




Original Article

Molecular epidemiology of aspergillosis in Magellanic penguins and susceptibility patterns of clinical isolates

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Abstract

Aspergillus section *Fumigati* is reported in up to 99% of aspergillosis cases in penguins. So far, no data regarding molecular epidemiology and azole resistance are available for *A. fumigatus* isolates collected from Magellanic penguins. The aim of this work was to perform molecular identification of *Aspergillus* section *Fumigati* at species level, to genotype those isolates using microsatellite markers, to evaluate the *in vitro* susceptibility patterns of *A. fumigatus* sensu stricto, and to characterize the *cyp51A* gene in clinical *A. fumigatus* strains isolated from Magellanic penguins with proven aspergillosis. All 34 isolates included in the study were identified as *A. fumigatus* sensu stricto. Analyzing the genetic diversity of the isolates of *A. fumigatus* sensu stricto, we identified two possible outbreaks in the rehabilitation center and we also observed the maintenance of clonal strains through the years. One *A. fumigatus* sensu stricto isolate was resistant to posaconazole, but the mutations found in the *cyp51A* gene of this isolate have not been described as conferring phenotypic resistance, suggesting that other mechanisms of resistance could be involved in the resistance of this isolate. With this study, we were able to understand the molecular diversity of *Aspergillus fumigatus* isolates collected from Magellanic penguins, to characterize them and to associate them with the described global population of *Aspergillus fumigatus*.

Lay summary

A. fumigatus sensu stricto is of great importance in penguins' aspergillosis. We could identify two outbreaks in the rehabilitation center and the maintenance of clonal strains through the years. Regarding antifungal prophylaxis, it may proceed, but preferably with surveillance for azole resistance.

Key words: molecular identification, microsatellite markers, genomic diversity, genotyping, *Aspergillus* resistance, mechanisms of resistance, penguins.

Introduction

Aspergillosis is one of the most important infectious diseases in captive penguins, and an important limiting factor for the rehabilitation of these animals. Rates of 7.3 per 100 penguins/month for incidence density, and of 48.5% for proportionate mortality are attributed to this mycosis in Magellanic penguins (*Spheniscus magellanicus*) during rehabilitation.^{1,2} As well known for other hosts,^{3,4} inhalation of fungal conidia is the main route of infection, and respiratory airways are the most affected anatomic site in penguins.⁵

The main infection source of aspergillosis in penguins in rehabilitation is not proven so far. A study with different avian species has shown the recovery center as a potential source of *Aspergillus* infection.⁶ However, a previous epidemiological study also raised the hypothesis that some captive penguins could have been colonized/infected by *Aspergillus* in nature, prior to their rescue to the rehabilitation center.¹ Although there are some studies describing aspergillosis in free-ranging birds,^{7–10} there is just one report of this mycosis in Magellanic penguins in nature.¹¹

Up to 99% of aspergillosis cases in penguins are attributed to *Aspergillus* section *Fumigati*.¹ *Aspergillus* identification at section level is achieved by the observation of macro- and microscopic characteristics, however, the identification at species level is only possible using molecular techniques.¹² Clinical implications for identifying cryptic species (species from the same section difficult to distinguish phenotypically from each other but different at molecular level) include their differences concerning intrinsic azole resistance^{13,14} and gliotoxin production.¹⁵ Birds are particularly susceptible to this mycotoxin when produced *in vivo*, since it induces immunosuppression.^{15,16}

Despite the intrinsic azole resistance of cryptic *Aspergillus* species, the emergence of azole resistance in *A. fumigatus* has become a global concern, resulting in increase of morbidity and mortality rates caused by aspergillosis.^{17–19} Understanding the molecular mechanisms involved in phenotypic resistance is important from an epidemiological perspective, since it can suggest the origin of the resistance. Mutations in the *cyp51* gene are reported as the most common molecular mechanisms involved in phenotypic azole resistance so far.^{20,21} Most of these mutations can be related to agriculture procedures, most likely associated with cross resistance from the use of crop fungicides.^{22,23} Alternatively, acquired resistance may be due to patients' long-term azole therapy.^{24,25}

Surveillance of *Aspergillus* azole-resistance has been recommended worldwide,^{26–31} however it is not a routine in veterinary medicine practice, despite the routine antifungal use in prophylaxis and/or treatment of fungal diseases.^{1,32} Specifically regarding the susceptibility patterns of *Aspergillus* isolated from avian species, few studies have been published, most of them with isolates from poultry.^{33–36} In addition, no data concerning molecular identification at the species level of *Aspergillus* isolates from Magellanic penguins are available so far. This penguin species re-

produces in Argentine and Chilean Patagonia and the Falklands Island, and migrates seasonally to the coast of Argentina, and through the Atlantic oceanside to other countries (Uruguay and Brazil).

