CURRENT STUDIES IN COMPARATIVE EDUCATION, SCIENCE AND TECHNOLOGY, VOLUME 4, NUMBER 1, 2017, PP. 81-93

Antifungal Resistance Surveillance: A Tool Necessary for Monitoring Azole Resistance Potentials in Candida Isolates from Niger Delta Communalities in Nigeria

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Abstract

Antifungal resistance surveillance is strategic for monitoring prophylaxis, empirical therapy, and treatment of *Candida* infections; most especially systemic candidiasis. Antifungal susceptibility testing of pathogenic fungi could help to manage the selection of adequate therapy; predict therapeutic outcome and therapeutic potentials of new antifungal agents. However, antifungal resistance is becoming a recurring phenomenon in today's management of *Candida* infections globally. This study attempts to determine the *in-vitro* susceptibilities of Azole against the *Candida* isolates evaluate resistance trend and advance for the need for regular antifungal resistance surveillance. Also, all *Candida* isolates were inoculated onto a Sarboraud dextrose agar plate to obtain a pure culture and used for formal saline microscopy; germ tube test and carbohydrate assimilation tests were used to confirm *Candida* species and antifungal susceptibility testing using the Kirby Bauer disc diffusion method accord. The result of this study showed 48.4% susceptibility, 22.6% susceptibility dose dependence and 29% resistance to ketoconazole among the community settings isolates while hospital settings showed 53.6% susceptible, 14.3% susceptibility dose dependent and 32.1% resistance to ketoconazole. However, the overall resistance percentage of all the isolates tested with ketoconazole stood at 30.5% while 18.6% were regarded as susceptibility dose dependent. Furthermore, itraconazole and ketoconazole had the highest population of resistant *Candida* isolates at 30.5% each while fluconazole showed the lowest percentage of resistant *Candida* isolates with 20.3% which is also high when compared with that of the control disc 3.4%. Resistance to azoles shown by *Candida* isolates can be halted by initiating early detection of susceptibility dose dependent *Candida* species and subsequently interfering with their progression to resistance. Conclusively, drug manufacturing and formulation companies should perform a routine periodic antifungal check to ensure a continuous update of the antifungal trend of fungal pathogen around the world with adequate information on specie susceptibility pattern and resistance trend against such drugs in our communities to guide healthcare practitioners in their usage and be more current in diagnosis and treatment of *Candida* infections.

Keywords: Azole, Antifungal, Resistance, *Candida* species, Niger Delta Communities.

Introduction

Candida infections has been an age long occurrence and antifungal drugs designed to kill them have been changing over time in response to research observations on the existing drugs' antifungal activity at a given time. Azole drugs emanated from this same solution driven research with their primary focus on the ability of antifungal agents to bind to the fungal cell membrane and inhibit sterol biosynthesis, which leads to fungal death (Abbey, 1991). But experimental observations indicate that there exists resistance to azole as they all bind to same point along the sterol synthesis pathway in the fungal cell membrane. Antifungal resistance surveillance serves as a major strategy for prophylaxis, empirical therapy, and treatment of *Candida* infections; most especially, systemic candidiasis (Badiee and Aborzi, 2001). The antifungal susceptibility testing of pathogenic fungi could help to manage the selection of adequate therapy and also provide an estimate of antifungal efficacy. Monitoring of drug resistance development could also help to predict therapeutic outcomes and therapeutic potentials of untested compounds (Pfaller *et al*., 2007). Similarly, key elements in the selection of the appropriate antifungal agent includes the patient involved, severity of immune suppression, history of prolonged exposure to antifungal drugs, and knowledge of the species of the infecting *Candida* pathogen and its typical susceptibility pattern (Richardson and Lass-Florl, 2008). Azoles are drugs produced to treat fungal infections just like antibiotics are meant to take care of bacterial infections. They belong to a class of five-member nitrogen heterocyclic ring compounds that contains at least one non-carbon which could be either sulfur, nitrogen or oxygen atom (Eicher and Hamptmann, 2003). The classification of the azole drug is somewhat complex as it entails so many criteria depending on the number of nitrogen atom present in the compound with or without one or more heteroatom (Eicher and Hauptmann, 2003). It had been estimated by Ringdahi *et al*., (2010), that candidiasis affects between 70 and 75% of adult women during their lifetime. Also, the Center for Disease Control and prevention (CDC, 2010), had estimated that *Candida* infections are the fourth most occurring cause of infections around the world. Predominantly in immunocompromised or individuals with poorly developed immune system. Nonetheless, azole antifungal surveillance is very important in the management of patients suffering from candidiasis. Hence, the determination of the changes in the distribution of *Candida* species and their sensitivity pattern to antifungal agents are apparently important (Badiee and Aborzi, 2001). Although, current medical practice guidelines stipulates that antifungal prophylaxis be administered to patients developing risk for systemic candidiasis since definitive early diagnosis was difficult; so empiric therapy of antifungal agents has become a standard of medical practice in immune-suppressed patients (Badiee and Aborzi, 2001). Given the fact that antifungal resistant strains are becoming a recurring phenomenon in today's management of *Candida* infections globally, especially in local communities where you could find poor health facilities, high rate of illiteracy and abject poverty re-

