Journal de Mycologie Médicale 28 (2018) 617–622

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Research Paper

Mucormycosis outbreak due to *Rhizopus microsporus* after arthroscopic anterior cruciate ligament reconstruction surgery evaluated by RAPD and MALDI-TOF Mass spectrometry

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ARTICLE INFO

Article history:
Received 17 April 2018
Received in revised form 6 July 2018
Accepted 4 September 2018
Available online 22 September 2018

Keywords:
Rhizopus
Cruciate ligament
Mucormycosis
Outbreak
Fingerprinting
MALDI-TOF strain typing
Healthcare-associated mucormycosis

ABSTRACT

Background. – *Rhizopus microsporus* is one of the main causative agents of mucormycosis. These mycoses are mostly described as isolated cases involving uncontrolled diabetes mellitus or immunosuppressed patients. In this work we report a nosocomial outbreak of mucormycosis due to *R. microsporum* involving three young immunocompetent patients whom underwent arthroscopic anterior cruciate ligament reconstruction surgery in a seven-month time span.
Procedures. – During the outbreak period, a total of 32 surgeries of this type were performed in the clinic (mucormycosis prevalence of 9.375%). The three patients presented healthcare-associated Mucormycosis comprising the bone surrounding one of the fixation screws (femoral or tibial). In addition to these three strains, another three *R. microsporus* strains isolated in the medical center during the same period of time were included in the study. One of these fungi was isolated from a skin lesion of a kidney transplant patient while the other two strains were isolated from environmental sources. Classical, mass spectrometry-based (MALDI-TOFF) and molecular identification were performed. Genetic relatedness was established by Rep-PCR (RAPD variant) and by single-linkage cluster analysis mass spectra. Cluster analysis was performed by unweighed pair group method with arithmetic mean (UPGMA).
Main findings. – All the strains were identified as *R. microsporum* by the used phenotypic and genetic tools. Clinical strains fell into 2 different clusters separating the renal transplant recipient strain from the three strains isolated post ACLR surgery, which clustered together.
Conclusions. – The established genetic/mass spectra relatedness between the three post-surgery isolates suggests that these cases may be considered a healthcare-associated mucormycosis outbreak.

1. Introduction

Rhizopus microsporus is one of the main causative agents of mucormycosis [1]. There were four known varieties of *R. microsporus* but only *R. microsporus* var. *microsporus* and *R. microsporus* var. *rhizopodiformis* were reported as human pathogens [1]. In 2014, the described varieties were reduced to synonyms based on the combination of mating test results and the analysis of different molecular, enzyme and protein markers [2]. Mucormycosis are mostly described as isolated cases involving uncontrolled diabetes mellitus or immunosuppressed patients [3] while nosocomial outbreaks are infrequently reported [4,5]. Here we describe a nosocomial outbreak of mucormycosis due to *R. microsporum* involving three patients whom underwent arthroscopic anterior cruciate ligament reconstruction (ACLR) surgery in a seven-month time span. We used a genetic and a proteomic approach to establish the isolates relatedness. Moreover, we confirmed the

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https://doi.org/10.1016/j.mycmed.2018.09.002
1156-5233/

infection by a PCR technique capable to diagnose mucormycosis in biopsies identifying the etiological agent to a species level.

2. Material and methods

2.1. Strains

Nine *R. microsporus* strains were studied in this work. Three of them were isolated from necrotic bone tissue obtained from ACLR surgery patients during a 7-month period (December 2010–July 2011). During the same time span, a forth clinical isolate was obtained from a surgical wound infection suffered by a renal transplant recipient and two more were isolated from the institution's environment. Surgery rooms' surfaces and pharmacy shelf surfaces in contact with the surgical material were sampled and cultured in malt extract agar with chloramphenicol searching for Mucorales. Fifteen air and surface samples were obtained a day after the second and third post-surgical infection confirmation. Two *R. microsporus* var. *rhizopodiformis* strains were obtained (one in each of the sampling days). Both strains were isolated from pharmacy shelves surfaces where the fixation screws and all the equipment used in the ACLR surgeries were stored. These strains were included in the study and were named as LMDM-168 and LMDM-167. The remaining three strains were used as outgroup controls and included *R. microsporus* CBS 536.80 and two clinical strains (LMDM-379 and LMDM-596) isolated in Buenos Aires and Salta cities (Argentina) in 2007 and 2014, respectively. To the best of our knowledge, there is no *R. microsporus* type strain. However, we used the strain CBS 536.80 as control since it was used as reference in earlier published reports [6,7]. Moreover, *Mucor circinelloides*, *Lichtheimia corymbifera* and *Syncephalastrum racemosus* strains (one each) were used as control strains for PCR identification. The *L. corymbifera* strain was also used as outgroup species for MALDI-TOF strain typing.

