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Multicenter Outbreak of Infections by *Saprochaete clavata*, an Unrecognized Opportunistic Fungal Pathogen

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ABSTRACT Rapidly fatal cases of invasive fungal infections due to a fungus later identified as *Saprochaete clavata* were reported in France in May 2012. The objectives of this study were to determine the clonal relatedness of the isolates and to investigate possible sources of contamination. A nationwide alert was launched to collect cases. Molecular identification methods, whole-genome sequencing (WGS), and clone-specific genotyping were used to analyze recent and historical isolates, and a case-case study was performed. Isolates from thirty cases (26 fungemias, 22 associated deaths at day 30) were collected between September 2011 and October 2012. Eighteen cases occurred within 8 weeks (outbreak) in 10 health care facilities, suggesting a common source of contamination, with potential secondary cases. Phylogenetic analysis identified one clade (clade A), which accounted for 16/18 outbreak cases. Results of microbiological investigations of environmental, drug, or food sources were negative. Analysis of exposures pointed to a medical device used for storage and infusion of blood products, but no fungal contamination was detected in the unused devices. Molecular identification of isolates from previous studies demonstrated that *S. clavata* can be found in dairy products and has already been involved in monocentric outbreaks in hematology wards. The possibility that *S. clavata* may transmit through contaminated medical devices or can be associated with dairy products as seen in previous European outbreaks is highly relevant for the management of future outbreaks due to this newly recognized pathogen. This report also underlines further the potential of WGS for investigation of outbreaks due to uncommon fungal pathogens.

IMPORTANCE Several cases of rapidly fatal infections due to the fungus *Saprochaete clavata* were reported in France within a short period of time in three health care facilities, suggesting a common source of contamination. A nationwide alert collected 30 cases over 1 year, including an outbreak of 18 cases over 8 weeks. Whole-genome sequencing (WGS) was used to analyze recent and historical isolates and to design a clade-specific genotyping method that uncovered a clone associated with the outbreak, thus allowing a case-case study to analyze the risk factors associated with infection by the clone. The possibility that *S. clavata* may transmit through contaminated medical devices or can be associated with dairy products as seen in previous European outbreaks is highly relevant for the management of future outbreaks due to this newly recognized pathogen.

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Invasive fungal infections are well described in immunocompromised patients and represent a still-underestimated public health burden (1, 2). They can also occur in the setting of natural disasters (3) or as sporadic cases or epidemic health care-associated infections, possibly traced to a common source (4). Until now, detection of fungal outbreaks has been hampered by the diversity of fungal species involved, the small number of genomes annotated, and the lack of reliable typing tools for every pathogenic species. These reasons often preclude detection of a clonal dissemination and its source, at least for rare species. Genomic comparisons using whole-genome sequencing (WGS)

have been successfully used to investigate outbreaks of bacterial pathogens (5) but have so far rarely been applied to fungal outbreaks (6, 7). As noted by Litvintseva and colleagues (8), who investigated by WGS the recent outbreak of fungal meningitis due to *Exserohilum rostratum*, rapid laboratory methods to establish the relatedness of different strains may be the critical factor in determining whether a cluster of rare fungal infections represents a true outbreak or just an unusual coincidence. We here report a case where WGS was also a powerful tool for the investigation of a multicenter outbreak of infections due to a rare fungal pathogen, *Saprochaeta clavata*.

