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Molecular epidemiology and antifungal susceptibility patterns of *Sporothrix schenckii* isolates from a cat-transmitted epidemic of sporotrichosis in Rio de Janeiro, Brazil

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Since 1998 a cat-transmitted epidemic of sporotrichosis has been observed in Rio de Janeiro (RJ), Brazil. Besides the lymphocutaneous and fixed forms, other presentations, such as disseminated cutaneous and mucosal involvement, as well as for the first time, erythema nodosum and erythema multiforme have been reported associated with sporotrichosis. This study investigates the phenotypes and genotypes of *Sporothrix schenckii* isolates recovered from different clinical forms of the disease noted as part of this epidemic. A total of 88 isolates recovered from 59 cases associated with the epidemic and 29 controls (from cases in other Brazilian regions and Spain) were included in this study. *In vitro* susceptibility testing was conducted as part of the phenotypic analysis, while the genotypic analysis involved a DNA fingerprinting method with primer M13 and ribosomal DNA sequencing of the internal transcribed spacer (ITS). MIC values of amphotericin B, itraconazole, posaconazole, ravuconazole, and terbinafine were found to be significantly lower ($P<0.01$) for isolates associated with the epidemic than for control strains. No differences in MIC values were observed related to clinical forms of the infection. Fingerprinting analysis showed that RJ epidemic strains were genetically related. Although nine subtypes were found, they were not associated with specific clinical forms. Similar results were obtained with the ITS sequence analysis. These data suggest that the strains isolated from the epidemic cases of sporotrichosis in RJ all originated from a common source.

Keywords *Sporothrix schenckii*, *in vitro* susceptibility, PCR fingerprinting, clinical forms, ITS region

Introduction

Sporotrichosis, a subacute or chronic subcutaneous infection caused by the dimorphic fungus *Sporothrix schenckii,* is frequently observed in Latin America countries. Classical infection is associated with traumatic subcutaneous inoculation of soil, plants, and organic matter contaminated with *S. schenckii*. Rare cases may result from transmission of the etiologic agent from infected animals [1,2].

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The most common clinical form of sporotrichosis is the cutaneous lymphatic disease, accounting for 75% of cases, followed by localized cutaneous infections (20%) [1]. Osteoarticular and pulmonary sites are among the most common extra-cutaneous forms of sporotrichosis. Mucosal involvement is uncommon, with the conjunctiva being preferentially affected [1,3]. Meningitis is one of the worst complications of *S. schenckii* infection, and several of the most recently reported cases describe AIDS as the underlying risk factor [4–6].

Recent molecular epidemiologic studies have addressed the question of the genetic diversity of *S. schenckii* [7–9]. Some of these investigations have demonstrated that clusters of *S. schenckii* strains are related to geographical origin. However, the association between genotyping and clinical forms is still controversial [10–13].

Since sporotrichosis is not reportable in most states of Brazil, there is little information concerning the incidence of the disease. It has been described primarily in São Paulo (SP) and Rio Grande do Sul, mostly in males engaged in activities which would expose them to the etiologic agent [14–16]. Isolated cases of sporotrichosis apparently involving the transmission of the etiologic agent from infected cats have been reported in SP [17,18].

Starting in 1998, a sporotrichosis epidemic affecting humans, cats, and dogs (in smaller proportions), has been described in the metropolitan region of Rio de Janeiro (RJ), mainly in areas with underprivileged socio-economic conditions and precarious health services [19–21]. Between 1998 and 2004, a total of 759 humans, 1,503 cats, and 64 dogs were diagnosed with sporotrichosis by isolation of *S. schenckii* from different types of samples [22] at the Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz. The most frequently affected individuals were homemakers taking care of cats with sporotrichosis [19]. There were peculiar aspects reported in this epidemic. Besides the most frequent lymphocutaneous and fixed forms of the disease, other presentations such as disseminated cutaneous sporotrichosis, without an underlying immunosuppressive condition, and mucosal involvement affecting nasal cavity or conjunctiva were observed [19,23,24]. In addition, erythema nodosum and erythema multiforme were reported associated with sporotrichosis cases from this epidemic [25,26]. Furthermore, symptoms of arthralgia and arthritis were also described in some cases. Spontaneous regression of the mycosis was observed although it has been rarely reported in the literature [19].We considered that one of the possibilities for these uncommon manifestations might be associated with the mechanism of transmission of sporotrichosis. Cats with sporotrichosis usually present a high fungal burden in their lesions, and as they are pets, they are in close contact in the household environment, leading to a greater exposure to the fungus [27]. Reis *et al*. studied 15 cases from this epidemic and demonstrated by molecular methods the relationship between the strains of *S. schenckii* recovered from patients and those isolated from their cats [28]. Here we describe the genotypic analysis and *in vitro* antifungal susceptibility of *S. schenckii* isolates recovered from patients presenting different clinical forms from the epidemic in Rio de Janeiro. A PCR based DNA fingerprinting method (amplified fragment length polymorphism) was used to identify different population strains. DNA sequencing of the ribosomal internal transcribed spacer (ITS) was performed as well, in order to determine the evolutionary relationship among different populations of *S. schenckii*.

