J Antimicrob Chemother 2012; **67**: 362–366
doi:10.1093/jac/dkr443 Advance Access publication 25 October 2011

Journal of Antimicrobial Chemotherapy

Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR/L98H mutations in the *cyp51A* gene in India

Anuradha Chowdhary[1]*, Shallu Kathuria[1], Harbans S. Randhawa[1], Shailendra N. Gaur[2], Corné H. Klaassen[3] and Jacques F. Meis[3,4]

[1]Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; [2]Department of Pulmonary Medicine, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; [3]Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands; [4]Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

*Corresponding author. Tel: +91-11-27667560; Fax: +91-11-27666549; E-mail: dranuradha@hotmail.com

Received 21 July 2011; returned 21 September 2011; revised 23 September 2011; accepted 26 September 2011

Objectives: Azole resistance in *Aspergillus fumigatus* isolates impacts on the management of aspergillosis since azoles are primary agents used for prophylaxis and therapy. We report the emergence of resistance to triazoles in two *A. fumigatus* isolates from patients in Delhi, India.

Methods: One hundred and three *A. fumigatus* isolates, collected from 85 patients suspected of bronchopulmonary aspergillosis during 2005–10, were investigated for susceptibility to itraconazole, voriconazole, posaconazole and isavuconazole. We undertook a mixed-format real-time PCR assay for the detection of mutations leading to triazole resistance in *A. fumigatus*. The resistant isolates were compared with 25 Dutch TR/L98H-positive isolates by microsatellite analysis.

Results: Of the 103 *A. fumigatus* isolates tested, only 2 had high MIC values of itraconazole (>16 mg/L), voriconazole (2 mg/L), posaconazole (2 mg/L) and isavuconazole (8 mg/L). The resistant *A. fumigatus* isolates exhibited the TR/L98H genotype and showed identical patterns by microsatellite typing, but were different from 25 Dutch TR/L98H isolates.

Conclusions: We report for the first time from India the occurrence of TR/L98H mutations in the *cyp51A* gene (responsible for reduced azole susceptibility) in two *A. fumigatus* isolates from patients with chronic respiratory disease who had not previously been exposed to azoles. The presence of TR/L98H is consistent with a route of resistance development through exposure to azole compounds in the environment. Given the emergence of azole resistance in environmental strains, continued surveillance of resistance in clinical *A. fumigatus* strains is desirable for successful therapy of aspergillosis.

Keywords: itraconazole, chronic respiratory disease, azole-naive patients

Introduction

Aspergillus fumigatus is the most common aetiological agent of invasive and chronic pulmonary aspergillosis. Azoles, such as itraconazole, voriconazole and posaconazole, are among the recommended first-line drugs in the treatment and prophylaxis of aspergillosis. Since the first report of resistance of three clinical *A. fumigatus* isolates to itraconazole in 1997 in the USA,[1] there have been several reports of therapeutic failure in aspergillosis treatment caused by triazole-resistant *A. fumigatus*.[2–4] Triazole-resistant *A. fumigatus* strains were isolated from both azole-exposed and azole-naive patients, and from the environment in The Netherlands and recently also in Denmark.[3,5–7] The most common mechanism of resistance involves changes in the amino acid sequence of the *cyp51A* gene encoding 14-α-demethylase, a component of the ergosterol pathway and the target of triazole antifungal drugs. A specific L98H alteration in combination with a tandem repeat in the promoter region (designated 'TR/L98H') was found to be the dominant resistance mechanism in Dutch *A. fumigatus* isolates. The emergence of multiple-triazole resistance in *A. fumigatus* may significantly impact on the therapeutic role of azoles in aspergillosis, as it would rule out the use of oral antifungals leaving only the option of intravenous amphotericin B or echinocandins. Given the emerging reports of azole resistance in environmental

strains, continued surveillance of the emergence of resistance in clinical *A. fumigatus* strains is desirable for more successful therapy of aspergillosis. Herein we report, for the first time from India, the occurrence of *cyp51A* mutations responsible for reduced azole susceptibility in two *A. fumigatus* isolates recovered from patients with chronic respiratory diseases.

