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Selection of *Aspergillus fumigatus* isolates carrying the G448S substitution in *CYP*51A gene after long-term treatment with voriconazole in an immunocompromised patient

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ABSTRACT

We present a case of a 55-year-old man with a heart transplant who acquired Invasive Aspergillosis by *Aspergillus fumigatus* with the focus in the kidney. During about two years of antifungal treatment, most of the time with voriconazole, it was possible to obtain nine isolates of *A. fumigatus,* with the same genotypic characteristics, but with an increase in MIC for several azoles. The two last isolates presented high MICs for Voriconazole (>8 μg/mL>). Sequencing of the *CYP*51A gene showed G448S amino acid substitution in the same two isolates. In long-term treatments with antifungals, it would be important to regularly evaluate the susceptibility of isolated strains, as resistance to azoles has been increasingly described around the world.

1. Introduction

Solid organ or bone marrow transplantation have come to play an important role in extending the estimated life span due medicine advances. The significant increase in the number of transplants placed the opportunistic fungus, *Aspergillus* spp., in a prominent place as a causative agent of invasive infections in immunocompromised patients [1]. *Aspergillus* species are associated with a wide variety of infections, being *Aspergillus fumigatus* the most isolated species and also responsible for the highest rate of Invasive Aspergillosis (IA) [2]. Invasive aspergillosis usually requires a long-term antifungal therapy. The first-line therapy are azole antifungals such as Voriconazole (VRC), however, these treatments often cause several side effects and may induce the selection of resistant isolates [2,3]. Resistance is usually attributed to mutations in the *CYP*51A gene, which encodes cytochrome P450 14-α-lanosterol demethylase [2,3]. The University Hospital of Campinas (HC-UNICAMP) performs an average of 400 transplants every year, therefore receiving many patients susceptible to opportunistic infections, such as IA. This manuscript reports the case of a patient who developed IA after heart transplantation. During two years of antifungal treatment, especially with VRC, it was possible to obtain nine *A. fumigatus* isolates, with the same genotypic characteristics in microsatellites analyses, but with distinguished Minimal Inhibitory Concentration (MICs) for several azoles, resulting in the detection of the G448S mutation in the *CYP*51A gene.

2. Case presentation

In 2018 a 55-year-old man was admitted to the HC-UNICAMP with intense pain in the right iliac fossa, and pain on deep palpation of the abdomen. The patient had a medical history of hypertension, diabetes mellitus, and heart transplant performed in 2015. He also presented multiple rejections episodes which demanded pulse therapy with methylprednisone and thymoglobulin. After physical examination, he underwent abdominal computed tomography (CT), which detected an abscess in the right kidney (Fig. 1). The first day of hospital admission was defined as “day 0” in this report. On day +12 since the first admission the abscess was punctured, and the aspirate material was sent to the Microbiology Laboratory. Results of culture, macroscopic microscopic and Spectrometric analysis (Maldi-Tof) identified *Aspergillus fumigatus*. The antifungal treatment was initiated using intravenous (IV) Micafungin (MCF) 100mg for 14 days and continued with IV VRC (200mg/12h) for 30 days. On day +43, the patient underwent a partial nephrectomy of the right kidney. The abscess content was submitted to