Materials and methods

Fungal specimens

Eighty-eight *S. schenckii* strains were included in this study, including 59 clinical isolates obtained between 1999 and 2004 from the IPEC, Fiocruz. These 'epidemic' strains are available in the culture collection of the Mycology Branch of Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz, Brazil. They were initially recovered from different anatomic sites such us skin, eyes, nose, and cerebrospinal fluid. All were identified as *S. schenckii* by their colony morphology on potato dextrose agar (PDA) and brain-heart infusion agar (BHI) as well as their microscopic appearance, i.e., septate hyaline hyphae, conidiophores, and typical conidia on culture media at 25°C. The dimorphic characteristic of the etiologic agents was demonstrated by converting it to the yeast form at 37°C and observing the typical oval to cigar-shaped yeast cells.

Twenty-nine control strains from different geographic origins were also included, i.e., 15 from the Adolpho Lutz Collection, SP, Brazil, a state 600 km from RJ, 11 from the Spanish National Center for Microbiology Mycelial Collection, and three strains from the Instituto Oswaldo Cruz /Fiocruz Filamentous Fungi Collection which had been isolated from RJ up to 72 years before the epidemic (1926, 1959, 1984).

Patients

Clinical and epidemiology features. The sporotrichosis clinical cases were classified according to Sampaio and Lacaz [29] and the characteristics of the patients are presented on Table 1. Twenty patients were male

Table 1 Clincal isolates of epidemic strains from Rio de Janeiro, Brazil according their year of isolation, clinical form and source of isolation

Case	Isolate	Internal identification	Year of identification	Clinical form	Isolate site
1	IPEC25348	M05-218	2004	Cutaneous lymphatic	Skin
2	IPEC25573	M05-219	2004	Cutaneous lymphatic	Skin
3	IPEC25758	M05-221	2004	Cutaneous lymphatic	Skin
4	IPEC25457	M05-222	2004	Cutaneous lymphatic	Skin
5	IPEC25810	M05-223	2004	Cutaneous lymphatic	Skin
6	IPEC25403	M05-224	2004	Cutaneous lymphatic	Skin
7	IPEC24923	M05-225	2004	Cutaneous lymphatic	Skin
8	IPEC25545	M05-226	2004	Cutaneous lymphatic	Skin
9	IPEC25294	M05-227	2004	Cutaneous lymphatic	Skin
10	IPEC16459	M05-228	2000	Cutaneous lymphatic	Skin
11	IPEC19777	M05-229	2001	Cutaneous lymphatic	Skin
12	IPEC16910	M05-230	1999	Cutaneous lymphatic	Skin
13	IPEC16672	M05-231	1999	Cutaneous lymphatic	Skin
14	IPEC25877	M05-232	2004	Cutaneous lymphatic	Skin
15	IPEC25976	M05-233	2004	Cutaneous lymphatic	Skin
16	IPEC15647	M05-234	1999	Cutaneous lymphatic	Skin
17	IPEC25329	M05-235	2003	Cutaneous lymphatic	Skin
18	IPEC16415	M05-236	1999	Cutaneous lymphatic	Skin
19	IPEC15383	M05-237	2000	Cutaneous lymphatic	Skin
20	IPEC17786	M05-238	2003	Cutaneous lymphatic	Skin
21	IPEC25032	M05-239	2004	Cutaneous lymphatic	Skin
22	IPEC25719	M05-240	2004	Cutaneous lymphatic	Skin
23	IPEC26034	M05-241	2004	Cutaneous lymphatic	Skin
24	IPEC19536	M05-242	2001	Cutaneous lymphatic	Skin
25	IPEC25011	M05-247	2004	Cutaneous lymphatic	Skin
26	IPEC25817	M05-249	2004	Cutaneous lymphatic	Skin
27	IPEC25494	M05-252	2004	Cutaneous lymphatic	Skin
28	IPEC25354	M05-253	2004	Cutaneous lymphatic	Skin
29	IPEC25357	M05-254	2004	Cutaneous lymphatic	Skin
30	IPEC25739	M05-255	2004	Cutaneous lymphatic	Skin
31	IPEC25738	M05-256	2004	Cutaneous lymphatic	Skin
32	IPEC25155	M05-257	2004	Cutaneous lymphatic	Skin
33	IPEC19778	M05-276	2001	Localized	Skin
34	IPEC26156	M05-272	2004	Localized	Skin
35	IPEC26449	M05-275	2004	Localized	Skin
36	IPEC25390	M05-273	2004	Localized	Skin
37	IPEC19540	M05-277	2001	Localized	Skin
38	IPEC19541	M05-278	2001	Localized	Skin
39	IPEC19481	M05-279	2001	Localized	Skin
40	IPEC25854	M05-280	2004	Localized	Skin
41	IPEC25644	M05-274	2004	Localized	Skin
42	IPEC26611	M05-281	2004	Localized	Skin
43	IPEC25319	M05-248	2004	Localized	Skin
44	IPEC21806	M05-251	2004	Localized	Skin
45	IPEC17878	M05-258	2000	Localized	Skin
46	IPEC25712	M05-259	2004	Cutaneous disseminated	Skin
47	IPEC25356	M05-260	2004	Cutaneous disseminated	Skin
48	IPEC25502	M05-261	2004	Cutaneous disseminated	Skin
49	IPEC25507	M05-262	2004	Cutaneous disseminated	Skin
50	IPEC25612	M05-263	2004	Cutaneous disseminated	Skin
51	IPEC25064	M05-264	2004	Cutaneous disseminated	Skin
52	IPEC25683	M05-265	2004	Cutaneous disseminated	Skin
53	IPEC17692	M05-266	2000	Cutaneous disseminated	Skin
54	IPEC25686	M05-250	2004	Cutaneous disseminated	Skin
55	IPEC25521	M05-244	2004	Cutaneous lymphatic+extracutaneous (nasal)	Nasal mucosa
56	IPEC25425	M05-243	2004	Cutaneous lymphatic+extracutaneous (conjunctival)	Conjunctival mucosa
57	IPEC17943	M05-267	1999	Extracutaneous (skin and meningitis)	Cerebrospinal fluid
58	IPEC25303	M05-268	2004	Extracutaneous (skin+bone+nasal)	Skin
59	IPEC22493	M05-269	2003	Cutaneous disseminated+extracutaneous (bone)	Skin