The study of the *Aspergillus* genetic diversity by the use of molecular typing methods is a valuable tool. Genotyping techniques are being used in molecular epidemiology studies for detection of outbreaks, identification of patients chronically colonized/infected by *Aspergillus* and for monitoring antifungal therapy efficacy in those patients with aspergillosis.^{37–39} Thus, this study aimed to perform molecular identification of clinical *Aspergillus* section *Fumigati* strains isolated from Magellanic penguins with proven aspergillosis, to understand the epidemiology of those *A. fumigatus* sensu stricto by its genotyping, to evaluate the susceptibility pattern of *A. fumigatus* sensu stricto to azole drugs, and to study possible mutations of the *cyp51A* gene associated with azole resistance.

Methods

Aspergillus isolates

A total of 34 *Aspergillus* isolates belonging to section *Fumigati* were recovered from fatal aspergillosis that occurred in Magellanic penguins between 2008 and 2019 (31 from captive penguins and three from free-ranging penguins)¹ in Southern Brazil. *Aspergillus* isolates were identified at section level by macro- and microscopic characteristics and were stored at the fungal collection of the Mycology Laboratory from the Hospital of the Federal University of Rio Grande, Rio Grande do Sul, Brazil.

DNA extraction

For the molecular techniques described below, genomic DNA of clinical isolates was extracted with the High Pure PCR Template Preparation KitTM (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Molecular identification of *Aspergillus* at species level

Partial sequencing of the calmodulin gene (*calM*),⁴⁰ using the primers cmd5 (5'-CCGAGTACAAGGAGGCCTTC3') and cmd6 (5'-CCGATAGAGGTCATAACGTGG3'), and/or partial sequencing of the β -tubulin gene (*benA*),⁴¹ using the primers β -tub1 (5'-AATTGGTGGCCGCTTTCTGG-3'), and β -tub2 (5'-AGTTGTCCGGACGGAATAG-3') were performed to the species identification of all isolates. PCR for both amplifications was carried out in a 25 μ l volume containing 0.2 μ M of each primer, 20–50 ng of *Aspergillus* genomic DNA; and 10 \times PCR buffer, 1 mM MgCl₂, 0.1 mM each of dATP, dGTP, dCTP, and dTTP, 1 U of Taq DNA polymerase (all Applied Biosystems Inc., Foster City, CA, USA).

For partial *calM* amplification, PCR was carried out in a thermal cycler as follows: after an initial denaturation step of 95°C

for 10 min, 38 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min. For partial *benA* amplification, PCR was carried out as follows: after an initial denaturation step of 94°C for 2 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. PCR products were confirmed by 2% agarose gel electrophoresis and the purification of the PCR product was made using the ExoSAP-IT enzyme system (USB Corporation, Cleveland, OH, USA), according to the manufacturer's instructions.

Sequencing was performed using the forward primers and the BigDye terminator v 1.1 Cycle sequencing kit (Applied Biosystems) in the thermal cycler with the following conditions: an initial denaturation at 96°C for 5 s, 30 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min, and a final extension at 72°C for 5 min. Sequences were analyzed with MEGA software version 10.0.5, and the obtained sequences were then compared with those deposited in the GenBank database (Bethesda, MD, USA) in order to achieve identification to species level.

Analysis of the genetic diversity of *A. fumigatus* sensu stricto

All the identified *A. fumigatus* sensu stricto isolates (n = 34) were evaluated regarding their genetic diversity. Isolates genotyping was performed based on microsatellite markers, using the multiplex PCR M3 combination previously described,⁴² amplifying three loci with trinucleotide repeats, with a calculated discriminatory power of 0.9968. The three forward primers (STRAf 3A, STRAf 3B, and STRAf 3C) were labeled at the 5' end with carboxyfluorescein (FAM), hexachloro carboxyfluorescein (HEX), or dichloro carboxyfluorescein (NED), respectively.

