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ARTICLE

European Confederation of Medical Mycology (ECMM) epidemiological survey on invasive infections due to *Fusarium* species in Europe

A. M. Tortorano · A. Prigitano · M. C. Esposto · V. Arsic Arsenijevic · J. Kolarovic · D. Ivanovic · L. Paripovic · L. Klingspor · I. Nordøy · P. Hamal · S. Arikan Akdagli · C. Ossi · A. Grancini · C. Cavanna · G. Lo Cascio · C. Scarparo · A. Candoni · M. Caira · M. Drogari Apiranthitou · On the behalf of the ECMM Working Group

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Abstract In order to better understand the epidemiology of fusariosis in Europe, a survey collecting information on the clinical characteristics of the patients infected by *Fusarium* as well as on the infecting isolates was launched. A total of 76 cases of invasive fusariosis occurring from January 2007 to June 2012 were collected and *Fusarium* isolates were identified by sequencing the translation elongation factor 1α (TEF) gene. Also, antifungal susceptibility was tested by broth

The ECMM Working Group
Czech Republic-Slovak Republic: E. Faber (Olomouc), A. Streharova (Trnava)
Greece: A. Argynopoulou, J. Baltadakis, M. Christofidou, E. Giamarellos-Bourboulis, N. Harahalakis, M. Marango, J. Meletiadis, K. Pananikolaou, M. Pirounaki, M. Stamouli (Athens)
Italy: M. Passera (Bergamo); S. Oliveri (Catania); C. Bonetti (Crema); S. Grandesso (Mestre); R. Milani, F. Ciceri, C. Verona, S. Malato, R. Chiesa, A. Puglisi, P. Manunta, E. Pajetta, F. Giglio, C.M. Rossi, A. Assanelli, B. Valeri, C. Boschetti, P. Tarsia, C. Annaloro, G. Viola (Milano); I. Casaroli (Monza); M. Sanguinetti (Roma); A. Sartor (Udine)
Turkey: O. Uzun, S. Alp (Ankara)

A. M. Tortorano (✉) · A. Prigitano · M. C. Esposto
Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milano, Italy
e-mail: annamaria.tortorano@unimi.it

V. Arsic Arsenijevic
Institut za Mikrobiologiju i Imunologiju, Univerziteta u Beogradu, Beograd, Serbia

J. Kolarovic
Department of Hematology/Oncology, Institute for Child and Youth Health Care of Vojvodina, Novi Sad, Republic of Serbia

D. Ivanovic
Mycology Laboratory, Institute of Public Health of Serbia, Beograd, Serbia

L. Paripovic
Institute of Oncology and Radiology, Beograd, Serbia

L. Klingspor
National and Karolinska University Hospital, Stockholm, Sweden

I. Nordøy
Institute of Microbiology, Rikshospitalet, Oslo, Norway

P. Hamal
Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

S. Arikan Akdagli
Hacettepe University Medical School, Ankara, Turkey

C. Ossi
Laboraf, Diagnostica e Ricerca San Raffaele, Milano, Italy

A. Grancini
Laboratorio di Microbiologia, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli, Regina Elena, Milano, Italy

C. Cavanna
Laboratorio di Microbiologia, IRCCS Policlinico S. Matteo, Pavia, Italy

microdilution according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Etest. Disseminated disease was considered proven in 46 cases and probable in 17 cases. Localised infection was seen in 13 cases. *Gibberella fujikuroi* species complex (SC), including *Fusarium verticillioides* and *F. proliferatum*, and *F. solani* SC were the most frequent aetiology of disseminated and localised infections, respectively. The crude mortality rate was 46 %, the highest associated with *F. solani* SC (67 %) and *F. proliferatum* (62.5 %). A wide range of antifungal susceptibilities was observed. Amphotericin B was the most potent antifungal in vitro, and itraconazole the least effective. The azoles exhibited lower minimum inhibitory concentrations (MICs) against *F. verticillioides* strains, with posaconazole having a slightly better performance, while *F. solani* SC isolates were resistant to all three azoles tested. The essential agreement between the Etest and the EUCAST method was 100 % for itraconazole and voriconazole, and 96 % for amphotericin B and posaconazole. In conclusion, we confirm that fusariosis is a rare but severe event in Europe, that *G. fujikuroi* SC is the predominant cause of deep infections and that different species have different antifungal in vitro susceptibility patterns.