2.2. Strain identification

Strains were received at the Micología y Diagnóstico Molecular Laboratory (Universidad Nacional del Litoral–CONICET) for identification confirmation and genotyping. Isolates were molecularly identified by sequencing of the 5.8S RNA gene and adjacent internal transcribed spacer 1 and 2 regions (ITS1 and ITS2) [8] and by a faster modified PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) method designed for the identification of the main etiological agents of Mucormycosis [7]. This last procedure includes a first step aimed to identify the Mucorales at genera level by 18S ribosomal subunit gene amplification (primers in Table 1) followed by restriction digestions for the identification of Mucorales at species level. Our modification consisted in replacing the restriction enzymes *BmgB1* and *CspCI* with *ClaI* and AseI (Promega-Biodynamics, Argentina). After these digestions, we were able to differentiate *R. stolonifer* (one *ClaI* digestion site), *R. microsporus*/*R. azigosporus* (one *AseI* site) and *R. oryzae* (no *AseI* nor *ClaI* restriction sites). Species identifications were based on band sizes predicted on restriction maps of the sequences published under the following accession numbers: AF113440.1 (for *R. oryzae*), AF113438.1 (*R. microsporus* var. *microsporus*), KM527234.1 (*R. microsporus* var. *rhizopodiformis*), AF157158.1 (*R. microsporus* var. *oligosporus*), FN182235.1 (*R. microsporus* var. *chinensis*), AF113441.1 (*R. stolonifer*) and AB250156.1 (*R. azigosporus*).

2.3. Clinical data and Mycology

The three ACLR patients have no immunosuppressive diseases and or treatment. These patient underwent ACLR surgeries after sport-related ($n = 2$, both male) or traffic accident ($n = 1$ female) lesions and showed similar epidemiological characteristics (e.g. healthy individuals of 20–30 years-old with no immunosuppression treatment or condition nor reported drug consumption, diabetes, etc.) (Table 2). Additionally, patients showed the same clinical features after the surgery including painful arthritis of the knee, negative synovial fluid cultures both for bacteria and fungi, marked bone rarefaction at the MRI surrounding the tibial or the femoral fixation screws. Debridement surgeries were performed and devitalized material was obtained. Total DNA was obtained using a phenol-based procedure with a proteinase K pre-treatment [9].

Table 1
Oligonucleotide primers used in this work.

Primer	Sequence (5′–3′)	Purpose
M13[a]	GAGGGTGGCGGTTCT	Fingerprinting
(GACA)4[a]	GACAGACAGACAGACA	Fingerprinting
OPC-05[b]	GATGACCGCC	Fingerprinting
Rpl1[c,d]	TGATCTACGTGACAAATTCT	Molecular diagnosis and Mucormycetes identification to genera
RmLl[c,d]	TGATCTACGCGAGCGAACAA	Molecular diagnosis and Mucormycetes identification to genera
MucL1[c,d]	TGATCTACGTGACATATTCT	Molecular diagnosis and Mucormycetes identification to genera
AbsL1[c,d]	TGATCTACACGGCATCAAAT	Molecular diagnosis and Mucormycetes identification to genera
Mr1[c,d]	AGTAGTTTGTCTTCGGKCAA	Molecular diagnosis and Mucormycetes identification to genera

[a] (15).
[b] (13).
[c] Specific sense primers for *Rhizopus* spp. (Rpl1), *Rhizomucor* spp. (RmL1), *Mucor* spp. (MucL1) and *Lichteimia* spp. (AbsL1) [7].
[d] Degenerate antisense primer for Mucormycetes [7].