(33.9%) and 39 female (66.1%) with a median age of 42.5 years within a range of 9–73 years. Thirty-two patients (54.2%) presented with the lymphocutaneous form, 13 (22.0%) localized form, 9 (15.2%) cutaneous disseminated form and 5 (8.5%) extracutaneous form. Erythema nodosum was seen in 7 patients (8.4%) and erythema multiforme in five (11.8%).

Treatment and outcomes of the patients with cutaneous sporotrichosis (lymphocutaneous and localized). In these patients (45) itraconazole (ITC) was the drug of the choice and was used at a dose of 100 mg/day in 36 patients, 200mg/day in 4 patients, and at 400 mg/day in one patient. Most patients (89%) were cured within a median period of 3 months (range 1–16 months) but treatment was unsuccessful in four patients (8.8%; cases 2, 16, 18 and 19). Two pregnant women (cases 15 and 21) were treated with the local application of heat and one patient was lost during the follow up.

Treatment and outcomes of the patients with cutaneous disseminated sporotrichosis. ITC was also the drug of choice in these patients (9) and was used in a dose of 100 mg/day in 5 patients, 200 mg/day in 2 patients and at 400 mg/day, in association with fluconazole (FLC) in one patient. One patient was treated with a combination of 725 mg amphotericin (AMB) and 400 mg ITC. Eight patients (88%) were cured in a median of 6 months (range 1–9 months) and one was lost in follow-up.

Treatment and outcomes of the patients with extracutaneous sporotrichosis. Two patients with lymphocutaneus form and mucosa involvement (nasal and conjunctival) were treated with 100 mg ITC for 5 (case 55) and 6 months (case 56). In the other three patients with these forms of the disease (cases 57–59), AMB was used with a cumulative dosage of from 1,080 to 5,890 mg, after which they received ITC 200 mg associated with FLC 200 mg (case 57) or 400 mg (case 58 and 59). All patients were cured in a median of 10 months (range 5–12 months).

Sampling and laboratory analyses were performed according to the Ethics Committee of the Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz.

Antifungal susceptibility testing

The susceptibility testing was conducted in accord with the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) M-38A recommendations, with minor modifications [30]. Briefly, the tests included RPMI supplemented with 2% glucose as the assay medium and an inoculum suspension of 1×10^5 to 5×10^5 CFU/ ml, adjusted by microscopic enumeration with a hematocytometer. The antifungal agents used in this study were AMB (Sigma-Aldrich Quimica SA, Madrid, Spain), FLC (Pfizer SA, Madrid, Spain), ITC (Janssen Pharmaceutica, Madrid, Spain), voriconazole (VRC) (Pfizer, Ltd., Sandwich, United Kingdom), ravuconazole (RVC) (Bristol-Myers Squibb, Princeton, NJ), posaconazole (POS) (Schering-Plough, Kenilworth, NJ), and terbinafine (TRB) (Novartis, Basel, Switzerland). *Aspergillus fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were included as control isolates in each set of experiments. The microplates were incubated for three days at 30°C and visually read with the help of a mirror. Minimum inhibitory concentrations (MIC) were defined as the lowest concentration showing complete inhibition of growth. The tests were performed in duplicate on two different days.

Differences in proportions were determined by Fisher's exact test or Chi-square analysis. The significance of the differences in MIC values was determined by Student's *t* test (unpaired, unequal variance). In order to approximate a normal distribution, all MIC were transformed to $\log_2$ values to establish differences in susceptibilities between the isolates and were expressed by Geometric Mean (GM). A P value <0.01 was considered significant. Statistical analysis was done with the Statistical Package for the Social Sciences (version 13.0; SPSS S.L, Madrid, Spain).