PCR reactions were carried out in a 25 µl volume containing 1 × PCR buffer (Applied Biosystems Inc.), 1 µM of each primer [STRAf 3A (Forward FAM-GCTTCGTAGAGCGGAATCAC and reverse GTACCGCTGCAAAGGACAGT), STRAf 3B (Forward HEX-CAACTTGGTGTCAGCGAAGA and reverse GAGGTACCACAACACAGCACA), and STRAf 3C (Forward NED-GGTTACATGGCTTGAGCAT and reverse GTACA-CAAAGGTGGGATGG)], 0.2 mM deoxynucleoside triphosphates, 3.0 mM MgCl₂, 1 U of Taq DNA polymerase (Applied Biosystems Inc.), and 1 ng of genomic DNA. PCR conditions were carried out in a thermal cycler as follows: after an initial denaturation step of 95°C for 10 min, 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. A 15 µl of ultrapure formamide mixture was added in 1 µl of PCR product, and then a denaturation at 95°C for 3 min was performed. After this step, analysis of the fragments was performed by capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems) instrument, using the molecular weight marker GeneScan 500 ROX Size Standard (Applied Biosystems), and the GeneMapper 6.0 software (Applied Biosystems) was used for data analysis.

Alleles were designated by the fragment length of the resulting PCR product. The number of repeats of the STRAf 3A, STRAf 3B, and STRAf 3C markers were determined according to de Valk et al.⁴² Simpson diversity index was calculated using untpb package, and the neighbor joining tree was constructed using the ape package, both in Rstudio® (Boston, MA, USA) software. The genotypes obtained were compared with genotypes of global population of *A. fumigatus* using the web-based programme AfumID (<https://afumid.shinyapps.io/afumID/>).

Detection of azole-resistant strains

The azole-resistance screening method was performed in all *A. fumigatus* sensu stricto strains based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) procedure.^{43,44} Three different supplemented Sabouraud dextrose agar (SDA) plates were prepared, containing 4 mg/l itraconazole (ICZ), 2 mg/l voriconazole (VCZ), and 0.5 mg/l posaconazole (PCZ) in each. A 0.5 McFarland turbidity inoculum suspension prepared from fresh conidia from a 5 to 7 day old culture was inoculated by dipping a sterile swab into the inoculum suspension and swabbing the agar surface. Plates were incubated at 35°C, and observation of fungal growth was performed after 48 h of incubation. A pan-azole resistant strain with known TR₃₄/L98H mutation⁴⁵ was used as resistance control, and the *A. fumigatus* strain ATCC 204305 was used as a susceptible control. Each isolate suspension tested was inoculated onto a plate containing Sabouraud Dextrose Agar without chloramphenicol and without any antifungal drug, as a growth control. Those isolates that were not able to grow in the plates with antifungal drugs were considered as 'susceptible', and those that showed any growth on antifungal-containing media, were selected for further investigation.

Broth microdilution antifungal susceptibility testing

Isolates that grew in screening agar azole plates were tested by broth microdilution test according to the M38 protocol of Clinical Laboratory Standards Institute (CLSI 3rd Edition).⁴⁶ Tested antifungal concentrations ranged from 0.0156 to 8 µg/ml for all drugs tested (ICZ, VCZ, PCZ). *A. flavus* strain ATCC 204 304 was used as control of drug potency. A standard conidial inoculum was prepared from a conidial suspension with optical density ranging from 0.09 and 0.11 (80–82% transmittance), then diluted 1:50 in RPMI-1640 media. An inoculum control was performed by plating 100 µl of 10⁻² dilution of the final suspension of the standard conidia inoculum and incubated at 30°C. Colony forming units (CFU) were enumerated after 48 h incubation at 25°C.

Tests were performed in a 96-well sterile microplate, in which 100 µl of the standard conidia inoculum was inoculated in each well containing 100 µl of distributed drug. In each microplate, a sterile media control and the inoculum growth control were