2.4. Antifungal susceptibility testing

Antifungal susceptibility testing was performed following the CLSI document M38 3rd ed. [10]. The antifungal drugs tested were purchased from Sigma-Aldrich as standard powder and included: Amphotericin B (AMB), Voriconazole (VRC), Posaconazole (PSC), Itraconazole (ITC) and Terbinafine (TRB). The susceptibility evaluation to the allylamine drug was performed following the same document despite it is not included in it. TRB was dissolved in DMSO and the range of concentrations tested were 0.015 to 8 μg/ml. Since there is no MIC limit ranges for microdilution tests for TRB, the produced plates were controlled by using *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 and TRB MIC ranges obtained by our group in an earlier work [11]. Strains were classified as susceptible or resistant to a particular drug using the epidemiologic cut-off values definitions [12].

2.5. Molecular typing by PCR fingerprinting

PCR amplification was used to fingerprint the *R. microsporus* strains. Fingerprinting primers (random primers for Random Amplified Polymorphic DNA) (Table 1) were chosen from previously reported ones used to type *R. stolonifer*, *Aspergillus flavus*, dermatophytes and fungi in general [13,14]. Firstly, we optimized the PCR reactions for each of the primers in order to obtain the maximum number of PCR bands using *R. microsporum* CBS 536.80 DNA. Amplifications were performed in a Quanta Biotech Q-cycler II thermocycler (Quanta Biotech, UK) which was programed for one initial step of 4 min at 94 °C followed by

Table 2
Clinical and demographic data of the ACLR patients.

Patient	Gender	Age (years)	Days after symptoms initiation	Affected bone
1	M	28	10	Tibia
2	F	21	12	Tibia
3	M	30	14	Femur

M: Male; F: Female.

30 cycles of 30 s at 95 °C, 30 s at 50 °C (when M13 was used) or 30 s at 35 °C (for $GACA_4$ and OPC05) (with a ramping temperature rate of 1.5 °C per second), and 2 min at 72 °C and then a final cycle of 10 min at 72 °C. The total volume of the PCR products were resolved by electrophoresis using a 2% agarose gel stained with gel green (Genbiotech, Argentina) for 5 h at 3 V/cm. These experiments were repeated three times in different days and only reproducible patterns with intense bands were analyzed. Blinded duplicates were included to ensure that each particular strain showed the same band pattern. After the electrophoresis, cluster analysis was performed following the CLSI Document MM11-A [15] with the software PyElph version 1.4 (http://pyelph.sourceforge.net/) using the unweighted pair group method with arithmetic mean (UPGMA). To avoid intergel variability, the parameters of the software were tuned up so a replicate from each gel (*R. microsporus* CBS 536.80) were considered identical upon blinded cluster analysis by the software.

2.6. Matrix-assisted laser desorption/ionization time-of-flight intact cell mass spectrometry identification and typing

2.6.1. Sample preparation

Fungal mycelia was obtained by growing *R. microsporus* strains in YPD broth (Yeast extract 1%, Bacteriological peptone 2% and Dextrose 2%) for 16 h at 35 °C and 150 rpm. Mycelial mats were washed, centrifuged and resuspended in 75%V/V ethanol. After a 2 min vortexing step, samples were centrifuged (10000 rpm) and the supernatant was discarded. The resulting pellet was treated with 50 μl of Formic acid (70%V/V) and 50 μl of acetonitrile. The samples were subjected to a last vortexing and centrifugation step and 1 μl of the supernatant was spotted to a MALDI-TOF MS plate (bioMérieux). The samples were air dried, overlaid with 1 μl of α-cyano-4-hydroxi-cinnamic acid matrix (CHCA) (bioMérieux) and air dried again before the analysis. Each sample was spotted in quadruplicate to confirm reproducibility.

2.6.2. MALDI-TOF analysis

The analysis was performed on an AXIMA Performance system (Shimadzu-Jenck – Argentina). The laser intensity, mass range and final spectra generation parameters were configured as proposed by manufacturer and described for other fungal species [16–18].

2.6.3. Spectral identification and analysis

Strain identification to species level was performed by comparison of the peak lists of individual strains with the SARAMIS database (SARAMISTM software package – AnagnosTec, Germany). Acceptance criteria was fixed at 90% mass similarity (out of at least 100 peaks) with species SuperSpectra. A dendrogram of spectral similarities between *R. microsporus* isolates were created using the same software package as described before [17]. Briefly, intraspecies similarity was expressed as the number of matching mass signals between individual spectra, after subjecting the data to a single link agglomerative clustering algorithm.