Molecular fingerprinting method

A subset of strains isolated from 41 clinical cases of sporotrichosis associated with the epidemic in RJ was included in this analysis. Seventeen strains were recovered from the lymphocutaneous form, 11 from the localized form, six from the cutaneous disseminated form and six from the extra-cutaneous form. Seven strains were from erythema nodosum-associated sporotrichosis cases, and four strains were from the erythema multiforme-associated sporotrichosis form (Table 2). The control population included seven strains, i.e., three from São Paulo, two from Spain and two historical ones from RJ before the beginning of the epidemic.

Mycelial mats were recovered and subjected to a DNA extraction protocol previously described [31]. After RNAse treatment, samples were subjected to proteinase K (Sigma-Aldrich Quimica), and DNAs were then purified by phenol/chloroform extraction, and ethanol precipitation. The DNA integrity was verified and its concentration determined by running serial DNA

Table 2 Control strain according the year of isolation and origin

Isolate	Internal identification	Year	Origin
IOC 1113	98371010	1926	Rio de Janeiro, Brazil
IOC 1226	98371011	1984	Rio de Janeiro, Brazil
IOC 2843	98371012	1959	Rio de Janeiro, Brazil
107 MA	M05-821	–	São Paulo, Brazil
131	M05-822	1970	São Paulo, Brazil
200	M05-823	1972	São Paulo, Brazil
237	M05-824	1975	São Paulo, Brazil
262 S	M05-825	2005	São Paulo, Brazil
287	M05-826	1976	São Paulo, Brazil
329	M05-827	1975	São Paulo, Brazil
424	M05-828	1970	São Paulo, Brazil
430	M05-829	1972	São Paulo, Brazil
440	M05-830	1958	São Paulo, Brazil
441	M05-831	1952	São Paulo, Brazil
444	M05-832	1967	São Paulo, Brazil
576	M05-833	1977	São Paulo, Brazil
579	M05-834	1974	São Paulo, Brazil
840	M05-835	2000	São Paulo, Brazil
45	M91-36	1991	Spain
412	M93-184	1993	Spain
503	M94-10	1994	Spain
521	M94-67	1994	Spain
660	–	–	Spain
776	M96-146	1996	Spain
938	M97-150	1997	Spain
1403	M99-333	1999	Spain
2022	M01-1034	2001	Spain
2988	M04-510	2004	Spain
3022	M04-644	2004	Spain

dilutions in 0.8% agarose gels along with a commercial bacteriophage lambda DNA of known concentration (Pharmacia, Barcelona, Spain). The DNA concentration was adjusted to 5ng/ml for typing methods.

A PCR technique using the minisatellite-core sequence oligonucleotides of the wild-type phage M13 (5′-GAGGGTGGCGGTTCT-3′) was employed as single primers to amplify hypervariable genomic DNA sequences from 47 representative *S. schenckii* strains [32]. Amplification reactions (25 µl) consisted of 25 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.2 mM (each) dNTPs (Invitrogen, RJ, Brazil), 3 mM magnesium acetate, 2.5 U of *Taq* polymerase (Invitrogen), and 30 ng of primers. Forty cycles of 20 s at 94°C, 1 min at 50°C, and 20 s at 72°C were performed, followed by a single extension cycle at 72°C for 6 min. In order to estimate PCR products size and to perform gel normalization, molecular weight markers consisting of a 100 bp DNA ladder (Invitrogen) were concurrently electrophoresed. The amplicons were analyzed on a 1.5% agarose gel containing .5 µg of ethidium bromide/ml at 60 V for 5 h, and were visualized under UV light. All visualized bands on the gel were counted, and data was scored for the presence or absence of amplification products, regardless of their intensity. The reproducibility of this method was confirmed when identical electrophoretic profiles were observed in PCR assays repeated at least three times under the same conditions. The similarity coefficient for each isolate was calculated using the Jaccard algorithm, with the NTSYSpc version 2.02 Applied Biostatistics Inc. For clustering, the unweighted paired-group method with arithmetic means (UPGMA) was used.

For sequencing and phylogenetic analyses DNA segments from epidemic and control strains, comprising ITS1, 5.8S and ITS-2 regions were amplified with primers ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-CCTCCGCTTATTGATATGC-3′) [34]. The reaction mixtures contained 0.5 M of each primer, 0.2 mM of each deoxynucleoside triphosphate, 5 µl of PCR (10X) buffer (Applied Biosystems, Madrid, Spain), 2.5 U *Taq* DNA polymerase (Amplitaq; Applied Biosystems), and 25 ng of DNA in a final volume of 50 µL. Samples were amplified in a GeneAmp PCR System 9700 (Applied Biosystems) by using the following cycling parameters; one initial cycle of 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 56°C, and 2 min at 72°C, with one final cycle of 5 min at 72°C. The reaction products were resolved in a 0.8% agarose gel and visualized with an UV lamp after ethidium bromide staining. Sequencing reactions were done using 4 µl of the sequencing kit (BigDye Terminator cycle sequencing ready reaction; Applied Biosystems), 1 µM of the primers (ITS1, ITS4) and 3 µl of the PCR product in a final volume of 10 µl.