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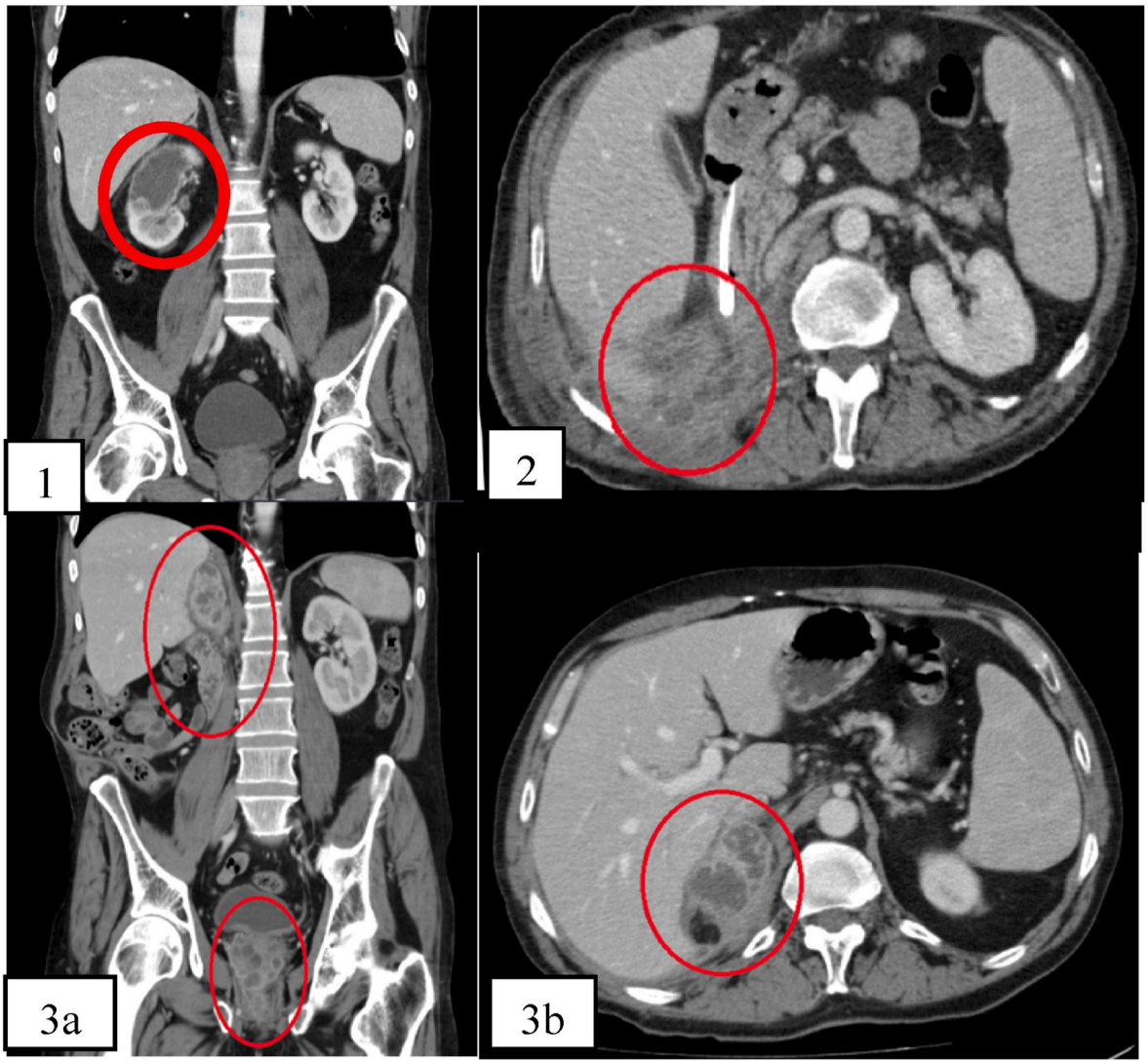


Fig. 1. (1) Sagittal image from abdominal CT on day 0 showing in the right kidney a cystic image, compatible with renal abscess. **(2)** Axial image from CT on day +183 indicates the presence of stores with chronic-looking collections (no change from the previous exam), extending to the soft parts of the right flank and right adrenal gland, with suspected enteric fistula. **(3a)** Axial and **(3b.)** sagittal CT images of the abdomen on day +456, showing abscesses in the right nephrectomy pocket and prostate.

new culture and *A. fumigatus* has identified again. On days +49, +56, +65, +73, blood galactomannan tests were performed and 1.27, 1.34, 0.99 and 0.03 OD values were obtained, respectively.

