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First azole-resistant *Aspergillus fumigatus* isolates with the environmental TR_{46}/Y121F/T289A mutation in Iran

Fatemeh Ahangarkani[1,2] | Ynze Puts[2] | Mojtaba Nabili[3] | Sadegh Khodavaisy[4] | Maryam Moazeni[5,6] | Zahra Salehi[7] | Melika Laal Kargar[7] | Hamid Badali[5,6,8] | Jacques F. Meis[2,9]

[1]Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran

[2]Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands

[3]Department of Medical Sciences, Sari Branch, Islamic Azad University, Sari, Iran

[4]Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[5]Department of Medical Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

[6]Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari, Iran

[7]Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

[8]Fungus Testing Laboratory, Department of Pathology and Laboratory Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA

[9]ECMM Excellence Center for Medical Mycology, Centre of Expertise in Mycology Radboudumc, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands

Correspondence
Jacques F. Meis, Center of Expertise in Mycology Radboudumc/CWZ, Nijmegen, The Netherlands.
Email: j.meis@radboudumc.nl

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Summary

Background: Azole resistance in *Aspergillus fumigatus* is an emerging problem and reported from all continents. As triazole antifungals are the mainstay of therapy in the management of invasive aspergillosis, azole-resistant *A fumigatus* has become a major medical concern and with complicated clinical management.

Objective: Screening of environmental presence of azole-resistant *A fumigatus* in Iran.

Methods: Compost from Northern Iran, collected between 2017 and 2018, was screened for the presence of azole-resistant *A fumigatus* with azole-containing agar. Phenotypic MICs were obtained from selected, molecularly confirmed isolates. *cyp51A* gene sequencing and genotyping of azole-resistant isolates were done.

Results: Among 300 compost samples, three *A fumigatus* isolates had high voriconazole MICs (≥16 mg/L) and harboured the TR_{46}/Y121F/T289A mutation in the *cyp51A* gene. Microsatellite typing of these isolates showed that two strains had the same allele across all nine examined microsatellite loci and were genotypically related to Indian azole-resistant strains. The other isolate had a different genotype.

Conclusion: This is the first report of *A fumigatus* with TR_{46}/Y121F/T289A mutation from the region. Monitoring and surveillance of antifungal susceptibility of clinical *A fumigatus* is warranted in Iran and elsewhere in the region.

KEYWORDS

Aspergillus fumigatus, azole resistance, compost, TR_{34}/L98H, TR46/Y121F/T289A

1 | INTRODUCTION

Invasive aspergillosis due to azole-resistant *A fumigatus* has become a major medical concern associated with high mortality in immunocompromised individuals.[1-3] Azole resistance in *A fumigatus* emerges due to long-term treatment with azole antifungals, but also extensive exposure of the fungus to azole compounds in the environment is a major driver of resistance selection.[4-7] Resistance to azole drugs in *A fumigatus* is mainly linked to multiple amino acid substitutions in the *cyp*51A gene. The most described *cyp51A*-mediated resistance mechanism is a 34-base-pair (bp) sequence tandem repeat (TR_{34}) in the promoter region of *cyp51A* gene combination with the L98H substitution followed by a double substitution combined with a 46 bp tandem duplication in the *cyp51A* promoter (TR_{46}/Y121F/T289A).[8,9] These mutations lead to high-level resistance to triazole antifungals of *A fumigatus* isolates from both azole-naive and azole-treated patients.[10] Furthermore, several institutions report a rise in resistant strains found in environmental niches such as soil samples, paddy fields, aerial samples of hospitals and compost.[11-19] Compost (decaying plant waste material) is an important source of *A fumigatus*. It is known that high concentrations of azole-resistant *A fumigatus* spores are released during incomplete composting processes, especially when azole residues from agricultural waste are present.[11,13-15,20-23] Azole-resistant *A fumigatus* with the TR_{34}/L98H mutation in the environment has been reported earlier in Iran.[24] Here, we report for the first time the presence of resistance due to the TR_{46}/Y121F/T289A mutation.

