Diversity of Azoles Resistant *Aspergillus* Species Isolated from Experience and Naïve Soils in Nairobi County and Naivasha Sub-County Kenya

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Abstract

New triazole antifungals voriconazole, itraconazole and posaconazole are recommended for prophylaxis and treatment of both invasive and chronic fungal infections such as aspergillosis and aspergilloma. Emergence of azole-resistant among *A. fumigatus* isolates have been reported in other countries including Tanzania ascribed to either previous antifungal treatment, prophylaxis or triazoles use in agriculture. The use of azole based fungicides in the robust horticulture in Kenya is a significant risk factor for antifungal resistance. The study proposes to analyze environmental isolates of *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus niger* for the presence of resistance against the triazoles antifungals. Fungicide naïve soils were obtained from uncultivated virgin fields while fungicide experience soils were collected from flower, agricultural and horticultural fields and greenhouses within Naivasha sub-county and Nairobi County. The fungal isolates were subjected to antifungal susceptibility to triazoles using broth micro dilution method. A total of 492 samples were analyzed in Nairobi, 52 isolates were identified and they resistance were as follow: *A. fumigatus* (32%), *A. niger* (26.09%), *A. flavus* (33.33%) and *A .terreus* (0%) and in Naivasha 44 isolates were isolated out of which 25 were *A. fumigatus* and its

resistance was at 36%. Data were analyses using student T test and showed they no different between resistant and susceptible isolates from the two location. Data generated will serve to inform on the current status of triazoles resistance pattern and to raise concern emerging antifungal resistance in clinical practice.

Keywords: Azoles resistant *Aspergillus, Environmental isolates, Aspergillus fumigatus*

Introduction

Aspergillus species are found worldwide, it has isolated in organic matter, soil and decaying organic matter, Most of the *Aspergillus* species sporulated highly (Balajee, 2009, Mann *et al*., 2003). The conidia hence is carried by wind and inhaled causing infection in individual whose immune system is compromised (Perfect *et al*., 2001). Azoles namely posaconazole, Voriconazole and intraconazole are drugs used as first line in treatment of aspergillosis (Walsh *et al*., 2008). Other drugs with sensitive against *Aspergillus* species are Anidulafungin, micafungin, caspofungin and amphotericin B. *Aspergillus fumigatus* which causes most of the chronic and invasive aspergillosis (Denning *et al*., 2003, Walsh *et al*., 2008) is normally sensitive to the three classes of antigungal. However the recent past cases of patient with aspergillosis caused by *A. fumigatus* resistant to azoles has been reported (33). *Aspergillus terreus* is resistant to amphotericin B while *Aspergillus pseudofischeri* and *A. lentulus* is resistant to azoles intrinsically (Alcazar *et al*., 2008).

Azole resistance in *Aspergillus* infection due to azole treatment failure is an emerging health problem (Van der Linden *et al*., 2013. Van der Linden *et al*., 2011, Vermeulen *et al*., 2013). Surveillance reports have showed geographical spread of azole resistant species in clinical and environment in Africa, Europe, Asia, North and South America and Middle East (Vermeulen *et al*., 2013, Chowdhary *et al*., 2014). In contrast Netherland surveillance studies showed that azole resistance is endemic, TR46/Y121F/T289A and TR34/L98H cyp51A gene mediated resistance been the most common (Van der Linden *et al*., 2011).Multicenter international surveillance network study reported 3.2% triazoles resistance. Majority of resistant isolates were *Aspergillus* species at 78% and 22% of the other sibling species (*A. lentulus, A. udagawae and A. thermomutatus*) (Lamoth, 2016, Van der Linden *et al*., 2015).