stricting people from assessing quality health care. Therefore, there is need for concerted efforts in research such as this which is aimed at determining the distribution of *Candida* species *in-vitro* susceptibilities to azole antifungal agents. The *Candida* species were isolated from the patients from Niger delta communities and processed at the medical microbiology department, University of Port Harcourt teaching hospital for laboratory investigations.

Materials and Methods

The study is designed for comparative analysis of *Candida* species isolated from community and hospital settings in Rivers State. It was carried out at the University of Port Harcourt teaching hospital, a tertiary hospital facility that accommodates referrals and out patients from all parts of the Rivers State (located 4°45′N 6°50′ E / 4.750°N 6.833°E) / and the South-South geopolitical zone of Nigeria called the Niger Delta. This region consists of six states including Akwa Ibom, Cross Rivers State, Delta State, Edo State, Bayelsa State and Rivers State, situated at Alakahia, a sub-burb of the metropolitan city of Port Harcourt, the Rivers State capital. All *Candida* isolates were inoculated onto a Saboraud dextrose agar plate to obtain a pure culture and then used for normal saline microscopy, germ tube test and carbohydrate assimilation tests to confirm *Candida* species and antifungal susceptibility testing using the Kirby Bauer disc diffusion method according to Ochei and Kolhatkar, (2000).

Exclusion Criteria

Candida isolates from patients who are using or have used antifungal drugs less than 14 days before submitting their samples for laboratory processing were excluded from being part of this study.

Inclusion Criteria

*Candida i*solates obtained from samples of persons who have not used antifungal drugs for a period of 14 days or more before submitting their samples for laboratory processing were included from being part of this study. *Candida* isolates from samples gotten from persons who have not visited the hospital or are admitted into a hospital facility or employed as staff of a hospital/laboratory/biomedical facility for a period not more than 48 hours before sample collection, were classified as community settings *Candida* isolates. Whereas *Candida* isolates derived from patient samples produced by patients who had been admitted into a hospital/biomedical facility or are staff working in a hospital/biomedical facility or patient relatives who are care givers to a patient admitted into a hospital/biomedical facility, were regarded as Hospital settings *Candida* isolates.

Processing

All *Candida* isolates were inoculated onto a Saboraud dextrose agar plate to obtain a pure culture. The inocula were prepared by growing the various *Candida* isolates on

separate Saboraud dextrose agar plates for purity which was then used for normal saline microscopy, germ tube test and carbohydrate assimilation tests to confirm *Candida* species and susceptibility testing using the Kirby Bauer disc diffusion method.

Preparation of Susceptibility Disc

The drugs used for this experiment were bought from pharmacy stores in Port Harcourt. These drugs include fluconazole 50mg capsule (Drugfield pharmaceuticals limited- Nigeria), ketoconazole 200mg tablet (Hovid Bhd-Malaysia), clotrimazole (Drugfield pharmaceuticals limited- Nigeria), and itraconazole 100mg (Hanmi pharmaceutical company limited-Korea). They were dissolved in sterile distilled water in a sterilized test tube to the required concentration. A Whatman filter paper 3 was perforated to 10mm diameter, placed in a glass Petri dish with lid closed, sterilized by autoclaving at 121^{o}C and 15psi for 15 minutes. The Petri dish and the sterilized perforated Whatman filter paper were allowed to dry in a hot air oven at 45^{o}C. Then, each azole solution of a known concentration and calculated volume is exposed to a calculated number of the sterile perforated Whatman paper for absorption. The soaked perforated Whatman paper discs were allowed to dry in a hot air oven at 45^{o}C. When dried, they were then used as antifungal susceptibility disc for each azole having a known quantity of the antifungal agent.

