Prevalence of Pulmonary Mycotic Lesions and Antifungal Susceptibility Pattern of Fungi Isolated from Nigerian Trade Cattle

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ABSTRACT

Pulmonary mycosis is a major cause of deaths in many livestock herds, especially in immune-compromised animals. A total of 138 lung samples (6mm in size) were collected from cattle slaughtered for meat at Nsukka abattoir. Swab sticks were dipped into each lung sample and streaked on freshly prepared Sabouraud Dextrose Agar (SDA) for fungal isolation. Isolated fungi were identified using morphological and microscopic features. Disc diffusion method was used to test the antifungal susceptibility pattern of a randomly selected isolates with voriconazole (1µg), fluconazole (25µg) and methanol extract of *Garcinia kola*, using disc diffusion method. The lung samples were examined for lesions using histopathological procedures. Out of the 138 lung samples, 23 had fungal growths while 115 had no growth of any sort. A total of 34 fungi were isolated as pure cultures. *Aspergillus spp.* had the highest frequency of isolation, 44.92%, followed by *Penicillium spp.* 11.76%, *Trichophyton spp.* 5.88%, *Conidiobolus* and *Sporothrix* species had 2.94% isolation rate each. About 29.6% of the isolated fungi could not be identified. Fungal positive lungs showed acute to sub-acute inflammatory responses with bronchiolar septal, alveolar interstitium and peribronchial inflammatory cell infiltration and thickening of the lung tissues. All the screened fungal isolates were sensitive to voriconazole with the zone of inhibition ranging from 12-27mm. The fungal isolates were all resistant to fluconazole and *G. kola* extract, except one that showed sensitivity to the extract. Voriconazole showed strong antifungal effect on all the fungi; but the efficacy of the *G. kola* on the fungal isolates was very low compared to voriconazole.

Key words: Fungi, Fluconazole, Cattle, Lesions, Mycosis, Pulmonary, Voriconazole.

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INTRODUCTION

In recent times, the livestock industry has experienced a huge rise in development. Due to the demand for meat and other livestock products, the sector has contributed to annual revenue of \$1.4 trillion in most developing countries. The livestock industry is a major source of employment generation in many countries, and it is also a source of livelihood to over 600 million people at a global level (Thornton et al. 2006; Thornton 2010).

In Nigeria, there are over 15.3 million cattle comprising of different breeds (Kubkomawa 2017; Ovharhe et al. 2020). Some of the popular breeds indigenous to Nigeria include *Sokoto Gudali*, *Muturu*, *Adamawa Gudali*, *Wadara*, *Azawak*, *Keteku*, *White Fulani*, *Red Bororo*, *Ndama* and *Kuri* (Babayemi et al. 2014; Kubkomawa 2017). Cattle provides animal proteins, beef, milk, hides and skin for many households, in most cases,

they are used for traction in some parts of the Nigeria (Kubkomawa 2017). However, cattle production in Nigeria is faced with challenges such as diseases, inadequate nutrition, lack of improved breeds, poor marketing strategies, structural problems, and social factors (Yesuf et al. 2012).

Pulmonary mycoses are important from public health and economic point of view as they lead to high mortality especially in immune-compromised animals and can be of great zoonotic importance. Although mycotic pneumonia is rare in cattle, it can be caused by *Coccidioides immitis*, *Aspergillus species* and other opportunistic fungal pathogens (Panciera and Confer 2010). In cattle, mycotic pneumonia has been associated with lesions such as epithelial metaplasia of the airways, bronchitis, bronchiectasis, interstitial fibroplasia, as well as bronchiolitis obliterans (Ramírez-Romero et al. 2012). The importance of fungal infections in both humans and