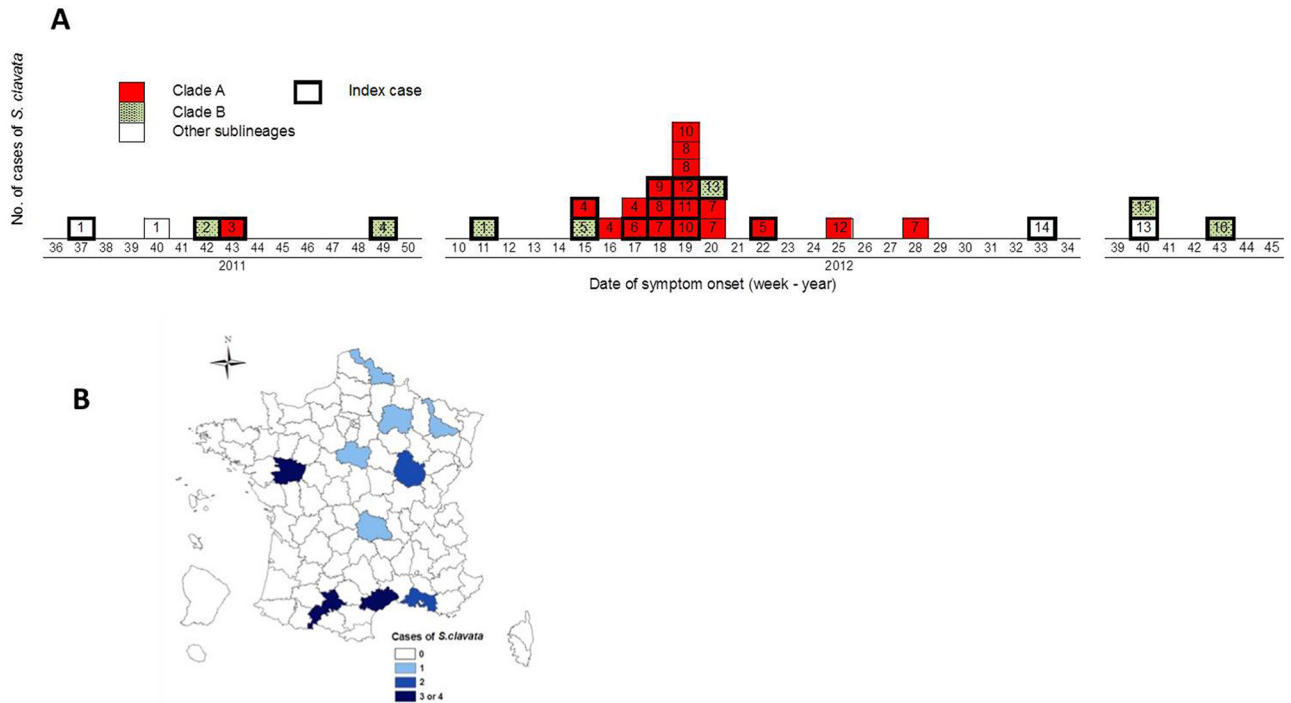


FIG 1 Cases of *Saprochaeta clavata* infections, France, September 2011 to October 2012. (A) Epidemiological curve of *S. clavata* infections in France. Centers are designated by numbers (see Table 1 for details), with boxes corresponding to the date of the first positive culture for cases infected by clade A, clade B, and other lineages. Index cases are outlined with thick black lines. (B) Administrative districts where the cases of *S. clavata* infections were diagnosed.

Saprochaeta clavata, an ascomycetous fungus, formerly called *Geotrichum clavatum*, is phylogenetically and phenotypically closely related to *Magnusiomyces capitatus*, formerly called *Geotrichum capitatum*. Both species are often misidentified (9). Both are intrinsically resistant to echinocandins, which are recommended in hematology wards in cases of *Candida* fungemia or febrile neutropenia of unknown origin (10). Sporadic cases and outbreaks of fungemia due to *M. capitatus* have been reported, mostly in Europe, in patients with acute hematological malignancies, and these outbreaks have sometimes been connected to contamination of dairy products (11–13) by the use of nonstringent typing methods (14). In contrast, *S. clavata* has rarely been reported to be responsible for infection (15).

RESULTS

Initial investigation. Of the 39 cases collected between September 2011 and October 2012, 30 were due to *S. clavata* and 9 to *M. capitatus*. A peak of 18 cases due to *S. clavata* was observed over 2 months in 2012 (week 15 [W15] to W22; Fig. 1A) in 10 health care facilities (HCF) located in 10 different regions (Fig. 1B).

Half of the 30 cases were male patients, with a median age of 63 years (Table 1). The majority (70%) of the patients were hospitalized for acute myeloid leukemia. *Saprochaete clavata* was recovered from blood (26/30, 87%) and bronchoalveolar fluid or tracheal aspirates (12/30, 40%). Overall, 18/30 (60%) patients had multiple body sites infected. Diarrhea before or associated with fungemia was reported for 16/26 (61.5%) cases. Stool cultures yielding a fungus exhibiting arthrospores were reported in 11/19 patients (57.9%). The case fatality rate at day 60 was 80%, with death occurring at a median of 7 days after diagnosis.

Microbiological investigation. Overall, 73 isolates of *S. clavata*, collected during this study and through the surveillance programs at the National Reference Centre for Invasive Mycoses & Antifungals (NRCMA) and including the European isolates and the type strain, were studied (see Table S1 in the supplemental material). All *S. clavata* isolates exhibited similar antifungal susceptibility profiles, with no significant difference according to period of isolation (Table 2). WGS was performed on a panel of 17 French *S. clavata* clinical isolates collected before ($n = 6$) and during ($n = 11$) the investigation period as well as on the type strain (see Table S1). In the 18 isolates, the gene sequences coding for a set of 175 fungal proteins as well as the 18S rRNA sequences (~324,000 aligned nucleotide characters) were totally identical (<0.00034% polymorphic sites), showing that *S. clavata* is a genetically highly monomorphic species. We therefore searched for single nucleotide polymorphisms (SNPs) over the entire genome sequence using a mapping approach, which led to the discovery of 312 high-quality SNPs. The resulting 103 phylogenetically informative characters were compatible with each other (homoplasy index = 0 [16]), therefore allowing a robust phylogenetic tree to be inferred (Fig. 2). Two particular clades, denoted A and B, were identified as homogeneous phylogenetic sublineages containing 7 and 5 of the 18 isolates, respectively. Clade A isolates differed by at most four SNPs among each other (Fig. 2), indicating that this clade is a young clone. Clades A and B were characterized by 15 and 9 unique characters, respectively. The clade-specific polymorphisms (see Table S2) were confirmed by Sanger sequencing, and eight of the SNPs were used to classify all isolates as belonging to clade A or B or to other lineages.

Among the 15 clade A-specific nucleotide changes, four were

TABLE 1 Characteristics of the *S. clavata* cases and major exposures prior to infection, France, September 2011 to October 2012