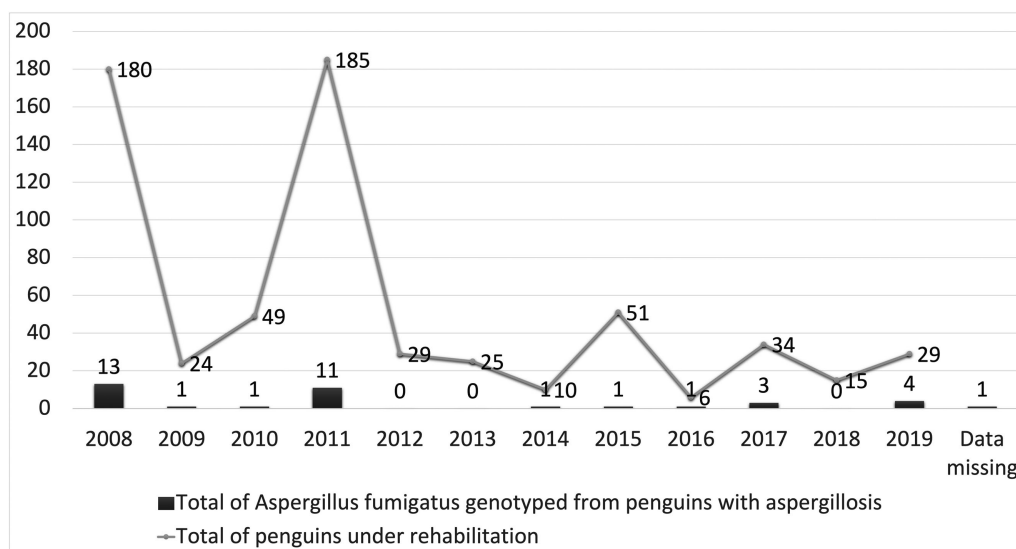


Figure 1. Year of obtaining the *Aspergillus* clinical isolates collected from Magellanic penguins who died with aspergillosis during rehabilitation.

included. Visual reading of the results was performed after 48 h incubation at 35°C, determining the minimum inhibitory concentration (MIC) as the lowest concentration able to inhibit 100% of fungal growth. Tests were performed at least in duplicate, and in case of conflicting results, a triplicate was performed. The determination of azole-resistant strains followed the MIC breakpoints for ICZ (>2 µg/ml), VCZ (>2 µg/ml), and PCZ (>0.5 µg/ml) proposed based on the EUCAST protocol.⁴⁷

Detection of mutations in the *cyp51A* gene conferring azole resistance

Isolates that grew in the screening agar azole plates were submitted to a multiplex real-time PCR assay for detection of azole-resistant markers TR₃₄/L98H and TR₄₆/Y121F/T289A (AsperGenius®, PathoNostics, Maastricht, Netherlands), performed on the RotorGene Q instrument (Qiagen, Hilden, Germany), following the manufacturer's instructions. In addition, the sequencing of the *cyp51A* gene and its promoter was performed from an amplification reaction in a final volume of 25 µl consisting of 20–50 ng of *Aspergillus* genomic DNA, 0.4 µM of each primer [CYP51A-AF1 (5'-ATGGTGCCGATGCTATGG-3') and CYP51AR2 (5'-AGTGAATAGAGGAGTGAATCC-3')], and 2.5× PCR buffer, 2 mM MgCl₂; 0.2 mM each of dATP, dGTP, dCTP, and dTTP; 1 U of Taq DNA polymerase (all Applied Biosystems). The PCR reaction was performed by an initial denaturation step of 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min.⁴⁸ Purification was performed as described above. For each PCR product, four sequencing reactions were performed, using two forward primers [CYP51AF1 and CYP51AF2 (5'-GACATCTCTGCGCAATGG-3')] and two reverse primers [CYP51AR2 and CYP51AR3(5'-

CCATTGCCGAGAGATGTC-3')] following the sequencing conditions described previously.

For sequencing the *cyp51A* promoter, the primers PA5 (5'TCTCTGCACGCAAAGAAGAAC3') and PA7 (5'TCATATGTTGCTCAGCGG3')⁴⁹ were used, following the same volume reactions as described for amplification of *cyp51A*. PCR conditions were an initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 s, 66°C for 45 s, and 72°C for 2 min, and a final extension at 72°C for 7 min. PCR product confirmation, purification, and sequencing were performed as described above, using both forward and reverse primers. The editing of nucleotide sequences, assembly of the consensus sequences and alignment, was performed using MEGA software version 10.0.5.

Results

Clinical isolates recovered from penguins

Aspergillus isolates were obtained from penguins that died from 2008 to 2019, with 70% of those isolates corresponding to the years 2008 (13/34) and 2011(11/34) (Fig. 1). From a total of 34 cases, at least (data regarding prophylaxis were missing in some cases) 39% (n = 12) of these penguins received ICZ at 20 mg/kg for 15 days during rehabilitation as an antifungal prophylaxis.