Methods

Fungal isolates and patients

One hundred and three *A. fumigatus* isolates were prospectively collected from 101 clinical specimens originating from 85 patients suspected of bronchopulmonary aspergillosis, admitted to the Clinical Research Centre of the Vallabhbhai Patel Chest Institute (VPCI), Delhi, India, during 2005–10. Of the 101 clinical specimens, 71 were sputum, 7 bronchoalveolar lavage, 14 endotracheal aspirate, 4 blood, 3 nasal polyps, 1 biopsy and 1 pleural fluid. Identification was based on macro- and micromorphological characteristics, and on growth at 50°C, which differentiates *A. fumigatus* from *Aspergillus lentulus*. Identification of the resistant isolates was confirmed by internal transcribed spacer (ITS) sequencing.

Antifungal susceptibility testing

The triazoles used for antifungal testing were itraconazole (Lee Pharma, Hyderabad, India; and the Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central Research, Sandwich, UK), posaconazole (Schering-Plough Corp., Kenilworth, NJ, USA; now Astellas), and isavuconazole (Basilea Pharmaceutica International AG, Basel, Switzerland). The MICs of itraconazole, voriconazole, posaconazole and isavuconazole were determined according to the CLSI M38-A2 broth microdilution method.[8]

Mixed-format real-time PCR assay to detect mutations

All of the *A. fumigatus* isolates for which the itraconazole MIC was ≥2 mg/L were subjected to a mixed-format real-time PCR assay as

