Azole-resistant Aspergillus fumigatus in the environment of northern Italy, May 2011 to June 2012

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In recent years acquired azole resistance in *Aspergillus fumigatus* has been increasingly reported and a dominant mechanism of resistance (TR_{34}/L98H) was found in clinical and environmental isolates. The aim of the present study was to investigate the prevalence of azole resistance in environmental *A. fumigatus* isolates collected in northern Italy. *A. fumigatus* grew from 29 of 47 soil samples analysed. Azole-resistant isolates were detected in 13% (6/47) of the soil samples and in 21% (6/29) of the soil samples containing *A. fumigatus*. High minimal inhibitory concentrations (MIC) of itraconazole (≥16 mg/L) and posaconazole (≥0.5 mg/L) were displayed by nine isolates from six different soil samples, namely apple orchard (1 sample), rose pot compost (2 samples), and cucurbit yields (3 samples). Seven isolates had a MIC=2 mg/L of voriconazole. Seven of nine itraconazole and posaconazole resistant isolates harboured the same TR_{34}/L98H mutation of *cyp51A*. These findings, together with the occurrence of resistant clinical isolates, suggest that azole resistance should be considered in primary patient care.

Introduction

Aspergillus fumigatus is a filamentous fungus that causes a broad spectrum of diseases. Invasive life-threatening infection affects subjects with compromised immune system, mainly patients with haematological malignancies, recipients of haematopoietic stem cells or solid organ transplantation, or patients under prolonged treatment with steroids. Chronic aspergillosis occurs in patients with pre-existing pulmonary or sinus disease, and locally invasive infection occurs as a result of trauma or surgery.

A. fumigatus is ubiquitous in the soil and in decaying organic matter, and produces asexual spores (conidia) that are continuously dispersed in the air. Most of the patients develop aspergillosis following inhalation of conidia into the alveoli or the upper airways. Triazole antifungals, itraconazole, posaconazole and voriconazole, are increasingly used in the treatment and prophylaxis of aspergillosis [1]. Voriconazole is recommended as first line treatment for invasive aspergillosis in European and American guidelines [2-5].

A. fumigatus is generally susceptible to these antifungals. However, in the recent years, azole resistance has been increasingly reported in patients under long-term antifungal treatment and also in azole naïve patients as well as in strains from the environment [1,6-8].

The most common mechanism of azole resistance is an alteration of lanosterol 14-α-demethylase, the key enzyme in the biosynthetic pathway of the ergosterol, a main component of the cell membrane. Different point mutations in the *cyp51A* gene, which encodes this enzyme, have been shown to confer resistance [1,9-11]. A dominant mechanism of resistance involving a 34-bp tandem repeat in the gene promoter region and a substitution of a leucine for a histidine at codon 98 (TR34/L98H) was initially found in clinical and environmental isolates from the Netherlands and a correlation to the use of azoles in the environment was suggested [12].