Species identification and microsatellite genotyping of the *Aspergillus* isolates

All clinical isolates, from free-ranging penguins (n = 3) and from captive penguins (n = 31), were molecularly identified as *A. fumigatus* sensu stricto, showing more than 99% homology with the reference sequence NC_007194 from the Genbank.

Table 1. Alleles of *Aspergillus fumigatus* sensu stricto isolated from Magellanic penguins with proven aspergillosis, obtained from three different loci (*STRAf3A*, *STRAf3B* and *STRAf3C*).

Microsatellite designation	Number of alleles	Minimum fragment size	Maximum fragment size	Number of repeats (range)
<i>STRAf3A</i>	16	128.89	295.34	6 - 61
<i>STRAf3B</i>	12	147.86	225.01	4 - 32
<i>STRAf3C</i>	18	70.85	171.39	3 - 35

Data for each allele are presented in fragment length and number of repeats.

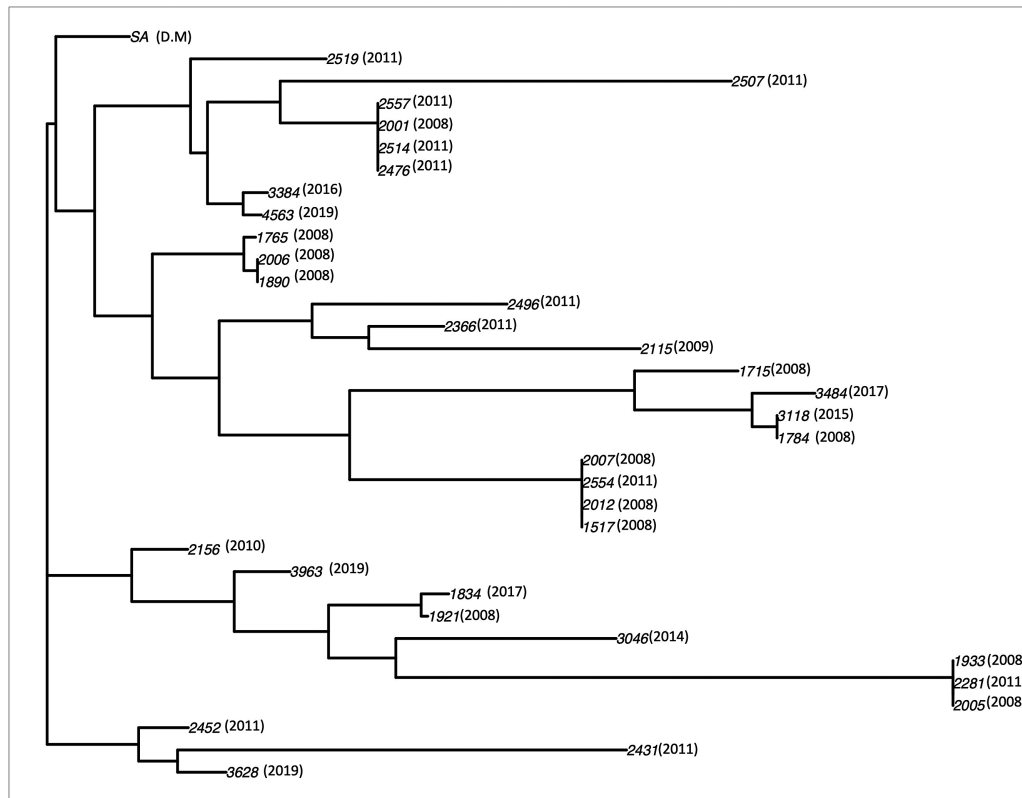


Figure 2. Dendrogram showing clustering of clinical *Aspergillus fumigatus* isolates obtained from penguins with aspergillosis, based on microsatellite multilocus genotyping. Genetic distances were calculated by ape package in Rstudio® software programme and clustering performed by using neighbor-joining tree estimation of Saitou and Nei (1987).⁶⁹ Isolate identification is followed by date of isolation in brackets. Isolates marked with * correspond to free-ranging penguins. D.M–Data missing.

Microsatellite data analysis showed 24 different multilocus genotypes (Simpson's diversity index = 0.9340463), obtained with 16 alleles for *STRAf3A* loci (Simpson's diversity index = 0.9322638), 12 alleles for *STRAf3B* loci (Simpson's diversity index = 0.8877005), and 18 alleles for *STRAf3C* loci (Simpson's diversity index = 0.9358289) (Table 1).

