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The Capability of Clinical Laboratories in Kenya to Diagnose Fungal Infections and as Well Conduct Antifungal Drug Susceptibility Testing on the Isolates

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Abstract: Antifungal drug resistance seems to be growing at a first rate and the life span of the current antifungal drugs may soon be shorter than its shelf life. The capacity to detect resistance is being hampered by the lack of the means to test and also by the misconceived perception that most fungi are still susceptible to current antifungals drugs, resulting in the abdication of the duty to test and profile the susceptibility patterns. In view of this, the study aimed at determining if clinically isolated fungi are exhibiting drug resistance patterns by subjecting a few yeast forms of fungi, *Candida albicans* isolated during a routine urinalysis procedure on subjects with vulvovaginitis attending a medical camp, to antifungal sensitivity tests. As observed, out of thirty two (32) urine cultures, ten (10) were confirmed to contain *Candida albicans*. Out of the 10 *Candida albicans* isolates, three (3) of them exhibited signs of drug resistance to fluconazole and clotrimazole; hence the estimated resistance rate is about 30%. In conclusion a significant population of *Candida albicans* are clinically resistance to both fluconazole and clotrimazole and we need to strengthen the capacity of our clinical laboratories to conduct antifungal drug susceptibility testing.

Keywords: Antifungal Agents, Clinical Laboratories, Sensitivity Tests, *Candida Albicans*

1. Introduction

Candida albicans is considered a polyphenic commensal that grows both as yeast and as filamentous cells and a causal agent of opportunistic urogenital infections in humans [1], but it is also a constituent of the normal gut flora comprising microorganisms that live in the human mouth and gastrointestinal tract [2]. As an important observation, a number of antifungals agents have been developed for the management of different mycoses caused by this pathogen [3]. However, over the years resistance to this agents and also cross-resistance [4-5] to other compounds with similar modes of action has become increasingly common with almost all clinically significant resistant strains reportedly seen in immunocompromised patients, especially those co-infected with HIV [6-8]. The evidence of such resistance pattern is often calculated based on values obtained from the clinical breakpoints and epidemiologic cut of values often estimated upon observation of poor clinical outcomes during therapy and also elevated minimum inhibitory concentrations in laboratory assays [9].

A couple of anti-mycotics are available for local use as creams, lotions, aerosol sprays, vaginal tablets, suppositories and coated tampons. The choice of which antifungal agents to use, to manage fungal infections depends upon the clinical status of the patient, the site of infection, and the pharmacokinetics and pharmacodynamics of the agent [3], [10]. Candida vaginitis is generally treated with either the vaginal administration of an imidazole or triazole antifungal agents or the prescription of oral fluconazole.

In every country, it is generally recommended that antimicrobial treatment regimens are often guided by laboratory supported evidence of drug susceptibility which in turn guide the choices of alternative antimicrobials to use whenever resistance is reported. This is religiously being

practice for antibiotics in most hospitals. The same principle should apply to antifungals but at present, testing is not being performed in the various accredited clinical laboratories and neither is the service being offered even in the research laboratories. Perhaps this is due to the perceived less severity of fungal infections as commonly expressed or due to perceived testing complexity as expressed in our clinics and hospitals which has led to the abdication of the responsibility for testing by laboratory personnel. In view of this we set up to pilot a study aimed at determining if clinically isolated fungi are exhibiting drug resistance patterns by simply and selectively subjecting a few yeast forms of fungi to antifungal sensitivity tests. In this study, we specifically evaluated the sensitivity of *Candida albicans* isolated during a routine urinalysis procedure in subjects with vulvovaginitis.

2. Materials and Methods

2.1. Study Design

This was a laboratory experimental study by design

2.2. Specimens

Mid-stream urine previously collected from female subjects who had been diagnosed to have vaginal thrush was refrigerated and stored at the Microbiology department.

2.3. Primary Culturing and Isolation

A loop of urine was streak inoculated onto Sabouraud's dextrose agar and incubated overnight at 37°C. The following day cream coloured pasty colonies with a distinctive yeast smell were observed to grow on the medium.

2.4. Gram Stain

The gram staining procedure was done as briefly described. A smear of a single colon was prepared, fixed with methanol and then air dried. The fixed smear was floated with crystal violet for 30-60 seconds, washed off with clean water, and then covered with lugol's Iodine for 60 seconds; the Iodine was washed off with running tap water. And finally stained with neutral red stain for one minute and the smear was examined microscopically, first with the 40x objective and then with the oil immersion objectives.