The common reported mechanism of azoles resistance is alteration of the azoles drugs target i.e. lanosterol 14-α-demethylase and mutation in gene *cyp51A* (Chen *et al*., 2005, Mellado *et al*., 2005, Mellado *et al*., 2004, Diaz-Guerra *et al*., 2003, Mann *et al*., 2003). Substitution usually occurs in codon

98 by usually by substitution of histidine with leucine usually in the promoter region i.e. TR34/L98H. Some authors have attribute widespread uses of azoles in agricultural may have led to selection of strain carrying the resistant gene (Snelders *et al*., 2012, Verweiji *et al*., 2009). The use of azole based fungicides in the robust horticulture in Kenya is a significant risk factor for antifungal resistance. Recently several authors have reported a novel CYP51A mediated resistance with high resistance level to Voriconazole, TR46/Y121F/T289A also has be isolated from clinical sources and environmental in Europe, Africa, Latin America and Indian (Verweij *et al*., 2015,Abdolrasouli *et al*., 2016, Le Pape *et al*., 2016, Fuhren *et al*., 2015). The aims of this study were to investigate the diversity and prevalence of azoles resistant *Aspergillus* species in Nairobi County and Naivasha sub-County.

Materials and methods

c. Sampling for environmental isolates

Approximately 5g dry top surface soils from agricultural site were collected into a sterile 15 ml Falcon tube using a sterile plastic spoon. Soil samples were transported in a leak proof packaging in a cool box to KEMRI-Center for Microbiology Research for mycological investigations.

d.

e. Fungal culture and identification

Approximately a gram of the soil sample were suspended and vortexed thoroughly in 5 ml (0.5% w/v) saponin. The debris allowed to settle and the supernatant transferred to a fresh tube. The resulting suspension were then centrifuged, and the pellet suspended in a final volume of 500 µl sterile NaCl. Hundred µicrolitres of the suspension were plated on Sabouraud dextrose agar containing; (a) no drug, (b) 1 µg/ml Itraconazole (c) 1 µg/ml Voriconazole. All the Plates were incubated 30^{o}C for 72 hours, checking daily for any growth. Colonies growing after incubation on triazoles free media were used as control and to access fungal diversity, while triazoles containing agar were used to determine resistant isolates.

Triazoles susceptibility testing

Minimum inhibitory concentrations to Itraconazole (ITZ), Voriconazole (VCZ) and Posaconazole (PSZ) were tested by broth micro dilution according to the EUCAST reference method (EUCAST, 2008) with minor modification.

Exactly 9.6mg of azoles (Itraconazole, Voriconazole, posaconazole) powder were weighed and dissolved in 3.0ml of DMSO to get 1600µg/ml stock. Ten sterile tubes were used for serial dilution in each tube 3mls of DMSO were added. Starting with the first tube 3mls of the stock solution

were added, mix and 3mls form the mixed of the first tube was transfer to second tube mixed and pipette 3mls from the second and transfer to the third tubes and the process were continued up to the ten tubes. The second serial dilution were done using ten tubes, in each of the tubes (From first serial dilution tubes) 4.9ml of RPMI were added to each of the ten tubes

From each tubes of the second dilution 200µl were dispensed to microtitre plates and inoculated with 10µl of the inoculums. Plates were then incubated at 30°C for 72 h. The MIC values of all drugs were determined visually as the lowest concentrations with no visible growth. EUCAST drug susceptible controls strains DSM819 and ATCC46645 were used while azole resistant control isolates allele TR/L98H) and (allele G54W, ITZR + PSZR86) will be used.

Results

A total of 492 of both Naïve and experience soils (246 Naïve soils and 246 experienced soils) were analyzed. At total 52 isolates were isolated from experience soil in Nairobi out which proportions of azoles resistance were as follows: *A. fumigatus* (32%), *A. niger* (26.09%), *A. flavus* (33.33%) and *A .terreus* (0%) as showed in table I. In Naivasha a total of 44 isolates were isolated, out of which 25 *A. fumigatus* were isolated and 36% were azole resistant as showed in Table 1.

Table 1: Showing isolates from experience soils both in Nairobi and Naivasha

Nairobi Experience soils isolates				Naivasha experienced soils Isolates		
Isolates	Total no.	Susceptible	Resistant's	Total no.	Susceptible	Resistant's
Fumigatus	25	17 (68.0%)	8 (32.0%)	25	14 (56.0%)	9 (36.0%)
Niger	23	17(73.91%)	6(26.09%)	13	10(76.92%)	3(23.08%)
flavus	3	2(66.67%)	1(33.33%)	3	2(66.67%)	1(33.33%)
terreus	1	1(100%)	0 (0.0%)	3	3 (100%)	0(0.0%)
Total	52	37	15	44	29	13

Out of 246 naïve soils analyzed In Nairobi a total of 40 isolates were isolated, of which resistance were as follows: *A. fumigatus* (19.23%), *A niger* (23.08%), *A. flavus* (0%), and *A. terreus* (0%) as showed in table 2. In Naivasha 50 *Aspergillus* species were isolated of which resistance were as follows: *A. fumigatus* (19.05%), *A niger* (15.38%), *A. flavus* (0%), and *A. terreus* (0%) as showed in the table 2 below.