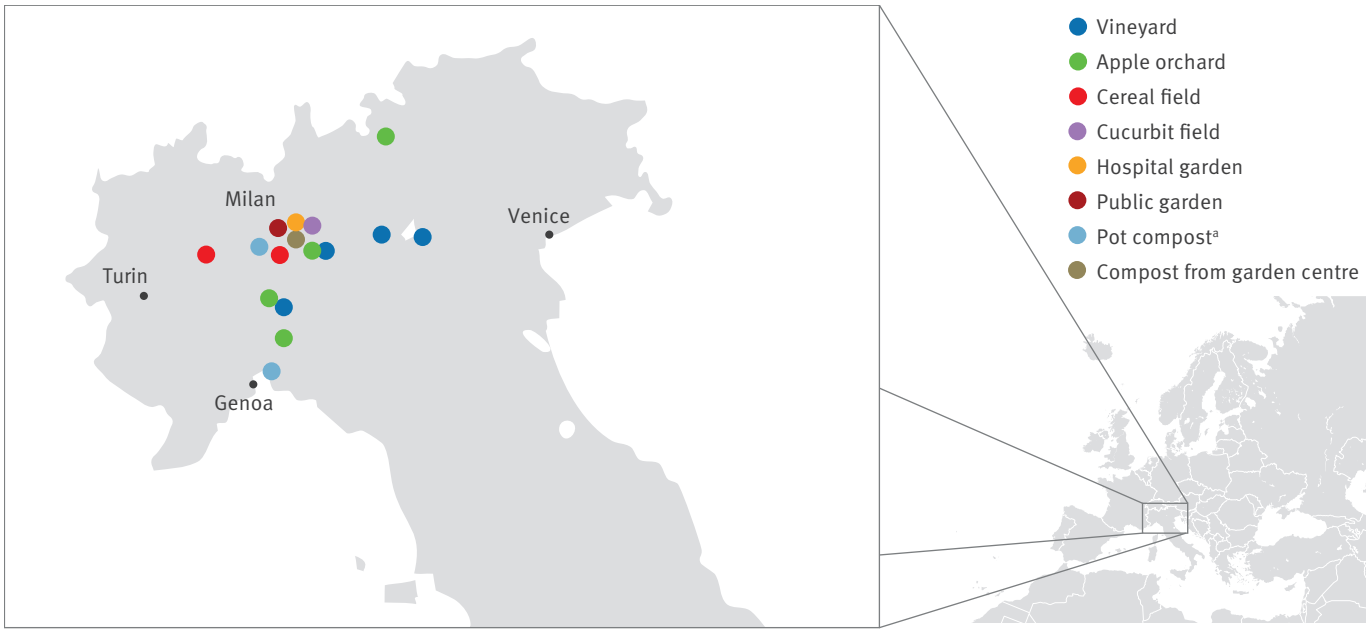
During the international surveillance of azole resistance in *A. fumigatus* clinical isolates (SCARE Network) intrinsic resistance was observed in four of 209 isolates collected in Italy [13] and the TR34/L98H mutation was detected in all these isolates. The aim of the present study was to investigate the prevalence of azole resistance in *A. fumigatus* environmental isolates collected in northern Italy.

Methods

Environmental sampling was carried out in northern Italy in the period between May 2011 and June 2012. A total of 47 soil samples, namely 12 samples from apple orchard, 12 from cucurbit fields, six from vineyards, five from cereals fields, five from pot composts (including rose and other flower pot compost), three from flowerbeds of public gardens, three from hospital gardens, and one from compost purchased from a commercial garden centre, were examined. The sampling sites are reported in the map (Figure 1). Azole fungicides are

Figure 1

Map of northern Italy reporting the sampling sites for *Aspergillus fumigatus*, May 2011—June 2012



Only sites where more than one sample could be analysed, are shown.

[a] Including rose and other flower pot compost.

used in all the sampled environments, except gardens. No information was available for composts.

The samples were treated according to the method previously described by Snelders et al. [12] with minor modifications. Briefly, 2 g of each sample were suspended in 8 mL of sterile distilled water added of 1% Tween 20 (Sigma, St. Louis USA) and chloramphenicol (0.5 g/L, Sigma) and vortexed. The suspension was stored at room temperature for 30 to 60 min and 100 µL of the supernatant was inoculated on two plates of Sabouraud dextrose agar (SDA, Biolife, Milan, Italy) supplemented with chloramphenicol (0.5 g/L) and on two plates of SDA supplemented with chloramphenicol and itraconazole (4 mg/L, Janssen, Beerse, Belgium). Control plates and itraconazole-containing plates were incubated at 37° C and at 42° C (to limit the fungal growth) and examined after 24, 48, and 72 hours of incubation. All the *A. fumigatus* isolates grown on itraconazole-containing agar and an equal number of isolates grown on control plates were selected and maintained on SDA medium.

The isolates were identified by macroscopic and microscopic morphology on Czapek agar medium (Difco, Becton Dickinson, Buccinasco, Italy) as *A. fumigatus* species complex.

A. fumigatus isolates grown in presence of itraconazole and an equal number of isolates grown on control plates were tested for antifungal susceptibility to itraconazole, posaconazole and voriconazole. Susceptibility testing was performed, within one month from the isolation, by broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [14]. The minimal inhibitory concentration (MIC) of all the azoles was determined visually as the lowest concentration of drug giving a complete inhibition of fungal growth. All tests were performed in duplicate. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as quality control in each test.

