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Incidence, antifungal resistance properties, and virulence traits of *Candida*species isolated from HIV/AIDS patients from county hospitals in Kenya

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Abstract

Introduction: *hospitalized patients with acquired immune deficiency syndrome usually suffer from candidiasis, which is the most common fungal infection resulting in mortality and morbidity. The study aimed to characterize, incidence, susceptibility, resistance genes, antibiofilm activity and virulence traits of Candidatypes isolated from HIV-infected patients.* ***Methods:*** *herein, one hundred and eighty-one samples collected were cultured on Sabouraud Dextrose Agar, by use of automated Vitek-2® Compact bioMérieux biochemical test was performed followed by susceptibility tests by use of various conventional antifungals against the isolates using standard procedures. Virulence factors, biofilm formations and resistance genes of Candida strains were determined.* ***Results:*** *out of the 181 samples, 46 were recognized to be of Candida spp., 20 C. albicans (43.5%), 6 C. tropicalis (13.0%), 8 C. krusei (17.4%), 4 C. glabrata (8.7%), 3 C. famata (6.5%), 3 C. parapsilosis (6.5%), and 2 C. guilliermondii (4.3%). All the Candida albicans isolated were gram-positive and the germ test tube tested positive. Eighteen (90%) of the isolates were prone to clotrimazole at a concentration of 5 µg/mL - 10 µg/mL followed by 17 (85%) isolates to panosoconazole at a concentration of 0.002 µg/mL - 5 µg/mL. Eight (40.0%) of the Candida albicans isolates possessed the gene Cerebellar Degeneration Related 1 (CDR1) that was observed at 286 bp. Virulence enzymes were determined in which 100% produced haemolysin, followed by proteinase (75.0%), phospholipase (50%), coagulase (50%), and lastly capsulase (25.0%). Fluconazole and Clotrimazole did not inhibit the growth of C. albicans at elevated concentrations but this study showed that they inhibit biofilm formation at lesser concentrations.* ***Conclusion:*** *C. albicans isolates were resistant to various antifungals as well as those frequently used in the management of HIV/AIDs patients. This attributed to resistant genes and produced various virulence factors that were found to be present in the isolates hence, these calls for a regular close watch on antifungal drug resistance.*

Introduction

The decline of CD4+T lymphocyte cells in HIV patients leads to a danger of Acquired Immunodeficiency Syndrome (AIDS) [1]. However, the use of antiretroviral therapy has a key impact, candidiasis remains a common opportunistic disease among people living with HIV. The high number of infections, the protracted use of antifungal to treat repeated infections, and the surfacing of antifungal resistance have shaped the need for antifungal susceptibility testing [2]. Information about the effects or load of antifungal resistance is not as much as that of antibiotic-resistant bacterial infections, which are extensively documented as a public health crisis [3]. This highlights the need to realize the reasons for their surfacing, create alertness among medical and public health communities about these infections, and better attention to prevent avert and manage them.

Candida species remains the main source of fungal infections globally more so to those living with HIV [3]. *Candida* species are common microbiota inside the gastrointestinal tract, respiratory tracts, vaginal region and oral cavity. Moreover, they have been classified as the major sexually transmitted diseases amongst sexually active folks [4]. *Candida* is a yeast development in all females and is usually controlled by bacteria. *Candida* species vary in their antifungal defenselessness and virulence factors. The genus is composed of a varied collection of organisms, and further 17 different *Candida* species are identified to be etiological agents of human infections; nevertheless, more than 90% of invasive infections are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. dubliniensis* and *C. lusitaniae* [5]. The yeast begins to attack and take control of the body tissues by releasing toxins like killer toxins which are proteins in nature that kill sensitive cells of the same or

related yeast genera into the bloodstream causing symptoms like stupor, persistent diarrhea, yeast vaginitis, bladder infections, muscle and joint ache, menstrual troubles, constipation and severe dejection [6-8]. Opportunistic fungi that most commonly affect humankind are the species of *Candida* and *Cryptococcus* [8,9], out of these two groups, the *Candida albicans* species is still the prime *Candida*species cause of *Candida* infections with cases that numbers more than half in the world [6]. An increase in the occurrence of yeast infections caused by non-albicans *Candida* like *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis* and *Candida krusei*, has also been reported in various parts of the globe [8] extended practice of antifungals in treating infectious diseases caused by *C. albicans* has led to the surfacing of azoles resistance. The azole resistance acquired in medical isolates of *C. albicans* regularly results in cross-resistance to numerous dissimilar drugs, a trend termed Multidrug Resistance (MDR) [8-11].

Etiology of fungal infections is more frequent in health practice, facilitated by the use of extreme antibiotics, frequently given in insufficient conditions of illness and taken in disproportionate doses, and also to the "misuse" of steroids, plus the high occurrence of grave devastating diseases (cancers, HIV infection, diabetes) which requires antagonistic treatment or amalgamation therapies that may have a harmful outcome by diminishing generally the body power [12,13]. Some *Candida spp.* strains on the other hand have been documented to form biofilms which is currently becoming a big problem in the management of fungal pathogens worldwide. Most *Candida spp.* are capable of producing other virulence factors (phospholipase, protease, hemolysin, coagulase factors, etc) which help the fungi to survive *in vivo*, dissemination to the mucous level and attach to the core epithelial cells, which enables them to evade the host defense system. Therefore, the study aimed at characterizing, incidence, susceptibility patterns, resistance genes, antibiofilm activity, as well as virulence traits of *Candida* isolates from HIV/AIDS patients attending a Kenyan medical facility-Muhoroni County Hospital.

Methods

Study design

This was a cross-sectional study conducted between January to August 2020 in the Department of Comprehensive Care Centre (CCC), Muhoroni County Hospital (MCH) a consultant and referral center for the sub-county. *Candida spp.* isolated from patients with oral thrush, vaginal candidiasis, candiduria, oesophageal candidiasis and candidemia were included and characterized. Candidemia was considered invasive candidiasis, while oral thrush, vaginal candidiasis, oesophageal candidiasis and candiduria were considered non-invasive candidiasis.

Study area

The study was conducted at Muhoroni County Hospital (0.15660°S, 35.19840°E) in Kisumu County (East to Muhoroni Sugar Company), Kenya. Muhoroni County Hospital is a government 100-bed capacity health facility situated in Muhoroni Town, Muhoroni Sub County in Kisumu County.

Study population

This study targeted total HIV-infected patients with Low Detectable Viral Load (LDL) who sought Medicare at Muhoroni County Hospital (MCH) between January to August 2020 showing the symptoms of candidiasis. Based on Harris *et al.* (1991) formulae a sample size of 181 sample size was utilized in this study.

Study samples

We targeted various clinical samples which included High Vaginal Swabs (HVS) from female patients, and urine and sputum from both female and male patients seeking medicare at the

comprehensive care center department within the study period. The samples were then kept in the laboratory under -18°C before the analysis.

Laboratory analysis

Candida spp. identification and preparation of inoculums

The main yeast isolation was done using the CHROMagar™ Candida (Difco) chromogenic differential medium in Petri dishes and incubated at 37°C for 48h using protocols that have been used previously [14]. *C. albicans* were identified by green colored colonies, *C. tropicalis* by blue-cobalt, *C. krusei* by pink or lilac, and other species were whitish-pink. The isolated yeasts from the chromogenic medium were picked and incubated at 35°C for 48h in tubes with Sabouraud Dextrose Agar (SDA) (SDA with chloramphenicol - Acumedia) medium and then stored at - 20°C for use in the study. The biochemical identification of the yeasts was performed using an automated method (VITEK-2 Compact bioMérieux, Marcy-l'Étoile, France). The bioMérieux VITEK-2® system includes the VITEK-2 cards that permit species recognition by comparing the biochemical profile with a wide-ranging database bioMérieux VITEK-2® extended its task in this area with a yeast susceptibility
test that determines the growth of *Candida* spectrophotometrically via VITEK-2 microbiology systems, performing wholly automated testing of susceptibility to 5-FC, AMB, FCZ, and VCZ [15]. For the preparation of the fungal inoculum (3 mL 0.45% saline + yeast colony), a McFarland scale 2 from the DensiChek-bioMérieux VITEK-2® method was used. This standardized suspension was aspirated into the identification cards, and then the cards were sealed and subjected to biochemical tests by an optical sensor reading. We used the Yeast identification card (YST) bioMérieux) to establish the genus and species of yeast. The investigation was considered absolute when the percentage of probability was ≥85% and there was no request for extra testing.

