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RESEARCH ARTICLE

ANTIFUNGAL EVALUATION OF MULTIDRUG-RESISTANT NON-DERMATOPHYTIC MOULDS OF BOVINE ORIGIN

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ABSTRACT

Background and aim: Fungi are eukaryotic, non- chlorophyllous saprophytic organisms that are ubiquitous in nature. They are observed to cause infections called mycosis, especially in animals for which antifungals are usually recommended. Though, unproductive treatment kept increasing, it leads to drug resistance or tolerance. In this study, the antifungal activities of four oxoid brand conventional antifungal drugs were evaluated for multi drug resistance on some selected fungal isolates recovered from bovine sources.

Methods: Fungal isolates were recovered from bovine and cultured using standard techniques, identified by direct microscopy and molecular techniques. Susceptibility test was evaluated using disc diffusion method. The oxoid brand discs of Ketoconazole 10 µg/ml, Fluconazole 25 µg/ml, Voriconazole 1 µg/ml and Amphotericin B 20 µg/ml were used for the analysis, incubated at 28 °C and zone of inhibition measured after 3-5 days.

Results: Seven fungal genera were identified from the bovine skin samples, out of which eleven species were used for susceptibility test. Ketoconazole was highly effective against some fungal isolates such as *Penicillium citrinum, Fusarium succisae, Cladosporium tenussimum, Curvularia kusanol, Fusarium solani*, Voriconazole could not clear the moulds tested completely and were tagged to be resistant mutant, all moulds tested were resistant to Fluconazole while Amphotericin B showed minimal inhibitory zones on the moulds.

Conclusion: This study has shown that several fungal isolates respond differently to antifungal drugs. The different non-dermatophytic moulds tested were highly susceptible to Ketoconazole when compared with other antifungals employed in this study and as such may be best recommended for the treatment of infections caused by these moulds.

Keywords: Bovine skin samples, fungal isolates, sensitivity, resistance, oxoid antifungals

INTRODUCTION

Fungi are pure environmental contaminants that are widely present in soil, air, and water infections called dermatomycosis [1]. These are superficial infections caused by a group of fungi called dermatophytes and non-dermatophytes such as *Trichophyton* species, *Microsporum* species, *Aspergillus* species, *Rhizopus* species and *Fusarium* species. These group of fungi have affinity for the surface keratin layer of keratinous tissues [2]. These infections can range from superficial, cutaneous, sub-cutaneous to systemic diseases. Recent studies have reported that non-dermatophytic moulds can replace dermatophytes in causing dermatophytosis [1,3,4]. Dermatophytosis caused by *Trichophyton floccosum*, *T. rubrum*, *T. mentagrophytes*, *Blastomyces dermatitidis*, and *Microsporum canis* are very common because it affects the skin of both immune competent and immune compromised hosts (man and animal) [4]. The fungus penetrates the host skin with its hyphae thereby secreting a wide range of lytic enzymes such as keratinase, protease, and lipase which act as a virulent factor [5]. In bovine breeding, skin infections are one of the biggest challenges faced by farmers, which may be due to the colonization of cattle skin by these fungi.

This colonization could be attributed to its close contact with the soil. The destruction of bovine hides also causes huge economic losses to farms and reduces the quality of raw hides required for processing industry and consumption [3, 6]. Conventional antifungal drugs with fungicidal and fungistatic mode of action usually recommended for treating these fungal infections have been shown to selectively eliminate fungal pathogens on the host with minimal or no toxicity [7]. They possess different mechanisms of actions including acting on the nucleic acids, cell membrane, processes of cell division or cell wall. The polyenes antifungals interact with sterols in cell membrane to form pores through which ions, small molecules and fluid in the cell leaks example Amphotericin B. Azoles inhibits the production of ergosterol which is the active component of the plasma membrane of the cell membrane. Azoles are grouped into two imidazole example ketoconazole, clotrimazole and triazoles example itraconazole, fluconazole. Allylamines inhibit the enzyme squalene epoxidase which also affects ergosterol biosynthesis example butenafine, amorolfine. Echinocandins inhibits beta-1-glucan synthase which is also necessary for fungal cell wall formation example Amphotericin B, Caspofungin [8].

In recent times, some of these fungi tend to express tolerance/ resistance to these antifungals which could be attributed to long term use, abuse or due to its associated side effects. At times overlap in mechanism of action of these drugs might also lead to phenotypic expression of multidrug resistance (MDR) by these fungi which invariably leads to treatment failure [8]. Microbial resistance means the ability of the fungus to withstand the inhibitory actions of the antifungals. There have been many studies on the effectiveness of antifungal agents against dermatophytes [9,10,11,12], but studies on non-dermatophytes are less extensive. Therefore, this study addressed this question by evaluating the phenotypic expression of some selected conventional antifungal agents against some non-dermatophytic moulds of bovine origin of the skin.