Genetic clustering, based on microsatellite multilocus genotyping, of clinical *A. fumigatus* isolates obtained from penguins who died of aspergillosis showed that five genotypes were common to more than one isolate. Two to four isolates were grouped and considered identical (Fig. 2).

Excluding one multilocus genotype (207.20/222.19/102.47), all the other identical multilocus genotypes were collected in captive penguins from the years 2008 and 2011. These were

the years when the highest number of isolates was obtained (Table 2). About 50% of the *A. fumigatus* multilocus genotypes described in 2008 were identified in more than one individual (two to three isolates sharing identical multilocus genotypes were collected from different penguins) and in 2011, three isolates shared the same multilocus genotype. All birds with *A. fumigatus* isolates with common multilocus genotypes shared the captivity facilities in the same period. Comparing the global population of *A. fumigatus* with our data, none of our isolates shared identical genotypes with *Aspergillus* reported in other countries. In addition, based on information obtained from AfumID' programme, 7 of our 24 multilocus genotypes were related with isolates from the resistant global population of *A. fumigatus* (Fig. 3).

Table 2. Microsatellite multilocus genotypes (fragment length) of *Aspergillus fumigatus* sensu stricto isolated from Magellanic penguins that died of aspergillosis, compared with global population.

Penguin ID	Year of rehabilitation/isolation of <i>Aspergillus</i>	<i>STRAf3A</i>	Number of repeats	Multilocus genotyping <i>STRAf3B</i>	Number of repeats	<i>STRAf3C</i>	Number of repeats	AfumID match
1517 ^D	2008	216.18	35	193.78	20	139.42	25	NM
1715	2008	195.20	28	216.60	28	86.59	8	NM*
1765	2008	198.13	29	182.30	16	102.27	13	NM
1784 ^C	2008	207.18	32	222.13	30	102.26	13	NM
1834	2016	207.21	32	159.14	8	149.73	28	NM
1890 ^B	2008	201.16	30	182.32	16	102.27	13	NM
1921	2008	207.16	32	159.27	8	146.36	27	NM
1933 ^E	2008	295.34	61	164.96	10	168.45	34	NM*
2001 ^A	2008	195.30	28	159.14	8	76.83	5	NM*
2005 ^E	2008	295.31	61	165.00	10	168.4	34	NM*
2006 ^B	2008	201.16	30	182.3	16	102.27	13	NM
2007 ^D	2008	216.24	35	193.84	20	139.41	25	NM
2012 ^D	2008	216.18	35	193.76	20	139.42	25	NM
2115	2009	128.89	6	165.10	10	139.41	25	NM
2156	2010	207.16	32	167.84	11	120.51	19	NM
2281 ^E	2011	295.32	61	164.98	10	168.49	34	NM*
2366	2011	148.79	13	179.49	15	123.74	20	NM
2431	2011	283.07	57	170.85	12	96.25	11	NM
2452	2011	216.12	35	167.91	11	96.06	11	NM
2476 ^A	2011	195.20	28	159.14	8	76.69	5	NM*
2496	2011	148.78	13	196.59	21	114.31	17	NM
2507	2011	136.58	9	147.86	4	70.85	3	NM*
2514 ^A	2011	195.22	28	159.14	8	76.70	5	NM*
2519	2011	161.58	17	165.68	10	105.34	14	NM*
2554 ^D	2011	216.20	35	193.78	20	139.42	25	NM
2557 ^A	2011	195.30	28	159.15	8	76.71	5	NM*
3046	2014	204.19	31	173.68	13	171.39	35	NM
3118 ^C	2015	207.20	32	222.19	30	102.47	13	NM
3384	2016	183.14	24	168.63	11	89.72	9	NM
3484	2017	210.19	33	225.01	32	108.39	15	NM
SA	D.M	198.12	29	165.00	10	105.15	14	NM*
3628	2019	225.16	38	165.07	10	102.37	13	NM*
3963	2019	198.11	29	164.89	10	136.09	24	NM
4563	2019	179.99	23	168.59	11	93.13	10	NM

Identical superscript letters correspond to identical multilocus genotype; D.M–Data missing; NM–No match with global population of *Aspergillus fumigatus*, isolate mostly associated with other isolates from the non-resistant population; NM*–No match with global population of *Aspergillus fumigatus*, isolate mostly associated with other isolates from the resistant population.