Characteristic(s) of the case	All cases (n = 30)	A cases (n = 19)	Non-A cases (n = 11)	P value	A index cases (n = 10)	Non-A index cases (n = 10)	P value
No. (%) of occurrences during the outbreak period (weeks 15–22, 2012)	18 (60.0)	16 (84.2)	2 (18.2)	0.001	9 (90.0)	2 (20.0)	0.005
Median age in yrs (95% CI) ^a	63 (55–65)	63 (49–69)	63 (36–69)	0.846	62 (48–70)	63.5 (34–73)	0.821
Male gender, n ^b (%)	15 (50.0)	7 (36.8)	8 (72.7)	0.128	4 (40.0)	7 (70.0)	0.37
Underlying disease, n (%)							
Acute myeloid leukemia	21 (70.0)	13 (68.4)	8 (72.7)	0.919	8 (80.0)	7 (70.0)	0.582
Acute lymphoid leukemia	6 (20.0)	3 (15.8)	3 (27.3)		1 (10.0)	3 (30.0)	
Chronic lymphoid leukemia	1 (3.3)	1 (5.3)			1 (10.0)		
Others	2 (6.7)	2 (10.5)					
Immune status							
Severe neutropenia (<500 neutrophils/mm ³), n (%)	27 (90.0)	17 (89.5)	10 (90.9)	1	10 (100.0)	10 (100.0)	1
Median duration (days) of neutropenia at the time of positive culture (95% CI)	14 (13–20) (n = 25)	14 (11–20) (n = 15)	15.5 (10–32) (n = 10)	0.579	13 (8–17)	15.5 (12–32)	1
Prolonged corticosteroid treatment without aplasia, n (%)	2 (6.7)	2 (10.5)			1 (9.1)		
Clinical signs at the time of positive culture, n (%)							
Diarrhea	16/26 (61.5)	8/15 (53.3)	8/11 (72.7)	0.428	4/9 (44.4)	7/10 (70.0)	0.37
Pulmonary symptoms	16 (53.3)	10 (66.7)	6/9 (66.7)	1	4/8 (50.0)	5/8 (62.5)	1
Positive culture (<i>S. clavata</i>), n (%)							
Blood	26 (86.7)	16 (84.2)	10 (90.9)	1	10 (100.0)	9 (90.0)	1
Respiratory tract (bronchoalveolar lavage, tracheal aspirate)	12 (40.0)	9 (47.4)	3 (27.3)	0.442	4 (40.0)	3 (30)	1
Stools ^c	11/19 (57.9)	7/10 (70.0)	4/9 (44.4)	0.370	2/5 (40.0)	4/8 (50.0)	1
Multiple noncontiguous sites	18 (60.0)	11 (57.9)	7 (63.6)	1	6 (60.0)	6 (60.0)	1
Median interval (days) between admission and first positive culture (95% CI)	21 (18–27)	22 (18–29)	20 (15–29)	0.667	24.5 (16–35)	22 (15–32)	0.647
Mortality							
Crude mortality at day 30, n (%)	22 (73.3)	15 (79.0)	7 (63.6)	0.417	9 (90.0)	6 (60.0)	0.303
Crude mortality at day 60, n (%)	24 (80.0)	16 (84.2)	8 (72.7)	0.641	9 (90.0)	7 (70.0)	0.582
Median interval (days) between first culture and death (95% CI)	7 (5–16) (n = 24)	8 (3.5–21) (n = 16)	6.5 (2–31) (n = 8)	0.668	7 (2–19) (n = 9)	7 (1–41) (n = 7)	0.958
Major exposure prior to infection, n (%)							
Echinocandins	16 (63.3)	10 (52.6)	6 (54.6)	1	6 (60.0)	5 (50.0)	1
Cytarabine ^d	18/23 (78.3)	13/17 (76.5)	5/6 (83)	1	8/9 (89)	4/6 (83)	0.5
Apheresis platelet concentrates (APC)	21/25 (84)	15/17 (88)	6/8 (75.0)	0.57	10 (100)	5 (50.0)	0.03
Prepared with medical device from manufacturer X	10/25 (40)	6/17 (35)	4/8 (50)	0.7	3/9 (33)	3/7 (43)	1
Prepared with medical device from manufacturer Y	19 (63.3)	14 (73.7)	5 (45.4)	0.238	10 (100.0)	5 (50.0)	0.03

^a 95% CI = 95% confidence interval.^b n where the denominator represents the number of patients for whom the information was available (not stated when it was available for all patients).^c Stools were not cultured for all patients.^d Analysis taking into account brands or producers gave only low exposure rates.

located in sequences homologous to those of protein-coding genes from *Yarrowia lipolytica*, the most closely related species with an annotated genome (17). All these changes impacted the deduced amino acid sequence, suggesting positive selection. SNP9 (see Table S2 in the supplemental material) caused a premature stop codon in a sequence similar to that of mycelial growth factor-1 from *Y. lipolytica*. The carbon assimilation pattern and

antifungal susceptibility profile and mycelium growth did not reveal clade-specific characteristics (data not shown).

Of the 30 cases recorded during the investigation period, 19 belonged to clade A, 7 to clade B, and 4 to other sublineages (Fig. 1). Of note, 18 of 19 cases belonging to clade A occurred in 2012 between weeks 15 and 28, and in that time frame, clade A was identified in 18/20 (90%) cases reported (including the 9 original

TABLE 2 MICs of the major antifungal drugs for all *Saprochaete clavata* isolates tested at the French National Reference Center for Invasive Mycoses & Antifungals

Isolate group	No. of isolates	MIC (mg/liter) (median [range])				
		Flucytosine	Voriconazole	Posaconazole	Amphotericin B	Caspofungin
All isolates	45	0.25 [<0.125 –1]	1 [0.06–2]	0.5 [0.125–1]	0.5 [0.125–1]	8 [1–8]
Isolates recovered before September 2011	15	0.25 [<0.125 –1]	1 [0.06–2]	0.5 [0.125–1]	0.5 [0.125–1]	8 [1–8]
Isolates recovered between September 2011 and October 2012	30	0.5 [<0.125 –1]	1 [0.06–2]	1 [0.125–1]	0.5 [0.25–1]	8 [1–8]
Clade A isolates	19	0.5 [<0.125 –0.5]	1 [0.5–2]	1 [0.25–1]	0.5 [0.25–1]	8 [1–8]
Non-clade A isolates	26	0.25 [<0.125 –1]	1 [0.06–2]	0.5 [0.125–1]	0.5 [0.125–1]	8 [1–8]

cases that alerted us to the issue; see Table S1 in the supplemental material). This allowed defining an outbreak period (W15 to W28). Isolates belonging to clade B or other sublineages have been responsible for sporadic cases since 2005 and occurred with no increase during the outbreak period.