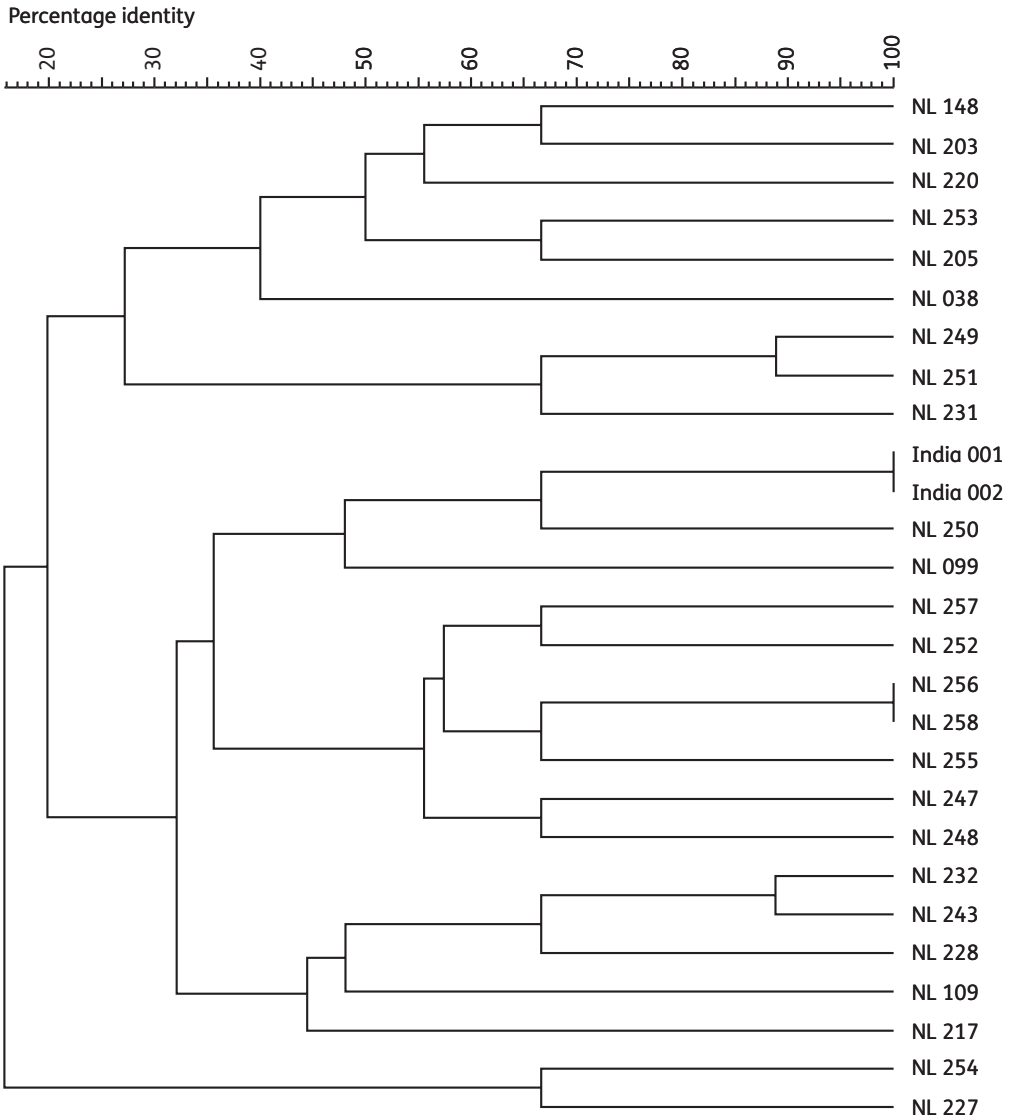

Figure 1. Genotypic relationship between the studied TR/L98H isolates. The dendrogram is based on a categorical analysis of nine microsatellite markers in combination with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering.

described previously for the detection of mutations leading to triazole resistance in *A. fumigatus*.[9]

Microsatellite genotypic analysis

Genotyping was performed with a panel of nine short tandem repeats (STRs) as described previously.[10] For phylogenetic analysis, 25 Dutch clinical and environmental isolates of *A. fumigatus* containing the TR/L98H genotype were included along with the Indian isolates.

Results

Of the 103 *A. fumigatus* isolates tested, only two had high MIC values of itraconazole (>16 mg/L), voriconazole (2 mg/L), posaconazole (2 mg/L) and isavuconazole (8 mg/L). Both of these *A. fumigatus* isolates exhibited the TR/L98H genotype and showed identical STR patterns by microsatellite typing (see dendrogram, Figure 1), but they differed from the 25 Dutch isolates. Isolate VPCI 1042/09 (India 001) originated from sputum of a 55-year-old male outpatient at the VPCI, first admitted in May 2008 and diagnosed with chronic obstructive pulmonary disease with bilateral bronchiectasis and cor pulmonale. His skin-prick test for antigens of four common species of *Aspergillus*, namely *A. fumigatus*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus tamarii*, was negative. Also, tests for specific IgG and IgE against *A. fumigatus* were negative. During his follow-up of 1 year, he received inhaled glucocorticoids, long-acting β-agonists and anti-cholinergic drugs, but no azole drugs. He was admitted to the Emergency Department due to exacerbations of his condition four times in a span of 1 year, during which he was administered nebulizations with salbutamol and ipratropium, and short courses of systemic glucocorticoids, antibiotics and methylxanthines, but no azoles. Microscopy of a solitary sputum specimen received in September 2009 revealed hyaline, septate and branching hyphae, and culture yielded multiple-triazole-resistant *A. fumigatus*. However, the aetiological significance of this observation remained undetermined as the patient was not available for follow-up.

The second multiple-triazole-resistant *A. fumigatus* isolate, VPCI 942/09 (India 002) originated from sputum of a 22-year-old male labourer who presented to the Outpatient Department of the VPCI in August 2009 with complaints of productive cough for 2 years and fever on-and-off for 1 year. His differential diagnosis included pulmonary tuberculosis and allergic bronchopulmonary aspergillosis. As in the preceding case, the patient was not available for follow-up in order to evaluate the aetiological significance of *A. fumigatus*.

