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Identificación molecular de aislamientos clínicos de *Fusarium* en Colombia

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ABSTRACT

Objective Identifying *Fusarium* isolates from mycosis symptomatic patients through molecular techniques as PCR and sequencing.

Methods In this study, samples were taken from 101 mycosis symptomatic patients in-between 2004-2006. To determine isolates belonging to the *Fusarium* genus, the DNAr 28S region was amplified through PCR and specific PCR primers further confirmed their identity to the species level. Additionally, in order to confirm the identity of the species of the isolates, 75 isolates of these were analyzed by partial sequencing of the 28S rDNA and the TEF1-α gene.

Results The 28S rDNA portion detected all 101 isolates as belonging to *Fusarium* and the PCR specific primers detected 52 and 29 isolates as *F. oxysporum* and *F. solani*, respectively; 34 and 41 of these, afterwards studied by partial sequencing of the 28S rDNA and TEF1-α genes respectively, were effectively identified by the technique.

Conclusion From all the molecular markers used to identify *Fusarium* isolates, the sequence of the TEF1-α gene provided the best resolution in the identification of species level; however it is possible to discriminate between *F. oxysporum* and *F. solani* isolates by PCR, in most of the cases, what is important considering the simplicity of the technique and a faster diagnosis.

Key Words: PCR; rDNA; sequence analysis; elongation factor *(source: MeSH, NLM)*.

RESUMEN

Objetivo Identificar aislamientos de *Fusarium* en pacientes con micosis por medio de las técnicas moleculares de PCR y secuenciación.

Métodos Se tomaron 101 muestras de pacientes con micosis sintomática, entre los años 2004 y 2006. Para la detección de aislamientos como pertenecientes al género *Fusarium*, se amplificó parcialmente por PCR la región 28S del DNAr; y posteriormente —para la detección de la especie de *Fusarium*— se utilizaron cebadores específicos para *F. oxysporum* y *F. solani*. La verificación de la identidad de la especie de los aislamientos se hizo por secuenciación parcial de los genes 28S DNAr y TEF1-α.

Resultados El total de 101 aislamientos fueron detectados como pertenecientes al género *Fusarium* utilizando un cebador universal de la region 28S DNAr; 52 y 29 aislamientos se detectaron como *F. oxysporum* o F. salani, respectivamente con los cebadores específicos, y la secuenciación parcial de los genes 28S rDNA o TEF1-α confirmó la identidad de las especies.

Conclusión La secuenciación parcial del gen TEF1-α es aún el mejor marcador molecular para identificar aislamientos de *Fusarium* a nivel de especie. Sin embargo, en la mayoría de los casos es posible discriminar entre aislamientos de *F. oxysporum* y *F. solani* por PCR con cebadores específicos, lo que proporciona una ventaja importante considerando la simplicidad de la técnica y el rápido diagnóstico.

Palabras Clave: ADN ribosómico; PCR; secuenciación; elongación *(fuente: DeCS, BIREME)*.

Fusarium disease is the second reason of fungal infection caused by filamentous fungi, after aspergillosis (1). The incidence of this fungal infection varies according to the region and is more common in regions with warm and humid weathers (2). *Fusarium* fungi can infect humans through the inhalation of micro particles from the polluted environment. Other less frequent forms of contamination occur through direct contact with skin structures damage, in-hospital contamination of water deposits and nearby construction sites, from which the dispersion of the conidia in micro particles is capable of reach the respiratory tract (1).

In humans some *Fusarium* species cause infections as onychomycosis, keratitis, endophthalmitis, fungemia by using catheters, peritonitis, skin and subcutaneous infections; and less frequent, osteomyelitis, arthritis, otitis, sinusitis and brain abscess, and invasive fungal infections (3-5).

The clinical form of fusariosis depends largely on the immune status of the host. Superficial and localized diseases occur mostly in immunocompetent patients and invasive and disseminated diseases affect immunocompromised patients (6). Unlike the immunocompetent host, where onychomycosis and keratitis are the most frequent manifestations, disseminated fusariosis is the most common form of presentation in severe immunosuppression states (1). The most commonly associated predisposing factors are: prolonged neutropenia especially in patients with leukemia or transplanted hematopoietic progenitors; therapy with corticosteroids or cytotoxic chemotherapy (7). The typical pattern is a granulocytopenic patient who has received a treatment prolonged with broad-spectrum antibiotics due to fever unknown etiology. A higher incidence has also been observed of infections by these filamentous fungi in patients with solid organ transplantation (7).

