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First Description of Azole-Resistant *Aspergillus fumigatus* Due to TR 46 /Y121F/T289A Mutation in France

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1 First description of azole-resistant *Aspergillus fumigatus* due to TR₄₆/Y121F/T289A mutation
2 in France

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16 **Running Head:** TR46 resistant *Aspergillus fumigatus* in France

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21 **Abstract:** Azole resistance in *Aspergillus fumigatus* is an emerging public health concern.
22 Recently, a novel fungicide-driven mutation in the *cyp51A* gene and its promoter,
23 TR₄₆/Y121F/T289A, leading to high-level resistance to voriconazole has been identified in
24 the Netherlands, Belgium, Germany, Denmark, Tanzania and India in both clinical and
25 environmental samples. Here we report the first description of *A. fumigatus* carrying this
26 mutation in France, in a cystic fibrosis patient, underlining the need for extensive monitoring
27 of *Aspergillus* resistance.

28 Azole-resistant *Aspergillus fumigatus* are increasingly reported in Europe since the late 2000s.
29 This emerging public health concern occurs through two distinct routes of acquisition: *in vivo*
30 selection of resistance as a consequence of long-term azole treatment or *de novo* acquisition
31 of a resistant isolate directly from the environment, linked to the widespread use of azole
32 fungicides in agriculture. Besides the TR₃₄/L98H mutation in the *cyp51A* gene first described
33 in the Netherlands, a novel fungicide-driven mutation, TR₄₆/Y121F/T289A has been recently
34 identified. Up to now, the TR₄₆/Y121F/T289A mutation has been reported in both
35 environmental and/or clinical samples in four countries across Europe (1-7), in Asia (8) and
36 more recently in Africa (9), suggesting a large geographical spread. Here we provide the first
37 description of *A. fumigatus* carrying TR₄₆/Y121F/T289A mutation in France, in a cystic
38 fibrosis patient.

39

40 A 23-year-old male cystic fibrosis patient with follow-up at the Pneumology Department at
41 Rouen University Hospital, France, was seen in consultation in March 2014. This patient had
42 high levels of total IgE and *Aspergillus*-specific IgE with positive *Aspergillus*-specific IgG
43 antibodies, being suggestive of diagnosis of allergic bronchopulmonary aspergillosis. He had
44 a history of *A. fumigatus* airway colonization and mold-active azoles exposure since 2002
45 (itraconazole and voriconazole). At the time of the consultation, he was treated with
46 voriconazole. Mycological cultures of the sputum collected during the consultation grew *A.*
47 *fumigatus* (strain 1). Species identification was obtained by both macroscopic and
48 microscopic characteristics on Sabouraud's agar medium together with sequencing of the
49 beta-tubulin gene (10). According to a local research protocol aiming at the surveillance of
50 azole resistance, this isolate was tested for antifungal susceptibility by the Etest[®] method
51 (bioMérieux, Marcy-l'Etoile, France). Unexpectedly, this strain exhibited a high-level
52 resistance to voriconazole (MIC>32µg/mL) in contrast with itraconazole (MIC=8µg/mL) and

53 posaconazole (MIC=1 µg/mL). Antifungal susceptibility was confirmed by the EUCAST broth
54 microdilution reference method (Table 1) (11, 12). Nucleotide sequencing of the *cyp51A* gene
55 and its promoter, using previously described primers (13, 14) and home-designed primers
56 (CYP51AF-F1: 5'-ATTTCCCTCATCACTGCAA, CYP51AF-R1: 5'-
57 CATCATGTGCGCAATCTCTT, CYP51AF-F2: 5'-AGAAGCGAGATGCTGCTCAT,
58 CYP51AF-R2: 5'-CCTTTGAAGTCCTCGATGGT), showed the TR₄₆/Y121F/T289A
59 mutation. Antifungal therapy was therefore switched to posaconazole in April 2014 then to
60 caspofungin (50 mg per day) in July 2014 because of pulmonary exacerbation.
61 Given these findings, we retrospectively analyzed all *A. fumigatus* strains previously isolated
62 from this patient since 2007 (n=13), for itraconazole and voriconazole susceptibility, *cyp51A*
63 sequencing and microsatellite genotyping. As shown in Table 1, our patient had already been
64 colonized by a TR₄₆/Y121F/T289A isolate one year before, in February 2013 (strain 3). All
65 remaining isolates collected before February 2013 were azole-susceptible being either wild-
66 type for the *cyp51A* gene or carrying mutations previously found in both azole-resistant and
67 azole-susceptible isolates (15). As a single colony was subjected to *in vitro* susceptibility
68 testing other azole-resistant isolates could have been missed. Microsatellite genotyping was
69 performed using a panel of nine short tandem repeats as described previously (16). As
70 illustrated in Table 1, both TR₄₆/Y121F/T289A isolates from our patient had the same
71 genotype as a strain previously isolated in Germany (7) (Table 1). To gain further insights into
72 the route of acquisition of this azole-resistant isolate in our patient, we conducted an
73 environmental study by soil samplings next to the patient's home as described previously
74 (17), as well as surface samplings (contact agar plates) in his office. Neither *A. fumigatus*
75 carrying TR₄₆/Y121F/T289A nor TR₃₄/L98H was identified.
76