Antifungal susceptibility patterns and molecular mechanisms of azole resistance

From all 34 *A. fumigatus* sensu stricto screened for resistance in azole-supplemented agar media, three (all of them from captive penguins) showed some minor growth in the ICZ-supplemented media (isolates #2007, #2507 and #3118), therefore selected for further characterization. Two of them were collected from penguins that received ICZ prophylaxis. By the microdilution broth test, one of these three isolates (isolate #3118, the one collected from a penguin without antifungal prophylaxis) showed resistance to PCZ (MIC = 2.0 mcg/ml to ICZ, 1.0 mcg/ml to PCZ, and 0.5 mcg/ml to VCZ). The other two were susceptible for all

the azoles tested (MIC = 1.0 mcg/ml to ICZ, 0.5 mcg/ml to PCZ, and 0.5 mcg/ml to VCZ, for both).

No mutations were detected in the multiplex real-time PCR assay for azole resistance markers in any of the three selected isolates. Analyzing the nucleotide sequencing of *cyp51A* and its promoter, only the PCZ-resistant strain (#3118) showed several nucleotide mutations, resulting in four amino acid substitutions F46Y/N248T/D255E/M172V.

Discussion

We performed a study of molecular epidemiology of *A. fumigatus* clinical isolates from Magellanic penguins. Microsatellite

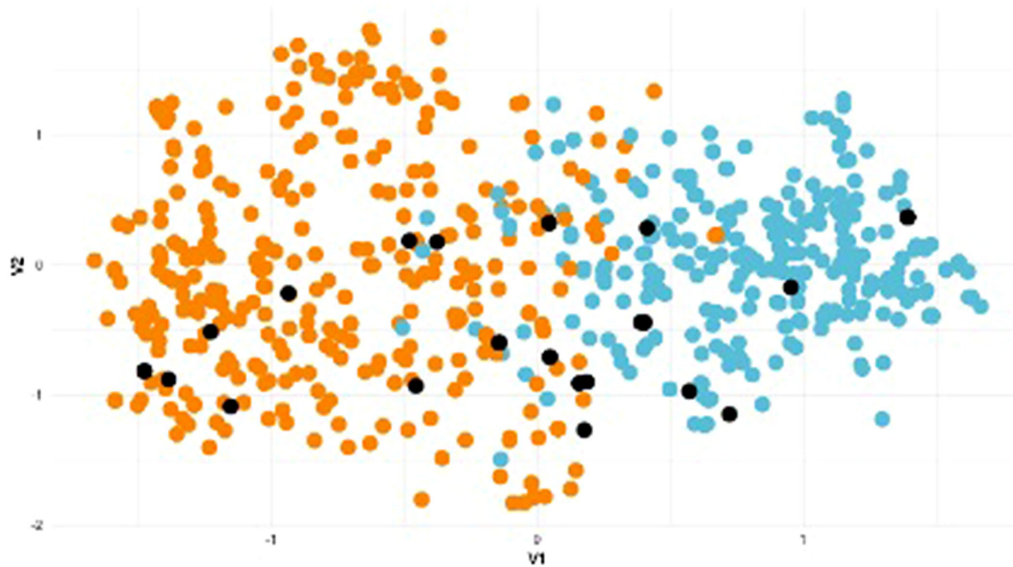


Figure 3. Distribution of *Aspergillus fumigatus* isolates from penguins with proven aspergillosis (in black), among a global population of 4049 (described by Sewell et al., 2019).⁷⁰ Isolates mostly associate with non-resistant population (in orange) and mostly associate with azole-resistant population (in blue). The figure was built using the web-based programme AfumID (<https://afumid.shinyapps.io/afumID/>).

genotyping of those isolates was performed, and the susceptibility profile to ICZ, VCZ, and PCZ was evaluated, with characterization of the molecular sequence of the *cyp51A* gene as well. To the best of our knowledge, this is the first work on molecular identification *Aspergillus* species collected from Magellanic penguins with proven aspergillosis and their microsatellite genotyping. These data are important for a better understanding of the molecular epidemiology of this disease in these animals and also in the *One Health Context*.⁵⁰