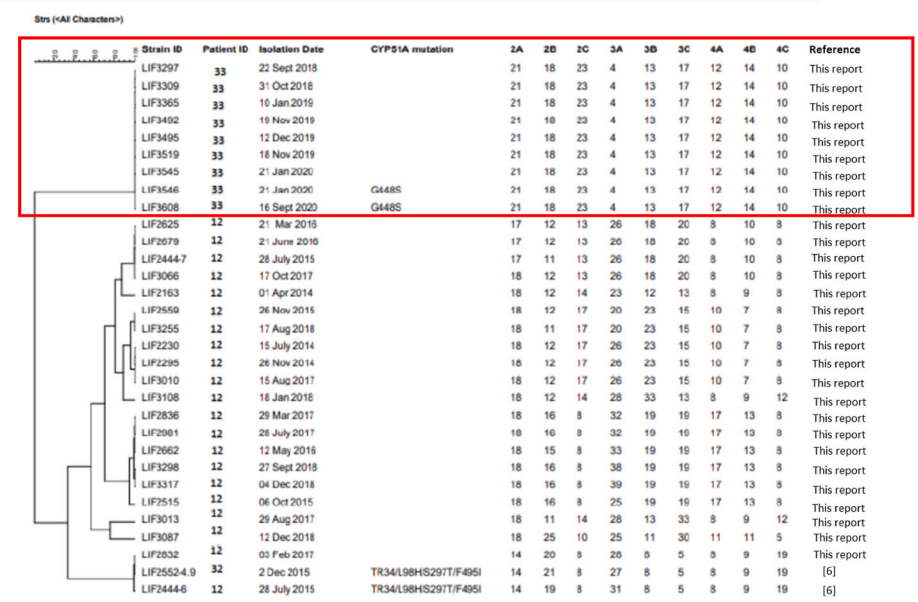
Another kidney puncture was performed, and *A. fumigatus* was identified. Under the intravenous administration of VRC 200mg twice a day, there was a significant improvement in the patient's condition, and he was discharged from the hospital. The continuous administration of oral VRC (200mg/12h) was maintained. On day +81 after the first admission, due to difficulty in acquiring VRC, it was necessary to change to oral Itraconazole (ITC) (200mg/12h) for 102 days. On day +183, the patient returned to the hospital complaining about dysuria, decreased urinary stream, and abdominal pain. Hospitalized again, an abdominal CT showed new abscesses in the right kidney and prostate (Fig. 1), the galactomannan marker was positive (1.71 OD). Transurethral resection of the prostate was performed. With the worsening of the condition with the use of oral ITC 200mg/12h, the treatment was substituted by oral VRC (200mg/12h).

As susceptibility testing is not routinely performed in HC-UNICAMP, a special request was made and broth microdilution tests for *A. fumigatus* isolated from the previous clinical specimens were performed (Fig. 1). Treatment with IV amphotericin B (AMB) lipid complex (1mg/kg/day) was initiated on day +456. During this period, the patient underwent a radical prostatectomy on day +489. Once again, the prostate material was positive for *A. fumigatus* and, to control the treatment, galactomannan was performed, showing positive (3.44 OD) higher results.

The patient evolved with clinical improvement. The blood serum level of galactomannan was negative (0.02 OD). Due to the impossibility of performing the AMB lipid complex at home, the patient was discharged from the hospital +605 days after the first hospitalization with a prescription of oral (20mg/kg/day) Posaconazole (POS). On +702 day, the patient was hospitalized again due to a pulmonary IA involvement, with right pleural effusion. The POS was then replaced by IV AMB lipid complex (1mg/kg/day). He underwent thoracentesis and the pleural fluid was sent to the laboratory, confirming *A. fumigatus* pulmonary infection. Pulmonary drainage and subsequent pleurotomy were performed. After using the AMB lipid complex, the patient showed a significant improvement and he was discharged from the hospital on +809 day, presenting negative galactomannan (0.04).

3. Discussion

All nine *A. fumigatus* isolates recovered from clinical samples were



Strain ID	Patient ID	Isolation Date	CYP51A mutation	2A	2B	2C	3A	3B	3C	4A	4B	4C	Reference
LIF3297	33	22 Sept 2018		21	18	23	4	13	17	12	14	10	This report
LIF3309	33	31 Oct 2018		21	18	23	4	13	17	12	14	10	This report
LIF3365	33	16 Jan 2019		21	18	23	4	13	17	12	14	10	This report
LIF3492	33	19 Nov 2019		21	18	23	4	13	17	12	14	10	This report
LIF3495	33	12 Dec 2019		21	18	23	4	13	17	12	14	10	This report
LIF3519	33	18 Nov 2019		21	18	23	4	13	17	12	14	10	This report
LIF3545	33	21 Jan 2020		21	18	23	4	13	17	12	14	10	This report
LIF3546	33	21 Jan 2020	G448S	21	18	23	4	13	17	12	14	10	This report
LIF3608	33	16 Sept 2020	G448S	21	18	23	4	13	17	12	14	10	This report
LIF2625	12	21 Mar 2016		17	12	13	26	18	20	8	10	8	This report
LIF2679	12	21 June 2016		17	12	13	26	18	20	8	10	8	This report
LIF2444-7	12	28 July 2015		17	11	13	26	18	20	8	10	8	This report
LIF3066	12	17 Oct 2017		18	12	13	26	18	20	8	10	8	This report
LIF2163	12	01 Apr 2014		18	12	14	23	12	13	8	9	8	This report
LIF2559	12	26 Nov 2015		18	12	17	20	23	15	10	7	8	This report
LIF3255	12	17 Aug 2018		18	11	17	20	23	15	10	7	8	This report
LIF2230	12	15 July 2014		18	12	17	26	23	15	10	7	8	This report
LIF2295	12	26 Nov 2014		18	12	17	26	23	15	10	7	8	This report
LIF3010	12	15 Aug 2017		18	12	17	26	23	15	10	7	8	This report
LIF3108	12	18 Jan 2018		18	12	14	28	33	13	8	9	12	This report
LIF2836	12	29 Mar 2017		18	16	8	32	19	19	17	13	8	This report
LIF2981	12	28 July 2017		18	16	8	32	19	19	17	13	8	This report
LIF2662	12	12 May 2016		18	15	8	33	19	19	17	13	8	This report
LIF3298	12	27 Sept 2018		18	16	8	38	19	19	17	13	8	This report
LIF3317	12	04 Dec 2018		18	16	8	39	19	19	17	13	8	This report
LIF2515	12	06 Oct 2015		18	16	8	25	19	19	17	13	8	This report
LIF3013	12	29 Aug 2017		18	11	14	28	13	33	8	9	12	This report
LIF3087	12	12 Dec 2018		18	25	10	25	11	30	11	11	5	This report
LIF2632	12	03 Feb 2017		14	20	8	26	8	5	8	9	19	This report
LIF25524.9	32	2 Dec 2015	TR34/L98HS297T/F495I	14	21	8	27	8	5	8	9	19	[6]
LIF2444-6	12	28 July 2015	TR34/L98HS297T/F495I	14	19	8	31	8	5	8	9	19	[6]