2.5. Germ Tube Test

In brief, 0.5ml of human serum was put into a small test tube. Using a sterile wire loop a yeast colony from the Sabouraud's plate was inoculated in the serum. The tubes were placed in an incubator at 37°C for 2-3hours. Then using a Pasteur pipette, a drop of the serum-yeast culture and a drop of lactophenol cotton blue was transferred on a glass slide and covered with a cover slip. The preparation was examined for presence of sprouting yeast cells using the 10x and 40x objectives with the condenser iris diaphragm closed sufficiently to give good contrast. If sprouting yeast cells were seen, the culture was reported as `*Candida albicans* isolated' and if the yeast cells did not show any sprouting the cultures were reported as `yeast other than *Candida albicans* isolated'.

2.6. Antifungal Drug Sensitivity Testing

In brief, standard investigative methods were employed using the NCCLS guidelines [11-13], clotrimazole was dissolved in sterile 1% (w/v) dimethyl sulfoxide (DMSO) and fluconazole in water then a doubling dilution of the drugs fluconazole and clotrimazole in normal saline was done in a series of test tubes with RPMI medium (Sigma). The yeast culture concentration to use was standardised by using a 0.5 McFarland tube to compare the turbidity. To each drug dilution 50 µl of the standardised inoculum of the test organism was added vortexed and the tubes incubated overnight at 37°C after which sub-culturing was done from each tube onto Sabouraud's dextrose agar.

The organisms were incubated overnight at 37°C after which the colonies were counted with a colony counter. The minimum inhibition concentration was reported as the lowest concentration of the antifungal preventing the growth of 50% of the yeast cells. All isolates were tested in duplicates. A tube was added to act as the positive control (yeast without either drug).

3. Results

3.1. Estimated Drug Resistance Rate

As observed, out of thirty two (32) urine cultures, ten (10) were confirmed to contain *Candida albicans*. Therefore the prevalence of the yeast infection was calculated to be about 31.3%. To estimate resistance levels, we compared the antifungal activities of the clinical isolates using these two concentrations; 15.6µg/ml for fluconazole and 0.3µg/ml of clotrimazole respectively. At these two concentrations all laboratory maintained isolates tested in the study were susceptible hence forming the reference points. For all the clinical isolates, the MICs of fluconazole above 15.6 µg/ml and those above 0.3µg/ml for clotrimazole was considered as showing decreased sensitivity (elevated MICs) to each of the drugs respectively. As a result, out of the 10 *Candida albicans* isolates, three (3) of them exhibited signs of drug resistance; hence we estimate the resistance to be about 30%.

3.2. Minimum Inhibitory Concentration of Clotrimazole

As observed in the study, the M. I. C_{50} for clotrimazole on isolates # 77 was 1.25µg/ml and that for isolates #32 and #70 was 10µg/ml. These concentrations were about 4 to 30 times above the reference point (0.3µg/ml) for this drug. The additional sensitivity reactions for the other concentrations can be deduced from figure 1 below.

3.3. Minimum Inhibitory Concentration of Fluconazole

An analysis of the M. I. C_{50} for clotrimazole on isolates #

77 was recorded to fall on 62.5µg/ml and that for isolates #32 and #70 were 31.25µg/ml. Thus the concentrations of this drug that could kill the three clinical isolates were then noted to be about 2 to 4 times above the reference point (15.6µg/ml) for this drug. The additional sensitivity reactions for the other concentrations can be deduced from figure 1 below.