Of the 31 European isolates sent as *M. capitatus*, reidentification at the NRCMA showed that *S. clavata* accounted for 12/17 clinical and 13/14 environmental sources, none belonging to clade A or B (see Table S1 in the supplemental material). The remaining isolates were confirmed to be *M. capitatus*. No notification of an outbreak of *S. clavata* infection was sent to the Centers for Disease Control in the United States (CDC) or Stockholm (eCDC).

Assessment of risk factors for infection. The case-case study compared 19 clade A cases to 11 non-clade A cases (Table 1). Characteristics of the patients did not differ significantly between the two groups except for a trend toward a lower proportion of men in the clade A group. Clade A was associated with more clusters (defined as 2 or more cases in a single HCF within 8 weeks) than other sublineages (5 clusters of 2 to 4 cases versus 1 cluster of 2 cases).

Analysis of exposures prior to positive culture did not show a significant difference between the two groups or identify a common source of contamination (see Table S3 in the supplemental material). All cases received blood products. Clade A cases (14/19, 74%) were more frequently exposed than non-clade A cases (5/11, 45%) to apheresis platelet concentrates (APCs) prepared by the use of manufacturer Y's medical device (not significant). This trend was significant for the group consisting solely of index cases (10/10 [100%] versus 5/10 [50%], respectively, $P = 0.033$). Of note, 10/13 (1 missing information) patients with clade A infection cases exposed to the suspected medical device were also exposed to cytarabine. The mean interval between the last infusion of APC prepared with this medical device and positive culture results was 3.0 days for clade A cases (2.0 days for index cases).

The market share in France of this specific medical device is high (50% to 60% of APC in France are prepared with it), which is consistent with the exposure rates recorded among non-clade A cases. Overall, 11 lots of this medical device—produced abroad on the same production line and distributed only in France—were linked to the 30 cases, and more than 12,300 APCs were prepared with the 11 suspected lots. Other lots were produced on the same line and distributed to other countries.

Applying precautionary principles, imposition of a quarantine was decided by health authorities in September 2012 for unused

blood products prepared with the suspected lots of the medical device, but very few were still stored. Two unused medical devices (see Fig. S1 in the supplemental material) as well as 3 APCs and 2 frozen plasma samples prepared with the suspected lots were analyzed and showed no evidence of fungal contamination. Results of local investigations of fungal contamination of food and environmental samples were also negative.

Experimental infections. Knowing the epidemiology of fungal infections helps in understanding their pathophysiology and the risk of person-to-person transmission. Here we had indirect evidence that index cases could have been contaminated through APC and we knew that *S. clavata* was recovered from stool specimens in humans, representing therefore a source for transmission to secondary cases. Translocation from gut to blood after local proliferation is well known and has been demonstrated, for example, for *Candida albicans* (18) in experimental models and in humans. The reverse process (translocation from blood to gut) is usually not a concern and has not been systematically studied for other fungal pathogens. To assess whether it was possible, intravenous inoculation of *S. clavata* clade A was performed in nonimmunocompromised mice and fungal growth recorded in blood and feces samples from infected animals (Fig. 3A and B). We first checked that no fungal colony was observed in samples from uninfected control mice. From infected mice, all stool specimens on days 2, 5, and 7, as well as 4/5 blood samples on days 2 and 5 and 3/5 blood samples on day 7, grew *S. clavata*.

We also compared the levels of virulence of clades A and B, which differed in mice after intravenous inoculation, and found a significant difference ($P = <0.001$, log-rank test) (Fig. 3C). All mice infected with clade B isolate died before day 25 after inoculation; no differences between males and females were seen. All female mice infected with clade A survived on day 30, whereas two male mice infected with clade A died (1 on day 10 and 1 on day 24).

DISCUSSION

While the French national surveillance of invasive fungal infections recorded only sporadic cases of *S. clavata* until 2012, the events observed in the spring of 2012 (epidemic curve and geographic distribution) suggested a common-source outbreak during which patients were exposed to a nationally distributed source over a relative brief period. This multicenter outbreak affected patients with severe hematological malignancies and was associated with a high short-term case fatality rate. As the potential