2 | MATERIAL AND METHODS

2.1 | Sample collection and identification of azole resistent *A fumigatus* isolates

Three hundred compost samples from different regions of Iran (Mazandaran province (n = 200) and Tehran province (n = 100), located about 300 km apart, were collected during 2017-2018. To recover *A fumigatus* strains, 1 cm^2 compost was dissolved in 5 mL sterile saline solution containing Tween 40 (0.05%), vortexed, and allowed to settle. Briefly, according to a previously described protocol, for primary screening of azole-resistant *A fumigatus* strains from the supernatant, 100 µL was plated on a Sabouraud dextrose agar plate (SDA; Difco), supplemented with 4 and 1 mg/L itraconazole and voriconazole, respectively, and incubated at 45°C for 72 hours in the dark.[23] Identification of *Aspergillus* section Fumigati was performed based on both macroscopic and microscopic characteristics. Subsequently, molecular identification with DNA sequencing of the partial b-tubulin gene, using TUB2a (5- TGACCCAGCAGATGTT-3) and TUB2b (5-GTTGTTGGGAATCCACTC-3), was done.[25]

2.2 | In vitro antifungal susceptibility testing

To detect azole-resistant *A fumigatus*, colonies that grew on screening media supplemented with itraconazole and voriconazole and confirmed by DNA sequencing as *A fumigatus* were selected and evaluated with microbroth dilution minimum inhibitory concentration (MIC) testing using the Clinical and Laboratory Standards Institute document for filamentous fungi.[26] *Paecilomyces variotii* (ATCC 22319) and *Candida parapsilosis* (ATCC 22019) were used as quality controls.

2.3 | Detection of *cyp51A* gene mutations

Isolates with reduced susceptibility to voriconazole (MIC >2 µg/mL) were tested with a mixed-format real-time PCR assay as described before.[27] Isolates with TR_{46}/Y121F/T289A mutations were further confirmed by sequencing the cyp51A gene as described previously.[28]

2.4 | Microsatellite typing

Genotyping of *A fumigatus* isolates was performed with a panel of nine short tandem repeats (STRs) loci (namely STR*Af* 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C), as previously described.[29]

Genetic relatedness between the environmental Iranian azole-resistant TR_{46}/Y121F/T289A isolates was compared with already barcoded clinical and environmental isolates from different countries (The Netherlands, India, Tanzania, France, Colombia, Ireland, China and Germany) using BioNumerics software version 7.6.1.(Applied Maths NV).

3 | RESULTS

A total of 63 samples from both Mazandaran (n = 44) and Tehran province (n = 19) yielded *A fumigatus* on SDA supplemented with itraconazole and voriconazole. A total of 57 *A fumigatus* isolates had high MICs level of itraconazole (>8 mg/L) (55/57) or voriconazole (>2 mg/L) (51/57) by in vitro antifungal susceptibility testing. Exploring the mechanisms of resistance by mixed-format real-time PCR and sequencing of *cyp51A* and its promoter in these isolates showed that resistant isolates had different resistance mechanisms including forty-four with TR_{34}/L98H and three isolates with TR_{46}/Y121F/T289A (1 isolate with TR_{46}/Y121F/M172I/T289A/G448S and two isolates with TR_{46}/Y121F/T289A mutations in the cyp51 region). All three isolates with TR_{46}/Y121F/T289A mutation in *cyp51A* and high voriconazole MIC (≥16 mg/L) were isolated from Mazandaran province. Microsatellite analysis revealed two different genotypes among isolates harbouring TR_{46}/Y121F/T289A. Two strains had the same alleles across all nine examined microsatellite loci and the other differed by seven loci (2C, 3A, 3B, 3C, 4A, 4B and