Sequences were assembled and edited by using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, Wis.). All phylogenetic analyses were conducted with Fingerprinting II Informatix software, version 3.0 (Bio-Rad Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2,000 simulations. Phylograms were rooted to outgroups comprising *Rhizopus oryzae* and *Rhizopus microsporum* belonging to the Spanish National Center for Microbiology Mycelial Collection.

Results

Antifungal susceptibility testing

Susceptibility testing results are presented in Table 3. Significant results were observed when MIC values were compared to the origin of the isolates. Initially,

Table 3 Susceptibility testing results in μg/ml of 88 *Sporothrix schenckii* isolates according to their origin

Origin	AMB		ITC		VRC		RVC		POS		FCL		TRB	
	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}
Epidemic strains from Rio de Janeiro $n=59$	0.79 (0.25–2.0)	1/2	0.44 (0.12–8.0)	0.5/1	12.5 (1–>8)	>8/>8	1.27 (0.12–8.0)	1/4	0.54 (0.06–2.0)	0.5/1	>64.0 (64)	>64/64	0.14 (0.02–1.0)	0.12/0.25
Control strains from Rio de Janeiro $n=3$	1 (0.5–2.0)	1/1	3.17 (1–>8)	2/>8	6.34 (1–>8)	>8/>8	4.0 (1–8)	8/8	2.51 (1.0–8.0)	2/8	–	–	0.25 (0.25)	0.25/0.25
Control strains from São Paulo $n=15$	1.28 (0.50–4.0)	1/2	2.29 (0.12–>8.0)	1/>8	12.4 (1–>8)	>8/>8	6.6 (1–>8)	8–>8	1.5 (0.25–>8.0)	1/8	>64.0 (64)	>64.0	0.44 (0.06–>16)	0.5/2
Control strains from Spain $n=11$	1.32 (0.12–2.0)	2/2	3.63 (0.25–>8.0)	>8/>8	10.2 (8–>8)	>8/>8	6.78 (1–>8)	8–>8	1.34 (1.0–8.0)	1/1	>64 (64)	>64.0	0.23 (0.06–1.0)	0.25/0.25

AMB, amphotericin; ITC, itraconazole; VRC, voriconazole; RVC, ravuconazole; POS, posaconazole; FCL, fluconazole; TRB, terbinafine; GM, geometric mean.

MIC for the 29 control strains were comparable and independent of origin of the strains, except for the terbinafine MICs which were somewhat higher for SP control strains than the control isolates from Spain and from RJ. However, significant differences ($P<0.05$) were found between epidemic and control strains, i.e., the MIC values for amphotericin B, itraconazole, ravuconazole, posaconazole and terbinafine of epidemic isolates were clearly lower than those of the control strains. Differences were particularly marked for itraconazole, ravuconazole, and posaconazole, as MIC values for epidemic strains were up to four times lower than those found with control strains.

No significant differences were found with respect to the MIC values of isolates recovered from different clinical forms of the disease of the epidemic (Table 4). Isolates causing cutaneous lymphatic sporotrichosis, localized infection, cutaneous disseminated disease, extra-cutaneous infection, erythema nodosum, and erythema multiforme exhibited comparable MIC values. Differences were not observed either relative to the clinical response of the respective patients.

Fingerprinting analysis

Representative DNA fingerprinting profiles obtained with M13 primer from *S. schenckii* samples are shown in Fig. 1a. Fifteen PCR fingerprinting patterns were generated, indicating a high discrimination among the *S. schenckii* strains isolated in different geographic regions.

Genetic relationships obtained with the UPGMA method are represented as a dendrogram (Fig. 1b). Three major clusters were distinguished at a similar distance of 0.10. Cluster I (the upper branch in Fig. 1a) was composed of three *S. schenckii* control isolates, two from Spain and one from RJ (IOC1113), with the two Spanish strains exhibiting 100% similarity. Cluster II was composed of four control isolates, two from SP and two from RJ with 33% to 82% of relatedness among them. Cluster III included 41 epidemic strains presenting 70% similarity. A clear separation into three subgroups (IIIa, IIIb, IIIc) was observed within this cluster. Subgroup IIIa accounted for six isolates presenting 90% to 100% relatedness. Subgroup IIIb was composed of 32 organisms, with a high degree of relatedness among them (96%), and included all cases of erythema nodosum (Fig. 1b). Three isolates comprised subgroup IIIc. The analysis of the 41 clinical isolates from the sporotrichosis epidemic in RJ revealed 9 genotypes not associated with distinct clinical manifestations.