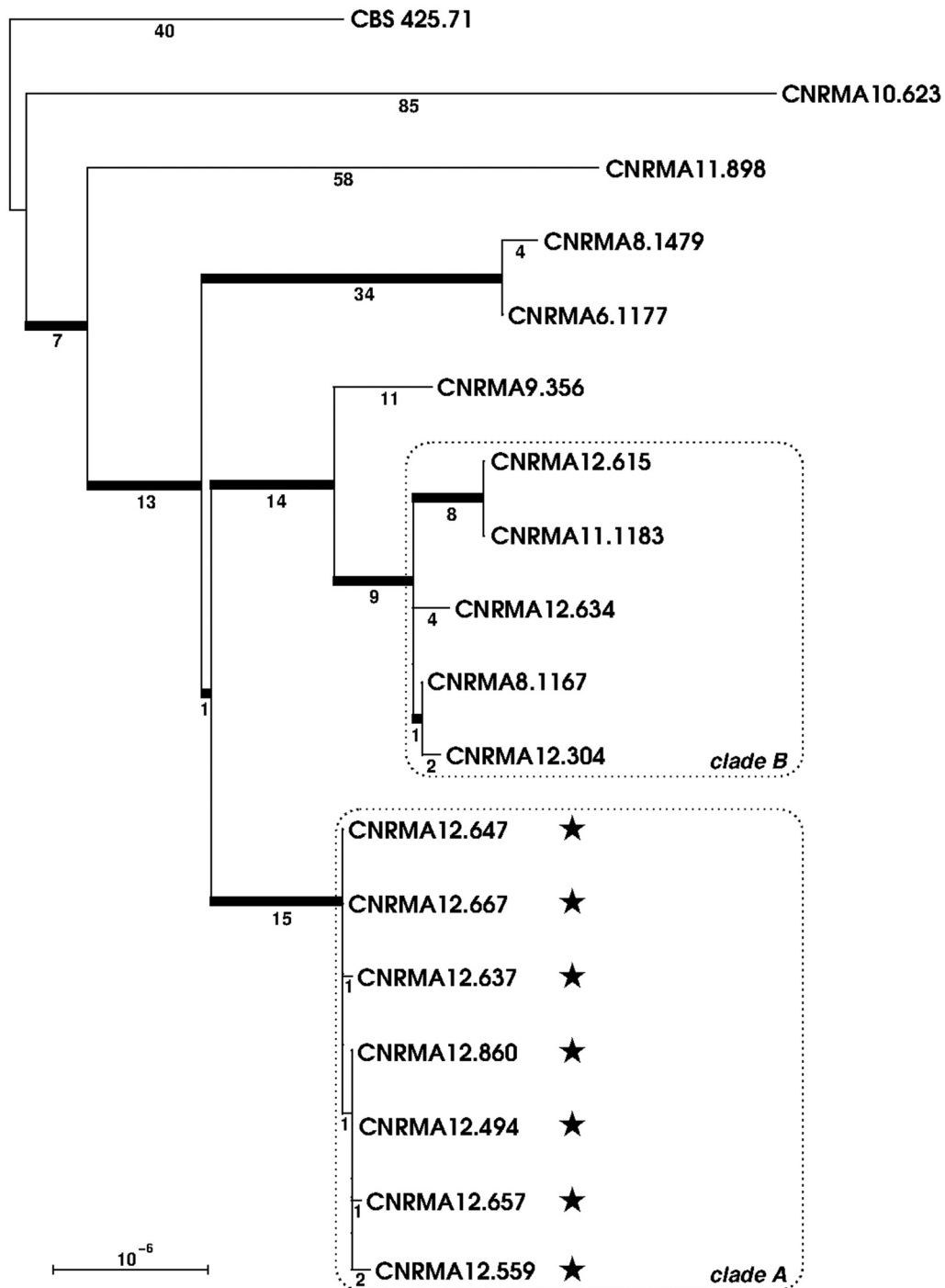


FIG 2 Phylogenetic tree inferred for 18 *Saprochaete clavata* isolates based on genome-wide SNPs. An unrooted BioNJ (40) tree constructed from pairwise nucleotide p-distances is shown. The number of nucleotide changes which were unique to the corresponding branch is indicated below each branch. The scale bar represents the SNP rate based on the estimated genome length (~17.5 Mb). Thick branches are 100% supported by bootstrap analysis results. Clades A and B are indicated with dashed boxes. Isolates recovered during the outbreak are indicated with a black star.

source of *S. clavata* was unknown, an investigation of food, drugs, and blood products was started based on the assumption that dairy products might be involved, as seen with *M. capitatus*, and that the incubation period should be short in cases of oral ingestion with digestive translocation.

Multilocus sequence typing (MLST) can now be replaced by

whole-genome SNP typing (whole-genome sequence typing [WGST]) for inferring the relatedness of both bacteria and fungi. Whole-genome sequencing is indeed a powerful approach to characterize microbial strains for outbreak investigation and molecular epidemiology purposes. Indeed, genomic sequencing provides maximal discriminatory power while uncovering at the same

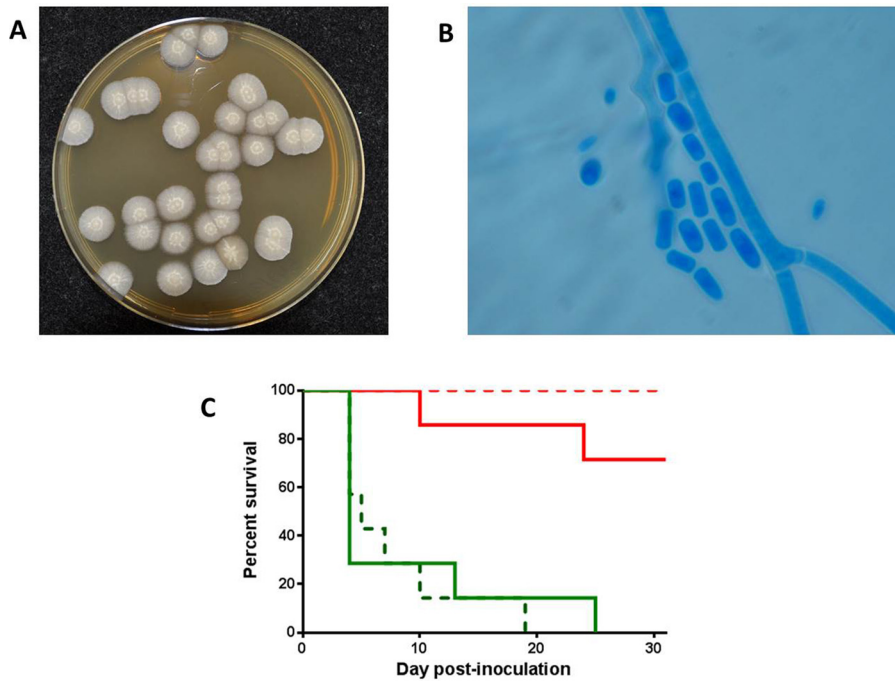


FIG 3 Experimental infections with *Saprochaete clavata*. (A) Macroscopic image of *S. clavata* on SDA petri dish after 48 h of incubation at 30°C. (B) *Saprochaete clavata* from a culture grown on 2% malt extract agar examined with Nomarski interference contrast ($\times 100$), after 48 h of incubation at 30°C. (C) Survival curves for OF1 mice (dashed lines for males, solid lines for females) infected intravenously by strains of *Saprochaete clavata* belonging to clade B (green lines) or clade A (red lines).

