fungal sinusitis. In fact, these agents are increasingly seen in a variety of clinical syndromes in both immunocompromised and normal hosts. They are, particularly, responsible for cutaneous infections, oculomycosis, invasive sinusitis and allergic fungal sinusitis [25]. These fungi grow slowly in conventional culture media and often species differentiation is morphologically difficult.

For environmental samples, PCR sequencing allowed as revising mycological identification in 18% of cases and gave the exact species identification in 22% of cases.

The ITS regions are the most commonly chosen genetic marker for the molecular identification of fungi in environmental and ecological studies [26].

They have been, widely, used in many environmental studies for identification and discrimination between *Aspergillus* and other molds [2,14,15,27–30].

A. flavus and *A. nidulans* were the most species isolated in our samples. *A. tamari* was also, identified but frequently confused with *A. flavus*. Although, *A. fumigatus* and *A. flavus* have been reported as the major causative agents of *Aspergillus* infections, *A. tamarii* is considered as an unusual opportunistic human pathogen. It was, recently, isolated from cutaneous infection, keratomycosis and onychomycosis [31–34].

In addition, PCR sequencing allowed us to identify a rare *Aspergillus* species in our environmental samples, such as *A. unguis*, *A. sepultus*, *A. pallidofulvus*, *A. quadrilineatus* and *A. chevaleri*. *A. unguis* was one of two most common fungi encountered in the homes of asthmatic children in Detroit, Michigan, reported from 72% of homes [35]. Currently, human infections were described for these fungi. Nail and skin lesions caused by *A. unguis* and *A. quadrilineatus* has been reported [36,37]. *A. chevalieri* was reported for the first time as a cause of fatal cerebral aspergillosis acquired by traumatic inoculation probably from the environment [38].

Furthermore, we have used ITS regions as a tool for phylogenic analysis of our fungi strains. It allowed us to identify distinct genetic groups among our different mold and intra-species diversity for majority of species. In the same way, many studies used the same markers to classify their fungal species [2,39].

As well, the phylogenetic tree confirmed the close relationship species of *Aspergillus* section flavi (*A. flavus*, *A. oryzae* and *A. tamarii*).

For the other rare isolated species, we found that *A. chevaleri* was closely related to *E. amstelodami.* These strains were assigned to belong to the teleomorph genus *Erotium.* It contains 20 species, four on which were commonly encountered: *E. amstelodami*, *E. chevalieri*, *E. herbariorum*, and *E. rubrum.* These species were, currently, accommodated in section *Aspergillus* of the genus *Aspergillus.*

A. unguis species were included in the *A. nidulans* group. In fact, *A. unguis* is the anamorph state of *Emericella unguis*; and was, historically, assigned to the *A. nidulans* group, due to the resemblance of its ascospores and cleistothecia to those of *Emericella nidulans*.

A. sepultus and *A. pallidofulvus* were clustered with *A. ochraceus* species. *A. ochraceus* belongs to the *Aspergillus* section *Circumdati.* This section was, currently, revised and 27 species are accepted, introducing seven new species, of which, *A. pallidofulvus*, and *A. sepultus* [40].

For the *Aspergillus* environmental samples, collected in the study of fungal flora in asthmatic patients, Trabelsi et al, found that some strains isolated from the clinical and environmental samples were identical and have the same genotype [41].

Concerning, the Mucorales fungi, high sequence dissimilarities were found among our isolates of *R. oryzae* consistently with what observed by Abe et al [42]. The same sequence of ITS region found in the two environmental and clinical isolates suggests that it is the same strain.

For dematiaceous fungi, *Alternaria* and *Curvilaria* species were closer than the *Cladosporium* genus. In fact, the tree genus belongs to the Dothideomycetes class but not included in the same order.

In conclusion, this study highlights the value of ITS sequencing as a diagnostic tool for correct mold identification. It allowed the accurate identification of species frequently misidentified by phenotypic methods. Indeed, the accurate identification of these fungi is important to determine etiology of the disease, to detect new pathogen species, to predict intrinsic resistance to antifungal agents, to initiate an early appropriate therapy and to detect clusters of nosocomial infection among hospitalized patients.

Disclosure of potential conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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