Discussion

To our knowledge, this is the first report from India on the occurrence of multiple-triazole-resistant *A. fumigatus* isolates carrying the TR/L98H genotype in patients with chronic respiratory diseases. The TR/L98H mutation associated with pan-azole resistance in *A. fumigatus* has been reported so far only from Europe and recently from China.[3,5–7,9,11–16] As both of our triazole-resistant *A. fumigatus* isolates were epidemiologically unrelated but showed identical STR genotypes, and were obtained from patients without a history of exposure to azoles or of travel to Europe, it is highly likely that the resistance was acquired from the environment in India. Both of our isolates were phylogenetically different from the 25 TR/L98H-containing Dutch isolates of *A. fumigatus*. Acquired azole-resistance can develop in different settings in patients (exhibiting chronic aspergillosis), notably aspergilloma, from

Table 1. Global distribution of multiple-triazole-resistant strains of *A. fumigatus* with TR/L98H mutations

Country	Source	Number of isolates with TR/L98H mutations/number tested	Reference
Europe			
Netherlands	clinical	14[a]/–[b]	11
Spain	clinical	17/393	12
Netherlands	clinical	30[c]/1912	5
Netherlands	environmental	13/248	6
UK	clinical	2/519	3
UK	clinical	1[d]	13
Netherlands	clinical	4/209	9
Denmark	environmental	4/55	7
Denmark	clinical	2/133[d]	14
Netherlands	clinical	74/2062	16
Asia			
China	clinical	8/497	15
India	clinical	2/103	present study

[a]Includes one isolate from Spain.
[b]Not given.
[c]Includes one isolate from Norway.
[d]Denotes patient number.

receiving azole therapy and also through environmental exposure to azole fungicides that are used in agriculture.[6] A possible explanation for the spread of this single-resistance mechanism (TR/L98H) in *A. fumigatus* isolates is the use of azole fungicides in the environment. The resistance mechanism seems to be indicative of the mode of resistance development. In patients on long-term azole treatment, induced azole-resistant *A. fumigatus* isolates with point mutations in codons 54, 138 and 220 have also been described.[4] However, the presence of a tandem repeat is an important mechanism found in plant pathogenic moulds where resistance to sterol demethylation inhibitor (DMI) fungicides is a well-known phenomenon.[17] Since the fungal plant pathogens share their natural environment with *A. fumigatus*, the fungus is exposed to a strong and persistent pressure from fungicides, including DMIs. The presence of *Aspergillus* species in the environment is considered the most important risk factor for invasive aspergillosis, and if TR/L98H is present in the environment, resistant conidia will be dispersed in the air and may cause infection through inhalation.[14] The occurrence of azole-resistant environmental *A. fumigatus* isolates in agricultural soil has been reported from The Netherlands and recently also from Denmark.[7] A recent report by Snelders *et al*.[6] revealed that TR/L98H and wild-type *A. fumigatus* isolates from both clinical and environmental sources showed cross-resistance to the azole fungicides tebuconazole and metconazole, supporting a role of fungicides in resistance development. Interestingly, in India various triazole fungicides, such as hexaconazole, penconazole and propiconazole, have been registered for crop protection against rust, powdery mildew and blight diseases of wheat, rice, tea and grapes. Triazole fungicides are recommended as foliar sprays for growing crops and sometimes as seed treatments. In general, triazole fungicides are characterized by their long persistence in soil. The estimated half-life of hexaconazole in soil is ~2 months. Of this, about 15% remains undegraded for up to 6 months. Singh and Dureja[18] reported that the longer persistence of hexaconazole in Indian soil was due to its hydrophobic nature. However, in contrast to the environmental origin of triazole-resistant *A. fumigatus*, studies from the UK indicated that the *cyp51A* mutations occurred primarily due to the pressure of azole therapy.[3] Currently, clinical *A. fumigatus* isolates with the TR/L98H mutation have been reported from The Netherlands, Spain, Denmark, the UK, Norway and China (Table 1). In the present study, 1.9% (2/103) of the *A. fumigatus* clinical isolates were multiple-triazole resistant, whereas in Dutch hospitals the corresponding figure was 6%–12.8%.[5,16] In contrast, a low prevalence (0.85%) of azole-resistant *A. fumigatus* isolates (not TR/L98H) was reported from a cohort of 89 patients with haematological malignancies in France.[19] Notwithstanding the fact that antifungal susceptibility testing of *A. fumigatus* isolates is not routinely performed in most clinical laboratories, reports of emerging resistance in Europe and China and the present study underscore the importance of susceptibility testing for monitoring azole resistance. Finally, patients with azole-resistant *Aspergillus* disease commonly fail to respond to azole therapy. Therefore, the need for enhanced understanding of the evolution of azole resistance and for effective measures to prevent the emergence of multiple-azole-resistant *A. fumigatus* strains in countries using fungicides cannot be over-emphasized.