Table 4 Susceptibility testing results in μg/ml of 59 *Sporothrix schenckii* isolates from epidemic cases of Rio de Janeiro according to different clinical form

Origin	AMB		ITC		VRC		RVC		POS		FCL		TRB	
	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}	GM (Range)	MIC_{50}/ MIC_{90}
Cutaneous* $n=45$	0.83 (0.25–2.0)	1/2	0.44 (0.12–8.0)	0.5/1	12.95 (1–>8)	>8/>8	1.26 (0.12–8.0)	1/4	0.51 (0.06–2.0)	0.5/1	>64.0 (64)	>64/64	0.14 (0.03–1.0)	0.12/0.25
Cutaneous disseminated* $n=9$	0.63 (0.25–1.0)	0.5/1	0.44 (0.25–1.0)	0.5/1	11.54 (8–>8)	>8/>8	1.33 (0.5–4.0)	1/4	0.63 (1.0–8.0)	0.5/1	–	–	0.12 (0.02–0.25)	0.12/0.25
Extra-cutaneous $n=5$	0.77 (0.25–1.0)	0.5/1	0.5 (0.25–1.0)	0.5/0.5	10.6 (1–>8)	>8/>8	1.2 (1–>8)	1/4	0.66 (0.25–>8.0)	0.5/1	>64.0 (64)	>64.0	0.24 (0.12–0.5)	0.25/0.5

AMB, amphotericin; ITC, itraconazole; VRC, voriconazole; RVC, ravuconazole; POS, posaconazole; FCL, fluconazole; TRB, terbinafine; GM, geometric mean. *Includes cases with erythema nodosum and multiforme.

Sequencing and phylogenetic analyses

Two groups were revealed in the phylogenetic trees of ITS regions obtained with the maximum parsimony clustering. The first group was composed of Spanish isolates and the second of Brazilian isolates. No distinction was observed among strains from SP and RJ. Spanish isolates exhibited 100% similarity, whereas Brazilian isolates showed 98.9%. Similarity among the two different groups was around 90% (Fig. 2). The sequences of some isolates were deposited in GenBank under the accession numbers: M05-240 (Bankit1013571, EU126940); M05-243 (Bankit1013600, EU126941); M05-247 (Bankit1013605, EU126942); M05-247 (Bankit1013605, EU126942); M05-252 (Bankit1013620, EU126943); M05-259 (Bankit1013653, EU126944); and M05-267(Bankit1013693, EU126945).

Discussion

The present study evaluated the phenotypic and genotypic characteristics of *S. schenckii* isolates obtained from 59 clinical cases, grouped by clinical manifestations, which were recovered as part of an 9 year epidemic of sporotrichosis in RJ. This sampling represents approximately 5% of cases diagnosed thus far in our Institution.

The *S. schenckii* isolates from RJ were clearly distinguished from control strains by their lower *in vitro* MICs compared to the most common antifungals. Overall, AMB and TRB were the most active agents *in vitro,* while FCL and VRC were inactive against both epidemic and control strains. ITC, RVC, and POS were active against most of the epidemic strains, whereas high MIC values were found with these same antifungal agents.

Most of our epidemic cases were treated with ITC, which has become the drug of choice for sporotrichosis due to the good susceptibility of the etiologic agent, easy administration (once a day), and limited toxicity [4,33–35]. Although AMB is less well tolerated due to toxicity, its use may be indicated in treating patients with extensive involvement or in those in which the use of other antifungals has proven unsuccessful. Recently, it was suggested that disseminated forms of sporotrichosis were less susceptible to ITC than cutaneous forms [35], but these data was not confirmed in this study. Our susceptibility data showed that TRB was the most active drug *in vitro* against *S. schenckii,* as has been previously reported [33]. However, one study demonstrated that elevated doses of the drug (500 mg to 1,000 mg) are needed to ensure clinical efficacy [36]. Further studies are necessary to confirm this data.