Gram staining

From the growth on SDA (Oxoid, UK) smears were prepared and left to air-dry, then heat-fixed and stained using the Gram staining technique after which examined microscopically as per a previously used protocol [16].

Germ tube test

Yeasts isolated from the specimens were all processed and identified to the species level using the germ tube test and API ID 32 C strip (bioMérieux, Marcy-l'Etoile, France) [14]. Briefly, a Pasteur pipette was used to collect 5.0ml of fresh human serum (from the serology laboratory at the Muhoroni County Hospital) and then set into labeled test tubes. Using a sterilized inoculating loop, a colony of yeasts was transferred into the serum in the labeled test tubes. The colony then was emulsified gently in the serum. Incubated at 37°C for about 3 hours. A drop of the suspension was taken from the test tube after incubation using a Pasteur pipette and placed on a slide which was clean and dry for examination. The suspension was then covered with a coverslip and examined microscopically under low power (10X objective lens) for germ tubes for the yeasts. A high-power objective (40X objective) was used to confirm the absence or presence of germ tubes. The presence of a short hyphal (filamentous) extension arising from a yeast cell indicated the presence of *C. albicans* [16]. Both the positive control isolates (*C. albicans*- ATCC 10231) and the negative control isolate (*C. glabrata*-ATCC 2001) were included in this test. All tests were done in triplicates independent of each other.

API ID 32 C kits

API ID 32 C kits were used to deduce the presence of *C. albicans* and to identify the yeast isolates as used before [17]. Briefly, by use of an inoculating loop which is sterile, one or several colonies from the Sabouraud's Dextrose Agar (Oxoid, UK) plates were put into 2 ml of API® suspension medium (bioMérieux, Marcy-l'Etoile, France). The colonies

were emulsified in the API® suspension medium to form a suspension of turbidity equivalent to 2 McFarland.

The suspension turbidity was measured with the Densimat (bioMérieux, Marcy-l'Etoile, France) device that came with the test kit. About 250 µl of the suspension (already prepared) was then transferred into 7ml of API C medium (bioMérieux, Marcy-l'Etoile, France) using an ATB™ Electronic Pipette (bioMérieux, Marcy-l'Etoile, France) supplied with the test kits. All tests were done in triplicates that are independent of each other.

In vitro antifungal susceptibility tests

Antifungal Susceptibility Tests (AFST) were done using the VITEK-2® automated system and Etest® (Biodisk AB, Solna, Sweden) as per the manufacturer's recommendation. These methods have been selected as they are simple to carry out and present the outcome in a short time [18]. Briefly, 180 µL of inoculum was standardized to the McFarland scale 2.0 using the DensiChek densitometer of the VITEK-2® system, placed in a tube containing 3 mL of 0.45% saline and aspirated into the AST-YSO1 card (bioMérieux). The listed below antimycotic were tested: Amphotericin B (10 µg/), Fluconazole (25 µg), Itraconazole (10 µg), Nystatin (50 µg), Clotrimazole (10 µg), and Panosoconazole (5 µg). The data analysis and understanding were performed according to M27-A3 and M27-S3 CLSI standards. For quality control, *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were used as standard strains. For mycotic strains that didn't react to the AST cards, an E-test® (Biodisk AB, Solna, Sweden) was carried out, which consisted of a gradient technique with predefined concentrations of FCZ and AMB in µg/mL. Under the method, colonies were seeded at a concentration of 0.5 McFarland on dishes with RPMI AGAR (Probac®, São Paulo, Brazil) supplemented with 2% glucose agar and then incubated at 35°C for 48h. Reading was done by assessing the point of intersection between the halo formed and the E-test strip. The Fluconazole MIC was determined as the lowest concentration that inhibited 80% of fungal strains, and the Amphotericin MIC was calculated as the lowest concentration with no fungal growth observed. The profile of the antifungal drug sensitivity was classified as sensitive (S), dose-dependent sensitivity (S-DD), and/or intermediate (I) and resistant (R).

The endpoints used to classify sensitivity, intermediate, and resistance for each variety were those defined by the Clinical and Laboratory Standards Institute (CLSI) (2022). The MIC values ≤ 8 µg/mL for Fluconazole were considered susceptible (S), 16 - 32 µg/mL was considered susceptible dose-dependent (SDD), and ≥64 µg/mL was resistant (R). For Amphotericin, MICs ≤ 1 µg/mL were considered to be S and ≥1 µg/mL was R. For Itraconazole, CIMs ≤ 4 µg/mL were considered to be S, 8 - 16 µg/mL was I, and ≥32 µg/mL was R. For Panosoconazole, MICs ≤ 0,125 µg/mL were S and ≥16 µg/mL were considered R. Fluconazole and amphotericin were selected for the tests for the reason that they have diverse mechanisms of action and are the major drugs preferred for the management of *Candida* infections [19]. Panosoconazole and Itraconazole were preferred as an alternative for species resistant to Fluconazole and Amphotericin.

Determination of biofilm formation by microtitration plate method

Candida spp. isolates formed in the SDB were adjusted to be 10^7 cfu / mL, and they were spread as 20 µL in each well of the 96-well plate to determine the formation of biofilm in the presence of antifungals using earlier used and established protocol [20]. Briefly, 180 µL Synthetic Dextrose Liquid (SDL) medium containing 2.5% glucose was transferred onto it and incubated for 48 hours at 35°C. After incubation, the plates were emptied and each well was washed 3 times with sterile physiological saline. The wells were fixed with 200 µL 99% methanol for 15 minutes. At the end of this period, the wells were emptied and left to dry. Subsequently, each well was stained with 200 µL 2% crystal violet for 15 minutes. After the

end of this period, the wells were washed with distilled water and dried for one hour. The wells were treated with 160 µL 33% glacial acetic acid after drying and assessed spectrophotometrically at 590 nm. For each experiment, background staining was corrected by subtracting the crystal violet bound to untreated controls (blank) from those of the tested sample. The experiments were done in triplicate and average OD_{590}nm values were calculated. To estimate the antibiofilm activity (Abf A) of a given antifungal the following equation will be used

$$Abf\ A\ (\%) \left[1 - \left(\frac{OD_{Test\ sample} - OD_{Blank}}{OD_{Untreated\ sample} - OD_{Blank}}\right)\right] \text{x } 100$$

Genome characterization for resistance genes

Polymerase Chain Reaction (PCR) for pathogenic and antibiotic-resistant genes

DNA was extracted from the isolates using a method described by Peano *et al.* and Hamza *et al.* [20,21]. The DNA for the resistant isolates was amplified using conventional Polymerase Chain Reaction (PCR) as used before [22] and was used in detecting the presence of *CDR1, CDR2, RTL3* and *MAL 2* resistance genes amongst the isolates. The lists of the primers used are presented in Table 1 below. The cycles were set at 94-98°C for 1-3 minutes for denaturing, depending on the DNA template, 55-72°C for 1-2 minutes for annealing and extension at a temperature of 68-72°C depending on the DNA polymerase rate and length of the target DNA.

Gel electrophoresis

Amplified *candida spp.* isolates genomes were separated using Pulsed-Field Gel Electrophoresis (PFGE) as used before [18] with minor changes. A two-block program with a primary block ramp time of 2 seconds to 10 seconds for 13 hours. At 6V/cm (for separation of smaller fragments) and subsequent block with a ramp time of 20 and 25 seconds for 6 hours (for separation of larger fragments) at 6V/cm were used. The NotI restriction enzyme was used to digest the chromosomal DNA. Restriction fragments were separated in a 1% Pulsed-Field Certified Agarose gel in 1xTBE (8.9 mM Tris base, 8.9 mM boric acid, and 0.25 mM disodium EDTA) by using a CHEF-DR II system (Bio-Rad). The CHEF DNA size standard of *Saccharomyces cerevisiae* (Bio-Rad Laboratories, Inc, CA) was then used as a molecular mass standard. Following electrophoresis, gels were stained for 20 min with ethidium bromide (2 µg/ml in 1% TBE buffer), and then stained.

