UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

FUSARIOSE: ASPECTOS EPIDEMIOLÓGICOS, DIVERSIDADE GENÉTICA E PERFIL DE SUSCEPTIBILIDADE

PRISCILA DALLÉ DA ROSA

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“Sonhos determinam o que você quer. Ação determina o que você conquista”.

(Aldo Novak)

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RESUMO

Introdução: Fusariose é uma micose causada por um fungo filamentoso hialino cosmopolita que acomete pacientes imunocompetentes e imunodeprimidos, principalmente com neoplasias hematológicas. Atualmente existe uma preocupação em estabelecer epidemiologia dessa doença, assim como a diversidade molecular das espécies de *Fusarium* e do seu perfil de susceptibilidade.

Objetivo: Caracterizar os aspectos epidemiológicos, da diversidade genética e do perfil de susceptibilidade do gênero *Fusarium* nas três diferentes formas clínicas de fusariose: onicomicose, ceratite, e fusariose invasiva.

Métodos: Foi realizado um estudo observacional, prospectivo e retrospectivo, em três diferentes formas clínicas de fusariose. Os dados epidemiológicos foram coletados a partir dos prontuários dos pacientes, e registrados em planilhas do Excel. A análise estatística dos dados quantitativos foi realizada pelo *software SPSS Statistics*, assim como os dados qualitativos da análise descritiva. A identificação fenotípica foi realizada pelo método clássico e a identificação molecular foi realizada através do sequenciamento dos genes: espaçador transcrito interno (*ITS*) do DNA ribossomal, do fator de elongação 1-alfa (*EF1-α*) e da subunidade II do RNA polimerase (*RPB2*). O teste de sensibilidade foi realizado com antifúngicos anfotericina-B, natamicina, terbinafina, fluconazol, itraconazol, voriconazol pelo método de microdiluição em caldo, conforme o protocolo *Clinical & Laboratory Standards Institute* (CLSI) M38-A2. A relação filogenética e a identificação das espécies de *Fusarium* foram definidas principalmente pelo *software* MEGA 7.0 e pelo sistema *Multilocus Sequence Typing* (MLST).

Resultados: O perfil dos pacientes variou nas três diferentes formas clínicas de fusariose, predominando o sexo feminino nos casos de onicomicose, masculino nos casos de ceratite, e de maneira igualitária nos casos de fusariose invasiva. A faixa etária também variou predominando a população adulta nas micoses localizadas, ceratite e onicomicose (22-74 anos), enquanto na fusariose invasiva ocorreu uma variação maior na faixa etária (2-73 anos). Na fusariose invasiva foi observada uma taxa de mortalidade de 40,7% e foram acometidos principalmente os pacientes com leucemia aguda (51,85%). Nesses pacientes, predominaram os isolados coletados de amostras do trato respiratório (40,7%), do tecido cutâneo (29%) e do sangue (19%). As espécies dos complexos *Fusarium solani* (FSSC) e *F. oxysporum* (FOSC) predominaram nas três formas clínicas de fusarioses, enquanto que, particularmente na ceratite, foram identificadas espécies do complexo do *F. chlamydosporum* e do complexo *F. fujikuroi*. A espécie mais prevalente foi *F. solani* nos isolados dos pacientes com ceratite e fusariose

invasiva, enquanto na onicomicose foi *F. keratoplasticum*. A variabilidade observada no perfil de susceptibilidade do *F. solani* para anfotericina-B (AMB; 1-16 µg/ml) foi semelhante nas três formas clínicas das fusarioses. Os isolados de onicomicose e de ceratite foram menos sensíveis ao voriconazol (VRC) que os isolados de fusariose invasiva. A maioria dos isolados das três formas clínicas apresentou resistência *in vitro* para fluconazol e itraconazol. Na ceratite, os isolados apresentaram um excelente perfil de sensibilidade para natamicina, sendo que na fusariose invasiva, AMB e VRC apresentaram os menores valores de concentração inibitória mínima no teste de susceptibilidade *in vitro*.

Conclusão: A fusariose invasiva acometeu pacientes imunodeprimidos, principalmente com leucemia aguda, apresentando uma incidência de 14,8 em leucemia linfoide aguda e 13,1 casos em leucemia mieloide em cada 1.000 pacientes no período de 2008-2017. Também foi descoberta uma nova espécie de *Fusarium* agente de rinossinusite, nomeada de *F. riograndense.* As fusarioses localizadas ocorreram principalmente em pacientes imunocompetentes, apresentando uma maior variabilidade dos complexos de *Fusarium* nos casos de ceratite e uma incidência de 57.47 casos por 1.000 casos diagnosticados com úlcera córnea no período de 2008-2017. Outro achado raro foi à descrição da espécie *Lasiodiplodia theobromae* agente de Ceratite e *Neocosmospora rubicola* agente de onicomicose (Primeiro caso). As técnicas moleculares além de aprimorarem o diagnóstico das fusarioses podem possibilitar a descoberta de novas espécies ou a descrição de casos raros de infeção, contribuindo para epidemiologia e consequentemente no planejamento dos recursos financeiros para atender a real necessidade tanto de tratamento quanto de prevenção. Sendo fundamental alinhar a técnica do perfil de susceptibilidade, a fim de evitar mecanismos de seleção e de resistência dentro das espécies de fungos e ainda direcionar o tratamento mais adequado, e dessa forma contribuir economicamente com os escassos recursos destinados aos serviços de saúde.

Palavras-chave: Complexo de espécies do *Fusarium*, EF1-α, Epidemiologia Molecular, Fusariose, Identificação Molecular, ITS, Perfil de Susceptibilidade, RPB2.

ABSTRACT

Introduction: Fusariosis is a mycosis caused by a cosmopolitan hyaline filamentous fungus that affects immunocompetent and immunosuppressed patients, mainly with haematological malignancies. Currently, there is a concern to establish the epidemiology of this disease, as well as the molecular diversity of *Fusarium* species and their susceptibility profile.

Objective: To characterize the epidemiological aspects, genetic diversity and susceptibility profile of the genus Fusarium in the three different clinical forms of fusariosis: onychomycosis, keratitis, and invasive fusariosis.

Methods: An observational, prospective and retrospective study was conducted in three different types of fusariosis. Epidemiological data were collected from medical records and recorded in Excel spreadsheets. Statistical analysis of the quantitative data was performed by the SPSS Statistics software, as well as the qualitative data of the descriptive analysis. The phenotypic identification was performed by the classical method and the molecular method was performed by sequencing the genes: internal transcribed spacer (ITS) of ribosomal DNA, translation elongation factor 1α (EF1-α) and a portion of the RNA polymerase II subunit (RPB2). The antifungal sensitivity test was performed by the broth microdilution method, according to the Clinical & Laboratory Standards Institute (CLSI) protocol M38-A2. The phylogenetic relationship and the identification of *Fusarium* species were defined mainly by the MEGA 7.0 software and by the Multilocus Sequence Typing (MLST) system.

Results: The profile of the patients varied in the three different clinical forms of fusariosis, predominantly females in cases of onychomycosis, male in cases of keratitis, and equally in cases of invasive fusariosis (IF). The age range also varied, with the adult population predominating in the localized mycoses, keratitis and onychomycosis (22-74 years), whereas in the case of invasive fusariose there was a greater variation in the age group (2-73 years). In invasive fusariosis, a mortality rate of 40.7% was observed and the patients with acute leukemia (51.85%) were affected. In these patients, the isolates collected from samples of the respiratory tract (40.7%), cutaneous tissue (29%) and blood (19%) predominated. *Fusarium solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC) predominated in the three clinical forms of fusariosis, while in the keratitis, species of the *F. chlamydosporum* complex and *F. fujikuroi* complex were identified. The most prevalent species was *F. solani* in the isolates of patients with keratitis and invasive fusariosis, while in onychomycosis it was *F. keratoplasticum*. The susceptibility profile of *F. solani* to amphotericin-B (AMB: 1-16 μg/ml)

was similar in the three clinical forms of fusariosis. Isolates of onychomycosis and keratitis were less sensitive to voriconazole (VRC) than invasive fusariosis isolates. Most isolates from the three clinical forms showed in vitro resistance to fluconazole and itraconazole. In the keratitis, the isolates showed an excellent sensitivity profile for natamycin, and in the invasive fusariosis, AMB and VRC had the lowest values of minimum inhibitory concentration in the in vitro susceptibility test.

Conclusion: Invasive fusariosis affected immunosuppressed patients, mainly with acute leukemia, with an incidence of 14.8 in acute lymphocytic leukemia and 13.1 cases in myeloid leukemia in every 1,000 patients in the period 2008-2017. Also discovered was a new species of *Fusarium* agent of rhinosinusitis, named *F. riograndense*. Localized fusariosis occurred mainly in immunocompetent patients, presenting a greater variability of *Fusarium* complexes in cases of keratitis and an incidence of 57.47 cases per 1,000 cases diagnosed with corneal ulcer in the period 2008-2017. Another rare finding was the description of the species *Lasiodiplodia theobromae* agent of Ceratite and *Neocosmospora rubicola* agent of onychomycosis (First case). Molecular techniques besides improving the diagnosis of fusarioses may allow the discovery of new species or the description of rare cases of infection, contributing to epidemiology and consequently in the planning of financial resources to meet the real need for both treatment and prevention. It is fundamental to align the technique of the susceptibility profile, in order to avoid selection and resistance mechanisms within fungi species and also to direct the most appropriate treatment, thus contributing economically with the scarce resources destined to health services.

Keywords: *Fusarium* species complex, EF1-α, Molecular Epidemiology, Fusariosis, Molecular Identification, ITS, Susceptibility Profile, RPB2.

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LISTA DE ABREVIATURAS E SIGLAS

AB	Agar Batata
AMB	*Amphotericin B* (anfotericina B)
AMB lip	Anfotericina-B lipídica
AFST	*Antifungal susceptibility testing*
BI	*Bayesian inference analysis*
BLASTn	*Basic Local Alignment Search Tool nucleotídeo*
BT2	β-tubulina
Cal	Calmodulina
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CBS	*Fungal Biodiversity Centre*
CF	Ceratite Fúngica
CIM	Concentração Inibitória Mínima
CINB	Código Internacional de Nomenclatura Botânica
CLA	*Carnation Leaf Agar*
CLSI	*Clinical & Laboratory Standards Institute* (Instituto de Padronização Clínica e Laboratorial)
DMSO	Dimetil-sulfóxido
DNA	Ácido desoxirribonucléico
EF-1α	*Translation elongation factor 1-α* (fator de elongação 1-alfa)
EUCAST	*European Committee on Antimicrobial Susceptibility Testing*
FND	Fungos não-dermatófitos
f. sp	*formae speciales*
FCSC	Complexo de espécies do *Fusarium chlamydosporum*
FCZ	*Fluconazole* (fluconazol)
FDSC	Complexo de espécies do *Fusarium dimerum*
FI	Fusariose invasiva
FIESC	Complexo de espécies do *Fusarium incarnatum equiseti*
FOSC	Complexo de espécies do *Fusarium oxysporum*
FRC	*Fusarium Research Center*
FS	*Fusarium solani*
FSSC	Complexo de espécies do *Fusarium solani*
FVSC	Complexo de espécies do *Fusarium ventricosum*

GenBank	Banco de dados de Nucleotídeos do NLM/NCBI
GFSC	Complexo de espécies do *Giberella fujikuroi*
HE	Hematoxilina-eosina
HCPA	Hospital de Clínicas de Porto Alegre
ITS	*Internal transcribed spacer* (Espaçador interno transcrito)
ITC	*Itraconazole* (itraconazol)
KNAW	*Westerdijk Fungal Biodiversity Institute*
LSU	*Large subunit of ribossomal* RNA
MEGA	*Molecular Evolutionary Genetics Analysis Software* (Programa para análise de evolução molecular gênica)
MFC	*Minimum fungicidal concentration*
MIC	*Minimum inhibitory concentration*
ML	*Maximum Likehood*
MLST	*Multilocus Locus Sequence Typing*
MOPS	Ácido 4-morfolino propano sulfônico
MP	Máxima Parcimônia
NAT	Natamicina
NCCLS	*National Committee for Clinical Laboratory Standards* (Comitê Nacional para Padronização de Laboratório Clínico)
NJ	*Neighbnor-joining*
NRRL	*ARS Culture Collection*
OTU	Unidade taxonômica operacional
PAS	Ácido periódico de schiff
PCR	Reação em cadeia da polimerase
PDA	*Potato Dextrose Agar*
RM	Ressonância magnética
RNA	*Ribonucleic acid* (ácido ribonucleico)
RPB2	Subunidade II da RNA polimerase
RS	Estado do Rio Grande do Sul, Brasil
SDA	*Sabouraud Dextrose Agar* (Ágar Sabouraud Dextrose)
SNA	*Synthetic Nutrient Agar*
SPSS	*Statistical Package for the Social Sciences*
TC	Tomografia computadorizada

TRB	*Terbinafine* (terbinafina)
UFC	Unidade formadora de colônia
UFRGS	Universidade Federal do Rio Grande do Sul
VRC	*Voriconazole* (voriconazol)

SUMÁRIO

1. INTRODUÇÃO

O avanço da medicina contribuiu para o aumento do número de pacientes imunocomprometidos, que reflete no crescimento de vários grupos de risco acometidos por fungos oportunistas, tais como os pacientes transplantados, com doenças autoimunes, HIV positivo, sob regime de quimioterápicos. Estima-se que a taxa de mortalidade alcança aproximadamente 90% em pacientes com neutropenia prolongada e grave [1].

O fungo filamentoso *Fusarium* sp. é um dos principais agentes de hialo-hifomicose, além de ser um saprófita do solo e um frequente fitopatógeno [2]. Existe mais de 200 espécies desse fungo, das quais 73 espécies foram isoladas de infecções em humanos [3]. O fungo do gênero *Fusarium* são agentes de um amplo espectro de infecções, denominadas fusarioses, acometendo pacientes imunodeprimidos, principalmente com neoplasias hematológicas, e imunocompetentes globalmente [4]. Em pacientes imunocompetentes, causa principalmente infecções localizadas como onicomicose e ceratite [5]. As fusarioses, em geral, são difíceis de tratar em função do alto grau de resistência das diferentes espécies de *Fusarium* aos antifúngicos [1,6–8], tornando primordial o ensaio de susceptibilidade para escolha mais adequada na terapêutica.

A espécie de maior virulência descrita é o *Fusarium solani* pertencente ao complexo de espécies de *F. solani* (FSSC) [9]. Outras espécies desse complexo, como *F. petroliphilum* (por exemplo, FSSC 1-a) e *F. keratoplasticum* (por exemplo, FSSC 2-d), são frequentemente encontradas no encanamento hídrico de hospitais [10]. Assim como os membros do complexo da espécie *F. oxysporum* (por exemplo, FOSC ST 33) [11], tornando-se um importante problema nosocomial [12]. Dessa forma, a identificação dos agentes envolvidos possibilita a geração do conhecimento a nível clinico epidemiológico. Desse modo, a identificação das espécies de *Fusarium* e o teste de sensibilidade aos antifúngicos na rotina laboratorial são importantes para o manejo clínico dos pacientes com as diferentes infecções por *Fusarium.*

2. REVISÃO DA LITERATURA

Esta revisão da literatura está focada nos principais aspectos relacionados à fusariose. Abordando os seguintes temas: Taxonomia e Evolução das espécies de *Fusarium*; Identificação Molecular; Filogenia; Marcadores Moleculares; Perfil de Susceptibilidade; Genes de Resistência; Definição da doença e Manifestações Clínicas; Aspectos Epidemiológicos; Tratamento e Diagnóstico. Essa estratégia de busca envolveu as seguintes bases de dados: PubMed, Scopus, SciELO e LILACS, com publicações até maio de 2018. Foram realizadas as buscas nas bases de dados entre os dias 01 a 23 de maio de 2018, através dos termos inseridos na tabela 1, na qual apresenta o esquema utilizado, a partir da combinação das palavras-chaves, o número de artigos encontrados e inseridos para cada tema.

Nessa revisão nas bases de dados observou que nunca foi realizado um estudo dessa magnitude no Rio Grande do Sul e que existem poucos estudos desse tipo no Brasil, embora exista uma grande preocupação mundial e um crescente aumento global relacionado a essa temática de publicação científica. Dessa forma, a problemática do nosso estudo envolve avaliar a incidência e a prevalência da fusariose regional, estudar o perfil dos pacientes acometidos, conhecer a diversidade molecular das espécies de *Fusarium* e relacionar os aspectos fenotípicos com os genotípicos, a fim de contribuir no aprimoramento no diagnóstico micológico.

Tabela 1: Esquema de busca com as palavras chaves nas bases de dados

Palavras-chaves	Pubmed	Scopus	Scielo	Lilacs	Artigos Inseridos
Fusariosis review (últimos 5 anos)	42	62	2	0	10
Fusariosis AND taxonomy	78	9	0	0	8
Fusariosis AND epidemiology	109	86	0	0	23
Fusariosis AND epidemiology AND Brazil	13	7	0	0	10
Fusariosis AND epidemiology AND South of Brazil	0	2	0	0	0
Fusariosis AND epidemiology AND antifungal sensitivity	15	6	0	0	15
Fusariosis AND epidemiology AND treatment AND antifungal sensitivity	10	2	0	0	10
Fusariosis AND clinical manifestation	6	42	0	0	20
Fusariosis AND molecular diagnosis	32	36	0	1	20
Fusariosis AND MLST	4	4	0	0	4
Fusariosis AND RPB2 AND EF-1α	7	8	0	0	8
Fusariosis AND Genotyping	5	5	0	0	5
Fusarium sp. nov. AND new species	33	30	0	0	11

2.1 *Fusarium* e Taxonomia

O gênero *Fusarium* é composto por uma grande variedade de espécies fitopatogênicas e sapróbias do solo. Sua posição taxonômica é definida atualmente como pertencente ao Reino *Fungi*, Filo *Ascomycota*, Classe *Sordariomycetes*, Ordem *Hypocreales*, Família *Nectriaceae*, Gênero *Fusarium* [13].

Dentro do grande gênero *Fusarium* pertencem mais de 200 espécies, e pelo menos sete complexos que compreendem múltiplas espécies causadoras de infecções em

humanos e em animais [14]. Desses principais complexos estão: o complexo de espécies do *Fusarium solani* (FSSC), o complexo de espécies do *F. oxysporum* (FOSC), o complexo de espécies do *F. incarnatum equiseti* (FIESC), o complexo de espécies do *Giberella fujikuroi* (GFSC), o complexo de espécies do *Fusarium chlamydosporum* (FCSC) e o complexo de espécies do *Fusarium dimerum* (FDSC) [7].

A taxonomia do gênero *Fusarium* ao longo dos anos sofreu várias mudanças, antigamente sua classificação a nível de espécie era baseada apenas nos critérios morfológicos e fisiológicos do fungo. Atualmente com utilização de técnicas moleculares, aliado a novos *softwares* e plataformas taxonômicas, uma nova compreensão da evolução filogenética vem sendo estabelecida e em consequência a sua taxonomia [15].

As espécies do gênero *Fusarium* apresenta grande importância econômica, pois são fitopatógenos de ampla distribuição mundial, causando devastadoras perdas na agricultura, como a podridão em vegetais, na qual na maioria das vezes a preocupação é identificar a presença de micotoxina no produto vegetal e quantificá-la, do que propriamente identificar e espécie causadora de doença. A característica fundamental para identificação desse gênero é a produção de conídios multiseptados, em forma de foice, com as extremidades afiladas [16].

A diferenciação na identificação dentro do gênero *Fusarium* é bastante complexa e se baseia na morfologia dos macro e microconídios, fiálides, conidióforos, clamidósporos e na disposição dos conídios no conidióforo, além das características macroscópicas de crescimento do seu micélio algodonoso-cotonoso e das suas diferentes tonalidades, como produção de pigmento, temperatura de crescimento, taxa de crescimento e entre outras características. Os microconídios podem ser unicelulares, com uma grande variedade de forma, apresentando formato elipsoidal a esférico, e podem ser encontrados agrupados ou de maneira isolada [17]. A taxonomia desse gênero é extremamente complexa devido ao grande número de espécies que existem na natureza e a sua complicada conidiogênese [7]. O reconhecimento é difícil, especialmente quando não se produzem macroconídios, podendo ser confundidos com outras espécies, como *Acremonium*, *Cylindrocarpon* ou *Verticillium* [16].

2.1.1 Evolução da Taxonomia do Fusarium

Em 1753, Linnaeus colocou todos os fungos no gênero Chaos. Somente depois de quase dois séculos foram classificar as espécies do gênero *Fusarium*, em 1935, Wollenwe & Reinking criaram uma nova classificação, a partir das diferenças morfológicas, dividiram em 65 espécies, 55 variedades e 22 formas. Posteriormente, duas escolas de taxonomistas de *Fusarium* se desenvolveram - os "divisores", principalmente na Europa; e os "lumpers" nos EUA, onde Snyder e Hansen reduziram o gênero a nove espécies em 1945. Essas nove espécies reconhecidas por Snyder e Hansen foram: *F. oxysporum, F. solani, F. moniliforme, F. roseum, F. lateritium, F. tricinctum, F. nivale, F. rigidiuscula e F. episphaeria* [17].

Outros sistemas taxonômicos foram propostos por Gerlach e Nirenberg (1982), Raillo (1950), Bilai (1955), Joffe (1974), Snyder e Hansen (1940), Messiaen e Cassini (1968), Matuo. (1972), Gordon (1952), Booth (1971) e Nelson et al. (1983). Atualmente todos os sistemas modernos de taxonomia do *Fusarium* são baseados no trabalho de Wollenweber e Reinking (1935) [18]. Em resumo, os taxonomistas de *Fusarium* podem ser divididos em "splitters", "lumpers" e "moderates", como apresentado na Figura 1.

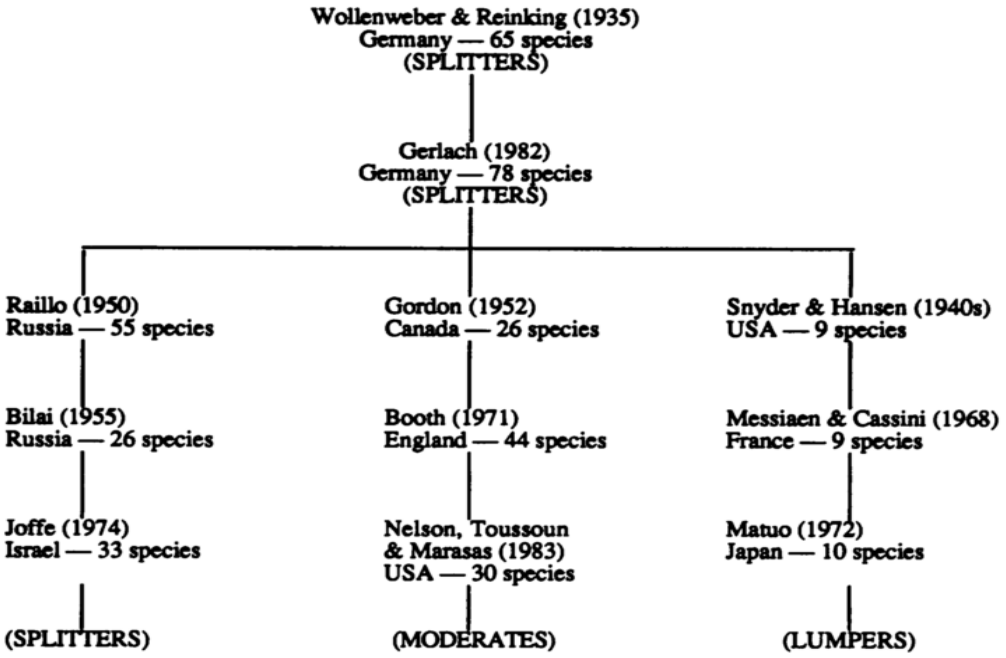


Figura 1: Relação da evolução dos vários sistemas taxonômicos de *Fusarium* spp. (Fonte: Nelson, [18]).

2.1.2 Nomenclatura do Fusarium

A nomenclatura dos fungos é regida pelo Código Internacional de Nomenclatura Botânica – CINB [19], sendo baseada na modalidade de propagação do esporo. Os fungos compreendem um estágio sexual (telemorfo), e um ou mais estádios assexuais (anamorfo); No entanto, o *Fusarium* sp. apresenta tanto a fase sexual, quanto assexual, o que acarretou em uma nomenclatura micológica também dual. Dessa forma, diferentes nomenclaturas foram inferidas para a mesma espécie.

Espécies de *Fusarium* (anamorfo) têm sido associadas a diferentes nomenclaturas telemórficas dos ascomicetos, como por exemplo, *Gibberella, Nectria, Neocosmospora, Haematonectria, Cyanonectria, Geejayessia e Albonectria*. E anamórficos de fitoparasitas, tais como *Bisfusarium* e *Rectifusarium*, os quais foram recentemente propostos, respectivamente, para membros do complexo *F. dimerum* (FDSC) e *F. ventricosum* (FVSC) [19]. A grande problemática era que os dois nomes diferentes eram utilizados em uma única espécie, ora para se referindo ao estágio anamorfo e ora se referindo ao estágio telemorfo. No entanto, em 1˚ de janeiro de 2013, o CINB proibiu a utilização da nomenclatura dupla. Portanto, para fungos pleomórficos, ou seja, aqueles que têm as fases assexuada e sexuada, apenas um nome deve ser escolhido. Em relação ao gênero de *Fusarium*, os especialistas propuseram recentemente que seja reconhecida apenas a nomenclatura anamórfica [7].

2.1.3 Nomenclatura formae speciales

A classificação *formae speciales* (f. sp) é denominada de acordo com hospedeiro que parasitam; e a denominação "raças" está baseadas na virulência. Essa classificação dos táxons (grupos) é para distinguir as formas patogênicas de uma espécie particular, que geralmente não são distinguíveis morfologicamente. Ou seja, constituem um grupo dentro das espécies distinguíveis e recebem o complemento no nome, estando esse relacionado ao nome científico do hospedeiro. Essa classificação é muito comum para a espécie de *F. oxysporum*, como por exemplo, *F. oxysporum* f. sp. *vasinfectum* que parasita o algodão; ou *F. oxysporum* f. sp. *cubense* que parasita a banana [17]. Também ocorre

dentro do FSSC, como *F. solani* f. sp. *cucurbitae* race 2, que infectam abóboras. A designação da "f. sp" não é específica de *Fusarium*, é também utilizada para outros fungos fitopatogênicos.

Genealogias multigênicas revelaram que muitos formatos f. sp dentro do FOSC são não-monofiléticos, ou seja, que não consiste exclusivamente numa espécie ancestral comum e todos os seus descendentes. O agente etiológico parasita da banana no Panamá, *F. oxysporum* f. sp., mostrou ter evoluído pelo menos cinco vezes independentes, indicando que a patogenicidade para este hospedeiro evoluiu de forma convergente [20].

2.2 Características Morfológicas

Fusarium é um fungo filamentoso hialino e que produz colônia bastante algodonosa, caracteriza-se pela produção de conídios robustos, hialinos, septados e em forma de canoa (conhecidas como macroconídios), que na maioria das espécies são produzidas em estruturas de frutificação chamadas esporodóquio. Além disso, algumas espécies também produzem microconídios na porção do micélio aéreo. Os clamidósporos também são produzidos por algumas espécies, seu formato e disposição são importantes para classificação da espécie. Outras características fundamentais para distinção morfológica entre as espécies de *Fusarium* são a descrição dos aspectos macroscópicos da colônia, tais como o crescimento, textura, cor do micélio aéreo, tanto anverso, como reverso; taxa de crescimento, formato do macroconídio e do esporodóquio, as dimensões dos macroconídios e a quantidade de septos, bem como o formato da célula basal e apical [17].

2.2.1 Macroconídios

O macroconídio (esporo assexuado) é a célula principal para identificação da espécie e geralmente tem formato de foice, podem ser retos e/ou finos. Em geral, possuem uma curvatura dorsiventral ao longo de todo seu comprimento ou pelo menos em parte do seu esporo (Figura 2). Esses esporos geralmente têm aproximadamente a mesma largura em todo o seu comprimento. Nas extremidades, o lado dorsal (superior) é distintamente mais curvo que o lado ventral (inferior), como pode ser observado na figura 2, geralmente o centro do macroconídio é mais largo do que as extremidades [21]. A

região apical da célula (ápice), corresponde a parte mais curva da célula, posicionada no lado esquerdo da célula. Ao passo que, a porção basal da célula, seria o pé da célula, está localizado no lado direito da célula, sendo ambas para classificação a nível de espécie [21].

Na Figura 3 podem ser visualizadas as quatro formas existentes de células apicais, denominadas de: forma de gancho (*hooked*), formato mamilo (*nipple-like*), arredondado (*blunt*), e afunilado (*conical*). Células que são particularmente alongadas também podem ter versões distorcidas desses modelos para sua célula apical. Os caracteres diagnósticos mais importantes são o grau de curvatura, o comprimento relativo e a forma geral.

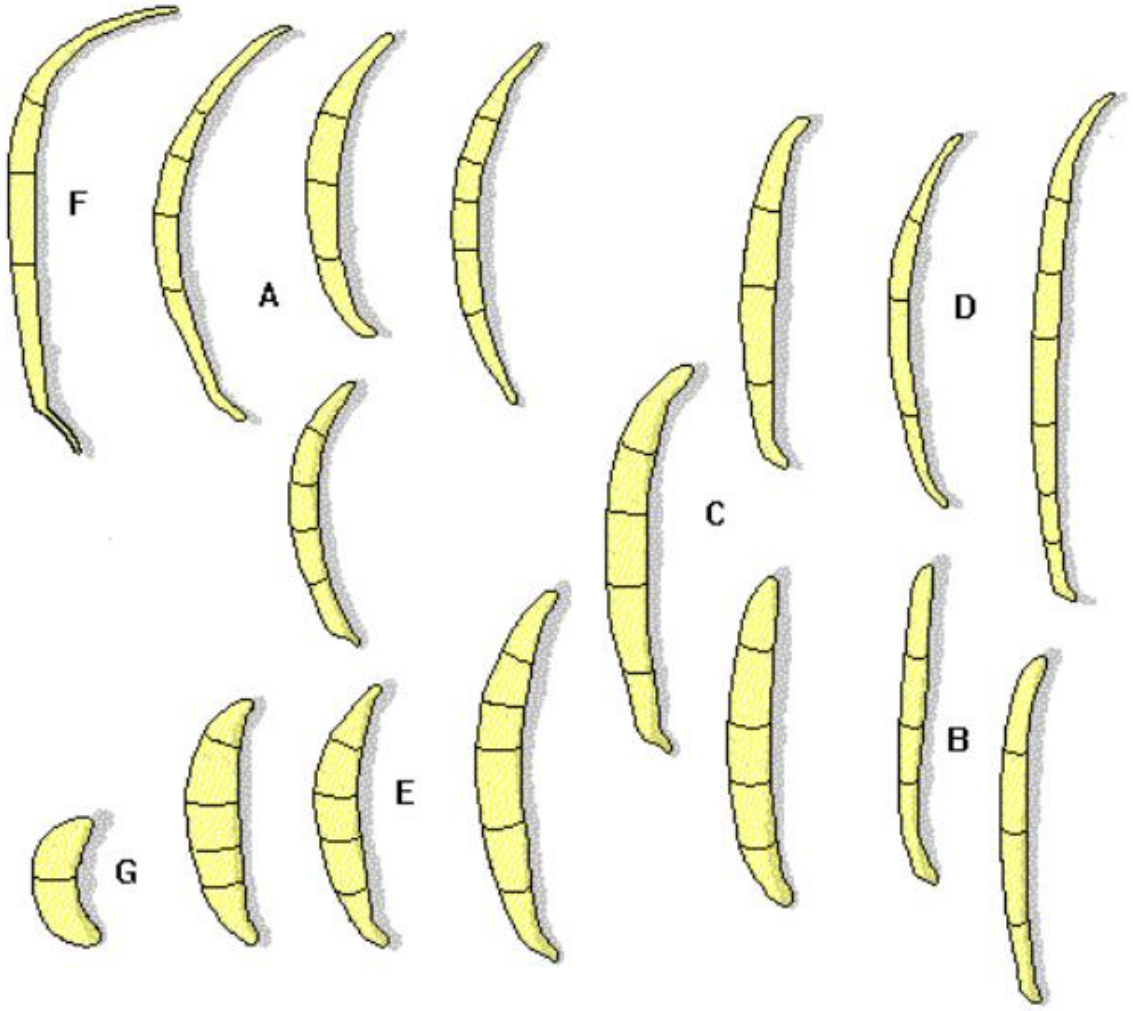


Figura 2: Formato dos macroconídios de *Fusarium* spp. (Fonte: Seifert K., [21]).

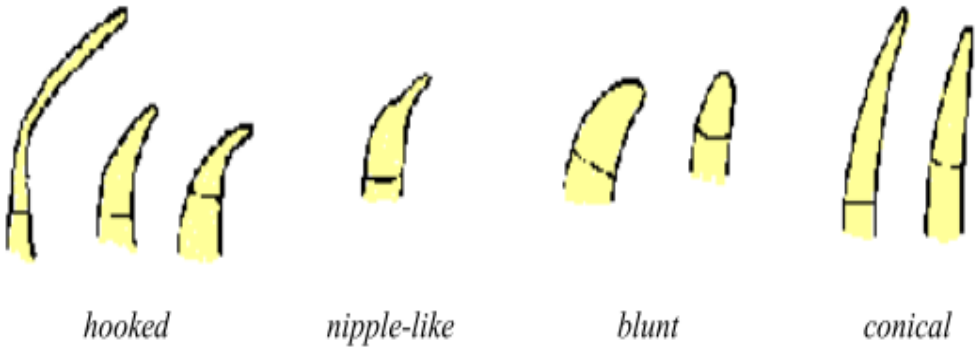


Figura 3: Formato da célula apical dos macroconídios de *Fusarium* spp. (Fonte: Seifert K., [21]).

A célula basal geralmente é a parte menos curvada no esporo. Existem também quatro formas gerais: sem corte (*not notched or blunt*), papilada (*papillate*), em formato de pé estendido (*extended foot*) e entalhado ou em forma de pé (*notched or foot-shaped*), apresentadas na Figura 4.

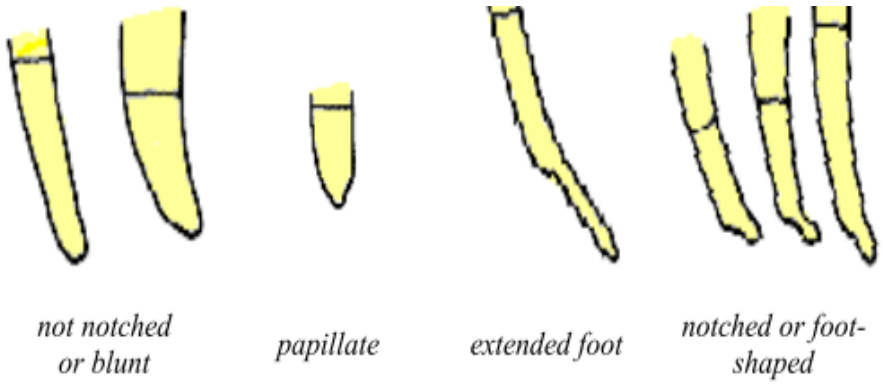


Figura 4: Formato basal dos macroconídios de *Fusarium* spp. (Fonte: Seifert K., [21]).

Muitos meios são utilizados para produção e avaliação dos macroconídios, tais como *Synthetic Nutrient Agar* (SNA, meio sintético), *Carnation Leaf Agar* (CLA, meio de ágar cravo) e *Potato Dextrose Agar* (PDA, meio de ágar batata), os quais são utilizados para avaliar o comprimento, formato, largura e curvatura, número de septos, formato das células apicais e basais dos macroconídios. Outro ponto importante observado é o número de septos e variação do número médio de septos por esporo [17].

2.2.2 Microconídio

O formato e o número de septos do microconídio deve ser observado. Normalmente são arredondados ou elípticos e apresentam 0 ou 1 septos. Entretanto, algumas espécies podem produzir conídios com dois septos. Deve-se determinar a média do comprimento e da largura, assim como sua variação [17].

As formas mais comuns são: oval (A), reniforme (B), obovóide (C), globosa, isto é, esférica (D), piriforme (E), napiforme, (F) e fusiforme, ou seja, em forma de fuso ou em forma de charuto (Figura 5) [18].

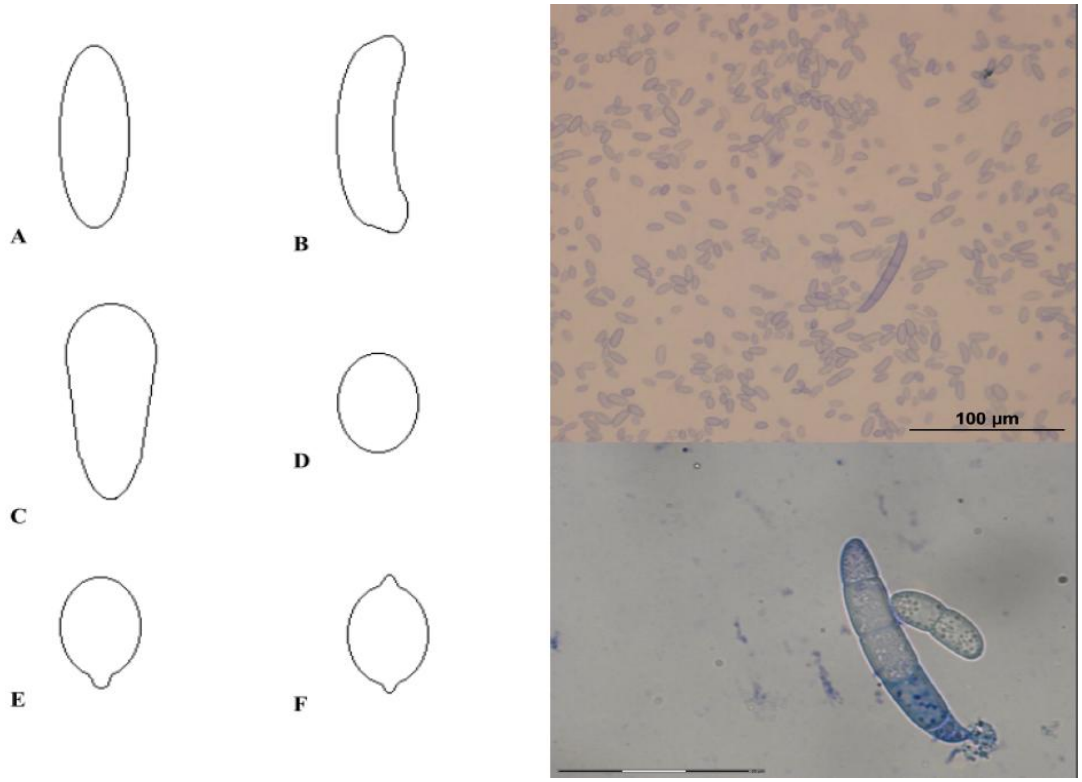


Figura 5: Formato dos oconídios de *Fusarium* spp., imagem à esquerda. Imagens de microconídios ao lado de um macroconídio, à direita (Fonte: autoria própria).

2.2.3. Fiálides

São células conidiogênicas dos fungos ascomicetes, também chamadas de esterigmas, em forma de haste, as quais produzem conídios hialinos ou pigmentados, globosos e algumas vezes rugosos. Micro-morfologicamente, as colônias apresentam fiálides perpendiculares aos filamentos fúngicos, de maneira isolada (monofiálides) ou

ramificada (polifiálides) [22]. O comprimento e o formato do micro-conidióforos, os quais estão localizados na porção terminal das fíalides também são descritos como essenciais para a classificação das espécies [17].

Monofiálide é uma haste isolada com conidióforo com apenas uma abertura ou poro através do qual são expelidos os endoconídios, enquanto um polifiálide possui duas ou mais aberturas ou poros [21]. Na Figura abaixo pode se observar diferentes formatos de monofiálides (Figura 6, nas imagens 14-17) e de polifiálides (Figura 6, nas imagens 18-21).

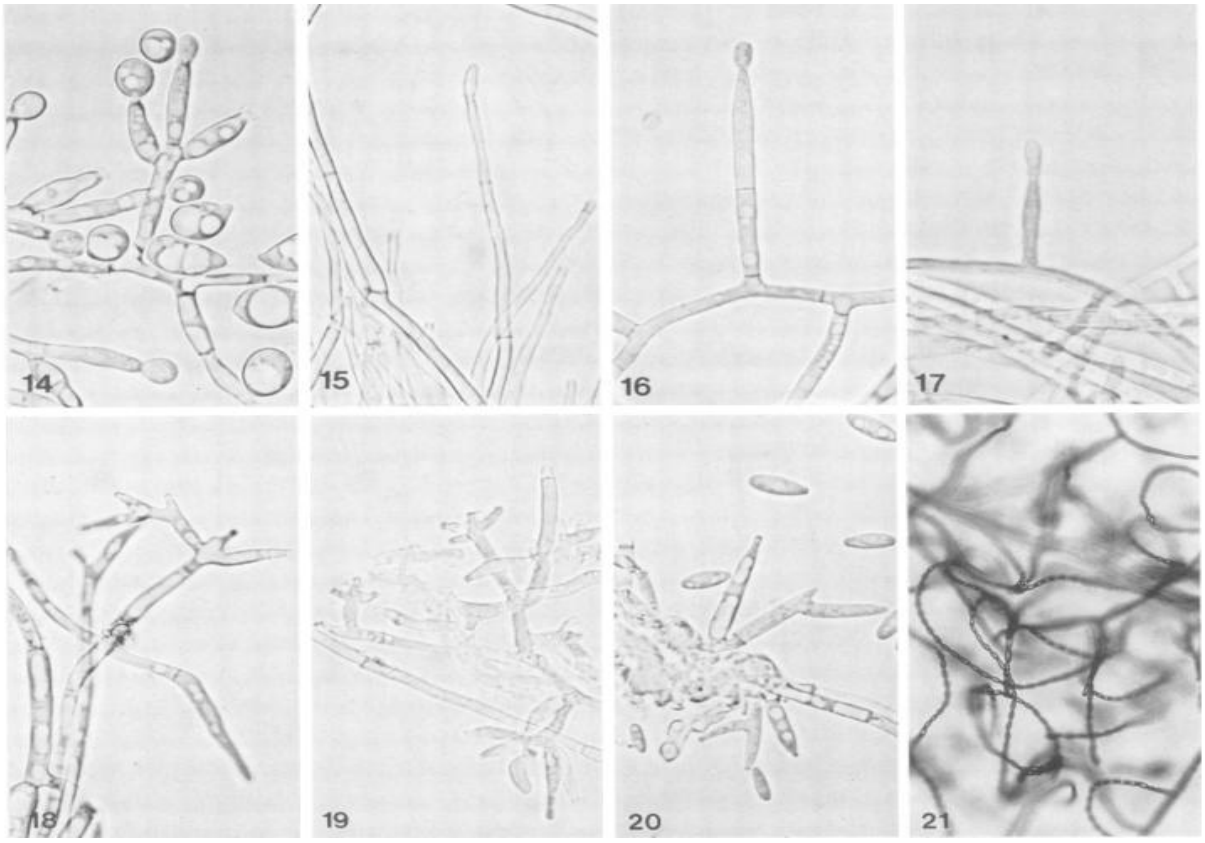


Figura 6: Diferentes formatos de fiálides das espécies de *Fusarium.* Imagem no aumento de 970X: Monofiálides de várias espécies de *Fusarium*, 14 (*F. poae*), 15 (*F. solani*), 16 (*F. moniliforme*), 17 (*F. oxysporum*). Polifiálides: 18 (*F. subglutinans*), 19 (*F. scirpi*), 20 (*F. proliferatum*) [18]; (Fonte: Nelson, [18]).