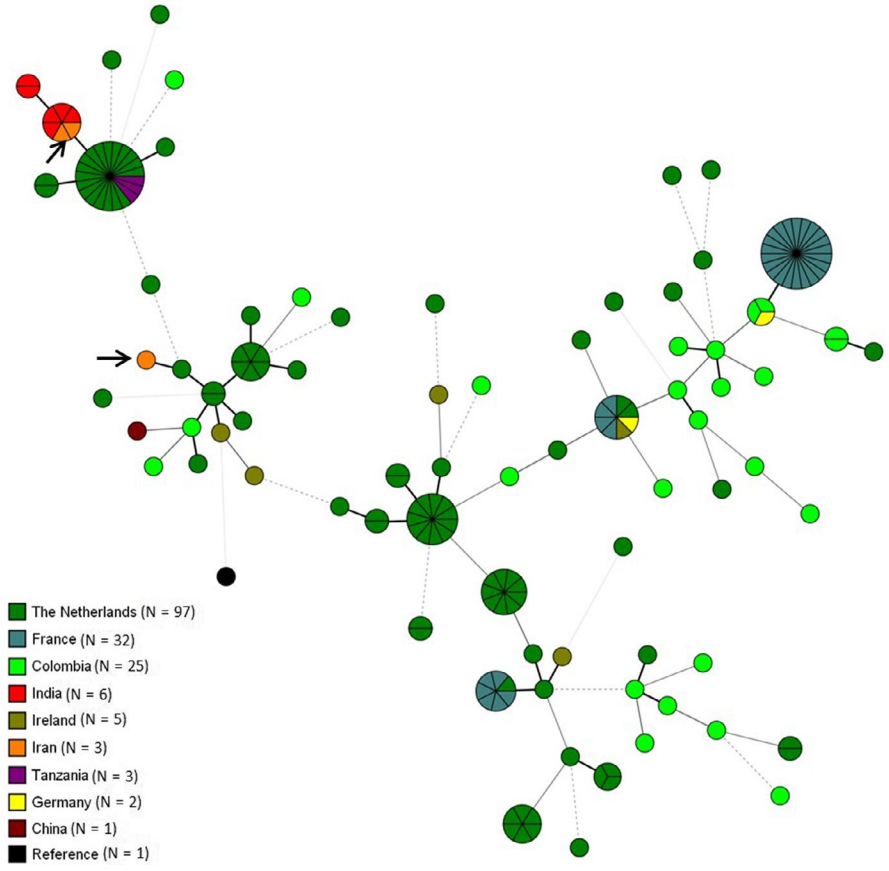

FIGURE 1 Minimum-spanning tree showing the genetic relationship of *A fumigatus* genotypes harbouring TR_{46}/Y121F/T289A mutation in cyp51A allele. Genotypic relationship of three Iranian isolates is illustrated by arrows

4C). For comparison of the genotype of these isolates with other isolates from different countries, a minimum-spanning tree was constructed based on nine STR*Af* loci of different azole-resistant isolates.[29] Two Iranian *A fumigatus* TR_{46}/Y121F/T289A isolates were related to resistant isolates originating from India while the third was genetically different from other strains (Figure 1).

4 | DISCUSSION

Azole resistance in *A fumigatus* due to TR_{34}/L98H and more recent TR_{46}/Y121F/T289A mutations in *cyp*51A has been described from wide geographical areas among both clinical and environmental isolates.[30,31] This is linked to the widespread use of azole fungicides in agriculture rather than the clinical use of antifungal drugs.[2-14,19-21] The fungicide-driven mutation TR_{46}/Y121F/T289A has been first detected in the Netherlands in 2009.[32] Since then, several reports have demonstrated that, particularly this mutation, corresponded to voriconazole resistance in *A fumigatus* in European, African, American and Asian countries. We report the presence of TR_{46}/Y121F/T289A now also from Iran, where until now only TR_{34}/L98H had been isolated.[24,33-35] This mutation, conferring voriconazole resistance, has been reported from both environmental (China,[17,21] Taiwan,[13] United Kingdom,[11] Colombia,[12,14] France,[16,36] Germany,[37] India,[19] the Netherlands[32] and Tanzania[18]) and clinical sources (Spain,[38,39] United Kingdom,[40] France,[41] Portugal,[42,43] Argentina,[44] Taiwan,[45] Germany,[46-49] China,[50,51] Japan,[52] United States,[53,54] Denmark,[10] Belgium [7,55-57] and the Netherlands[7,32,58-60]) (Table 1). All previous studies conducted in Iran for monitoring the mechanism of resistance among azole-resistant *A fumigatus* in both clinical and environmental samples showed that the TR_{34}/L98H mutation was reported with increasing frequency from 3.3% in 2013 to 6.6% in 2016.[24,34] Microsatellite typing of the current isolates showed that two *A fumigatus* isolates with TR_{46}/Y121F/T289A isolates were related to resistant isolates from India in 2014. It is noteworthy that the first isolates with TR_{34}/L98H in Iran in 2013 were also genotypically related to some resistant clinical and environmental TR_{34}/L98H isolates from India and the Netherlands.[24] It is anticipated that strains with the TR_{46}/Y121F/T289A or TR_{34}/L98H mutation will spread rapidly to other geographical regions especially due to the use of azole-based agricultural fungicides which is a significant factor in the increase of multiple-triazole-resistant *A fumigatus*.[4] In our study, most of the azole-resistant *A fumigatus* isolated from Iranian compost were from Mazandaran province located in Northern Iran where agricultural activity and subsequently the usage of fungicides are generally higher than in other regions of Iran. Compost is a main ecological niche for *A fumigatus*, and azole residues have been reported to be present in commercial compost.[20] Snelders et al[61] showed that 86% of triazole-resistant *A fumigatus* isolates recovered from environmental sources such as compost had the same resistance mechanism as found in clinical isolates. Compost is used widely in gardens and indoor plants in Iran, and has a key role in the exposure of azole-resistant *A fumigatus*