Detection of other virulence factors

Extracellular phospholipase activity

Phospholipases were screened for by measuring the zone size of precipitation after the growth of the isolates on egg yolk agar using recognized methods as used earlier [13]. The plates were read with the help of a computerized image analysis structure (Quantimet 500 Qwin, Leica), which measures the diameter of the colonies relative to the precipitation zones on a magnified scale. Phospholipase activity was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone (in mm) [20]. All tests will be done in triplicates that are independent of each other.

Determination of haemolysin production activity

The β-hemolytic action was tested for on-base agar (Himedia, India) in supplement with 7% sheep erythrocytes for 18-24 hours as described by Rodríguez-Cerdeira *et al.* [22]. Briefly, one colony for the night culture was emulsified in 1 ml of sterile normal saline then 10 µl of suspension was inoculated onto an SDA plate supplemented with 3% glucose and 7% sheep blood. Plates then were incubated aerobically at 37°C for 72 hours. Hemolysin actions were determined by measuring the diameter of the translucent halo in the region of the inoculum site when viewed with transmitted light. *Streptococcus pyogenes* ATCC12384 was used as the positive control. All

tests were done in triplicates that are independent of each other.

Determination of coagulase action

Coagulase actions were detected and interpreted by the use of coagulase biochemical tests as described earlier [22]. Briefly, *Candida* cells were inoculated into Sabouraud's dextrose broth and incubated aerobically at 37°C for 18-24 hours. A total of 100 µl of the all-night broth was aseptically placed into a test tube containing 0.5 ml of EDTA rabbit plasma and incubated aerobically at 37°C for 4 hours. *Staphylococcus aureus* ATCC 25923 was used as positive control while *Staphylococcus epidermidis* ATCC 14990 was used as the negative control. All tests were done in triplicates that are independent of each other.

Determination of capsulase formation ability

Assessment of capsule formation was done by culturing the *Candida* strains on Congo red agar (CRA) as described by Zaranza *et al.* [23] with slight modification. The Congo red agar was prepared by mixing 36 g saccharose (Sigma Chemical Company, Lezennes, France) with 0.8 g Congo red in 1 L of on Tryptic Soy Agar (TSA, Difco, City, Spain) supplemented with NaCl (1.0%). The plates were then inoculated with the *Candida spp.* and incubated aerobically for 24 h at 37°C followed by a further 24 hours at 30°C. After incubation, black colonies were considered as capsule producers, whereas red colonies were considered as non-producers [23]. All tests were done in triplicates that are independent of each other.

Determination of proteinase production activity

The assessment of Extracellular protease activity was done by plating the *Candida* isolates on bovine serum albumin agar (BSA) with 1% KH_2PO_4, 0.05% $MgSO_4$ (LOBACHEMIE-Mumbai, India), 2% agar, 0.01% yeast extract (Finkem), and 0.2% BSA (AppliChem, Germany) adjusted to pH of 4.5 as previously described in the study of Rodríguez-Cerdeira *et al.* [22]. The clearance zone around the colonies as a result of the cleavage of the Bovine Serum Albumin (BSA) by proteases was indicative of the positive protease activities. The diameter clearance was measured and recorded after 72 h of growth. All tests were done in triplicates that are independent of each other.

Validity and reliability

All experiments were done in triplicates that were independent of each other to validate reproducibility.

Data analysis

Statistical analysis was performed using Stata Software. Data on socio-demographics were summarized by frequencies and percentages. All values of diameter zones of inhibition are reported as mean ± standard error.

Ethical consideration

Confidentiality and privacy were strictly adhered to and no names of individuals were recorded or made known in the collection or reporting of information. The study was granted ethical clearance by the Board of Postgraduate Studies (BPS) of Kisii University Ref no. KSU/R&E/03/5/513 and ethical approval to conduct the study was sought from the Institutional Research Ethics Committee (IREC) at Moi University/Moi Teaching and Referral Hospital (MTRH) Ref. No. IREC/2019/111 and the National Commission of Science, Technology and Innovations (NACOSTI) Ref. No. NACOSTI/P/20/3219.

Results

Demographics and incidence of isolates

From 181 samples analyzed, 46 (25.4%) fungal isolates were identified from them in which more than one species were isolated from a particular clinical sample in some cases. Almost 83% of patients participating in the study had oropharyngeal candidiasis, and 52.2% had used

azoles. Of these, 75% were using both Fluconazole and Nystatin and 8.3% used Fluconazole, Nystatin, and Amphotericin. Among the samples, 46 (25.4%) grew in CHROMagar™ *Candida* medium and 135(74.6%) were negative. Using the automated VITEK-2® Compact bioMérieux system, 46 isolates were identified as *Candida spp.* which included 20 (43.5%) *C. albicans*, 6 (13.0%) *C. tropicalis*, 8 (17.4%) *C. krusei*, 4 (8.7%) *C. glabrata*, 3 (6.5%) *C. famata*, 3 (6.5%) *C. parapsilosis*and 2 (4.3%) *C. guilliermondii* (Table 2). Looking into the distribution of isolates of *Candida spp.* by patients' gender, we observed that the figure of isolates obtained from the women patients 40 (87%) were higher than that from the men 6 (13%) as shown in Table 2.

Characterization of *C. albicans*

Of the 20 *Candida albicans* isolated, gram staining technique, germ test tube and sub culture on SDA media was performed for confirmation of the species of interest and they both revealed that 100% of the isolates were *Candida albicans*. For instance, in gram staining every isolates were established to be gram positive in cocci. Also, for the germ tube test the 20 isolates proved to be positive by producing yeast cell budding like shapes. Regarding culturing on SDA media, the isolates produced white to cream with even & yeast like manifestation colonies.

The isolates were further characterized using API ID 32 C kits and all 20 isolates were confirmed to be *C. albicans* by turning out to be positive to SAC, RAF, TRH, ROR, and RHA and Negative to ARA, CEL MAL MEL, and MAN as shown in Table 3.

Distribution of *C. albicans* among the age and gender in the study sites

The age cluster with the utmost frequency of *Candida albicans* isolates was 16 - 25 years at 8 (40%), followed by age group of 26-35 years at 7 (35%), age group 5-15 years at 4 (20%), age group 36-45 years at 1 (5%) and lastly age group >46 years at 0 (0%). From the samples analyzed, there were high isolates on HVS samples n=12/76 followed by urine samples (n=8/90) and lastly sputum samples at 0/15 as shown in Table 4.

Antifungal susceptibility pattern

The positive isolates for *C. albicans* were screened for their susceptibility to a variety of antifungal drugs to deal with candidiasis infections. Eighteen (90%) isolates were susceptible to Clotrimazole at a concentration of 5 µg/mL-10 µg/mL followed by 17 (85%) isolates to Panosoconazole at a concentration of 0.002 µg/mL-5 µg/mL. However, there was a reduced susceptibility to Fluconazole 16 (80%) which is commonly used for *Candida* management at a concentration of 0.016 µg/mL-25 µg/mL. In all, 14 (70%) of the *C. albicans* isolates were susceptible to both Amphotericin B and Nystatin at a concentration of 0.016 µg/ mL-10 µg/mL and 0.016 µg/mL-50 µg/mL respectively, and *C. albicans* ATCC 10231-PC showed 100% susceptibility to almost all antifungal agent used in this study except to Fluconazole and Nystatin which shows 95% together at the aforementioned concentrations as shown in Table 5.

Detection of other virulence factors

The study investigated the formation of a variety of virulence enzymes like proteinase, phospholipase, capsulase formation, coagulase and haemolysin (Table 6) on the four isolates, which were established to be resistant to all azoles. It was discovered that 3/4 (75.0 %) of these isolates of *C. albicans* produce protease enzymes. Also, it was confirmed that two out of four isolates (50.0%) produce phospholipases. More findings also point out that out of the four isolates two of them (50.0%) could produce coagulase and only one isolate (25.0%) produced capsules. Finally, it was determined that each one of the four isolates was capable of producing the haemolysin by haemolysing the sheep red blood cells resulting in beta (β) haemolysis. Haemolysin hence was produced as the largest part of virulence factor by these isolates (100%), followed by proteinase (75.0%), phospholipase (50%), coagulase (50%),

and lastly capsulase (25.0%). Out of the four isolates, one isolate (MCH/16/20) produced the entire virulence traits studied, one isolate (MCH/05/20) produced at least three virulence traits, and two isolates (MCH/75/20, MCH/47/20) produced at least one of the virulence traits as shown in Table 6.