time genomic variations that can be linked to increased pathogenic potential. Here, in the absence of validated methods for genotyping *S. clavata* isolates, the first complete genome sequence of this fungal species was determined and used to design a novel genotyping method. Comparison of outbreak and sporadic isolates clearly demonstrated that the outbreak involved one single strain (clade A), which represents a recently emerged clone, while other lineages were responsible for sporadic cases in France and for monocentric outbreaks in Europe (12, 14, 19), outbreaks previously attributed to *M. capitatus*. Given the small amount of polymorphism uncovered, any classical typing approach was deemed likely to fail to recognize the outbreak clone, underlining the importance of WGS as a novel tool for outbreak investigations of fungal pathogens.

Four clade A-specific nucleotide changes were located in sequences homologous to those of protein-coding genes from *Y. lipolytica* and impacted the deduced amino acid sequence, suggesting positive selection. SNP9 caused a premature stop codon in a sequence similar to that of mycelial growth factor-1 from *Y. lipolytica*. The issue of whether these changes conferred a selective advantage to clade A isolates or increased their pathogenic potential was intriguing. Carbon assimilation pattern and mycelium growth analyses did not reveal clade-specific characteristics. We ruled out major changes in antifungal susceptibility profiles that could explain the emergence of clade A. A nonsignificant increase in the voriconazole MIC was observed for clade A isolates (1 dilution) that may have impacted its fitness. However, this was not confirmed by *in vivo* experiments, where clade A was less virulent in mice than clade B. It must be kept in mind, though, that we used intravenous inoculation of immunocompetent mice and not neu-

tropenic mice or mice with damaged intestinal tract or disturbed microflora (18).

The discovery that a single clade of *S. clavata* was associated with the outbreak and was distinct from sporadic isolates provides, however, additional if not definite evidence for a common source of contamination. Clade A was associated with a higher number of clusters than other sublineages, indicating high exposure to a common source or a higher capability of person-to-person transmission. The majority (almost 80%) of cases were also exposed to cytarabine, which is known to alter digestive mucosa. Diarrhea associated with fungemia was reported in more than half of the patients. Furthermore, gut colonization was recorded in approximately 60% of patients for whom we had the relevant information. Gut colonization may have been underestimated, given the knowledge that *Galactomyces candidus* (synonym of *Geotrichum candidum*), which has the same morphology in culture as *S. clavata*, is considered a contaminant and is not always reported. We found here that, after experimental intravenous inoculation of *S. clavata* into immunocompetent mice, *S. clavata* can translocate from the blood to the gut even when the gut mucosa is intact. Even if the proportion of patients recorded with diarrhea and positive stools was relatively low, our observations suggested that index patients with fungemia could have experienced subsequent gut translocation due to cytarabine or diarrhea. These findings are compatible with cross-contamination within HCF.

We considered the possibility of a source of contamination that was ingested orally, given the high frequency of diarrhea and positive stool specimen results. Furthermore, we discovered that *S. clavata* can be recovered from dairy products (12, 19) and dish-

washers (20) as determined on the basis of reidentification of isolates previously labeled as *M. capitatus*. Finally, the low percentage of men infected with clade A (37%), which is different from the usual male predominance recorded during periods of yeast fungemia (60.7% [2,317/3,818] for all yeast species and 77.8% [7/9] for non-clade A *S. clavata* fungemia in our YEASTS surveillance program [2] [NRCMA, unpublished data]), could suggest the involvement of a product preferentially consumed by women, as in the recent Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany (21). However, epidemiological and microbiological investigations did not lead to a documented source of infection.

Case-case comparisons have been instrumental in deciphering, for example, outbreaks of listeriosis (22, 23). Our genotyping technique allowed differentiation of the cases into two meaningful groups (clade A versus other sublineages), thus allowing a case-case study. All patients came from the same source population and were susceptible to *S. clavata* infection. A classical case-control approach selecting controls among patients in the same wards might have introduced bias due to similar probabilities of exposure to contaminating sources. A significantly higher exposure frequency among clade A index cases than among non-clade A index cases led to the hypothesis of a role for APC as an infection source and of possible transmission to secondary cases as described above. The end of the nationwide outbreak coincided with the exhaustion of the APC stock before recall of the suspected lots, consistent with our hypothesis. Exposure to the medical device used for APC collection could explain 75% of the cases. However, the APC contamination hypothesis is challenged by the fact that the same production line was used to produce medical devices that were exported to various countries, which makes the possibility that contamination occurred only in lots distributed to France less likely. Neither the European CDC nor the U.S. CDC recorded outbreaks due to *S. clavata*, and no fungal contamination was evidenced in the medical devices of the same lots and in the blood products tested.