Our study showed that all penguins suffered from aspergillosis caused by *A. fumigatus* sensu stricto, reinforcing the relevance of this species as the main fungal pathogen for penguins, as already described for other birds and humans.^{51,52} No cryptic species belonging to section *Fumigati* were identified in this study, including the three isolates recovered from free-living penguins. Several factors may favor the infection of the penguins by this species, including the higher thermotolerance of *A. fumigatus* sensu stricto.⁵³ The body temperature of these birds normally varies between 39 and 41 degrees Celsius, which favors the growth of *A. fumigatus* sensu stricto. In addition, this fungal species produces high levels of gliotoxin, to which its greater virulence is attributed.^{15,54}

Regarding the genotyping of those *A. fumigatus* sensu stricto isolates, it was observed that the majority of clonal *A. fumigatus* were isolated from penguins that shared the captivity facilities at the same period. This finding suggests the occurrence of outbreaks during the penguins' rehabilitation. Of those clonal isolates, 70% were recovered during the 2 years with the highest density of penguins at the rehabilitation center,¹ which would increase the load of environmental *Aspergillus*, due to higher avail-

ability of organic matter (penguins feces), higher humidity due to the systematic cleaning of the facilities, and maintenance of a warm temperature to better acclimatize the penguins.

One of the major limitations of this study is the impossibility to characterize and genotype the environmental *A. fumigatus* isolates collected during the penguins' rehabilitation. Those isolates were collected^{55,56} but are not available anymore. However, the occurrence of clonal strains among penguins' clinical isolates reinforces the idea that the rehabilitation environment can be considered a potential source of infection.⁶ The probable maintenance of some clonal strains in the environment of the rehabilitation center for a long period, verified by the detection of two multilocus genotypes (195/159/76 and 216/193/139) in 2008 and again in 2011, and of another one (207/222/102) in 2008 and again in 2015, is suggested by epidemiological studies in other environments.^{39,57} Even with the adoption of measures to control the fungal load in the environment,^{2,58} it is impossible to achieve a total success, in particular in these places where several animals are kept at the same time, and with different management areas.

Regarding the occurrence of the identical multilocus genotype (207/222/102) in penguins #1784 (wild-type) and #3118 (resistant to PCZ) (both died within 2 days in captivity) we could raise two hypotheses. The first one is that these were related strains that had undergone mutations related to antifungal resistance,⁵⁹ considering the long time between isolations (7 years); or, despite the high discriminatory power of the genotyping method applied (0.9968), they could be unrelated isolates that the technique was not able to discriminate.⁴²

Although almost half of the penguins had received antifungals as prophylaxis, no phenotypic resistance was observed in

the majority of the isolated *A. fumigatus* sensu stricto strains. In Magellanic penguins, guidelines for rehabilitation include prophylaxis for aspergillosis, with ICZ at 20 mg/kg/day for 15 days from the beginning of the rehabilitation process.¹ The use of azoles as prophylaxis in distressed penguins, as well as the treatment of aspergillosis in penguins in captivity, are very common.^{60,61} Data obtained in this study suggest that the prophylaxis applied to these animals may not have been a relevant factor contributing to the emergence of resistance, maybe because, unlike treatment, prophylaxis is not performed for long periods.

Although seven genotypes described in this study were mostly associated with azole-resistant global populations, none of the isolates showed phenotypic pan-azole resistance, and in accordance with these findings, no TR mutation¹⁹ was detected in real-time PCR or by *cyp51A* sequencing. The amino acid substitutions in *cyp51A* gene demonstrated in the *A. fumigatus* sensu stricto isolate resistant to PCZ have already been reported in a pan-azole-resistant isolate.⁶² However, the combination of these substitutions does not seem to be related to azole resistance, since they were also found several times in different susceptible strains.^{62–64} Thus, probably other mechanisms, such as efflux pumps,⁶⁵ or mutations in other genes^{66–68} could be associated with the lower susceptibility of this isolate to PCZ.

This study showed that *A. fumigatus* sensu stricto is the most important etiologic agent of aspergillosis in Magellanic penguins. The outbreaks detected during penguins' rehabilitation and the observation of the environmental maintenance of some *Aspergillus* multilocus genotypes through the years are novel in the scientific literature. This report also contributes important data for a better understanding of the molecular epidemiology of aspergillosis in penguins. Data regarding antifungal resistance profiles reported in this study suggest that prophylaxis performed in these animals during rehabilitation may not markedly impact the emergence of resistance. Since prophylaxis could be related to better outcomes in penguin rehabilitation, its use may proceed, but preferably with a systematic surveillance for azole resistance.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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