2.2.4. Caracteristicas morfológicas do complexo de espécies do Fusarium solani

A morfologia dos macroconídios do FSSC são em formato de canoa, retos, de estatura média ou robusta, frequentemente apresentam células basais e apicais bastante arredondadas. Os microconídios são abundantes e na maioria elipsoidal, produzidos a partir de monofiálides longas, estão localizados na porção aérea do micélio, no exame microscópico observam-se frequentemente falsas cabeças, ou seja, microconídios dispostos em forma circular e amontoados. As células conidiogênicas estão posicionadas a partir de monofiálides. Clamidósporos são produzidos frequentemente isolados ou em pares, ocasionalmente em cadeias curtas com aparência globosa ou oval, podendo apresentar paredes lisas ou ásperas [21] (Figura 7). Esses geralmente são formados de maneira abundante e rápida, geralmente dentro de 2-4 semanas no meio de cultura CLA. Também podem se apresentar intercalados nas hifas ou em ramos laterais terminais e mais curtos [17].

Geralmente as culturas em PDA são brancas a creme com micélio esparso, embora exista uma grande variação na coloração dentro deste complexo e seus esporodóquio muitas vezes são produzidos em abundância e pode ser creme, azul ou verde. Essa estrutura é responsável pela produção de macroconídios. Também pode haver a pigmentação do meio, tornando-o amarronzado ou avermelhado [23].

Figura 7: Estruturas fúngicas do FSSC: sc=macroconídio, mc=microconídio, sp=esporodóquio, chl=clamidósporo, mp=monofiálide (Fonte: Seifert K., [21]/autoria própria).

2.2.5.Caracteristicas morfológicas do complexo Fusarium oxysporum

Os macroconídios deste complexo são formados em esporodóquio de cor laranja pálido e geralmente abundante; possuem um comprimento relativamente curto, em formato falciforme, quase reto, de paredes finas e geralmente com 3-septos. A célula apical é curta e ligeiramente em formato de gancho em alguns isolados. A célula basal é entalhada ou em forma de pé. Os macroconídios são originados a partir de monofiálides em conidióforos ramificados em esporodóquio e, em menor extensão a partir das monofiálides, continuação da hifa. Os microconídios geralmente apresentam 0-septos, podem ser ovais, elípticos ou reniformes (forma de rim), e são formados de forma abundante em falsas cabeças em monofiálides curtos (Figura 8).

A morfologia de colônia em PDA varia amplamente. O micélio pode ser flocoso, esparso ou abundante e variando sua tonalidade do branco ao violeta. Em alguns isolados, os macroconídios são abundantes de cor laranja pálido ou violeta-claro, produzidos em uma massa central de esporos. Pequenos escleródios, que são estruturas de resistência, as quais são formado macroscopicamente, semelhantes a gotículas, apresentando tonalidade de marrom-pálido, azul a azul-escuro ou violeta; podem ser produzidos abundantemente por alguns isolados de *F. oxysporum.* A pigmentação do meio também pode ocorrer neste complexo [17].

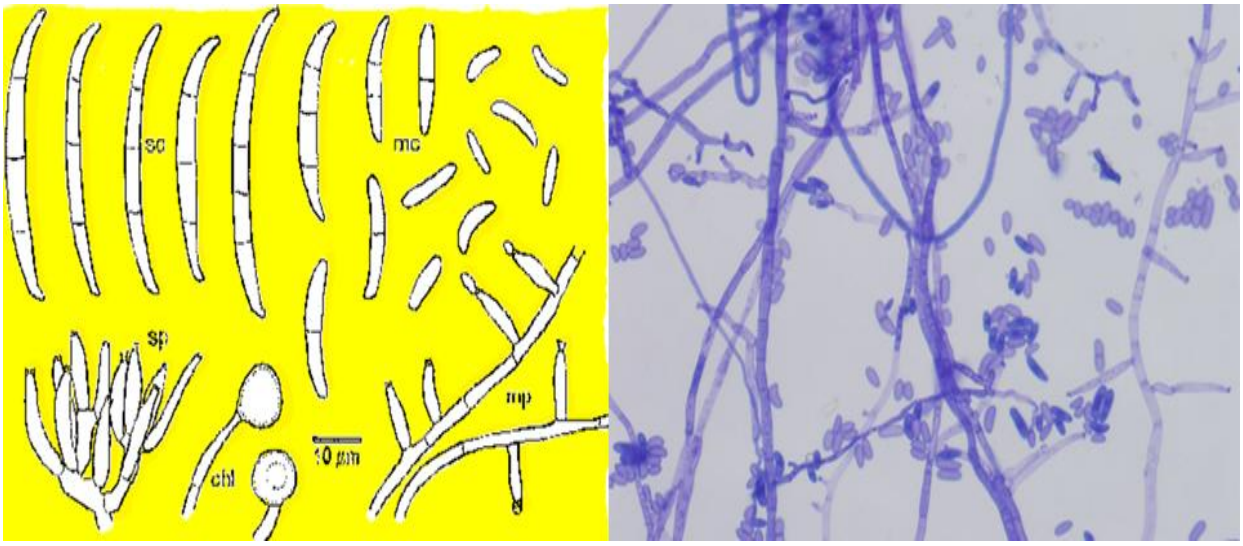


Figura 8: Estruturas fúngicas do FOSC: sc=macroconídio, mc=microconídio, sp=esporodóquio, chl=clamidósporo, mp=monofiálide (Fonte: Seifert K., [21]/autoria própria).

Os clamidósporos geralmente são produzidos isoladamente ou em pares. Esta espécie é dividida em muitas *formae especialis,* que não podem ser distinguidos usando critérios morfológicos. Essa espécie se distingue facilmente de *F. solani* no micélio aéreo pelas fiálides mais curtas [21]**,** outras características diferenciais estão resumidas na tabela 2.

Tabela 2: Características fundamentais para diferenciação das espécies do complexo *F.solani* (FSSC) e *F. oxysporum* (FOSC).

Macroconídio	**FSSC**	**FOSC**
Morfologia apical	Arredondada.	Cônico e curvo, às vezes com um ligeiro gancho.
Morfologia Basal	Quase cilíndrico, geralmente com uma extremidade entalhada ou arredondada.	Em forma de pé estendido.
Número de septos	5 a 7	3
Microconídia		
Forma	Oval, elíptica, em forma de rim e fusiforme.	Oval, elíptica ou em forma de rim.
Septo	0 ou 1; ocasionalmente 2 septos.	0

2.3 Diagnóstico Micológico Laboratorial

O diagnóstico laboratorial é a partir da coleta do material clínico, que pode ser secreção, aspirado de lavado brônquico alveolar, biópsia de tecido, sangue, raspado da córnea, swab nasal, líquido de ascite ou cavitário; raspados de unha e outros tipos de amostras clínicas onde pode estar presente o fungo. A identificação clássica é realizada a partir da amostra clínica utilizando uma lâmina para o exame direto a fresco e/ou corado; e paralelamente parte do material clínico é reservado para a semeadura em meios de cultura específicos para o crescimento de fungos, sendo geralmente semeado em dois tubos de ágar Sabouraud inclinado, e cada um é mantido a uma temperatura de incubação

para o crescimento (25 ˚C e 35 ˚C). Nas amostras de biópsia, ainda é realizada a investigação histopatológica e/ou citopatológica, geralmente com os corantes hematoxilina-eosina (HE), Grocott-Gomori e ácido periódico de schiff (PAS) [24].

Muitas vezes o exame direto, que é a preditividade do diagnóstico, apresenta-se negativo, ou seja, não há presença de hifas hialinas septadas. Embora, cresça no tubo ou em placa de ágar semeado. Uma forma para não descartar que houve uma possível contaminação, é investigar a história clínica do paciente. Por isso, é fundamental um controle e treinamento dos profissionais, sendo decisiva a primeira fase da coleta de amostra clínica no paciente, para que não ocorra variantes no diagnóstico que resultem em um falso positivo ou um falso negativo.

Nos casos de onicomicose, o exame micológico direto positivo com a presença de hifas septadas irregulares de diâmetros retos e com clamidósporos é considerada preditora de *Fusarium* sp. [25].

Os isolados de *Fusarium* sp. crescem com facilidade e rapidez na maioria dos meios sem cicloheximida, sendo que em três dias é possível caracterizá-los macroscopicamente pelo seu aspecto algodonoso e hialino. Embora, o gênero ou o complexo possam ser identificados microscopicamente pelas características da produção e do aspecto dos macroconídios hialinos em forma de banana com múltiplos septos, a identificação de espécie ainda é difícil e pode requerer métodos moleculares [14].

Outra possibilidade no diagnóstico da fusariose é a detecção sérica ou plasmática de 1,3-µ-D-glucano (BDG), que está presente na constituição da parede celular de fungos, no entanto não consegue distinguir das outras espécies que causam fungemia (*Candida, Aspergillus, Trichosporon* e outras) [9].

2.4 Diagnóstico Molecular

A identificação pelas técnicas tradicionais a nível de espécie do *Fusarium* é trabalhosa e requer o profissional experiente na área. Nos últimos anos, procedimentos moleculares de diagnóstico, como sequenciamento de DNA, reação em cadeia da polimerase (PCR), RFLP, nested PCR e PCR em tempo real, tornaram-se ferramentas importantes na identificação dos fungos [26].

O gênero do *Fusarium* é cada vez mais significativo para a saúde humana, particularmente pelas elevadas taxas de mortalidade descritas na fusariose invasiva.

Estudos taxonômicos moleculares têm focado na resolução do relacionamento evolutivo dentro dos complexos de espécies (clados) do *Fusarium* [27]. Bem como, o estudo dos lócus gênicos para avaliar a evolução entre as espécies [28].

Vários lócus gênicos foram testados para avaliar a relação filogenética do *Fusarium* spp., incluindo o DNA ribossômico (18S, 28S, ITS e IGS), DNA mitocondrial (mtSSU e mtLSU) [20] e os genes codificadores de proteínas e de enzimas, como RNA polimerase (RPB2), β-tubulina (BT2) e fator de alongamento 1-alfa (EF1-α) [27]. O gene EF1-α foi eleito o "código de barras" dentro do gênero *Fusarium*, pois tem alta utilidade filogenética pelas seguintes razões: (i) é altamente informativo a nível da espécie; (ii) possui cópias não-ortólogas do gene; e (iii) é alinhável em todo o gênero *Fusarium*. Outro forte candidato ao código de barras do gênero é o gene codificador de proteína, tanto o RPB1 quanto o RPB2 [28].

O gênero *Fusarium* inclui mais de 200 espécies, das quais 73 foram isoladas de infecções em humanos, apresentando-se frequentemente como patógenos oportunistas com etiologia variável. A identificação das espécies é otimizada quando realizada a combinação de genes informativos de ancestralidade, os quais apresentam variedade dentre as espécies (regiões polimórficas), como o EF1-α, o RPB2 e/ou o gene parcial da BT2. Os espaçadores transcritos internos (ITS) do RNAr também têm sido utilizados, no entanto, o ITS não tem poder de discriminar entre várias espécies estreitamente relacionadas e, ainda possui cópias não-ortólogas. Recentemente foi demonstrado outros dois locus gênicos, como potenciais "código de barras"para identificar precisamente a nível de espécie o *Fusarium*, o gene da topoisomerase I (TOP1) e fosfoglicerato quinase (PGK) [3].

Atualmente o método molecular recomendado é o sequenciamento por *Multilocus Sequence Type* (MLST), baseado EF1-α e no RPB2, sendo o mais fidedigno para discriminar entre as espécies dos complexos do *Fusarium* [29]. Dessa forma, várias espécies oportunistas têm sido descobertas, como é o caso do *Fusarium temperatum*, na qual as análises filogenéticas mostraram pertencer ao complexo de espécies de *Fusarium fujikuroi* [30]. Por conseguinte, é essencial a identificação molecular, pois além de contribuir no aprimoramento do diagnóstico, auxilia no conhecimento epidemiológico de cada região. Na tabela 3 foram reunidos os genes que foram utilizados nas últimas duas décadas para descrever novas espécies de *Fusarium*.

Muitos pesquisadores defendem que para ter uma terapia medicamentosa bem-sucedida depende da identificação precisa do patógeno ao nível da espécie, tornando

imprescindível o uso de ferramentas moleculares para que isso ocorra de maneira adequada [41]. Outra vantagem é possibilidade de revelação do envolvimento de novas espécies fúngicas.

Tabela 3: Marcadores moleculares para descrição de novas espécie de *Fusarium.*

Espécie Nova	**Meio**	**Marcador filogenético**	**Autor/ano**
Gibberella fugikuroi	NI*	BT2, mtSSUrDNA, ITS, 28S rDNA	O'Donnell et al. 1998 [20]
Gibberella Konza	CLA/PDA/ SDA/SNA	AFLP BT2.	Zeller et al. 2003 [31]
F. commune	PDA/SNA	mtSSUrDNA	Skovgaard et al. 2003 [32]
F. foetens	PDA/SNA	BT2, EF1-α, mtSSUrDNA	Schroers et al. 2004 [33]
F. dimerum	CLA/SNA	BT2, EF1-α, LSUrDNA, mtSSUrDNA, ITS	O'Donnell et al. 2009 [34]
F. mexicanum	PDA/SNA	BT2, calmodulina, EF1-α, IGSrDNA, histona h3/ RAPD	Colina et al. 2010 [35]
F. temperatum		AFLP BT2, EF1-α	Scauflaire et al. 2011 [36]
F. torreyae	PDA/SNA	RPB1, RPB2, EF1-α	Aoki et al. 2012 [37]
F. keratoplasticum e F. petroliphilum	CLA/PDA	MAT/RPB2, rDNA, EF1α	Short et al. 2013 [38]
F. secorum	CLA/PDA	BT2, calmodulina, mtSSUrDNA	Secor et al. 2014 [39]
F. paranaense	PDA/SNA	RPB2, EF1-α	Costa et al. 2015 [40]

NI*= Não informado

Existe uma extensa gama de *sites* e de *softwares* para auxiliar na identificação dos fungos (Tabela 4), cuja a bioinformática é uma importante aliada. Assim, a facilidade do acesso aos meios digitais favorece o aumento crescente dos dados consultados e depositados, que são alimentados constantemente e globalmente [15]

Tabela 4: Bancos de dados e ferramentas para classificação e identificação baseada em sequências moleculares.

General identification tools and data repositories	
BOLD	http://www.boldsystems.org/
CBS-KNAW	http://www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx—contains 30 BLASTn searchable databases
Dryad	http://datadryad.org/
FUSARIUM-ID	http://isolate.fusariumdb.org/
GreenGenes	http://greengenes.lbl.gov/cgi-bin/nph-index.cgi
Maarj*AM*	http://maarjam.botany.ut.ee/
Mothur	http://www.mothur.org/
Naïve Bayesian Classifier	http://aem.asm.org/content/73/16/5261.short?rss=1&ssource=mfc
Open Tree of Life	http://www.opentreeoflife.org/
QIIME	http://qiime.org/
RefSeq Targeted Loci	http://www.ncbi.nlm.nih.gov/refseq/targetedloci/
Ribosomal Database Project (RDP)	https://rdp.cme.msu.edu/
Silva	http://www.arb-silva.de/
TreeBASE	https://treebase.org/
TrichoBLAST	http://www.isth.info/tools/blast/
UNITE	https://unite.ut.ee/
Data standards	
BIOM	http://biom-format.org/
MIMARKS	http://www.nature.com/nbt/journal/v29/n5/full/nbt.1823.html
Darwin Core	http://rs.tdwg.org/dwc/
1000 Fungal Genomes Project (1KFG)	http://1000.fungalgenomes.org/home/
FungiDB	http://fungidb.org/fungidb/
GEBA	http://jgi.doe.gov/our-science/science-programs/microbial-genomics/phylogenetic-diversity/
MycoCosm	http://genome.jgi.doe.gov/programs/fungi/index.jsf
Functional database	
FUNGuild	https://github.com/UMNFuN/FUNGuild
Nomenclature and nomenclatural databases and organizations	
Catalogue of Life (COL)	http://www.catalogueoflife.org/
Index Fungorum	http://www.indexfungorum.org/
International code of nomenclature for algae, fungi, and plants (ICNAFP)	http://www.iapt-taxon.org/nomen/main.php
International Commission on the Taxonomy of Fungi (ICTF)	http://www.fungaltaxonomy.org/
List of prokaryotic names with standing in nomenclature (LPSN)	http://www.bacterio.net/
MycoBank	http://www.mycobank.org/
Biodiversity collections databases	
Global Biodiversity Information Facility (GBIF)	http://www.gbif.org/
iDigBio	https://www.idigbio.org/
MycoPortal	http://mycoportal.org/portal/index.php
World Federation of Culture Collections (WFCC)	http://www.wfcc.info/
Citizen science resources	
Encyclopedia of Life	http://eol.org/
Mushroom Observer	http://mushroomobserver.org/

(Fonte: Hibbett *et al*.,[15])

2.4.1 Multilocus Sequence Type (MLST)

Atualmente o padrão-ouro para o diagnóstico molecular é a técnica de *Multilocus Sequence Typing* (MLST), a qual também é aplicada para o entendimento da biodiversidade e da evolução do fungo [42]. O MLST é um método altamente preciso usado para distinguir entre isolados de espécies microbianas, e foi inicialmente desenvolvido para facilitar os estudos de epidemiologia e organizar a estrutura populacional de microrganismos procariontes [43]. E tem como vantagem a facilidade do acesso dos dados em uma escala global através de sites de acesso livre, e ainda possibilita que múltiplos usuários comparem seus resultados. Sendo atualmente utilizada para identificação de fungos patogênicos em humanos.

Os principais bancos de dados das sequências gênicas de *Fusarium* spp. são o FUSARIUM-MLST da CBS-KNAW do Centro de Biodiversidade fúngica (http://www.cbs.knaw.nl/Fusarium) e o FUSARIUM-ID da Universidade Estadual da Pensilvânia (http://isolate.fusariumdb.org), os quais foram construídos para facilitar a identificação de *Fusarium* sp. tanto na agricultura quanto clinicamente [44,45]. Dessa forma, devido à sua crescente relevância clínica vários estudos moleculares estão sendo realizados envolvendo tipagem desse fungo, com aumento expressivo de publicações mundialmente a fim de estabelecer as espécies locais de fusariose, assim como os complexos de espécies mais prevalentes, os sítios clínicos da onde são isolados, e o perfil de sensibilidade aos antifúngicos [12,44,45].

O site do FUSARIUM-MLST do CBS-KNAW *Fungal Biodiversity Centre* (http://www.cbs.knaw.nl/Fusarium) foi construído para facilitar a identificação de isolados agrícolas e clínicos através da realização de consultas dentro dessa base de dados, com objetivo de comparar com as sequências nucleotídicas de DNA depositadas [46]. Estão disponíveis para consultas nesse banco de dados, tanto consultas simples, quanto avançadas do BLASTn, em que é possível fazer a busca com várias sequências de genes de maneira simultânea. O algoritmo de alinhamento de sequência única compara a sequência de um desconhecido (dado do isolado fúngico) com as que estão presentes no banco de dados de referência do *Fusarium* MLST [47]. Essa base de dados tem maior segurança em relação ao BLAST, porque apenas o curador dela é responsável por

depositar novas sequências, enquanto que no BLAST qualquer pessoa pode fazer o depósito de informações sobre as sequências.

As consultas com múltiplas sequências estão representadas na Figura 9, na qual existe a possibilidade de inserir dez diferentes genes, essa ferramentas é possível devido ao desenvolvimento do programa BioloMICS. Recomenda-se utilizar esse sistema de multilocus, porque ele garante uma identificação mais confiável [46].

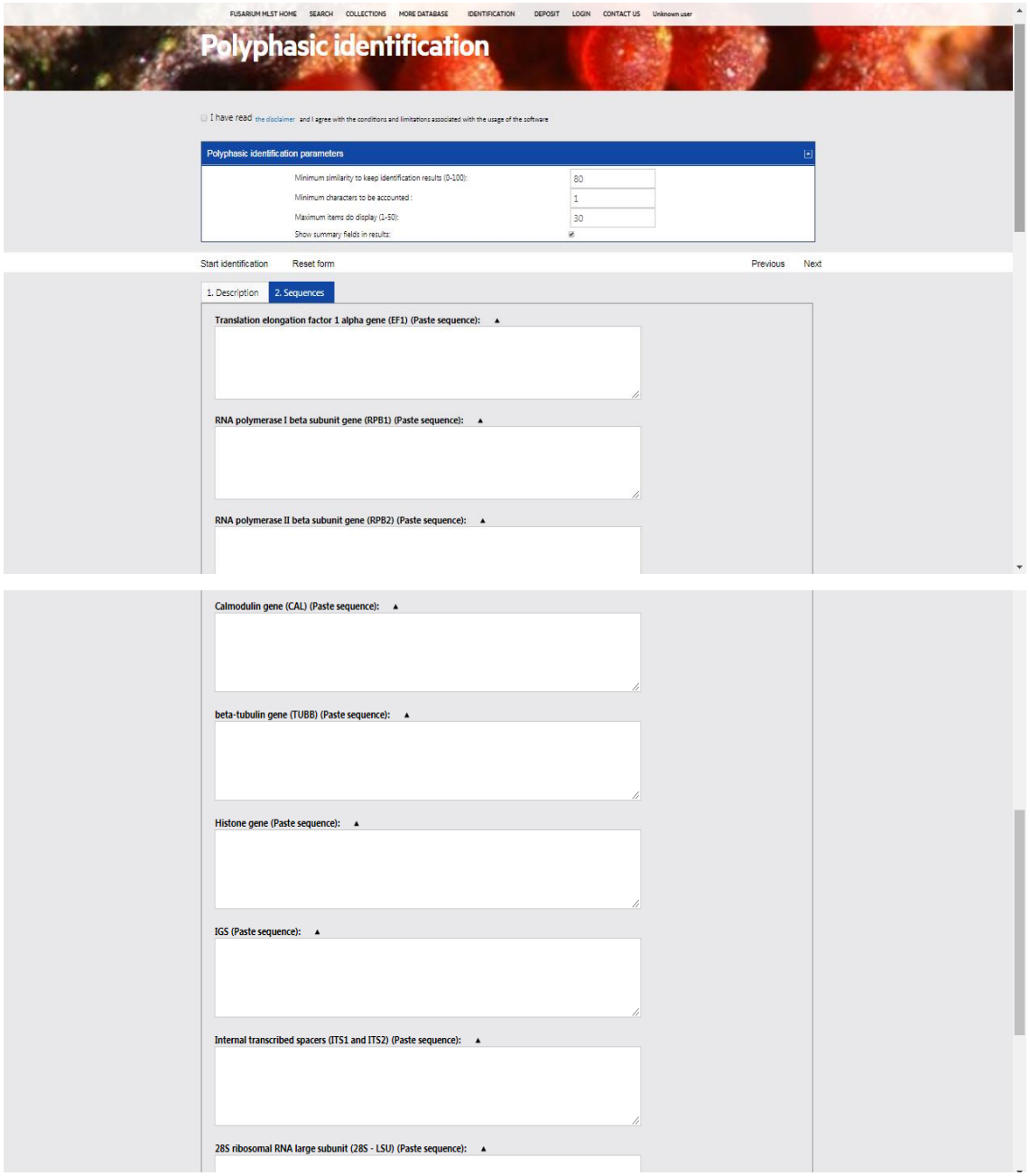


Figura 9: Página de busca *on line* na base de dados do MLST

O'Donnell K. [47] fornece um guia contemporâneo com passo a passo dos três recursos para identificação das espécies de *Fusarium*: FUSARIUM-ID (http://isolate.fusariumdb.org/; FUSARIUM-MLST (http: // www.cbs.knaw.nl/fusarium); e NCBI GenBank (http: //www.ncbi. nlm.nih.gov/).

A interpretação da identificação precisa da espécie está relacionada à porcentagem de similaridade, apresentando uma similaridade de 99-100% na cobertura com a sequência nucleotídica, podendo assim ser inferido com segurança. Para que as seguintes interpretações sejam verdadeiras, é assumido que as seqüências consultadas dos genes EF-1α, RPB1 e / ou RPB2 no FUSARIUM-MLST foram cuidadosamente editadas, como exemplificadas na Figura 10.

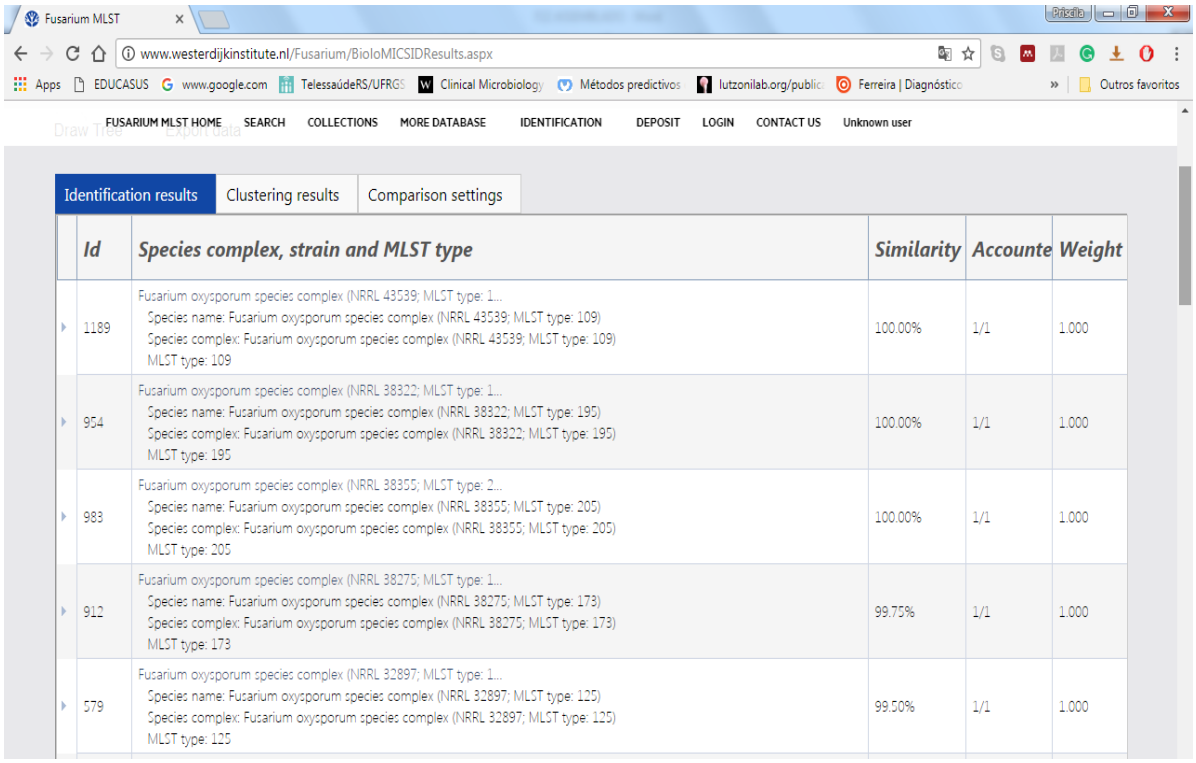


Figura 10: Demonstração de resultado da base de dados do MLST.

A quantidade de informação nesse banco de dados deve aumentar significativamente à medida que os membros da comunidade de microbiologia clínica depositam seus isolados nestas coleções de cultura acessíveis internacionalmente e

publicam seus achados. Além das culturas, podem ser solicitado o deposito dos resultados dos cromatogramas e dos dados das sequencias nucleotídicas [46].

Atualmente, pelo menos 300 espécies filogeneticamente distintas foram resolvidas com as linhagens genealógicas. As espécies de *Fusarium* estão sendo constantemente depositadas nas micotecas internacionais, como ARS Culture Collection (NRRL), no CBS-KNAW Biodiversity Center (CBS) e no *Fusarium* Research Center (FRC), e constantemente estão sendo aproveitadas para as análises filogenéticas. No entanto, existem ainda espécies que não foram nomeadas e muitas delas são morfologicamente enigmáticas [19].

2.4.2 Análise Filogenética

Atualmente, abordagens morfológicas, aliadas as análises de métodos de estudos de filogenia, como a construção de árvores filogenéticas, são utilizadas para definir a relação da evolução entre as espécies. Quando existe a possibilidade de identificação de uma nova espécie, recomenda-se realizar uma investigação cuidadosa com mais de um gene informativo, como os genes EF1-α e RPB2 para identificar espécies de *Fusarium*, bem como realizar a consulta no bando de dados do FUSARIUM-MLST para definir seus haplótipos [3].

A filogenia avalia as relações de ancestralidade comum entre os táxons (grupos), na qual existe a unidade taxonômica operacional (OTU). A relação filogenética é baseada na distância evolutiva, e essa é quantificada por algoritmos nos *softwares* especializados, como o MEGA 7.0. Essa relação pode ser realizada pela avaliação dos *clusters* (grupos) pelo método do UPGMA, por exemplo, ou pelo método *Minimum evolution*, formando cladogramas, que são uma matriz de distância evolutiva.

O dado *p-distance* é a distância baseado na fração (possibilidade) de posições nucleotídicas nas quais as duas ou mais sequências analisadas divergem (amostra nucleotídica de isolado). Esse dado subestima a verdadeira distância genética derivada da ocorrência de eventos das múltiplas substituições nucleotídicas ao longo do processo evolutivo [48].

Na Figura 11 estão apresentadas diversas ferramentas para a aquisição de árvores filogenéticas, as quais representam modelos matemáticos implementados pelo *software*

MEGA. O primeiro na lista, *Maximum Likehood* (ML) ou Máxima Verossimilhança, é baseado em um modelo de evolução explícito [49]. O segundo modelo é o Neighbor-joining (NJ), que está baseado nas relações entre as distâncias das sequências, não avaliando cada caractere separadamente da sequência de DNA, mas sim em uma matriz de distância que é produzida previamente com o modelo matemático de distância determinado [50]

E o último método, da Máxima Parcimônia (MP), que não é baseado em um modelo de evolução explícito e sim no modelo de hipótese mais simples, ou seja, o que representa o menor número de variação das possibilidades de construção de árvores [48]. Teoricamente, MP e ML precisam computar todas as topologias possíveis; o que seria computacionalmente dispendioso em questão de tempo. Ambas analisam cada sítio independentemente. Adicionalmente, a ML utiliza modelos evolutivos de substituição de nucleotídeos, o que é mais robusto, sendo uma vantagem do método em comparação aos outros modelos apresentados [51].

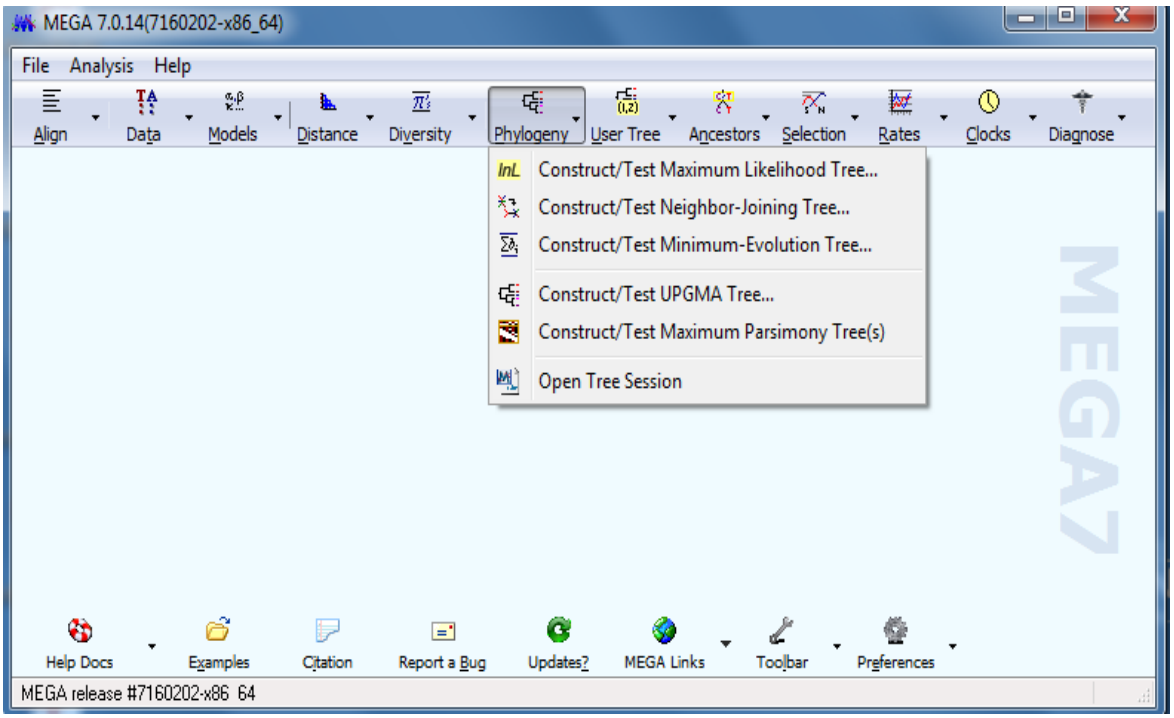


Figura 11: Software MEGA, sinalizando as opções de construção de árvores filogenéticas.

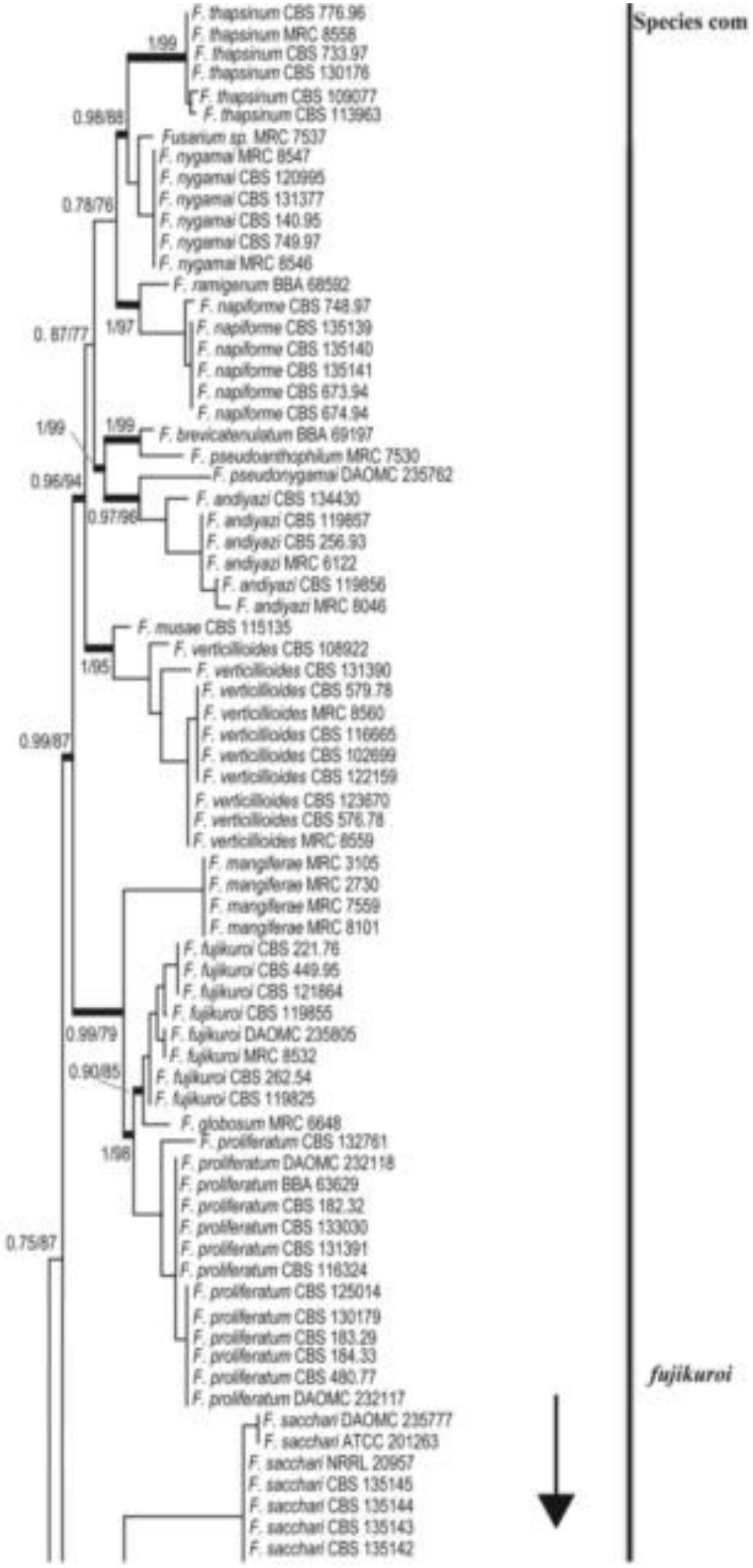
Infelizmente, os dados para tais análises de alinhamentos e filogenias não estão totalmente disponíveis. Um estudo do *Open Tree of Life Project* descobriu que apenas

cerca de 17% das árvores filogenéticas de fungos estão disponíveis em formato de acesso aberto (*on line*). Embora muitas revistas micológicas exijam que os conjuntos de dados e árvores sejam submetidos a TreeBASE ou Dryad, ainda falta um controle rigoroso na fiscalização e segurança desses depósitos [15].

A Tabela 5 apresenta a realizada uma revisão nos modelos das árvores filogenéticas mais utilizadas nas publicações de novas espécie de *Fusarium* spp., sendo que 8/13 utilizaram o modelo matemático ML, 7/13 o modelo MP, 3/13 o modelo Bayesian inference analysis (BI) e 2/13 o NJ. Na Figura 12**,** está representada uma árvore filogenética obtida por inferência bayesiana (BI) e máxima likelihood (ML) para classificação dos diferentes complexos de *Fusarium* [3].