Introduction

Fusarium species are ubiquitous moulds that cause a broad spectrum of infections in humans, including superficial, locally invasive and disseminated infection [1]. The clinical presentation largely depends on the immune status of the host and the fungal portal of entry [1, 2]. In the immunocompetent or in patients with immune function partially preserved, *Fusarium* spp. cause superficial infections, such as keratitis and onychomycosis. Occasionally, they may cause localised infections, such as allergic or invasive sinusitis [1, 2]. In contrast, in immunocompromised patients, invasive and disseminated infections occur, mainly associated with prolonged and profound neutropaenia or severe T-cell immunodeficiency [1].

Fusariosis represents between the second and third most frequent mould infection in patients with haematological malignancies and in haematopoietic stem cell transplantation (HSCT) recipients. The acquisition of infection is through the inhalation of airborne conidia, breaks in the skin due to trauma, vascular access or onychomycosis. In such patient populations, it occurs as a severe disseminated infection with mortality rates of up to 75 % [1, 2].

Approximately 200 species, grouped into ten species complexes (SCs), are included in the *Fusarium* genus. Most of them are soil inhabitants or plant pathogens, and about 70 species, mainly identified by multilocus sequence typing (MLST), have been involved in human or animal infections [3, 4]. Isolates more frequently encountered in human disease belong to three groups: the *Fusarium solani* SC, the *F. oxysporum* SC and the *Gibberella fujikuroi* SC [1, 3].

F. solani is reported as the cause of approximately 50 % of the infections, followed by *F. oxysporum* (20 %), *F. verticillioides* (10 %) and *F. moniliforme* (now classified as *F. verticillioides*, belonging to *G. fujikuroi* SC, 10 %) [1, 5]. In contrast with data from the literature [1, 3], in Italy, *F. verticillioides* is the most prevalent species, followed by *F. solani* (25 %). In particular, *F. verticillioides* was the most frequent species (57 %) in deep-seated infections and *F. solani* was more common in superficial infections (46 %) [6].

Fusarium species are, in vitro, relatively resistant to the most common systemic antifungal agents. However, different species have been shown to have different patterns of susceptibility, with the majority of *F. solani* isolates exhibiting reduced susceptibility to azoles [6–11]. The clinical relevance of these in vitro data is unclear, as no in vitro–in vivo correlation has yet been demonstrated for the antifungal management of fusariosis [1, 12].

In order to better understand the epidemiology of fusariosis in Europe, the European Confederation of Medical Mycology (ECMM) launched a survey collecting information on the clinical characteristics of patients infected by *Fusarium*, as well as on the infecting isolates.

In the present report, cases of invasive fusariosis were analysed with respect to their molecularly identified infecting isolates and their in vitro susceptibility to antifungal agents. Data concerning superficial infections, including keratitis, onychomycosis and intertrigo, will be the subject of another report.

Methods

Cases of invasive fusariosis, for which the infecting isolate was available, were recorded on a questionnaire form. The collected data included: demographics, type of infection, underlying diseases and predisposing factors, antifungal treatment and outcome, and mycological data.

Data were collected prospectively from January 2009 to June 2012, and retrospectively for the years 2007 and 2008.

G. Lo Cascio
Servizio di Microbiologia, Azienda Ospedaliera di Verona, Verona, Italy

C. Scarparo · A. Candoni
Azienda Ospedaliero-Universitaria S. Maria della Misericordia, Udine, Italy

M. Caira
Università Cattolica del S. Cuore, Roma, Italy

M. Drogari Apiranthitou
National and Kapodistrian University of Athens, Attikon Hospital, Athens, Greece

Disseminated fusariosis was defined as proven or probable according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) definitions [13].

The *Fusarium* isolates—identified in the respective hospitals on the basis of morphological characteristics or by matrix-assisted laser desorption/ionisation (MALDI-TOF) or by sequencing—were sent to the Laboratory of Medical Mycology of the University of Milan, Italy, for further identification and susceptibility testing. The isolates were stored as freeze-dried, on potato agar slants and in distilled water. Isolates were identified to the genus level using morphological characteristics on potato dextrose agar (PDA).