3. Results

3.1. Strains identification and molecular diagnosis of Mucormycosis

During a seven-month period, 32 ACLR surgeries were performed in the clinic and three presented post-surgery bone Mucormycosis (9.38% prevalence). In addition, necrotic tissue was received for molecular diagnostics. Biopsies were stained using Grocott gomori methenamine silver stain and non-septated hyphae were observed (Fig. 1). When cultured, fast-growing dark grey colonies with a colorless reverse were obtained. Unbranched sporangiophores measuring 500 to 1000 μm in length (predominantly 500–600 μm) and simple pigmented rhizoids originated opposing each other from stolons were seen at the microscope. Sporangiophores appeared in clusters mostly of three and terminate in unique rounded sporangium measuring 50 to

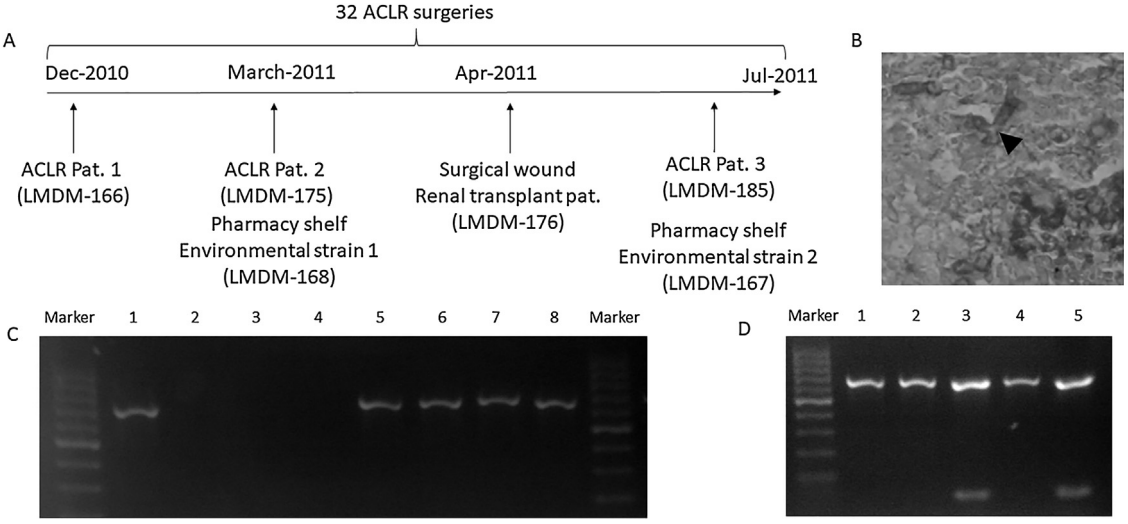

Fig. 1. A). Time course of the outbreak of mucormycosis due to *R. microsporus* in ACLR patients. B). *R. microsporus* in necrotic tissue section stained with GMS. Arrowhead shows the 90° angle branching. C). *Rhizopus* identification to genera level using the primers published by Machouart et al. [7] and DNAs from *Rhizopus oryzae* (lane 1), *Mucor circineloides* (lane 2), *Syncephalastrum racemosus* (lane 3), *L. corymbifera* (lane 4), *R. microsporus* strains LMDM-166, LMDM-176 and LMDM-175 (lanes 5 to 7, respectively) necrotic bone biopsy sample (lane 8). D) *Rhizopus* species identification using PCR-RFLP (PCR with Rpl1 and MR1 primers followed by AseI and ClaI digestions). *R. oryzae* - AseI digestión (Lane 1). *R. microsporus* LMDM-166 - ClaI and AseI digestión (lanes 2 and 3, respectively) and total DNA from the biopsy sample where LMDM-166 strain was isolated - ClaI and AseI digestion (Lanes 4 and 5, respectively)