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animals have been emphasized in some studies due to the rising interest in the diseases caused by fungi (Seyedmousavi et al. 2018). In healthy animals with intact immunity, fungi rarely cause diseases, however, opportunistic fungal pathogens can cause infections in immunocompromised animals (Seyedmousavi et al. 2018; Imran et al. 2020). Despite improvements in therapeutic options and new diagnostic tools, fungal infections are major cause of mortalities observed in cattle (Alastruey-Izquierdo et al. 2015). Therefore, it is important to initiate the correct antifungal therapy for a better treatment outcome (Patel et al. 2009). However, the problem of fungal resistance to the available antifungal drugs is a huge challenge to treatment of fungal diseases. This situation has been attributed to indiscriminate use of antifungal agents, coupled with the increase in opportunistic fungal infections. Resistance to antifungal therapy can occur due to clinical or microbiological dynamics. When a fungal infection is treated with the optimal dosage of the appropriate antifungal drug and the infection persists, then the infection is said to be clinically resistant to the therapeutic agent (Sanglard 2011). Careless use of antimicrobials has resulted in microbial resistance with high level of morbidity and mortality in animals (McEwen and Fedorka-Cray 2002; Feifei et al. 2020). Various cases of antimicrobial resistance reported in humans have been traced to resistant microbes that are thought to have originated from livestock (Paphitou 2013; Mamun et al. 2017). As one of the strategies to stem this rising challenge, efforts are being made to tap into medicinal plants as the alternative to the conventional antimicrobial agents.

Garcinia kola (bitter kola) is a medicinal plant with high economic value, and it is commonly found in countries in West Africa. The medicinal value of the plant has been found to reside in the seeds, nuts, fruits, and bark; and each part has been demonstrated to be effective in the treatment of diseases (Okoli 1991). Dimeric flavonoid molecules fused together with biflavonoid, xanthenes and benzophenones have been shown to constitute the bioactive constituents of the plant (Seanego and Ndip 2012). *G. kola* is eaten in most homes in suspected cases of consumption of bacteria-contaminated food, an indication that the plant has antibacterial property (Icheke et al. 2018). The antimicrobial, anti-inflammatory, antiviral, antidiabetic, and antioxidant properties of *G. kola* is thought to be due to the phenolic compounds present in the plant (Oluyemi et al. 2007; Emily et al. 2020). The alcoholic extract of *G. kola* has been reported to show inhibitory activities against numerous fungi and bacteria (Ezeifeke et al. 2003). The antifungal activity of *G. kola* has been attributed to the presence of hydroxybiflavonoids (Seanego and Ndip 2012). Exploring the antimicrobial properties of this plant in the treatment of pulmonary mycosis will help to reduce the burden posed by resistance to the available antifungal agents.

The present study investigated the prevalence of pulmonary mycosis and its attendant lesions in cows slaughtered for meat at Nsukka Abattoir in Enugu State, Nigeria. The study also compared the antifungal pattern of two conventional antifungal agents (fluconazole and voriconazole) with the medicinal plant, *G. kola* on the isolated fungal agents.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the institutional head of the Faculty of Veterinary Medicine University of Nigeria, Nsukka and the findings from the study were reported with strict compliance to the procedures outlined in ARRIVE guidelines for reporting in vivo experiments in animal research (Karp et al. 2015).

Sampling and Data Collection

A total of 138 lung samples of slaughtered cattle were examined by palpation before collection for fungal and histopathology investigations. Collected samples for mycological investigation were placed in polythene bags, labelled appropriately, and transported in a cooler with ice to the Laboratory for further processing.

Fungal Isolation and Identification

Smears of each lung sample was streaked evenly on a freshly prepared Sabouraud Dextrose Agar (SDA). The SDA plates were incubated at room temperature (25-30°C) for 3-4 weeks. Fungal growths were sub-cultured on fresh SDA plates for purification and identification according to Sephavard et al. (2009).

Further identification using microscopic features was done following the methods by Aneke et al. 2018. The fungi were classified into genus and species levels using both morphologic and microscopic features (Ellis et al. 2000; Baron et al. 2003). Each fungal isolate was stocked on fresh SDA for further analysis.

Methanol Extraction of *Garcinia kola*

The fresh seeds of *G. kola* were purchased from Ogige market, Nsukka and identified at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The seeds were dried under open air for two weeks and crushed using electric blender. The crushed samples (50g) were poured into an extraction chamber (Soxhlet extractor) connected to a 1L round-bottom flask and a condenser. The extraction of the active constituents of the plant was done with 300mL of methanol added to the Soxhlet extractor. The extraction time was 6 h. A rotary evaporator operated under reduced pressure at 45°C was used to evaporate the solvent (methanol) after the extraction of the plant's bioactive component.