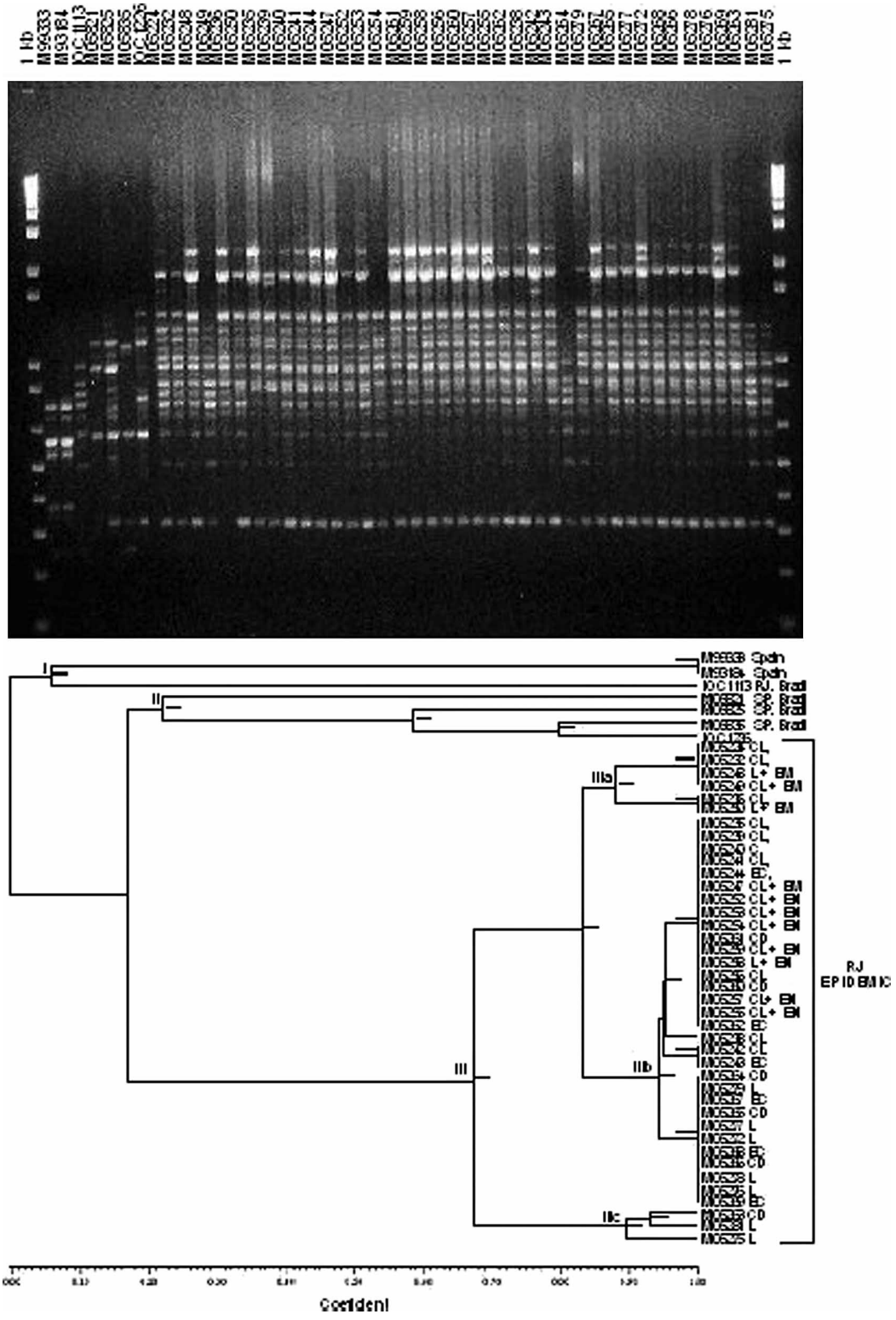

Fig. 1 (a) DNA fingerprinting profiles obtained with primer M13 of the 49 isolates included in this study. DNA molecular marker (DNA ladder, Invitrogen) was loaded in first and last wells. (b) Dendogram of S. schenckii strains based on the UPGMA method derived from analysis of the DNA profiles generated with primer M 13. Distinct clusters are designed by roman numerals. The numbers on the right are internal strains designations. Percentages of similarity (Sab) are shown on the x axis. CL cutaneous lymphatic; L, localized; CD, cutaneous disseminated; EC extracutaneous; EN, erythema nodosum; EM, erythema multiforme.