***C. albicans* isolates resistant genes profiling**

Upon screening of the resistant genes, it was deduced that eight (40.0%) of the *Candida albicans* isolates possessed the gene CDR1 that was observed at 286 bp as shown in Figure 1 plate A below. Analysis of the CDR2 gene revealed that 5 (25.0%) harbored the CDR2 gene at 364 bp as shown in Figure 1 plate B. It was also revealed that 4 (20.0%) of the isolates harbor the gene encoded by RTL3 gene at 201 bp as shown in Figure 1 plate C above. The minority, 3 (15.0%) were confirmed to possess the gene mal2 at 204 bp as shown in Figure 1 plate D.

The summary of the frequencies of the resistant genes profiling of *C. albicans* isolates is presented in Table 7 below.

Biofilm formation inhibitory effects of chosen antifungals against the *C. albicans* strains

Biofilm formation inhibitory effects of chosen antifungal drugs against the *C. albicans* strains that were resistant drugs towards the four isolates (Fluconazole, Panosoconazole, Itraconazole, Amphotericin B, Nystatin, and Clotrimazole) (36) were used as treatments for the antibiofilm formation assay in a 96-well microtiter plate. The outcome showed that the biofilm formation inhibitory effects of the range of concentrations (0.5, 0.25, 0.125, 0.0625, and 0.03125 mgml-1) were notably lesser than that of the positive control, a suggestion that biofilm formation was inhibited at these concentrations (Figure 2, Figure 3, Figure 4, Figure 5). As much as such inhibitory effects were recorded, these results reveal that the four isolates that proved to be resistant to regularly used antifungals can form biofilms.

Antibiofilm development action against isolating MCH/05/20 of *C. albicans* for various antifungals was low (Figure 2). Interestingly, the concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg ml^{-1} were able to inhibit biofilm formation to a large extent compared with the positive control. Many biofilm inhibitory effects were observed with Nystatin as compared to other antifungals. Additionally, more inhibitory activities were observed at a lesser dosage for this isolate as compared to a higher dosage of the antifungals used as treatments.

Antibiofilm formation action against isolate MCH/16/20 of *C. albicans* against a range of antifungals was observed in all the antifungals, with no implication difference on Fluconazole at all the concentration as compared with the positive control (Figure 3). The rest of the antifungals produced significant antibiofilm activity: Amphotericin B, Nystatin, and Clotrimazole also showed significant inhibitory activity against this isolate MCH/16/20 at lower concentrations. Also, for this isolate many inhibitory effects were observed at lower dosages of the antifungals used except for Itraconazole.

Isolate MCH/47/20 was more prone to the inhibitory effects of a range of dosages of the various azoles as compared to other isolates. Significant differences were also observed in a good number of the antifungals used compared with the positive control (Figure 4). Also, for this isolate much inhibitory effects were observed at lower dosages as compared to higher dosages of the antimycotic used except for Itraconazole with nystatin producing more inhibitory effects at 0.03125 mg/ml.

Antibiofilm formation action against isolated MCH/75/20 of *C. albicans* against a range of antimycotic agents was observed in all the antifungals (Figure 5), with a few significant differences at a variety of dosages. Panosoconazole does not have good inhibitory effects against isolated MCH/75/20. Whereas, Nystatin showed an opposite action with high

inhibitory effects being observed at 0.03125 mg ml^{-1} as compared with higher concentrations. Similar findings were also realized at lower dosages in comparison with elevated dosages of the other antifungals used apart from Itraconazole.

Discussion

Oropharyngeal candidiasis is the main opportunistic disease among HIV-seropositive patients and in those with AIDS, and it poses a serious treatment challenge. For this reason, therefore, it is recommended to establish the isolate implicated in the infection and its azoles susceptibility. This study showed that 51 samples were from men and 130 were women, resulting in a ratio of 1.3 cases in women for every 1 case in men. The outcomes direct us to reflect on whether the incidence of AIDS in women is rising and compared with the incidence in males. Additionally, the disparities in terms of gender could be attributed to the high rate of the incidence [24]. Our findings however do not concur with findings from a study done in Brazil, and was reported by Bulletin in 2011, which showed that in 2011, the ratio of 1.7 cases in men for every 1 case in women was significantly diminished when compared to data from 2008, when there were about six cases of AIDS in men for every case in women [24]. In the current study, we too establish that the main widespread age group with candidiasis was 16-25 years, followed by the 26-35 years age group. These findings show similarity to a previous study done in Brazil [24] and other countries [21]. The findings on the age group with the most prevalent *Candida spp.* isolates could be attributed to high rates of sexual practice among women [25].

During the study period, 46 (25.4%) out of the 181 study participants were *Candida spp.* positive after the culture test. This outcome is related to that was established in other studies, which showed that 20-35% of the patients contracted one or more fungal infections for the period of their illness [26-28]. In the study, it was established that *C. albicans*was the major prevalent species at 20 (43.5%), followed by *C. krusei* 8 (17.4%), *C. tropicalis* 6 (13.0%), *C. glabrata* 4 (8.7%), *C. famata* and *C. parapsilosis* at 3 (6.5%) both, and lastly *C. guilliermondii* 2 (4.3%) which is in harmony with other studies [29,30]. This outcome could be attributed to the fact that *C. albicans* is sometimes a normal flora in our systems and hence the likelihood of isolating it in higher numbers could be higher. Other studies have also documented the same findings. For instance, in a study done in India, they did document that *C. albicans* was the major prevalent species at 30 (48.5%) [31].

In the study, a case of a patient was identified with four *Candida spp.* isolates that were recognized in a particular clinical sample; the first species was *C. glabrata*, which was resistant to fluconazole together with Amphotericin, and further *C. krusei*, a second species was resistant to Fluconazole. This outcome is relevant for the reason that Fluconazole is the drug of preference for candidiasis treatment in AIDS patients even though it has a fungistatic mode of action [32,33], and both fluconazole and Amphotericin were being used by a few of the patients who participated in the research. Four patients had dual colonization, and an individual among them had colonization by *C. krusei* resistant to Fluconazole. The coexistence of diverse species in a similar clinical sample has also been reported in other studies [31,34]. The occurrence of oropharyngeal or esophageal candidiasis is acknowledged as a marker of immune suppression and is mainly observed in patients with CD4 T lymphocyte (CD4) cell counts <200 cells/mm^3, with esophageal disease typically occurring at lower CD4 counts than oropharyngeal disease [34].

In the study, all isolates of *C. albicans* (n D 29), *C. tropicalis* (n D 6), *C. parapsilosis* (n D 2) and *C. guilliermondii* (n D 2) showed sensitivity to all of the Azoles tested, which is steady with the broad pattern of susceptibility of the NCLS M-27 process and with the outcome of other studies [35-38].

Ninety percent of the *C. albicans* studied were sensitive to clotrimazole given 5-FC, an elevated sensitivity was realized in this study possibly because of this antifungal action, basically by destroying the permeability wall in the fungal cytoplasmic membrane [39,40]. Clotrimazole thereby inhibits the biosynthesis of ergosterol in a concentration-dependent manner by inhibiting the demethylation of 14 alpha lanosterol [41], followed by 85% isolates to Panosoconazole, 80% isolates to fluconazole which interact with 14-demethylase, a cytochrome P-450 enzyme responsible for catalyzing the conversion of lanosterol to ergosterol [40]. As ergosterol forms a significant part of the fungal cell membrane, Fluconazole inhibits the synthesis of ergosterol to raise cellular permeability, 70% isolates to both Amphotericin B which binds to ergosterol in the fungal cell membrane, which leads to the development of pores, ion outflow and ultimately fungal cell fatality and Nystatin that acts by binding to sterols in the plasma membranes of fungi causing the cells to seep out, ultimately leading to fungal cell fatality [41]. Lastly, 50% of the isolate demonstrates sensitivity to Itraconazol which inhibits the fungal-mediated synthesis of ergosterol, via inhibition of lanosterol 14α-demethylase [42]. These outcomes are related to those experimented by other researchers [21,37]. Resistance to Itraconazole was observed in 10 (50%) isolates of *C. albicans*followed by 6 (30%) to both Amphotericin and Nystatin, 4 (20%) isolants to Fluconazole, 3 (15%) isolants to Panosoconazole and lastly 2 (10%) isolates to Clotrimazole was observed in this study. The outcome of this study has the same opinion as several studies that confirmed the natural resistance of *C. albicans*to Fluconazole which is the commonly used antifungal among those living with HIV/AIDs [21,30,31,37,41].