Susceptibility Testing Procedure (Kirby Bauer's Disc Diffusion Method)

Two to three colonies from the pure culture plate were transferred with a sterilized inoculating loop into 3ml of sterile normal saline broth in a sterilized bijou bottle. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Saboraud dextrose agar plate was evenly inoculated with the organisms using a sterile swab. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid. Using the wet swab inoculation was done in the Saboraud agar by evenly streaking across the surface of the plate with the swab three times, turning the plate at angle 60^{o} between each streaking. Then, the inoculated plates are allowed to dry for 10 minutes with the lid in place. By means of a sterilized forceps and different antifungal azole discs were applied onto the surface of the inoculated Saboraud agar and the plates were incubated overnight at 37^{o}C. The zone of growth inhibition diameter observed was measured and compared to that of the control.

Results

This study involved the isolation of 59 *Candida* isolates obtained from the community and hospital settings of Niger delta communities. Community setting isolates showed 25.8% (8) susceptibility to itraconazole, 41.9%(13) susceptibility dose dependence and resistance of 32.3%. However, hospital setting isolates showed 35.5% (10) susceptibility, 35.7% (10) susceptibility dose dependence and 28.6% (8) resistance. Cumulatively, 30.5% of the *Candida* isolates used for this study were resistant to itraconazole while 39% were susceptible dose dependent and 30.5% were

susceptible. Table 4 tried to express the distribution of zones of inhibition of fluconazole against *Candida* isolates from community and hospital settings. Hospital setting isolates showed a cumulative resistance frequency of 21.4% while 21.4% were susceptibility dose dependent and 57.1% susceptibility was also observed. Community setting showed a susceptibility of 54.8%, susceptibility dose dependence of 25.8% and 19.4% resistance to fluconazole. There was observed 48.4% susceptibility, 22.6% susceptibility dose dependence and 29% resistance to ketoconazole among the community settings isolates while hospital settings showed 53.6% susceptible, 14.3% susceptibility dose dependent and 32.1% resistance to ketoconazole. However, the overall resistance percentage of all the isolates tested to ketoconazole stood at 30.5% while 18.6% were regarded as susceptibility dose dependent and 50.8% were susceptible. The control disc produced a susceptibility percentage of 90.3% for community settings and 82.1% for hospital settings isolates and a cumulative susceptibility percentage for the 59 tested *Candida* isolates to be 88.1% while community settings produced 9.7% susceptibility dose dependence whereas the hospital settings isolates produced 7.1%. Similarly, the community settings *Candida* isolates showed no resistance to the control disc while the hospital settings showed 10.7% resistance to the control disc. Nevertheless, the 59 *Candida* isolates showed overall 3.4% resistance, 8.5% susceptibility dose dependence and 88.1% susceptibility to the control disc antifungal agent.

Table 1: Distribution of Zones of Inhibition of Itraconazole in Community and Hospital Settings

Settings	**Zones of inhibition**						**Total**
	S	%	SDD	%	R	%	
Community	8	25.8	13	41.9	10	32.3	**32**
Hospital	10	35.7	10	35.7	8	28.6	**27**
Total	**18**	**30.5**	**23**	**39**	**18**	**30.5**	**59**

***Note**: S-susceptible, SDD- Susceptibility dose dependent, R-resistant, % - percentage frequency*

Table 2: Distribution of Zone of Inhibition of Fluconazole in Hospital and Community settings

Settings	**Zones of inhibition**						**Total**
	S	%	SDD	%	R	%	
Community	17	54.8	8	25.8	6	19.4	**32**
Hospital	16	57.1	6	21.4	6	21.4	**27**
Total	**33**	**55.9**	**14**	**23.7**	**12**	**20.3**	**59**

***Note**: S-susceptible, SDD-Susceptibility dose dependent, R-resistant, %-percentage frequency*

Table 3: Distribution of Zone of Inhibition of Clotrimazole in Hospital and Community Settings