Fig. 2. Genotypic relationship among *Aspergillus fumigatus* isolates from patient 33 (described in this case report, in red), isolates from patient 12, and the resistant isolate, already published, from patient 32 (LIF 2444-6 and LIF 2552-4.9) [6]. The dendrogram is based on a categorical analysis of nine microsatellite markers in combination with the arithmetic mean unweighted pair group clustering method (UPGMA) using Phyloviz 2.0a. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

analyzed. These isolates were submitted to molecular identification using beta-tubulin (β-tubulin 2A/B) primers [4]. The gold standard susceptibility test (broth microdilution) was performed [5] evidencing two resistant isolates, LIF3546 (MZ673598) and LIF3608 (MZ673599), presenting MICs: VRC>8 μg/mL. The *CYP*51A gene mutations search [6] showed both resistant isolates carrying G1413A mutation, which is responsible for a G448S amino acid substitution. Microsatellite genotyping analysis [6,7], performed at the Laboratory of Molecular Epidemiology of Infectious Diseases (LEMDI-UNICAMP), demonstrated the same microsatellite pattern in all isolates.

Invasive Aspergillosis is the second leading cause of morbidity and mortality among transplant recipients [8]. In heart transplant recipients, in addition to rejection problems, this kind of infection remains one of the main complications reported. The infection causes approximately 20% of deaths in the first year after transplantation and *Aspergillus* spp. has been reported as the most frequent invasive fungal infection, causing pneumonia with high attributable mortality, ranging from 53% to 78% in transplant patients [9–11].

In recent years, mutations found in the *CYP*51A gene became a concern in all diagnostic centers worldwide [12–14]. In Brazil, until now, there is only one study attributing azole resistance to the TR48/L98H/S297T/F495I mutation in the *CYP*51A gene. Our research group identified two *A. fumigatus* isolates carrying this mutation and presenting resistance to ITC. Although those isolates had been obtained from inpatients, they were considered only colonizers [6]. Fig. 2 presents a dendrogram comparing the microsatellite characteristics of the present case (patient ID 3) and those isolates from the previously published case (patient ID 12 and 32) presenting the mutation (TR48/L98H/S297T/F495I) and other susceptible isolates not previously described of patient 12. In the dendrogram, it is possible to observe that there is no genetic correlation among the isolates.

This case report has some limitations, therapeutic drug monitoring (TDM) for VRC was not performed. Unfortunately, HC-UNICAMP does not perform TDM for VRC, preventing to obtain actual serum levels of VRC in the patient and therefore low doses may have been administered, leading to progressive IA and resistance to the treatment. Another important limitation is the lack of other national publications to compare the genotypic characteristics of our *Aspergillus fumigatus* isolates.

The patient had continuous isolates recovered from the renal abscess since the second week of hospitalization. The non-effectiveness of VRC and ITC led *A. fumigatus* spread to the prostate and then to the lungs.