77 *Aspergillus fumigatus* isolates carrying the TR₄₆/Y121F/T289A mutation have been first
78 described in December 2009 in the Netherlands (2). Since then, such isolates have been
79 evidenced in three others European countries namely Belgium (1, 5), Germany (3, 7) and
80 Denmark (4) and recently in India (8) and Tanzania (9) (Table 2, Figure 1). Taken together,
81 these findings suggest, as discussed previously for TR₃₄/L98H isolates, a large geographical
82 spread of this resistance mechanism. Several lines of evidence indicate that, as for
83 TR₃₄/L98H, TR₄₆/Y121F/T289A has emerged through a fungicide-driven route (18), such
84 isolates being found in both azole-naïve (1, 2, 6, 7) and azole-exposed patients (2, 3, 5, 7) as
85 well as from the environment (2, 8, 9). Here we report the first description of *A. fumigatus*
86 carrying the TR₄₆/Y121F/T289A mutation isolated from a French patient.

87 Interestingly, our patient organized trips to the Netherlands as a tour operator. For these
88 working purposes, he traveled to Amsterdam in November 2012, three months before the first
89 isolation of the TR₄₆/Y121F/T289A strain from his sputum (February 2013). Moreover he
90 regularly received advertising postal packages from Dutch flower producers which were
91 opened in his office. Three hypotheses can explain the route of acquisition of this
92 TR₄₆/Y121F/T289A strain in our patient: (i) inhalation of spores carrying TR₄₆/Y121F/T289A
93 during his trip to the Netherlands (2); (ii) colonization after inhaling spores carrying
94 TR₄₆/Y121F/T289A from his environment in France. Our environmental study conducted
95 next to the patient's home, less than 100 km from Belgium (where TR₄₆/Y121F/T289A strains
96 have been recently identified (1)), failed to detect TR₄₆/Y121F/T289A environmental isolates.
97 Nevertheless, environmental isolates carrying this mutation have been recently identified by
98 our team in the same region in France supporting this hypothesis (unpublished data). (iii) The
99 last hypothesis is colonization after inhaling *A. fumigatus* spores carrying
100 TR₄₆/Y121F/T289A, escaping while opening the packages received from the Netherlands.
101 Though the French strains are genetically indistinguishable from the German and genetically

102 different from Dutch isolates, the route of acquisition in our patient is unclear, as spores
103 probably follow an airborne migration as hypothesized previously for TR₃₄/L98H (19, 20).

104 The present report now provides evidence that *A. fumigatus* voriconazole-resistant isolates
105 carrying the TR₄₆/Y121F/T289A mutation can be now isolated from clinical samples in
106 France. As observed with TR₃₄/L98H, a geographical spread of this resistance mechanism is
107 ongoing across Europe and possibly worldwide. These findings together with the high-level
108 voriconazole resistance of the TR₄₆/Y121F/T289A strains both *in vitro* and *in vivo* (1, 2, 4-6)
109 underline the need for intensive investigations to determine its prevalence in both clinical and
110 environmental samples. In line with this, as recommended by the ECDC Technical report
111 (18), antifungal susceptibility testing of triazoles should be performed on all clinical *A.*
112 *fumigatus* isolates before starting antifungal therapy.

113

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117

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122 Gilead.