Antifungal Activity of the Methanol Extract of *Garcinia kola*

The disc diffusion procedure as described by Bauer et al. (1966) was employed for the antifungal susceptibility test. Two antifungal agents; fluconazole (25µg) and voriconazole (1µg) purchased from Oxoid®, and *G. kola* extract (50µl) were used for the antifungal susceptibility test. The assay was done using 3-5 days old cultures of the fungal isolates. About 3mm agar plugs of each randomly selected fungus was put a sterile test tube containing 5ml distilled water. The sample was properly vortexed after hand shaking and allowed to stand at room temperature for 15 min. The supernatant was transferred to a fresh test tube to harvest the fungal spores; this was adjusted to 0.5 McFarland's standards (1.5 x 10⁸ spores per ml) using normal saline (McFarland 1907). The spore preparation was spread gently and evenly on the surface of freshly

prepared SDA and allowed to dry. Antifungal discs containing fluconazole (25µg/ml) and voriconazole (1µg/ml) were placed on the surface of the plates containing the fungal spores. Approximately 50µl of *G. kola* extract was applied into 3mm holes bored with sterile borer on each agar plate and the plates were incubated at room temperature for 3-10 days. The zones of inhibition were recorded in millimeters.

Histopathological Analysis

Lung samples thought to have lesions were fixed in 10% neutral buffered formalin. The fixed tissues were dehydrated in graded alcohol and stained with hematoxylin and eosin (Bancroft and Gamble 2002). The slides were viewed with light microscope and photomicrographs of the lesions were taken using a Motic Images plus 2.0 digital cameras (Motic China Group Ltd. 1999-2004).

Statistical Analysis

The data from the fungal assay and identification were analyzed using descriptive statistics and were presented as percentages.

RESULTS

Fungal Isolation

One hundred and thirty-eight (138) lung samples were cultured on SDA, but only 23 samples had fungal growths. Thirty-four fungal isolates were obtained after sub-culturing to get distinct cultures (Fig. 1), some of the lung samples had more than one fungal growth. The pure cultures were matched with pictures of fungal cultures from previous works and were identified further using microscopic structures (Fig. 2). Eight *Aspergillus* spp. were identified, four *Penicillium* spp., two *Trichophyton* spp., one *Pythium* spp., one *Conidiobolus* spp., one *Sporothrix* spp. were also identified. Others could not be identified. The *Aspergillus* spp. had the highest rate of occurrence 44.92%, followed by *Penicillium* spp. 11.76%, *Trichophyton* spp. 5.88%, *Conidiobolus* spp., and *Sporothrix* spp. had 2.94% rate of occurrence each. The unidentified fungi had 29.41% rate of occurrence.

Antifungal Sensitivity Test

The fungal isolates were screened with two antifungal agents (Voriconazole and Fluconazole) and a methanol extract from *G. kola* to ascertain their antifungal susceptibility patterns. Voriconazole had the highest activity against the fungal organisms, followed by the *G. kola* extract (Table 1). Fluconazole had no activity as the organisms were resistant to the drug. All the fungal isolates screened for antifungal susceptibility were sensitive to voriconazole, with the zone of inhibition ranging from 12-27mm (Table 1). The fungi were all resistant to fluconazole, while only one isolate was sensitive to the *G. kola* methanol extract. All the *Aspergillus* species screened in this study showed sensitivity to voriconazole but the zone of inhibition of each isolate was variable (Table 1).

Histopathology

Most of the analyzed tissues showed no observable histopathological lesions. The few sections of fungal positive lungs showed acute to sub-acute inflammatory response (acute necrohemorrhagic lesions) comprising of

Fig. 1: Morphological presentation of some fungi isolated in this study. Smears of lung sample were cultured in SDA and sub-cultured in fresh SDA after 1-4 weeks incubation, (a-c) *Aspergillus* species (d) Unidentified.

Fig. 2: Microscopic features of a selected fungal species identified in this study. Microscopic examination was done using the lactophenol cotton blue and wet mount methods, (a-d) *Aspergillus* species.

neutrophils and macrophages with few lymphocytes (Fig. 3). Bronchiolar septal, alveolar interstitium and peribronchial inflammatory cell infiltration and thickening were very prominent (Fig. 4). These were more common in the *Aspergillus* infected lungs. The lungs with negative fungal growths showed normal lung tissues with typical peribronchial lymphoid cells (Fig. 5).