The distinctive feature of disseminated fusariosis is the appearance of purpuric skin nodules with an area of central necrosis. In general, the biopsy of these nodules reveals the presence of septate hyaline hyphae with branches that invade the dermal blood vessels. The cultures of the biopsy material and the blood cultures (positive in more than 75%) are useful in the diagnosis of *Fusarium* infection. Clinically, it manifests with fever and large ulcerated skin lesions that progress to necrosis. This appearance complicates the distinction of this fungus with *Aspergillus*. However, unlike aspergillosis, patients with disseminated fusariosis usually have positive blood cultures (7). The prognosis is reserved since the mortality of the disseminated form is around 80% (1).

Fusarium houses about 100 species, most reported as human pathogens are *F. solani* (Mart.) Sacc and *F. oxysporum* Schlechtendal (8-10) and less frequently *F. vertici-*

llioides (Sacc.) Nirenberg (9,10), *F. proliferatum* (Matsush) (11,12), *F. dimerum Penz* (13) and *F. sacchari* (EJ Butler & Hafiz Khan) W. Gams (14); however, in most clinical cases the species is not reported because it is one of the most heterogeneous and difficult to classify fungi (15). Isolates of *F. solani* are more resistant to most of the antimicotic available than those of *F. oxysporum*.

Recently, the use of molecular methods based on sequence analysis of multiple genes has contributed to a more accurate identification of *Fusarium* and has showed great inter -and intra- species diversity, that in some cases represent complexes of species (16,17). Among the most important molecular markers used to identify *Fusarium* are the 5' region of the 28s rDNA subunit (18) and the gene encoding the elongation factor of translation 1α (TEF1-α), considered latter as the most informative gene for identification to species or species complex of the genus (19).

In Colombia, the identification of clinical isolates of *Fusarium* to the species level is incipient. However, there are studies that suggest *Fusarium* spp. as the main causative agent of onychomycosis, among non- dermatophyte fungi (20-22); and, cases of invasive fusariosis (1), a single pulmonary nodule in an immunocompetent patient (23) and keratitis (24) has been reported.

Having into account the simplicity of the PCR technique, the objectives of this study were to detect clinical isolates belonging to the genus *Fusarium* and the species *F. oxysporum*, *F. solani*, *F. verticillioides* and *F. subglutinans*, by PCR with specific primers. Also, to identify the isolates by partial sequencing of the gene 28s rDNA and the TEF1-α, in order to validate the PCR technique to detect the most common species of *Fusarium*. This study will contribute to a faster and more accurate diagnosis that is essential for timely and accurate treatment, taking into account the high resistance of *Fusarium* to most of the antifungal agents available.

MATERIALS AND METHODS

Origin of isolates

101 clinical isolates were obtained from symptomatic individuals, submitted to the laboratory of Medical Mycology and Experimental (MME) of the Corporation for Biological Research (CIB) in the period between 2004 and 2006. Samples were taken from nails and toes, cornea, sinuses, skin, or discharge; they were cultured in Sabouraud Agar, PDA and Mycosel media and incubated at room temperature for a period of seven to 14 days.

Preliminary identification of *Fusarium*

The initial identification of isolates of *Fusarium* spp. was performed by observation at the microscopic of plate with

lacto phenol blue, direct examination with 20% KOH Chinese ink and by description of the macroscopic features of colonies after a period of incubation of eight to ten days at 23 °C. Pure colonies identified as *Fusarium* spp. were stored in vials with sterile water.

DNA extraction from monosporic culture

The monosporic culture of each isolate of *Fusarium* spp. was carried out by Giraldo D. in 2010 (25). A portion of mycelium (approx. 100 mg) was taken and it was macerated with liquid nitrogen and equal volume of lysis buffer (1 mM EDTA, 10 mM Tris HCl, pH 8, sodium acetate was added 2M, 100 mM NaCl, Triton X - 100 2% and SDS 1%), for DNA extraction (26).

Detection by PCR of *Fusarium* to the genus and species level

PCR detection of isolates belonging to the genus *Fusarium* was performed with the primer pair Fus1/Fus2, which have as target the 28S rDNA subunit (27). Detection of *F. oxysporum* was performed with the primer pair OX31/OX32 (28). *F. solani* isolates were detected with the primer pair Fusorev and Fusofor, which have as target the 18S rDNA subunit (29). The previous thermocycler conditions used were those reported (25,27–29).

