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AZOLE RESISTANT *ASPERGILLUS FUMIGATUS* WITH TR34/L98H MUTATION FROM CLINICAL ISOLATES IN, KENYA

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AZOLE RESISTANT *ASPERGILLUS FUMIGATUS* WITH TR34/L98H MUTATION FROM CLINICAL ISOLATES IN, KENYA

E.K. Kemoi, A. Nyerere, O. Mashedi and C. Bii

ABSTRACT

***Objective*: The aim of the study was to characterize *aspergillus* species from clinical samples.**

***Design*: Cross sectional study.**

***Setting*: The study was conducted at Kenya Medical Research Institute using archived *aspergillus* isolates.**

***Subject*: The study was performed on 54 archived clinical samples.**

***Results*: Nine *Aspergillus fumigatus* and two *Aspergillus flavus* were isolated out of which two *A. fumigatus* had TR34/L98H mutation.**

***Conclusion*: Although no information on prior azole exposure was available, the present study reports the first triazole resistant *aspergillus* in Kenya and calls for more surveillance and measures to contain the emergence of azole resistance in clinical practice.**

INTRODUCTION

In last decade many studies have reported the detection of azole resistance in *Aspergillus fumigatus* as an emerging health concern. Acquisition of azole resistance can be ascribed to use of agricultural fungicides or long-term azole therapy (1, 2). Most of Azole resistance is due to mutation in cyp51A gene (TR46/Y121F/T289A and TR34/L98H) (3). TR34/98H has been reported in *Aspergillus fumigatus* isolated from environmental and clinical samples worldwide (4). Currently there are few reported cases of *Aspergillus fumigatus* with TR34/L98H resistance in patients with invasive aspergillosis (5, 6, 7). Emergence of azole resistant *Aspergillus fumigatus* has a negative impact on the use of oral antifungals leaving only the option of intravenous echinocandins and amphotericin. Voriconazole, itraconazole, and posaconazole are used in the management of aspergillosis as first-line medical triazoles (8). Azole resistant *Aspergillus fumigatus* has been increasingly reported in most middle and high-income countries (1, 9). Researchers in Asia (Iran,

Kuwait), Europe (Germany, Denmark, Netherlands, Belgium) and African (Kenya, Tanzania) have reported *Aspergillus fumigatus* resistant to azole from different sources both clinical and environment (10, 11). *Aspergillus fumigatus* resistance prevalence against itraconazole has reported being 10% and 17-20% in The Netherlands and the United Kingdom respectively (10, 12).

Resistance in *Aspergillus* species involves mechanism of cyp51A gene point mutation (3, 12). Resistance in *Aspergillus fumigatus* to medical azoles has been linked to the used of agricultural azole-based fungicides in agriculture. Presence of azole in the environment results in TR46/Y121F/T289A and TR34/L98H point mutation in the cyp51A genes in Europe and United States (13). Since 1970s, agricultural fungicides which are demethylation inhibitor have been intensively used in the environment to protect crops from fungal diseases (14). This practice has since been replicated in Kenya especially in the robust agricultural and horticultural industry in which the long term is unknown.

MATERIALS AND METHODS

Samples collections

The study was performed on 54 clinical samples out of which nine *Aspergillus fumigatus* and two were *Aspergillus flavus* were isolated. The samples analyzed were archived specimens at the Mycology laboratory, Kenya Medical Research Institute from the sputum of patients suspected of aspergillosis. Identification of the suspected *aspergillus* species was done using macro and micro morphological identifications according to (15).

Azole resistance screening test

Approximately hundred microliters of the suspension were cultured onto three Sabouraud dextrose agar (SDA) media plates: SDA with no azoles was used to determine the diversity of *Aspergillus* species while SDA with Itraconazole (1µg/ml) and Voriconazole (1µg/ml) to determine resistant *Aspergillus* then incubated at 30°C. Colonies growing on SDA media containing azoles were subcultured onto another media without azole for further tests.

Susceptibility test

All the *Aspergillus* isolates growing on media containing azole were subjected to broth dilution susceptibility test using EUCAST protocol.

Detection of TR34 mutation

The presence or the absence of the TR34 mutation was determined by PCR amplification using: AFCYPPR (5′-TGGTATGCTGGAACTACACCTT-3′) and AFCYPPR (5′-AATAATCGCAGCACCACTTC-3′) primers (Invitrogen, UK). The PCR carried out in 50µl vol. containing, AmpliTaq DNA polymerase, 0.1mM each dNTP, 2µl DNA, 4 pmol each for primers and 1× amplitap PCR buffer. PCR 35 cycles: denaturation at 95°C for 1 min, Annealing at 60°C for the 30s and extension at 72°C for 1 min. initial denaturation at 95°C for 5min and final extension step at 72°C for 10 min. The amplicons were detected using 2% agarose gels according to Al-Wathiqi *et al*., (16).