Molecular identification was performed by sequencing the translation elongation factor 1α (TEF) gene [14]. Genomic DNA was extracted using the PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA). A standard polymerase chain reaction (PCR) was used to amplify the TEF gene region using the primers ef1 (ATGG GTAAGGAGGACAAGAC) and ef2 (GGAAGTTACCAG TGATCATGTT). The TEF PCR products (≈700 bp) were visualised on 2 % agarose gel stained with ethidium bromide and used as a template for DNA sequencing using BigDye Terminators (Applied Biosystems) in a 310 ABI PRISM sequencer (Applied Biosystems). Nucleotide sequences were analysed using FinchTV software version 1.4.0. and blasted in the FUSARIUM-ID server at http://fusarium.cbio.psu.edu [4, 15, 16].

Isolates were tested for in vitro susceptibility to itraconazole (Janssen, Beerse, Belgium), posaconazole (Merck Sharp & Dohme, White House Station, NJ, USA), voriconazole (Molekula Ltd., Wimborne, Dorset, UK) and amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), both by the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [17] and the Etest.

Broth microdilution assay was performed in RPMI 1640 with glutamine, without bicarbonate (Sigma) supplemented with glucose to a final concentration of 2 %. Inoculum suspensions were prepared from 2–5-day-old cultures. The conidia suspensions were counted in a haemocytometer chamber and diluted to a final working inoculum of 2–5 × 10^5 cfu/mL. Plates were incubated at 35 °C for 48–72 h. The minimum inhibitory concentration (MIC) value was the concentration of drug yielding no fungal growth at visual reading. All tests were performed in duplicate. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as quality controls in each run.

Susceptibility was also determined using Etest strips (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. The surface of RPMI agar medium was inoculated using a sterile swab dipped in an inoculum suspension of 0.5–5 × 10^6 conidia/mL. Plates were incubated at 35 °C for 48–72 h. The MIC was the lowest drug concentration at which the elliptical inhibition zone intercepted the scale on the antifungal strip. When needed, MICs determined by the Etest were elevated to the next two-fold dilution concentration of the broth microdilution test to allow comparison.

The MIC values obtained with the two methods were compared to assess the essential agreement defined as differences of ±2 two-fold dilutions between the results obtained by the two techniques.

Results

A total of 76 cases were collected from seven European countries. The contribution of each country is reported in Table 1.

Table 2 summarises the characteristics of patients affected by *Fusarium* infection. According to the EORTC/MSG criteria [13], disseminated disease was considered proven in 46 cases, as documented by at least two positive blood cultures (29 cases) or one positive blood culture, together with skin or another organ involvement proven by culture and microscopy or histology (10 cases) or by positive culture, combined with hyphae at microscopy or histology, of skin biopsy in the presence of multiple lesions (4) or lung biopsy (2) or pleural fluid (in the absence of a broncho-pleural fistula).

Infection was considered probable in 17 cases, as a *Fusarium* species was isolated from bronchoalveolar lavage or sinuses in the presence of host factors, clinical signs or symptoms, and hyphae seen at microscopy.

Localised infection was seen in 13 cases. *Fusarium* spp. grew from pleural fluid in the presence of bronchopleural fistula in one case and from peritoneal fluid in the presence of peritoneal dialysis in another. In three haematological malignancy patients, two of whom were recipients of an HSCT, the fungus was repeatedly cultured from urine in the absence of signs of renal disease. In another case, the fungus was considered the aetiology of a lung mycetoma, as hyphae were seen at histology and only *F. proliferatum* was cultured from the surgical sample. Hyphae were seen at direct microscopy and *Fusarium* spp. were repeatedly cultured from skin biopsy from accidental traumatic lesion (three cases) or diabetic ulcer (two cases), or from a single skin lesion in a leukaemic patient.

Disseminated infections were mainly associated with haematological malignancies (47 out of 63 cases). Seventeen patients received an allo-HSCT.