DISCUSSION

Although infection of cattle by pathogenic fungi is self-limiting, they are however the cause of most contagious diseases in livestock. Pulmonary mycosis is a common disease of livestock in the tropics and by extension,

Table 1: The antifungal susceptibility pattern of randomly selected fungi screened in this study

Fungal Isolate	Voriconazole Diameter (mm)	Fluconazole Diameter (mm)	<i>Garcinia kola</i> Extract Diameter (mm)
<i>Aspergillus species</i>	16	R	R
<i>Sporothrix schenckii</i>	21	R	R
<i>Conidiobolus species</i>	21	R	R
<i>Aspergillus species</i>	14	R	R
<i>Aspergillus species</i>	14	R	R
<i>Aspergillus species</i>	25	R	R
<i>Aspergillus species</i>	27	R	R
<i>Aspergillus species</i>	17	R	R
<i>Aspergillus species</i>	22	R	R
<i>Aspergillus species</i>	12	R	14

Fungal spores harvested from 3-5 days old culture were evenly spread on freshly prepared SDA. Antifungal discs containing fluconazole (25µg/ml) and voriconazole (1µg/ml) were placed on the surface of the inoculated plates, 50µl of *Garcinia kola* extract was put into 3mm hole made with sterile borer on the media and the plates were incubated at room temperature for 3-10 days for measurement of zone of inhibition. R=resistance. Zone of inhibition measured in millimeter as the diameter of the area where the drugs prevented the growth of the fungi.

Fig. 3: Photomicrograph of the fungal positive lungs showing alveolo- interstitial hemorrhages, bronchiolar septal, alveolar interstitium and peribronchial inflammatory cell infiltration (Thin arrows) and thickening X10, H&E. Inset-Inflammatory cells which were mainly Lymphocytes, neutrophils and macrophages.

Fig. 4: Photomicrograph of the fungal positive lungs showing interlobular septal oedematous thickening (thin arrows), alveolar interstitium infiltration by inflammatory cell infiltration (A) and Emphysema (Thick arrows) X10 H&E

a public health issues. In the present study, we investigated the presence of mycotic organisms in the lungs of cattle brought for slaughter in Nsukka Abattoir of Enugu State.

Fig. 5: Photomicrograph of the fungal negative lungs showing normal features with typical peri-bronchial lymphoid cells (arrows). H&E X40.

We also screened for the antifungal susceptibility pattern of the isolated fungal agents against two conventional antifungal agents and a methanol extract of *G. kola*. *Aspergillus* species had the highest rate of occurrence (44.92%) in all the lung samples screened. *Aspergillus* species are respiratory pathogens responsible for infection in livestock. The genus *Aspergillus* causes a mycotic disease known as aspergillosis. Some known species implicated in aspergillosis in animals include *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. glaucus*, and *A. nidulans*. *A. fumigatus* is a life-threatening opportunistic pathogen (Arne and Deville 2010). The spores of *A. fumigatus* (conidia) if inhaled into the lungs of immunocompromised animals may cause pulmonary aspergillosis. Aspergillosis manifests itself as pulmonary infections in birds (Arne and Deville 2010), mammary gland infections and mycotic abortion in cattle (Puntenney et al. 2003) and guttural pouch mycosis in horses (Dobesova et al. 2012). In a similar study, Chihaya et al. (1991) reported 52.6% isolation rate of *Aspergillus* species from the lungs samples of infected cattle. *Penicillium*, *Conidiobolus species* and *Sporothrix species* cause infections in animals and humans, especially when the immune system of the animal is compromised, therefore they could be health risk to the animal handlers (Chan et al. 2013; Schlecht et al. 2015). *Penicillium* also produces a

mycotoxin known as ochratoxin which has adverse effects to the liver of most livestock and domestic animals (Seifi et al. 2018).

Sporotrichosis is considered a neglected fungal disease of humans and animals. Animals are exposed to infected plant materials and soil, which can be considered as the source of infection (Rodrigues et al. 2014). *S. scenkii* has been found to have many clinical forms such as fixed cutaneous, mucocutaneous, lymphocutaneous, pulmonary, osteoarticular and disseminated forms observed in human beings (Bonifaz and Tirado-Sánchez 2017). However, there has been no literature support for pulmonary infection in cattle where it is known to cause a cutaneous infection. This may be because the disease is more serious in cats than other animal species (Marimon et al. 2007).