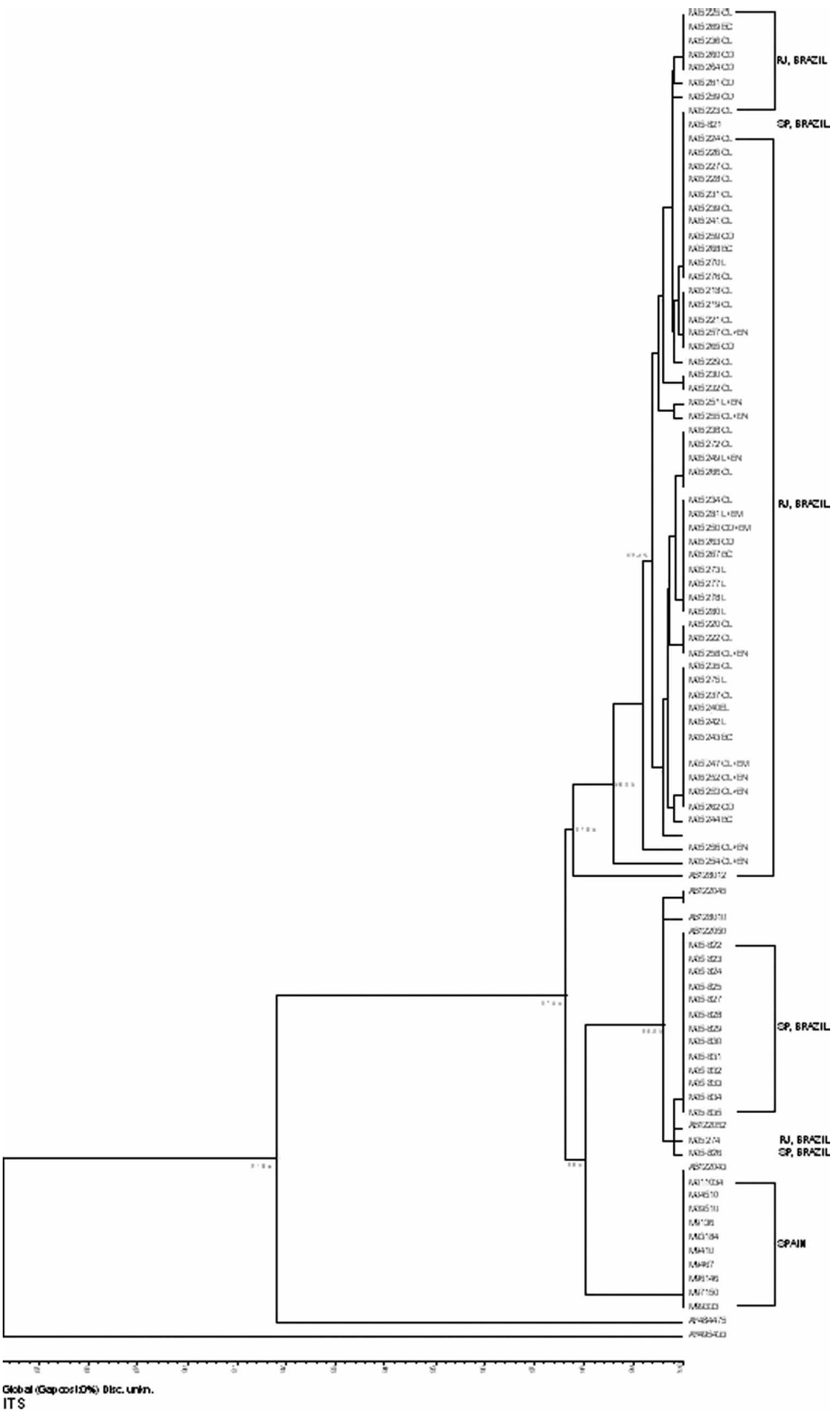

Fig. 2 Phylogram expressing distances (UPMG) between ITS2 sequences of strains of different origins. Control strains: *Sporothrix schenckii* (AB122043, AB122050, AB128010, AB122045); *Sporothrix schenckii* var. *luriei* (128012); *Ophiostoma stenoceras* (AF 484475), *Sporothrix inflata* (AY 495433).RJ, Rio de Janeiro; SP, São Paulo; CL, cutaneous lymphatic; L, localized; CD, cutaneous disseminated; EC extracutaneous; EN, erythema nodosum; EM, erythema multiforme.

Although *S. schenckii* can be resistant to FLC, it has been used in treatment of sporotrichosis at doses of 800 mg daily for patients who do not tolerate ITC or AMB [4]. In this study, FLC was inactive *in vitro* against all strains but it was used successfully in combination with ITC in one patient.

Overall, Rio de Janeiro epidemic *S. schenckii* isolates were separated from control strains by all molecular methods used in this study. Although the phylogenetic analyses showed great similarity among isolates of different origin, control strains (Spain and Brazil) presented higher genetic relatedness between them than with RJ epidemic strains. This may suggest that epidemic strains may be environmentally/geographically related.

In PCR fingerprinting analysis, 9 subtypes were generated for the epidemic strains from RJ. However, this diversity was not correlated with clinical forms of sporotrichosis, suggesting that the main factor determining sporotrichosis clinical forms is more likely related to the immune condition of the patient [37] and the route of transmission, rather than the strain genotype. Three extra-cutaneous forms presented here (Table 1, cases 57–59) were associated with AIDS (2 cases) and with nocardiosis (1 case). However, it is interesting to note that there was only one genotype associated with all cases of erythema nodosum. Fungal cells often contain a wide range of allergens and this particular genotype might be more allergenic than others, which could contribute to the development of erythema nodosum.

Recently, Guarro *et al*. [38] described 18 clinical isolates obtained from 11 patients with distinct clinical manifestations from this outbreak and found 6 highly related distinct genotypes. Other studies have confirmed that *S. schenckii* isolates were not associated with specific clinical forms of sporotrichosis [10,11]. However, Xiaoming found substantial differences in RAPDs profiles when he compared one isolate recovered from a patient with the disseminated form to three isolated from patients with fixed cutaneous or lymphatic forms [13]. Tachibana *et al*. reported that the proprieties of clinical isolates and the routes of infection are critical for the establishment and the development of systemic infection. This manifestation was observed only with *S. schenckii* isolates obtained from a human lung lesion after intravenous or intraperitonial injection in BALB/c mice [39]. Another experimental study confirmed that different genotypes were related to clinical forms of sporotrichosis [40].

Our phenotypic and genotypic analyses have concluded that isolates from the Rio de Janeiro epidemic have a high genetic similarity, which is suggestive of a common niche. It was possible to discriminate 9 genotypes. The high percentage of genetic similarity demonstrated among these isolates suggests the possibility that only one virulent genetic population was present in this micro ennvironment. These different genotypes were not clearly associated with distinct clinical form of sporotrichosis and notably, the strains did not change during the five year period that these strains were collected.

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