80 μm. Columellae were elongated and well-defined apophyses were observed. Sporangiospores were subglobose and measured 5 μm in average. Turning to thermo-tolerance evaluation, the three isolates obtained from the necrotic tissue showed a radial growth of 42 mm after 5 days of incubation at 50 °C. Similarly, the other 6 studied isolates (including the CBS strain) showed colonies with a radial growth of 40 ± 4 mm. These phenotypic characteristics suggested the identification of the studied fungi as *R. microsporus* while the thermotolerance at 50 °C identify the isolates as belonging to the formerly know *rhizopodiformis* variety [19]. This identification to species level was molecularly confirmed by *ITS* sequencing and by PCR-RFLP [7,8]. All the isolates showed identical *ITS* sequence than the published under the GenBank accession number HM999971.1 corresponding to a *R. microsporus* var. *rhizopodiformis* CBS 536.80 (used as control strain in this work). Moreover, the PCR-RFLP performed following the modified Machouart et al. protocol let us identify all the strains as *R. microsporus* since all the isolates showed two bands (753 and 77 bp) after *AseI* digestion while no *ClaI* digestion was obtained (one 827 pb band) (Fig. 1). The same PCR-RFLP protocol was used for Mucormycosis diagnosis using necrotic tissue biopsy total DNA. All the studied samples showed positive PCR reaction only when Rlp1 and Mr1 primer pair were used (Fig. 1). After the *AseI* and *ClaI* digestion, the same digestion pattern (one restriction site and none, respectively) were obtained confirming that the agent of the Mucormycosis was *R. microsporus* (Fig. 1).

3.2. Molecular typing

The tested primers (GACA4, M13 and OPC-05, Table 1) were able differentiate strains as different individuals (different genotypes). Genetic relatedness was established by dendograms obtained by using UPGMA algorithm. Strains clustered into 6 genotype groups for all the tested primers. All the control strains have different genotypes and clustered into different groups. Environmental strains showed low relatedness while clinical strains fail into 2 different clusters, which clearly separate the isolates from the surgical wound of the renal transplant, recipient and the three strains isolated post ACLR surgery. These last three strains were considered identical between each other when $GACA_4$ and OPC05 primers were used while for fingerprinting with M13 two were identical and the third one was considered strongly related (Fig. 2). This third strain was isolated from the last patient infected in the 7-month period.

3.3. MALDI-TOF-based strain identification and typing

R. microsporus strains were subjected to MALDI-TOF analysis in quadruplicate. The identification at species level was in total agreement with the pheno and genotypic methods. Replicates always showed less than 5% difference in confidence levels of identifications. The SARAMIS software conventional algorithm was used to obtain the spectra-based dendogram (Fig. 2D). Low mass similarity (lower than 20%) was obtained among *R. microsporus* clusters and the chosen out-group species (*L. corymbifera*). When mass spectra profiles within the *R. microsporus* cluster were analyzed, strains were arranged into seven subclusters with average mass similarities values between 65 to > 85%. The three *R. microsporus* strains used as epidemiologically non-related controls (Buenos Aires and Salta city isolates and CBS536.80) were clearly separated in different clusters. On the other hand, the three post ACLR strains clustered together while the two environmental and transplant strains showed low mass similarities with the ACLR strain (especially this last one with similarity lower than 70%) despite that all six strains were isolated in the clinic.

3.4. Antifungal susceptibility testing and fungal osteomyelitis treatment

The most active antifungal drugs against the nine *R. microsporus* strains tested were TRB and ITC followed by PSC and AMB (Geometric means 0.14, 0.18, 0.54 and 0.63 μg/ml, respectively). There were no important differences in MIC values between environmental, clinical and control strains and all the strains were