Specificity of the primers used in the detection of the genus *Fusarium* and the species *F. oxysporum* and *F. solani*

The specificity of the primers used for the detection of *Fusarium* and *F. oxysporum* and *F. solani* species was assessed by PCR with DNA of: *Acremonium* sp., *Alternaria* sp, *Aspergillus flavus, A. fumigatus, A. niger, A. terreus, A. versicolor, Candida albicans, C. tropicalis, Malassezia furfur, Neoscytalidium dimidiatum, Penicillium* sp., *Scedosporium apiospermun* and *Trichosporon asahii,* fungi commonly diagnosed in the Medical Mycology Unit of the CIB.

Partial sequencing of 28S rDNA

Partial sequencing of the 28S rDNA with the primer pair Fus1/Fus2 was performed for 34 isolates. The selection of the isolates was carried out as follows: 10 isolates identified as *F. oxysporum* with OX31/OX32 primers; five isolates, identified as *F. solani* with the primers Fusorev/Fusofor; and 16 isolates, whose species could not be detected with any of the primer pairs OX31/OX32, Fusofor/Fusorev. Additionally, three isolates (31 791, 32 892 and 32 989) from the collection the Universidad de los Andes Laboratory of Microbiology and Plant Pathology (LAMFU) were included. The PCR products were sent to

Macrogen (South Korea) for sequencing in both directions, in an ABI Prism 3730XL.

The sequences were edited with Genious Pro software (version 4.5 Biomatter Inc). A multiple alignment was performed with Clustal W using the consensus sequence of each isolate; it included five sequences of the 28S rDNA of different *Fusarium* species, reported in the GenBank database (accession number FJ614650.1F, AYO97318.1, AYO97316.1, DQ236682.1 and EU926284.1, http://www.ncbi.nih.gov/GenBank/). A phylogenetic tree was constructed by Neighbor–Joining, with 1 000 replicates and a supported Bootstrap above 50%, by the Jukes-Cantor model with the option "pairwise deletion" with the MEGA 6 software. In addition, each of the consensus sequences was identified by BLAST (www.ncbi.nlm.nih.gov/ blast/) analysis to confirm their identity.

Partial sequencing of TEF1-α

41 isolates selected from the results obtained by detection and sequencing of the 28S rDNA gene, were also analyzed by partial sequencing of TEF1-α gene. The PCR amplification was done with the EF1-EF2 primers (30), in a thermocycler T3000 (Biometra), with the following parameters: initial denaturation at 94 °C for five min., followed by 35 cycles of 95 °C for 30 s., 57 °C for one min., 72 °C for one min., and a final extension at 72 °C for seven min. The amplified products were purified with the QIAquick PCR Purification kit (QIAGEN, Germany). Sequencing was done in both directions with the primer pair EF3 and EF22T (31), with the Big Dye Terminator Cycle methodology (Applied Biosystems, USA), in an ABI Prism 3730XL sequencer of Macrogen (South Korea).

The species complex (hereafter the species) of each isolated was determined with the consensus sequence by BLAST on *Fusarium* ID database (http://www.*fusarium*db.org/ , table 1). With all sequences, and eight sequences of *Fusarium* ID database and eight sequences of the GenBank; a multiple alignment was performed using the algorithm Clustal W. A phylogenetic tree was constructed by Neighbor-Joining, with 1 000 replicates and a supported Bootstrap above 50%, by the Kimura-2 parameter model with the option "pairwise deletion" with the MEGA 6 software.

Electrophoresis

All PCR-amplified fragments were observed on agarose gel electrophoresis in 1,5% (w/v), stained with ethidium bromide (0.5 µl/10 ml), the size of the fragments was verified by comparison with the molecular weight marker 100 bp GeneRuler Plus (Fermentas).