In addition, the study investigated the capability of these test strains to make a variety of virulence factors, which may have a key function in their pathogenicity. Along with the virulence traits examined consisting of enzymes like proteinase, phospholipase, capsulase, coagulase, and hemolysin on the four isolates, which were established to be resistant to all antifungal revealed that 3/4 (75.0 %) of these isolates of *C. albicans*formed protease enzyme, these outcome is in agreement with the findings of an earlier study [42], which demonstrated that the majority isolates were protease positive, as well as protease enzyme has restricted effect on the pathogenesis of this antifungals. Results from the present study concur with a study that acknowledged that all isolates could produce protease [43]. Production of proteases by *C. albicans* has a significant task in pathogenicity, as they are in charge of hydrolysis of numerous physiologically essential proteins such as mucin, fibronectin and lactoferrin [44]. It might also proteolytically trigger genes and hemolysin, consequently making this pathogen more dangerous [45].

It was also confirmed that two out of four isolates (50.0%) produce phospholipases, these results further agree with earlier study outcomes in which out of 20 isolates, 10 (50%) isolates were found to have phospholipase production potential [46]. Our results are also in tandem with [47] study results for phospholipase presence. Phospholipases are lipolytic enzymes that hydrolyze phospholipid substrates at specific ester bonds. Phospholipases are extensive in the environment and take part in very various roles from attack in snake venom to signal transduction, lipid mediator production, and metabolite digestion in humans [48]. Additional outcomes also indicate that out of the four isolates, two of them (50.0%) could produce coagulase and only one isolate (25.0%) produced capsulase. Our results also agree with other studies, which showed that 50% of isolates obtained in the study could produce coagulase and only 20% produced capsulase which protects fungi from engulfment by host macrophages [48]. Coagulase enzymes catalyze the hydrolysis of the ester bonds of triacylglycerols and could have an important function in *C. albicans* pathogenicity or nourishment acquisition. The making of a surplus

amount of coagulase allows antifungal to infiltrate fatty tissue w resulting in the formation of abscess [49].

Consequently, the manufacture of these enzymes by the isolates may be a sign of the occurrence of the genetic organization of a discrete genetic element, which encodes three genes accountable for the production of proteinase, coagulase, and phospholipase. This organization possibly will be part of a pathogenic island, encoding a product capable of destroying host cells and being implicated in nutrient acquisition [49]. Finally, it was determined that every one of the four isolates was capable of producing the hemolysin by haemolysing the sheep red blood cells leading to beta (β) hemolysis which became resistance to the immune system of the host, tissue destruction, and lethality, either by direct action or by motivation of inflammatory mediators and signal transduction pathways. Haemolysin consequently was the main virulence factor produced by these isolates (100%) also was followed by proteinase (75.0%), followed by phospholipase and coagulase at (50%) and lastly capsulase (25.0%). Out of the four isolates, one isolate (MCH/16/20) formed all the virulence traits studied, one isolate (MCH/05/20) formed at least three virulence traits, and two isolates (MCH/75/20, MCH/47/20) formed at least one of the virulence traits. This finding concurs with another study which reported nearly the values as in this report which reveals that out of the 10 isolates all produced proteinase, phospholipase, and coagulase while only 2 (20%) were not able to produce capsulase [50].

From this study, it was revealed that four isolates that had shown resistance to commonly used antifungals indeed possess different factors that make them possible either to be resistant or provoke diverse infections in humans, which leads to modification of drug efflux which is one of the important mechanisms of resistance in fungus. The *C. albicans CDR1* gene is a homolog of *S. cerevisiae* PDR5, which encodes a multidrug efflux pump, and *CDR1* is the gene most frequently linked with energy-dependent drug efflux in FLU-resistant clinical isolates [50]. The MDR phenotype in *C. albicans* has before been revealed to be connected to proteins encoded by *CDR1, CDR2, RTL3* and *MAL2* genes. These proteins perform membrane-localized efflux pumps that pump drugs from the fungal cells [41]. A number of these traits are controlled at the gene level for that reason therefore, they can be moved from a fungal cell to a new one through conjugation or otherwise [26]. On analysis of the resistant genes, eight (40.0%) isolates possessed the gene *CDR1* and 5 (25.0 %) harbored the *CDR2* gene. It was also deduced that a variety of pathogenic and antifungal resistance genes like 4 (20.0%) of the isolates harbored the gene encoded by the *RTL3* gene. The minority, 3 (15.0%) were confirmed to possess the gene MAL2. This finding concurs with another study that reported nearly the same values as this report [31].

Most studies have recommended that Fluconazole and Clotrimazole do hinder development at elevated concentrations [38] however as of our study, it was concluded that they hamper biofilm formation at lesser concentrations. A similar situation was also noted in a good number of antifungals like Panosoconazole, Itraconazole, Amphotericin Nystatin, and others with such a "Goldilocks" effect. A probable account of the fewer actions observed at larger doses may be linked to the aggregation effects of the antifungal agent at the point of entrance into the fungal cell particularly at elevated dosages, something that is not observed at lesser dosages. Aggregation can probably favor biofilm development as antifungal agents fight to get to the point of action and consequently, the fungi will go on to flourish and thus form more biofilms [45]. This result agrees with the preceding studies on biofilm inhibitions by Boyken *et al.* [46], which showed higher biofilm inhibitory at lesser dosage concentration in opposition to the positive control. Nevertheless, it did not correspond with the earlier study on biofilm inhibition by Goldman *et al.* [47], which showed that Fluconazole growth is inhibitory at raised concentration as well as biofilm inhibitory

at high concentration as compared with positive control [47]. On the other hand, Panosoconazole, Itraconazole, Amphotericin, and Nystatin were established to restrain biofilm formation at elevated concentrations in several isolates and against the positive control. Conversely, it should be realized that they did not entirely hamper the biofilm formation capability of the test isolates, an apparent sign that appropriate antifungals ought to be used in the treatment of cases that are a result of these isolates.

Conclusion

It was found that *C. albicans* was resistant to Itraconazole, Amphotericin B and Nystatin antifungal, drugs commonly used in the management of HIV/AIDs patients in care in Muhoroni County Hospital. From this study, it can also be concluded that the clinical isolates at Muhoroni County Hospital had resistant genes and produced various virulence factors studied: proteinase, phospholipase, capsulase, coagulase and hemolysin. Even though inhibitory effects were recorded, the study result revealed that the isolates were resistant to frequently used antifungals as well as they make biofilms. These results, therefore, add significance to the facility on the management of the patients who are in care in the facility. Taken jointly, there is a need to carry out regular surveillance on antifungal drug resistance for the period of the outbreak.

What is known about this topic

- *Patients with acquired immune deficiency syndrome usually suffer from candidiasis;*
- *Candidiasis is the most common fungal infection resulting in mortality and morbidity;*
- *The fungi characterize, incidence and susceptibility.*

What this study adds

- *The resistance genes against the isolated C. albicans;*
- *Antibiofilm activity against the resistance isolated C. albicans;*
- *The virulence traits against the resistance isolated C. albicans.*

Competing interests

The authors declare no competing interests.

Authors' contributions

All authors contributed to this work, in which the corresponding author Haron Miruka did the conceptualization, data curation, formal analysis, investigation, methodology, resources, project administration, software, validation, visualization, and writing the original draft. While other authors Eric Omori Omwenga, Stanslaus Musyoki and Silas Onyango Awuor contribute to data curation, formal analysis, methodology, validation, visualization, supervision and writing review and editing. All authors have read and agreed to the final manuscript.