Tabela 5: Genes utilizados como marcadores genético do *Fusarium.*

Marcador	**Análise Filogenética**	**Autor/ano**
ITS, EF-1α, e RPB2	ML	Debourgogne et al. 2010 [52]
EF-1α, e RPB2	MP e BI	Costa et al. 2016 [40]
ITS, EF-1α, RPB2, D1 e D2 (LSU) rDNA	MP	Migheli, Quirico 2010 [8]
ITS, EF-1α, RPB2, D1 e D2 (LSU) rDNA	NJ, ML e MP	Scheel, Christina M 2013[53]
ITS, EF-1α, e RPB2, D1 e D2 (LSU) rDNA	MP	Short 2013 [38]
EF-1α, RPB2, e ITS α 28S rDNA	MP	Edupuganti, S et al. 2011 [54]
ITS, EF-1α, RPB2, D1 e D2 (LSU) rDNA	ML	O'Donnell, K 2008 [42]
ITS, EF-1α, e RPB2	ML e BI	Guevara-Suarez, M 2016 [55]
ITS, EF-1α, e RPB2	ML e BI	Diepeningen, A 2015 [56]
RPB1 e RPB 2	ML e MP	Aoki 2012 [37]
ITS, EF-1α, RPB2 e cal	ML e MP	O'Donnell, K 2009 [57]
BT2 e EF-1α	ML	Homa, M 2013 [58]
ITS, EF-1α, e RPB2	NJ e MP	O'Donnell, K 2007 [59]


Species com
F. thapsinum CBS 776.96
F. thapsinum MRC 8558
F. thapsinum CBS 733.97
F. thapsinum CBS 130176
F. thapsinum CBS 109077
F. thapsinum CBS 113963
Fusarium sp. MRC 7537
F. nygamai MRC 8547
F. nygamai CBS 120995
F. nygamai CBS 131377
F. nygamai CBS 140.95
F. nygamai CBS 749.97
F. nygamai MRC 8546
F. ramigenum BBA 68592
F. napiforme CBS 748.97
F. napiforme CBS 135139
F. napiforme CBS 135140
F. napiforme CBS 135141
F. napiforme CBS 673.94
F. napiforme CBS 674.94
F. brevicatenulatum BBA 69197
F. pseudoanthophilum MRC 7530
F. pseudonygamai DAOMC 235762
F. andiyazi CBS 134430
F. andiyazi CBS 119857
F. andiyazi CBS 256.93
F. andiyazi MRC 6122
F. andiyazi CBS 119856
F. andiyazi MRC 8046
F. musae CBS 115135
F. verticillioides CBS 108922
F. verticillioides CBS 131390
F. verticillioides CBS 579.78
F. verticillioides MRC 8560
F. verticillioides CBS 116665
F. verticillioides CBS 102699
F. verticillioides CBS 122159
F. verticillioides CBS 123670
F. verticillioides CBS 576.78
F. verticillioides MRC 8559
F. mangiferae MRC 3105
F. mangiferae MRC 2730
F. mangiferae MRC 7559
F. mangiferae MRC 8101
F. fujikuroi CBS 221.76
F. fujikuroi CBS 449.95
F. fujikuroi CBS 121864
F. fujikuroi CBS 119855
F. fujikuroi DAOMC 235805
F. fujikuroi MRC 8532
F. fujikuroi CBS 262.54
F. fujikuroi CBS 119825
F. globosum MRC 6648
F. proliferatum CBS 132761
F. proliferatum DAOMC 232118
F. proliferatum BBA 63629
F. proliferatum CBS 182.32
F. proliferatum CBS 133030
F. proliferatum CBS 131391
F. proliferatum CBS 116324
F. proliferatum CBS 125014
F. proliferatum CBS 130179
F. proliferatum CBS 183.29
F. proliferatum CBS 184.33
F. proliferatum CBS 480.77
F. proliferatum DAOMC 232117
F. sacchari DAOMC 235777
F. sacchari ATCC 201263
F. sacchari NRRL 20957
F. sacchari CBS 135145
F. sacchari CBS 135144
F. sacchari CBS 135143
F. sacchari CBS 135142
1/99
0.98/88
0.78/76
0. 87/77
1/97
1/99
1/99
0.96/94
0.97/96
1/95
0.99/87
0.99/79
0.90/85
1/96
0.75/87
fujikuroi

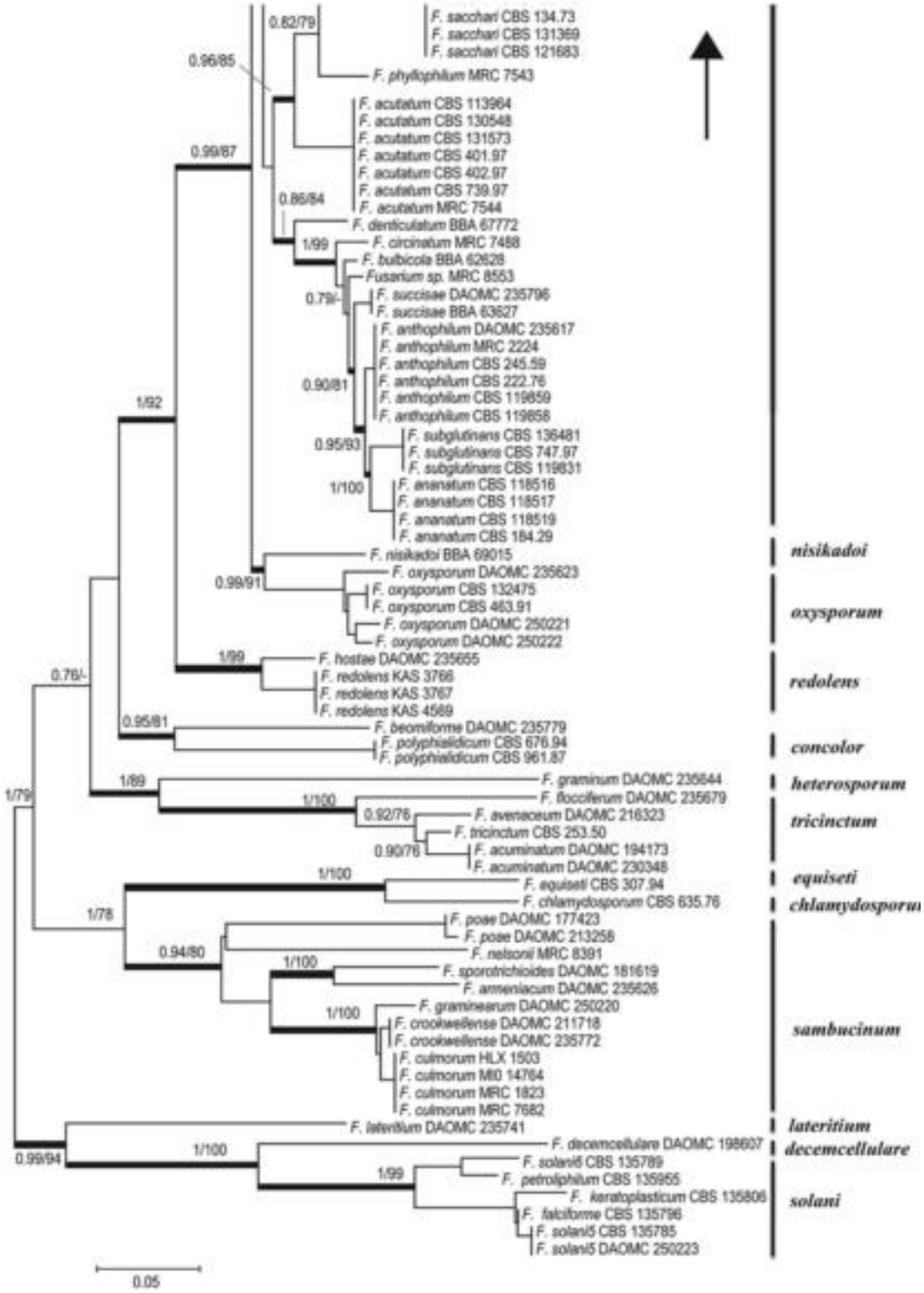


Figura 12: Árvore filogenética gerada com inferência bayesiana (BI) e máxima likelihood (ML) com o gene EF1-α, 589 caracteres, 10000000 gerações a partir 144 isolados. Os números dos ramos são probabilidades posteriores bayesianas (PP) e percentagens de 1000 replicações bootstrap de máxima verossimilhança MEGA6 (PP / ML). A árvore foi enraizada pelo complexo de *F. solani* (Fonte: Al-Hatmi, [3]).

2.5 Perfil de Susceptibilidade

O fungo *Fusarium* spp. vem surgindo com maior relevância na micologia médica, estando associados à baixas susceptibilidade aos antifúngicos disponíveis e alta taxa de mortalidade, principalmente em pacientes imunocomprometidos. Esses fatos destacam a importância de desenvolver testes de diagnósticos mais rápidos e precisos, e assim contribuir para o tratamento adequado [60].

As espécies do gênero *Fusarium* geralmente apresentam os maiores valores de concentrações inibitórias mínimas (CIMs) para anfotericina-B, nistatina, cetoconazol, voriconazol e posaconazol em relação aos outros fungos [61]. Além disso, tem sido relatada susceptibilidade variável entre as diferentes espécies de *Fusarium*. Também foram descritos que as cepas de *F. solani* são resistentes a todos os azólicos, mas suscetíveis à anfotericina-B [54]. Em oposição, as espécies de *F. verticillioides* apresentam susceptibilidade variável aos azólicos, com CIMs menores para voriconazol e posaconazol. Uma possibilidade de tornar o fungo mais suscetível ao tratamento é a combinação de dois agentes antifúngicos ou a combinação com outras classes de fármacos [6,62].

Recentemente, foi realizado um estudo multicêntrico que envolveu sete países, dentre eles o Brasil, o qual utilizaram 1.150 isolados, a fim de determinarem o ponto de corte para os principais complexos de *Fusarium* agentes de infecções clínicas. Dessa forma, conduziram o estudo de acordo com as diretrizes do CLSI, o qual preconiza que para determinar o ponto de corte é necessário que 97,5% dos valores dos CIMs estejam dentro da distribuição do modelo estatístico. Assim, o ponto de corte determinado para anfotericina-B foi de 4 µg/ml para a espécie de *F. verticillioides* e de 8 µg/ml para os ambos complexo de espécies do *F. oxysporum* e do *F. solani*; Para os triazólicos posaconazol foi de 2µg/mL para espécies de *F. verticillioides*, de 8 µg/mL para complexo de espécies do *F. oxysporum* e 32 µg/mL para o complexo de espécies do *F. solani*; e para o voriconazol, foi de 4µg/mL para a espécie de *F. verticillioides*, de 16 µg/mL para o complexo de espécies do *F. oxysporum* SC e 32 µg/mL para o complexo de espécies do *F. solani* SC; e para itraconazol foi de 32 µg/ml para ambos os complexo de espécies do *F. oxysporum* e *F. solani*. Para as espécies de *F. verticillioides* não foi definido [63].

Em vários estudos foi descrito que todas as espécies de *Fusarium* apresentavam resistência ao fluconazol, com CIM >64 µg/mL. Essa resistência pode estar relacionada ao amplo uso na agricultura de antifúngicos com mecanismo de ação semelhante ao fluconazol, e ao uso exacerbado como profilaxia clínica para tratar fungemias, certamente isso possibilita que ocorra uma pressão seletiva nos genes de resistência [64].

O estudo demonstrou que apenas os novos triazólicos, como o voriconazol e o posaconazol, apresentam atividade moderada, com o valor das CIMs variando de 2 a 8 µg/mL e 0,5 a 8 µg/mL, respectivamente; e dependendo do complexo da espécie. Contudo, o valor mais frequente de MIC neste estudo para anfotericina-B foi de 2 µg/mL, independentemente do complexo da espécie e para as equinocandinas foi de CIM > 16 µg/mL [64].

Noutro estudo também foi demonstrado que os perfis de susceptibilidade diferiram entre as espécies, *Fusarium verticillioides* apresentou os menores CIM e *Fusarium nygamai* os maiores CIM para A anfotericina-B (AMB). AMB foi o fármaco mais ativo, seguido pelo voriconazol (VRC), posaconazol (POS), isavuconazol (ISC) e natamicina (NAT). Os demais antifúngicos (fluconazol, itraconazol e micafungina) apresentaram baixa atividade, com valores de CIM ≥ 32, ≥16 e 8 µg/mL, respectivamente [65].

Em relação ao perfil de susceptibilidade do *Fusarium* spp. isolados de casos de onicomicose de um estudo na India, apresentaram uma variação na sensibilidade dos antifúngicos entre as espécies, demonstrando a importância do diagnóstico à nível de espécie, bem como do teste de sensibilidade para o tratamento adequado. Os resultados de susceptibilidade antifúngica indicaram que a anfotericina-B foi o antifúngico mais eficaz para todos os isolados (CIM; 0,5 a 2 µg/mL), seguido pelo voriconazol (CIM; 1 a 8 µg/mL). No entanto, uma grande variação foi mostrada na susceptibilidade ao posaconazol (MIC variando de 0,5 a 16 µg/ml) [66].

Nos casos dos isolados de ceratite, esses apresentam uma ampla resistência dentro do complexo de espécies *Fusarium solani* (FSSC) [42], essa resistência pode estar relacionada a formação de biofilme, como foi demonstrada no estudo de Mukherjee et al. [67] com 27 isolados de *Fusarium* spp.. Foi observado um aumento expressivo dos valores da CIM quando configurado a produção do biofilme (Tabela 6), ambos os isolados de *F. solani* apresentaram CIM maior para AMB, enquanto os isolados de *F.oxysporum* apresentaram um CIM maior para VRC.

Tabela 6: Perfil de susceptibilidade de células livres e em biofilme do *Fusarium* spp. (Fonte: Mukherjee et al. [67]).

	Células livres CIM (µg/mL)			**Biofilme CIM (µg/mL)**		
Cepa	**AMB**	**NAT**	**VCZ**	**AMB**	**NAT**	**VCZ**
F. solani B6914	1	4	8	128	4	2
F. solani B6970	1	2	4	16	4	8
F. oxysporum B6908	1	4	4	0,5	4	256
F. oxysporum B6936	1	4	4	1	4	256

Sobre o perfil de sensibilidade dos isolados de fusariose invasiva foi realizado um estudo entre sete países da Europa com 76 isolados de *Fusarium* spp., que foram isolados no período de janeiro de 2007 a junho de 2012, na qual observaram uma ampla gama de susceptibilidades antifúngicas. Em geral, a anfotericina-B foi o antifúngico mais potente *in vitro* e o itraconazol o menos efetivo. Os azólicos apresentaram concentrações inibitórias mínimas (CIMs) mais baixas contra os isolados das espécies de *F. verticillioides*, contudo o posaconazol apresentou um desempenho ligeiramente melhor, enquanto os isolados de *F. solani* foram resistentes aos três azólicos avaliados, o fluconazol, o itraconazol e o voriconazol [68].

Outro estudo de susceptibilidade realizado com isolados de *Fusarium* spp. de pacientes imunocomprometidos com fusariose invasiva apresentou resistência às equinocandinas: caspofungina e micafungina (CIM >16 µg/ml), fluconazol (CIM >64 µg/ml) e itraconazol (CIM 8 µg/ml). Os isolados também apresentaram valores mais elevados de CIM para anfotericina-B (CIM 2-4 µg/ml) e posaconazol (CIM 2-8 µg/ml), mas foram sensíveis ao voriconazol (0,25-0,5 µg/ml) [69].

2.5.1 Efeito Sinérgico dos Antifúngicos no teste de sensibilidade

Existe uma enorme preocupação com a resistência dos fungos frente ao arsenal terapêutico disponível tanto na área agrícola quanto na área médica, e muitos estudos estão avaliando a possibilidade do efeito sinérgico entre os antifúngicos ou na combinação com outros fármacos para solucionar essa problemática. Um exemplo disso

foi o estudo da combinação de terbinafina com voriconazol, no qual testaram contra 29 isolados de *Fusarium* spp. (15 *F. solani*, 7 *F. oxysporum*, 2 *Fusarium decemcellulare*, 2 *Fusarium dimerum* e 3 *Fusarium* sp.), e conseguiram resultados promissores para inibir a espécie *F. solani*. Os resultados das CIMs das drogas testadas sozinhas foram: 256 µg/mL para a terbinafina, e 2-16 µg/mL para o voriconazol. Enquanto que na combinação dessas drogas, as CIMs tiveram uma redução para 1 µg/mL e 0,25-1 µg/mL, respectivamente [62].

Em outro estudo, Spader *et al.* [70] obteve resultados notáveis entre as interações sinérgicas avaliadas (% de isolados de *Fusarium* spp. inibidos pelo efeito sinérgico), nas quais foram testadas as combinações de anfotericina-B (AMB) com caspofungina (68,7%), AMB com 5-flucitosina (59,3%) e AMB com voriconazol (37,5%). Dois anos depois Spader *et al.* [71] avaliou a combinação de voriconazol com terbinafina e obteve sinergismo em 84% dos seus isolados de *Fusarium* spp.

No estudo de Li et al. [72], as CIM_{90} de cada fármaco utilizado isoladamente foram: anfotericina-B, 4 µg/mL; terbinafina, 8 µg/mL; natamicina, 16 µg/mL; itraconazol, > 16 µg/mL e fluconazol, > 64 µg/mL para as amostras das espécies de *Fusarium* spp. O sinergismo foi obtido com as combinações de anfotericina-B com terbinafina (81,6%), e na anfotericina-B com itraconazol (84,2%). No entanto, apresentou antagonismo com natamicina em combinação com azólicos, e da natamicina em combinação com terbinafina em 52,6% a 60,5%, respectivamente.

Uma possível explicação dessas interações que obtiveram resultados sinérgicos é aos diferentes mecanismos de ação que esses fármacos possuem, por exemplo, AMB e a natamicina agem na membrana celular, criando poros e em consequência o extravasamento do conteúdo interno da célula do fungo, levando a morte deste. Ao passo, que a caspofungina age na proteína FKS, desencadeando mecanismos de sinalização para a desestruturação da parede celular do fungo. Outro diferente mecanismo é o da 5-flucitosina, que age no material nucléico do fungo, agindo como um antimetabólico e impedindo a síntese de DNA para novas células fúngicas. Por fim, o voriconazol, o itraconazol e a terbinafina agem nas enzimas percurssoras da via do ergosterol, interrompendo ou diminuindo a produção do constuinte fundamental da membrana do fungo.

2.6 Genes de Resistência do *Fusarium*

Os mecanismos exatos de resistência em *Fusarium* sp. não estão totalmente estabelecidos, mas podem estar relacionados com as combinações de alterações de aminoácido na proteína que codifica a enzima CYP51A e/ou superexpressão do gene CYP51A, que metabolizaria o antifúngico mais aceleradamente [64]. Recentemente, Fan et al. [31] descreveu em *Fusarium* a existência de três enzimas parálogas de metabolização (CYP51A, CYP51B e CYP51C), e é conhecido que a deleção no gene do CYP51A geralmente causa a resistência secundária em fungos, assim como evidenciado no *A. fumigatus* [25].

Os dados da análise do transcriptoma em *F. graminearum* [9] demonstraram fortes respostas para alguns genes da via de biossíntese dos esteróis, como FgCyp51A a FgCyp51C que codificam a enzima do citocromo P450 esterol 14alpha-desmetilase, que é o alvo molecular dos antifúngicos azólicos. Além disso, 15 dos 54 genes que codificam os transportadores ABC foram mais do que duas vezes regulados pelo fármaco do estudo [73]. Neste estudo, eles descobriram que os mutantes com a deleção DFgABC3 e DFgABC4 adquiriram uma maior sensibilidade a vários fungicidas. Notavelmente, a deleção de FgABC1 e FgABC3 causou uma forte redução de virulência em três culturas economicamente importantes, do trigo, da cevada e do milho.

Descobertas recentes sobre os mecanismos de resistência do gene FKS1 em *Fusarium* sp. indicaram que quando ocorre uma mutação, implica diretamente na resistência as equinocandinas. Para entender melhor a resistência a equinocandina nas espécies de *Fusarium*, várias pesquisas estão estudando o gene FsFKS1, que codifica a enzima (1,3)-D-glucano sintase, responsável pela integridade da parede celular do fungo. Quando silenciado esse gene, observou-se a redução da viabilidade dos esporos quanto das hifas, associado ao processo de lise [74].

Outro tipo de resistência estudada é a resistência cruzada do fluconazol com o itraconazol, esse tipo de resistência cruzada também foi descrita entre os três antifúngicos da classe das equinocandinas. Dados *in vitro* mostraram que existe uma potencial resistência cruzada entre os azólicos, as equinocandinas e os poliênicos, mas nenhuma informação clínica sobre esse fenômeno está disponível [74].

2.7 Fusariose: Definição das Formas Clínicas

Fusariose é uma doença causada por fungos do gênero *Fusarium* que afeta plantas e animais, inclusive humanos. Em humanos pode causar um amplo espectro de doenças, desde uma micose superficial ou subcutânea a uma micose sistêmica. Agressividade da infecção invasiva dependerá do estado de imunidade do paciente, podendo apresentar manifestações clínicas de sinusite, de pneumonia, ou com infecções cutâneas profundas e disseminadas; e frequentemente em pacientes imunocomprometidos se manifesta com febre não responsiva a medicamentos antimicrobianos. Os fatores de risco para o desenvolvimento de fusariose invasiva são neutropenia, déficits na imunidade celular, quimioterapia de indução para leucemia e transplante de células hematopoiéticas [75]. Outras infecções em pacientes imunocomprometidos incluem tromboflebite, fungemia com ou sem envolvimento de órgãos, endoftalmite, artrite séptica, osteomielite e peritonite em pacientes que recebem diálise peritoneal ambulatorial contínua [76].

Outro tipo de micose causada pelo *Fusarium* sp. é a subcutânea, conhecida como micetoma, na qual se desenvolve nos tecidos cutâneos ou subcutâneos após um implante traumático do agente etiológico. O micetoma nos membros inferiores é o mais comum, mas também outras partes do corpo podem ser afetadas. As principais características do micetoma são a tríade da tumefação, a formação de fístulas e a presença de grãos nos exsudatos (Figura 13 A e D).

No eumicetoma, os grãos são hifas compactadas do fungo e são cobertos por uma matriz extracelular cimentiforme dura. A cor do grão é determinada pelos pigmentos presentes ou excretada pelo agente causador, sendo os grãos de eumicetoma incolores a amarelos, ou negros, estando relacionado com o agente causador da infecção; Se hialinos ou melanizados, a população mais afetada é aquela que vive em áreas rurais e suas atividades de trabalho as colocam em risco de lesão traumática; e, assim, ter maior exposição aos agentes causadores, além de ter acesso limitado aos serviços de saúde. O diagnóstico é realizado a partir da biópsia pelo exame histopatológico (Figura 13 B e C) e visualização macro e microscópica do grão [77], aliado ao exame direto e confirmação do agente pelo exame cultural. Existem outras lesões cutâneas que não são micetomas, como por exemplo, a lesão nodular localizada no nariz do paciente da Figura 13 E, na qual obteve remissão total com o tratamento com itraconazol [78].

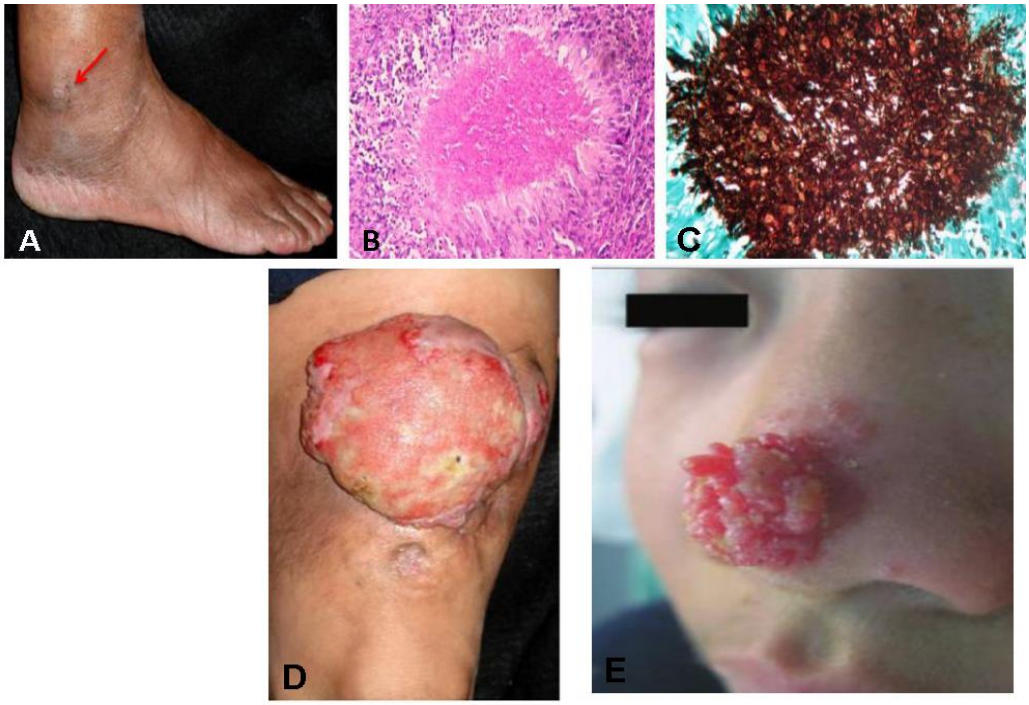


Figura 13: Eumicetoma causado por *Fusarium* sp. no pé direito do paciente após tratamento e cirurgia (A). Lesão inflamatória caracterizada por infiltrado linfo-histiocitário e eosinofílico corado com HE (B). Micetoma como agente o *F. pseudensiforme* corado com Grocott (C). Lesões semelhantes a tumores na coxa esquerda (D) [77]; (E) Lesão nodular no nariz pelo *F.moniliforme* [78].

Por outro lado, a fusariose em pacientes imunocompetentes se apresenta mais frequentemente como infecções superficiais, como ceratite e onicomicose. A micose superficial é a conseqüência da quebra da barreira de proteção da pele, ocasionadas por trauma mecânico, inclusive queimaduras e feridas [76].

2.7.1 Onicomicose

Onicomicose é uma micose que afeta a unha e sua manifestação clínica pelo *Fusarium* não é patognomônica da espécie, em geral aparece a lesão fúngica quando existe um descolamento da lâmina ungueal, frequentemente ocasionado por algum tipo de acidente mecânico, onde o fungo é inoculado. Alguns autores retrataram que este fungo causa uma lesão do tipo paroníquia (Figura 14 A e B), com presença de leuconíquia [79] (Figura 14 B), ou onicólise acompanhado de hiperqueratose [80] (Figura 14 C). Podendo causar distrofia total da unha [81] (Figura 14 D), ou hiperqueratose com amarelamento da placa ungueal, e frequentemente está localizado na porção distal da unha [82] (Figura 14 E).

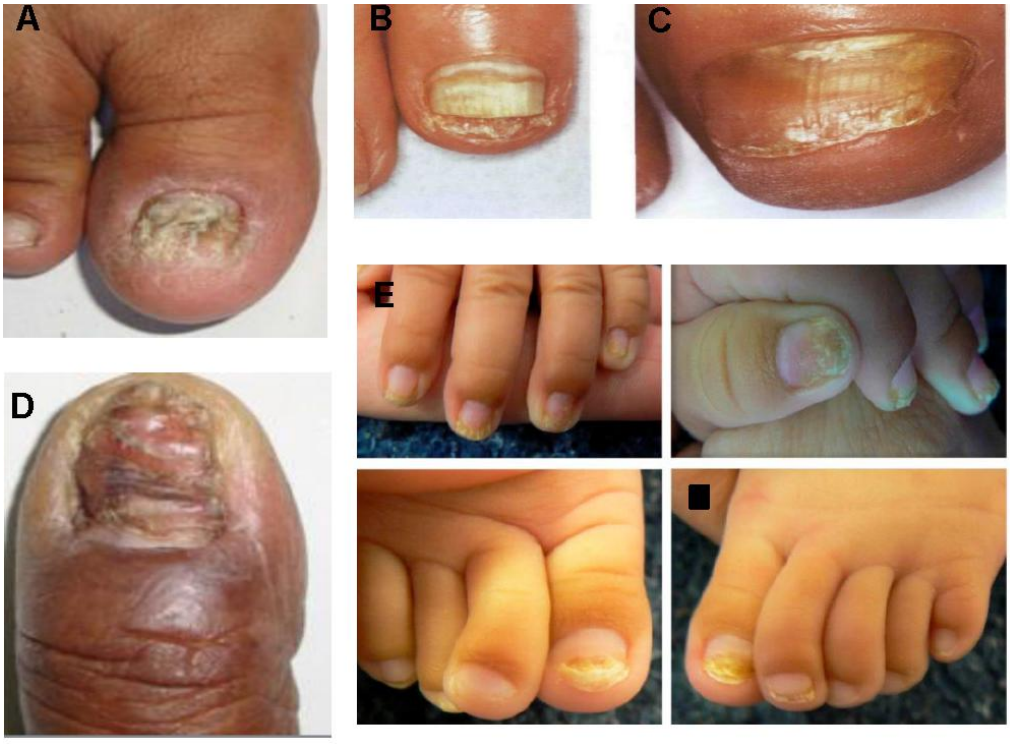


Figura 14: Onicomicose causada por *Fusarium* sp.. Paroníquia na unha direita (A); Leuconíquia na placa proximal, onicólise, hiperqueratose e presença de eritema (B); Paroníquia aguda na dobra subungueal proximal (C); Onicodistrofia total sem paroníquia envolvendo apenas unha do polegar esquerdo há 4 anos (D); Unhas com descoloração amarelada, hiperqueratose e rugosidade (E).

As unhas dos dedos do pé são frequentemente as mais afetadas, principalmente as do hálux; geralmente após um trauma ou anormalidades distróficas ou a outras condições predisponentes, como *diabetes mellitus*, história familiar e imunossupressão [83]. A presença de infecções envolvendo pele ou unha deve ser cuidadosamente investigada antes de iniciar a terapia imunossupressora, uma vez que foi demonstrada que tais lesões podem ser uma porta de entrada para a infecção disseminada [84]. Na Figura 15 pode-se observar lesões cutâneas primárias em pacientes com fusariose invasiva por *Fusarium* sp.; onicomicose no hálux direito (A); úlcera (B); intertrigo (C); intertrigo evoluindo para linfangite antes da disseminação (D); necrose em uma lesão de intertrigo (E) [84]; Também foi descrito um caso de onicomicose (G) em paciente com história clínica de insuficiência renal crônica e *diabetes mellitus*, também apresentou uma úlcera dolorosa em seu pé direito (F) [83].

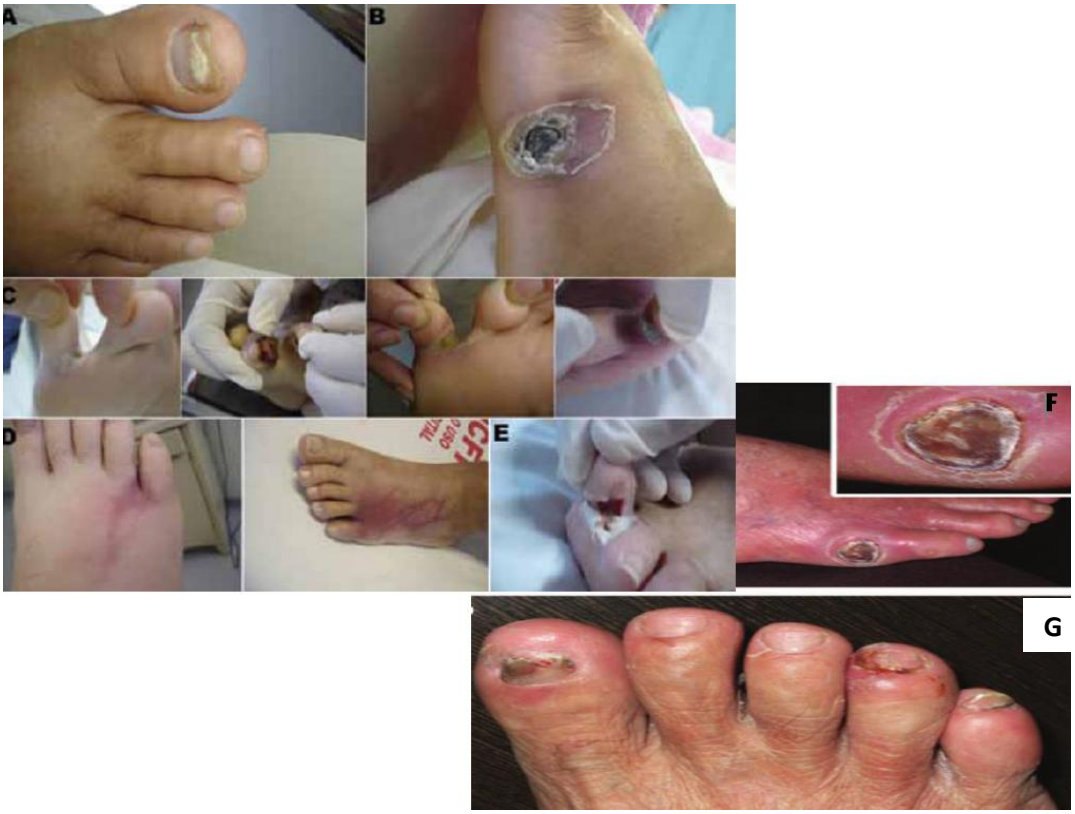


Figura 15: Lesões cutâneas primárias por *Fusarium* sp. evolução para fusariose invasiva - Lesões cutâneas primárias em pacientes com fusariose invasiva: Onicomicose (A), úlcera (B), intertrigo (C), intertrigo evoluindo para linfangite antes da disseminação (D), Necrose em uma lesão de intertrigo (E); úlcera dolorosa em pé direito após infecção ungueal (F), onicomicose no hálux direito (G).

As espécies mais frequentes de onicomicoses por fungos não-dermatófitos são as espécies do complexo *F. solani* e do complexo *F. oxysporum* [44], as quais provocam comprometimento proximal da unha associado com dor e inflamação periungueal. A unha afetada apresenta cor branca-amarelada e, frequentemente, superfície opaca. A dobra proximal da unha e a cutícula tornam-se de cor branca-amarelada, indicando a origem proximal da infecção [85]. A unha distal apresenta coloração amarelada quando há progressão da micose. A evolução da onicomicose por *Fusarium* sp. é de 1 mês a 15 anos (média de 3 anos) [16].

A onicomicose é uma onicopatia comum responsável por aproximadamente 50% de todos os problemas de unhas, e sua prevalência na população mundial varia entre 2 e 18% [25]. As onicomicoses por fungos não-dermatófitos (FND) têm aumentado rapidamente, sobretudo na Europa, onde são responsáveis por 1,6% a 6% dos casos [86], nos Estados Unidos é estimada em 13%, e no Brasil revela um aumento da incidência de

onicomicose causada por FND (incluindo *Fusarium* spp.), provavelmente devido ao clima quente e úmido, e ao grande número de agricultores, sendo um país que vive economicamente do setor primário [87].

Em um estudo epidemiológico em 2003 no sul do Brasil (no período de um ano) apresentaram 360 casos positivos de onicomicose, os quais foram confirmados apenas pelo método convencional de identificação microscópica, sem fazer a identificação molecular. De modo que, as espécies de *Fusarium* spp. foram responsáveis por 7,5% (n = 27) dos casos, tornando o gênero *Fusarium* o mais isolado entre os fungos filamentosos não-dermatófitos. A espécie mais frequente foi *F. oxysporum* (33,3%) [25].
A epidemiologia e a etiologia da onicomicose por *Fusarium* apresentam diferenças geográficas, especialmente na frequência de cada grupo de fungos responsáveis pelas infecções. Isso está relacionado com a microbiota fúngica, que é alterada no mundo periodicamente em sua composição quantitativa e qualitativa, em função de fatores ambientais, como o desenvolvimento urbano, industrialização, localização geográfica, e condições climáticas [88].

A onicomicose por *Fusarium* sp. é difícil de tratar, e apresenta altas taxas de falha, mesmo após longos cursos de antifúngicos sistêmicos ou terapia tópica, com ou sem remoção das unhas [71,81]. Apesar da limitada atividade *in vitro* ao itraconazol e à terbinafina, ambas são comumente utilizadas na terapia sistêmica para onicomicose por *Fusarium* sp. [16], apresentando eficácia em alguns casos de pacientes imunocompetentes [89].

É sabido que os novos triazólicos (voriconazol e posaconazol) apresentam maior atividade *in vitro* contra *Fusarium* spp. [90]. O tratamento para onicomicose com voriconazol é composto por uma dose de ataque de 400 mg por via oral, ou por duas doses, com intervalo de 12 horas, seguida por doses de manutenção de 200 mg por via oral, a cada 12 horas [91]. A remoção da unha aliado ao tratamento com anfotericina-B tópica pode ser feita em pacientes que não respondem à terapia antifúngica sistêmica, mas geralmente é evitada em pacientes imunocomprometidos [75], devido a infecção geralmente requer meses de terapia e a duração depende do estado imunológico geral do paciente e da resposta terapêutica.

O ciclopirox olamina também tem sido usado em combinação com agentes orais, existem estudos que comprovam seu efeito potencializador no tratamento, como é o caso

da sua combinação com terbinafina oral, onde obtiveram uma taxa de cura micótica de 88% e uma taxa de cura completa de 68%, enquanto a terbinafina sozinha tinha uma taxa de cura micótica de 65% e uma taxa de cura completa de 50% [92]. Na Figura 16 está apresentando um algoritmo geral de tratamento para onicomicose [93].

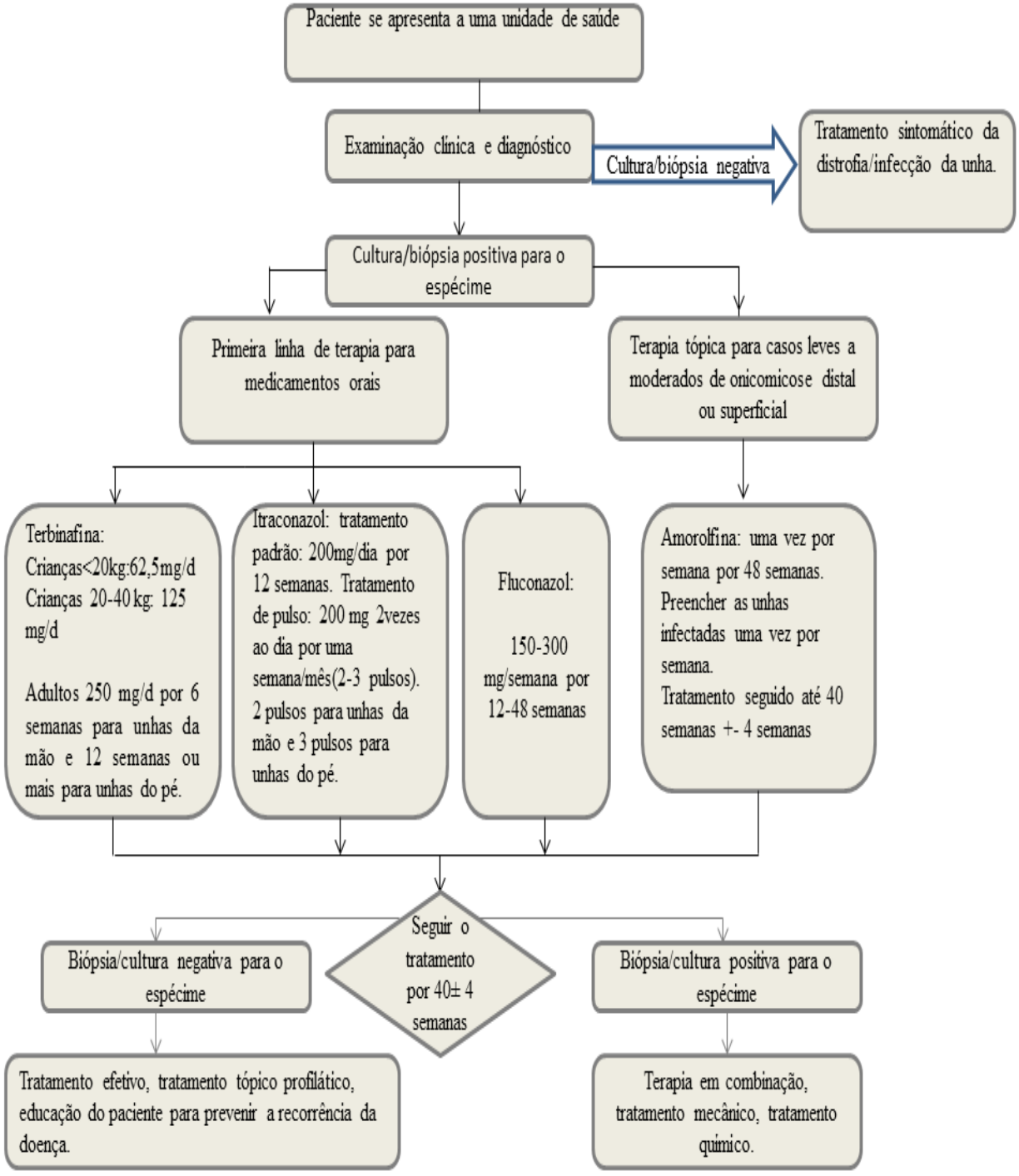


Figura 16: Algoritmo de tratamento de onicomicose para tratar todos os fungos (Adaptada de Thomas et. al. [93]).

O manejo clínico da onicomicose inclui prescrição tratamentos com antifúngicos sistêmicos e tópicos. Os antifúngicos mais urilizados atualmente incluem a terbinafina (uma alilamina), amorolfina (morfolina), itraconazol (triazólico) e ciclopirox (hidroxitriptofano). Ciclopirox, itraconazol e terbinafina são aprovados nos Estados Unidos e em todo o mundo para o tratamento de onicomicose, enquanto a amorolfina e o fluconazol são aprovados na Europa [94]. No Brasil todos esses fármacos são utilizados na terapêutica.

2.7.2 Ceratite Fúngica

A ceratite fúngica pode acometer todas as camadas da córnea até as mais profundas. Esse tipo de infecção causa ulceração da córnea, opacidade do globo ocular e uma inflação intensa da conjuntiva, caracterizada como uma infecção ocular de alta gravidade, que pode resultar em grande deficiência visual e perda do globo ocular [95].

A virulência do fungo é devido a sua capacidade de resistir às defesas do hospedeiro e de penetrar através da membrana lesionada por um trauma mecânico, atingindo a câmera anterior ocular. Pode ainda causar necrose tecidual através da produção de enzima, contribuindo para a resposta inflamatória resultando em dano tecidual [96].

Neovascularização pode ocorrer como resultado da inflamação (Figura 17 A) [97]. Os sinais associados que indicam a gravidade da inflamação incluem a presença do fungo na córnea envolvendo regiões da esclera (Figura 17 B) [30], muitas vezes atribuído ao uso de lentes de contato (Figura 17 C). Gravemente ocorre a descemetocele (Figura 17 D) [98] ou a produção de hipópio (pus na câmara anterior) [99]. Os fungos podem invadir em poucos dias o estroma profundo com grande rapidez e podem ter acesso à câmara anterior [100].

As infecções fúngicas quase sempre se apresentam de maneira insidiosa. Se não tratadas, os sinais inflamatórios progridem gradualmente, causando a degradação permanente do epitélio, ulceração estromal ou formação de descemetocele (afinamento da córnea). A córnea pode eventualmente perfurar, formando uma úlcera temporal densa de córnea com bordas mal definidas e hipónio de 1 mm (Figura 17 E). É frequente a

recorrência da infecção mesmo após a ceratoplastia penetrante excêntrica, como pode ser observado na Figura 17 F e G [101].

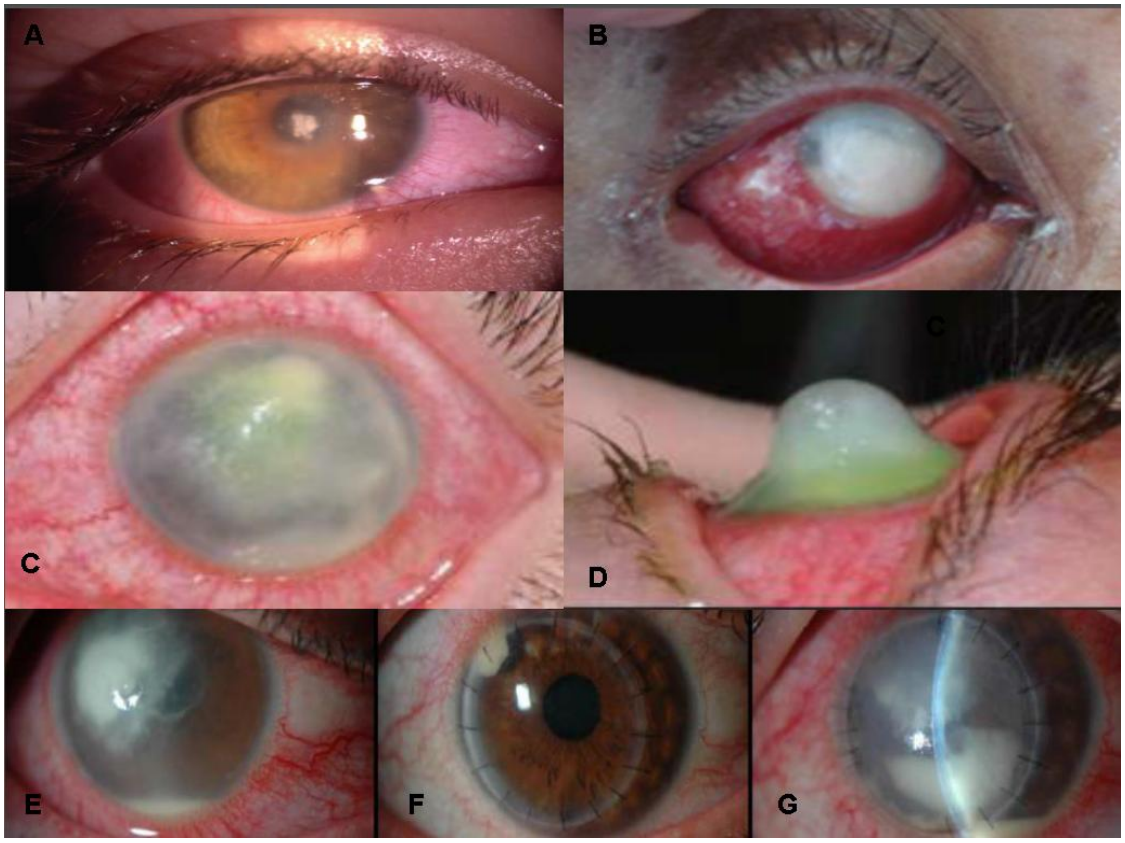


Figura 17: Ceratite causada por *Fusarium* sp. Abscesso da córnea no olho direito (A); Córnea infectada envolvendo regiões da esclera (B); Ceratite grave por *Fusarium* em usuários de lentes de contato (C); Descemetocele ocorrendo 5 dias após o exame inicial (D); Imagem mostrando úlcera temporal densa de córnea com bordas mal definidas e hipópio de 1 mm (E); Cinco semanas após a primeira ceratoplastia penetrante excêntrica, um infiltrado corneano (F); Infiltrados micóticos no transplante de córnea e produção hipópio (G).