MATERIALS and METHODS

***Collection of samples*:** The surface areas of the skin of the bovine showing signs of lesion were cleaned with swab pad dipped in alcohol. With a sterile scalpel, the sample was collected by scrapping the area gently

and this was placed in a sterile white envelope. These envelopes were labelled accordingly.

***Isolation and identification of fungal isolates*:** Skin scrapings were spread on sterile Sabouraud dextrose agar (SDA) plates which was supplemented with chloramphenicol (0.05 mg/l) to inhibit bacterial growth and was incubated at 28 °C for 3- 5 days. Isolates were identified by colonial morphology, microscopic examination using direct microscopy and slide culture techniques and characterised molecularly using DNA polymerase chain reaction (PCR) and sequencing. Direct microscopic examination was carried out by placing a small piece of the cattle skin sample on a drop of 10% KOH on a microscopic slide which was covered with a coverslip. It was examined under the microscope using x10 and x40 objectives. Both arthrospores and hyphae were checked for and note was taken of whether infection was located within or outside.

For slide culture technique about 4mm square block of Sabouraud dextrose agar was cut and placed on a sterile glass slide after which a small fragment of the pure culture of isolates were sub-inoculated on the four edges of the block. The inoculated glass slide was covered with a coverslip and the whole slide was placed in a petri dish which was supported under with sterile glass slide. This was incubated for two weeks at room temperature. When there was clearly visible growth the block was discarded in a disinfectant and the coverslip was removed and placed on a drop of lactophenol cotton blue. on a new slide while a drop of lactophenol cotton blue was added to the growth on the slide and covered with a new coverslip. Both preparations were examined microscopically using x10 and x40 objectives.

For DNA polymerase chain reaction, the ITS region of the rRNA genes of the isolates were amplified using the ITS1F:5'-CTTGGTCATTTAGAGGAAGTAA 3' and ITS4: 5' TCCTCCGCTTATTGATAT GC-3'. Primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40µl for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix, the primers at a concentration of 0.8µM and the extracted DNA template. The PCR conditions were as follows: Initial denaturation, 95 ºC for 5 min; denaturation, 95 ºC for 30 seconds; annealing, 53 ºC for 30 s; extension, 72 ºC for 30 seconds for 35 cycles and final extension, 72 ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 25 min and visualized on a blue transilluminator.

***Sequencing*:** Sequencing was carried out using the Big Dye Terminator kit on a 3510 ABI sequencer. The sequencing was observed at a final volume of 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 10 µM Primer PCR primer, 2.25 µl of 5 x BigDye sequencing buffer and 2-10 ng PCR template per 100bp. The condition for sequencing were as follows 32 cycles of 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min.

***Phenotyphic and susceptibility testing of antifungal agents*:** The chemotherapeutic agents which includes Ketoconazole, Fluconazole, Voriconazole and Amphotericin B were tested on the isolates using Kirby Bauer disc diffusion method. A cell suspension of the organisms' equivalent to 0.5% Mcfarland standard was employed [13].

***Inoculum Preparation*:** Colonies of fungi were selected from 5 days to 1week old agar plate culture depending on the fungi maturation. The top of each colony was touched with a sterile loop, and the growth was transferred into a tube containing 4 ml of

normal saline and compared with 0.5% McFarland standard. The turbidity was adjusted with sterile saline to obtain a turbidity optically comparable to that of the 0.5% Mcfarland standard. Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, 0.1 ml of the suspension was dispensed on the dried surface of the Sabouraud dextrose agar plates supplemented with chloramphenicol. The dispensed inoculums on the dried surface of the Sabouraud dextrose agar plates were evenly spread on its surface using a hockey stick [14].

***Application of discs to inoculated agar plates*:** The oxoid brand antimicrobial discs made in United Kingdom (Ketoconazole 10 µg/ml, Fluconazole 25 µg/ml, Amphotericin B 20µg/ml, Voriconazole 1 µg/ml) were dispensed onto the surface of the inoculated agar plates. Each disc was pressed down to ensure complete contact with the agar surface. Drug diffuses almost instantaneously; a disc was not relocated once it comes in contact with the agar surface. The plates were inverted and placed in an incubator set to 28 °C within 15 min after the discs were applied. After 3-5 days of incubation depending on the fungi proliferation, each plate was examined for zone of inhibition. The susceptibility of the isolates was based on the break point of some drugs used against non- dermatophytes (for disc: Sensitive ≥ 19 mm, susceptibility test dependance (SDD) = 15- 18 mm, and 16- 32 µg/ml of drugs used was taken as susceptible, R≤ 14 mm).