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Figure 1: gel electrophoresis result for confirmation of different resistance genes extracted from the 20 isolates of C. albicans isolates Plate A: representing the CDR2 gene in clinical C. albicans isolate. Plate B: Representing CDR1 genes in clinical C. albicans isolate. Plate C: representing RTL3 genes in clinical C. albicans isolate and Plate D: representing MAL2 genes in clinical C. albicans isolate

Figure 2: antibiofilm formation action against isolate MCH/05/20 of *C. albicans* in opposition to a range of antifungals

Figure 3: antibiofilm formation action against isolate MCH/16/20 of *C. albicans* against various antimycotic agents

Figure 4: antibiofilm formation action against isolate MCH/47/20 of *C. albicans* against various antifungals

Figure 5: antibiofilm formation action against isolate MCH/75/20 of *C. albicans* against various antifungals

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Table 1: sequences, conditions, and amplificons of oligonucleotide primers in PCR used for the study

Primer, genes and sequences (5'-3')	Amplificans size IN (bp)	Condition for PCR in °C	References
CDR1 F: 5'-TTTATCAACTTGTCACACCAGA-3' R: 5'-GGTCAAAGTTTGAAGATATACGT-3'	354	86.55 melting	[20]
CDR2 F :5'-TTGGGTTTGCTTGAAAGATGAT-3 R :5'-AAAGTTTGAAGAATAAAATGGC-3'	119	82.75 melting	[22]
RTA3 F: TTGTCTGAGCTCGGAGAGAG R: TTCTTTTCCTCCGCTTATTG	929	94.0 melting	[22]
MAL2 F: GATTTGCTTAATTGCCCCAC R: GTCAAACTTGGTCATTTA	583	65	[22]
CPAR F: GTCAACCGATTATTTAATAG R: GTCAACCGATTATTTAATAG	560	95.5 melting	[22]

Table 2: quantity and incidence of the Candida strains recognized by the Vitek-2® system, and their distribution according to patient sexual category			
Species	**Female**	**Male**	**Total No. of isolates**
Candida albicans	20 (43.5%)	0 (0%)	20
Candida tropicalis	5 (4.3%)	1 (2.2%)	6
Candida krusei	6 (13.0%)	2 (4.3%)	8
Candida glabrata	2 (4.3%)	2 (4.3%)	4
Candida guilliermondii	2 (4.3%)	0 (0%)	2
Candida parapsilosis	2 (4.3%)	1(2.2%)	3
Candida famata	3 (6.5%)	0 (0%)	3
Total	**40 (87%)**	**6 (13%)**	**46**

Table 3: biochemical characterization of the isolate to API ID 32 C kits										
Isolate	**API 32C KIT**									
	SAC	**ARA**	**CEL**	**RAF**	**MAL**	**TRH**	**SOR**	**RHA**	**MEL**	**MAN**
MCH/05/20	+	-	-	+	-	+	+	+	-	-
MCH/06/20	+	-	-	+	-	+	+	+	-	-
MCH/07/20	+	-	-	+	-	+	+	+	-	-
MCH/13/20	+	-	-	+	-	+	+	+	-	-
MCH/14/20	+	-	-	+	-	+	+	+	-	-
MCH/16/20	+	-	-	+	-	+	+	+	-	-
MCH/33/20	+	-	-	+	-	+	+	+	-	-
MCH/34/20	+	-	-	+	-	+	+	+	-	-
MCH/36/20	+	-	-	+	-	+	+	+	-	-
MCH/37/20	+	-	-	+	-	+	+	+	-	-
MCH/38/20	+	-	-	+	-	+	+	+	-	-
MCH/39/20	+	-	-	+	-	+	+	+	-	-
MCH/47/20	+	-	-	+	-	+	+	+	-	-
MCH/62/20	+	-	-	+	-	+	+	+	-	-
MCH/67/20	+	-	-	+	-	+	+	+	-	-
MCH/74/20	+	+	+	-	+	+	+	+	+	+
MCH/75/20	+	+	+	+	+	+	+	+	+	+
MCH/82/20	+	+	-	+	+	+	+	-	+	+
MCH/123/20	+	+	+	+	+	+	+	+	+	+
KEY: SAC = D -Saccharose, ARA = L - Arabinose, CEL = D -Cellobiose, RAF = D - Rafimose, MAL = D - Maltose, TRF = D- Trihalose, SOR = D - Sorbitol, RHA = L - Rhamnose, MEL = D - Mellobiose, MAN = D - Manitol, +=Positive and -= Negative										

Table 4: distribution of isolated C. albicans isolates from the analyzed samples by age groups						
Age group	**Urine sample (n=90)**		**Sputum sample (n=15)**		**Hvs sample (n=76)**	
	Frequency	**Percentage**	**Frequency**	**Percentage**	**Frequency**	**Percentage**
5-15	3	33.3%	0	0%	1	1.3%
16-25	2	22.2%	0	0%	6	7.9%
26-35	3	33.3%	0	0%	4	5.3%
36-45	0	0.0%	0	0%	1	1.3%
>46	0	0.0%	0	0%	0	0%
Total	**8**		**0**		**12**	

Table 5: susceptibility pattern of C. albicans isolates against frequently used antifungals drugs to deal with candidiasis (n=20)												
Antifungal agents	**Zone of inhibition (C. albicans)**						**Zone of inhibition (C. albicans ATCC 10231-PC)**					
	S		**I**		**R**		**S**		**I**		**R**	
	Zone (mm)	**N (%)**	**Zone (mm)**	**N (%)**	**Zone (mm)**	**N (%)**	**Zone (mm)**	**N (%)**	**Zone (mm)**	**N (%)**	**Zone (mm)**	**N (%)**
FL (25µg)	≥19	16(80)	18-15 DD	0	≤14	4(20)	≥19	19(95)	18-15 DD	0	≤14	1(5)
PA (5µg)	≥17	17(85)	16-14 DD	0	≤13	3(15)	≥17	20(100)	16-14 DD	0	≤13	0
IT (10µg)	≥23	10(50)	22-14 DD	0	<13	10(50)	≥23	20(100)	22-14 DD	0	<13	0
AM (10µg)	≥15	14(70)	14-10	0	<10	6(30)	≥15	20(100)	14-10	0	<10	0
NY (50µg)	≥15	14(70)	10-14	0	0	6(30)	≥15	19(95)	10-14	0	0	1(5)
CL (10µg)	≥20	18(90)	12-19	0	≤11	2(10)	≥20	20(100)	12-19	0	<11	0
Key; FL= Fluconazole, PA= Panosoconazole, IT= Itraconazole, AM= Amphotericin B, NY= Nystatin and CL= Clotrimazole, DD =Disk Diffusion												

Table 6: distribution of the four isolates versus the various virulence traits they produced					
Isolates	Haemolysis	Proteinase	Phospholipase	Coagulase	Capsulase
MCH/75/20	+	-	-	+	-
MCH/47/20	+	+	-	-	-
MCH/16/20	+	+	+	+	+
MCH/05/20	+	+	+	-	-
Key; MCH=Muhoroni County Hospital**; (+) =** Positive**; (-) =** Negative					

Table 7: analysis of pathogenic and antifungal resistance genes by Polymerase Chain Reaction in *C. albicans* isolates			
Target gene	**Positive N (%)**	**Negative N (%)**	**Total**
CDR1	8 (40%)	12 (60%)	20
CDR2	5 (25%)	15 (75%)	20
RTL3	4 (20%)	16 (80%)	20
MAL2	3 (15%)	17 (85%)	20