Susceptibility testing was also performed using Etest strips (BioMérieux, Bagno a Ripoli, Italy) on RPMI 1640 agar supplemented with 2% glucose, according to the manufactory indications. Isolates with MIC of itraconazole and voriconazole >2mg/L and those with MIC of posaconazole >0.25mg/L were considered resistant, according to the EUCAST breakpoints [15-17].

Genomic DNA was extracted from the nine azole-resistant and four susceptible isolates using the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA). Identification of the isolates as *A. fumigatus in sensu stricto* was confirmed by the amplification and sequencing of a portion of the beta-tubulin

Table 1

Environmental origin of itraconazole-resistant *Aspergillus fumigatus* isolates, Italy, May 2011–June 2012

Sample source	Period of sampling	Examined samples (n)	Samples with growth of *A. fumigatus* on control plates (n)	Samples with growth of *A. fumigatus* on itraconazole medium (n)	*A. fumigatus* isolates on itraconazole medium (n)	*A. fumigatus* isolates with confirmed itraconazole resistance (n)
Apple orchard	Oct–Dec 2011	12	3	1	1	1[a]
Cereal fields	Mar–Apr 2012	5	5	0	0	0
Compost from garden centre	Apr 2012	1	0	0	0	0
Cucurbit fields	May and Sep 2011	12	10	8	28	6[b]
Hospital gardens	Mar–Jun 2012	3	3	0	0	0
Pot compost	May 2011	5	5	2	29	2[c]
Public gardens	Apr–May 2012	3	3	0	0	0
Vineyards	Oct 2011	6	0	0	0	0
Total	–	**47**	**29**	**11**	**58**	**9**

[a] The isolate corresponds to one sample collected in the Lombardy region near the northern Italian border.

[b] The isolates were derived from three samples respectively collected in different fields and/or dates near the city of Milan.

[c] The isolates were derived from two samples from two different rose pots collected near the city of Genoa.

gene using the primers described elsewhere [9]. The sequences obtained were compared to the sequences present in the GenBank database (www.ncbi.nlm.nih.gov) by basic local alignment search tool (BLAST) analysis and the identification was confirmed if a 99 to 100% sequence identity was observed.

In addition, two further DNA regions, one inside the *cyp51A* gene and one inside its promoter, were sequenced to detect the presence of the point mutation t364a , which leads to the L98H substitution at the protein level, and the 34-bp tandem repeat, both specific for azole resistance. The *cyp51A* gene fragment (1168 bp) was amplified by polymerase chain reaction (PCR) using the two primers, P450-A1 [11] and *Cyp51A*R2 (5'-AGTGAATAGAGGAGTGAATCC-3'). PCR was performed in a 50-mL volume containing 10X buffer (10 mM Tris-HCL, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; Qiagen, Venlo, Netherlands), 1.5 mM $MgCl_2$, 200 mM of each of the four deoxynucleotides, 20 pmol of each primer, 2.5 U of Taq polymerase (Qiagen), and 2 mL of genomic DNA. The thermal cycling profile included an initial step at 94°C for 5 min, 30 cycles consisting in denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR product was then sequenced using two forward primers, P450-A1 and *Cyp51A*F2 (5'-GACATCTCTGCGGCAATGG-3'), and two reverse primers, *Cyp51A*R2 and *Cyp51A*R3 (5'-CCATTGCCGCAGAGATGTC-3'), to reach the complete sequencing coverage for both strands of the fragment. The *cyp51A* gene promoter was amplified and sequenced using the primers PA5 and PA7 as previously described [10].

All sequences were determined by an ABI PRISM 3100 genetic analyser (Applied Biosystems), electropherograms were analysed by FinchTV software (www.geospiza.com), and sequence alignment was performed using ClustalW algorithm (www.ebi.ac.uk). The *cyp51A* sequence from *A. fumigatus* strain 237 (GenBank accession number: AF338659) was used as wild type reference.