Table 1. Molecular identification of *Fusarium* isolates obtained from clinical samples

Code of the strain	Gender	PCR OX31 / OX32	PCR Fusofor / Fusorev	Identification by rDNA sequencing	Identification by TEF1-ᵛ sequencing
55347	F	N	Y		*F. solani*
55349	F	Y	N	*F. oxysporum*	
55399	F	N	Y		
55444	M	Y	N		
55466	M	Y	N		
55496₪	F	N	N	*Fusarium spp*	
55498	F	Y	N		
55529	M	N	Y		
55583	M	Y	N	*F. oxysporum*	*F. oxysporum*
55585	F	Y	N		
55588	F	Y	N		*F. oxysporum*
55639	F	Y	N		
55760	F	Y	N		
55762	F	Y	N		*F. oxysporum*
55787₪	F	N	N	*F. oxysporum*	*F. oxysporum*
55827	F	Y	N		
55861	F	Y	N	*F. oxysporum*	*F. oxysporum*
55882	F	Y	N		
55945	F	N	Y	*F. solani*	*F. oxysporum*
55979	M	Y	N		*F. oxysporum*
56016	F	Y	N		
56054	M	N	Y		*F. solani**
56094	F	Y	N		*F. oxysporum**
56104	F	Y	N		
56212	F	Y	N	*F. oxysporum*	*F. oxysporum*
56240	F	N	Y	*F. solani*	*F. solani*
56242	M	Y	N		
56301	F	N	Y	*F. solani*	
56320	F	Y	N		
56321	F	N	Y		
56323	F	Y	N		*F.oxysporum*
56337	F	N	Y		
56340	F	N	Y		*F. solani*
56363	F	Y	N	*F. oxysporum*	*F.oxysporum*
56375	F	Y	N		
56604	F	N	Y		
56665₪	F	N	N	*Fusarium spp*	*Fusarium spp*
56762	F	Y	N		
56780₪	F	N	N	*F. solani*	*F. solani*
56797	F	Y	N		
56848	F	Y	N		
56868	F	N	Y		
56891	M	N	Y		
56894	M	Y	N		*F.solani*
56988	M	N	Y	*F. solani*	*F.solani*
57034	F	Y	N		
57076	F	Y	N		
57081	F	Y	N		
57221	F	Y	N	*F. oxysporum*	*F.oxysporum*
57228	F	Y	N	*F. oxysporum*	*F.oxysporum*
57239	M	Y	N		
57250	F	N	Y		
57335	F	N	Y		
57502	F	N	Y		
57538	F	Y	N		
57560	F	Y	N	*F. oxysporum*	*F.oxysporum*
57721	F	N	Y	*F. solani*	*F.solani*
57855	F	N	Y		
57885₪	M	N	N	*F. oxysporum*	*F.oxysporum*
57949	F	Y	N		*F.oxysporum*
57952	F	N	N		

Code of the strain	Gender	PCR OX31 / OX32	PCR Fusofor / Fusorev	Identification by rDNA sequencing	Identification by TEF1-sequencing
58023	F	N	Y		F. solani
58025	M	Y	N	F. oxysporum	F. solani
58474₪	M	N	N	F. solani	
58756₪	F	N	N	F. solani	
62079₪	F	N	N	F. dimerum	
62698₪	M	N	N	F. solani	F.oxysporum
62802	F	Y	N		F.oxysporum
63051₪	M	N	N	F. solani	
63145₪	F	N	N	F. oxysporum	F.oxysporum
63200₪	F	N	N	F. solani	F. solani
63230₪	F	N	N	F. solani	
63316	F	N	N		F.oxysporum
63353	F	N	N		
63392	F	N	N		
63414₪	M	N	N	F. oxysporum	F.oxysorum
63447₪	F	N	N	F. solani	F.solani
63507₪	F	N	N	Fusarium spp	
63550	F	Y	N		
63613	F	Y	N		
63635	F	N	N		F.solani
63648	F	Y	N		
63649	F	Y	N	F. oxysporum	F.oxysporum
63666	F	N	Y		F.oxysporum
63746	M	Y	N		
63749	M	Y	N		
63768	F	Y	N		F. oxysporum
63783	F	N	Y		
63786	F	Y	N		
63857	F	Y	N		
63868	F	Y	N		
63880	F	Y	N		
63901	F	Y	N		
63917	F	Y	N		F.solani
63946	F	N	Y		
63962	M	Y	N		
64051	M	N	Y		
64765	F	N	Y		F.solani
64927	F	N	Y		
64938	M	N	Y		
65068	F	N	Y		F.solani
31791	-	N	Y	F. solani	
32892	-	Y	N	F. oxysporum	
32989	-	N	N	Fusarium spp	
Control 1	-	Y	N		
Control 2	-	N	Y		

* Identification obtained by comparison to the GenBank database using the BLAST tool. ** Identification obtained by comparison to Fusarium ID database using the BLAST tool. ₪ Unamplified strains with any of the primers

RESULTS

Preliminary identification of *Fusarium* isolates
101 clinical isolates of *Fusarium* were studied in this research. Macroscopically, most colonies were characterized by their cottony aspect, salmon pigmentation, purple, lilac, light brown, yellow or gray. Microscopically with hyaline hyphae, septate and filamentous; septate macroconidia, fusiform with characteristic appearance of alantoespora, oval microconidia and in some cases chlamydoconidia of thick wall (25).