A ceratite fúngica é uma infecção destrutiva da córnea com um alto nível de morbidade ocular. Essa patologia tem grande distribuição mundial, sendo mais frequente em áreas subtropicais, assim como em pacientes com o comprometimento da integridade corneana. Atualmente, o número anual de úlceras corneais registradas está rapidamente se aproximando entre 1,5 e 2 milhões, e o número real é provável que seja maior. Para a maioria destas infecções, o resultado final é geralmente opacidade da córnea, ou possivelmente os resultados sejam ainda mais devastadores, como perfuração da córnea, endoftalmite ou enucleação do olho [102].

O fator mais comum de predisposição à ceratite por fungos foi o trauma associado ao material vegetal. Outros fatores de risco que influenciaram nesta patologia foram o uso prolongado de corticosteróides e o estado de imunidade dos pacientes [103].

O Brasil apresenta um número significativo de casos de ceratite fúngica (CF), variando de 11% a 56% de todas com cultura positiva para infecção fúngica [104]. A espécie mais frequente de CF é *F. solani* (encontrado em até 91% dos isolados), seguido por *F. oxysporum* e *F. dimerum* [104]. Relata-se que as infecções oculares de *F. solani* têm o prognóstico mais grave devido à sua rápida progressão, apresentando perfuração da córnea em poucas semanas [105].

A ceratite é geralmente tratada com antifúngicos tópicos, pois as drogas sistêmicas não apresentam uma boa ação ocular, e a natamicina é a droga de escolha. Recentemente foi relatado o sucesso do tratamento com voriconazol tópico combinado com o oral [9].

A administração sistêmica da AMB apresenta pouca penetração nos tecidos oculares, não atingindo níveis terapêuticos na córnea, aquoso ou vítreo. Além disso, seus efeitos colaterais desestimulam o uso dessa via, sendo a administração direta *in situ* acaba sendo a principal forma de tratamento. É uma das poucas drogas que apresenta descrição na literatura do uso subconjuntival, intraestromal, intracameral e intravítreo, além do seu uso tópico [106]. O tratamento dos casos de ceratite fúngica está exemplificando de maneira esquemática na figura 18 [107].

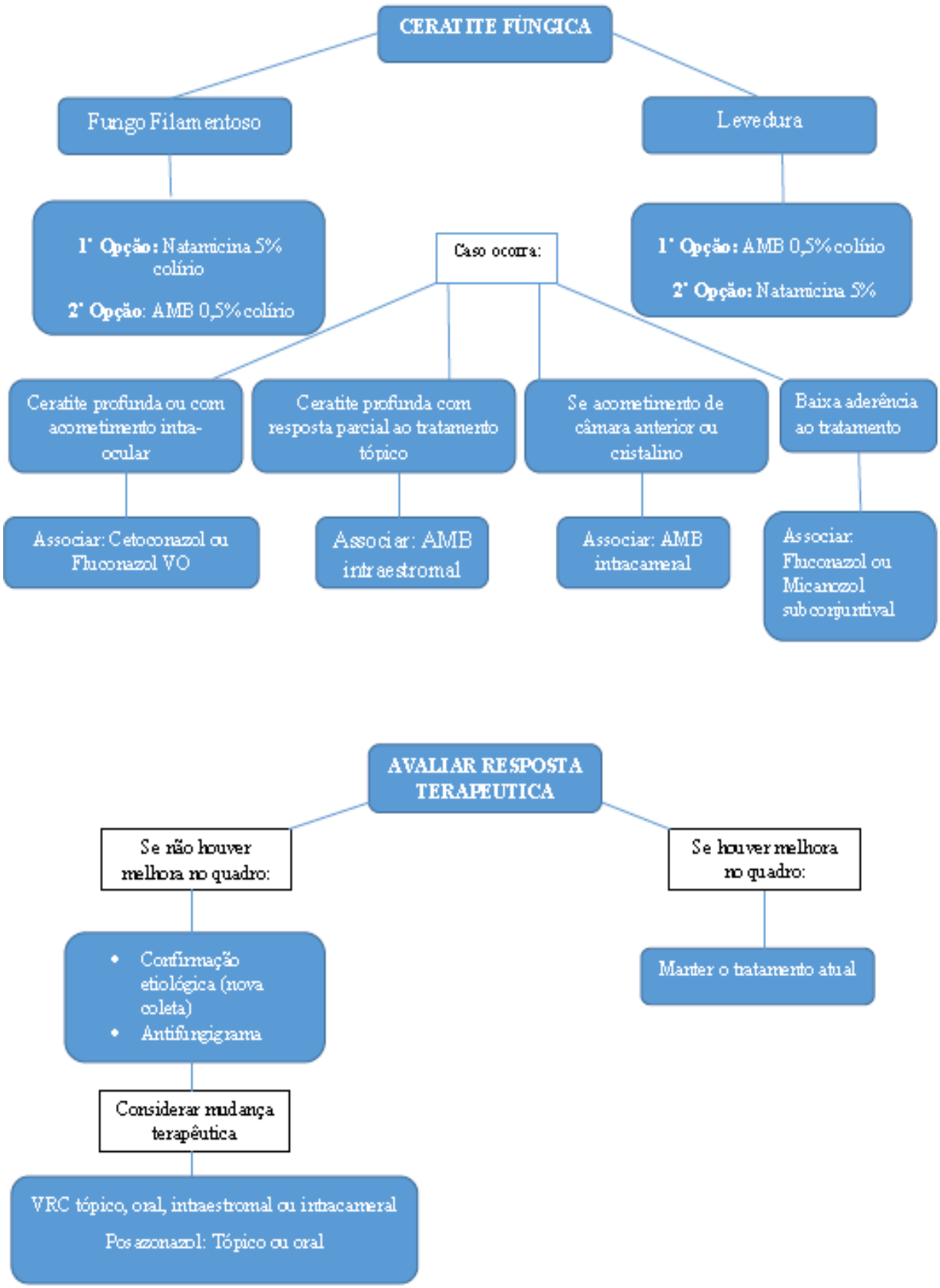


Figura 18: Algoritmo de tratamento para ceratite fúngica (Adaptada de Muller et al. [107]).

2.7.3 Fusariose invasiva

A fusariose invasiva apresenta elevada mortalidade e ausência de um protocolo de ensaios clínico (*guidelines*) para o manejo adequado nos casos de infecção invasiva, contribuindo para o crescente interesse no estudo desta micose. Essa infecção é caracterizada por febre persistente, que é refratária ao tratamento antibiótico de amplo espectro, e por lesões de pele com necrose central [7]. As manifestações clínicas da infecção por *Fusarium* incluem sinusite, pneumonia, lesões localizadas na pele, fungemia e infecções disseminadas em pacientes imunocomprometidos [38].

As infecções fúngicas invasivas são as principais causas de mortalidade e morbidade em pacientes com neoplasias hematológicas e neutropenia prolongada após a quimioterapia [108]. A manifestação clínica da fusariose depende em grande parte do estado imunológico do paciente e da porta de entrada, que incluem seios paranasais, pulmões e pele [109].

O envolvimento da pele na fusariose pode representar um local primário de infecção, geralmente uma celulite dos dedos dos pés, ou uma manifestação de infecção metastática em pacientes com fusariose disseminada [9]. As lesões cutâneas podem envolver qualquer sítio da pele, com predomínio nas extremidades. A infecção por *Fusarium* apresenta-se mais frequentemente como lesões cutâneas metastáticas, do tipo papulonodulares eritêmato-violáceas, algumas purpúricas, nos membros inferiores (Figura 19 A e B) [110], com apresentação inicial de lesões subcutâneas, indurações eritematosas, seguidas por lesões necróticas centrais, tipo em alvo (Figura 19 C, D e E) [111]. As lesões são formadas rapidamente dentro de um a cinco dias, em vários estágios de evolução e ocasionalmente com mialgias. As biópsias de pele são extremamente fáceis de realizar e confirmam a suspeita clínica. No entanto, as imagens histopatológicas das lesões cutâneas assemelham-se às das infecções por *Aspergillus*. As duas infecções exibem invasão vascular e hifas septadas ramificadas; portanto, a diferenciação pode ser difícil [108].

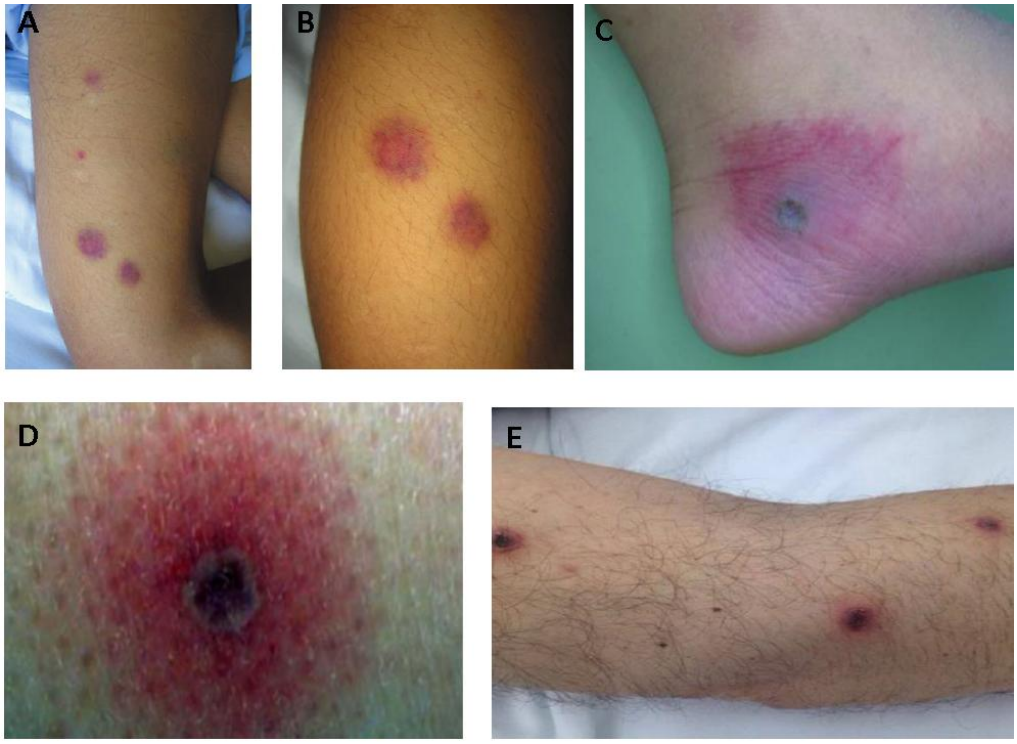


Figura 19: Lesões nos membros inferiores (A e B); Lesão clássica com pápula eritematosa endurecida e necrose central, única (C); Lesões cutâneas causadas por *F. solani* (D e E).

O envolvimento pulmonar é comum na fusariose invasiva, quadro de pneumonia, geralmente se manifestando como lesões nodulares e cavitárias (Figura 20). Como esperado, o envolvimento pulmonar está associado a maior mortalidade, mesmo após o controle do estado imunológico. A apresentação clínica é inespecífica, com alguns casos apresentando quadro clínico semelhante à aspergilose invasiva, com tosse seca, dor torácica pleurítica e falta de ar [112].

Quando há suspeita de rinosinusite fúngica aguda sugere-se a endoscopia nasal. Este exame demonstra a presença de sinais típicos, tais como mucosa avermelhada e edema no estágio inicial da doença; palidez e descoloração da mucosa em acometimento no estágio mais avançado da doença, podendo ser observada na Figura 20 C e D, além da evidente presença do fungo [112]. Outras fermentas que auxiliam no diagnóstico da infecção invasiva são a tomografia computadorizada (TC) (Figura 20 A), a ressonância magnética (RM) (Figura 20 B, E e F), assim como raio-x (Figura 20 G), [113].

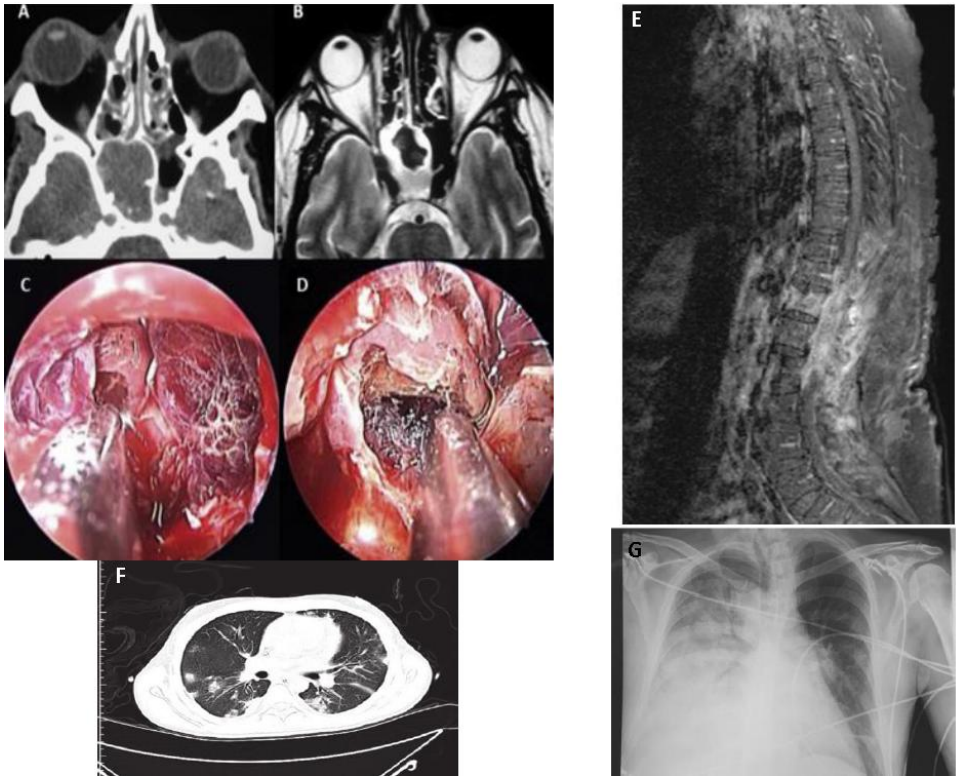


Figura 20: Rinossinusite fúngica invasiva por *Fusarium* sp., tomografia computadorizada e ressonância magnética revelaram opacificação do seio esfenoide direito, com manchas desinformadoras (A, B). Endoscopia para o desbridamento do tecido necrótico (C, D). Osteomelite detectado por RM (E). Observações radiológicas podem indicar infiltrados inespecíficos na formação nodular e / ou cavitária (F). Radiografia de tórax com lesões consolidativas com lucidez central envolvendo os lobos basal e medial do pulmão direito (G).

Durante a última década, o número de casos de fusariose invasiva (FI) aumentou em todo o mundo, entretanto existem poucos estudos descrevendo a epidemiologia molecular. Na Itália, um estudo realizado com 18 centros de hematologia relatou 15 casos de fusariose invasiva. Encontraram entre 11.802 pacientes com neoplasia maligna hematológica (prevalência de 0,1%), e os pacientes com leucemia mielóide aguda tiveram a maior incidência, 13 casos em 3.012 pacientes (prevalência de 0,4%) [84]. Nesse país, a espécie mais prevalente foi a *F. verticillioides*, seguido por *F. solani* (25%). Ao contrário do que acontece em Paris, na França, onde aproximadamente um terço das espécies é causada por FSSC, seguido por FOSC, complexo de espécies de *F. dimerum* (FDSC), e raramente pelo complexo de espécies *F. incarnatum-F. equiseti* (FIESC) [114].

Horn et al. [115] descreveram 65 casos do Registro *North American Path Alliance*, de 2004 a 2008. A maioria dos pacientes tinha malignidade hematológica (72,3%) e 83,1% tinham fusariose comprovada. Mohammed et al. [75] relataram 26 casos dos Estados Unidos, sendo o fator de risco mais importante encontrado para a fusariose invasiva , o comprometimento do sistema imunológico, e o local mais comumente infectado foi a pele; *F. solani* foi a espécie mais frequente (49% dos casos). Em um estudo prospectivo realizado em 22 centros na Argentina, a fusariose invasiva foi responsável por 14% dos casos [76]. A taxa bruta de mortalidade relatada na América Latina é de 66%, mas em pacientes com fusariose invasiva e imunodeficiência persistente, a mortalidade chega a 100%. Resultados semelhantes foram relatados no banco de dados da Rede americana de Vigilância de Infecções Associadas a Transplante (TRANSNET - *Transplant-Associated Infection Surveillance Network Database*) [116].

As taxas de mortalidade entre os pacientes com fusariose invasiva, cutânea e pulmonar foram 50%, 40% e 37,5%, respectivamente, em um estudo no Hospital Geral de Massachusetts, Boston, MA, e a espécie mais frequente foi a *F. solani* [75]. Nesta espécie é observada a maioria dos casos de resistência aos azólicos e aos poliênicos. Contudo, a espécie de *F. oxysporum* é mais suscetível ao voriconazol e ao posaconazol [69]. Noutro estudo epidemiológico realizado na Itália, observaram que os isolados estavam quase que uniformemente divididos entre o complexo de espécies de *Gibberella (Fusarium) fujikuroi,* o FSSC e o FSCO [8].

As condutas terapêuticas corretas recomendadas para os quadros de fusariose não são muito claras e são de difícil interpretação em razão do pequeno número de casos diagnosticados e tratados. Assim, a eficácia terapêutica foi avaliada a partir dos resultados dos relatos clínicos e dos estudos retrospectivos [7]. A remoção cirúrgica do tecido infectado associada ao uso de anfotericina-B tem sido relatada.

Recentemente, as diretrizes diagnósticas, *European Society of Clinical Microbiology and Infectious Diseases* (ESCMID) e *European Confederation of Medical Mycology* (ECMM), sugerem que a terapia precoce com voriconazol (VRC) ou anfotericina-B lipídica (AMBlip) com intervenção cirúrgica seja aplicada para prevenir a disseminação da doença fúngica; e que de forma alguma se deva ser administrado equinocandinas. Em espécies de *Fusarium* que não são suscetíveis a AMB e VRC, o medicamento de escolha indicado seria o posaconazol (POS) [109].

3. MARCO CONCEITUAL

O conceito da pesquisa foi investigar a diversidade das espécies de *Fusarium* agentes das fusarioses da região Sul do Brasil, a fim de verificar a possibilidade de diferença entre as espécies causadoras de onicomicose, de ceratite e de fusariose invasiva, e entre outras regiões; avaliar a possibilidade de um padrão de resistência e suas singularidades; conhecer os fatores e os pacientes de risco, com intuito de estabelecer um conhecimento sólido sobre o perfil epidemiológico regional. Na figura 21 está representado o conceito deste estudo, apresentando um gráfico que retrata a crescente publicação deste tema nos últimos anos, tanto em pacientes imunocomprometidos quanto em pacientes imunocompetentes. No período de 2000-2010, dobraram as publicações sobre fusariose, e também sobre investigações dos complexos de espécies mais prevalentes de *Fusarium*. Outra crescente preocupação é o desenvolvimento de um protocolo clínico específico para fusariose, pois no caso de ceratite e onicomicose não são específicos para tratar a espécie de *Fusarium*; e no caso de fusariose invasiva o protocolo clínico proposto recentemente em 2014 foi baseado em relatos de caso e em estudos observacionais. O grande consenso dos pesquisadores é a recomendação da realização do perfil de susceptibilidade, sendo extremamente relevante saber a concentração inibitória mínima e tentar aplicá-la no tratamento clínico, da mesma forma que é importante mensurar sua concentração plasmática, para inferir o quanto estará disponível no foco da infecção.

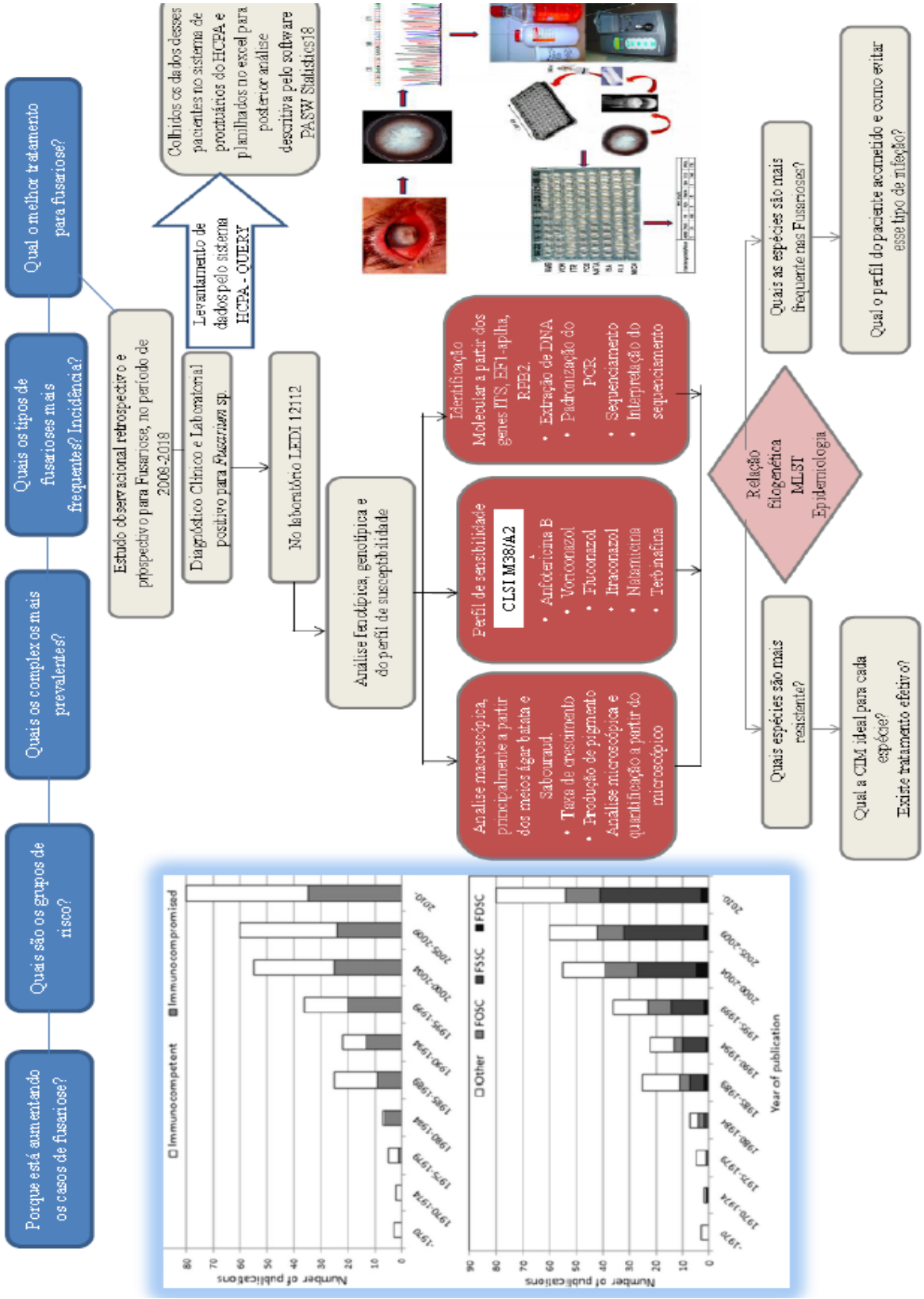


Figura 21: Mapa conceitual da tese de doutorado sobre Fusariose.

4. JUSTIFICATIVA

Estudos epidemiológicos são fundamentais para aprimorar o conhecimento sobre doenças emergentes, estabelecer prioridades, alocar recursos, definir novas diretrizes e protocolos clínicos para o tratamento e/ou profilaxia. Na última década, aumentaram globalmente os estudos epidemiológicos sobre fusarioses. Assim como, a preocupação em classificar a diversidade das espécies e dos complexos do *Fusarium*, a fim de conhecer todos os aspectos relacionados a natureza desse fungo, bem como os grupos de risco em que está causando infecção. Dessa forma, desenvolvemos um estudo com objetivo de caracterizar a epidemiologia da fusariose da região Sul do Brasil, para assim definir um conhecimento sólido sobre as espécies de *Fusarium*, tal como seu padrão de sensibilidade aos antifúngicos e por consequencia poder auxiliar na melhor classificação dessa doença e igualmente na orientação ao clínico com o tratamento mais adequado.

5. OBJETIVOS

5.1 Objetivo primário

Realizar um estudo observacional retrospectivo e prospectivo de três fusarioses: onicomicose, ceratite e fusariose invasiva, investigando os aspectos epidemiológicos, a diversidade genética e os perfis de susceptibilidade aos antifúngicos.

5.2 Objetivos secundários

- Caracterizar fenotipicamente os isolados de *Fusarium* spp., descrevendo suas características morfológicas macroscópicas e microscópicas;
- Caracterizar genotipicamente os agentes etiológicos das fusarioses, determinando as sequencias nucleotídicas, espécie, haplótipo e complexo;
- Investigar a relação filogenética entre as espécies de *Fusarium* agente de fusariose;
- Analisar o perfil de susceptibilidade entre as espécies de *Fusarium*;
- Verificar a existência de um padrão de sensibilidade entre os complexos de espécies nas diferentes infeções por *Fusarium*.
- Descrever o perfil dos pacientes de risco acometidos por fusariose.

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7. ARTIGOS

Artigo 1: Systematic review of onychomycosis caused by *Fusarium* species (*BJID*; FI=2,09)

Systematic review of onychomycosis caused by *Fusarium* species

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Short running title: *Fusarium* in onychomycosis

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ABSTRACT

Onychomycosis is a fungal infection of the nail and is the most common nail disease in the general population. Onychomycosis caused by the genus *Fusarium* have emerged over the past decades in immucompromised individuals, with disseminated infection and high mortality rates. The prevalence of this type of infection varies in the literature because of geographical differences in mould distribution and diagnostic methods. *Fusarium* onychomycosis is a difficult-to-treat infection, because little is known about the genetic variability and susceptibility pattern of *Fusarium* spp. in this setting. This article reviews our current understanding of *Fusarium* onychomycosis. We reviewed the main epidemiological, diagnostic and therapeutic aspects, including molecular identification and in vitro antifungal susceptibility testing of *Fusarium* species in onychomycosis.

Keywords: Onychomycosis, Molecular techniques, Susceptibility profile, *Fusarium* spp., Epidemiology.

INTRODUCTION

Onychomycosis is a fungal infection of the nail plate or nail bed, leading to the gradual destruction of the nail plate that causes discoloration, thickening, and separation from the nail bed. It occurs in 10% of the general population, 20% of persons older than 60 years, and 50% of those older than 70 years. Accurate diagnosis involves physical and microscopic examination and culture. Histologic evaluation using periodic acid–Schiff staining increases sensitivity for detecting infection [1].

A typical clinical manifestation of the affected toenails is leukonychia or distal subungual hyperkeratosis with yellowish brown coloration [2,3]. The infection freuquently affects the great toe nail after trauma or dystrophic abnormalities and other predisposing conditions like diabetes mellitus, peripheral circulatory diseases, walking bare feet, family history, and immunosuppression [4]**.** The presence of infections involving skin or nail should be carefully investigated before initiating immunosuppressive therapy since it has been shown that such lesions can be a focus for fungal dissemination [5].

Non-dermatophytic molds (NDM) onychomycosis accounts for 2% to 12% of all nail fungal infections and can be caused by a wide range of fungi, among them the *Fusarium oxysporum* and *Fusarium solani* [6]. *Fusarium* onychomycosis is a difficult-to-treat infection, even though there are several potent antifungal agents available for patient use. Little is known about the genetic variability and susceptibility pattern of *Fusarium* spp. in this setting. This article reviews our current understanding of *Fusarium* onychomycosis. Scopus and PubMed, databases search were conducted for this review. "Nail Fusarium" and "Fusarium onychomycosis" were the search terms used for all databases. Without restricting the search period. Eligible studies included aspects of epidemiology, diagnosis, and treatment including molecular identification and susceptibility profile of *Fusarium* species, and treatment.

EPIDEMIOLOGY

Onychomycosis is a nail infection caused by a variety of fungal agents, and which affects approximately 5 % of the population worldwide, representing up to 50 % of all

nail diseases [7]. The prevalence of onychomycosis by *Fusarium* in North America is approximate1y 4 %, varying from 2 to 18% in the world's population [8]. It can infect up to 50% of people older than 70 years of age. In a previous study, NDM was isolated in 13.8% of the lesions and *Fusarium spp*. (50.0%) was the most frequently observed in a reference hospital [9]. In a recent study in Brazil, 35 *Fusarium* spp. isolates were isolated from patients with onychomycosis, predominantly from female Caucasians. The most frequent anatomical location was the nail of the hallux and 71.4% of the isolates belonged to the *F. solani* species complex, followed by *F. oxysporum* species complex (28.5%) [3]. The infection is more prevalent in females [10–13] and adult age, especially over 45 (2,4,23-27). *F. oxysporum* is considered an agent of fingernail and toenail onychomycosis infections, whereas *F. solani* is more often isolated from toe-nails [2]. Proximal subungual onychomycosis with paronychia and distal onychomycosis has been associated with *F. oxysporum* [4]. *F. oxysporum* invariably affects the great toe nail after trauma or dystrophic abnormalities and other predisposing conditions like diabetes mellitus, walking bare feet, family history and immunosuppression.

Special aspects in case reports and case series *of Fusarium* onychomycosis

Case reports of Fusarium onychomycosis are listed in Table 1. The first reported case of *Fusarium* onychomycosis was Richie & Pinkerton (1959) [16]. *F. oxysporum and F. solani* species complex has been described as a the most prevalent *Fusarium* species causing onychomycosis, its incidence has been increasing in immunocompetent hosts. In a study by Godoy et al. in Brazil, *F. solani* and *F. oxysporum* were the common species isolated in patients with onychomycosis. Report eight cases of onychomycosis these species were isolated from toenails in all cases. The presence of the fungi in direct microscopic examinations and in histopathological examinations and the repeated isolation of the same organisms [2]. Additional case reports *Fusarium* onychomycosis due to *F. oxysporum* species have been published [8,17–19]. Onychomycosis caused by *Fusarium proliferatum* have been described in patients that were successfuly treated fully with oral ITC, even though the minimum inhibitory concentration of ITC was relatively high [20]. In another case report, a 73-year-old healthy female that exhibited a discoloration and thickening of the right big toenail was unsuccessfuly treated with TRB for 25.5 months by a primary physician. Direct microscopy revealed chlamydoconidia and hyphae, and periodic acid-Schiff-stained nail specimen showed septate hyphae. On

the basis of these morphological features and gene analysis, *F. proliferatum* was identified. Topical application of 10% efinaconazole solution cured the disease in 10 months [6].

Interestingly, Carvalho *et al*., [19] described the first case of congenital onychomycosis in a child caused by *F. oxysporum*. The infection being acquired in utero was proven by molecular methods with the identification of the fungus both in the nail and placenta, most probably as an ascending contamination/infection in an HIV-positive, immunosuppressed mother.

The most frequent species identified were *F. oxysporum* (36.5 %), *F. solani* (31.8 %), and *F. subglutinans* (8.3 %). FLC was not active against *Fusarium* spp., and the response to TRB varied according to species [21].

MYCOLOGY

Traditional mycology remains the gold standard for diagnosing NDM onychomycosis, which includes obtaining positive results from KOH and culture. Repeated isolations (2 or 3) in the absence of a dermatophyte increase the probability of accurate identification of the causative NDM [36]. Diagnosis is based on the following criteria: 1. nail abnormalities consistent with the diagnosis; 2. positive potassium hydroxide preparation with presence of hyphae in the nail keratin; 3. growth of the same mold in duplicate cultures; 4. failure to isolate dermatophytes or yeasts in culture; 5. colony morphology and sporulation pattern [37]. All patients who were diagnosed with onychomycosis caused by *Fusarium* spp. experienced pain, with nails that were hyperkeratotic and presented deformities and paronychia, according to observations in the literature [21].

The colonies of *F. solani* species complex (FSSC) present a cream-coloured mycelium with a green to bluish brown reverse. Microscopically, numerous microconidia on long phialides and the presence of macroconidia with indistinct foot-cells confirm the diagnosis [2]. Typically causes superficial white onychomycosis [6]. Only monophialides are present, microconidia abundant, in false heads only, sporodochia cream or blue-green to blue and chlamydospores single or in chains [5]. The colonies of *F. oxysporum* species complex (FOSC) grow rapidly. The colonies consist of white colonies, which gradually

become purple with a dark blue or dark purple reverse. Microscopic examinations revealed microconidia on short and often lateral phialides and macroconidia with distinct foot-cells [2]. Only monophialides are present, macroconidia very thin, needle-like with thin walls. Microconidia are abundant, with false heads, sporodochia are cream or orange to yellow or tan, and chlamydospores single or in chains [5]. *F. proliferatum* is morphologically similar to *F. oxysporum*, but is distinguished from *F. oxysporum* by the formation of polyphialides [6].

MOLECULAR

Fusarium species are characterized by their most distinguishable character, the fusiform or banana-shaped macroconidia. However, morphology is insufficient to differentiate between species. A PCR detection based on the intergenic spacer (IGS) region has been developed for different agricultural important *F.* species (complexes) that can also distinguish clinical species complexes like *F. equiseti* and *F. sporotrichioides* because different-sized fragments are produced [38].

In addition, clinical *Fusarium* strains often degenerate phenotypically. The rDNA internal transcribed spacer (ITS) is insufficient for distinguishing many species of this fungus. This genetic region is further complicated by the presence of highly divergent ITS2 rDNA paralogs or xenologs within every strain tested within six closely related species complexes [39]. Therefore, the current state-of-the-art diagnostic technique is multilocus sequence typing [40]. They used multilocus sequence analysis, the gold standard, to identify known lineages in the *F. solani* and *F. oxysporum* species complexes and also identified two new lineages with members capable of human infection [41]. Based, including a portion of the DNA-directed RNA polymerase II (rpb2), rDNA ITS and partial translation elongation factor (tef1-a) [42]. Technological and theoretical advances have been key to the greatly accelerated species discovery within *Fusarium* over the past two decades.

Most of the identified opportunistic *Fusarium* pathogens belong to the FSSC, the FOSC and *Fusarium fujikuroi* species complex (FFSC). Less frequently encountered are members of the *Fusarium incarnatum-equiseti* (FIESC), *Fusarium dimerum* (FDSC) and

Fusarium chlamydosporum species complexes (FCSC) or species such as *Fusarium sporotrichioides* [38].

Based on the molecular data, our *Fusarium* isolates proved to be derived from two *Fusarium* species complexes observed in human infections frequently: FSSC and FOSC. The FSSC is commonly seen as the major cause of human fusariosis worldwide. Within this complex, we observed etiological agents from two main human pathogenic lineages: *F. keratoplasticum* (haplotype 2) and *F. falciforme* (haplotypes 3-4) [43].

Table 2 summarizes the identification of *Fusarium* species that cause onychomycosis, and describes the genes used in molecular identification.

IN VITRO ANTIFUNGAL SUSCEPTIBILITY

Fusarium spp. and usually shows resistance to the generally applied antifungal agents. Susceptibility testing for different *Fusarium* isolates causing onychomycosis are described in table 3. However, different species may have different patterns of susceptibility. *F. solani* and *F. verticillioides* are usually resistant to azoles and exhibit higher MICs for amphotericin B than other *Fusarium* spp. [51]. Absence of activity of *Fusarium* strains studied was observed for flucytosine (MIC > 32 μg/mL), FLC (MIC > 64 μg/mL) and ITC (MIC > 16 μg/mL) when tested alone [52]. In a previous study, the antifungal susceptibility of *Fusarium* isolates in onychomycosis showed that AMB was the most effective antifungal agent against the majority of the isolates (60%, MIC ≤4 μg/mL), followed by VRC (34.2%, MIC ≤4 μg/mL). In general, those isolates had MIC values >64 μg/mL for FLC, ITC and TRB. Statistical analysis revealed FOSC complexes are significantly more sensitive than FSSC to VRC (P=.012) by the non- parametric Mann-Whitney test [3].

Antifungal susceptibility test results agree with the recently published guidelines for Epidemiological Cutoff Value (ECV) setting, ECVs encompassing >97.5% of pooled statistically modeled MIC distributions were as for different Fusarium species (AMB, 8 μg/mL to FOSC and FSSC; for VCR, 16 to FOSC and 32 μg mL^{-1} to FSSC; and for ITC, 32 μg/mL to FOSC and FSSC) [53].

TREATMENT

Systemic antifungals are the most effective treatment showing mycotic cure rates of 76% for TRB, 63% for ITC with pulse dosing, 59% for ITC with continuous dosing, and 48% for FLC. Concomitant nail debridement further increases cure rates. Topical therapy with ciclopirox is less effective; it has a failure rate exceeding 60%. Several nonprescription treatments have also been evaluated. Laser and photodynamic therapies show promise based on the in-vitro evaluation, but more clinical studies are needed. Despite treatment, the recurrence rate of onychomycosis is 10% to 50% as a result of reinfection or lack of mycotic cure [1]. Traditional antifungal treatments used for the dermatophyte borne disease are less effective against onychomycoses caused by NDM. Although some laser and light treatments have demonstrated clinical efficacy against onychomycosis, their US Food and Drug Administration (FDA) approval as "first-line" therapy is pending, partly due to the lack of well- demonstrated fungicidal activity in a reliable in vitro model [7]. First-line onychomycosis management strategies include oral administration of TRB, ITC or FLC [54]**.** FLC has no action against filamentous fungi including *Fusarium*, and if used in neutropenic patients it would only act on *Candida* spp**.** [55]. Although ketoconazole and FLC are often prescribed for the treatment of onychomycosis, only griseofulvin, ITC and TRB are approved by the US Food and Drug Administration (FDA) and currently licensed for the treatment of this condition in the United States and United Kingdom [54].

Onychomycosis due to *Fusarium* species is a difficult-to-treat infection and combined therapy with oral ITC or TRB, chemical and physical removal of the affected nail, and topical application of antifungal agents have been recommended [6]. Published articles on its efficacy on *Fusarium* or other NDFF onychomycosis are scarce and rely mostly in case series. Fifteen of 17 patients with onychomycosis due to NDM were treated with ITC pulse achieved clinical and mycologic. Of the fifteen patients, only 2 cases were due to *Fusarium*. Randomized, doubleblind trials showed that oral TRB 250 mg daily for 12 (Table 4) or 16 weeks was more efficacious than ITC, FLC, and griseofulvin in dermatophyte onychomycosis of the toenails [37]. In onychomycosis due to *Fusarium* species, ITC pulse therapy (400 mg/day for 1 week a month for 6 months) provided complete cure in two of two cases treated, ITC daily therapy (200 mg for 6-12 weeks) (Table 4) resulted in complete cure in one patient (28). Additionally, daily oral TRB (250 mg/day for 3-4 months) produced no mycological or clinical cure in one patient

(28). Infections with *F. oxysporum* have been sucessfully treated with difficulty with ITC, TRB, and imidazole but not with flucytosine [4].

Several studies have shown that ITC pulse therapy is effective on *Fusarium* onychomycosis, showing clinical cure in 40–88% of cases. In the study conducted by Ranawaka et al. 2015 on six cases of *F.* onychomycosis, oral ITC pulse therapy showed 60% clinical cure at 12 months follow-up, and the mycological cure was 100%.

NDM generally do not respond well to the usual systemic medications, but the removal of the diseased nail, followed by treatment with topical antifungals or the use of new-generation azoles may improve therapeutic outcome [56].

Systemic Antifungal agents

Fluconazole has been effective in treating onychomycosis by dermatophytes (150mg weekly), with levels being achieved in the stratum corneum of 7.1µg/mL after 7 days oral administration of 150mg [57]. For the management of onychomycosis, pulse therapy with 150, 300 or 450 mg once a week is recommended for up to 12 months. In vitro data show high activity against dermatophytes and yeasts [58]**.** This drug is known to be resistant to *F.* specimens and most MICs are > 64µg / mL in the case of these isolates [59–61]. Griseofulvin was the first oral antifungal drug approved for the treatment of onychomycosis. It exhibits a fungistatic mode of action by interacting with microtubule-associated proteins and results in the inhibition of fungal cell division [58].

Itraconazole is mainly fungistatic. In vitro data demonstrate its broad spectrum of activity against dermatophytes and yeasts, as well as other molds. It is metabolized by the liver and many early studies have highlighted the hepatic toxicity of ITC [54]**.** ITC 400 mg daily for 1 week a month was given to 21 patients, and TRB 250 mg daily for 4 months was prescribed to 6 patients. Treatment was continued for 2 months in fingernail infection and for 4 months in toenail infection [14]**.** Terbinafine (TRB) was the first oral synthetic alkylamine approved for the treatment of onychomycosis [54]**.** TRB was suggested the treatment of choice for dermatophyte onychomycosis [37]**.** With regard to molds, in the *F. oxysporum* case, the response to TRB and topical imidazole was good and led to clinical and mycological recovery from an infection which is often refractory to

antimycotics [62]**.** Systemic treatment 1 week per month with either TRB (250 mg/d) and is prescribed in association with topical treatment [15]**.**

Topical antifungal agents

Ciclopirox has a mycotic cure rate of 29% to 36%, and a clinical cure rate of 6% to 9% in fungal onychomycosis. It is a member of the hydroxypyridine family. Is believed to work by inhibiting metal-dependent enzymes by chelating the polyvalent cations (Fe3+ or Al3+). It also inhibits fungal nutrient uptake, resulting in decreased nucleotides and a reduction in protein synthesis. The solution is applied daily covering the entire nail plate and approximately 5 mm of surrounding skin for 12 months. Combined results from clinical studies indicate a 29% to 36% mycologic cure rate [56]**.** Briefly the treatment is described in table 4.