Settings	**Zones of inhibition**						**Total**
	S	%	SDD	%	R	%	
Community	25	80.6	1	3.2	5	16.1	**32**
Hospital	17	60.7	3	10.7	8	28.6	**27**
Total	**42**	**71.2**	**4**	**6.8**	**13**	**22**	**59**

Note: S-susceptible, SDD-Susceptibility dose dependent, R-resistant, %-percentage frequency

Table 4: Distribution of Zones of Inhibition of Ketoconazole in Hospital and Community Settings

Settings	**Zones of inhibition**	**Total**					
	S	%	SDD	%	R	%	
Community	15	48.4	7	22.6	9	29.0	**32**
Hospital	15	53.6	4	14.3	9	32.1	**27**
Total	**30**	**50.8**	**11**	**18.6**	**18**	**30.5**	**59**

Note: S-susceptible, SDD-Susceptibility dose dependent, R-resistant, %-percentage frequency,

Table 5: Summary of interpretative data of zones of inhibition of control disc (fluconazole disc from Oxoid ltd)

Settings	**Zones of inhibition**	**Total**					
	S	%	SDD	%	R	%	
Community	28	90.3	3	9.7	0	0	**32**
Hospital	23	82.1	2	7.1	3	10.7	**27**
Total	**52**	**88.1**	**5**	**8.5**	**3**	**3.4**	**59**

***Note**: S-susceptible, SDD-Susceptibility dose dependent, R-resistant, %- percentage frequency*

Discussion

In this present study, the results showed a disparity in susceptibility among the five antifungal discs used. Comparing *Candida* isolates' resistance to the four test azole discs and the control disc, it was observed that there was difference in their overall antifungal activity with the overall resistance percentage of all the isolates tested increases as you move from the community to hospital settings. However, itraconazole and ketoconazole group had the highest population of resistant *Candida* isolates at 30.5% each while fluconazole showed the lowest percentage frequency of resistant *Candida* isolates with 20.3% which is also high when compared with that of

the control 3.4%. This is higher than the 7.14% resistant *Candida* isolates reported by Pam *et al*., (2012).

Nevertheless, when comparing the fact that fluconazole was the active ingredient in the fluconazole disc as well as in the control disc, one may ask several questions bordering on quality to efficacy. Obviously, this research had observed variations in the antifungal trend of the various azole used and it can be deduced that resistant *Candida* isolates were observed more in the hospital settings except for itraconazole which had a higher percentage frequency of *Candida* isolates showing resistance among the community setting isolates which incidentally is the highest resistance frequency percentage 32.3% but the lowest resistance frequency percentage 16.1% was observed among the community settings *Candida* isolates tested with clotrimazole.

Although, this is low compared to the control disc's 0% percentage frequency of resistance, it agreed with published reports of other researchers like Stein *et al.*, 1991. Therefore, to answer the rhetorical questions posed by the variations seen in the antifungal activity of tested azole, one may argue that it could be a factor encouraging this resistance trend may have been the disparity in measurements, parallax error, as well as standard quantity or concentration of the active ingredient (azole) embedded in each individual azole discs used in this study Michael *et al*., (2008). Secondly, we may also assume that each of the azole purchased from the local pharmacy shops may have been produced below the prescribed standard concentration enshrined in the drug information.

Thirdly, there could be some loss in the active azole ingredient in each drug solution in the course of preparation of the antifungal discs used for this study as evident in the variation of resistance, susceptible dose dependent and susceptible *Candida* isolates to fluconazole and control disc, which are suppose to have equal concentration of the drug fluconazole. Similarly, the resultant increase in the incidence of resistant *Candida* species may have been due to the intake of these drugs below the required concentration needed for absolute killing of infecting *Candida* and subsequent development of resistant genes to the azole, an assumption in line with the work of Pam *et al*., (2012).

In the same vein, the incidence of a high population of *Candida* isolates from the community settings resistant to itraconazole may have been a resultant effect of self medication and non-adherence to antifungal drug regimen due to lack of the financial muscle to buy complete set of the required drugs in cases of treated community candidiasis. All of these speak in favor of periodic antifungal surveillance in patients undergoing treatment for serious *Candida* infections and for routine hospital quality control. Also, auto-infection with *Candida* species from one body site to another as a result of poor hygienic practices could be a factor responsible for this trend. However, the distribution of zones of inhibition of clotrimazole against *Candida* isolates in hospital settings recorded more resistant (28.6%) *Candida* strains than community settings 16.1%. Nevertheless, cumulative percentages resistant strains (22%) for clotrimazole against *Candida* isolates and 6.8% indicates are susceptibility dose dependent which is comparable with the work of Pam *et al.,*(2012).