PCR detection of clinical isolates of *Fusarium*
PCR detection of *Fusarium* isolates with the primers Fus1/Fus2 allowed to obtain PCR products of the expected size for all 101 isolates. The primers showed that they are specific to the genus *Fusarium*, since they did not amplify any fragment from the DNA of all the other fungus genera evaluated.

PCR detection of *F. oxysporum* and *F. solani* isolates with the primer pairs Fusofor/Fusorev and OX31/OX31, respectively, revealed the expected fragment from 52 (51.4%) and 28 (27,7%) out of 101 isolates, respectively.

The specificity of these two pairs of primers showed no amplification of any DNA fragment from the other genera of fungi evaluated.

Partial sequencing of the 28s rDNA gene and TEF1-α
A total of 34 and 41 isolates were analyzed by partial sequencing of the 28s rDNA and TEF1-α genes, respectively. Besides 10 isolates being detected by PCR as *F. oxysporum*, the rDNA also allowed to identify them as *F. oxysporum*; likewise, TEF1-α sequencing confirmed the identity of all

as *F. oxysporum*, except for the isolate 58 025, which was identified as *F. solani*. In the dendrogram constructed from rDNA sequences, the 58 025 was allocated in the cluster I, with 100% of identity with strain ATCC MYA- 3970 of *F. oxysporum* (Figure 1), but in the dendrogram constructed with sequences obtained from TEF-α, it was located in one of the cluster of *F. solani* (Figure 2).

Similarly, of five isolates detected by PCR as *F. solani*, rDNA sequencing indicated its identity as *F. solani*; the TEF1-α gene confirmed the identity of the isolates as *F.*

Figure 1. Dendrogram of the 28S rDNA region of 34 clinical isolates of *Fusarium* spp., and five sequences of *Fusarium* spp., taken from the GenBank, constructed by Neighbor Joining with Bootstrap support > 50%, with 1000 replications
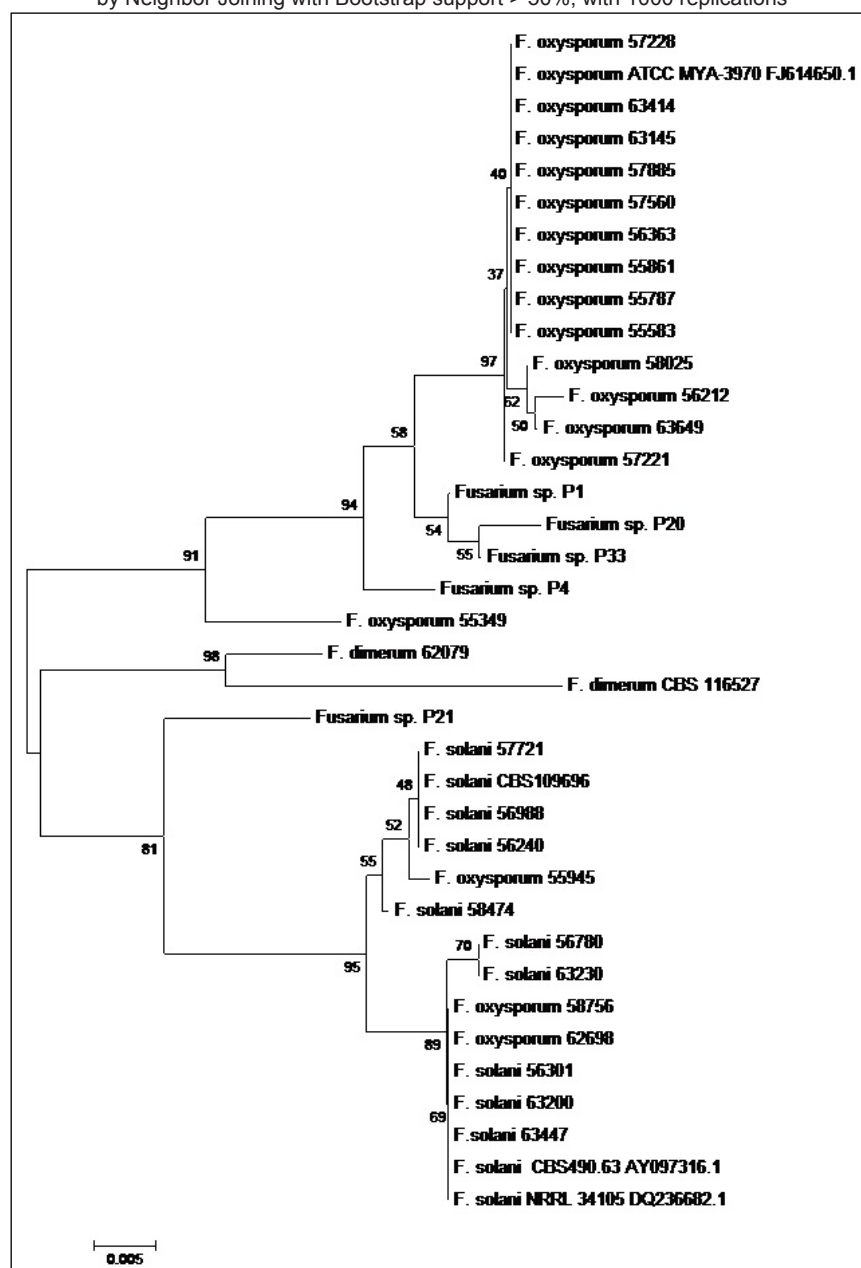
solani, but not to 55 945 which was identified within the complex *F. oxysporum*. In the dendrogram constructed by rDNA sequences such isolate was placed inside the *F. solani* group, close related to the *F. solani* CBS109696 strain (Figure 1); contrarily, in the dendrogram constructed with the TEF1-α sequences, this isolate was placed with 100% identity with the *F. oxysporum* strains, NRRL38515 and MYC163 -05 (Figure 2).