Table 2: Showing isolates from Naïve soils both in Nairobi and Naivasha

Nairobi Naïve soils isolates				Naivasha Naïve soils Isolates		
Isolates	Total no.	Susceptible	Resistant's	Total no.	Susceptible	Resistant's
fumigatus	26	21(80.77%)	5(19.23%)	21	17(80.95%)	4(19.05%)
niger	13	10(76.92%)	3(23.08%)	26	22(84.62%)	4(15.38%)

flavus	0	0(0.0%)	0(0.0%)	3	3 (100%)	0(0.0%)
terreus	1	1 (100%)	0(0.0%)	0(0.0%)	0(0.0%)	0 (0.0%)
Total	40	32(80.0%)	8(20.0%)	50	42(84.0%)	8(16.0%)

T-test comparison of the resistant samples of Nairobi Naïve soils isolates to the Nairobi Experience soils isolates

The data was first normalized by getting the percentage resistance of the fungi. These were therefore used in testing for the variations. Using two-sample t-test, there was no significant difference in percentage resistant isolates in Nairobi naïve samples (mean 6.2%) to the percentage resistance isolates in Nairobi experience isolates (mean 7.8%), (T = 1.24, P = 0.271).

Two-Sample T-Test and CI: Nairobi Naive, Nairobi experience

Two-sample T for Nairobi Naive soils vs Nairobi experience soils

	N	Mean	StDev	SE Mean
Nairobi Naive	4	10.6	12.3	6.2
Nairobi experience	4	22.9	15.6	7.8

Difference = mu N. Naive - mu N. experience
Estimate for difference: -12.28
95% CI for difference: (-37.78, 13.23)
T-Test of difference = 0 (vs not =): T-Value = -1.24 P-Value = 0.271 DF = 5

T-test comparison of the resistant samples of Nairobi Naïve soils isolates to the Naivasha naïve soil isolates

The result showed that there was no significant difference in percentage resistant isolates in Nairobi naïve samples (mean 6.2%) to the percentage resistant isolates in Naivasha naive isolates (mean 5.0%), (T = 0.25, P = 0.814).

Two-Sample T-Test and CI: Nairobi Naive, Naivasha naive

Two-sample T for Nairobi Naive soils vs Naivasha naïve soils

	N	Mean	StDev	SE Mean
Nairobi Naive	4	10.6	12.3	6.2
Naivasha Naïve	4	8.6	10.1	5.0

Difference = mu N. Naive - mu Naivasha naive
Estimate for difference: 1.97
95% CI for difference: (-18.46, 22.40)
T-Test of difference = 0 (vs not =): T-Value = 0.25 P-Value = 0.814 DF = 5

T-test comparison of the resistant samples of Naivasha Naïve soil isolates to the Naivasha experience soil isolates

The result showed that there was no significant difference in percentage resistant isolates in Naivasha naïve samples (mean 5.0%) to the percentage resistant isolates in Naivasha experience isolates (mean 8.2%), (T = 1.51, P = 0.206).

Two-Sample T-Test and CI: Naivasha experience, Naivasha naive

Two-sample T for Naivasha experience vs Naivasha naive

	N	Mean	StDev	SE Mean
Naivasha experience	4	23.1	16.4	8.2
Naivasha naive	4	8.6	10.1	5.0

Difference = mu Naivasha experience - mu Naivasha naive
Estimate for difference: 14.50
95% CI for difference: (-12.18, 41.17)
T-Test of difference = 0 (vs not =): T-Value = 1.51 P-Value = 0.206 DF = 4

T-test comparison of the resistant samples of Naivasha Naïve soil isolates to the Nairobi experience soil isolates

The result showed that there was no significant difference in percentage resistant isolates in Naivasha naïve samples (mean 5.0%) to the percentage resistant isolates in Nairobi experience isolates (mean 7.8%), (T = 1.54, P = 0.185).