Obviously, from these observations, clotrimazole appears to be the most efficient among the azole tested as statistical analysis with Fisher's exert also showed P=0.000 (F/statistic/=14.692) less than 0.05. Clotrimazole also showed a low per-

centage resistance and a lower percentage of susceptibility dose dependent isolates than the control, as susceptibility dose dependent organisms are tending towards resistance and will require a higher amount of azole for absolute killing to be effected (Pam *et al*., 2012). It can also be said that for systemic infections, since clotrimazole is not suitable, Fluconazole still remains the drug of choice as shown in this study using Fisher's exert statistical tool (F/statistic/=11.002) with P=0.001. This was in agreement with Pam *et al*., (2012).

Finally, Itraconazole drifted towards resistance more than any other azole used in this study with the highest susceptible dose dependent *Candida* isolates which was comparable with the report of Haria *et al*., (1996). In conclusion, even though examination of *Candida* isolates by culture methods is accepted as the current universal `gold standard for candidiasis diagnosis, no single method for estimation of antifungal activity of azoles against *Candida* had been fixed, as such, different methods were used by all investigators under comparison, which could have been responsible for the variations in data between different studies.

Conclusion

This study has shown the need for a robust policy on antifungal resistance surveillance as a standard for antifungal drug efficacy monitoring and antifungal resistance surveys; which will help save lives and prevent recurrent *Candida* and other yeast-like infections from gaining hold of our body system or transforming into superbugs. Antifungal resistance surveillance policy framework when developed and implemented, could help to initiate early detection of susceptibility dose dependent or intermediately susceptible strains of *Candida* and other yeast-like infections and halting their progression to resistance within a given infection treatment course period/ facility/community.

Nevertheless, drug manufacturing and formulation should be followed with periodic antifungal surveillance to ensure a continuous update of the antifungal trend of fungal pathogens around the world with adequate information on specie susceptibility/resistant pattern to compliment empiric therapy with such drugs in our communities in order to guide healthcare practitioners in their usage and be more current in diagnosis and treatment of *Candida* infections. Therefore, over dependence on foreign made drugs far above locally made drugs, may have also contributed to the growing trend of resistance to azoles, since, there may have been reduction in these drug efficacy due to transportation, storage and movement from one place to another and also bearing in mind the cost implications. Hence, the domestication of medicines around the worldwide, will in no small measure, impact positively on the national economics of smaller countries and also improve or reduce trade imbalance between them and the industrialized nations in favor of impoverished and largely consumptive regions of the world like the Niger Delta as more pharmaceutical companies when in production, would imply that more jobs are created, reduction in the cost burden of purchasing drugs and the easier to monitor drug failures. These companies when in business, would sponsor researches and encourage such regular drug resistance surveillance, in order to track their drug failures and also ensure that they improve on their efficacy. When this is implemented, it will help reduce the burden

of health care costs on households in the Niger Delta region of Nigeria and to a larger extent the government.

Nevertheless, a change in attitude from self medication to adherence to prescribed drug regimens could also be very crucial in arresting the trend of resistance to azoles, as it remains a key factor that could be responsible for increased incidence of *Candida* species' resistance to azole therapy. Therefore, a change in favor of adherence to prescribed medicine, when encouraged by health personnel, care givers and biomedical organizations, would enhance the monitoring of drug efficacy and their clinical outcomes. Also, the general consciousness of the people about, the abuse of drugs and the need to generally consult the physician on issues of drug administration will have to be increased if the above outlined approaches would make any meaningful impact. This would involve education and re-education of both the learned and the unlearned groups of the population through the use of both short and lengthy messages in different broadcasting media; print, radio, audiovisual, social media and local village town criers. However, for such messages to reach each target group with optimum impact, it must be segmented and tailored to reach a target audience, win their attention and meet set goals. To achieve this, planners of such messages, must take into cognizance the following; understanding their targets' specific language, their catching point(s) or center of attraction, cultural diversities, religious inclinations and their unique niche in the society. That is in addition to a clear thought out planned research and execution guideline that is transparent, flexible, interpretable, sensitive, reproducible and practicable.

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