Acknowledgements

We acknowledge the Indian National Science Academy, New Delhi, for the award of an Honorary Scientist position to H. S. R.

Funding

This work was partly supported by the Indian Council of Medical Research, New Delhi (HIV/50/107/2008). Part of this work was done during a research fellowship of A. C. at the Canisius Wilhelmina Hospital, Nijmegen, The Netherlands, which was sponsored by Labland BV, Wijchen, The Netherlands.

Transparency declarations

C. H. K. has received a research grant from Pfizer. J. F. M. has received grants form Astellas, Merck, Basilea and Schering-Plough. He has been a consultant to Basilea and Merck and received speaker's fees from Merck, Pfizer, Schering-Plough, Gilead and Janssen Pharmaceutica. All other authors: no potential conflicts of interest.

References

1 Denning DW, Radford SA, Oakley KL *et al*. Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection. *J Antimicrob Chemother* 1997; **40**: 401–14.

2 Howard SJ, Webster I, Moore CB *et al*. Multi-azole resistance in *Aspergillus fumigatus*. *Int J Antimicrob Agents* 2006; **28**: 450–3.

3 Howard SJ, Cerar D, Anderson MJ *et al*. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg Infect Dis* 2009; **15**: 1068–76.

4 Arendrup MC, Mavridou E, Mortensen KL *et al*. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS One* 2010; **5**: e10080.

5 Snelders E, van der Lee HA, Kuijpers J *et al*. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med* 2008; **5**: e219.

6 Snelders E, Huis in 't Veld RA, Rijs AJ *et al*. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* 2009; **75**: 4053–7.

7 Mortensen KL, Mellado E, Lass-Flörl C *et al*. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother* 2010; **54**: 4545–9.

8 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antimicrobial Susceptibility Testing of Filamentous Fungi—Second Edition: Approved Standard M38-A2*. CLSI, Wayne, PA, USA, 2008.

9 Klaassen CH, de Valk HA, Curfs-Breuker IM *et al*. Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in *Aspergillus fumigatus* and prevalence of multi-triazole resistance among clinical isolates in the Netherlands. *J Antimicrob Chemother* 2010; **65**: 901–5.

10 de Valk HA, Meis JF, Curfs-Breuker IM *et al*. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J Clin Microbiol* 2005; **43**: 4112–20.

11 Mellado E, Garcia-Effron G, Alcázar-Fuoli L *et al*. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob Agents Chemother* 2007; **51**: 1897–904.

12 Rodriguez-Tudela JL, Alcazar-Fuoli L, Mellado E *et al*. Epidemiological cut-offs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 2008; **52**: 2468–72.

13 Howard SJ, Pasqualotto AC, Denning DW. Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus* bronchitis. *Clin Microbiol Infect* 2010; **16**: 683–8.

14 Mortensen KL, Jensen RH, Johansen HK *et al*. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J Clin Microbiol* 2011; **49**: 2243–51.

15 Lockhart SR, Frade JP, Etienne KA *et al*. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the cyp51A gene. *Antimicrob Agents Chemother* 2011; **55**: 4465–8.

16 van der Linden JW, Snelders E, Kampinga GA *et al*. Clinical implications of azole resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009. *Emerg Infect Dis* 2011; **17**: 1846–54.

17 Snelders E, Melchers WJ, Verweij PE. Azole resistance in *Aspergillus fumigatus*: a new challenge in the management of invasive aspergillosis? *Future Microbiol* 2011; **6**: 335–47.

18 Singh N, Dureja P. Persistence of hexaconazole, a triazole fungicide in soils. *J Environ Sci Health B* 2000; **35**: 549–58.

19 Alanio A, Sitterlé E, Liance M *et al*. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *J Antimicrob Chemother* 2011; **66**: 371–4.