The *Fusarium* species responsible for infection are listed in Table 3. *G. fujikuroi* SC was the most frequent

Table 1 Contribution of different countries to the European Confederation of Medical Mycology (ECMM) *Fusarium* Working Group

Country	Collected cases			
	Total	Disseminated infection		Localised infection
		Proven	Probable	
Czech/Slovak Republic	3	1	1	1
Greece	9	8	1	
Italy	46	20	14	12
Norway	3	2	1	
Serbia	10	10		
Sweden	3	3		
Turkey	2	2		
Total	76	46	17	13

aetiology of proven or probable disseminated infections (36/63, 57 %), namely, *F. verticillioides* caused 19 cases (30 %) and *F. proliferatum* 17 cases (27 %). *F. solani* SC was involved in 11 (17 %) proven or probable infections and *F. oxysporum* SC in 9 (14 %). In contrast, *F. solani* SC predominated in localised infections.

F. proliferatum isolates were collected from five countries (12 from Italy, four from Serbia, two from Greece and one each from Norway and Sweden), *F. verticillioides* from Italy (14), Serbia (5), Greece (2) and Czech Republic (1). *F. oxysporum* isolates were composed of five from Italy, three from Greece, one from Serbia and one from Sweden. *F. solani* were found in Italy (11 cases, mainly as the cause of localised infections), Turkey (2), Greece, Sweden and Czech Republic, one case each.

Skin involvement occurred in 18 cases of disseminated proven or probable infections. *F. proliferatum* caused skin lesions in 7 out of 17 (41 %) invasive infections, *F. solani* SC in 6 out of 11 cases (54.5 %), *F. verticillioides* in two cases (10 %) and *F. oxysporum* SC in only one. *F. solani* SC was responsible for four out of six skin localised infections.

During the study period, five clusters of cases were observed. Four cases of *F. verticillioides* fungaemia occurred in the same centre in a two-month period and four cases of *F. proliferatum* disseminated infections were diagnosed in another centre during a 3-day interval. In other hospitals, two to three cases of fusariosis, caused by different species, were observed within a restricted period of time.

Data concerning antifungal treatment were available for 49 patients with disseminated *Fusarium* infection (38 proven and 11 probable). Twenty-six patients received monotherapy, mainly voriconazole given to 13 patients and lipid-based amphotericin B given to eight patients. Eighteen patients were treated with two drugs, mainly lipid-based amphotericin B associated with voriconazole, and the other three patients received three antifungals.

Table 2 Characteristics of the 76 patients affected with *Fusarium* infection

	Fusarium infection		
	Disseminated infection		Localised infection
	Proven	Probable	
Age in years, median (range)	45 (5–86)	57 (20–90)	65 (15–77)
Gender, male/female	26/20	11/6	10/3
Underlying conditions			
Haematological malignancies	32	15	
Acute myeloid leukaemia	11	2	2
Acute lymphoid leukaemia	7		
Chronic myeloid leukaemia		2	
Unspecified leukaemia	5	6	1
Lymphoma	5	1	
Refractory anaemia with excess blasts	1	1	
Myelodysplastic syndrome		1	
Other	3[a]	2[b]	
HSCT			
Allo	10	7	1
Auto			1
Solid cancer	8[c]	3[d]	2[e]
Other	4[f]		7[g]

[a] Aplastic anemia; promyelocytic leukaemia; paroxysmal nocturnal hemoglobinuria

[b] Severe neutropenia

[c] Osteosarcoma (4); lung (2); astrocytoma (1); liver (1)

[d] Lung (2); prostate (1)

[e] Lung (1); kidney (1)

[f] Chronic granulomatous disease; chronic mastoiditis; hemodialysis; liver transplantation

[g] Diabetes (3); accidental trauma (3); peritoneal dialysis (1)

Table 3 Distribution of isolated *Fusarium* species according to infection type

	Fusarium infection			
	Total	Disseminated infection		Localised infection
		Proven	Probable	
Gibberella fujikuroi SC	43			
F. verticillioides	22	15	4	3
F. proliferatum	20	14	3	3
F. andiyazi	1			1
F. solani SC	16	7	4	5
F. oxysporum SC	10	7	2	1
F. dimerum SC	3		3	
F. globosum	1		1	
F. sporotrichoides	1	1		
Not identified[a]	2	2		

[a] Due to contamination of the original isolate

The outcome at day 30 was available for 37 proven and 11 probable infections. The crude mortality rate was 46 % (22/48), namely, 40.5 % and 63.3 % in proven and probable disseminated infections, respectively. The highest mortality was associated with *F. solani* SC (6 out of 9, 67 %) and *F. proliferatum* (10 dead out of 16 infections, 62.5 %). *F. verticillioides* infection had a 19 % crude mortality.