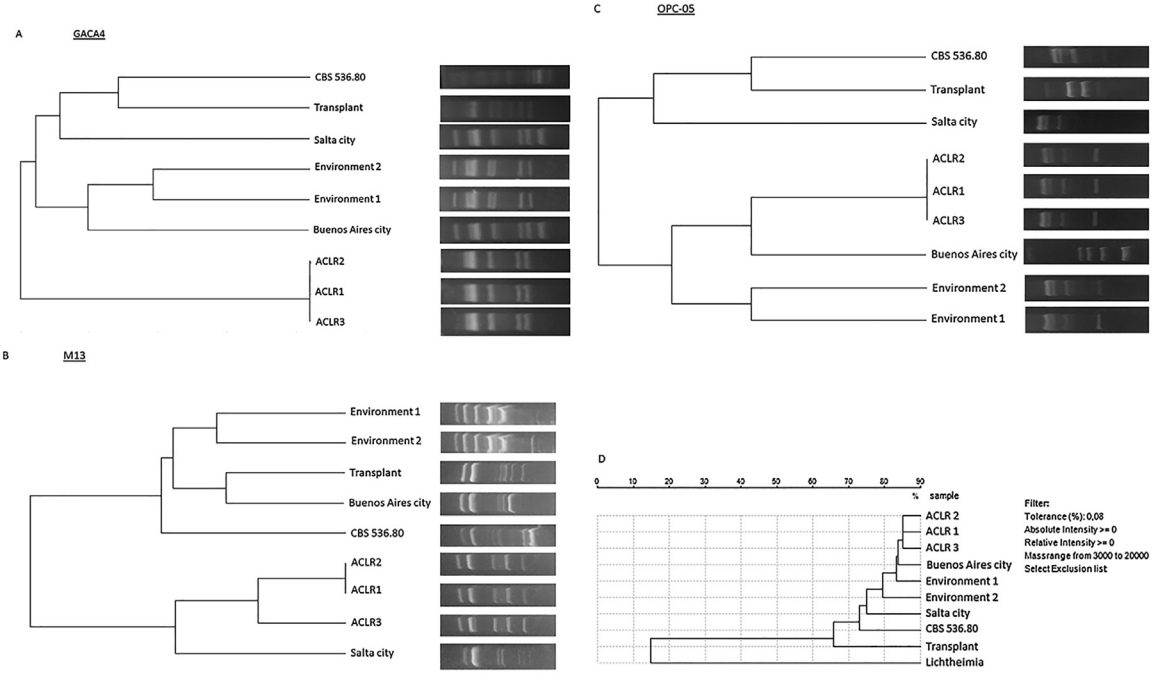

Fig. 2. UPGMA algorithm derived dendograms of the 9 *R. microsporus* strains studied using GACA4 (A), M13 (B) and OPC-05 (C) primers. Band profiles are presented (D). Dendrogram obtained from single-linkage cluster analysis mass spectra (obtained by MALDI-TOF analysis) of the nine *R. microsporus* strains and the *L. corymbifera* used as out-group strain. Percentage of mass similarity was used as distance measurement units

Table 3
Susceptibility testing results of the *R. microsporus* var. *rhizopodiformis* strains studied.

Strain	Origen/Isolation site	MIC (mg/L)[a]			
		AMB	PSC	ITC	TRB
LMDM-166	ACLR 1[b]	0.50	0.50	0.50	0.12
LMDM-175	ACLR 2[b]	0.50	0.50	0.50	0.12
LMDM-185	ACLR 3[b]	0.50	0.50	0.50	0.12
LMDM-165	Surgical wound[c]	1.00	0.25	0.25	0.06
LMDM-168	Environment 1[d]	1.00	1.00	0.12	0.12
LMDM-167	Environment 2[e]	1.00	0.25	0.12	0.25
LMDM-158	CBS 536.80[f]	0.50	0.50	0.50	0.25
LMDM-379	Buenos Aires[f]	0.50	1.00	0.25	0.25
LMDM-596	Salta[f]	0.50	1.00	0.25	0.12
GM (Range)	0.63 (0.5–1.0)	0.54 (0.25–1.00)	0.18 (0.12–0.5)	0.14 (0.06–0.25)	

[a] Geometric means (GM) of at least 3 repetitions performed on different days. AMB: amphotericin B; PSC: posaconazole; ITC: itraconazole and TRB: terbinafine.
[b] ACLR: anterior cruciate ligament reconstruction. Strains isolated from necrotic bone tissue after arthroscopic knee ligament surgery.
[c] Isolated from the surgical wound of a renal transplant recipient during the 7-month time span of the outbreak.
[d] Isolated from institution's operation room after the second ACLR surgery-related mucormycosis.
[e] Isolated from institution's pharmacy shelf after the third ACLR surgery-related mucormycosis.
[f] Epidemiologically non-related strains used as control.

classified as susceptible to AMB, PSC and ITC by using the epidemiologic cut-off values definitions [12]. The three strains isolated from ACLR surgery patients showed the same exact MIC pattern (Table 3). The three post-ACRL osteomyelitis patients receives the same treatment including: bone debridement with massive bone resection (10–12 cm) followed by intravenous antifungal treatment with 1 mg/kg/day AMB deoxicholate for six weeks (two patients were rotated after a week of treatment to liposomal AMB after renal toxicity). Knee reconstruction was done with an allograft-prosthesis composite using amphotericin B loaded bone cement beads [20]. All patients showed no signs of clinical infection as for April 2018.