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192

193 Table 1- Overview of the characteristics of all *Aspergillus fumigatus* strains isolated from
194 sputum samples of the patient

195 Table 2- Literature review of all studies reporting TR₄₆/Y121F/T289A *A. fumigatus* isolates

196 Figure 1- Geographical spread of the TR₄₆/Y121F/T289A resistance mechanism (for each
197 strain, the exact location and origin (clinical or environmental) is indicated)

198 Figure 2- STRAf dendrogram highlighting the genetic relatedness between *Aspergillus*
199 *fumigatus* isolated from our patient and previously reported TR₄₆/Y121F/T289A isolates

Table 1- Overview of the characteristics of all *Aspergillus fumigatus* strains isolated from sputum samples of the patient

Strain number	Reference in the dendrogram	Month/Year of isolation	MICs mg/L (EUCAST)		<i>Cyp51A</i> mutations
			ITC	VRC	
1	14-105-2468	Mar 2014	8	>8	TR ₄₆ /Y121F/T289A
2	14-148-2457	Nov 2013	0.5	0.5	Wild-type
3	14-148-2460	Feb 2013	>8	>8	TR ₄₆ /Y121F/T289A
4	141428-459	Jan 2013	0.5	1	Wild-type
5	14-148-2458	Jan 2013	0.25	0.25	Wild-type
6	14-148-2456	Dec 2010	Not performed		F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C
7	14-148-2455	Sept 2010	Not performed		F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C
8	14-148-2454	Jul 2010	Not performed		F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C
9	Not performed	Jul 2009	Not performed		F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C
10	14-148-2450	Jul 2009	0.25	1	Wild-type
11	14-148-2448	Mar 2009	0.25	1	Wild-type
12	14-148-2447	Dec 2007	0.25	1	Wild-type
13	14-148-2445	May 2007	0.5	2	Wild-type
14	Not performed	Feb 2007	0.5	1	F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C

Table 2- Literature review of all studies reporting TR₄₆/Y121F/T289A *A. fumigatus* isolates

Reference	Date	Type of sample	Underlying condition	Infection	Antifungal susceptibility CLSI (EUCAST*)			Outcome	Country		
					VRC	ITC	POS				
(1)	July 2012	BAL fluid	HSCT	Probable IA	>16	4	1	Death	Belgium		
	Dec 2009	Sputum	HSCT	Probable IA	>16	4	0.25	Persistent infection			
	Jan 2010	Ear	Chronic otitis externals/sinusitis	IA	>16	>16	2	Persistent infection			
	Jan 2010	Abdominal abscess	SOT	Proven IA	>16	2	0.5	Death			
	Feb 2010	Sputum	Cystic fibrosis	No IA	>16	4	0.5	Alive			
	Feb 2010	Sputum	Lung carcinoma	No IA	>16	>16	2	Alive			
	March 2010	Sputum	HSCT	Probable IA	>16	1	0.25	Death			
	March 2010	Sputum	Cystic fibrosis, SOT	Proven IA	>16	>16	0.5	Alive			
	May 2010	Biopsy	Chronic otitis, surgery	Proven IA	>16	4	1	Alive			
	May 2010	Sputum	Lung fibrosis	None	>16	>16	1	Alive			
	June 2010	Sputum	Traumatism	None	>16	1	0.25	Death			
	July 2010	Brain biopsy	Beta thalassemia, diabetes mellitus	Proven IA	>16	4	1	Death			
	Sept 2010	Sputum	Cystic fibrosis	ABPA	>16	2	0.5	Alive			
	Oct 2010	Sputum	COPD, SOT	None	>16	>16	2	Alive			
(2)	Nov 2010	Sputum	COPD	No IA	>16	>16	2	Alive	The Netherlands		
	Jan 2011	Sputum	HSCT	Probable IA	>16	>16	1	Death			
	Dec 2009 to Jan 2011	Air sampling			ND	ND	ND				
	(8)	2012-2013	Soil sampling		>16	1-2	0.25-0.5			India	
	(3)	Sept 2012	Sputum	Cystic fibrosis	Colonization	>8*	>8*	2*		Alive	Germany
	(4)	Jan 2014	Sputum	Bruton's agammaglobulinemia, SOT	Probable IA	>4*	0.25-0.5*	0.125-0.25*		Death	Denmark
	(9)		Soil sampling			16->16	1-2	0.25-0.5		Tanzania	
	(5)	Nov 2013	BAL fluid	HSCT	Probable IA	>8	>16	1		Death	Belgium
	(7)	Sept 2012	BAL fluid	HSCT	Probable IA	16*	>16*	0.5*		Death	Germany
		July 2012	BAL fluid	HSCT	Proven IA	1*	>16*	0.5*		Death	
	Present report	Feb 2013	Sputum	Cystic fibrosis	Colonization	>8*	>8*	ND		Alive	France
		March 2014				>8*	8*				

POS: posaconazole

BAL: bronchoalveolar lavage; HSCT: hematopoietic stem cell transplantation; IA: invasive aspergillosis; SOT: solid organ transplantation; ABPA: allergic bronchopulmonary aspergillosis; COPD: chronic obstructive pulmonary disease; ND: Not determined