A total of 54 isolates were tested for in vitro antifungal susceptibility. The distribution of MIC values for amphotericin B and azoles is shown in Table 4. The geometric mean of the MICs (G-MICs) of amphotericin B for all 54 isolates was 1.51, ranging from 0.71 for *F. dimerum* to 2.89 for *F. verticillioides*. The MICs of azoles showed variable values: the G-MICs ranged from 3.02 for posaconazole to 7.50 for itraconazole. In addition, a large interspecies variability was observed: G-MICs ranged from 0.35 to 1.74 for *F. verticillioides* isolates, while considerably high azole MICs were observed for *F. solani* SC isolates (G-MICs >13 for all three azoles).

The essential agreement between the Etest and the EUCAST method was 100 % for itraconazole and voriconazole, and 96 % for amphotericin B and posaconazole. MIC values obtained by the Etest were generally lower than those obtained by the EUCAST method, even when the values were in the ±2 dilutions range.

Discussion

This is the first study reporting a multinational series of patients with microbiologically proven fusariosis from Europe. The identification of the fungus grown in culture is required for the diagnosis as histopathology, although an essential method for the diagnosis is not specific, since hyaline septate hyphae in tissue could correspond to different filamentous fungi, including *Aspergillus* [18].

Most of the cases (39 cases) in the present survey were proven by positive blood cultures. The high yield of blood cultures in case of disseminated fusariosis is explained by the adventitious sporulation of *Fusarium* in tissues [19]. Besides fungaemia, the most common clinical manifestations of fusariosis in our patients included multiple skin lesions (18 cases) and sino-pulmonary involvement (24 cases).

Consistent with the observations of the literature, most patients in this survey had haematologic malignancy or an HSCT as underlying conditions [1, 2, 12]. *Fusarium* infection in such patients results in a poor prognosis, with death rates of up to 75 % [1, 2, 12]. In the current study, the crude mortality was 46 %, but there were insufficient evidences to evaluate mortality attributed to *Fusarium* infection.

F. verticillioides and *F. proliferatum*, both belonging to the same phylogenetic SC *G. fujikuroi*, were the species most frequently identified as the cause of both disseminated and localised infections, causing 57 % and 54 % of cases, respectively. *F. solani* SC was identified as the aetiology in 17 % and 38 % of disseminated and localised infections, respectively, but this difference was not statistically significant (p=0.13, Fisher's exact test). It seems, therefore, that clinical manifestation is not species related.

These data confirm the distribution of *Fusarium* species identified by molecular methods detected in our previous experience from Italy, where *F. verticillioides* was ranked as the first cause of deep-seated fusariosis [6]. Our data differ from those of the literature reporting *F. solani* as the cause of approximately 50 % of the infections. However, in most of the reported clinical cases of fusariosis, *Fusarium* isolates were not speciated [2, 12, 20, 21]. In addition, the identification of the isolates were mostly carried out by morphology that can

lead to the misidentification of *F. oxysporum* as *F. solani*, due to the morphological similarities shared by these two species [22]. In general, molecular phylogenetic studies have shown that morphological species recognition within *Fusarium* greatly underestimates its species diversity [4]. Spread of the different aetiological species over the different European areas may not be feasible due the limited number of isolates from some countries.

Among the clusters of cases of fusariosis detected in five hospitals during the study period, only two could be considered an outbreak as the same species; *F. verticillioides* in one centre and *F. proliferatum* in another hospital were identified as the cause of four cases of fungaemia in a restricted period of time. The other clusters of cases involving different species could be attributed to episodic local favourable climatic conditions rather than a common source of infection.

Interpretative MIC breakpoints for *Fusarium* spp. have not yet been identified. However, the MIC determination gives an overview of in vitro resistance and, therefore, may support the choice of antifungal treatment. Fluconazole and echinocandins have apparently no effect, while amphotericin B, voriconazole and posaconazole have lower MIC values, even if voriconazole exhibits high MICs for *F. solani*. In contrast, *F. oxysporum* and *F. verticillioides* are more susceptible to voriconazole and posaconazole [1, 6, 23–25].