Figure 2. Dendrogram of the TEF1-α region of 41 clinical isolates of Fusarium spp, eight sequences of *Fusarium* spp., taken from the GenBank and eight sequences from Fusarium ID, constructed by Neighbor-Joining, waith supporting Bootstrap, 1000 replications, under the model of Jukes-Cantor
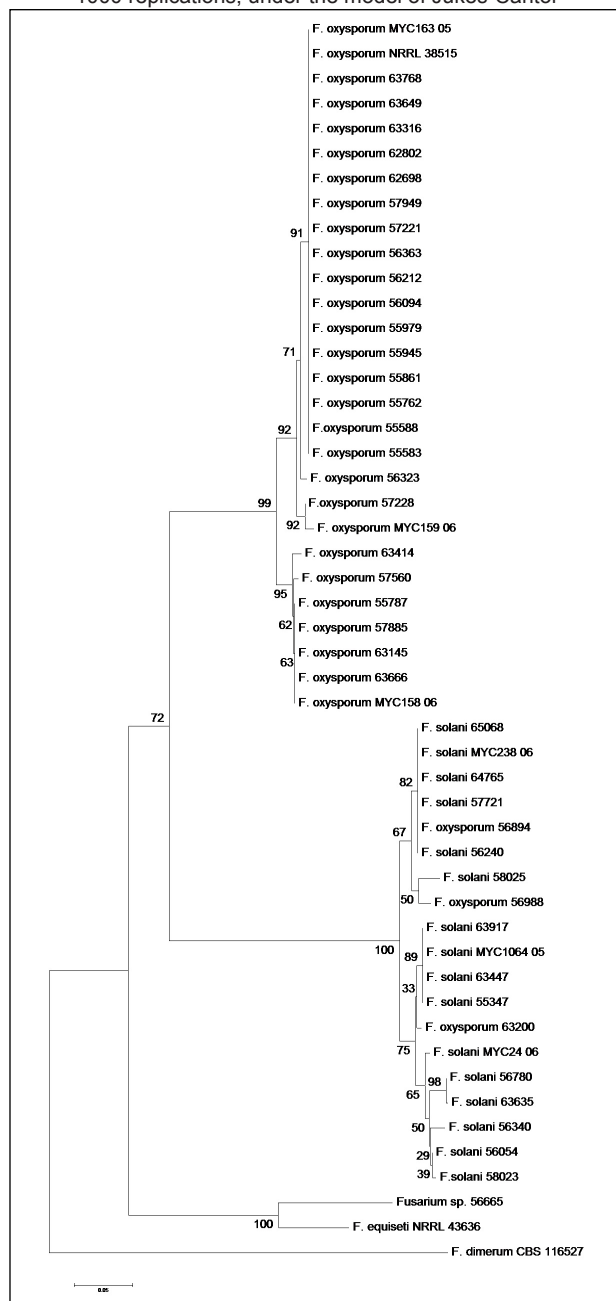
Out of 21 isolates not detected by PCR with any of the two pairs of primers used to detect *F. oxysporum, F. solani*, eight were analyzed by partial sequencing of the genes 28S rDNA and/or TEF1-α. Of those isolates, three were identified as *F. oxysporum* and four as *F. solani* by the two genes. The isolate 56 665 was only identified as *Fusarium* spp., for both genes. The isolate 62 698, not detected by PCR with any of the four primer pairs, was identified by sequencing of the gene 28S rDNA as *F. solani*, with 100% of identity with strain CBS 490.63 of *F. solani*, and by the TEF1-α gene - as *F. oxysporum*, with 100% of identity with the sequences of strains NRRL38515 and MYC163 -05 of *F. oxysporum* (Figures 1 and 2).