The proposed association between APCs and *S. clavata* cases remains hypothetical, as no cases occurred in HCF outside the study area that also used products from the same lots; only a few cases were identified despite widespread use of the device, and no fungal contamination of blood products or associated with the storage or processing of medical devices was uncovered. Our study included only a small number of cases, which lowered the power of the analysis and limited the choice of the controls. The retrospective nature of the investigation precluded collection of all data and microbiological testing of all potential sources of contamination (especially food). Overall, the source of the contamination remains unidentified.

Further studies are needed to provide insight into the process of how a microorganism probably ingested or inoculated with a low inoculum can cause severe invasive disease once critical components of the host immune system are compromised. However, the possibility that *S. clavata* may be transmitted through contaminated medical devices or can be associated with dairy products as in previous European outbreaks is highly relevant for the management of future outbreaks due to this newly recognized pathogen.

MATERIALS AND METHODS

Epidemiological investigation. The French National Institute for Public Health Surveillance (Institut de veille sanitaire [InVS]) coordinates na-

tional surveillance for health care-associated infections (24). During the spring of 2012, three clusters of three cases each of invasive infections due to *M. capitatus* were reported to InVS from 3 HCF located in three regions (25). The isolates were subsequently identified as *S. clavata* at the National Reference Centre for Invasive Mycoses & Antifungals (NRCMA).

The occurrence of nine cases in three remote centers over a limited period suggested a multicentric outbreak, possibly associated with a common source. Indeed, only sporadic infections due to these species (15 cases between January 2005 and September 2011) had been recorded through the nationwide ongoing surveillance programs on invasive fungal infections implemented by the NRCMA. These programs include active surveillance of fungemia (including those due to *M. capitatus* and *S. clavata*) in the Paris area (YEASTS program [26]) and passive nationwide surveillance of rare invasive fungal infections, collecting both clinical information and isolates (https://epidemiologie.pasteur.fr/resomyc_portail/).

Thus, because *S. clavata* epidemiology and ecology were largely unknown and to confirm the outbreak and identify its onset, a national alert was launched in all hematology wards and HCF laboratories in metropolitan France, with the objective to identify all cases due to one of the two species (investigation period, September 2011 to October 2012). A standardized questionnaire was used to collect information from treating physicians. The cases were defined as patients with a positive culture for a fungus identified as *M. capitatus* or *S. clavata* or *Geotrichum* sp. from a sterile site or from the respiratory tract when it was associated with clinical and/or radiological symptoms in a patient with underlying predisposing conditions (27). All isolates were to be sent to NRCMA for further analysis.

The objectives were to describe case characteristics, medical histories, and outcomes and to identify risk factors, including a potential common source. Data corresponding to food intake, including but not limited to dairy products, and drug administration, including mouthwashes, medical fluids, and drugs, within 1 week (1 month for antifungals) before positive culture were recorded. Exposure within 1 month to blood products was specifically investigated with the cooperation of the French national agencies for blood products (Etablissement Français du Sang) and for drug safety (Agence de Sécurité du Médicament et Produits de Santé). The single-use medical devices used to collect and store blood products (see Fig. S1 for an example) and the specific lots used for the infused blood products were also recorded. In parallel, the Centers for Disease Control in the United States (CDC) and in Stockholm (eCDC) were informed and asked if similar events had occurred recently in other countries.

This investigation was considered to be a public health response and deemed to be nonresearch; thus, requirements for approval by an institutional review board or for written informed consent from patients were waived. Epidemiological data were recorded anonymously except when tracking blood products lots (approval 341194v42 of the French Commission for Data Protection [CNIL]).

Laboratory investigation. Food samples (samples from similar dishes prepared at the same time as those offered to the patients were blended and plated on Sabouraud dextrose agar [SDA] plates) and environmental samples (swabs on hard surfaces, air samples plated on SDA) were analyzed in a few HCF. Because of the high fatality rate, these investigations were limited to a few cases. At the NRCMA, washes of unused medical devices and blood products from the incriminated lots were tested for fungal contamination by culture (pellets of centrifugation incubated for 30 days on Sabouraud agar at 30°C) and amplification of internal transcribed spacer (ITS) regions with panfungal primers after DNA extraction was performed (28).

Strains studied. European colleagues who previously identified *M. capitatus* from clinical sources (blood) or environmental sources (dishwashers, milk, or containers) (12, 19, 20) were contacted and asked to share their isolates for comparisons. All these “European” isolates together with all the French isolates collected during and before the investigation period through the surveillance programs were reidentified at the NRCMA using nucleotide sequencing of the D1D2 and ITS1-5.8S-ITS2

regions (9, 29–31). Antifungal susceptibility testing was performed according to the EUCAST method (32). Type strains of *S. clavata* (CBS425.71) and *M. capitatus* (CBS162.80) (see Table S1 in the supplemental material) were studied.

Illumina sequencing and data preprocessing. Since genotyping methods for *S. clavata* were lacking, WGS was performed to compare isolates and allowed the subsequent design of a genotyping method by PCR and Sanger sequencing.