In accordance to the above findings, our results revealed a wide range of susceptibility. They showed that amphotericin B

Table 4 In vitro susceptibilities of the 54 *Fusarium* spp. isolates determined by broth microdilution according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology

Fusarium (no. of tested isolates)	Antifungal	No. of isolates with an MIC (mg/L) of:								G-MIC	MIC_{50}	MIC_{90}
		0.12	0.25	0.5	1	2	4	8	≥16			
proliferatum (16)	Amphotericin B			1	9	4	2			1.35	1	2
	Itraconazole				1			1	14	12.88	≥16	≥16
	Posaconazole		3			1	1		11	5.91	≥16	≥16
	Voriconazole					4	3	3	6	6.44	8	≥16
verticillioides (15)	Amphotericin B					7	8			2.89	4	4
	Itraconazole				9	4			2	1.74	1	2
	Posaconazole		8	7						0.35	0.25	0.5
	Voriconazole			1	9	5				1.20	1	2
solani (14)	Amphotericin B			1	11	2				1.05	1	2
	Itraconazole								14	16.00	≥16	≥16
	Posaconazole				1	1			12	13.79	≥16	≥16
	Voriconazole						1	2	11	13.12	≥16	≥16
oxysporum (6)	Amphotericin B				5	1				1.12	1	2
	Itraconazole								6	16.00	≥16	≥16
	Posaconazole				3				3	4.00	1	≥16
	Voriconazole				2	1	2	1		2.52	2	8
dimerum (2)	Amphotericin B			1	1					0.71		
	Itraconazole								2	16.00		
	Posaconazole								2	16.00		
	Voriconazole						1	1		5.70		
andiyazi (1)	Amphotericin B					1						
	Itraconazole			1								
	Posaconazole			1								
	Voriconazole			1								
All species (54)	Amphotericin B			3	26	15	10			1.51	1	4
	Itraconazole			1	10	4		1	38	7.60	≥16	≥16
	Posaconazole		11	8	4	2	1		28	3.01	4	≥16
	Voriconazole			2	11	10	7	7	17	4.16	4	≥16

G-MIC: geometric mean of the MIC (mg/L)

MIC_{50}: MIC at which 50 % of isolates are inhibited

MIC_{90}: MIC at which 90 % of isolates are inhibited

was the most potent antifungal in vitro, and itraconazole the least effective. Amphotericin B inhibited the fungal growth of most isolates, including those belonging to the *F. solani* SC at an MIC of ≤1 mg/L, but it had higher MICs against the *F. verticillioides* strains. The azoles, instead, exhibited lower MICs against the *F. verticillioides* strains, with posaconazole having a slightly better performance, whereas the opposite was true for *F. solani*, which had high MICs to all three azoles tested. The true meaning of these MICs in clinical practice is not known [26]. Voriconazole, the most often used azole in recent years, appears clinically effective, despite the high MIC values, especially against *F. solani* [12, 27].

Posaconazole, which is licensed as salvage therapy against invasive fusariosis, also seems to be clinically effective, but it has not been extensively studied and there are no studies to compare it with voriconazole [28].

The Etest was evaluated in comparison with the EUCAST broth microdilution method and an overall essential agreement of 96–100 % for amphotericin B and azoles was obtained.

A limitation of the study is the poor participation of the European countries in this survey, to which the Italian contribution accounted for more than 60 % of the cases. The real burden of this disease is not known, as the incidence rate cannot be calculated due to the lack of denominators.

Another limitation is that the isolates were identified by the sequencing of a single locus (elongation factor 1α) instead of three different loci recommended for species identification [4]. This approach, adopted for cost containment, allows a correct identification at the SC level, but does not always allow the identification of the cryptic species. However, the identification of phylogenetic species in a complex, for example, 60 species within *F. solani* SC, most of which are named with an alphanumerical system of nomenclature, could not have a medical relevance [29].

In conclusion, we confirm that fusariosis is a rare but severe event in Europe, that *G. fujikuroi* SC is the predominant cause of deep infections and that different species have different antifungal in vitro susceptibility patterns. At this point, we would like to highlight the need for more studies to identify reservoirs of *Fusarium* spp. in the hospital wards, to elucidate the correlation between in vitro and in vivo susceptibilities. In addition, we have a large collection of *Fusarium* clinical isolates, coupled with clinical information, available for further studies.

Conflict of interest The authors declare that they have no conflict of interest.

The manuscript does not contain clinical studies.

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