Applications of amorolfine 5% have been indicated until the affected nail tissue has grown out, which is approximately 9 to 12 months in toenails and 6 months in fingernails [56]. Once weekly lacquer for up to 24 weeks is reported to have 60–71% mycological cure rates in randomized clinical studies, for the management of fungal onychomycosis [54]**.**

This review article gathered several important information for epidemiology, diagnosis and diversity of species of *Fusarium* onychomycosis agents. In summary, only 30% of the cases reviewed in the literature reported success in treatment, and no unanimous indication was found in the therapeutic indication. Most of the cases described were of nail infection of the foot, and 54.2% of these were exclusively caused by *F. oxysporum* species and 21% by *F. solani*. The diagnosis at the species level using molecular techniques was present in less than the metadata of the analyzed cases, as well as the histological examination (44.4%). The succession profile is very variable, showing a low sensitivity to most antifungal agents.

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List of Tables

Table 1. Summary of case reports of onychomycosis by *Fusarium* spp.

Species	**Case Report**	**Site**	**Treatment**	**Infection**	**cure**	**Author and year**
F. oxysporum	2	FN	Thiomersal	G	NI	Richie & Pinkerton, 1959 [16]
F. oxysporum	1	FN	NI	NI	NI	Gordon, 1960 [22]
F. oxysporum	1	TN	NI	NI	NI	Zaias *et al.*, 1966 [23]
Fusarium spp.	2	TN	NI	NI	NI	Walshe & English, 1966 [24]
F. oxysporum	1	TN	Glutaraldehyde	NI	S	Suringa, 1970 [25]
F. oxysporum	53	TN	R and pimaricin	CP and DP	S	Rush-Munro *et al.*, 1971[23]
F. oxysporum	5	TN	NI	NI	NI	Zaias *et al.*, 1972 [26]
F. oxysporum	1	FN	R	RT	S	Young & Meyers 1979 [27]
F. oxysporum	1	TN	NI	T	NI	DiSalvo & Fickling 1980 [28]
F. solani	3	TN	AMB	L	S	Merz *et al.*, 1988 [29]
F. solani	1	TN	ITC	T	S	Feuilhade *et al.*, 1989 [16]
Fusarium spp.	1	TN	R	L and T	IS	Nadler *et al.*, 1990 [30]
F. solani	1	TN	AMB	L and G	S	Robertson *et al.*, 1991 [31]

F. solani	1	TN	FLC AMB	A	IS	Gimenia *et al.*, 1992 [32]
F. oxysporum	1	TN	EFC	IP	S	Toutous-Trellu *et al.*, 1995 [17]
F. oxysporum	3	TN and FN	NI	NI	NI	Luque *et al.*, 1995 [16]
F. oxysporum	1	TN	CP and B	CP	IM	Dordain-Bigot *et al.*, 1996 [33]
F. oxysporum	3	PSR	R and CP and B	CP	IM	Baran *et al.*, 1997 [17]
Fusarium	4	FN, TN, FN and TN	NI	NI	NI	Bokhari, 1999 [13]
F. oxysporum	9	II and FN	ITC (4), CP (2), ITC and TRB(1)	NI	NI	Romano *et al.*, 1998 [34]
Fusarium spp.	26	DS (5) and PSR (21)	ITC,TRB, R and CP	NI	40% cure	Tosti *et al.*, 1998 [14]
F. solani	1	FN and TN	ITC	CP	IM	Lee *et al.*, 2002 [35]
F. oxysporum and *F. solani*	8		NI	NI	NI	Godoy 2004[2]
F. oxysporum (6) and *F. solani* (3)	9		R and CP or amorolfine	NI	NI	Piraccini & Tosti, 2004[15]

*B= Bifonazole, CP= Immunocompetent patient, CP=Ciclopirox, DP = Diabetic Patient, DS=Distal Subungual, EFC= Efinaconazole, FN=fingernail, G=Gardening, T=trauma, II=Interdigital Intertrigo, IM = Improviment, IP = Immunocompromised Patient, IS = Insusess, L= Leucemia, A=Aplastic Anemia, NI =

Uninformed, PSR= Proximal Subungual Region, R= Nail Removal, RT= Renal transplant patient, S = success, TN= Toe Nail.

Table 2. Diagnostic methods used in the diagnosis of onychomycosis of *Fusarium.* species

Species	**Isolates**	**Examen direct**	**PCR (gene)**	**Histo-logie**	**Country**	**Reference**
F. oxysporum	3	Yes	no	Yes	Italy	[17]
Fusarium sp.	26	Yes	no	Yes	Italy	[18]
F. oxysporum	7	Yes	no	Yes	Italy	[34]
Fusarium sp.	2	Yes	no	Yes	Brazil	[32]
F. solani and F. oxysporum	8	Yes	no	no	Brazil	[2]
F. proliferatum	2	Yes	cytochrome B	no	Japan	[20]
Fusarium sp.	128	no	28S rDNA	yes	Switzerland	[44]
Fusarium sp., *F. solani*	7	Yes	no	no	Brazil	[45]
F. oxysporum	27	Yes	no	yes	Brazil	[8]
F. solani	167	Yes	no	no	Brazil	[46]
F. solani and *F. oxysporum*	58	no	MLST	no	Italy	[47]
Fusarium sp.	4	Yes	no	no	Brazil	[48]
F. oxysporum	1	Yes	ITS	yes	Brazil	[19]
F. fujikuroi and *F. solani*	47	no	ITS	no	Turkey	[49]
F. solani, F. oxysporum and *F. incarnatum-equiseti*	44	Yes	TEF and RPB2	yes	Thailand	[38]
F. solani and *F. oxysporum*	89	no	TEF and RPB2	no	Colombia	[50]
F. fujikuroi and *F. solani*	46	Yes	TEF and RPB2	no	India	[43]
F. oxysporum	1	Yes	no	no	India	[4]

Table 3. Antifungal susceptibility of *Fusarium.* spp. causing onychomycosis MIC (µg/mL)

EFC	FLC	GRI	KTC	ITC	PSC	VCR	TRB	Reference
	>256					0.25		[57]
			0.5–16	1–>16		0.125–1	0.25–8	[8]
				>16		2–16	2 ->64	[63]
	4–64			0.03–16		0.007–8	0.03–16	[64]
				4–16		2–8		[65]
0.5–2				>4			1–4	[66]
	16 - >64	16 - >64	4 - >16	4 - >16		1 - >16	4 - >16	[9]
				2->16	0.125–>16	0.5–>16		[67]
	>64			>16	4–>16	2–>16		[50]
		32->128		0.5–>64		0.125–>32	16->64	[3]

*EFC Efinaconazole, FLC Fluconazole, GRI, griseofulvin, ITC itraconazole, KTC ketoconazole, PSC posaconazole, TBF terbinafine, VRC voriconazole

Table 4. Summary of recommended treatments for *Fusarium* onychomycosis

Agent	Dose	Duration
Amorolfine	apply once or twice a week	Remove lacquer before each new application. Toenails: 9–12 months. Fingernails: 6 months
Ciclopirox	apply once per day apply	Remove lacquer once per week. Treat for up to 48 weeks
Fluconazole	300–450 mg or mg 150–300	Toenails: once/week for 9–12 months or Fingernails: once/week for 4–6 months
Itraconazole	200 mg or pulse therapy	Toenails: once per day for 12 weeks or 200 mg twice per day for 1 week/no treatment for 3 weeks. Repeat for 3–4 months
Terbinafine	250 mg 200	Toenails: once per day for 12 weeks

Artigo 2: Genetic diversity e antifungal susceptibility of *Fusarium* isolates in onychomycosis (*Mycoses;* FI=2,9)

Genetic diversity and antifungal susceptibility of *Fusarium*isolates in onychomycosis

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Summary

Fusarium species have emerged as an important human pathogen in skin disease, onychomycosis, keratitis and invasive disease. Onychomycosis caused by *Fusarium* spp. The infection has been increasingly described in the immunocompetent and immunosuppressed hosts. Considering onychomycosis is a difficult to treat infection, and little is known about the genetic variability and susceptibility pattern of *Fusarium* spp., further studies are necessary to understand the pathogenesis and better to define the appropriate antifungal treatment for this infection. Accordingly, the objective of this study was to describe the in vitro susceptibility to different antifungal agents and the genetic diversity of 35 *Fusarium* isolated from patients with onychomycosis. *Fusarium* spp. were isolated predominantly from female Caucasians, and the most frequent anatomical location was the nail of the hallux. Results revealed that 25 (71.4%) of isolates belonged to the *Fusarium solani* species complex, followed by 10 (28.5%) isolates from the *Fusarium oxysporum* species complex. Noteworthy, the authors report the first case of *Neocosmospora rubicola* isolated from a patient with onychomycosis. Amphotericin B was the most effective antifungal agent against the majority of isolates (60%, MIC ≤4 μg/mL), followed by voriconazole (34.2%, MIC ≤4 μg/mL). In general, *Fusarium* species presented MIC values >64 μg/mL for fluconazole, itraconazole and terbinafine. Accurate pathogen identification, characterisation and susceptibility testing provide a better understanding of pathogenesis of *Fusarium* in onychomycosis.

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ORIGINAL ARTICLE

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Genetic diversity and antifungal susceptibility of *Fusarium* isolates in onychomycosis

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Summary

Fusarium species have emerged as an important human pathogen in skin disease, onychomycosis, keratitis and invasive disease. Onychomycosis caused by *Fusarium* spp. The infection has been increasingly described in the immunocompetent and immunosuppressed hosts. Considering onychomycosis is a difficult to treat infection, and little is known about the genetic variability and susceptibility pattern of *Fusarium* spp., further studies are necessary to understand the pathogenesis and better to define the appropriate antifungal treatment for this infection. Accordingly, the objective of this study was to describe the in vitro susceptibility to different antifungal agents and the genetic diversity of 35 *Fusarium* isolated from patients with onychomycosis. *Fusarium* spp. were isolated predominantly from female Caucasians, and the most frequent anatomical location was the nail of the hallux. Results revealed that 25 (71.4%) of isolates belonged to the *Fusarium solani* species complex, followed by 10 (28.5%) isolates from the *Fusarium oxysporum* species complex. Noteworthy, the authors report the first case of *Neocosmospora rubicola* isolated from a patient with onychomycosis. Amphotericin B was the most effective antifungal agent against the majority of isolates (60%, MIC ≤4 μg/mL), followed by voriconazole (34.2%, MIC ≤4 μg/mL). In general, *Fusarium* species presented MIC values >64 μg/mL for fluconazole, itraconazole and terbinafine. Accurate pathogen identification, characterisation and susceptibility testing provide a better understanding of pathogenesis of *Fusarium* in onychomycosis.

KEYWORDS

Fusarium, molecular phylogenetics, multidrug resistant, *Neocosmospora rubicola*, onychomycosis, species complex

1 | INTRODUCTION

Fusarium species have emerged as an important pathogen in immunocompromised and immunocompetent patients and usually acquired with trauma or through inhalation of airborne conidia. In immunocompromised patients, *Fusarium* infections affect haematopoietic stem cell recipients and manifest as severe disseminated infection, with mortality rates of up to 75%,[1,2] largely due to delay in diagnosis, a difficulty of treatment and resistance of *Fusarium* spp. to most available antifungal agents.[3,4] Fusariosis presents mostly as superficial and localised infections, such as skin infections, keratitis and onychomycosis, in the immunocompetent.[5–8]

Onychomycoses due to *Fusarium* spp. almost always involve the great toenails, especially those affected by traumatic and dystrophic abnormalities, and/or nails already infected by dermatophytes.[9] In an epidemiological study in Brazil, 17 (7.5%) of 560 cases of

onychomycosis caused by *Fusarium* spp. were confirmed during 1 year period, making it the most isolated genus among non-dermatophyte filamentous fungi (NDFF). Nine *Fusarium* species were isolated, and *F. oxysporum* (55.6%) was the most frequent species isolated. None of the patients diagnosed with onychomycosis caused by *Fusarium* spp. were immunocompromised.[6]

Considering that in general onychomycosis are infections difficult to treat effectively and little is known about the genetic variability and susceptibility pattern of *Fusarium* spp., further studies are necessary to understand the pathogenesis and better to define the appropriate antifungal treatment for onychomycosis caused by *Fusarium* spp. In fact, *Fusarium* species can exhibit variable susceptibility patterns,[7,8] and therefore it is important to follow an appropriate susceptibility profile definition and identification at the species level.[9–10] Internal transcribed spacer (ITS) region of the nuclear ribosomal DNA has been used to identify the species level, since it was selected as the official 'barcode' locus for the Fungi.[11] Several studies have shown that ITS1 and ITS2 are useful targets for detection and identification *Fusarium* species.[12–14] The objective of this study was to describe the in vitro susceptibility to different antifungal agents and the genetic diversity of 55 *Fusarium* isolated from patients with onychomycosis.

2 | METHODS

2.1 | Fungal isolates

Isolates from the nails of the hand and foot were obtained from patients with onychomycosis presenting at dermatologist consulting service between July and September 2015 in a tertiary care hospital in southern Brazil. The diagnosis was made based on repeated direct microscopic examination of a nail sample with potassium hydroxide (KOH 20%) revealing hyaline, septate, branched hyphae and fungal culture positive for *Fusarium* spp. (Sabouraud dextrose agar). The strains were maintained in monthly subculture on Sabouraud dextrose agar (Himedia®, Mumbai, India). Potato dextrose agar (Liofilchem®, Roseto degli Abruzzi, Italy) was used for phenotypic identification and antifungal susceptibility testing.[17]

2.2 | Antifungal susceptibility

Conidia were obtained by gently probing colonies with a sterile Pasteur pipette tip to dislodge them from the hyphal mat. They were then suspended in a 0.85% NaCl solution, counted in a Neubauer chamber and adjusted to a concentration of 5×10^4 conidia/mL, according to the Clinical and Laboratory Standards Institute broth microdilution method for filamentous fungi (M38-A2).[18] Subsequently, conidia were resuspended in RPMI Medium 1640 (Gibco®, Paisley, UK) with L-glutamine (without sodium bicarbonate) and buffered with 0.165 mol/L 3-morpholinopropanesulfonic acid (MOPS; Sigma-Aldrich, St. Louis, MO, USA) at pH 7.0. The final suspension was distributed onto microdilution plates containing predefined incremental concentrations (64 μg/mL) of amphotericin B (AMB; Sigma-Aldrich), voriconazole (VRC; Sigma-Aldrich) and terbinafine (TRB; Sigma-Aldrich), and concentrations of 256 μg/mL for itraconazole (ITC; Sigma-Aldrich) and fluconazole (FLC; Sigma-Aldrich). The plates were incubated at 35°C for 48 h. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that caused 100% of inhibition of visible fungal growth at 48 h. The tests were performed in duplicate. The American Type Culture Collection (ATCC) strains *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control.

2.3 | Statistical analysis

Data analysis was performed with SPSS statistical package release 11.01. Groups were compared with chi-squared or adjusted residual test for categorical variables and the Mann-Whitney test for non-parametric continuous variables. Chi-squared tests were used to test for differences in molecular identification and phenotypic identification. The Mann-Whitney test was used to evaluate if there was any difference in MIC values against the different antifungal agents tested in comparison between the FSSC and FOSC complexes. A significance level of <0.05 was used to test all hypotheses.

2.4 | Molecular phylogenetics

Isolates were inoculated in GYP broth incubated at 25°C for 72 h (Himedia®) for DNA extraction. One millilitre of the medium with mycelia was collected by centrifuged at 15 000 *g* for 5 min. The pellet was suspended in 1 mL of distilled water and centrifuged under the same conditions and genomic DNA was extracted with a commercially available kit (PureLink™ Genomic DNA kit, Invitrogen Life Sciences, Carlsbad, CA, USA). The internal transcribed spacer (ITS) 1-5.8S to ITS2 region was amplified using ITS primers ITS1 and ITS4 under the following conditions: one initial (ie denaturation) cycle at 94°C for 5 min; followed by 35 cycles at 94°C for 30, 45 s at 56°C and 2 min at 72°C, with one final cycle at 72°C for 5 min.[19] Amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and were sequenced using a genetic analyzer (ABI 3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. To identify *Fusarium* isolates obtained from patients with onychomycosis, at least to the species complex level, a phylogeny was inferred by DNA sequence analyses based on datasets of ribosomal ITS. Based on that loci, DNA sequence databases were constructed then compared via BLAST with sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and pairwise sequence alignment with the *Fusarium* MLST database (http://www.cbs.knaw.nl/Fusarium/).

DNA sequences were aligned using the ClustalW function and manually edited in MEGA 6.0 for Windows (Microsoft Corporation, USA).[20] A phylogenetic reconstruction was determined using the UPGMA cluster analysis method with a bootstrap of 1000 replicates. The cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic averages (UPGMA).

Phylogenetic trees were drawn to scale, with branch length representing the number of changes over the entire sequence. Clades were labelled with the statistical support of 70 or higher. The sequences of

Trichoderma reesei (GenBank, ITS JN704548) were used as the outgroup reference.

3 | RESULTS

All patients were previously healthy adults and presented with distal subungual dystrophic or acute paronychia associated with proximal or total leuconychia of the affected nails. There were 54 (97.14%) females and1 (2.86%) male enrolled in the study. Age ranged from 22 to 74 years. The toenails, especially the left hallux (51.45%), were more frequently affected (97.14%) than the fingernails (Table 1).

A residual is a difference between the observed and expected values for a cell. The larger the residual, the greater the contribution of the cell to the magnitude of the resulting chi-squared obtained value (≥2.0). Adjusted residual of phenotypic identification with *F. oxysporum* was of 2.0 by CP/MY and of 4.1 by WC/MY. *F. solani* was of 5.0 with SC/MB. *F. keratoplasticum* was of 2.8 with WC/MH. *Neocosmospora rubicola* was of 5.9 with GC/MY. A *P*-value of .05, considered statistically significant.

The clinical appearance of the nail of the hallux included a yellowish white discoloration, onycholysis and roughness of the distal and lateral nail plates, which was the typical clinical manifestation of onychomycosis caused by *F. oxysporum* (HCF11) (Figure 1).

As shown in Table 2, *Fusarium solani* species complex (FSSC) was the most common group of *Fusarium* found in this study (71.45% of isolates), and was divided into the following species: *F. keratoplasticum*, *F. solani*, *F. petroliphilum*, *F. falciforme* and *Neocosmospora rubicola*. Ten isolates were identified as FOSC (28.57% of isolates). The results

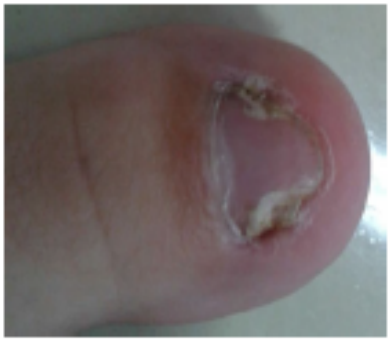

FIGURE 1 Hallux-right with yellowish white discoloration, onycholysis and roughness of the distal and lateral nail plates.

TABLE 1 Clinical and epidemiological characteristics of 55 patients infected by *Fusarium solani* species complex (FSSC) and *Fusarium oxysporum* species complex (FOSC)

	Species complex					
	FSSC					FOSC
	F. keratoplasticum	*F. solani*	*F. petroliphilum*	*F. falciforme*	*Neocosmospora rubicola*	*F. oxysporum*
Patients/Isolates	17	5	2	2	1	10
Female	17	5	2	1	1	10
Male				1		
Age, years, median	47.55	56.55	57.00	57.00	50.00	55.00
Caucasian	17	5	2	2	1	9
Black						1
Phenotypic identification[a]						
WC/MH	10	1	2	1		1
SC/MB	7	1		1		
GC/MY					1	
WC/MY		1				6
CP/MY						2
PC/MY						1
Site of isolation						
Halluxleft	8	2		1	1	6
Halluxright	6		1			1
Hallux	5					
Toenail	5	1		1		5
Fingernails			1			

[a]WC/MY white colony and medium transparent yellow; SC/MB suede colony and medium brown, WC/MH white colony and medium honey; CP/MY pink colony and medium transparent yellow; PC/MY purple colony and medium transparent yellow; WC/MY white colony and medium transparent yellow.

TABLE 2 Minimum inhibitory concentrations (MICs) of antifungal agents for *Fusarium solani* species complex and *Fusarium oxysporum* species complex

Species (isolates, n)	MIC (μg/mL)											
	Amphotericin B			Voriconazole			Itraconazole			Fluconazole			Terbinafine		
	Range	GM	Mode	Range	GM	Mode	Range	GM	Mode	Range	GM	Mode	Range	GM	Mode
FSSC															
Fusarium keratoplasticum (17)	1->16	4.88	4	0.5->32	20.09	>32	>64	64.00	>64	32->128	121.14	>128	>64	64.00	>64
Fusarium solani (10)	0.125-16	0.71	4	0.5->32	8.17	>16	1->64	43.00	>64	32->128	112.00	>128	>64	64.00	>64
Fusarium petroliphilum (2)	1-8	4.90		8-16	12.00		1->64	32.50		>128	128.00	>128	>64	64.00	>64
Fusarium falciforme (2)	4-8	6.00		0.5-16	8.25		>64	64.00	>64	>128	128.00	>128	>64	64.00	>64
Neocosmospora rubicola (1)	4			8			>64.00			>128			>64		
FOSC															
Fusarium oxysporum (10)	0.5-16	8.25	8	0.125-32	5.86	4	0.5->64	51.35	>64	64->128	121.60	>128	16->64	52.80	>64
Total (35)	0.125-16	5.90	4	0.125->32	14.23	>32	0.5->64	54.79	>64	32->128	117.03	>128	16->64	53.49	>64

compared via BLAST with sequences in GenBank, and *Fusarium* MLST database searches were confirmed by phylogenetic analysis. One isolate (HCF45), which was identified previously as an isolate of *Fusarium oxysporum* species complex (FOSC) by MLST, in fact, belongs to FSSC, which was confirmed using BLAST, with 99% query cover and identity with type strain *N. rubicola* CBS 101018.

Two distinct isolate groups were identified following UPGMA analysis. The first group included most of the FSSC isolates, and the second group included all of the isolates of FOSC. The distinctness of all two groups was supported by bootstrap values of 92%–99% in 1000 replicates (Figure 2). UPGMA similarity between the main group of *F. oxysporum* KC577176 and HCF36, HCF37, HCF46, HCF47, HCF48, HCF49 was high (86%). Considering the under two clusters more external of *F. oxysporum* isolates that included HCF 11 and HCF 22, there was 99% and 92% of similarity respectively.

As shown in Table 2, in vitro susceptibility testing of *Fusarium* spp. to antifungal drugs tested presented, in general, high MICs values. AMB was the most effective antifungal agent against the majority of the isolates (60%, MIC ≤4 μg/mL), followed by VRC (54.2%, MIC ≤4 μg/mL). In general, *Fusarium* isolates had MIC values >64 μg/mL for FLC, ITC and TRB. The VRC mode for *F. oxysporum* SC (five species/10 FSSC=4 μg/mL) was 1 dilution less than *F. solani* SC (six species/25 FSSC=8 μg/mL), whereas the VRC mode was >32 for six species of 14 *F. keratoplasticum* and two species of six *F. solani* (data not shown in Table 2). Statistical analysis revealed FOSC complexes are significantly more sensitive than FSSC to voriconazole ($P=.012$) by the non-parametric Mann-Whitney test.

4 | DISCUSSION

Some studies have reported that up to 10% of onychomycosis are caused by *Fusarium* spp. when aetiological agents are typed to the species level.[21] The prevalence of onychomycosis caused by *Fusarium* spp. in North America is 15.8%, and varies from 2% to 18% globally.[22] In Colombia, its prevalence (estimated to be 15% to 50%) is significantly higher than in North America.[23] FOSC is the most prevalent group of *Fusarium* (range 65% to 86%), followed by species of FSSC (31% to 68% of cases) in Europe. In contrast, species of FSSC form the most prevalent group of *Fusarium* (65% to 76%) followed by FOSC (24% to 68%) in the Americas and Asia,[24] which is consistent with our data (71.45% FSSC and 28.57% FOSC). In a study from Thailand by van Diepeningen et al., [24] *F. falciforme* and *F. keratoplasticum* were the most common *Fusarium* spp. isolated from patients with onychomycosis. A recent study from Colombia by Guevara-Suarez et al. [25] identified five phylogenetic species of FSSC (*F. falciforme*, *F. keratoplasticum*, *F. lichenicola*, *F. petroliphilum*) and FOSC.

In our study, the predominant species were *F. keratoplasticum* and *F. oxysporum*. Similarly, in a study from Brazil De Araújo et al. [25] and Godoy et al. [1] found *F. solani* and *F. oxysporum* to be the most prevalent *Fusarium* spp. in patients with onychomycosis. In another study from northeast Brazil, Lima et al. [26] reported a predominance of *F. solani* in patients with onychomycosis. In addition, Guilhermetti

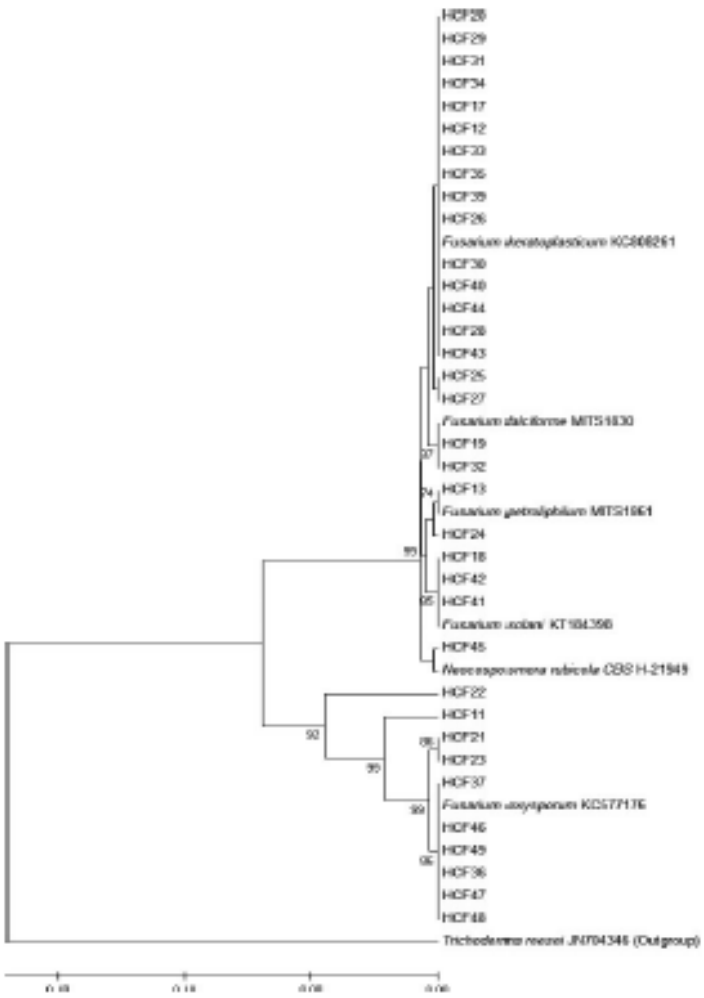

FIGURE 2 Phylogenetic tree of the subset of isolates included in this study obtained using UPGMA cluster analysis and based on internal transcribed spacer sequences.

et al. [6] from southern Brazil reported that *Fusarium* spp. accounted for 7.5% (n=27) of isolates from patients with onychomycosis. Nine species of this genus were identified, with *F. oxysporum* the most frequent *Fusarium* spp. isolated (55.5%). However, the identification of *Fusarium* in the majority of these studies was based on microscopic and macroscopic morphological features instead of molecular analysis.

Considering specific location, the nail of the hallux and the fifth finger are more likely to develop a fungus. This may be partly due to the fact that they constantly are more frequently exposed to trauma exposure.[27] In fact, NDFF was frequently associated with the infection of traumatized nails in aged patients.[28] *N. rubicola* was described as a new species in the genus *Neocosmospora* from the plant host *Rubus*idaeus. Sequence comparisons on the FUSARIUM-ID (http://isolate.fusariumdb.org) and *Fusarium* MLST (http://www.cbs.knaw.nl/fusarium) databases were inconclusive, identifying both isolates as part of the *F. solani* complex.[29] Interestingly, isolate HCF 45 was grouped with the strain *N. rubicola* CBS H21949 of BLAST nucleotide base, which had never been reported as a clinical isolate.

In a review of 259 published cases of invasive fusariosis, onychomycosis as the primary lesion before dissemination. Therefore, it is necessary to monitor the evolution of patients with immunodeficiency and onychomycosis caused by *Fusarium* spp.[30] NDFF are difficult to eradicate by using and combining different systemic antifungal treatments. *Fusarium* onychomycosis was eradicated in only 40% of adult patients in a study performed in Italy.[31] In this aspect, toenails are most affected (61.8%) and involvement of the nail is less severe in immunocompetent hosts with long evolution without complications.[32] Therefore, a thorough diagnostic work-up for onychomycosis by *Fusarium* spp. is essential for more specific and appropriate treatment.

According to the CLSI guidelines for Epidemiological Cutoff Value (ECV) setting, ECVs encompassing >97.5% of pooled statistically modelled MIC distributions were as follows: for amphotericin B, 8 μg/mL to *F. oxysporum* SC and *F. solani* SC; for voriconazole, 16 μg/mL to *F. oxysporum* SC and 32 μg/mL to *F. solani* SC; and for itraconazole, 32 μg/mL to *F. oxysporum* SC and *F. solani* SC.[10] In general, MIC values of 8 μg/mL for AMB followed the proposed ECV values,[10] and were comparable in 10 of 35 isolates of *Fusarium* spp. (7 of 25 FSSC and 3 of 10 FOSC isolates) (Table 2). Overall, our findings on in vitro activities of the main oral triazole antifungal agents demonstrated high MIC for *Fusarium* species causing onychomycosis, which are in agreement with previous studies where antifungal agents including FLC, ITC diminished activity against different *Fusarium* species.[8,22,23] In our study, VRC was the most effective agents against Fusarium species. According to the study Gupta et al, the antifungals ITC, FLC, flucytosine, caspofungin and anidulafungin were not effective in any of the tested concentrations.[22] AMB showed the highest in vitro activity (ranging 0.5-2 μg/mL) against all species. The triazole VRC had greater in vitro activity (MIC ranging 1-8 μg/mL). We demonstrated that TRB showed poor activity against *Fusarium* species causing onychomycosis. Previous studies have shown a significant variation of the pattern of TRB susceptibility against *Fusarium* where 25% of the isolates showed MICs equal to 2.0 μg/mL, 25% had MICs of 4.0 μg/mL and 50% had MICs greater than or equal to 16 μg/mL.[24] In another study, the highest MICs of most of the antifungal agents including TRB were those against *Fusarium* spp., except for griseofulvin, whose MICs against all the others NDFF were >64 μg/mL.[25] Indeed, patients with *Fusarium* onychomycosis that were not responsive to systemic terbinafine were successfully treated by topical amphotericin B.[26,27]

In summary, treatment of fungal nail infections accounts for significant health care resource use and expenditures. Some authors consider this particular superficial fungal infection to be the most difficult to treat and, even in cases in which the antifungal agent is appropriate for the identified aetiological agent, the cure is not always achieved and recurrences are frequent.[28] Considering onychomycosis, *Fusarium* is usually was resistant to the older azoles (eg ITC and FLC), and has variable resistance to VCR and AMB.[9] In our study, TRB, ITC and FLC showed very poor in vitro activity against 25 phylogenetically diverse isolates of FSSC and 10 isolates of FOSC. VRC had a tendency to be more effective for *F. oxysporum*, given that it inhibited 80% of the isolates and more than one-half of these isolates had an MIC ≤8 μg/mL.AMB was the most effective drug for *Fusarium* spp. infection. In fact, AMB and VRC have been indicated for localized and disseminated fusariosis in a recent guideline.[9] Additional studies are necessary for investigating *Fusarium* spp. diversity and sensitivitivity to novel antifungal agents for the treatment of onychomycosis.

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DISCLOSURES

The authors have no financial disclosures or conflicts of interest to declare.

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Artigo 3: *Fusarium solani* multiresistant isolated from fungal keratitis

(*Acta Ophthalmologica*; FI=3,3)

Fusarium solani multiresistant isolated from fungal keratitis

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Abstract

A case report of fungal keratitis of a isolate multiresistant *Fusarium solani* from South of Brazil. A 55-year-old men with a history of trauma presented with keratitis in left eye. The patient has a complicated clinical course and failed to respond to local and systemic antifungal treatment, requiring eye enucleation. Despite multiple topical, intraocular and systemic antifungal treatments, hyphal infiltration repeatedly penetrated the corneal transplant causing continuous recurrences. The cultures of a corneal biopsy scraping were positive for *Fusarium* spp. The organism was identified to species level by multi-locus sequencing for translation elongation factor 1 alpha (EF-1α), and RNA polymerase II subunit (RPB2). Also, was tested for in vitro antifungal susceptibility by the broth microdilution method, according to CLSI M38-A2, which was susceptible to natamycin and demonstrated resistance to amphotericin B, voriconazole, itraconazole and fluconazole. Due to numerous resistances against antimycotic drugs the eye had to be enucleated to prevent the pathogens from spreading. Unfortunately, the management of fungal keratitis remains a therapeutic challenge the optimal treatment for *F. solani* infection has not yet been established.

Keywords: Keratitis, Corneal ulcer, MLST, *Fusarium solani*, Multiresistant

Introduction

Fusarium spp. is a filamentous fungus do rarely penetrate an intact epithelium and are often secondary to epithelial defects caused by trauma [1], mainly vegetable traumas, since it is common to find this fungus in soils and in different types of vegetables. Usually, the incidence of *Fusarium* keratitis peaks are during harvest seasons when farm workers at are more risk of corneal injury and exposure to airborne spores [2]. Studies show that 45%–67% of fungal keratitis in Brazil is caused by this microorganism [3].

Fungal keratitis appears as ulcerative lesions and is usually managed using topical antifungal medications, occasionally integrated with subconjunctival injections, although therapeutic keratoplasty may be necessary for patients whose corneal infection persists [4]. The species most commonly associated with human infections is specie *F. solani* (50%) [5] and, also are the most resistant the available antifungal drugs [6]. This fungal may completely destroy an eye in a few weeks since the infection is usually severe, and perforation, deep extension, and malignant glaucoma may supervene [7]. Another important point already mentioned above is most *Fusarium* exhibit broad resistance to the spectrum of antifungals currently available, including amphotericin B, azoles, echinocandins, and terbinafine, which typically show high MICs in vitro [8].

We describe the clinical course of a *Fusarium* keratitis, which failed to respond to systemic and local antimycotic treatment, and experienced a progression to a severe keratitis.

Case Report

Male, 55 years old, with penetrant corneal transplant (TX) for keratoconus on left eye (LE) 32 years ago. Consult for blunt trauma on LE 10 days ago, presenting corneal ulcer with melting, hypopyon and vitreal commitment visualized on ultrasound, in treatment with eye drops ketorolac 5 mg/mL e gatifloxacin 0.3%. The corneal button was subjected to microbiological and histopathological examination. Samples were inoculated onto Sabouraud dextrose agar (SDA) plates and incubated for 7 days at 25°C. Pale yellowish colony pigmentation, cottony, rapidly growth and aerial mycelium white

(Figure 1 A and B), which microscopically revealed septated hyphae, half-moon-shaped macroconidium, and microconidia (Figure 1 C). On the basis of culture and microscopy, the fungus was phenotypically identified as *Fusarium* spp. The morphological identification could be confirmed for multilocus sequence typing (MLST)[9]. This method is based on two-locus DNA sequence-based typing schemes, included portions of translation elongation factor 1α (EF-1α) and second-largest subunit of RNA polymerase (RPB2). Our isolate was identified as *Fusarium solani* species complex (FSSC). Also, antifungal susceptibility testing was performed by broth microdilution method, CLSI document M38-A2[10]. The fungal showed susceptible to natamycin with MIC value of 2 µg/ml, and reduced sensitivity to voriconazole and amphotericin B, with higher MIC values of 32 and 16 µg/ml, respectively. Resistance was demonstrated against itraconazole and fluconazole (> 64 µg/ml).

The patient was treated with intravenous amphotericin B and voriconazole,and intravitreal injection of voriconazole. Despite aggressive treatment, the infection persisted due to the high resistance of this organism to usual antifungal drugs; Therefore, it was decided to eviscerate the LE.

Discussion

Keratitis is the most common ocular manifestation in immunocompetent individuals and this may be complicated by irreversible visual deterioration[11]. Globally, the FSSC is the most common group encountered in human infections and is present in approximately 50 % of patients with fungal keratitis [12,13], the species *F. solani* is the most common (found in up to 91% of isolates) pathogenic to the eye [3]. This has been associated with a higher rate of therapeutic penetrating keratoplasty [14]. Most infections have been reported in farmers, and the infections are often preceded by trauma. These data support our finding, our patient was a grown man, a farmer who suffered trauma to his left eye and developed an aggressive fungal keratitis [15].

Given the generally resistant nature of *Fusarium* species to antifungal agents, a combination of compounds with moderate activity should be considered. Most antifungal drugs are fungistatic, and their poor penetration, when applied as topical medications, hampers the treatment of deep fungal keratitis [6].

Amphotericin B has been used by systemic, topical (eye drops), intracameral, intrastromal, and intravitreal routes in the treatment of fungal keratitis and endophthalmitis. For members of the FSSC, a high dose amphotericin B preparation is considered to be the treatment of choice and for other species, amphotericin B and/or voriconazole can be administered[16]. However, these antifungal options were not effective for our patient.

The antifungal activity test *in vitro* showed resistant to almost all antifungal agents available, except for natamycin. Unfortunately, access to this medication was not available to the patient, and when he was able to use it, it was too late. *F. solani* is usually resistant to azoles and exhibit higher amphotericin B MICs than other species of *Fusarium* [8].

Second-generation triazoles such as posaconazole appear to be promising for the treatment of fungal infections of the eye. Posaconazole was shown to penetrate the vitreous humor as well as the aqueous humor in a patient with a *F. solani* keratitis and endophthalmitis [15]. Unfortunately, *Fusarium* species, in general, show high degrees of resistance to most antifungals. Many larger studies have been done and consensus has not been reached on the best drugs to treat *Fusarium* keratitis. The topical drug of choice in cases of *Fusarium* keratitis is natamycin (also known as pimaricin) 5%, but delayed diagnosis may lead to an insufficient response because the penetration of natamycin through the corneal epithelium is poor [17].

MLST used molecular identification to identify important fungal genera and species, including *Fusarium*, is very important to predict therapeutic outcome and the emergence of these Fungi. Contribute greatly to the understanding of the epidemiology of infections and the evolution of pathogens. They are therefore important for monitoring infectious disease outbreaks. Our isolate was identified as *F. solani*. Unfortunately, the optimal treatment for *Fusarium* infection has not yet been established, and numerous cases of fungal keratitis worldwide end with the loss of the eye.

In conclusion, the management of fungal keratitis remains a therapeutic challenge and requires correct and prompt diagnosis as well as antifungal susceptibility testing. Furthermore, the monitoring of local epidemiological data is of importance in guiding the clinical follow up.

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Figures

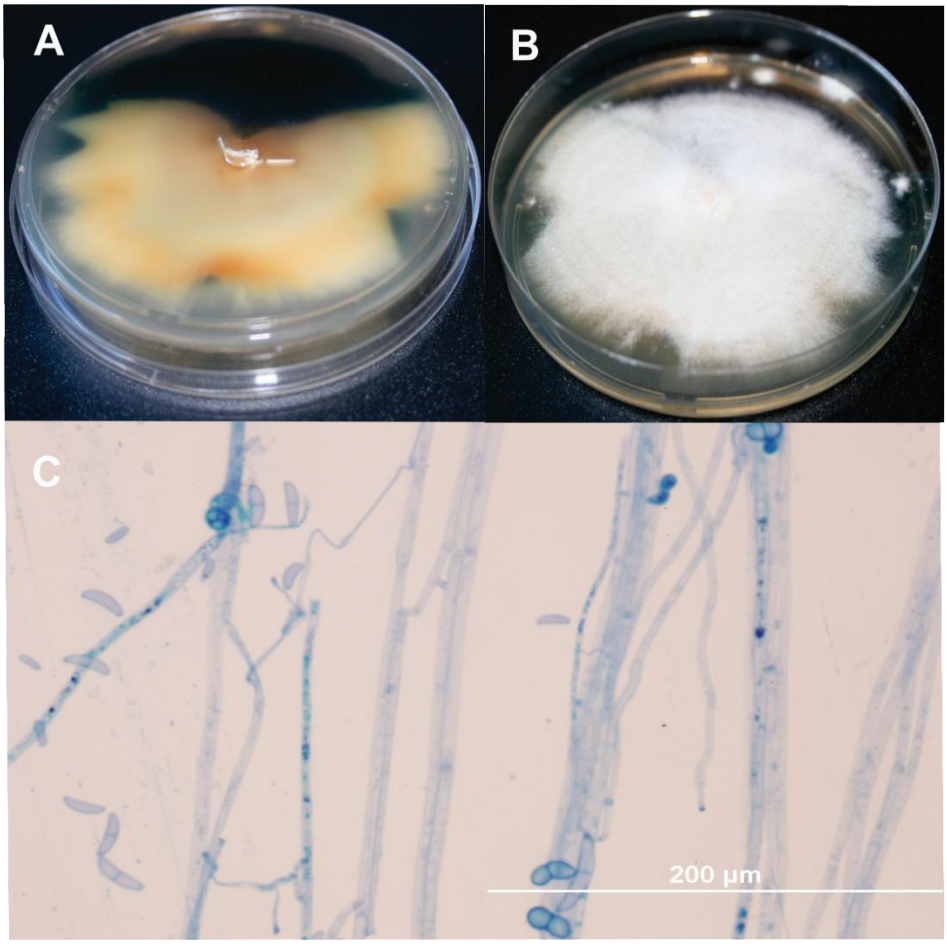

Fig. 1. Colony reverse (A) and surface of the colony (B) on SDA was with incubation at 25°C for 1 week. *F. solani* in microscopy (light micrograph, lactophenol cotton-blue stain) with abundant macroconidia and microconidia ellipsoidal (0-1-septate) observed by microcultivation from a 7-day old culture: conidiophores and conidia from sporodochial (C), x400

Artigo 4: In vitro susceptibility e multilocus sequence typing of *Fusarium* isolates causing keratitis (*Journal de Mycologie Médicale*; FI=1,6)

In vitro susceptibility and multilocus sequence typing of *Fusarium* isolates causing keratitis

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A B S T R A C T

Fungal keratitis is recognized as a significant cause of ocular morbidity and blindness especially in developing countries. In this study, we aimed to present the molecular identification and susceptibility of *Fusarium* isolates causing fungal keratitis in a university hospital in southern Brazil. The samples were identified using the second largest subunit of the RNA polymerase gene (*RPB2*) and the translation elongation factor 1-alpha (*TEF1*), while the antifungal susceptibility was tested by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) methodology. The majority of the isolates belonged to the Fusarium solani species complex (*F. solani, F. keratoplasticum* and *F. falciforme*) and *Fusarium oxysporum* species complex. Antifungal susceptibility has shown that amphotericin B and natamycin were the most effective antifungals across all isolates, followed by voriconazole. Variation among Fusarium complexes in their antifungal sensitivities was observed in our study. The identification of *Fusarium* species from human samples is important not only from an epidemiological viewpoint, but also for choosing the appropriate antifungal agent for difficult-to-treat Fusarium infections such as keratitis.