Azole-resistant as well as five susceptible isolates were genotyped by microsatellite analysis using the primers STRAf3A, STRAf3B, STRAf3C, STRAf4A, STRAf4B, and STRAf4C as described elsewhere [18].

Results

A. fumigatus grew in 29 of 47 soil samples (62%), mainly form cucurbit and cereal fields and rose pot composts (Table 1). No *A. fumigatus* was isolated from vineyards and compost from garden centre. A total of 58 isolates grew on itraconazole-containing agar.

Broth microdilution and Etest confirmed itraconazole resistance in nine isolates (MIC ≥16 mg/L). All these isolates were also posaconazole resistant (MIC ≥0.5 mg/L). Seven isolates showed an intermediate susceptibility to voriconazole (MIC =2 mg/L) by broth microdilution method according to EUCAST (Table 2). Etest MIC values were lower but MIC values obtained by broth microdilution and Etest were in the +/- 2 dilution range. The *A. fumigatus* control isolates were susceptible to itraconazole (range of MICs: 0.06 to 0.12 mg/L), posaconazole (range of MICs: ≤0.03 to 0.06 mg/L), and voriconazole (range of MICs: 0.06 to 0.25 mg/L). Identical results were obtained in tests performed in duplicate.

Table 2

Results of EUCAST and Etest susceptibility testing, and analysis of mutations in *cyp51A*, Italy, May 2011–June 2012

Isolate number	Sample source	MIC (mg/L) determined by EUCAST			MIC (mg/L) determined by Etest			Mutation in *cyp51A*
		ITZ[a]	POS[a]	VRC[a]	ITZ[a]	POS[a]	VRC[a]	
11-0087A	Rose pot compost	>16	1	2	>32	1	1	TR34/L98H
11-0088E	Rose pot compost	>16	2	1	16	0.5	1	None
11-0099A	Cucurbit fields	>16	2	2	>32	1	2	TR34/L98H
11-0104A	Cucurbit fields	>16	1	2	>32	0.5	2	TR34/L98H
11-0104B	Cucurbit fields	>16	1	2	>32	0.5	2	F46Y; M172V; N248T; D255E
11-0104D	Cucurbit fields	>16	1	2	>32	1	1	TR34/L98H
11-0317C	Cucurbit fields	>16	2	2	>32	0.5	0.5	TR34/L98H
11-0317D	Cucurbit fields	>16	1	1	>32	0.5	1	TR34/L98H
11-0396	Apple orchard	>16	2	2	>32	0.5	1	TR34/L98H

EUCAST: European Committee on Antimicrobial Susceptibility Testing; MIC: minimal inhibitory concentration.

Isolates with MIC of itraconazole and voriconazole >2mg/L and those with MIC of posaconazole >0.25mg/L by broth microdilution method according to EUCAST, were considered resistant [15-17]. Data obtained by EUCAST and Etest differed, however results in a +/- 2 dilution range are considered acceptable.

[a] The azoles used for susceptibility testing were ITZ (itraconazole), POS (posaconazole), VRC (voriconazole).

Figure 2

Alignment of three amino acid sequences derived from the *Cyp51A* genetic sequence from itraconazole susceptible and resistant isolates, Italy, May 2011–June 2012

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11-0087C (S)    MVPMLWLTAYMAVAVLTAILLNVVYQLFFRLWNRTEPPMVFHWVPFLGSTISYGIDPYKF 60
11-0099A (R)    ************************************************************ 60
11-0104B (R)    *******************************************Y****************** 60

11-0087C (S)    FFACREKYGDIFTFILLGQKTTVYLGVQGNEFILNGKLKDVNAEEVYSPLTTPVFGSDVV 120
11-0099A (R)    ************************************H*********************** 120
11-0104B (R)    ************************************************************ 120

11-0087C (S)    YDCPNSKLMEQKKFIKYGLTQSALESHVPLIEKEVLDYLRDSPNFQGSSGRMDISAAMAE 180
11-0099A (R)    ************************************************************ 180
11-0104B (R)    *************************************************V******** 180