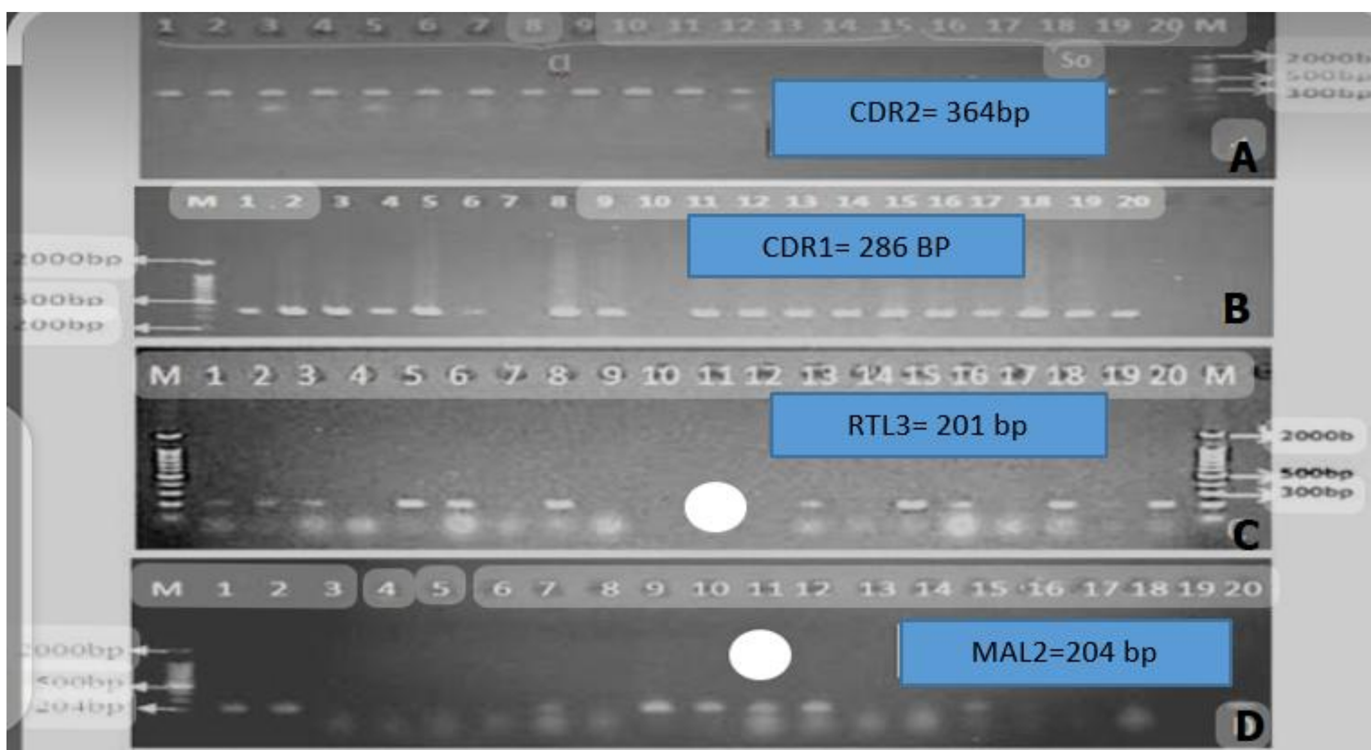

Figure 1: gel electrophoresis result for confirmation of different resistance genes extracted from the 20 isolates of C. albicans isolates Plate A: representing the CDR2 gene in clinical C. albicans isolate. Plate B: Representing CDR1 genes in clinical C. albicans isolate. Plate C: representing RTL3 genes in clinical C. albicans isolate and Plate D: representing MAL2 genes in clinical C. albicans isolate

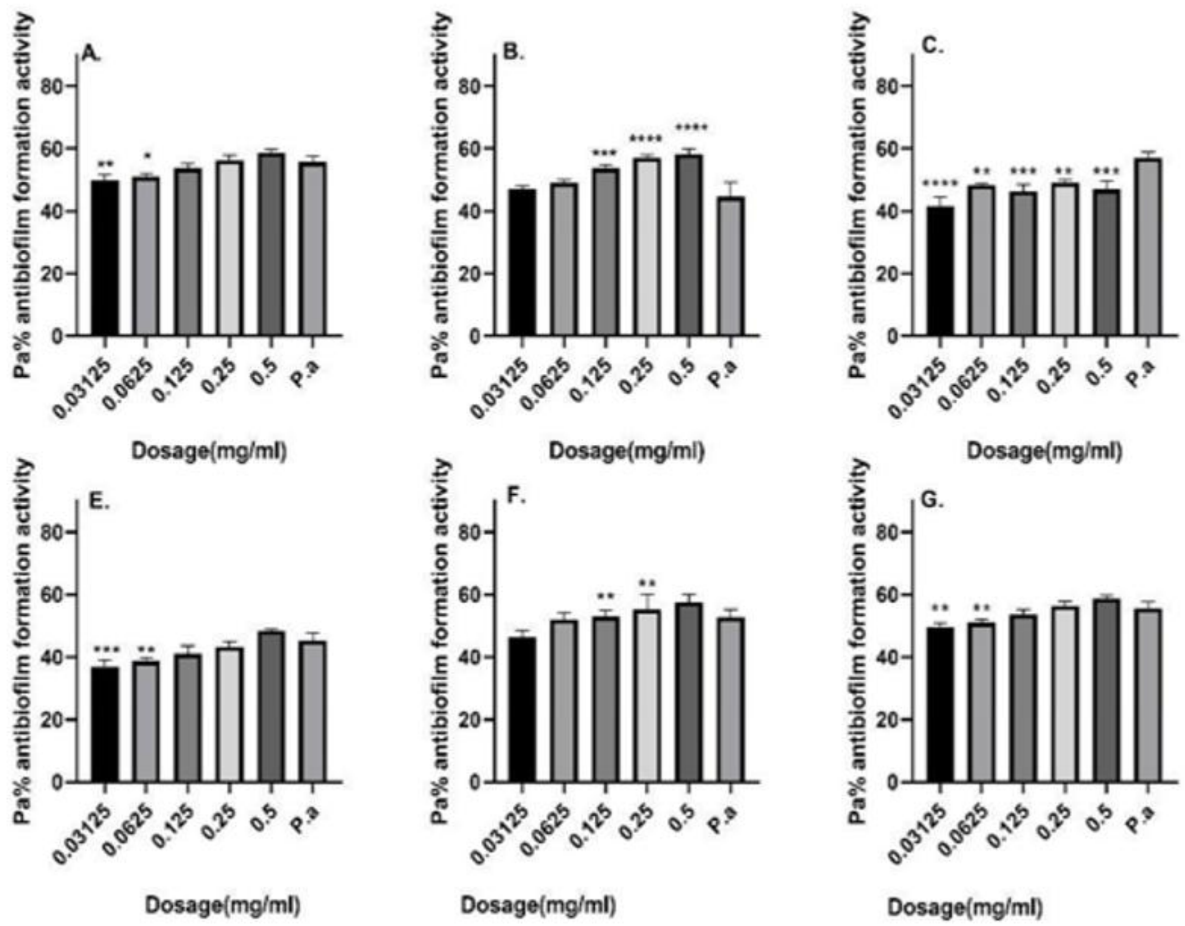

Figure 2: antibiofilm formation action against isolate MCH/05/20 of C. albicans in opposition to a range of antifungals