DISCUSSION

Fusarium disease is the second reason of fungal infection caused by filamentous fungi, after aspergillosis, mainly affecting immunocompromised patients. Clinical presentation depends on the route of entry of the fungus, the intensity and duration of immunosuppression. The prognosis is reserved since the mortality of the disseminated form is around 80% (1,7).

In recent years, there has been a remarkable increase of reports of these fungal infections, possibly, due to the alteration of the bacterial flora product of the overuse of topical antibiotics, the extensive use of corticosteroids and immunosuppressive drugs, as well as the improvement of diagnostic methods (7). Climate changes are predisposed to this type of fungal diseases (7).

About 15 species of *Fusarium* have been reported as causative agents of human diseases, among the most common are *F. solani* and *F. oxysporum*. (7,14,21,32,33). In Colombia, the identity of *Fusarium* isolates to the species level is rarely reported, although the cases are in rise. Between January 1980 and September 1989, 87 cases of dermal and ocular *Fusarium* infection were diagnosed in the CIB (20) and over 2003 and 2004 a total of 128 isolates from 137 patients with onychomycosis caused by *Fusarium* spp. were reported in the Laboratorio Especializado de Micología Médica (LEMM) in Bogotá (22).

Identification of the species level of fungi belonging to *Fusarium* using classical methods is difficult. It is time consuming and it has limitations in terms of sensitivity and specificity (34,35). On the other hand, *Fusarium* taxonomy complexity is widely known (18,31,36-41).

Molecular diagnostic methods allow faster and higher resolution and sensitivity. The correct and rapid diagnosis of the agent involved is essential for the implementation of appropriate treatment, which if done correctly, helps to reduce the high mortality rates of high-risk patients (34).

This study validates the use of two pair of PCR primers for fast and precise detection of *Fusarium* isolates belonging to *F. oxysporum* or *F. solani*, which represents more than 90% of the isolates involved in human fusariosis (21,22,34,42-44). In our work, *F. oxysporum* was the most common specie, followed by *F. solani*. These results are in agreement with those found in another city of Colombia, Medellin, by partial sequencing of TEF1-α and RPB2 genes, (45) although some other works report *F. solani* as the most common specie (21,22,46).

Partial sequencing of the 28S rDNA gene has suggested the usefulness of the primers OX31/OX32 and Fusorfor/Fusorev to detect isolates of *F. oxysporum* and *F. solani*. Several authors report the ribosomal regions as uninformative for differentiation of *Fusarium* species (11), however this study highlights its usefulness in detecting the genus and the species complex *F. oxysporum* and *F. solani*, in concordance with other reports (44,47,48).

The TEFF-α sequence, considered the best molecular marker to identify *Fusarium* isolates (31), also confirmed the detection of the PCR primers OX31/OX32 and Fusorfor/Fusorev *F. oxysporum*; besides, as expected, the TEFF-α sequences identified 16 isolates that were not detected with any of the specific primers.

The isolate 56 665 identified only as *Fusarium* spp., both by sequencing of the 28S rDNA and by the TEF1-α gene, should be considered in a subsequent study ♠

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