RESULTS

A total of fifteen samples (30%) were collected from the head region, ten (20%) from the body, twenty (40%) from the leg region and five (10%) from the tail region. The fungal isolates occurrences and their distribution were classified into seven different genera as follows: *Penicillium* spp. (10), *Aspergillus* spp. (25), *Fusarium* spp. (15), *Curvularia* spp. (5), *Cladosporium* spp. (10), *Pestalotiopsis* spp. (5) and *Absidia* spp. (10). These isolates were further subjected to antifungal sensitivity screening using Clinical and Laboratory Standard Institute standard.

Four antifungal drugs which includes Ketoconazole (10 µg/ml), Voriconazole (1 µg/ml), Fluconazole (25 µg/ml) and Amphotericin B (20 µg/ml) were tested on eleven specific characterised non-dermatophytic moulds which include *Penicillium citrinum*, *Aspergillus fumigatus*, *Aspergillus welwitschiae*, *Aspergillus aculeatus*, *Fusarium succisae*, *Curvularia kusanol*, *Cladosporium tenuissimum*, *Pestalotiopsis microspora*, *Fusarium solani*, *Fusarium lichenicola* and *Absidia* species. Table 1 shows the results from the antifungal drug patterns. Figure 1 shows the phenotypic descriptions from the antifungal drug patterns (resistant mutant A, resistant B, highly sensitive C and mildly sensitive D).

DISCUSSION

Bovine is a major source of protein to the world's populace; its skin is used in preparing a stable delicacy ("kpomo") in different parts of Nigeria. It can also act as a source of skins and hides for production of leather and wool in the production industries. Damage to the skin can also lead to a lot of economic losses in the farm. In this study fungal isolates associated with bovine skin lesions were evaluated and results revealed area showing lesions were encountered more from the leg region followed by the head and then the body region, this could be attributed to the fact that these animals lay and roll on the ground. For the fungal isolates, *Aspergillus* species were more prevalent, followed by *Penicillium* species. This could be related to

the fact that these organisms are widely disseminated in the environment reported by [15,16].

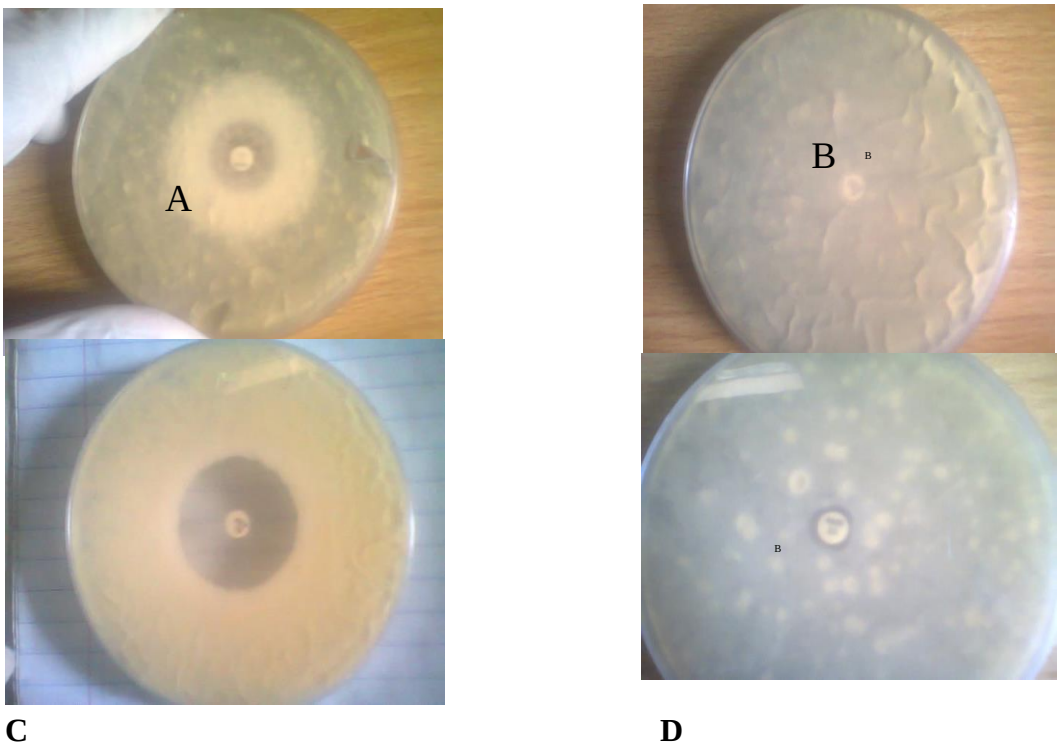

Figure 1: Ketoconazole on *Fusarium succisae* (A), Voriconazole on *Aspergillus welwitshiae* (B), Fluconazole on *Aspergillus fumigatus* (C) and Amphotericin-B on *Aspergillus fumigatus* (D)