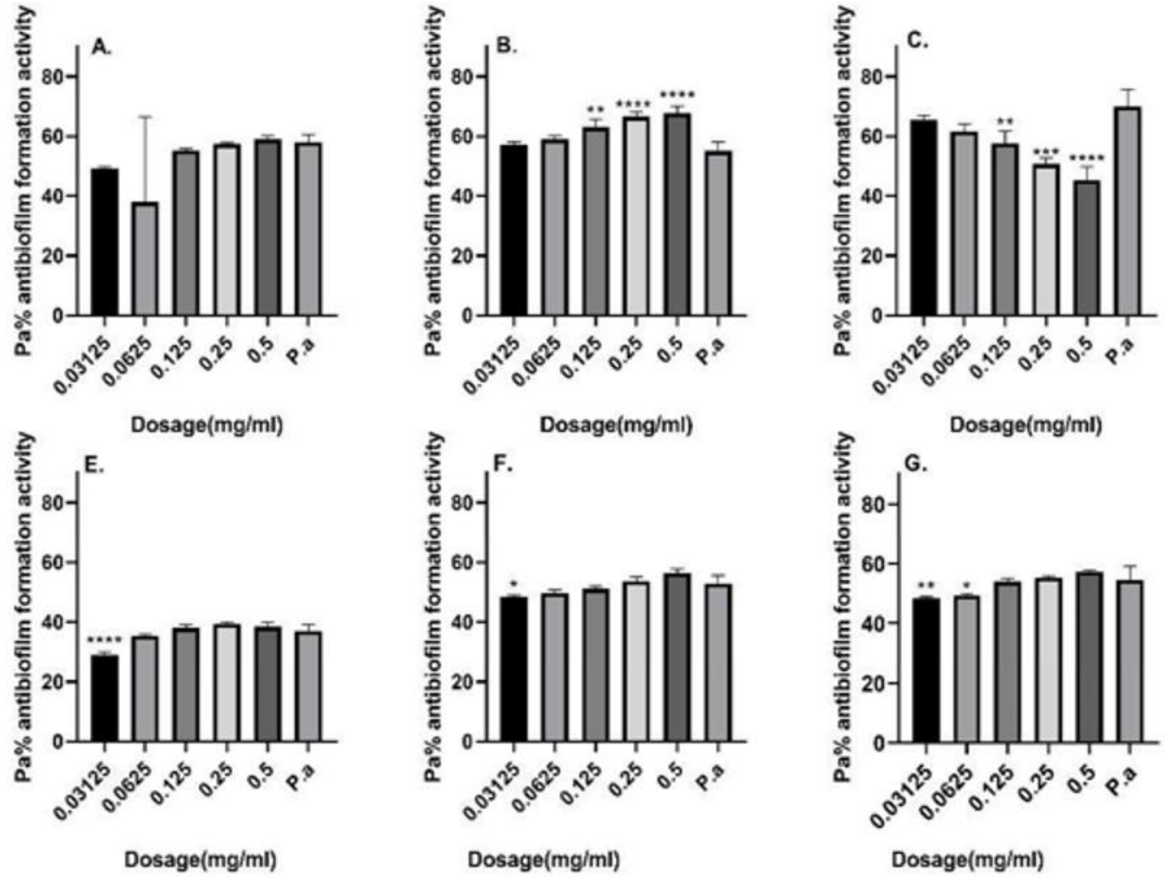

Figure 3: antibiofilm formation action against isolate MCH/16/20 of C. albicans against various antimycotic agents

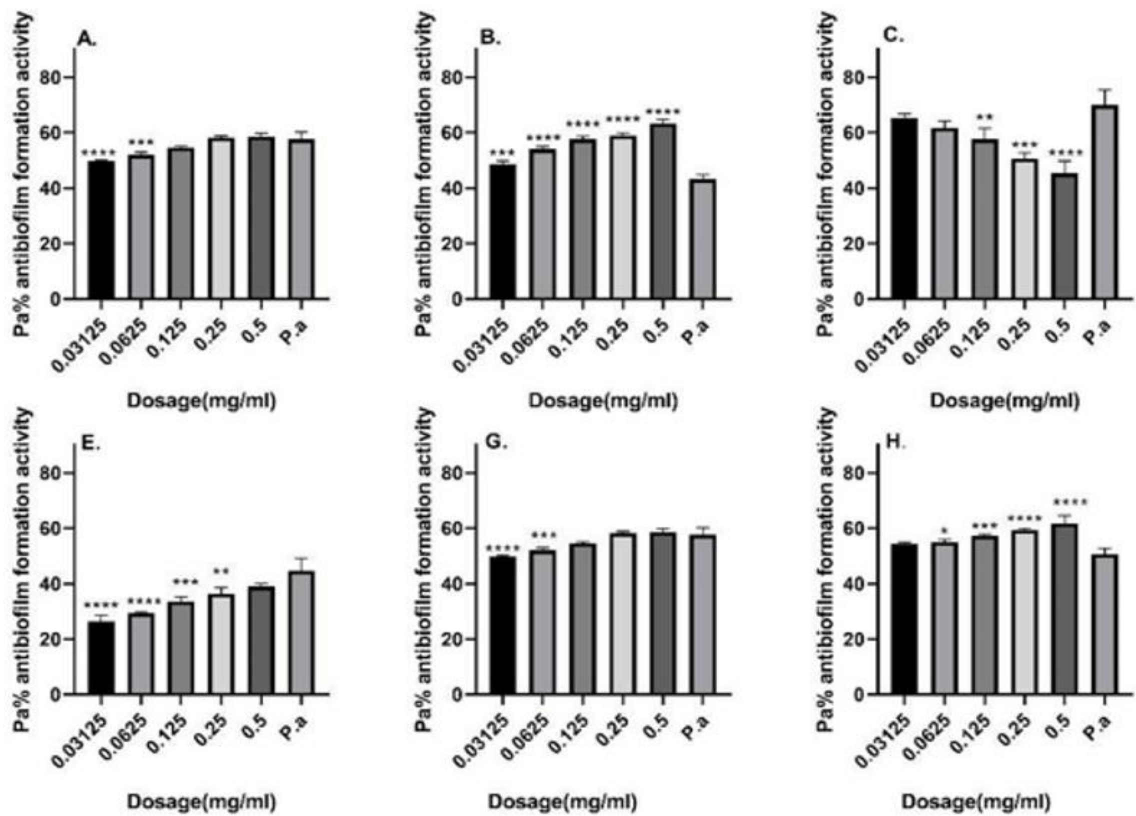

Figure 4: antibiofilm formation action against isolate MCH/47/20 of C. albicans against various antifungals

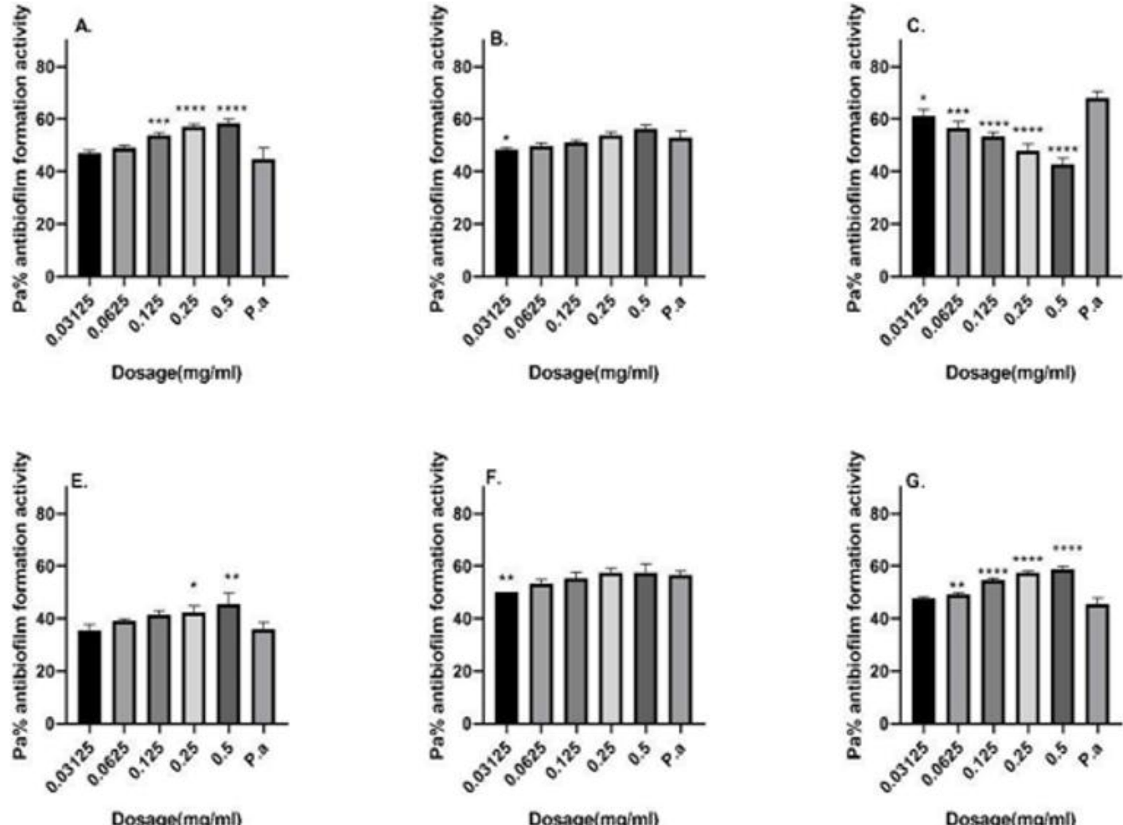

Figure 5: antibiofilm formation action against isolate MCH/75/20 of C. albicans against various antifungals