A first batch of 11 clinical isolates, including 5 outbreak isolates (see Table S1 in the supplemental material), was sequenced in July 2012. Libraries were constructed using a Nextera DNA sample preparation kit (Illumina) and sequenced on an Illumina HiSeq 2000 sequencer with a 100-nucleotide single-end protocol. Two lanes from these tagged libraries resulted in ~26 million reads per strain on average. Genomic libraries for a second batch of 6 clinical isolates plus the *S. clavata* type strain were prepared in August 2012 following the same protocol, except that the libraries, with an insertion size of ~250 bp, were sequenced with a paired-end protocol that generated $2 \times \sim 7$ million reads of 100 bases per strain on average. Finally, to improve genome assembly, one isolate (CNRMA12.647; see Table S1) was resequenced using an Illumina TruSeq library with a $2 \times$ 100-nucleotide paired-end module (insertion size of ~400 bp), leading to $2 \times \sim 190$ million reads. All reads were preprocessed to remove low-quality or artifactual nucleotides. First, all nucleotides occurring at the 5' and 3' ends and supported by a Phred quality score of <28 were removed using the program Sickle version 1.33 (GitHub Inc., San Francisco, CA, USA). Second, contaminant oligonucleotides (i.e., library adaptors) were detected and removed using the program AlienTrimmer (33). Third, reads that were below 45 nucleotides in length after performance of the cleaning steps described above were discarded, as were those containing more than 5% of the remaining nucleotides with a Phred score of <28. Finally, the program fqduplicate (<http://ftp.pasteur.fr/pub/gensoft/projects/fqtools>) was used to discard every duplicate single- or paired-end read.

Detection of high-quality SNPs among strains. Whole-genome *de novo* assembly for each strain was performed using the program *clc_novo_assemble* (CLC Bio, Aarhus, Denmark). A set of 175 conserved and universally distributed fungal protein sequences (34, 35) as well as 18S rRNA regions were extracted from each set of contigs and aligned using MUSCLE (36, 37). As a distinct approach, sequence variation was mined following a read-mapping procedure. To obtain a high-quality reference, all filtered reads from the first batch of 11 strains were pooled and assembled, resulting in 4,618 contigs of >500 nucleotides in length (~3,668-nucleotide length on average, ~17.5 Mb in total). The read sets of each of the 18 isolates were then mapped on the reference using the Burrows-Wheeler Alignment (BWA) tool (38). Information on coverage (the number of reads that map to each reference sequence position) and polymorphisms was extracted using SAMtools (39). To select high-quality SNPs, we retained those that (i) were covered in all strains by at least four reads mapped in each orientation, (ii) had a minimum mapping quality of 30, and (iii) were supported by at least 80% of reads mapped at this position. In complement, SNPs occurring in regions of microrepeats or less than 100 nucleotides from any contig end were discarded.

Phylogenetic analysis. All the inferred high-quality SNPs were merged into a set of 312 variable characters. Among these characters, 103 were parsimony informative (i.e., contained more than one type of nucleotides, with at least two of them occurring in at least two isolates, therefore supporting phylogenetic groupings). Phylogenetic inference was performed with the algorithm BioNJ on the pairwise nucleotide *p*-distances, and confidence supports were estimated for each internal branch with a bootstrap procedure based on 10,000 replicates (40). As expected given that the 312 characters were compatible, the same tree topology was obtained using alternative tree inference methods, including maximum parsimony and maximum likelihood.

De novo genome assembly of strain CNRMA12.647. Scaffolded contig sequences of isolate CNRMA12.647 (belonging to clade A) were pro-

duced from filtered reads by *de novo* assembling with parameters selected to maximize the average scaffold length. This resulted in 339 scaffold sequences of a total length of ~17.5 Mb, which were used to position the specific SNPs of clades A and B identified previously (see Table S3 in the supplemental material).

Virulence tests in mice. The mouse experiments had two objectives: (i) to study the potential translocation of *S. clavata* from blood to the digestive tract and (ii) to compare the levels of virulence of *S. clavata* in normal hosts according to host gender and clades of *S. clavata*. For the survival experiments, 6-week-old OF1 male or female mice (Charles River, France) were inoculated intravenously with 100 μ l of a suspension of *S. clavata* at 4×10^6 CFU/ml. Groups of 7 animals were inoculated with either clade A (CNRMA12.647) or clade B (CNRMA12.304). Animals were observed daily. Those that appeared moribund or in pain were sacrificed by CO₂ inhalation. Survival rates were compared using the log-rank test and GraphPad Prism version 6 (GraphPad Software, Inc.).

For the gut translocation study, 3 groups of 5 OF1 female mice were infected intravenously with 100 μ l of a suspension of *S. clavata* clade A (CNRMA12.647) at 3×10^6 CFU/ml. Five mice were sacrificed on days 2, 5, and 7 postinoculation. Blood (500 μ l obtained by cardiac puncture) was cultured on SDA. Feces samples were diluted in 500 μ l sterile water on SDA plates containing chloramphenicol and incubated for 5 days at 30°C. The identification of the fungus growing from the feces obtained on day 2 was confirmed by sequencing the ITS. All subsequent colonies were identified by morphology. Animal experiments were approved by the national ethical committee (CETEA 2013-0135) and the local ethical committee (CHSCT 14.135).

Statistical analysis. The first descriptive analysis focused on all cases of *S. clavata* recorded during the investigation period. Following the identification of clade A, a case-case study (41) was performed to identify exposure and factors associated with infection with clade A (see above for a description of how clade A was identified) compared to those due to other sublineages. “A” cases were defined as all cases belonging to clade A and “non-A” cases as all cases belonging to other lineages that occurred in France during the investigation period. An index case was defined as the first case infected (positive culture) by either clade A or other lineages in a given HCF. Differences in categorical variables were compared using Fisher’s exact test and differences in quantitative variables using the Mann-Whitney test. Data analysis was performed using Stata 9.2 (Stats Corp., College Station, TX).

Nucleotide sequence accession numbers. Assembled sequences of isolate CNRMA 12.647 were submitted to the EMBL database and are accessible under accession numbers CBXB01000001 to CBXB010000339.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02309-14/-/DCSupplemental>.

Figure S1, JPG file, 0.1 MB.

Table S1, PDF file, .01 MB.

Table S2, PDF file, 0.02 MB.

Table S3, PDF file, 0.01 MB.

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