Table 1: Antifungal sensitivity test on the non-dermatophytes (RM = resistant mutant)

Fungal isolates	**Antibiotics (zone of inhibition, mm)**			
	Ketoconazole	Voriconazole	Fluconazole	Amphotericin B
Penicillum citrinum	30	0	0	0
Aspergillus fumigatus	10	16^{RM}	0	10
Aspergillus welwitschiae	18	20^{RM}	0	9
Aspergillus aculeatus	0	30^{RM}	0	0
Fusarium succisae	30	20^{RM}	0	12
Curvularia kusanol	45	40^{RM}	0	7
Cladosporium tenussimum	53	35^{RM}	0	0
Pestalotiopsis microspora	34	10^{RM}	0	15
Fusarium solani	50	0	0	0
Fusarium lichenicola	40	0	0	12
Absidia spp.	10	0	0	7

In their report they stated that isolation of *Aspergillus* and *Penicillium* species were 44% and 60.9% respectively, when they evaluated antifungal activity against non-dermatophytic moulds causing onychomycosis.

In our study it is very significant to note that all non-dermatophytic moulds were completely resistant to Fluconazole which agrees with a similar study carried out by Keyvan [16], whereas Amphotericin B expressed very minimal inhibition zones on seven isolates followed by a complete resistant expression by other isolates. The minimal sensitivity expressed by these isolates against Amphotericin B, could be attributed to the fact that the drug composition might not be strong enough to clear the isolates. It is also an indication that sensitivity tests are very important as this will help in selecting the right choice of drug in treating some of these infections caused by these fungi since several fungus tends to respond differently to various drugs. In the case of Voriconazole, the drug was only able to inhibit the sporulation of the isolates without clearing the mycelium strands. This could be attributed to the fact that the isolates might have acquired some genetic characteristics that made them difficult to be completely eliminated or the drug concentration was low since its just 1ug/ml. Our study is partially in line with another study that found that *Aspergillus* isolated from skin samples was highly susceptible to Ketoconazole, Voriconazole, Fluconazole, and Terbinafine [17]. Another study showed that Amphotericin B and Clotrimazole were the most sensitive antifungal drugs against all moulds except *Fusarium* [18]. The later contradicts our results.

In another study Voriconazole expressed high efficacy against *Aspergillus* and *Fusarium* [19] yet in another study [20], Voriconazole showed excellent activity against dimorphic fungi and opportunistic moulds (*Aspergillus* spp., *Fusarium* spp.) *in vitro*, these findings were in contrary to our results.

Stainslaw *et al* [17] and Pearce *et al* [21] reported that Voriconazole and Miconazole were more effective against *Aspergillus* spp. and *Fusarium* spp. when compared with Fluconazole and Ketoconazole. Emenuga and Oyeka [2] also reported that Fluconazole was more effective against *Fusarium* spp. and *Aspergillus* spp. than Ketoconazole. These findings were in contradiction with our study. Moreover, our findings are in agreement with that of Brooks *et al*. [22]. In their work, Ketoconazole was more sensitive than Fluconazole against fungal isolates (*Aspergillus* spp., *Fusarium* spp., *Penicillium* spp.) obtained from horses with ulcers in Florida. Keith *et al*. [23] also showed that Amphotericin B was very effective against *Curvularia* spp. isolated from clinical samples, while Voriconazole was not. This result was also consistent with our findings.

Treating bovine skin lesions is necessary to prevent secondary bacterial infection, reduce cosmetics damage and drastic economic losses in the farm and also maintain and preserve healthy skin for industrial purposes. The choice of drug could be determined by different reasons such as the degree of severity of the lesion, the causative agent or due to success or failure of previous treatment. Selecting the right drug of choice will also help reduce abuse and long-term use of drugs.

CONCLUSION

This study has shown that different fungal isolates expressed specific antifungal activity against a variety of antifungals. Based on our

analysis, Ketoconazole was the most sensitive drug against non-dermatophytic moulds. and is recommended for the treatment of infections caused by these moulds. It cannot eliminate the fact that resistance to Ketoconazole might arise in subsequent studies. Nevertheless, the weak and complete resistant activity of the moulds against Amphotericin B, Voriconazole and Fluconazole is not a final revelation that most of these drugs may not be the drug of choice for the treatment of fungal diseases or mycosis from the bovine origin, especially Fluconazole in some areas.

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