11-0087C (S)    ITIFTAARALQGQEVRSKLTAEFADLYHDLDKGFTPINFMLPWAPLPHNKKRDAAHARMR 240
11-0099A (R)    ************************************************************ 240
11-0104B (R)    ************************************************************ 240

11-0087C (S)    SIYVDIINQRRLDGDKDSQKSDMIWNLMNCTYKNGQQVPDKEIAHMMITLLMAGQHSSSS 300
11-0099A (R)    ************************************************************ 300
11-0104B (R)    *******T******E***************************************** 300

11-0087C (S)    ISAWIMLRLASQPKVLEELYQEQLANLGPAGPDGSLPPLQYKDLDKLPFHQHVIRETLRI 360
11-0099A (R)    ************************************************************ 360
11-0104B (R)    ************************************************************ 360

11-0087C (S)    HSSIH 365
11-0099A (R)    ***** 365
11-0104B (R)    ***** 365
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An asterisk (*) indicates that the amino acid in the sequence is identical to that of the sequence presented at the top of the alignment.

The sequence 11-0087C is derived from an itraconazole susceptible (S) isolate, while the sequences 11-0099A, with the resulting L98H mutation, and 11-0104B, with other resulting amino acid substitutions, are from respective itraconazole resistant (R) isolates.

Table 3

Allelic profiles at six microsatellite loci of itraconazole resistant (n= 8) and susceptible (n=5) environmental isolates, Italy, May 2011–June 2012

Isolate	R/S	Fragment size as bp (number of repeats)					
		STRAf3A	STRAf3B	STRAf3C	STRAf4A	STRAf4B	STRAf4C
11-0087A	R	292 (60)	164 (10)	78 (5)	182 (9)	185 (9)	185 (10)
11-0088E	R	190 (27)	169 (12)	80 (6)	231 (20)	184 (9)	175 (7)
11-0099A	R	310 (66)	158 (8)	78 (5)	179 (8)	185 (9)	185 (10)
11-0104A	R	323 (70)	158 (8)	86 (8)	179 (8)	185 (9)	175 (7)
11-0104B	R	161 (18)	162 (9)	108 (15)	201 (13)	184 (9)	175 (7)
11-0104D	R	179 (23)	160 (9)	78 (5)	183 (9)	184 (9)	185 (10)
11-0317C	R	203 (31)	164 (10)	75 (4)	186 (10)	180 (8)	224 (20)
11-0396	R	310 (66)	158 (8)	80 (6)	178 (8)	184 (9)	185 (10)
11-0023B	S	212 (34)	164 (10)	194 (42)	196 (12)	184 (9)	175 (7)
11-0034A	S	212 (34)	156 (7)	80 (6)	182 (9)	180 (8)	242 (26)
11-0036C	S	222 (37)	164 (10)	80 (6)	196 (12)	175 (7)	162 (5)
11-0087C	S	190 (27)	162 (9)	118 (18)	182 (9)	196 (12)	162 (5)
11-0104E	S	219 (36)	169 (12)	80 (6)	185 (10)	192 (11)	181 (9)

R: itraconazole resistant; S: itraconazole susceptible.

The nine itraconazole- and posaconazole-resistant isolates were recovered from pot composts (2 isolates from 2 different rose pots from Genoa), from apple orchard (1 isolate from an apple orchard near the northern border of Italy), and from cucurbit fields (6 isolates). These last six isolates were from three different samples, 11-0099, 11-0104 and 11-0317, collected in different fields and/or dates.

All nine resistant isolates were identified as *A. fumigatus sensu stricto* by amplification of a fraction of the beta-tubulin gene.

Sequence analysis of *cyp51A* gene showed the t364a point mutation, which results in the L98H substitution, combined with the 34-bp tandem repeat in the promoter region, in seven of nine itraconazole- and posaconazole-resistant isolates (Table 2). The TR_{34}/L98H alteration was neither detected in the four azole susceptible isolates used as control nor in the resistant isolates 11-0088E and 11-0104B. No mutation in the *cyp51A* gene was found in the recovered sequence of 11-0088E. The other resistant isolate (11-0104B) lacking the mutation leading to the L98H substitution had four other inferred amino acid changes at codons 46, 172, 248 and 255, as reported in Figure 2.