In this manuscript, we report the finding of the G448S mutation in *A. fumigatus* isolated in the renal capsule. Kidney infections caused by *Aspergillus* spp. are rare and is interesting that after about two years of antifungal treatment, especially with VRC, it was possible to obtain nine isolates of *A. fumigatus*, with the same microsatellites' genotypic characteristics, but with an increase in azoles MICs until the detection of the G448S mutation in the *CYP*51A gene. The G448S substitution was found in the second last (LIF 3546) and in the last (LIF 3608) isolates recovered respectively after +489 and + 740 days. LIF 3545 (recovered from the prostate) and LIF 3546 (recovered from the renal capsule) were isolated

Table 1
In vitro antifungal susceptibility test results and presence of mutation detected on *CYP*51A gene sequence of the nine isolates analyzed.

Isolate	Days after first hospitalization	Clinical specimen	CLSI MIC (μg/mL)						*CYP*51A mutation
			MCFG	CPFG	AMPH-B	ITC	VRC	POS	
LIF 3297	+12	Renal abscess	0.03	0.25	1	0.5	2	0.25	None
LIF 3309	+43	Renal abscess	0.015	0.12	2	0.5	2	0.25	None
LIF 3365	+114	Renal abscess	0.015	0.5	2	0.5	4	0.5	None
LIF 3492	+426	Prostate	0.015	0.25	2	0.5	2	0.25	None
LIF 3519	+427	Prostate	0.015	0.12	2	0.5	1	0.25	None
LIF 3495	+456	Prostate	0.015	0.25	1	0.5	1	0.25	None
LIF 3545	+489	Prostate	0.015	0.12	1	1	4	0.25	None
LIF 3546	+489	Renal capsule	0.015	0.12	2	2	>8	0.5	G448S
LIF 3608	+740	Pleural fluid	0.015	0.25	2	2	>8	1	G448S

MICs were determined using CLSI method M38-A2. MCFG, micafungin; CPFG, caspofungin; AMPH-B, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole.

on the same date (+489), but from different body sites, which suggests that the microorganism developed the mutation in the host, or the resistant ones were selected there due to a high antifungal concentration. Invasive prostatic aspergillosis (IPRA) is a rare condition with a few cases in the literature. Moreover, the prostate is considered a reservoir of microorganisms, where higher doses of VRC are recommended for treatment [15,16]. Table 1 shows increasing MICs for VRC according to the isolates date. *Aspergillus fumigatus* carrying G448S mutation usually presents high MIC for VRC (>8 μg/mL) [20].

The G448S is considered a clinical mutation acquired after a long time of VRC exposure [17,18]. This substitution has already been reported in clinical cases of *A. fumigatus* infection [19,20]. G448 is conserved in all P450 cytochromes encoded by ERG11/CYP51 of yeasts and filamentous fungi. In *Candida albicans* and *Cryptococcus neoformans*, this substitution is related to Fluconazole resistance [21,22].

To our knowledge, this is the first case report of an *A. fumigatus* with G448S substitution causing IA in an immunocompromised patient in our institution. It is possible to confirm that the resistance to VRC observed *in vitro* led to therapeutic failure. This case report suggests that in all long-term treatments with antifungals, especially azoles, it would be important to regularly evaluate the susceptibility of isolated strains and to perform TDM of antifungal levels monitoring, although this practice is not common, and is also not available in our institution. The data presented here confirms the role of this mutation in resistance to VRC, since genotyping results of all isolates have demonstrated to be equal from the first to the last isolate. *A. fumigatus* azole resistance surveillance has become essential within the clinical laboratory, as resistance to azoles is increasingly detected around the world.

Ethical form

The present study was approved by the local Ethics Committee (CAAE51794615.0.0000.54.

Medical Mycology Case Reports requires full disclosure of all sources of funding and potential conflicts of interest. The journal also requires a declaration that the author(s) have obtained written and signed consent to publish the case report from the patient or legal guardian(s).

If you have nothing to declare in any of these categories then this should be stated. Funding Source.

All sources of funding should be acknowledged and you should declare any extra funding you have received for academic research of this work. If there are none state ‘there are none’.

Please state any competing interests

None.

Consent

Please declare that you have obtained written and signed consent to publish the case report from the patient or legal guardian(s).

Please state that consent has been obtained from the patient or legal guardian(s)

Written informed consent was obtained from the patient or legal guardian(s) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

As corresponding author, I hereby declare that I sign this document on behalf of all the authors of the above mentioned manuscript.

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Declaration of competing interest

Please declare any financial or personal interests that might be potentially viewed to influence the work presented. Interests could include consultancies, honoraria, patent ownership or other. If there are none state ‘there are none’.

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