Trichophyton spp. had a prevalence rate of (5.88%) in the sampled lungs. Their presence in the lungs where no visible clinical signs of pulmonary mycosis were seen may be because this species causes a cutaneous form of mycosis (Dahl 1994). It is also possible that these group of fungi were contaminants in the samples screened in this study, as *Trichophyton* are mainly dermatophytes in most domestic animals. Pythiosis is a granulomatous fungal infection caused by the fungus, *Pythium insidiosum*. The disease has been reported in dogs, cats, horses, cattle and humans (Pier et al. 1994). The low isolation rate of this fungus in this study may be due to environmental factors and the rearing pattern of the animals.

The acute to sub-acute inflammatory response (acute necrohaemorrhagic lesions) and its disseminated localization as observed in the histological section of lungs with positive fungal growth is thought to be associated with the hematogenous route of spread (Jensen et al. 1992). This acute inflammation is an indication of early onset of the fungal agents and the gradual establishment of infection in the lung tissues. The bronchiolar septal, alveolar interstitium, peribronchial inflammatory cell infiltration and thickening observed in the lung tissues did not agree with granulomatous lesions and hyphae seen in previous works by (Jensen et al. 1990). This may be due to the fact that mycotic infections in this present study did not have a chronic course. Similar observation of bronchitis and bronchiectasis were reported by Ramírez-Romero et al. (2012).

The positive fungal lesions were more prominent in all the *Aspergillus* infected lungs which could be an evidence of pulmonary aspergillosis. *Aspergillus* infection is a major cause of deaths in immunocompromised animals (Puntenney et al. 2003). Dairy cattle in early lactation and animals in intense antimicrobial therapy are more predisposed to *Aspergillus* infections (Jensen et al. 1994). However, most of the cattle used in this study were normal or apparently healthy cattle, so it is expected that the observed lesions will be minimal as the animals were not in immune compromised states.

For the antifungal susceptibility test, it was not surprising to see the high activity of voriconazole over the two other antifungal agents. Voriconazole is a broad-spectrum antifungal agent, and it is a drug of choice for invasive aspergillosis (Johnson and Kauffman 2003; Cecil and Wenzel 2009; Rosowski et al. 2020). However, cases of strong resistance to voriconazole in invasive

aspergillosis caused by *A. fumigatus* species have been reported (Pieter et al. 2019). Voriconazole showed a large protective effect over Amphotericin B in a randomized, multicentre trial (Herbrecht 2002). The *G. kola* showed very low activity in comparison with voriconazole. In other studies, the diethyl ether extract and the N-hexane extract of *G. kola* showed low antifungal activity against *Candida albicans* (Ibedu et al. 2018). Furthermore, a comparison between the aqueous and the methanolic extracts of *G. kola* showed that the methanolic extract showed higher antifungal activity (Penduka et al. 2011). This may be because; methanol as an organic solvent, dissolves organic compounds in the *G. kola* thereby liberating the active ingredients required for antimicrobial activity (Penduka et al. 2011). The antifungal activity of *G. kola* was earlier reported to be due to its hydroxybiflavonoids and quercetin content (Ezeifeke et al., 2003; Seanego and Ndip 2012; Adamu et al. 2020). All the isolated fungal species showed resistance to fluconazole. In separate and independent studies, *Candida albicans* isolated from the lungs of cattle showed similar resistance to fluconazole (Pfaller et al. 2000). *Candida* resistance to fluconazole has been linked to the expression of MRR1 gene, which encodes a transcription factor capable of inducing fluconazole (FLZ) resistance in *Candida* species (Elora et al. 2018). However, all the fungi were sensitive to voriconazole. The *Aspergillus* species had variable zones of inhibitions on the voriconazole, an indication that each species responded differently to the antifungal agent.

Conclusion

In conclusion, different fungal species were isolated from the lungs of the screened cattle with *Aspergillus* being the most prevalent fungi in the sampled lungs. Voriconazole had a more efficacious effect than fluconazole but the efficacy of the *G. kola* on the fungal isolates was too low.

Author's Contribution

ACM conceptualized the study, designed it, supervised it and drafted the manuscript. AA did part of the laboratory work. ICU helped in the manuscript preparation. WE did the histopathological analysis. OIR helped with the histopathological work and also in the manuscript preparation.

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