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In vitro susceptibility and multilocus sequence typing of *Fusarium* isolates causing keratitis

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ABSTRACT

Fungal keratitis is recognized as a significant cause of ocular morbidity and blindness especially in developing countries. In this study, we aimed to present the molecular identification and susceptibility of *Fusarium* isolates causing fungal keratitis in a university hospital in southern Brazil. The samples were identified using the second largest subunit of the RNA polymerase gene (*RPB2*) and the translation elongation factor 1-alpha (TEF1), while the antifungal susceptibility was tested by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) methodology. The majority of the isolates belonged to the *Fusarium solani* species complex (*F. solani*, *F. keratoplasticum* and *F. falciforme*) and *Fusarium oxysporum* species complex. Antifungal susceptibility has shown that amphotericin B and natamycin were the most effective antifungals across all isolates, followed by voriconazole. Variation among *Fusarium* complexes in their antifungal sensitivities was observed in our study. The identification of *Fusarium* species from human samples is important not only from an epidemiological viewpoint, but also for choosing the appropriate antifungal agent for difficult-to-treat *Fusarium* infections such as keratitis.

1. Introduction

Fungal keratitis is a very common cause of corneal infection in developing countries, and recognized as a significant cause of ocular morbidity and blindness [1]. Keratitis caused by *Fusarium solani* and *F. oxysporum* species complexes (*FSSC* and *FOSC*, respectively) is among the most refractory and common causes of fungal keratitis, and its incidence is increasing in many areas of the world, including the United States and Northern Europe [2,3]. This type of infection is more common in tropical or subtropical areas, especially in countries such as India, China, and Brazil [4–6]. *Fusarium* species are prevalent, accounting for 36%–67% of all cases of fungal keratitis in tropical areas following traumatic eye infection by vegetable matter contaminated by fungi among agricultural workers [7,8]. Whereas, the use of contact lenses is an increasing cause of fungal keratitis by *Fusarium* in developed countries in temperate climates.

Currently, fungal keratitis treatment is largely empirical, with no consensus on the role of susceptibility testing in guiding treatment decisions [9]. [10]. For instance, natamycin is the drug of choice for therapy of fungal keratitis in many countries particularly for keratitis due to filamentous fungi. Although treatment protocols for keratitis have changed over the last decade, with a decrease in the use of amphotericin B and an increase in natamycin and voriconazole therapy, it is necessary to establish a treatment protocol in severe cases of fungal keratitis by *Fusarium* [7].

The main goal of the present study was to phenotypically and molecularly characterize a set of *Fusarium* species isolated from patients with keratitis attending a Brazilian university hospital in order to ensure the appropriate management of this particularly infection. In addition, this study aimed to investigate the epidemiology and clinical characteristics of *Fusarium* keratitis of patients living in a subtropical climate region.

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2. Materials and methods

2.1. Fungal isolates and patients

Ten different isolates of fungi were collected from 10 patients with suspected clinical diagnosis of fungal keratitis attending a tertiary care hospital in southern Brazil between July 2008 and September 2017. The diagnosis was made based on repeated direct microscopic examination of a corneal scraping sample with potassium hydroxide (KOH 20%) revealing hyaline hyphae and fungal culture positive for *Fusarium* spp. (Sabouraud dextrose agar). The isolated were maintained in monthly subculture on Sabouraud dextrose agar (Himedia®, Mumbai, India). Potato dextrose agar (Uoffichem®, Roseto degli Abruzzi, Italy) was used for phenotypic identification and antifungal susceptibility testing. The details of the patients including age, sex, and relevant history were recorded.

2.2. DNA extraction and MLST

Genomic DNA of the isolates was extracted from cultures by using Pure link®Genomic DNA Mini kit (USA) followed by DNA quantification in NanoDrop 2000 (Thermo Scientific, Wilmington, USA).

A multilocus sequence typing was performed in order to classify strains in the clade. The procedure was used according to the *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium/). The portion of the *translation elongation factor 1-alpha* (*TEF1*) gene and a second largest subunit of RNA polymerase (RPB2) were amplified according to O'Donnell et al. [12]. PCR products were purified and sequenced using an ABI3730xl DNA analyzer and an ABI3500 Genetic analyzer (Applied Biosystems, USA). Raw sequences were assembled and manually edited. After, the consensus sequence was compared with reference sequences deposited in FUSARIUM MLST (http://www.cbs.knaw.nl/fusarium/) and via BLAST to sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.3. Antifungal susceptibility

The clinical isolates were evaluated for susceptibility to voriconazole (VRC; Sigma-Aldrich, USA), itraconazole (ITC; Sigma-Aldrich, USA), amphotericin B (AMB; Sigma-Aldrich, USA), natamycin (NTM; Sigma-Aldrich, USA), ketoconazole (KTZ; Sigma-Aldrich, USA) and fluconazole (FLC; Sigma-Aldrich, USA). Minimal inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method for filamentous fungi, M38-A2 [13]. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth as compared to the drug-free control well, and the tests were done in duplicate. The quality control (QC) isolates *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were included as positive growth controls, and MIC ranges were within established limits.

3. Results

We identified 10 patients with *Fusarium* keratitis, diagnosed from 2008 to 2017. Only one patient was immunocompromised, with acute lymphoid leukemia. The main characteristic features of our patients are described in Table 1. The most frequent predisposing factor was trauma (70%). The majority of the patients (60%) were treated with a single antifungal, 40% of them with combination of topical and systemic antifungal medications, and 30% of the patients underwent corneal transplantation (keratoplasty). Except for two patients, the majority of patients (80%) were successfully treated with active antifungal agents including amphotericin B, natamycin and voriconazole.

As shown in Table 2, the majority of the causative agents (n = 6) were found to belong to the FSSC, one isolate was *F. keratoplasticum*, two were *F. falciforme* and three were *F. solani*. The other etiological agents isolated were members of the FOSC, (n = 2), FIESC (n = 1), and FFSC, (n = 1).

Table 1
Characteristics of the patients and epidemiological profile of Fusarium species causing keratitis.

Isolate	Year	Morphological	Sex	Age	Race	Residence (city)	Profession	Treatment	Keratoplasty	Cure
IF2	2008	FSSC	M	38	W	Cerro Largo	Farmer	IC and IS VRC	Yes	Yes
IF9	2011	FSSC	M	59	B	Arroio Grande	Military	IC AMB	No	Yes
IF16	2012	FIESC	M	61	W	Tramandaí	Retired	Topical SR NTM and KTZ	Yes	Yes
IF20	2015	FSSC	M	70	W	Rosário do Sul	Farmer	IC AMB and KTZ	No	Yes
IF21	2015	FSSC	F	20	W	Santa Maria	Student	IC and IS VRC	No	Yes
IF24	2015	FOSC	M	41	W	Anta Gorda	Farmer	ITC/VRC (IV) and IS VRC	No	Yes
IF28	2015	FSSC	M	72	NI	Porto Alegre	Farmer	IC AMB and VRC	No	No
IF33	2017	FSSC	F	41	W	Santa Maria	Farmer	IC AMB	No	Yes
IF34	2017	FSSC	M	55	W	Santa Cruz do Sul	Farmer	IC and IS AMB and VRC	Yes	No
IF35	2017	FOSC	F	52	W	Rosa Sales	Farmer	Topical SR NTM	No	Yes

M: male; F: female; W: white; B: black; IC: intracorneal; IS: intrastromal; FSSC: *Fusarium solani* species complex; FOSC: *Fusarium oxysporum* species complex; FIESC: *Fusarium incarnatum-equiseti* species complex; VRC: voriconazole; AMB: amphotericin B; NTM: natamycin; KTZ: ketoconazole; ITC: itraconazole.

Table 2
Genotypes and in vitro susceptibilities (μg/mL) of the 10 isolates of Fusarium spp. tested in the study.

Identification			GenBank		MLST	In vitro susceptibility (MIC, μg/mL)					
Isolate	Morphological	Molecular	TEF1	RPB2	TEF1 + RPB2	AMB	NTM	VRC	FLC	KTZ	ITC
IF9	FSSC	*F. falciforme*	KM827951.1	KM828067.1	NRRL 32754; type: 3+4-ww	2	2	16	>128	>128	>128
IF20	FSSC	*F. falciforme*	KP020508.1	KM828050.1	NRRL 32786; type: 3+4-hh	2	4	8	>128	>128	>128
IF21	FSSC	*F. keratoplasticum*	KM827965.1	KJ653812.1	NRRL 22661, CBS H-12716	0,5	2	1	128	>128	>128
IF28	FSSC	*F. solani*	KY078212.1	EF469958.1	NRRL 22142; type: 1-b	1	1	16	>128	>128	>128
IF33	FSSC	*F. solani*	KM827973.1	KM828056.1	NRRL 22142; type: 1-b	4	4	32	128	>128	128
IF34	FSSC	*F. solani*	KF939495.1	KT313524.1	NRRL 32741; type: 5-n	16	4	32	>128	>128	>128
IF24	FOSC	*F. oxysporum*	KT258757.1	AB186568.1	NRRL 20403; type: 2	4	4	16	>128	>128	2
IF35	FOSC	*F. oxysporum*	KP966488.0.1	JX885464.1	NRRL 20403; type: 2	>16	4	4	>128	>128	>128
IF16	FIESC	*F. incarnatum*	KF601580.1	KU894469.1	NRRL 32599	1	2	2	>128	>128	>128
IF2	FSSC	*F. proliferatum*	KM816153.1	HM579027.1	NRRL 13592; type: none	4	8	1	>128	>128	>128

VRC: voriconazole; AMB: amphotericin B; NTM: natamycin; KTZ: ketoconazole; ITC: itraconazole; FLC: fluconazole.

All the isolates produced detectable growth after 48 h of incubation at 35 °C. The MIC of the quality control strain was within the reference ranges for each test. The MIC values of voriconazole, itraconazole, fluconazole, amphotericin B, natamycin, and ketoconazole against the isolates are summarized in Table 2. MIC for amphotericin B ranged from 0.5 to >16 μg/mL (GM, geometric mean = 3.83 μg/mL), and 50% of the isolates had high in vitro MIC values > 2 μg/mL. The MIC ranges for natamycin and voriconazole were 1–8 μg/mL (GM = 3.5 μg/mL) and 1–32 (GM = 12.8 μg/mL), respectively. In fact, 60% of the *Fusarium* isolates showed high MIC values for voriconazole and natamycin (> 2 μg/mL). *Fusarium* species in general showed higher MIC values for fluconazole, ketoconazole, and itraconazole (> 128 μg/mL).

4. Discussion

Among immunocompetent patients, tissue breakdown caused by trauma, contact lens, severe burns or foreign body, is the risk factor for fungal keratitis. Ocular manifestations including pain and irreversible visual deterioration, are usually diagnosed in men involved in soil-related work [14–19]. The most common filamentous fungi isolated in patients with fungal keratitis include *Aspergillus*, *Fusarium*, and *Curvularia* species [15].

Identification of *Fusarium* species from human samples is important not only from an epidemiological viewpoint, but also for choosing the appropriate antifungal agent for difficult-to- treat infections, such as fungal keratitis. MLST has previously been shown to be highly resolutive for epidemiological and population structure analysis of several fungi including *Fusarium* [20]. Specific regions of TEF1 and RPB2 have good discriminatory power and are often used in MLST schemes [21,22]. Worldwide, the FSSC is the most common group associated with human diseases and is present in approximately 50% of the patients with fusariosis [23]. Similar to previous studies in Brazil, and other countries, such as Argentina, Denmark, and India, our results showed that the FSSC is a major cause human including *F. keratoplasticum* and *F. falciforme* [3,4,6,18,19,24,25]. In fact, Oechsler et al. reported that 41 isolates in patients with *Fusarium* keratitis were genotypically classified by internal transcribed spacer region (ITS) as *F. solani* species complex (36 isolates, 88%), *F. oxysporum* species complex (two isolates, 5%), *Fusarium dimerum* species complex (one isolate, 2%) [6]. Two isolates did not group into any of the species complexes. In another study, the most frequent species identified by ERIC-PCR were *F. solani* (62 patients), followed by *F. verticillioides* (3 patients), *F. oxysporum* (2 patients), and *F. dimerum* (1 patient) [19].

Our preliminary data suggest that the predominant source of contamination of keratitis by *Fusarium* spp. is through direct contact with vegetables considering that 70% of the patients were farmers. We also noticed that a very large number of cases were caused by a great diversity of *Fusarium* species, but in particularly haplotype 1b of *F. solani* and the MLST type 2 haplotype of *F. oxysporum*. As expected, our data have shown that itraconazole, fluconazole, ketoconazole demonstrated little in vitro activity against *Fusarium*. All strains proved to be resistant to drugs that belong to azoles, a major class of antifungal agents. In fact, Córdoba et al. have shown that 29 *Fusarium* spp. isolates showed resistance to itraconazole [26]. In addition, previous studies demonstrated high MICs for itraconazole and/or fluconazole (> 64 μg/mL) [4,27–29]. In general, high MICs against *Fusarium* species have been also shown for ketoconazole in several studies [11,30,31]. As expected, our *Fusarium* isolates presented sensitivity for voriconazole with apparently some differences between FOSC and FSSC groups. Variation among *Fusarium* complexes in their antifungal sensitivities has been described in previous studies [27,32]. Nevertheless, resistance to voriconazole has been previously described for *Fusarium* sp., and attributed to the long-term azole therapy in patients with invasive fusariosis, in addition to cross-resistance to the agricultural azoles, and intrinsic resistance of this species [33].

Similar to our findings, amphotericin B and natamycin proved to be the most effective antifungal agents against the *Fusarium* spp. [4,6,24] Although natamycin exhibits relatively poor penetration through the corneal layers, its bioavailability is considered sufficient for antifungal activity and for effective treatment of *Fusarium* keratitis in vivo [15].

Unfortunately, clinical interpretative breakpoints for in vitro antimicrobial susceptible testing for *Fusarium* are not yet established [34]. When the only piece of data available is the MIC distribution, a breakpoint cannot be determined and an ECV is the only available tool that may provide some guidance for treatment. A multicentric study established epidemiological cutoff values (ECVs) for *Fusarium* spp., which we can predict the low sensitivity to the drug in relation to the high value of MIC, superior to the ECV [32]. The data encompassed >97.5% of pooled statistically modelled MIC distributions for *Fusarium* spp. amphotericin B, 8 μg/mL to *F. oxysporum* SC and *F. solani* SC; for voriconazole, 16 μg/mL to *F. oxysporum* SC and 32 μg/mL to *F. solani* SC; and for itraconazole, 32 μg/mL to *F. oxysporum* SC and *F. solani* SC [32]. We can observe that these ECV values are very high in relation to the MICs of our *Fusarium* isolates and other species of fungi.

In summary, *Fusarium* spp. is an important cause of keratitis. Variations within its species are associated with different susceptibility profiles to antifungal agents. FOSC, particularly *F. solani*, are the most common group causing human *Fusarium* keratitis. Natamycin, amphotericin B and voriconazole are the most active antifungal agents for topical and systemic treatment of *Fusarium* keratitis. Considering our results, we could not find a species-specific susceptibility pattern for the *Fusarium* species isolated from patients with keratitis. Treatment should be instituted promptly with topical and oral antifungal agents for *Fusarium* keratitis. Subconjunctival injections may be used in patients with severe keratitis. Patients who do not respond to medical treatment with topical and oral antifungal medications usually require surgical intervention, including corneal transplantation. Continuous monitoring of local epidemiological data is important in guiding treatment for this difficult-to-treat infection.

Disclosure of interest

The authors declare that they have no competing interest.

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Artigo 5: *Fusarium riograndense* sp. nov., a new species in the *Fusarium solani* species comples causing fungal rhinosinusitis (*Journal de Mycologie Médical*; FI=1,6).

Fusarium riograndense sp. nov., a new species in the _Fusarium solani_ species complex causing fungal rhinosinusitis

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S U M M A R Y

Invasive fusariosis has a high mortality and is predominantly observed in patients with leukemia. We report the first case of a novel species of *Fusarium*, *Fusarium riograndense* sp. nov, isolated from a lesion in the nasal cavity lesion of a patient with acute lymphoblastic leukemia. The etiological agent was identified by Multilocus Sequencing Typing (MLST), including *RPB2*, *TEF-1a*, and *ITS-LSU* sequences, the gold standard technique to identify new species of *Fusarium*. MLST and phenotypic data strongly supported its inclusion in the *F. solani* species complex (FSSC). The new species produced a red pigment in the Sabouraud Dextrose Agar similar to other members of the complex. The macroconiodia developed from phialides on multibranched conidiophores which merge to form effuse sporodochia with a basal foot-cell instead of papilla in basal cell shape. The microconidia were ellipsoidal, 0–1-septated, produced from long monophialides. Chlamydospores were produced singly or in pairs. Amphotericin B (MIC 1 mg/ mL) was the most active drug, followed by voriconazole (MIC 8 mg/mL). The patient was successfully treated with voriconazole. Our findings indicate another lineage within FSSC capable causing of invasive human infection.

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Fusarium riograndense sp. nov., a new species in the *Fusarium solani* species complex causing fungal rhinosinusitis

Fusarium riograndense sp. nov., *une nouvelle espèce dans le complexe d'espèces de* Fusarium solani *causant une rhinosinusite fongique*

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SUMMARY

Invasive fusariosis has a high mortality and is predominantly observed in patients with leukemia. We report the first case of a novel species of *Fusarium*, *Fusarium riograndense* sp. nov, isolated from a lesion in the nasal cavity lesion of a patient with acute lymphoblastic leukemia. The etiological agent was identified by Multilocus Sequencing Typing (MLST), including RPB2, TEF-1α, and ITS-LSU sequences, the gold standard technique to identify new species of *Fusarium*. MLST and phenotypic data strongly supported its inclusion in the *F. solani* species complex (FSSC). The new species produced a red pigment in the Sabouraud Dextrose Agar similar to other members of the complex. The macroconiodia developed from phialides on multibranched conidiophores which merge to form effuse sporodochia with a basal foot-cell instead of papilla in basal cell shape. The microconidia were ellipsoidal, 0–1-septated, produced from long monophialides. Chlamydospores were produced singly or in pairs. Amphotericin B (MIC 1 μg/mL) was the most active drug, followed by voriconazole (MIC 8 μg/mL). The patient was successfully treated with voriconazole. Our findings indicate another lineage within FSSC capable causing of invasive human infection.

Mots clés :
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RÉSUMÉ

La fusariose invasive a une mortalité élevée et est principalement observée chez les patients atteints de leucémie. Nous rapportons le premier cas d'une nouvelle espèce de *Fusarium*, *Fusarium riograndense sp. nov*, isolée de la lésion de la cavité nasale d'un patient atteint de leucémie lymphoblastique aiguë. L'agent étiologique a été identifié par le typage par séquençage multilocus (MLST), incluant les séquences RPB2, TEF-1α et ITS-LSU, la technique de référence pour identifier de nouvelles espèces de *Fusarium*. Le MLST et les données phénotypiques ont fortement soutenu son inclusion dans le complexe d'espèces *F. solani* (FSSC). La nouvelle espèce a produit un pigment rouge dans la gélose Sabouraud Dextrose similaire aux autres membres du complexe. Les macroconidies sont produites par des phialides portées par des conidiophores multi-branchés rassemblés en sporodochies. Elles sont falciformes, dorsiventrales, de stature robuste, 2-5-septées (surtout 5-septés), avec une cellule apicale en pointe arrondie et une cellule

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basale pédiculée. Les microconidies sont ellipsoïdes, 0-1-cloisonnées, produites à partir de longues monophialides. Les chlamydospores sont produites isolément ou par paires. Amphotéricine B (CIM 1 μg/mL) est suivie du voriconazole (CIM 8 μg/mL). Le patient a été traité avec succès avec du voriconazole. Nos résultats indiquent une autre lignée au sein de FSSC capable de provoquer une infection humaine invasive.

Introduction

The genus *Fusarium* includes more than 200 species of which 73 have been isolated from human infections [1]. The majoritary causes of most of the identified *Fusarium* pathogens in human invasive fusariosis belong to members of the *Fusarium solani* species complex (FSSC) [2]. *Fusarium* spp. causes a broad spectrum of infections in humans. Immunologically competent hosts show mainly localized skin infections, whereas disseminated fusariosis occurs almost exclusively in immunocompromised patients. Invasive fungal rhinosinusitis is an uncommon disease with high mortality rates in patients with hematological malignancies. There is currently no consensus on the best treatment timing [3]. Fungal rhinosinusitis can be categorized into non-invasive and invasive groups. While non-invasive fungal rhinosinusitis does not exhibit the penetration of mucosa by hyphae, in invasive fungal sinusitis hyphae do invade the mucosa. Acute invasive fungal rhinosinusitis (AIFRS) is considered the most aggressive form of fungal sinusitis [4,5].

The aim of our study is to describe a new species of FSSC causing invasive rhinosinusitis in a immunocompromised patient, featuring its morphological and molecular aspects and susceptibility profile against the main antifungal agents currently used to treat this type of infection.

Case report

A 11-year-old man child presented to our facility with a 2-week history of fever, epiphora and bilateral nasal obstruction with the presence of hyaline rhinorrhea in August 2014. The patient was diagnosed with acute lymphoblastic leukemia, precursor B-cell type in 2011. The patient completed induction chemotherapy with Hyper-CVAD protocol (cyclophosphamide, vincristine, doxorubicin, and dexamethasone), maintenance treatment with metotrexate, vincristine, and prednisone lasted until September 2013. On physical examination, a black-crusted lesion was observed in right nasal fossa. Blood tests on admission revealed neutropenia with a neutrophil count of 300/mm³. Biopsy of the nasal lesion revealed on fresh microscopic examination (KOH 10%) the presence of septate hyaline hyphae. The histopathologic examination using hematoxylin and eosin (HE) (Fig. 1) and Gomori's methenamine silver staining revealed presence of invasive hyphae of the nasal tissue. Paranasal sinus and chest Computed tomography (CT) were normal. The culture of the biopsy fragment was inoculated on Sabouraud dextrose agar (SDA; Oxoid®, UK) with chloramphenicol (50 μg/mL) (Sigma-Aldrich®, USA) in 9 cm plastic Petridishes for morphological identification. SDA plates were incubated at 25 °C and 35 °C for 1 week and were examined daily. Cultures showed whitish colonies, which became dirty brown. Microscopic examination (KOH 10%) have shown robust and sickle-shaped macroconidia, ellipsoid microconidia and chlamydospores. The preliminary morphological identification of this fungus was *Fusarium* sp. (HCF3). Patient was started treatment with intravenous voriconazole (20 mL, 10 mg/mL), IV of 12/12 h for 10 days, and replaced by voriconazole oral tablet 200 mg in 12/12 h for 12 weeks. The patient was successfully treated under this therapeutic regimen with complete regression of the lesion.

Materials and methods

Fungal isolate

Fungal isolate (HCF3) was deposited in the Collection of Microorganisms, DNA and Cells of Minas Gerais Federal University (A Coleção de Micro-organismos, DNA e Células da Universidade Federal de Minas Gerais, UFMG, http://www2.icb.ufmg.br/cmufmg/) under accession number UFMG-CM F12570, and at the mycology collection of Pernambuco Federal University (Micoteca URM da Universidade Federal de Pernambuco, UFPE, http://www.ufpe.br/micoteca) under accession number URM-7361. Isolates were grown on potato-dextrose agar (PDA; Liofilchem, Italy) carnation leaf agar (CLA), synthetic nutrient-poor agar (SNA) and on SDA with chloramphenicol (50 μg/mL) (Sigma-Aldrich, USA) at 25 °C in the dark by 7 to 10 days' growth [6]. The description of the morphological characteristic was according to with Aoki et al. [7]. All microcultures were incubated at 25 °C in dark environment for 10 days. Measurements of minimal and maximal sizes of conidia, chlamydospores, and phialides were performed, and characteristic morphological traits were photographed using a ZEISS PALM MicroBeam microscope, digital camera and PALMRobo 4.6 Pro software (Table 1).

DNA extraction and MLST

Genomic DNA was extracted by using Purelink™Genomic DNA Mini kit (USA) followed by DNA quantification in NanoDrop 2000 (Thermo Scientific, Wilmington, USA). A multi-locus sequence typing was performed in order to classify HCF3 in the FSSC clade. The procedure was used according to the *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium/). The Internal Transcribed Spacer (ITS) region and Large Subunit (LSU) rDNA, a portion of the translation elongation factor 1-alpha (TEF-1α) gene, and a second largest subunit of RNA polymerase (RPB2) were amplified according to O'Donnell et al. [8]. PCR products were purified and sequenced using an ABI3730xl DNA analyzer and an ABI3500

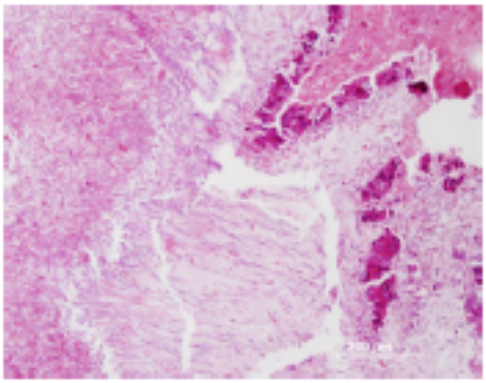

Fig. 1. Hematoxylin and Eosin (H&E) stained nasal tissue section showing an invasive mass of fungal hyphae (× 20, bar 200 μm).

Table 1
Macroscopic and microscopic characteristics of *Fusarium nigrandense* isolated from a nasal lesion of a patient with acute lymphoblastic leukemia.

Morphological characterization	FSSC			
	CLA	SNA	PDA	SDA
Macroscopic characteristics[a]	Soft mycelium, almost transparent, smooth	Sparsemyceliumgrown quite rapidly and smooth	Grown quite rapidly with a dense aerial myelium	Slower growth
Colony colour	Cream	White	White to cream	Pink-gray
Colony color reverse	Cream	White	Cream	Brown to dark
Pigmentation	No	No	No	Red
Growth rate (mm/day)	3.57 ± 1.54	9.36 ± 0.99	7.36 ± 1.75	0.5 ± 0.15
Growth total (mm)	35	80	67	40
Microscopic characteristics[b]				
Mean length of macroconidia	41.78 ± 5.89	37.93 ± 5.88	43.55 ± 4.14	
Mean width of macroconidia (µm)	4.29 ± 0.48	5.39 ± 0.88	6.01 ± 0.37	
Number of septa in macroconidia	2 to 5	2 to 5	2 to 5	
Mean length of microconidia (µm)	17.19 ± 3.56	15.47 ± 3.37	17.68 ± 3.29	
Mean width of microconidia (µm)	4.43 ± 0.62	4.06 ± 0.69	4.40 ± 0.79	
Number of septa in microconidia	0 to 1	0 to 1	0 to 1	

Mean values of length and width of 50 randomly picked macroconidia ± standard deviation.
[a] After 7 days of incubation at 25 °C.
[b] After 10 days of incubation at 25 °C.

Genetic analyzer (Applied Biosystems, USA). Raw sequences were assembled and manually edited. After, the consensus sequence was compared with reference sequences deposited in Fusarium-ID (http://isolate.fusariumdb.org/index.php), FUSARIUM MLST (http://www.cbs.knaw.nl/fusarium/) and via BLAST to sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The obtained sequences were deposited in the Genbank database under the accession numbers KT18636 (ITS), KX534001 (LSU), KX534002 (TEF-1α), and KX534003 (RPB2).

Phylogenetic analysis

Multiple sequences alignments of TEF-1α and RPB2 genes were performed and manually edited for 231 strain sequences. Phylogenetic analyses were conducted using the combined genes through Bayesian inference analysis (BI) (Beast v1.8.3 and featured Beauty v1.8.3 software). Each locus was aligned separately in MEGA version 7 using the CLUSTALW algorithm and analyzed separately before analyzing the combined two-loci. The best substitution model for all gene matrices was estimated by jmodelTest v2.1.3. Parameters were fixed in the BI using 50000000 Markov chain Monte Carlo (MCMC) generations and samples were recorded every 1000 generations. The TreeAnnotator v1.8.3 software was used to select the 50% majority-rule consensus tree and we discarded the first 25% of the samples. Posterior probability values (PP) were calculated and *P* values > 0.95 were considered significant. The final tree was plotted and edited in Figtree v1.4.2 software. *Rectifusarium robinianum* CBS 830.85 was used as out-group. Aligned datasets were deposited in TreeBASE (accession number: 20164).

Antifungal susceptibility

The clinical isolates were evaluated for susceptibility to voriconazole (VRC; Sigma-Aldrich, USA), itraconazole (ITC; Sigma-Aldrich, USA), amphotericin B (AMB; Sigma-Aldrich, USA), and fluconazole (FLC; Sigma-Aldrich, USA). Minimal inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method for filamentous fungi, M38-A2. [9]. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth as compared to the drug-free control well. The quality control (QC) isolates *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019), were included to monitor the general performance of the broth microdilution test.

Results

Molecular analysis

The four loci of the HCF3 isolate (ITS, LSU, TEF-1α and RPB2) were compared using BLAST with Fusarium-ID, FUSARIUM MLST and Genbank databanks, and the identity permitted identify the genus *Fusarium*, inside the *F. solani* complex (FSSC). The Genbank identity with individual sequences was low (97%). We repeated the BLAST of these four sequences using the "Fusarium MLST database" (www.cbs.knaw.nl/fusarium/BioloMICS.aspx) and showed 98.62% of identity with cured sequences of FSSC. These results suggested that HCF3 is a novel species within the *F. solani* species complex (Fig. 2). Multilocus DNA sequence data was used to assess the phylogenetic relationships and species limits of a comprehensive collection of clinical and environmental important isolates within the FSSC (Fig. 2). The aligned TEF-1α and RPB2 genes consisted of 724 bp, and 1838 bp characters, respectively, totalling 2562 bp of aligned DNA sequence per isolate. All sequences used for comparison were retrieved from *Fusarium* MLST or GenBank databases. A Multilocus Sequence Typing (MLST) analysis was used to confirm the independent BLAST comparisons and to find the genotype inside the FSSC. A phylogenetic tree was constructed using 231 Fusarium sequences from TEF-1α and RPB2 two-loci datasets and FUSARIUM MLST or Genbank databanks, and contained three highly supported clusters. The combined TEF-1α and RPB2 genes consisted of 2562 bp (724 bp, and 1838 bp characters, respectively) of aligned DNA sequence per isolate. The new phylogenetic species identified in this study, named as *F. nigrandense* (HCF3), was supported using Bayesian inference (BI) analysis, located at the base of the clade 3, closely related to four phytopathogenic strains of *Fusarium*: NRRL 22178, isolated from dicot tree in Venezuela; NRRL 22153 and 22098, *Fusarium* sp. f. sp. *Cucurbitae* (non-formal species names), isolated from cucurbit in California, USA; and NRRL 22570, isolated from *Piper nigrum* in Brazil (Fig. 2). The number of nucleotide substitutions varied in a range of 23-33 for TEF1-α gene, 35-66 for RPB2 gene, 10-35 for ITS region and 5-16 for LSU region, which suggests a variability greater than 1% for all the sequences analyzed in this clade (Table 2). The pairwise comparisons of nucleotides, in addition to the HCF3 with NRRL numbers, were examined for NRRL numbers to have a better view of the sequences divergences between the 5 isolates. Considering that NRRL numbers were placed in different phylogenetic species, this could be additional information to strengthen the description of a new species for HCF3.1 in this clade, the number

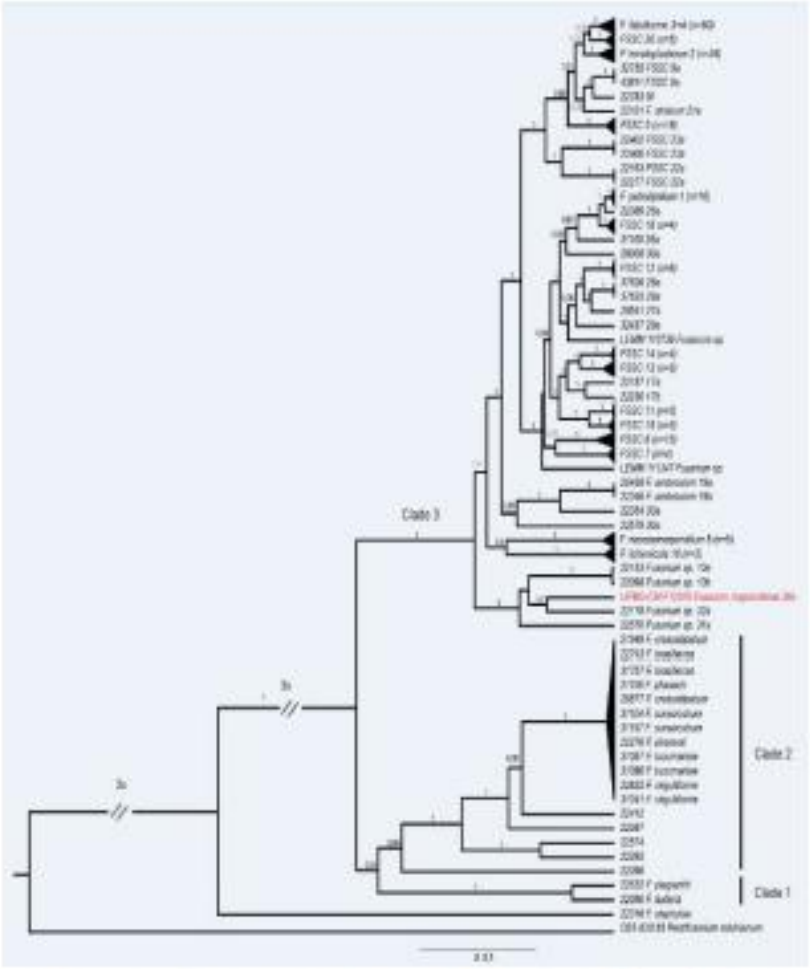

Fig. 2. Phylogenetic diversity within the *Fusarium solani* species complex (FSSC) using Bayesian inference (BI) analysis of the combined TEF-1α (724 bp), and RPB2 (1038 bp) sequence data (n = 231 sequences). *Neocosmospora rubriciannum* CBS 1030.85 was used as rooted out-group. Support values are above branches and represent posterior probabilities, where values >0.95 was considered significant. All sequences were retrieved from the *Fusarium* MLST database or GenBank database; FSSC codes indicate haplotype; the 5-digit number indicates NRRL strain numbers.

of nucleotide substitutions varied between 22 and 109 nucleotides, depending on the locus assessed for all five sequences. When we looked a pairwise comparison between HCF3 and other NRRL strains, the variability was 23–33 for TEF1α gene, 35–66 for RPB2 gene, 10–35 for ITS region and 5–16 for LSU region. These results demonstrated good variability (greater than 1%) for all the sequences analyzed in this clade and support the hypotheses that at least two strains, including HCF3, belong to different species.

Morphological analysis

Colonies on PDA showed an average radial mycelial growth of 7.4 ± 1.75 mm per day at 25 °C in the dark, and presented a diameter of 8 cm after 10 days of incubation. Aerial mycelia was cream and mostly floccose. The mycelium grew much sparser in the SNA and CLA media (Fig. 3). Microscopically, the microconidia were oval or elliptical, usually 0-septate, and aerial conidia hyaline had

Table 2
Pairwise comparisons of nucleotide variability between HCF3 and closely related sequences in the tree (Fig. 2).

Strains analyzed	TEF1 α (724 bp)	RPB2 (1038 bp)	ITS (626 bp)	LSU (515 bp)
HCF3-NRRL 22178	23	35	10	5
HCF3-NRRL 22088	26	66	31	16
HCF3-NRRL 22153	27	66	35	16
HCF3-NRRL 22570	33	45	22	7
HCF3-all NRRL	62	109	47	22
Between NRRL (excluding HCF3)	57	95	43	21

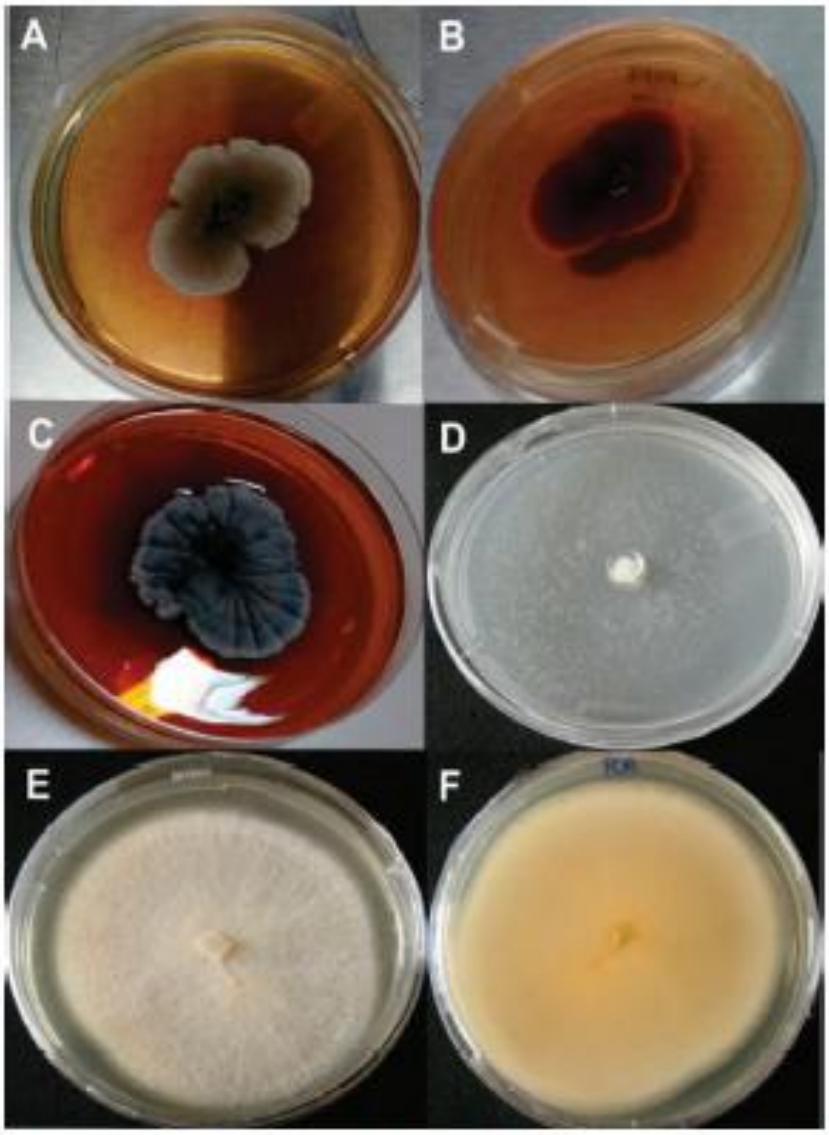

Fig. 3. Colony surface (A and C) and reverse of the colony (B) on SDA. Colony surface (D) on SNA. Colony surface (E) and reverse of the colony (F) on PDA, all cultures were incubated at 25 °C for 1 to 2 weeks.

15.5 ± 3.4–17.7 ± 3.3 μm in length, and 4.1 ± 0.7–4.43 ± 0.6 μm in width (Fig. 4). Macroconidia was falciform and robust, with 2- to 5-septate. Apical Cell morphology was blunt and rounded, and basal cell morphology cylindrical, usually with a notched or a rounded end. The wall of the macroconidia acquired a more globular form between one septum and another in the SNA medium. Sporodochial conidia were 37.9 ± 5.8–43.5 ± 4.1 μm (range length SNA, CLA and PDA) × 5.4 ± 0.8–6.0 ± 0.4 μm (width), branched or unbranched. Monophialides were often quite long, generally 40 μm. Chlamydospores, single or in chains, were observed daily for growth up to 10 days (Fig. 4).

This study used the GCPSR-based MLST schemes for the two bootstrapped single-locus genealogies (TEF + RPB2). In addition, bootstrapping revealed that none of the individual genealogies contradicted the monophyly of the species. GCPSR-based analyses indicated that HCF3 comprises a phylogenetically distinct species, strongly supported using Bayesian inference (BI) analysis, located at the base of the clade 3.

Taxonomy

Fusarium riograndense Rosa, Ramírez-Castrillón, Valente, Fuentefria, van Diepeningen, Goldani, sp. nov. The type strain is UFMG-CM F12570, deposited at the Microorganisms, DNA and Cells Collection of Minas Gerais Federal University. Also, was deposited at the mycology collection of Pernambuco Federal University under accession number URM-7361. The Mycobank number is MB 814515.

Etymology

The name of the new species is related to the geographical location of the type strain (Rio Grande do Sul state, Brazil), causing a human infection.