Eight of nine resistant isolates (one was no more vital) and five additional susceptible environmental isolates were genotyped by microsatellite analysis. The results showed that all the isolates presented genotypes different from each other (Table 3).

Discussion

Acquired resistance to azoles develops in response to exposure of fungi to azole compounds in patients and in agricultural settings and is favoured by long duration of exposure to these compounds and high numbers of reproducing fungi [1,19,20]. Acquisition of resistance in patients is characterised by a variety of resistance mechanisms. The dominance of the TR_{34}/L98H resistance mechanism in unrelated clinical isolates in a large Dutch culture collection suggested that isolates with this mechanism might be present in the environment, favoured by azole fungicides used in agriculture [20]. The detection of the TR_{34}/L98H resistance mechanism also in Italy, in four *A. fumigatus* isolates from two azole-naïve patients among the 209 isolates tested in the SCARE project [13] and in an additional isolate from an azole-exposed patient, lead to investigate the presence of azole-resistant *A. fumigatus* in the environment in our country.

Nine of the 58 isolates obtained by screening soil samples collected in northern Italy on an itraconazole containing medium were confirmed to be resistant to itraconazole and posaconazole by MIC determined with broth microdilution and Etest. Seven of these nine had also a reduced susceptibility to voriconazole when tested with broth microdilution. The discrepancy between the numbers of isolates on the screening medium containing itraconazole at a concentration of 4 mg/L and the numbers of confirmed resistant isolates could be attributed to trailing of organic compounds present in the soil.

Azole-resistant *A. fumigatus sensu stricto* were detected in 13% (6/47) of the soil samples collected and in 21% (6/29) of the soil samples confirmed as containing *A. fumigatus*. These results are in agreement with the data from other European countries: resistant isolates were detected in 8% of soil samples and in 11% of samples containing *A. fumigatus* in Denmark [8] and in 20.4% of *A. fumigatus* positive soil samples in the Netherlands [12]. Due to the limited number of samples it was impossibile to note a seasonal variation

Molecular analysis showed the presence of the TR_{34}/L98H resistance mechanism in seven of nine resistant isolates. This resistance mechanism has also been reported in clinical isolates from several European and Asian countries [1,7,9,13,21-25]. To explain the prevalence of this resistance mechanism it was hypothesised that TR_{34}/L98H isolates may have an advantage with respect to fitness compared to isolates with other mutations [6]. Other point mutations (in codons 46, 172, 248, 255) were detected in one of the remaining resistant isolate. These mutations have been reported by other authors, and were found in both azole-susceptible and resistant strains [7,26,27]. No mutation was observed in the sequenced *cyp51A* gene of the other resistant isolate. These two resistant isolates would suggest the existence of other mechanisms of resistance.

As shown by molecular typing, all the resistant isolates were characterised as different strains confirming that resistance does not arise by a clonal expansion of a mutant but it is likely induced by the selective pressure resulting from the presence of azoles in the environment.

In conclusion this study provides evidence that azole-resistant *A. fumigatus* are detected in the environment also in Italy, in soil or composts exposed to azole fungicides, and confirms the presence of the TR_{34}/L98H mutation in several countries in Europe. Susceptibility testing of filamentous fungi is not routinely carried out in most medical microbiology laboratories, however the risk for patients to acquire multi-azole-resistant strains from the environment could have a serious impact on the management of life-threatening invasive infections. Finally, the possible selection of resistant fungal pathogens should lead applying azoles in agriculture with caution.

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Conflict of interest

None declared.

Authors’ contributions

Prigitano Anna, Venier Valentina, Cogliati Massimo, Esposto Maria Carmela contributed to the planning of the research, performed sampling and laboratory tests, and contributed to the manuscript preparation. De Lorenzis Gabriella contributed to microsatellite analysis tests and contributed to the manuscript preparation. Tortorano Anna Maria planned the reaserch and prepared the manuscript.

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