4. Discussion

We are reporting three cases of bone healthcare-associated mucormycosis due to *R. microsporus* arisen after ACLR surgery in non-immunosuppressed individuals. The genetic and proteomic relatedness between the three isolated molds allow us to suggest that these three cases may be considered a mucormycosis outbreak. The epidemiological, genetic, proteomic and microbiological data presented here are almost unique if we consider that (i) ACLR is a common orthopedic procedure with low infection rates, (ii) fungal infections post-ACLR surgeries are almost nonexistent [20–22], (iii) immunosuppression is a risk factor observed in a high percentage of the mucormycosis cases [3] and (iv) healthcare-associated mucormycosis outbreaks due to *R. microsporus* are rare events [4,5,20,23].

Few authors have described typing methodologies for *Rhizopus* spp.[4,14]. Most of the previously reported outbreaks due to Mucorales were considered as such by identifying the strains to specie or variety level [5,14,23] or establishing partial genetic relatedness [4]. In this work we use 3 fingerprinting primers (random primers for Random Amplified Polymorphic DNA) to assess the genetic relatedness of the *R. microsporus* strains isolated in the clinic after ACLR surgeries. Our results, as any fingerprinting data, have limitations [24]. In order to reduce them, we use a set of completely unrelated isolates (knowing that they have no epidemiological relationship whatsoever) to test the method capacity to cluster them separately [24]. Moreover, we combined the data generated by three primers in order to increase the discriminatory power of the technique. However, no indexes (e.g. Simpson's index) were obtained since a small control population was studied [24]. To confirm our results and circumvent the described limitations we use a proteomic based typing (MALDI-TOF). The obtained results were in agreement with the fingerprinting analysis demonstrating that MALDI-TOF could be a rapid and accurate alternative tool for typing. There is scant data about the applicability of MALDI-TOF as a tool for intraspecies typing in fungal outbreak situations [25,26]. The few examples in the literature include a *C. parapsilosis* outbreak and the evaluation of the typing capability of this technique in *Trichosphyton rubrum*, *C. auris* and other *Candida* spp. in strain collections [16–18]. To the best of our knowledge, this is the first report showing the potential utility of MALDI-TOF to typify *Rhizopus* spp.

The epidemiological importance of our finding is reinforced by the fact that in a recent systematic review of post-ACLR infections, only 7 fungal infections were reported, 6 of which were due to *R. microsporus* [22]. Notoriously, all the six *R. microsporus* infections included in Stucken et al. review [22], were reported in an Argentinian hospital that received patient derived from other national centers [27]. Moreover, the named Argentinian hospital reported other nine cases of post-ACLR surgeries infections due to *R. microsporus* not included in the cited review [28,29]. What was more disturbing was the fact that in 2011, more than 30 *R. microsporus* post-ACRL osteomyelitis cases occurring in 10 different Argentinian provinces were presented at the meeting of the Argentinian Society for Mycology [29,30]. All the Argentinian cases share the same clinical characteristics with our patients including the occurrence in immunocompetent patients; only one of the bones (tibia or femur) was affected showing bone rarefaction surrounding one of the fixation screws [27,29,30]. The genetic relatedness of these Argentinian *R. microsporus* strains was not established and the discovery of a common source of infection was never achieved. Thus, this is the first study regarding the relatedness of the Argentinian *R. microsporus* isolated from post-ACLR surgeries.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

This study was supported in part by Sanatorio La Entrerriana and by Fondo para la Investigación Científica y Tecnológica (FONCYT) grant [PICT 2013-1571] to G.G.E. C.D., D.M. and F.L. have a fellowship from CONICET. M.C. is a PhD candidate at the Methodology of Biomedical Research and Public Health programme, Universitat Autònoma de Barcelona, Spain. *Mucor*

circinelloides, *Lichtheimia corymbifera* and *Syncephalastrum racemosus* used as control strains were kindly provided by Dr. Gustavo Giusiano.

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