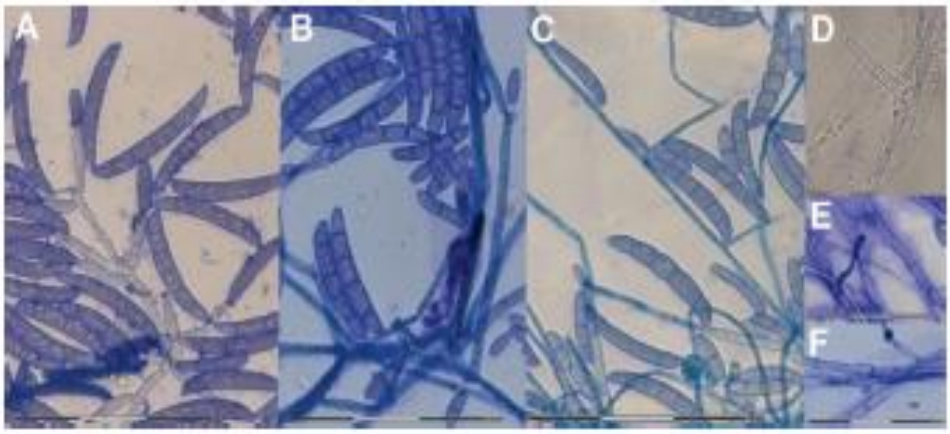

Fig. 4. *F. nisigrandense* n.sp. nov in microscopy (light micrograph, lactophenol cotton blue stain) with abundant macroconidia (2–5–septate) and some microconidia ellipsoidal (0–1–septate) observed by microcultivation from a 10-day-old culture: conidiophores and conidia from sporodochia on PDA(A), microconidia forms with blunt apical and barely notched basal cell on CLA (B), some macroconidia showed more rounded shape on the limitation of the septa on SNA(C) (× 400 bar 75 μm). Macroconidia 4 × greater than microconidia in length and monophialides on SNA (D) (× 1000 bar 30 μm). Chlamydospore in pairs (E) or singly (F) on SNA (× 400 bar 75 μm).

Antifungal susceptibility

In vitro studies demonstrated that AMB was the most active drug, followed by VRC, ITC and FLC. The values of minimum inhibitory concentrations were 1 μg/ml, 8 μg/ml, > 64 μg/ml. and > 64 μg/ml. respectively. Antifungal activity is similar to the species of its complex, showing low sensitivity to the first generation triazole, and good sensitivity to the second generation triazole and polyene (Figs. 3 and 4, Table 1) antifungal agents.

Discussion

Fusarium species may cause invasive sinusitis in immunocompromised host. The clinical manifestations of fusarial sinusitis are indistinguishable from those caused by *Aspergillus* or Mucor species. Necrosis of the mucosa is a hallmark and is a consequence of the angioinvasive nature of these mycoses [10]. Risk factors for severe fusariosis include prolonged neutropenia and T-cell immunodeficiency. The principal portal of entry for *Fusarium* spp. is the airways, followed by the skin at site of tissue breakdown and possibly the mucosal membranes.

The present study identified and describes *F. nisigrandense* sp. nov, a novel species pathogenic to humans in a patient with leukemia. We combined morphological and molecular characters to describe the new species, including MSLT with RPB2, TEF-1α and ITS-LSU regions. All queries showed similarity below 98%, indicating the queries sequences are from a novel species within the *F. solani* species complex. TEF-1α and RPB2 genes were used for phylogenetic reconstruction because they are the most informative genes for *Fusarium* [11]. Furthermore, several studies have shown that TEF-1α and RPB2 have the highest discriminatory power in delineating FSSC using comparative sequence analyses [12–14]. These techniques were used to delimit species and to estimate the genetic and phylogenetic relatedness of the isolate, avoiding the ITS or LSU regions [11,15].

Traditionally, plant pathogens within FSSC were named after their host plant as *forma specialis* (f.sp.) on that host. These *forma especialis* in FSSC are assumed to correspond to biologically and phylogenetically distinct species [16]. Some species of FSSC are known to be weak plant pathogens that cause fruit rot of cucurbits, and are equivalent to the group known historically as "*Fusarium* sp. f. sp. cucurbitae race 2", along with the species "*Fusarium* sp. f. sp. *cucurbitae* Race 1", which is an unrelated species that infects roots, stems and fruits [17]. Interestingly, plant pathogenicity experiments have established that FSSC isolates from human infections are often pathogenic to cucurbits [18]. Therefore, considering the conservative criteria of genealogical concordance for phylogenetic species recognition and its importance as a human pathogen, we formally describe a new *Fusarium* species, and designated as *Fusarium nisigrandense*. This new species belongs to clade 3 of the FSSC, phylogenetic analyses provide clear evidence for an ingroup relationship of *F. nisigrandense* (HCF3) with FSSC 10, a and b (= NRRL 22153 and 22098), which were previously described as pathogenic fungi to humans and plants [19]. The phylogenetic tree was constructed using 231 Fusarium sequences and two-loci datasets, TEF-1α and RPB2, and formed three highly supported clusters, designated 1, 2, and 3. The clade 3 comprises several clinically important species, such as *F. falciforme*, *F. petroliphilum*, *F. keratoplasticum*, and *F. lichenicola*, as reported in other studies [12,19,20]. Zhang et al. found that all the FSSC isolates from humans were members of a previously defined major clade within the complex ("clade 3") [12]. Only two species within clade 3 have been previously shown to be pathogenic to both humans and plants, and these include FSSC 1 (informally known the *Fusarium* sp. f. sp Cucurbitae race 2) and FSSC 8 (*Fusarium nanoconsporiellum* = *Nanoconspora vasinfecta*) [19].

After the 2011 meeting of the Nomenclature Session of the Botanical Congress in Melbourne, it was decided that there should be one name for each fungus, with the banishment of the dual nomenclatural system for anamorph and teleomorph fungi [21]. We decided to follow the arguments by Geiser et al. to maintain *Fusarium* as the sole name for the genus. Therefore, the new species is described as *F. nisigrandense*, and it belongs to the FSSC [22].

The shared characteristics of the new species with those of the FSSC consist of the development of conidia on long monophialides of the aerial mycelium. This microscopic characteristic is routinely used in order to identify fungi isolated from clinical samples, but the overall shortage of diagnostic morphology characters complicates the separation of similar species and the description of new species in the FSSC based on morphology alone [23].

Considering all these aspects, description of a novel species in complex *F. solani* including *F. nisigrandense* relies on a combination of characteristics including, phenotypic and molecular characteristics (Table 1). *F. nisigrandense* produced conidia and conidiophores with shapes and dimensions typical for the classic morphological concept of *F. solani* [24]. Other members of the *F. solani* family were shown to have several characteristics in

common, such as the growth rate of the colony at 24 h and after 72 h of cultivation in PDA, as well as the macroscopic characteristics in SDA [25]. *F. nigrum*inense and other *Fusarium* spp. synthesizes a red pigment, which was previously described as bikaverin in *F. fujikuroi*, aurofusarin in *F. graminearum*, and in other FSSC members [24].

Invasive *Fusarium* infections are extremely difficult to treat, with high mortality rates. Amphotericin B and voriconazole are the drugs of choice recommended for treatment of deep and disseminated *Fusarium* infections [26–28]. In general, *Fusarium* spp. shows high in vitro resistance to available antifungal drugs, not only azoles, but also echinocandins and polyenes. In our study, we have observed high MICs for itraconazole and fluconazole. Amphotericin B followed by voriconazole were the most active antifungal agent against our new species of *Fusarium*, consistent with previous studies for most of the *Fusarium* species. However, previous studies have shown that species- and strain-specific differences in antifungal susceptibility exist within *Fusarium*. Susceptibility testing is important in this setting and may improve the prognosis of these infections [29].

In summary, we described phenotypic and molecular aspects, and susceptibility profile of a new pathogenic *Fusarium* species isolated from a clinical sample of an immunocompromised host. Further assessment of the ecologic, epidemiologic, and clinical features of infections caused by this new species is required to facilitate its distinction from other known infections caused by different *Fusarium* species.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

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Artigo 6:

Invasive Fusariosis: correlation between morphological e molecular *Fusarium* species (*Medical Mycology*; FI=2,8).

Invasive Fusariosis: correlation between morphological and molecular *Fusarium* species

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Abstract

Invasive fusariosis (IF) is considered an emerging fungal disease and an important problem worldwide, affecting increasingly immunocompromised individuals, mainly patients with hematologic malignancies. The aim of this study is to characterize morphologically and molecularly the isolates of *Fusarium* species, in order to acquire the understanding of the phylogeny that genus and establish a solid taxonomic platform for the causative agents of IF, mainly in order to facilitate identification in the laboratory routine. Multilocus sequence typing (MLST) was performed on the following genes: *second largest subunit of the RNA polymerase gene (RPB2)* and the *elongation factor 1 alpha (EF-1α)*. Morphological and molecular phylogenetic analyzes revealed that these isolates were *F. solani* species complex (FSSC, n = 16) and *F. oxysporum* species complex (FOSC, n = 5); FSSC-2h, FSSC-1and FOSC-183 were the most frequent sequence type. In conclusion, our study indicated that FSSC was the most common etiological agent of IF in Brazil, and the distribution of phylogenetic species in FSSC isolates from patients with IF had different spectra. Our data will help reveal the infection characteristics of fusariosis, which will be helpful for appropriate diagnosis to achieve better prognosis.

Key words: *Fusarium*; genotyping; MLST; molecular epidemiology; morphology

Introduction

Invasive Fusariosis (IF) is a mycosis caused by infection with *Fusarium* spp., that affects immunocompromised patients [1], mainly patients with hematologic malignancies and hematopoietic cell transplant recipients [2]. Thereby, the increasing cases of this infections pose a significant challenge to infectious disease specialists because, except posaconazole [3], most species show high levels of resistance to all antifungals currently available [4].

The genus *Fusarium* is a large group of hyaline fungi that are ubiquitously present in the soil, water and air, both in temperate and in tropical climates [5], The potential of nosocomial transmission has recently been raised, especially concerning the high mortality rate of ~90% in patients with prolonged and severe neutropenia [6].

The diagnosis of *Fusarium* in the laboratory includes some criteria such a positive direct mycological examination showing typical septated hyphae branching at 45˚. However, the identification this fungal to the species level is often difficult and requires a specialized laboratory and skilled personnel. *Fusarium* cultures are characterized by their usually fast-growing, pale or bright-colored, colonies with sparse and floccose to lanose aerial mycelium. The diameter of the colonies after 7-10 days incubation at 25 ˚C and their gross morphology and pigmentation are important characteristics in the identification of the taxa. Most *Fusarium* species are best identified from cultures grown on potato dextrose agar (PDA). Recent studies have also shown the value of media with low nutrient concentrations such as Synthetic Nutrient Agar (SNA) for good development of microscopic features and it is also the medium of choice of several taxonomists [7–9]. The cultures need incubation at 25 ˚C. The presence of chlamydospores, characteristic of some species, at times, requires longer periods of incubation [10].

However, numerous recent studies have shown that morphology alone is not always enough to determine and characterize cryptic species, and identification of a *Fusarium* isolate by its phenotype or only to section level might be erroneous in as many as 50 % of cases [11]. MLST is a highly accurate method used to distinguish between isolates of microbial species, which are available in Fusarium-ID and Fusarium-MLST databases [12,13]. O'Donnell et al. [14] constructed several multilocus DNA sequence datasets to assess the phylogenetic diversity and have been widely used to investigate phylogenetic relationships at the interspecific level. However, the gene *EF-1α* is already

in use as a barcode in Hypocreales, e.g. *Fusarium* [15]. This gene combined with *RPB2* were more effectively barcoding markers for precise identification of cultures known to represent *Fusarium* species [16].

Molecular biology techniques might be helpful for the definitive diagnosis [17], such as DNA sequencing, in order to improve identification and are recommended for epidemiological studies and where morphological identification does not establish species [18]. In addition, identifying the etiological agent is important for initiating the proper treatment. The aim of this study is to characterize morphologically and molecularly the isolates of *Fusarium* species, in order to acquire the understanding of the phylogeny that genus and establish a solid taxonomic platform for the causative agents of IF, mainly in order to facilitate identification in the laboratory routine. Besides that, to characterize the epidemiological profile of the most prevalent *Fusarium* species in this group of patients from Southern Brazil and verify if there is some epidemiological links between the *Fusarium* strains or suggest to common source of contamination.

Materials and methods

Isolates analyzed. A total of 21 strains collected from clinical samples were analyzed, as skin biopsy of the leg, arm, fingers, bone fragment, blood, nasal cavity, and ascites fluid, all from immunosuppressed patients. Stock cultures were maintained on Sabouraud dextrose agar (SDA; Kasvi, Italy) plus chloramphenicol at 25 °C in the Laboratory of the Special Laboratory of Infectious Diseases in the Hospital of Clinics of Porto Alegre (HCPA). The data sources of isolation are listed in Table 1.

Morphological characterization. To study the pigmentation, growth rates, colors and characteristics of the colony, all strains were transferred onto fresh plates 8.5 cm Potato Dextrose Agar (PDA; Liofilchem, Italy) and SDA incubated in growth chambers at 25 °C. Cultures were grown under 12 h light–dark (l/d) cycles daylight colour fluorescent lights, which were observed daily for growth up to 10 days, colony diameters were measured using a ruler and the average growth rate per species was calculated and expressed as colony growth rate per 24 h.

For microscopic observations, all strains were transferred to synthetic nutrient-poor agar (SNA, home-made at HCPA; KH_2PO_4 0.1%, KNO_3 0.1%, $MgSO_4.7H_2O$ 0.05%, KCl 0.05%, Glucose 0.02%, Sucrose 0.02%, agar 2.0%) plates and incubated

under 12 h alternating light (black/white) at 25°C for 10 days. Conidia were measured from images taken using a using a ZEISS *PALM MicroBeam* microscope, digital camera and *PALMRobo 4.6 Pro software.* Sporodochial conidium (macroconidium) and aerial conidium (microconidia) mean length and width were calculated for individual isolates from measurements of 25 spores.

Molecular characterization. Fungal mycelium was grown in SDA medium, and a small amount of the mycelia, 5 mm^2, was subsequently suspended in 1 mL 0.85% NaCl solution by extraction of genomic DNA, using Pure link™Genomic DNA Mini kit (USA) according to the manufacturer's instructions, followed by DNA quantification in NanoDrop 2000 (Thermo Scientific, Wilmington, USA).

PCR amplification for multilocus DNA sequencing with *EF-1α* and *RPB2*. The amplification was performed with the *Veriti* Thermal Cycler (Applied Biosystems, USA) using a volume of 15 µL, containing 1.46 µL of 10× PCR buffer, 1.0 $MgCl_2$ µL (50mM), 10.94 µL Distilled Water UltraPure™ (Gibco, USA), 0.58 µL of dNTP mix (2.5mM), 0.18 µL of each primer (10 pmol), 0.09 µL of *Platinum® Taq* polymerase (5 U/µL), and 1 µL of template DNA (100 ng/µL). The cycling conditions included 34 cycles of 30 s at 94 °C, 45 s at 62 °C and 1.0 min at 72 °C, a post elongation step of 5min at 72 °C for EF-1α (EF1, EF2). The PCR reactions were performed in a volume of 15 µL containing 1.5 µL of 10× PCR buffer, 0.3 of $MgCl_2$ (50mM), 0.9 µL of dNTP mix (10 µM), 0.35 µL of each primer (7Cf /11AR 20 µM) or 0.48 each primer (5F2/7CR 10µM), 0.12 µL of *Platinum® Taq* DNA polymerase (5 U/µL, Invitrogen, Brazil), and Distilled Water UltraPure™ (Gibco, USA) to complete a volume of 14 µL, and more 1 µL of template DNA (100 ng/µL). Pre-denaturation for 5 min at 94 °C, 5 cycles of 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a post elongation step of 5 min at 72 °C for RPB2. The PCR products were visualized by electrophoresis on 1.5% (w/v) agarose gels. Amplicons were purified using Exo-SAP-IT (USB Corp., Cleveland, OH) according to manufacturer's instructions, followed by sequencing with locus-specific primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.) in an ABI3500 Genetic analyzer (Applied Biosystems, USA). Raw sequences were assembled and manually edited in ChromasPro 2.1.6 (http://technelysium.com.au/wp/chromas/). After, the consensus sequence was compared with reference sequences deposited in Fusarium-MLST

(http://www.cbs.knaw.nl/fusarium/) and via BLAST to sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Results

Table 1 and 2 summarizes the most relevant morphological features useful for distinguishing these species, which can help those laboratories that have no molecular facilities for the identification of clinical isolates. According to culture characteristics, the aerial mycelium which was predominated cream in FSSC. Also presented aerial mycelium abundant, dense floccose, white (Fig. 1 and 3-5), occasionally greenish (Fig.1, F5). We observe a great diversity in the colors of the mycelium, with a tendency to be darker in the center (Fig. 1 and Fig. 5 A). Peach colony appears to be typical of the *Fusarium falciforme* and *Fusarium keratoplasticum* species (Fig. 3 and 4 A). Some isolates showed brown pigment (F26, F27, and F30) and, others reddish pigment (F10, F17, F18, F22 and F31) in the SDA culture medium after 14 days. After 21 days, also pigment on SDA the isolates F5, F6, F7, F8, F10 and F13. Cultures grew fast, the growth rate (mm⁄day) on PDA ranged from 3.89±2.76 to 10.71±8.10 mm⁄day and on SDA 4.44±2.19 to 10.71±3.57 mm⁄day (Table 1). In general, the reverse color of the colony was different in the media sown. FSSC isolates showed the darkest center in the SDA medium (Fig. 1 and 5A), however this is observed in the FOSC in PDA (Fig. 6 A).

The hyphae initially hyaline and mycelium became yellowish white; green to bluish-gray, and purple in reverse after 10 days. The isolates F6, F8, F17, F30 and F32 abundant sickle macroconidia. Sporodochial conidium (macroconidia) and aerial conidium (microconidia) length and width ranged between, 25-61 x 5.1-6.4 µm and 4.9-8.6 x 1.5- 3.3µm, respectively (Table 2). The macroconidia *F. falciforme* are more delicate and falcate, mostly with 4 septa and chlamydoconidium in pairs (Fig. 3 B, i.e. F6). In general, macroconidia long, slender, slightly curved, parallel dorso-ventral sides, blunt or conical apical cells, papillate basal cells, 3 to 5 septate, mostly 3 septate (Fig. 2 and Fig. 5). The shape of microconidia oval, reniform, elongated oval, and mostly 0-septate (Fig. 2-5), equal to the FOSC. Hyphae on SNA 1.5-5 µm wide, long monophialides and chlamydospores in pairs and in single. Some samples did not produce macroconidium in 10 days in the SNA, however, in the PDA medium, the presence of macroconidium in 21 days was verified for samples F 10, F22 and F27.

In the FOSC the majority did not present macroconidia, however abundant microconidia, in false heads clumping at the end in short monophialides (Fig. 6 B, i.e. F1, and F11). In general, aerial mycelium cottony, initially pinkish white and later becoming

light purple in the center. Reverse slightly darker purple (Fig. 6 A). Mesoconidia and microconidia, length and width ranged between, 5-17 x 2.7-4.6 µm and 3.5-6.2 x 1.4-3.5µm, respectively (Table 2).

DNA sequence data were used to assess the phylogenetic relationships and species limits of a comprehensive collection of 21 isolates of clinica, demonstrated in the Table 1. The FSSC was most frequent agent group (16 cases, 76.2%), followed by the FOSC (5 cases, 23.8%). Within the FSSC, 10 isolates were *F. solani* and two were *F. falciforme* and *F. keratoplasticum,* one were *F. riograndense and F. petroliphilum*. The most frequent isolates were nasal cavity tissue (n=9) and skin (n=7). Our isolates presented 13 different haplotypes, predominating the haplotype FSSC-2h (n=5), FSSC-1(n=2) and FOSC-183(n=2).

Discussion

Identification of fungal isolates has long been time based on morphological characteristics of cultures, and for many laboratories, this is still the standard for identification. However, many *Fusarium* species look similar in culture and have been shown to represent species complexes instead of single species [19]. Important references for the morphological identification of *Fusarium* is the classical work of Leslie and Summerell [7], which classifies the mycelium of FOSC as floccose, sparse or abundant and range in color from white to pale violet and sporodochia may be sparse or non-existent, as well as our four isolates (F1, F4, F10 and F11). The only sample that presented the smallest growth was white mycelium (F4), however, there are reports with the same growth rate 4.3± 0.7[20]. The isolated F23 showed macroconidia morphology straight and relatively slender, tapered and curved with apical cell and basal in pointed, being in agreement with the study of Hafizi et al.[20].

The characteristics described for the *F. keratoplasticum* (FSSC) are colony color on PDA in shades of white, salmon, peach, vinaceous grey and pale olivaceous grey; reverse pigmentation in shades of pale olivaceous grey, flesh, salmon, olivaceous buff, ochreous and pale luteous. Colony margin entire to undulate. Aerial mycelium is ranging from sparse to dense floccose [21]. We found fairly cottony colonies and the reverse peach in both culture media in our *F. keratoplasticum* isolates (F27 and F31), similar to what has been described by Short et al [21]. Microconidia in *F. petroliphilum* tended to be slightly rounder than those of *F. keratoplasticum* at 25˚C; previous reports indicate

that microconidial roundness is enhanced at 37 ˚C in *F. petroliphilum.* This is the predominant species in locally invasive infections in a study carried out at the university hospital in Turkey [22]. It also presented higher incidence together with *F. solani* in a study in Qatar [23] and showed not effective by azoles and echinocandins, leaving only amphotericin B as potentially effective treatment [24]. *F. falciforme* (n=14/127 cases; 11%) was the dominant species in a study in Utrecht, being one of the main species isolated from keratitis cases in Brazil, India, and Mexico [6]. Among the different species of FSSC, we also found *Fusarium riograndense* , which exhibits sickle and robust macroconidia [25] .

In FSSC, exist a greater variation of tones, cream to greenish or orange [20,26,27]. Cultures grew fast, the growth rate (mm⁄day) on PDA at 25 °C in intermittent light ranged from 7.8 to 8.6 mm⁄day. The hyphae initially hyaline and mycelium became yellowish white and green to bluish-gray in reverse after 1–2 weeks [28]. Sporodochia often are produced in abundance, produce pigments in the agar although some violet or brown pigments may be observed [29,30]. Similarly, 13/16 of our FSSC isolates. Both complexes have colonies growing rapidly [31]. Shape macroconidia are rounded and curved size 30–50 x 3.5–5.7 µm in *F. solani* [30]. Similar to *F. keratoplasticum,* which are notched and blunt 32-37 x 4.6-4.9 µm [28]. However, *F. falciforme* the macroconidia are barely notched and pointed or curved, 3- and 4-septate, 39 - 43 x 4.5-5.5 µm [32]. Most species of this complex produce pigments as secondary metabolites [21]. All data in the literature are in agreement with that of our study.

In relation to the macroconidium of this complex, general morphology is relatively wide, straight, stout and robust, with 5- to 7-septate. Monophialides, often quite long [7]. Apical Cell morphology is blunt and/or rounded and basal cell morphology cylindrical is usually with a notched or a rounded end (Barely notched and papillate curved) [32]. Microconidia shape oval, elongated oval, clavate, reniform and fusiform with 0 or 1, rarely 2 septate [32,33].

The FOSC appears to be particularly different from the FSSC in two main aspects, first with its lilac mycelium and secondly by the abundance of microconidia and absence of sickle macroconidia. Microscopically, *F. oxysporum* have chlamydospore shape smooth, microconidia on short and often lateral phialides [10]; and in general their microconidia are abundant [31], have the very similar format, as oval or elliptical shaped

[30] and usually 0-septate [7]. Also, aerial mycelium presentation false heads, short monophialides, according Leslie & Summerell [7].

In this study, biological characterization, including culture morphology, and molecular study were used to compare isolates of *Fusarium* associated IF. The respiratory tract (nasopharynx, 42.8%) was significantly more involved among patients, as well as in other studies [34,35]. One of the interesting features of fusarial infection was the involvement of skin (33.3%), especially the legs (83.3%). It has also been reported that skin involvement in fusariosis can represent a primary site of infection [36,37]. Skin involvement in fusariosis was present in 181 patients (70%) among 259 published cases of fusariosis (232 immunocompromised and 27 immunocompetent) [2]. It has been recommended that clinicians treating patients at high risk for invasive fusariosis undergo a thorough evaluation and treat skin lesions (particularly onychomycosis) before commencing antineoplastic therapy [37].

Other studies have shown that approximately 60% of all human infections are caused by members of the *F. solani* species complex (FSSC) and 20 % by the *F. oxysporum* species complex (FOSC) [31], similarly to our findings. The FSSC contains many opportunistic species (e.g., *F. falciforme*, *F. keratoplasticum*, and *F. petroliphilum*) with high prevalence, but other *Fusarium* groups are also important, such as *F. oxysporum, F. verticillioides* and *F. proliferatum* [38]. Clinically important sequence types (STs) within *F. keratoplasticum* and other fusaria appear to be ubiquitous in indoor plumbing [39]. Most frequent haplotypes or STs described in the Southeast region of Brazil for IF are FSSC 2-d and FSSC 2-f, in the immunosuppressed patients in the bone marrow transplant unit and dermatology outpatients [40]. In contrast, we found FSSC-2h, followed by FSSC-1a.

Members of the *F. oxysporum* species complex (e.g., FOSC ST 33) and the FSSC, specifically *F. petroliphilum* (e.g., FSSC 1-a) and *F. keratoplasticum* (e.g., FSSC 2-d) are the fusaria most commonly identified in hospital water systems employing MLST[39]. Distribution of the phylogenetic species of FSSC isolates from patients with IF in Japan most frequent are *F. keratoplasticum*（FSSC 2）and *Fusarium* sp.（FSSC5）[41].

In 2005 and 2006, outbreaks of *Fusarium* keratitis in U.S. states and Puerto Rico, the members of the FSSC comprised 61.8% of the isolates, specifically, FSSC haplotypes 1-a and 2-d [42]. In keratitis in Brazil, the most common haplotypes are FSSC 1-b and

FOSC-2 [43]. In onychomycosis in Colombia, the most common haplotypes are FSSC 2-d and FSSC 2-k [11]. More than 14 haplotypes were described as more frequent in mycoses of humans and other vertebrates and exhibited transoceanic distributions (i.e., FSSC 1-a, 2-f, 2-h, 2-i, 3 + 4-bb, 3 + 4-hh , 3 + 4-vv, 3 + 4-w, 5-e, 5-f, 5-h, 5-n, 6-j, and 9-a) [44].

In this way, we perform the identification and characterization through morphological approaches and MLST scheme to determine the genetic diversity of isolates and it was possible to distinguish the isolates in two large complexes. Unfortunately, the classification system based only on morphology has not provided an accurate tool for the identification of *Fusarium* at the species level within the FSSC. So, the molecular approach is promising in establishing accuracy in the diagnosis of medical mycology. The FSSC group with mycelium varying from cream to brownish greenish tones and with macroconidia, and the second FOSC group observed mycelial tones ranging from white to purple, predominantly microconidia and short phialides. Molecularly we observed a predominance of haplotype FSSC 2-h, which exhibit mycelium of similar color tones. However, the other predominant haplotype had no macroscopically in common feature (FSSC 1-a). The predominance of the isolates were from the nasal cavity and the skin, we are led to believe that the source of contamination is through the air, being acquired through breathing. And the second form of infection is possibly the direct contact with the inoculating source through a mechanical accident, breaking the physical barrier of the skin.

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Figures and Tables

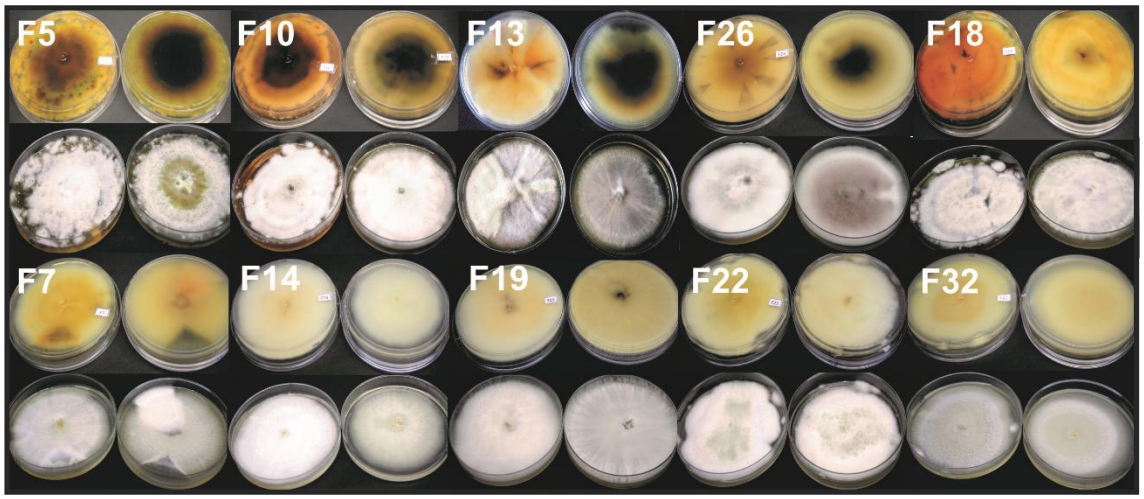

Fig. 1. Colony pigmentation of ten isolates of *Fusarium solani* grown on PDA (left) and on SDA (ride). Reverse view and top view, respectively.

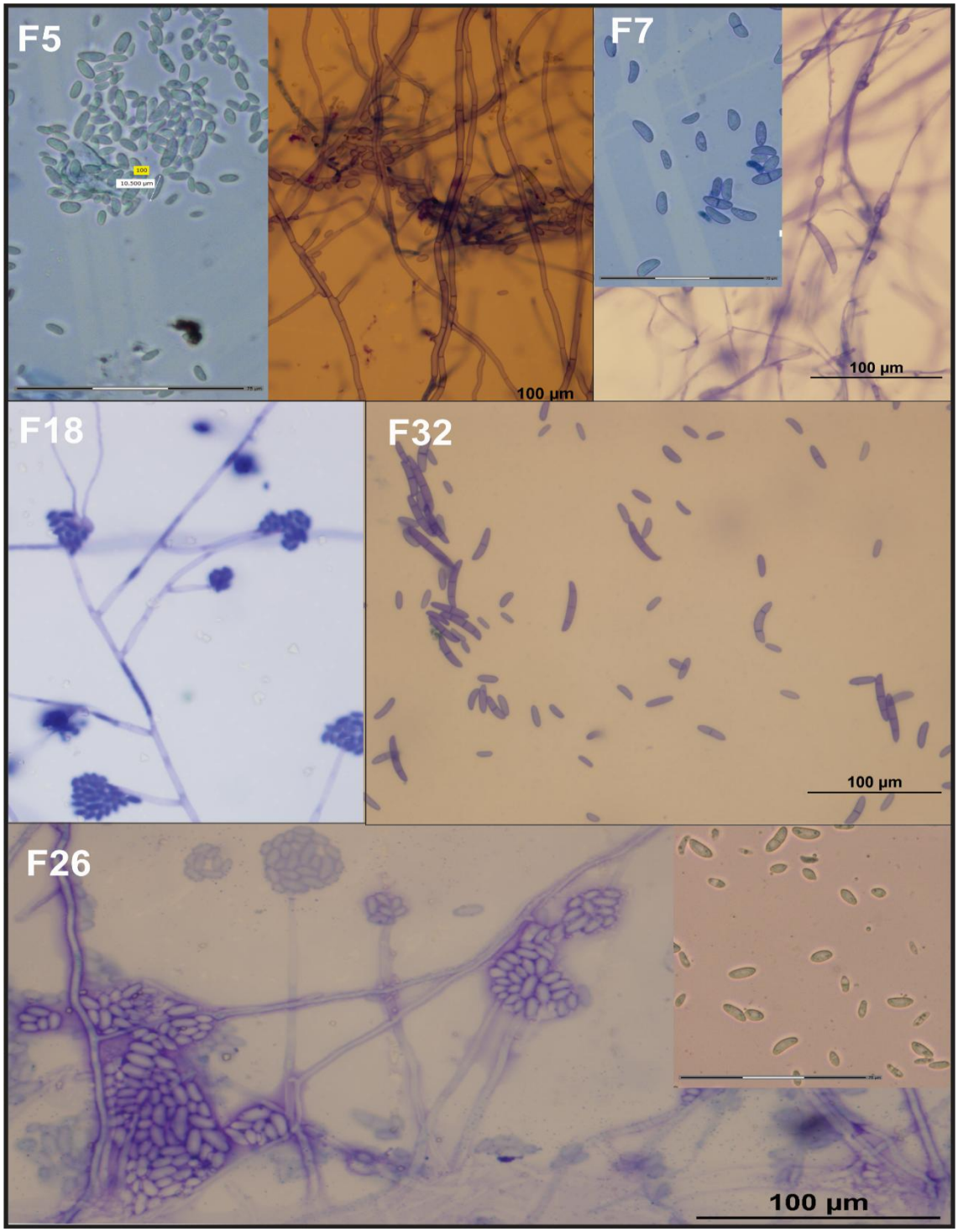

Fig. 2. Hyphae, macroconidia apical blunt and basal papillate (F5, F7, and F32), microconidia oval and reniform of *Fusarium solani* (F5, F7 and F26). Microconidia in round false heads on relatively long monophialides (F18 and F26). Macroconidium abundant 3-septate and also, in the format of the letter "C", microconidia elongated oval 0-1 septo (F32). All from 10 d old SNA cultures. Mounted in lactic acid/cotton blue. Majority scale bars is 100 µm, all others in 75 µm.

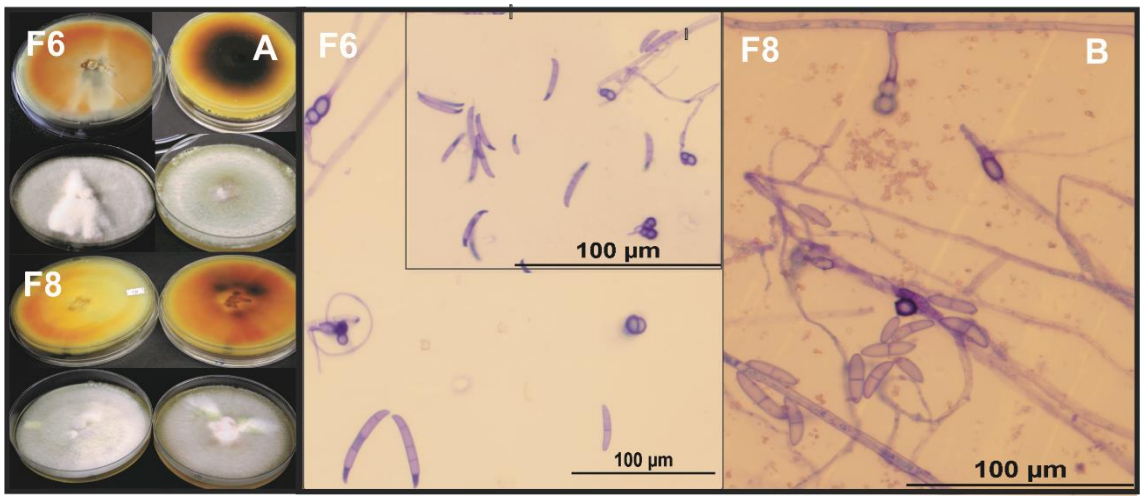

Fig. 3. Colony pigmentation of isolates of *Fusarium falciforme* grown on PDA (left) and on SDA (ride). Reverse view and top view, respectively (A). Hyphae, falciforme macroconidia and chlamydospores in pairs (F6; B). Microconidia elongated oval and reniform, 0-1septate, and microconidia in situ (F8, B). All from 10 d old SNA cultures. Mounted in lactic acid/cotton blue. Scale bars is 100 µm.

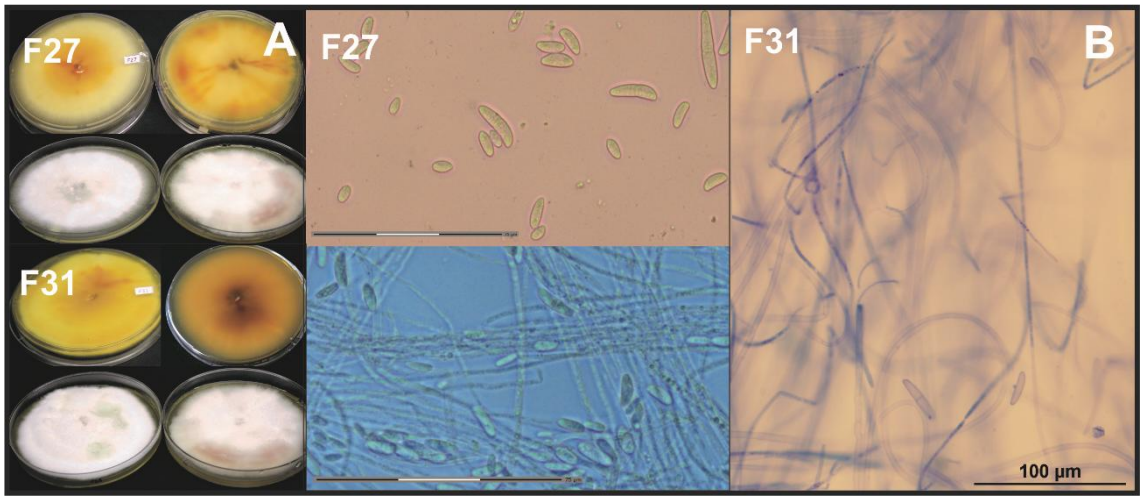

Fig. 4. Colony pigmentation of isolates of *Fusarium keratoplasticum* grown on PDA (left) and on SDA (ride). Reverse view and top view, respectively. Hyphae and microconidia oval, elongated oval and reniform, 0-1septate. All from 10 d old SNA cultures. Mounted in saline solution and in lactic acid/cotton blue. Scale bars is 100 µm and 75 µm (B).

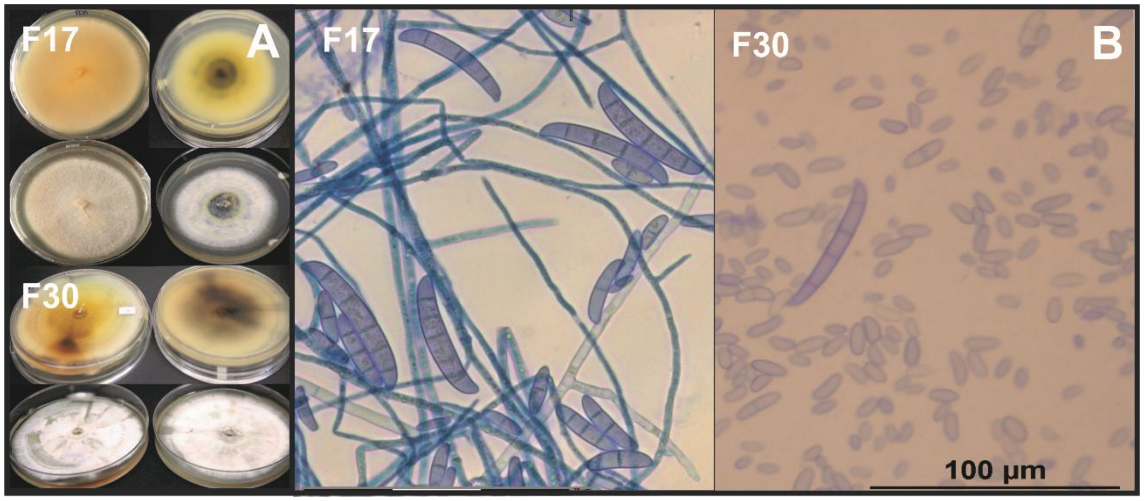

Fig. 5. Colony pigmentation of isolates of *Fusarium riograndense* (F17) *and Fusarium petroliphilum* grown (F30) on PDA (left) and on SDA (ride). Reverse view and top view, respectively (A). Hyphae, macroconidia with apical cell morphology blunt, 3-5-septate. Microconidia oval and ellipsoid with 0-1 septate. All from 10 d old SNA cultures. Mounted in lactic acid/cotton blue. Scale bars is 100 µm and 75 µm (B).

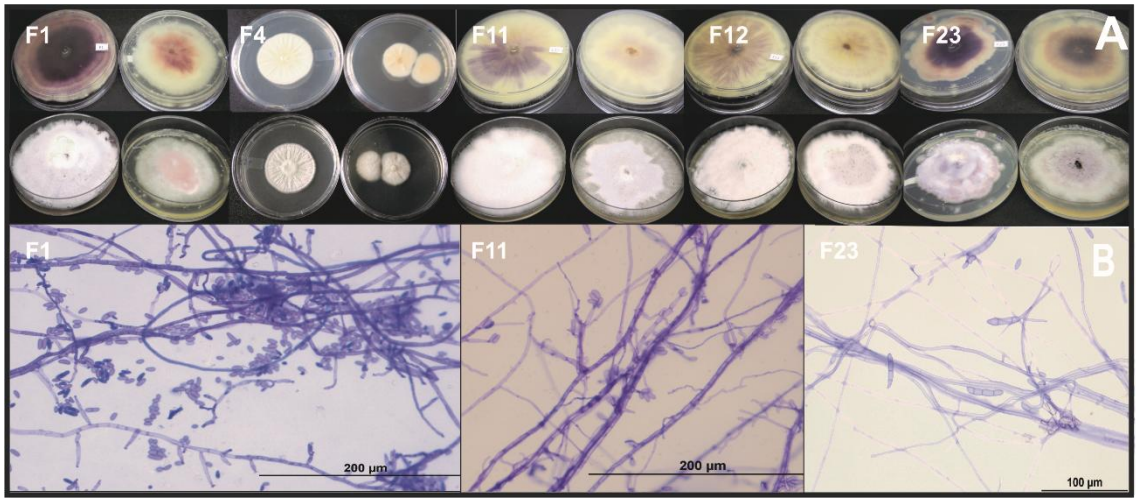

Fig. 6. Colony pigmentation of five isolates of *Fusarium oxysporum* complex grown on PDA (left) and on SDA (ride). Reverse view and top view, respectively. Short monophialides that arise directly from hyphae and ovoidal to ellipsoidal microconidia formed in heads by monophialides dispersed in the aerial mycelium (F1 and F11; B). Hyphae, macroconidia, and microconidia (F23; B). All from 10 d old SNA cultures. Mounted in lactic acid/cotton blue. Scale bars is 100 µm and 200 µm.