TABLE 1 Reports of azole resistant *Aspergillus fumigatus* isolates carrying TR_{46}/Y121F/T289A mutation

Number	Country	Year of publishing	Number of environmental isolates	Number of clinical isolates
Present report	Iran	2020	3	–
1	China	2020[21]	6	–
2	Taiwan	2019[13]	3	–
3	Spain	2019[38]	–	1
4	Belgium - Netherlands	2019[7]	–	12
5	United kingdom	2019[11]	6	–
6	France	2019[41]	–	2
7	Colombia	2019[14]	8	–
8	Netherlands	2018[58]	–	5
9	France	2018[16]	9	–
10	Portugal	2018[42]	–	1
11	Argentina	2018[44]	–	1
12	Taiwan	2018[45]	–	3
13	Portugal	2018[43]	–	1
14	Germany	2018[46]	–	1
15	Germany	2017[47]	–	1
16	Belgium	2017[55]	–	4
17	Colombia	2017[12]	17	–
18	United kingdom	2017[40]	–	1
19	France	2017[36]	21	1
20	China	2016[17]	2	–
21	Netherlands	2016[59]	–	4
22	Japan	2016[52]	–	1
23	China	2016[50]	–	1
24	United States	2016[53]	–	2
25	United States	2015[54]	–	1
26	China	2015[51]	–	1
27	Spain	2015[39]	–	1
28	Germany	2015[37]	6	–
29	France	2015[41]	–	2
30	Germany	2015[48]	–	2
31	Netherlands	2015[60]	–	1
32	Belgium	2014[56]	–	1
33	Denmark	2014[10]	–	1
34	Germany	2014[49]	–	1
35	Tanzania	2014[18]	4	–
36	India	2014[19]	6	–
37	Netherlands	2013[32]	14	21
38	Belgium	2012[57]	–	1

to susceptible hosts. Although CLSI and EUCAST have published standardised antifungal susceptibility testing methods to determine the in vitro efficacy of antifungal drugs, most clinical microbiology laboratories in Iran do not routinely perform antifungal susceptibility testing of *Aspergillus* isolates as recommended by ESCMID guidelines.[62] Therefore, the true prevalence of resistance and mechanism of resistance in clinical *A fumigatus* isolates in Iran is unknown. Reports of clinical isolates harbouring this mutation are mainly limited

to developed countries in Europe, East Asia and the USA, maybe because of the lack of routine antifungal susceptibility testing in less developed countries (Table 1). With this background in mind, the emergence of a new azole resistance mechanism in *A fumigatus* isolated from environmental sources in Iran is concerning as this mechanism has been associated with therapeutic failure with voriconazole, the first-line treatment for invasive aspergillosis. Nevertheless, correlations between in vitro susceptibility results and clinical outcomes are not really estimated for *A fumigatus*. Compost used for planting surroundings of immunocompromised patient's houses could be a source of invasive aspergillosis due to azole-resistant *A fumigatus*.[21,22] Our study reinforces the importance of surveillance studies to monitor antifungal susceptibility of clinical isolates in Iran.

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CONFLICT OF INTEREST

JFM received grants from Pulmozyme and F2G. He has been a consultant to Scynexis and received speaker's fees from United Medical, TEVA and Gilead Sciences. The other authors report no conflicts of interest.

AUTHORS CONTRIBUTIONS

FA and HB conceived this study. MN, SK, MM, ZS and MLK were responsible for collection of the isolates and data acquisition. FA and YP did the molecular analysis. FA and JFM wrote the concept paper and all authors critically reviewed the manuscript prior to submission.

ETHICS APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article related to micro-organisms.

ORCID

Fatemeh Ahangarkani https://orcid.org/0000-0002-3629-7446
Mojtaba Nabili https://orcid.org/0000-0002-1461-4164
Sadegh Khodavaisy https://orcid.org/0000-0001-8039-4991
Maryam Moazeni https://orcid.org/0000-0001-6581-2139
Hamid Badali https://orcid.org/0000-0002-6010-8414
Jacques F. Meis https://orcid.org/0000-0003-3253-6080

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