Two-Sample T-Test and CI: Nairobi experience, Naivasha naive

Two-sample T for N. experience vs Naivasha naive

	N	Mean	St Dev	SE Mean
Nairobi experience	4	22.9	15.6	7.8
Naivasha Naïve	4	8.6	10.1	5.0

Difference = mu Nairobi experience - mu Naivasha naive
Estimate for difference: 14.25
95% CI for difference: (-9.56, 38.06)
T-Test of difference = 0 (vs not =): T-Value = 1.54 P-Value = 0.185 DF = 5

Discussion

We report the detection of environmental azole resistance *Aspergillus* species from both Naïve and experience soil in Nairobi County and Naivasha sub-County, Kenya. In experience soils, there were high percentages in resistance from Naivasha isolates this may be due to irrational used of

fungicides in large scale horticultural farming as compare to Nairobi which was mostly small-scale farming

In the recent decades, there has an increase in opportunities infection one of them is aspergillosis (Walsh *et al*., 2008). Invasive fungal infection diseases are associated by high mortality and morbidity, partially because of failure to diagnosis early resulting in delay treatment. Most of the cross resistance in Aspergillus species by several authors have be reported infrequently, which mean they are infrequent to date. Low prevalence is due to variation in laboratory testing between different laboratories.

The detection of azole resistance of *Aspergillus* species was done based on medium containing azoles and micro dilution susceptibility testing. The uses of azole containing in screening environmental samples for the presence of resistance were first described using Itraconazole at a concentration of 4mg/liter (Snelders et al., 2009). Since then breakpoint have been proposed to be 1mg/liter for Itraconazole (Pfaller *et al*., 2009, Verweji *et al*., 2009). Which raises question that resistance isolates may go undetected. However it has been reported by several authors the MIC of most isolates against Itraconazole is 8mg/liter or more (Howard et al., 2009,). In the study we further included Voriconazole (1mg/liter) and posaconazole (1mg/liter) SDA agar. Student T test showed that they were no significant difference in percentage resistance (P= 0.271) of isolates from Nairobi naïve and experience soils isolates as well as in Naivasha from naïve and experience soils (P= 0.206).

The used of azole containing agar, hypothetically may results in laboratory generation of resistance in the recover isolates though expose. However it is believe that since de novo short azole exposure (Almost 72hr exposure) is for short time it may not cause resistance and detection of cyp51A alterations which cause necessary (Mellado *et al*., 2007). Acquire azole resistance usually develops due to response of fungi to azole exposure in patients or in agricultural (Chen *et al*., 2005, Howard *et al*., 2006, Verweij *et al*., 2009). Drug expose over a long periods and high numbers of reproducing fungi are some of the factors that played a role in the selection of resistance (Anderson, 2005). This condition is met in soil when agricultural azole applied remained in soils over a year and in patients with Cavitary *Aspergillus* (Hof, 2001).

Demethylation inhibitors and Azoles fungicides are recommend for used in plant protection (Hof, 2001). In Netherlands the used for fungicides in agricultural has double since mid1990s (Verweji *et al*., 2009). Similar trends has been observed in Denmark. Resistant *Aspergillus fumigatus* is selected by used of azole fungicides or it can be introduced via importation of commercial compost to the environment, are one of the key factors in development of resistance (Verweji *et al*., 2009). In our study most of the

sites with resistant Aspergillus were previously exposed to azoles fungicides before. This is always hard to management aspergillosis because most laboratories do not perform sensitivity test and due to negative cultures. Hence the prevalence of azoles resistant *Aspergillus* species may be underestimated with risk of inappropriate therapy. We believe in our study and emerging reports on azole related resistance, we suggest that susceptibility testing for *Aspergillus* species should be performed routinely.

Conclusion

The study found out that they are triazoles resistance Aspergillus species in the two county. High resistance in Naivasha sub county soils show that resistance could be catalyst by application of fungicides in agriculture. This posed a serious health public concern and more surveillance should done in the country.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: The study was approved by Kenya Medical Research Institute, Scientific and ethics Review Unit (KEMRI/SERU/P000602/3031).

Informed consent: There were no human participation or direct human contact. However Approved permission to access the farms were obtained from relevant institution or flower farms owner or individual farm owner.

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