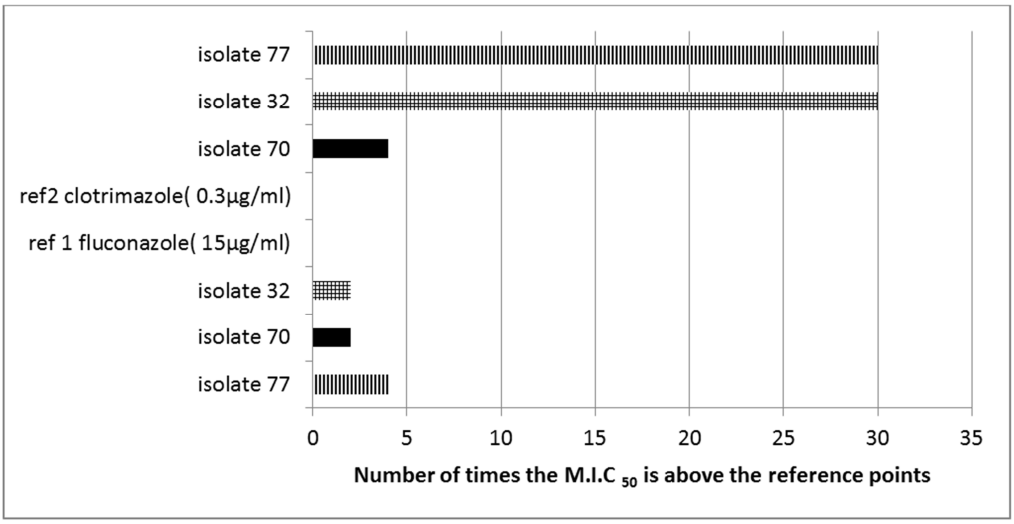

Figure 1. *Comparison of the number of times the M. I. C_{50} for fluconazole and clotrimazole were above the reference concentrations in three Candida albican isolates. (Ref: M. I. C reference values, µg/ml: Micrograms per millilitre).*

4. Discussion

The life span of the current antifungal drugs may soon be shorter than its shelf life. A time is coming when we will have to abandon the once we are using at the moment. Antifungal drug resistance seems to be growing at a first rate but our enthusiasm to detect it is being hampered not by the lack of the means to tests for it but the misconceived perception that most fungi are still susceptible to what is at the moment the drugs of choice to such pathogen, which results in the abdication of the duty to test and profile the susceptibility patterns. In reality we already have drug resisting fungi circulating in our population and the traits of resistance in fungi are similar to those observed in bacteria where mechanisms such as usage of efflux pumps and alteration in targeted biomolecules [10], [14] seem to be the causes of this resistance.

Fluconazole and Clotrimazole cross-resistant strains of the yeast *Candida albican* are circulating in Kenya and such strains can be isolated in clinically ill patients seeking the outpatient services and probably the hospitalised patients too. Similar observations have been reported worldwide [15-17]. A significant population of the yeasts we isolated showed resistance to both fluconazole and clotrimazole. Moreover, antifungal resistance was observed more with clotrimazole than with fluconazole in the laboratory investigations. This observation may greatly influence our choice of drugs considering that clotrimazole pessaries are often the most widely used medication to treat urinary tract infections in our hospitals and clinics but this treatment is often done without any consideration of the likelihood of the patient not responding to the treatment due to the acquired insensitivity of the target pathogen.

Our study was not able to determine any pervious or current usage of the two antifungals drugs by the patients from whom the isolates were acquired this is mainly due to the nature of study design for instance we could not retrieve or obtain any interview information on whether or not the subjects might have been using such drugs prior to sampling or their HIV status. This would have been important in order to collaborate the in vitro laboratory finding and confirm exposure (clinical vs. microbiological resistance). However, this does not in any way negate the fact that drug resistant strains of the yeast candida is causing clinically significant infections in our communities and that we should strongly encourage the routine screening for drug resistance in most cases of frequent mycoses. Moreover, empirical treatment without obtaining cultures by the clinician and the physicians and frequent self-diagnoses and self-medication with over the counter antifungals by the patient should be discouraged as this may antagonise efforts to minimize the occurrence of resistance.

Our clinical laboratories need to be supported with resources, and in training on how to perform drug sensitivity testing for fungal pathogens. Antifungal testing is just as equally cheap or as equally expensive as doing antibiotic tests, meaning that it will just cost the same to do both kind of tests, and were financially constraints limit such a venture this can be done through partnership with research facilities specializing in mycological investigations and universities especially where such institutions are acting as both teaching and referral centres. In addition, continuous surveillance and appropriate refresher training also has to be undertaken in consideration of challenges and difference of testing and screening for drug resistance in prokaryotic and eukaryotic microorganism as reviewed by [18].

5. Conclusion

A significant population of the yeast form of *Candida albicans* are clinically resistance to both fluconazole and clotrimazole and in general as an observation on the way diagnostic laboratories are handling fungal investigations it is important that we strengthen the capability of the clinical laboratory to diagnose fungal infections and at the same time be able to conduct antifungal drug susceptibility testing on the isolates through training on the current available methodologies.

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