Detection of L98H mutations

The presence or the absence of L98H mutation at cyp51A98 was done using DNA amplification using; AFCYP98F (5′-CAAGTTCTTCTTTGCGTGCAGA-3′) and AFCYP98R (5′-ATAAGTGGCACATGAGACTCT-3′) primers. The PCR reaction and cycling condition was as described above for TR34 mutations. The DNA was purified using Qiagen purification

kit according to the manufactured instruction. The purified DNA was digested in AluI (New England Bio-Labs) and electrophoresed for 5 hr at 37°C. The digest was detected by 2% agarose
gels to generate PCR-restriction fragment length polymorphism (10). For quality control know *A. fumigatus* containing TR_{34} in the promoter region and wild type were included. Results

Five *Aspergillus fumigatus* were found to be resistant to at least one triazole (Table 1). According to EUCAST (20,21) susceptibility break points, MIC of ≥ 0.5mg/l, ≤0.12mg/l are considered resistant and susceptible against Posaconazole respectively, while MIC between 0.12mg/l - 0.5mg/l are considered intermediate. Voriconazole and Itraconazole ≥4mg/l resistant, MIC 1mg/l – 4mg/l Intermediate and ≤1mg/l are susceptible (17).

Table 1

Minimum Inhibitory Concentration of the test isolates against the three Triazoles

Clinical sample No.	Isolate identification	Minimum Inhibitory Concentrations (ug/ml)		
		ITZ	VRZ	PSZ
C01	*Aspergillus fumigatus*	1	1	0.5
C02	*Aspergillus flavus*	0.5	0.25	0.5
C03	*Aspergillus flavus*	0.5	0.03	0.06
C04	*Aspergillus fumigatus*	0.5	1	0.13
C05	*A. fumigatus*	1	0.25	0.5
C06	*A. fumigatus*	0.5	0.5	0.5
C07	*A. fumigatus**	1	16	1
C08	*A. fumigatus*	0.5	2	0.13
C09	*A. fumigatus*	1	1	0.125
C10	*A. fumigatus**	4	0.5	0.25
C11	*A. fumigatus*	2	2	1

*ITZ=Itraconazole, VRZ=Voriconazole, PSZ=Posaconazole, * Sequenced isolates*

Two isolates C07 and C010 of *Aspergillus fumigatus* showing elevated MIC to any three of the tested triazoles were subjected to sequencing to determine the type of mutation (Table 2).

Table 2

Sequenced Aspergillus fumigatus isolates showing TR34/L98H mutation

Strain	Species	CYP51A	2A	2B	2C	3A	3B	3C	4A	4B	4C
C07	*A. fumigatus*	TR34/L98H	14	21	8	28	9	6	8	10	18
C14	*A. fumigatus*	TR34/L98H	14	23	8	30	9	6	8	10	20

DISCUSSION

In the study 35.7% of *Aspergillus* species isolated from archived clinical isolates were found to be resistant to at least one of the test azoles. The high resistant percentage could be due to small samples size used in the study. We reported detections of TR34/L98H point mutation from archived clinical isolates in Kenya. The TR34/L98H mutation have been isolated from the environment and linked to fungicide use in agriculture (18). Azole

resistant *Aspergillus fumigatus* with TR34/L98H mutation have been isolated also from clinical samples (18, 19) and in Japan, from azole naïve patients (20). It is hypothesized that azole resistant genotypes can be acquired by patient from the environment as fungal spores are known to be wind dispersed over long distance. The study reports the prevalence of *aspergillus* at 10.6% which agreed with the prevalence reported in the Netherlands at 10% and United Kingdom at 17-20% (10, 12).

In India, the variation among azole-resistant *Aspergillus fumigatus* posed a big challenge in the management of patients (21). Although in Kenya, randomized studies have not been carried out. It is now clear there is a high risk of therapy failure in some patients with azole-resistant aspergillosis, this may be due to azoles resistant *aspergillus fumigatus*. In a Dutch study eight patients diagnosed with azole-resistant invasive aspergillosis, seven died in less than two months after diagnosis (21). However, factors like underlying leukemia in already advanced aspergillosis infections, insufficient triazoles are some of the other factors that may cause treatment failure (22). As a result, at the moment it not clear what factors contribute to high MIC in treatment failure. In most developing countries especially Africa, Kenya included prompt diagnosis and treatment of aspergillosis is still a big challenge due to lack of enough resources. This is further complicated by high numbers of immunosuppressed individuals suffering from cancers, HIV/AIDS, tuberculosis, etc. at risk of contracting aspergillosis.

The emergence of azole resistant fungi is a health sector challenge requiring changes in strategies for management of fungal infection or prophylaxis (23, 24). Voriconazole has been reported by several authors as a superior azole for treatment of fungal infection (25, 22, 26). However, there is need for quick identification of the *Aspergillus* species, followed by susceptibility test before treatment commence (27, 18). The triazoles used in medical and agriculture practice have similar chemical structures e.g. voriconazole and itraconazole have similar structures to tebuconazole and epoxiconazole) but a different mode of action against *Aspergillus* (19, 28). Thus, the study supported the hypothesis that use of agriculturally based azoles in the environment may induce resistance in *Aspergillus fumigatus.* In resource constrained setting, this is not practical as infrastructural capabilities for diagnosis and susceptibility testing of fungi are limited or outright nonexistence. Use of molecular techniques to detect resistant strains is highly recommended since culturing have low sensitivity.

CONCLUSION AND RECOMMENDATION

The study isolated eleven azole resistant *Aspergillus fumigatus* from archival samples, the two isolates showed elevated minimum inhibitory concentration value to any of the three azoles (itraconazole, voriconazole and posaconazole), After sequencing TR34/L98H mutations were detected. Long term used of azole in management of patient should be stopped and rational use of fungicide in agriculture should be implemented through rigorous agriculture policies aimed at curbing the spread of resistance to clinical practice. More studies are recommended to determine the actual morbidity and mortality associated with triazoles resistant aspergillosis in Kenya.

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