TABLE 1. Comparison of growth rates and cultural characteristics of *Fusarium* species used in the current study subjected to DNA MLST

				BLAST	GenBank		MLST
Culture	Isolate source	Color in PDA	Growth rate Color in SDA	Growth rate Species identified	EF-1α	RPB2	EF-1α +RPB2[a]
F1	leg skin	purple	7.50±3.84 white with pink center	8.33±4.92 *F. oxysporum*	KP964859.1	AB986568.1	43431; 183
F4	blood	white	4.44±2.19 white	3.89±2.76 *F. oxysporum*	JF740724.1	XM_018390099.1	43431; 183
F5	nasal tissue	greenish orange with cream	8.33±3.54 degrade from brown to greenish	9.38±3.42 *F. solani*	KJ648656.1	DQ790576.1	32770;7-d
F6	nasal tissue	orange	9.38±5.60 degrade from brown to cream	9.38±3.97 *F. falciforme*	KF020508.1	JN985498.1	28549; 3-4-l
F7	leg skin	cream and with a dark triangle	7.50±2.04 cream and with a dark triangle	9.38±4.67 *F. solani*	KM096408.1	JN985499.1	43433; 2-a
F8	leg skin	cream with orange	9.38±5.07 orange	10.71±8.10 *F. falciforme*	AB817153.1	MF467484.1	32709; 3-4-w
F10	leg skin	brown in the center with cream	8.33±3.77 cream with a greenish center	8.22±2.66 *F. solani*	KX001802.1	KP696751.1	28546; 1-a
F11	nasal tissue	purple with white	9.38±4.10 rosette with white	8.33±6.08 *F. oxysporum*	KF624780.1	LN828106.1	26033; 40
F12	nasal tissue	purple with white	8.33±4.49 rosette with white	9.38±7.98 *F. oxysporum*	DQ837692.1	LN828106.1	43668; 237
F13	arm skin	peach with cream	8.92±1.46 greenish brown center	7.58±3.56 *F. solani*	KF429214.1	JN985499.1	43532; 2-h
F14	nasal tissue	cream	7.50±3.78 cream	8.22±2.66 *F. solani*	KM580555.1	KC808354.1	37625; 27-a
F17	nasal tissue	cream	7.36 ± 1.75 cream with greenish brown center	8.13±2.38 *F. riograndense*	KX534002.1	KX534003.1	25388; 5-g*
F18	leg skin	orange	10.71±4.50 cream with brown center	9.36±1.83 *F. solani*	LN827985.1	JN985499.1	43532; 2-h
F19	leg skin	cream	10.71±3.57 cream with brown center	10.71±8.10 *F. solani*	KX001802.1	KP696751.1	28546; 1-a
F22	tibia fragment biopsy cream		8.33±5.74 cream	8.33±3.10 *F. solani*	KF429214.1	JN985499.1	43532; 2-h
F23	ascites fluid	purple with white	7.5±3.68 purple with white	7.50±3.68 *F. oxysporum*	KF575347.1	XM_018390099.1	43539; 109
F26	nasal tissue	cream with brown center	8.33±2.65 white with greenish center	9.38±1.41 *F. solani*	KM096385.1	KF255523.1	28559; 3-4-o*
F27	blood	peach	8.33±4.37 peach	8.33±2.82 *F. keratoplasticum*	LN827985.1	JN985499.1	22791; 2-h
F30	nasal tissue	yellow tone gradient	9.19±1.31 brown tone gradient	8.33±4.13 *F. petroliphilum*	KX001802.1	MF467492.1	28546; 1-a
F31	toes	peach	8.33±3.87 dark peach	8.33±2.09 *F. keratoplasticum*	KT716218.1	LN828067.1	22791; 2-h
F32	nasal tissue	cream	9.38±3.43 cream	8.33±3.65 *F. solani*	KX901847.1	EF469984.1	32812; 5-c

Colony color reverse in PDA and SDA; Growth rate (mm/day);

[a] NRRL Agricultural Research Service Culture Collection, Peoria, USA (* similarity < 99%).

TABLE 2. Morphological characteristics of isolated *Fusarium* spp. the agent of Invasive Fusariosis

Isolate	Morfological	Macroconidia (3-5septos)	Length	Width	Mesoconidia (1-2septo)	Length	Width	Microconidia (0-septo)	Length	Width
F1	FOSC				EO	14.7±2.8	4.1± 0.6	oval and R	5.6±0.7	1.9±0.5
F4	FOSC				EO	7.6± 1.9	3.2± 0.6	EO	4.1± 0.6	1.3±0.5
F5	FSSC	AB and BP	38.4±5.9	5.6± 0.6	oval and R	11.0±±1.6	3.2± 0.6	EO	5.0±0.7	2.0±0.5
F6	FSSC	AC and BP	33.3±11.0	5.0± 0.6	EC	16.6±5.3	2.8± 0.6	EC	8.8± 1.5	2.3±0.5
F7	FSSC	AC and BB	35.5±3.5	5.6± 0.6	EO and R	13.3± 1.2	7.1± 0.6	EO	8.3± 0.9	5.3±0.5
F8	FSSC	AC and BP	31.4±3.0	5.6± 0.6	EO and R	28.1± 3.7	4.1± 0.6	EO and R	7.7±1.2	2.2±0.8
F10	FSSC				EO and R	14.2± 1.9	4.1± 0.6	EO and R	6.7±1.0	3.0±0.4
F11	FOSC				elongated	10.9± 1.6	2.1± 0.6	elongated	5.4± 1.0	2.0±0.5
F12	FOSC				EO	9.5± 1.5	4.1± 0.6	EO	5.0± 0.9	2.3±0.5
F13	FSSC	AC and BB	37.5±3.0	5.8± 0.6	EC	14.6±2.4	3.6±0.5	EO	6.4± 1.0	2.9±0.4
F14	FSSC	AC and BB	33.3±5.0	5.8± 0.6	R	12.2± 1.1	6.6±0.5	EO	6.6± 0.8	2.90±0.3
F17	FSSC	AB and BP	43.5 ± 4.1	5.8± 0.6	EC	15.0± 1.1	3.6±0.5	EC	6.9± 0.9	3.9±0.4
F18	FSSC	AB and BP	28.0±3.0	5.8± 0.6	EC	18.7± 2.0	4.3±0.8	EC	8.52±1.29	2.9±0.4
F19	FSSC	AB and BP	30.7±5.1	5.8± 0.6	EC	13.6± 2.4	4.4±0.3	EO and oval	5.51± 0.80	3.6±0.6
F22	FSSC				EC	11.7±2.4	4.4±0.3	EO	3.4± 0.5	3.6±0.6
F23	FOSC	AD and BD	43.3±9.10	4.1± 0.5	EC	11.2± 2.1	3.2±0.5	EO	4.3± 0.8	3.3±0.5
F26	FSSC				EC	12.9±2.4	3.2±0.5	EO	5.8±0.9	2.3±0.5
F27	FSSC				EO and R	12.1±1.9	3.2±0.5	EO	8.6± 0.9	3.9±0.5
F30	FSSC	AC and BP	55.0±12.89	5.8± 0.6	EC	18.5± 3.1	4.8±0.5	EO	7.7±0.9	2.2±0.4
F31	FSSC	AB and BP	57.0±3.04	5.8± 0.6	EC	22.4±2.0	4.6±0.5	EO and R	8.6± 0.9	2.3±0.5
F32	FSSC	AB and BP	35.0±5.10	5.8± 0.6	EC	25.1±2.8	4.6±0.5	EO	11.0±1.9	2.3±0.5

SNA at 25 ±2 °C for 10 days. Dimension of the length and width in µm; AB=Apical Blunt, AC=Apical Conical, AD=Apical Curved, BB=basal blunt, BP=Basal Papillate, BD=Basal Pointed, EO=Elongated Oval, EC=Elongated Curved, R=Reniform.

Artigo 7:

Epidemiology of Invasive Fusariosis in the South of Brazil: predominance of two complexes of human opportunists (*Mycopathologia;* FI=1,81)

Epidemiology of Invasive Fusariosis in the South of Brazil: predominance of two complexes of human opportunists

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ABSTRACT

Recently, invasive fusariosis (IF) have increased dramatically in immunocompromised hosts, mainly in patients with haematological malignancies and in transplant recipients. The remarkable intrinsic resistance of *Fusarium* species to most antifungal agents results in high mortality rates in this patient population. The aim of this work was improved understanding of IF epidemiology, describe clinical characteristics, shedding light on sources of infection, and determine correlations between clinical outcomes and antifungal susceptibility. A total of 27 cases of IF occurring from January 2008 to June 2017 in a reference hospital in south of Brazil. Antifungal susceptibility testing of isolates was performed by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) methodology. Hematologic malignancy was the predominant underlying condition. The nasopharynx and skin were the predominant sites of infection. The *Fusarium solani* species complex (FSSC) were the most frequent species, followed by the *Fusarium oxysporum* species complex (FOSC) isolated in those patients. Voriconazole (VRC) had the best activity for *Fusarium* spp., followed by amphotericin B (AMB). Itraconazole (ITC) and fluconazole (FLC) showed high MIC values, displaying in vitro resistance. Clinical isolates FSSC were significantly ($P < 0.05$) more resistant to AMB and VRC, than the FOSC isolates. The present antifungal susceptibility profiles showed that species- and strain-specific differences in antifungal susceptibility exist within *Fusarium* in this setting. Susceptibility testing is an important tool for appropriate management in patients with invasive fusariosis and improve of their prognosis.

Keywords: Antifungal Susceptibility, *Fusarium solani* species complex, *F. oxysporum* species complex, Invasive Fusariosis

INTRODUCTION

Invasive fungal infections are a major medical problem, particularly among immunocompromised hosts such as patients with hematological malignancies[1,2] or solid organ transplant or peritoneal dialysis [3,4]. This reflects in severe morbidity and the high mortality, with the rate of ~90% in patients with prolonged and grave neutropenia by *Fusarium* infections [5]. Fusariosis is acquired by inhalation of conidia, with subsequent hematogenous dissemination[6]. In addition, the skin may occasionally be a portal of entry, from tissue breakdown or onychomycosis [7–11].

Over the past decade, the number of cases of invasive fusariosis (IF) has increased worldwide [10] (Fig. 1). A total of 76 cases were collected from seven European countries, to which the Italian contribution accounted for more than 60 % of the cases, that *G. fujikuroi* SC is the predominant cause of deep infections and that different species have different antifungal in vitro susceptibility patterns[12]. In Paris, France, one-third of cases is caused by FSSC, followed by FOSC, *F. dimerum* species complex (FDSC), or rarely by *F. incarnatum-F. equiseti* species complex (FIESC) [13]. Another important point is the high *in vitro* resistance to antifungal agents and intrinsic resistance. Besides, different species have been shown to have different patterns of susceptibility [12]. *Fusarium* species are usually resistant to most antifungals; in vitro studies have shown lower MICs of amphotericin B, nystatin, ketoconazole, voriconazole and posaconazole [14].

Fusarium species are intrinsically resistant to azole antifungals [15]. *F. solani,* in particular, is intrinsically resistant to echinocandins [16]. Recently, diagnostic guidelines recommend amphotericin B and voriconazole as the preferred drugs of choice for treatment of deep and disseminated infections [13,17]. However, *F. nygamai* and *F. thapsinum* had high MICs of all drugs other than amphotericin B and natamycin, emphasizing that identification and susceptibility testing of aetiological agents is essential for initiating therapy[18].

Invasive fusariosis is a major cosmopolitan problem, there are few studies on the epidemiology of this disease in Brazil, and have no studies in the region in Southern of Brazil. The objective of our study is improved understanding of IF epidemiology, describe clinical characteristics, shedding light on sources of infection, and determine correlations between clinical outcomes and antifungal susceptibility.

MATERIALS AND METHODS

Patients and methods

This study was conducted at the University Hospital, Hospital de Clínicas de Porto Alegre (HCPA), Brazil. January 2008 to June 2017 by searching the records of the clinical microbiology laboratory with total of 27 patients with invasive fusariosis (one case in 2013 and 2017; two cases in 2015, three cases in 2008 and 2010, four cases in 2009, 2014 and 2016; five cases in 2011). We collected the data by reviewing the medical records of the patients considering their baseline characteristics, underlying diseases, treatment modalities, and outcome, and mycological cultures positive for *Fusarium*. The isolates were obtained from a variety of clinical specimens. Nineteen strains were isolated from as skin biopsy of the leg, arm, fingers, bone fragment, blood, nasal cavity, and ascites fluid, all from immunosuppressed patients (Table 1). Isolates were of the species *F. oxysporum* (n=5), *F. solani* (n=10), *F. keratoplasticum* (n=2), *F. falciforme* (n=2), *F.petroliphilum* (n=1), *F. riograndense* (n=1) and *Fusarium* sp. (n=6).

Antifungal susceptibility

The clinical isolates were evaluated for susceptibility to voriconazole (VRC; Sigma-Aldrich, USA), itraconazole (ITC; Sigma-Aldrich, USA), amphotericin B (AMB; Sigma-Aldrich, USA), and fluconazole (FLC; Sigma-Aldrich, USA). Minimal inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method for filamentous fungi, M38-A2 [19]. The MIC was defined as the lowest concentration exhibiting 100 % visual inhibition of growth as compared to the drug-free control well [19], and the tests were done in duplicate. The quality control (QC) isolates *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019), were included as controls of methodology with MIC ranges established limits [19]. The interpretation of our results are in accordance with Espinel-Ingroff et al. [20], which followed the CLSI guidelines for epidemiological cutoff values (ECVs). Therefore, considering > 97.5% of pooled statistically modeled MIC distributions and defined the ECVs: for amphotericin B, 8 µg/ml (FOSC and FSSC); for voriconazole, 16 µg / ml (FOSC), and 32 µg / ml (FSSC); and for itraconazole, 32 µg / ml (FOSC and FSSC).

Statistical analysis

Data analysis was performed with SPSS statistical package release 11.01. Groups (FSSC and FOSC) were compared with chi-squared or adjusted residual test for categorical variables and the Mann-Whitney test for non- parametric continuous variables. Epidemiological data were compared using the Chi-square test. The Mann-Whitney test was used to evaluate if there was any difference in MIC values against the different antifungal agents tested in the comparison between the FSSC and FOSC complexes; differences were considered statistically significant at a *p*-value of ≤ 0.05.

RESULTS

During the last ten years, 27 cases of invasive fusariosis were obtained. All patients were identified with invasive fusariosis, see Table 1. Most were descendants of Caucasians (88.9%) living in the metropolitan region of Porto Alegre located in southern Brazil. The mean age was 22.7 years (2-73 years). The infection was more prevalent in males (56%) than in females (44%). The nasopharynx (33%) and skin (33%) especially the legs (62.5%) were significantly more involved among patients. Blood culture was positive in 18.5 % of the patients.

The underlying causes of the immunodeficiency were hematologic malignancies (63%), including acute myeloid leukema (AML, n=7) and acute lymphoid leukemia (ALL, n=7). The prevalence of fusariosis was 14.8 cases per 1,000 in patients with ALL and 13.1 cases per 1,000 in patients with AML in this period from 2008-2017.

Most of the patients were treated with voriconazole and amphotercin B, with an overall mortality rate of 40%. Duration of treatment ranged from 5 to 120 days (mean, 22.6 days). Specific therapy was unrecorded for three patients.

The FSSC was most frequent agent *Fusarium* species (16 cases, 76.2%), followed by the FOSC (5 cases, 23.8%). Members of the FFSC were the most frequent etiological agents in patients with hematological disease (14/16, 87.5%). Within the FSSC, 10 isolates were *F. solani*, two were *F. falciforme* and *F. keratoplasticum*; One were *F. petroliphilum* and *F. riograndense*.

Geometric means (GMs), mode and MIC ranges of four antifungal agents are shown in Table 2. Clinical strains displayed resistance to AMB (n=5/16; 31.25%), with EVC >8 μg/mL (Fig. 2 A). All strains showed high MICs for fluconazole and itraconazole. VRC had the best activity, followed by AMB, the MIC ranges for VRC and AMB were 0.25-32 μg/ml and 1-16, respectively (Table 2). Clinical isolates FSSC were significantly ($P < 0.05$) more resistant to AMB and VRC, than the FOSC isolates (Fig. 2). Twenty seven clinical isolates were susceptible to AMB; AMB MICs >8 μg/ml were documented for *F. falciforme* (2 of 2), *F. solani* (9 of 12), *F. oxysporum* (3 of 5).

DISCUSSION

We analyzed the relationship of *Fusarium* species with their in vitro susceptibility profile for four different antifungal agents in patients with invasive fusariosis. The determination of the pathogen at the species level and its susceptibility to the main antifungal agents are important for epidemiological studies and appropriate management for this difficult-to-treat infection. FSSC was the most frequent agent group (16 cases, 76.2%), followed by the FOSC (5 cases, 23.8%) isolated in our patients. Within the FSSC, 10 isolates were *F. solani*, two isolates were *F. falciforme* and two isolates *F. keratoplasticum*. Previous studies have identified FSSC as the predominant species complex in hematologic patients with invasive fusariosis in Brazil [21,22]. Also, in Italy[1,2], in Israeli[3] and in Frace[13]. In recent study in a turkish university hospital, *F. fujikuroi* species complex (FFSC) proved to be the most frequent agent group (17 cases; 51 %) in patients with invasive fusariosis, followed by FSSC (14 cases; 42 %) [23]. In an epidemiological survey of *Fusarium* infections in Europe, disseminated disease was considered proven in 46 cases and probable in 17 cases. Localised infection was seen in 13 cases *F. fujikuroi* species complex (SC), including *F. verticillioides and F. proliferatum*, and *F. solani* SC were the most frequent etiology of disseminated and localised infections, respectively [12].

Joint guidelines on the treatment of hyalohyphomycoses recommend AMB and VRC for the treatment of systemic fusariosis [23]. In the present study, treatments voriconazole combined with amphotericin B (IV) and voriconazole were most effective, they presented the highest rates if treatment survival. A similar proportion of patients received monotherapy vs. combination therapy as initial therapy. For monotherapy,

voriconazole formulation was most commonly used. For combination therapy, voriconazole with an amphotericin B formulation was most commonly administered, with the highest survival rates, 77.8 and 66.7%, respectively. By making a connection between the MIC, treatment and evolution of the patient, we had two cases of patients who died, F13 and F22, infected by *F.solani* and who received treatment with amphotericin and voriconazole. Isolates of the same were shown resistant in the susceptibility profile test, or with reduced susceptibility to the VRC. In general, patients who presented a positive evolution to the treatment also showed a sensitivity to these drugs (83.3%), presenting MIC values below the cut-off point suggested to their complexes.

Voriconazole was approved for the therapy of *Fusarium* infections in 2002 [13]. This medication is used prophylactically against *Fusarium* spp. in these study patients. In addition to antifungal treatment, strategies to improve the host defenses and surgical intervention to remove necrotic tissue are important measures that may improve the prognosis of these patients. Resistant profiles to AMB and VRC, which are the currently recommended agents in the guidelines for treatments, and a late diagnosis may be associated with the high mortality rate in immunocompromised patients [24].

It is essential to evaluate the susceptibility profile of *Fusarium* species in patients with invasive fusariosis because most of the antifungals currently available exhibit such poor in vitro activity and fusarioses involving hematogenous dissemination results in a high mortality [2]. Considering the azoles susceptibility, FSSC isolates have higher MICs for VRC than FOSC [20,25]. In our study, FSSC complexes were also more resistant than FOSC to voriconazole. As expected, our *Fusarium* isolates did not show activity for FLC and ITC. In fact, only 5 isolates presented MIC $\leq$ 64 µg/mL for ITC. Previous studies have shown that FLC and ITC have no activity against *Fusarium* species [20,2]. Similar to previous described, *F. solani* isolated in our patients exhibited higher amphotericin B MICs than other *Fusarium* spp.

Consistent with observations in the literature, most patients with invasive fusariosis in this analysis had hematologic malignancy. One of the interesting features of fusarial infection in our study was the involvement of skin, nasopharynx, and a significant number of positive blood cultures as previously described in studies [26]. Only 7.4% of our patients were HIV positive, which is an uncommon event in this setting. In fact, the first case report of disseminated fusarial infection in an HIV-positive patient described in

the year 2000 [27]. Isolation of *Fusarium* in the respiratory tract was rare among our patients. In fact, fusarial pneumonia occurs almost exclusively in severely immunocompromised patients, especially patients with acute leucemia, and recipients of allogeneic cell transplantation.

Because *Fusarium* species are intrinsically resistant to most antifungal drugs, new approaches are needed for this difficult-to-treat opportunistic mycosis. This study was relevant to establish epidemiological features the of invasive fusariosis, and more importantly, to identify *Fusarium i*solates at species-level and testing them for antifungal susceptibility in the context of invasive fusariosis. Antifungal susceptibilities of isolates causing invasive fusariosis should be determined in all cases to ensure the accurate choice of drug enabling the successful therapy and proper management of the patient. It is important to improve the knowledge and to promote the importance of mycological tests in the aim of an appropriate treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure and Table

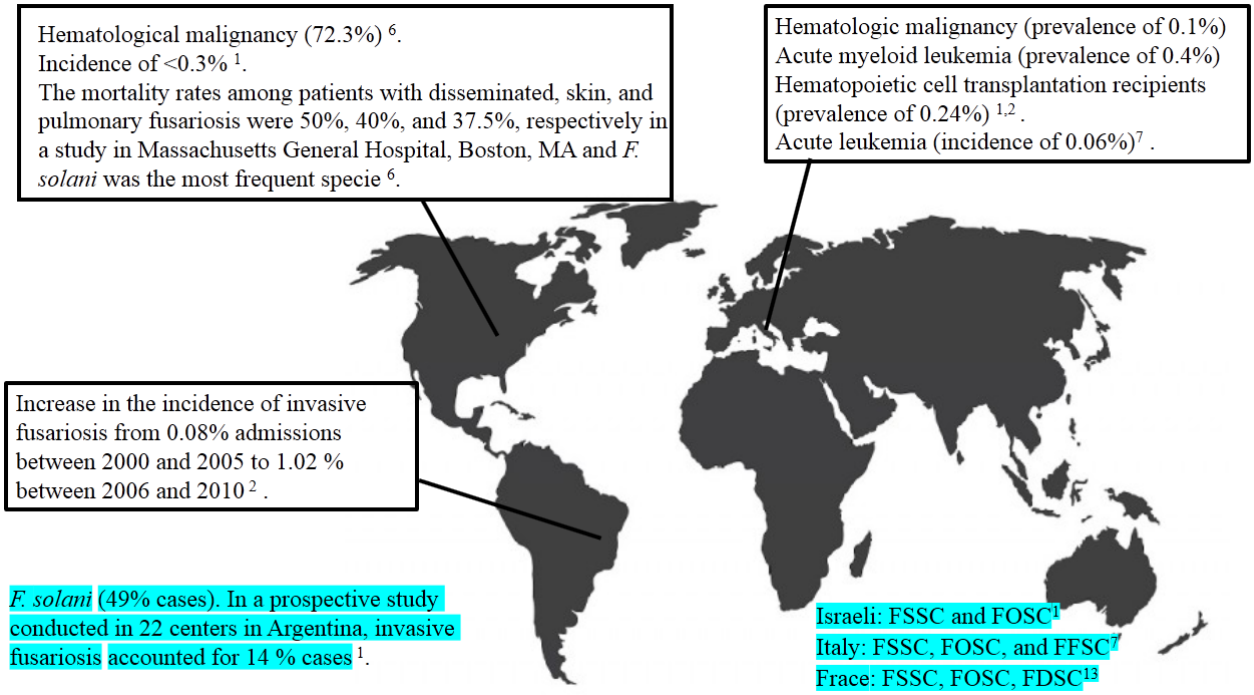

FIG. 1: Epidemiological characteristics of invasive fusariosis

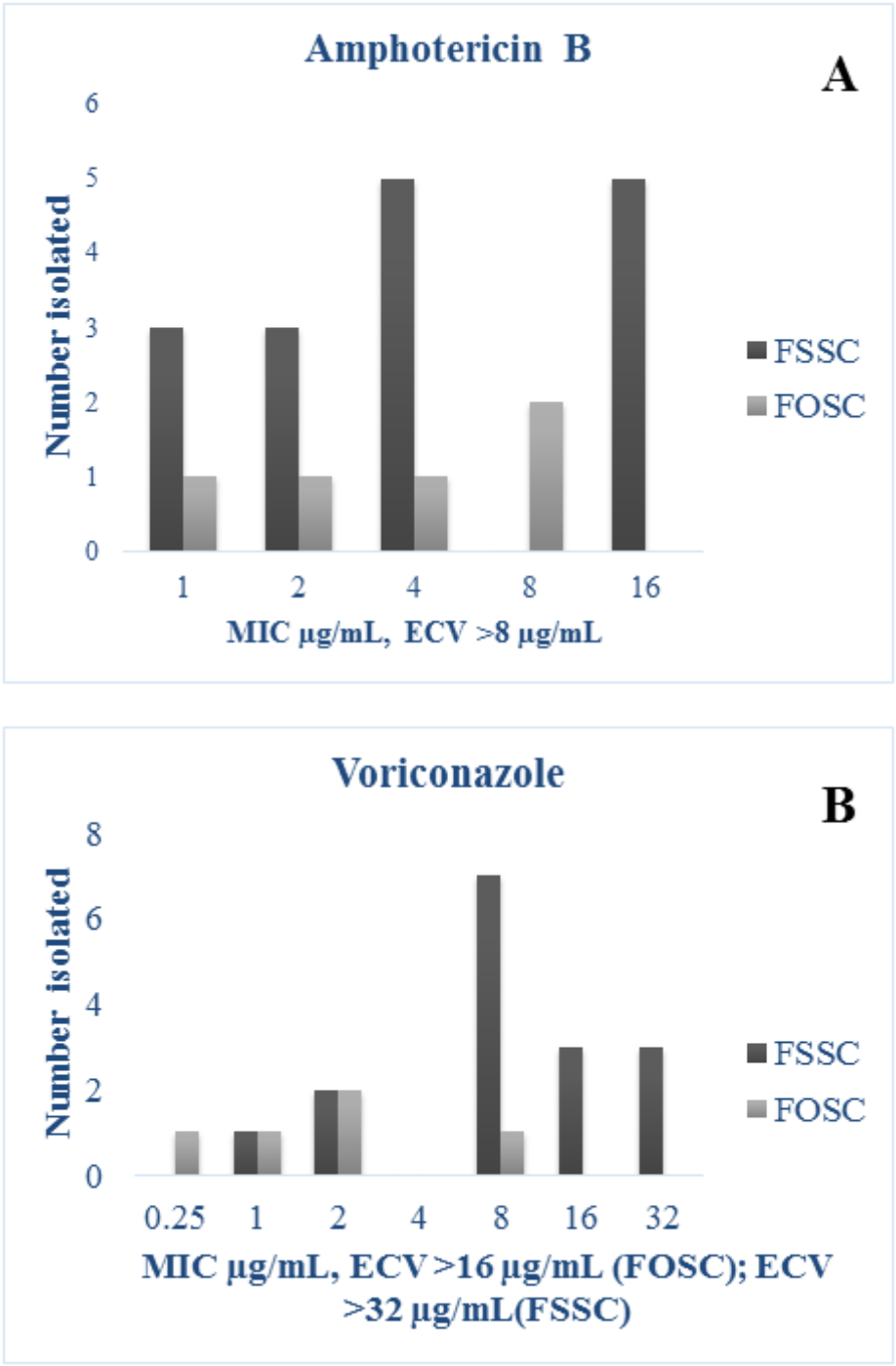

FIG. 2:

Susceptibility profile of *Fusarium solani* species complex (FSSC) and *Fusarium oxysporum* complex (FOSC) for amphoterina and voricolazole, following by Espinel-Ingroff et al. 2016 proposed epidemiological cutoff values (ECVs) of *Fusarium* spp.

TABLE 1: Epidemiological profile of *Fusarium* species as Invasive Fusariosis agent and characteristics of patients living in South of Brazil.

Species complex	Code	Sex	Race	Patient Origin
FSSC				
F. solani	F5	M	C	Porto Alegre
F. solani	F7	M	C	Campo Novo
F. solani	F10	M	C	Novo Hamburgo
F. solani	F13	F	C	Porto Alegre
F. solani	F14	F	C	Santa Cruz do Sul
F. riograndense	F17	M	C	Sapucaia do Sul
F. solani	F18	M	C	Imbé
F. solani	F19	M	A	Porto Alegre
F. solani	F22	F	C	Porto Alegre
F. solani	F26	M	C	Uruguaiana
F. petroliphilum	F30	F	C	São Leopoldo
F. solani	F32	F	A	Porto Alegre
F. keratoplasticum	F27	F	C	Novo Hamburgo
F. keratoplasticum	F31	H	C	Feliz
F. falciforme	F6	M	B	São Jerônimo
F. falciforme	F8	F	C	Santo Antônio da Patrulha
FOSC				
F. oxysporum	F1	M	C	Porto Alegre
F. oxysporum	F4	F	C	Putinga
F. oxysporum	F11	M	C	Viamao
F. oxysporum	F12	M	C	Jabotão dos Guarapés
F. oxysporum	F23	M	C	Porto Alegre
Fusarium sp.	A1	F	C	São Lourenço do Sul
Fusarium sp.	A2	F	C	Porto Alegre
Fusarium sp.	A3	M	C	Novo Hamburgo
Fusarium sp.	A4	F	C	Nova Santa Rita
Fusarium sp.	A5	F	C	Guaíba
Fusarium sp.	A6	M	C	Glorinha

*F=femea; M=male; C=caucasian; A=afrodescendant; B=brown; ABL=Alveolar bronchial lavage; AML=Acute myeloid leukemia; ALL=Acute leukemia; HL=Hodgkin lymphoma; NHL=Non-Hodgkin lymphoma; CML=Cronic myeloid leukemia; MN=Malignant neoplasm; SEL= spinal epidural *lymp*

TABLE 2. Minimum Inhibitory Concentrations (MICs) of antifungals agents for *Fusarium solani* species complex (FSSC) and *Fusarium oxysporum* complex (FOSC)

Species (n° of isolates)	Amphotericin B			Voriconazole			Itraconazole			Fluconazole		
FSSC	Range	GM	Mode	Range	GM	Mode	Range	GM	Mode	Range	GM	Mode
Fusarium solani (10)	1.0 - 16	6.9	4	1.0 - 32	10.1	8	8.0 - >128	98.4	128	>128	128	>128
Fusarium keratoplasticum (2)	16	16		16 - 32	24		16 - >128	72		>128	128	>128
Fusarium falciforme (2)	2.0 - 4.0	3		8	8		>128	128		>128	128	>128
Fusarium petroliphilum (1)	1.0			16			64			>128		
Fusarium riograndense (1)	1.0			8			>128			>128		
FOSC												
Fusarium oxysporum (5)	1.0 - 8.0	8.25	8	0.25- 8	2.65	2	32 - >128	108.8	128	64->128	115.2	>128

8.CONSIDERAÇÕES FINAIS

Neste estudo identificaram-se genotipicamente e fenotipicamente 66 isolados Clínicos de *Fusarium* spp., bem como seu perfil de susceptibilidade aos antifúngicos rotineiramente utilizados na terapêutica. Além disso, decreveu-se o perfil dos pacientes acometidos por onicomicose, ceratite fúngica e fusariose invasiva. Ainda definiu-se a incidência da fusariose invasiva na leucemia linfoide aguda e leucemia mieloide aguda. Também avaliou-se a incidência fúngica nos casos de ceratite. O perfil dos pacientes variou nos três tipos de micose, predominando o sexo feminino nos casos de onicomicose; masculino, nos casos de ceratite e, de maneira igualitária, nos casos de fusariose invasiva; a faixa etária também variou nos três tipos de infecção, predominando a população adulta nas micoses localizadas, onicomicose e ceratite; e apresentando uma variação maior na idade dentro da fusariose invasiva (2-73 anos). Na fusariose invasiva, os pacientes foram principalmente acometidos com leucemia aguda, sendo que predominaram os isolados coletados do trato respiratório (40,7%), do tecido cutâneo (29%) e do sangue (~19%).

Neste estudo constatou-se que existem diferenças entre as espécies e as variedades de complexos. No entanto, o FSSC predominou nas três fusarioses, similarmente ao padrão de prevalência mundial. Nos casos de ceratite fúngica e fusariose invasiva, a espécie mais prevalente foi *F. solani*. Conquanto, em onicomicose foi *F. keratoplasticum* (17/35). Na ceratite fúngica, a maioria dos isolados pertencia ao FSSC (*F. solani*, *F. keratoplasticum e F. falciforme*) e ao FOSC assim como nas outras duas micoses avaliadas. Entretanto, foram identificadas espécies do FIESC, e FFSC. Esse estudo foi descrito no artigo de número 4, no qual 90% dos casos de ceratite eram de pacientes com o sistema imune íntegro, apenas um paciente era imunocomprometido, com leucemia linfóide aguda. O fator predisponente para infecção foi o trauma mecânico (70%). Verificou-se que a maioria dos pacientes (60%) foi tratada apenas com um único antifúngico, 40% com a combinação de antifúngicos tópicos e sistêmicos, e 30% dos pacientes foram necessários o transplante de córnea (ceratoplastia). A sensibilidade aos antifúngicos demonstrou que AMB e natamicina foram os antifúngicos mais efetivos para inibir o fungo *in vitro* (https://www.ncbi.nlm.nih.gov/pubmed/29779647). Ambos agem na membrana plasmática do fungo, alterando a permeabilidade seletiva desta, o que é essencial à sobrevivência do microrganismo.

A análise realizada, unificando os dados do perfil de sensibilidade para três fusarioses, demonstrou estatisticamente que os isolados do FSSC são mais resistente que os do FOSC para o VRC, considerando o cut-off ≥ 32 µg/mL. Outro dado significativo pelo teste do Qui-quadrado (X^2) foi o perfil de sensibilidade dos isolados de fusariose invasiva, o qual foi mais sensível ao voriconazol quando comparado com as outras duas fusarioses.

Houve uma tendência à resistência nos isolados de FOSC no perfil da anfotericina-B, sobre os quais a metade apresentou o MIC maior ou igual ao cut-off (≥ 8 µg/mL), enquanto no FSSC foi apenas 27,7%, no entanto não apresentou diferença estatística entre os complexos. Quando avaliada a relação da resistência nas três fusarioses, observou-se valores acima do cut-off em 31,8%, 25% e 37,1% dos isolados pelo teste do Qui-quadrado (X^2), respectivamente para fusariose invasiva, ceratite e onicomicose.

O valor cut-off do VRC é superior ao do antifúngico da AMB para espécies de *Fusarium*, sendo duas diluições posteriores na microplaca do teste de susceptibilidade. Ao comparar o valor do cut-off do *Aspergillus*, que é outro fungo filamentoso-hialino, cuja resistência é atribuído a valores iguais ou superiores a 2 µg/mL. Dessa forma, as espécies do FSSC seriam resistentes em 88,2% dos casos de fusariose invasiva e de onicomicose para VRC; Enquanto para AMB seriam em 81,8% e em 75% nas mesmas doenças, considerando os complexos FSSC e FOSC.

A fusariose invasiva foi descrita nos artigos 6 e 7, tema esse relevante devido as altas taxas de mortalidade em pacientes imunocomprometidos; as espécies mais isoladas foram, *F. solani* (n=10*), F. oxysporum* (n=5), *F. keratoplasticum* (n=2), *F. falciforme* (n=2), *F.petroliphilum* (n=1), *F. riograndense* (n=1) e *Fusarium* sp. (n=6).

Em relação ao perfil de susceptibilidade para anfotericina-B, a espécie *F. solani* foi semelhante nos três tipos de fusariose (AMB; 1-16 µg/ml). De maneira geral, o perfil de susceptibilidade para voriconazol (VRC) dos isolados dos casos de onicomicose e de ceratite fúngica foram mais resistentes do que os isolados dos pacientes com fusariose invasiva. Estatisticamente o FSSC foi mais resistente que o FOSC em todas as micoses estudadas. Verificou-se resistência *in vitro* para fluconazol e itraconazol nas três fusarioses. Observou-se que a medicação mais apropriada para tratar onicomicose foi o ciclopirox, para ceratite foi a natamicina e para fusariose invasiva foi VRC combinado com AMB. Todos corroborando com os menores valores de CIM no teste de susceptibilidade *in vitro*.

A partir da identificação molecular, foi possível verificar a existência de uma nova espéci183

e pertencente ao FSSC, a qual foi denominada de *Fusarium riograndense*. Esse foi o 5˚ artigo apresentado nesta tese, publicado na revista *Journal de Mycologie Médicale* (https://www.ncbi.nlm.nih.gov/pubmed/?term=Fusarium+riograndense) .

Caracterizaram-e fenotipicamente as espécies, a fim de criar um padrão em relação ao perfil molecular para facilitar na identificação, foi apresentado esse estudo no artigo de número 7. Outro caso que surgiu pela possibilidade do sequenciamento foi a identificação do fungo filamentoso *Lasiodiplodia theobromae* (https://www.ncbi.nlm.nih.gov/pubmed/29383575), isolado de ceratite, no qual existiam apenas 14 publicações no PubMed. Também descreveu-se um relato de caso de um isolado de *F. solani* multirresistente, tanto aos poliênicos quanto aos azólicos, que infelizmente levou a enucleação do globo ocular do paciente (Artigo 3).

O primeiro artigo foi uma revisão de onicomicose, e o segundo foi uma investigação do aumento de casos de onicomicose, em que se definiu que 71,4% dos isolados pertenciam ao FSSC, seguidos por 28,5% dos isolados ao FOSC. Inesperadamente, relatou-se o primeiro caso de *Neocosmospora rubicola* isolado de um paciente com onicomicose (https://www.ncbi.nlm.nih.gov/pubmed/28657120). A anfotericina-B foi o antifúngico mais eficaz *in vitro* (60%, CIM ≤ 4 μg/mL), seguido pelo voriconazol (34,2%, CIM ≤ 4 μg/mL). Em geral, as espécies de *Fusarium* apresentaram valores de CIM> 64 μg/mL para fluconazol, itraconazol e terbinafina.

A investigação das espécies de *Fusarium* mais prevalentes na Região Sul é extremamente relevante, não só como um dado epidemiológico, mas a fim de esclarecer uma panorâmica das espécies emergentes, que até então eram consideradas apenas fitopatógenos e hoje causam infecção em humanos e são tão resistentes, que não somente se tornaram um desafio, como uma ameaça à saúde pública.

9. PERSPECTIVAS FUTURAS

Em relação ao presente projeto, visto que alguns materiais e dados podem ser (re)utilizados em outros projetos. Elencamos as seguintes perspectivas:

- Desenvolver um biobanco e um biorepositório das espécies de *Fusarium* do RS;
- Avaliar o efeito sinérgico dos antifúngicos disponíveis e de outros fármacos pela técnica do *Checkboard* frente a esses isolados de *Fusarium* spp.;
- Testar novos compostos, tais como extrato bruto, óleo vegetal ou animal, a fim de avaliar alguma atividade inibitória sobre esses fungos;
- Avaliar os fenótipos de resistência e verificar a partir de protocolos já propostos para outros fungos a presença e expressão de genes de resistência na espécie de *Fusarium*;
- Avaliar a produção de Biofilme das cepas de *Fusarium* spp. resistentes;
- Estudar os mecanismos de resistência aos azólicos e poliênicos;
- Avaliar a aplicação de RNA de interferência a fim de silenciar os mecanismos de resistência;
- Descobrir novas espécies a partir do sequenciamento;
- Estabelecer parcerias com outros grupos de pesquisa, visando o compartilhamento de conhecimento e de estrutura laboratorial para novos projetos. Aumentar o número de publicações.

É simplesmente inesgotável os diversos campos que podem ser investidos na pesquisa com essa espécie. Muitos estudos ainda são necessários para que se desenvolvam terapias mais efetivas contra o *Fusarium* sp., o ideal seriam estudos multicêntricos para assim definir novas diretrizes e protocolos de tratamento.

10. ANEXOS E/OU APÊNDICES

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (paciente ou responsável)
Nº do projeto GPPG ou CAAE: 52251115.4.0000.5327

Título do Projeto: CARACTERÍSTICAS EPIDEMIOLÓGICAS NO PERFIL DE INFECÇÕES POR FUSARIOSE INVASIVA EVIDENCIADO PELA TÉCNICA DE *MULTILOCUS SEQUENCE TYPING* (MLST)

Você (ou a pessoa responsável) está sendo convidado a participar de uma pesquisa cujo objetivo é melhorar o entendimento sobre a epidemiologia desta espécie de fungo *Fusarium* spp. e sua prevalência a nível de espécie nos casos de Fusariose invasiva , além de aperfeiçoar o entendimento do seu antifungigrama e no seu perfil de susceptibilidade as drogas testadas. Esta pesquisa está sendo realizada pelo Laboratório Especial de Doenças Infecciosas do CPE do Hospital de Clínicas de Porto Alegre (HCPA).

Se você aceitar participar da pesquisa, os procedimentos envolvidos em sua participação são os seguintes: apenas autorizar por escrito a pesquisa dos seus dados no prontuário.

Não existem possíveis riscos ou desconfortos decorrentes da participação na pesquisa.

Os possíveis benefícios decorrentes da participação na pesquisa são indiretamente, pois a participação na pesquisa não trará benefícios diretos aos participantes, porém, contribuirá para o aumento do conhecimento sobre o assunto estudado, e, poderá beneficiar futuros pacientes em relação à terapia com antifúngicos.

Sua participação na pesquisa é totalmente voluntária, ou seja, não é obrigatória. Caso você decida não participar, ou ainda, desistir de participar e retirar seu consentimento, não haverá nenhum prejuízo ao atendimento que você recebe ou possa vir a receber na instituição.

Não está previsto nenhum tipo de pagamento pela sua participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos.

Caso ocorra alguma intercorrência ou dano, resultante de sua participação na pesquisa, você receberá todo o atendimento necessário, sem nenhum custo pessoal.

Os dados coletados durante a pesquisa serão sempre tratados confidencialmente. Os resultados serão apresentados de forma conjunta, sem a identificação dos participantes, ou seja, o seu nome não aparecerá na publicação dos resultados.

Caso você tenha dúvidas, poderá entrar em contato com o pesquisador responsável Priscila Dallé da Rosa, pelo telefone 51 33598836, ou com o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 33597640, ou no 2º andar do HCPA, sala 2227, de segunda à sexta, das 8h às 17h.

Esse Termo é assinado em duas vias, sendo uma para o participante e outra para os pesquisadores.

_____

Nome do participante da pesquisa

_____

Assinatura

_____

Nome do pesquisador que aplicou o Termo

_____

Assinatura

Local e Data: _____