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Research Article
Identification, Antifungal Susceptibility and Phylogenetic Comparison of Fungi in Poultry Environment in Nigeria

[1]A.C. Mgbeahuruike, [1]K. Aliyoo, [2]M. Karlsson and [1]K.F. Chah

[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria
[2]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden

Abstract
Objective: This study was conducted to investigate the fungal community structure in Nigerian poultry environments. **Materials and Methods:** In ten (layer and broiler) farms, samples were collected from drinkers, doors, feeders, floors, poles, roofs, walls and window nets. Fungal isolation was done on Sabouraud dextrose agar (SDA) followed by identification using morphological and microscopic features. The fungal identities were confirmed by sequencing the internal transcribed spacer region, followed by phylogenetic analysis. Antifungal susceptibility testing was done using nystatin (100 μg mL^{-1}), fluconazole (25 μg mL^{-1}) and voriconazole (1 μg mL^{-1}). **Results:** A total of 244 fungi were identified in all the locations. In the layers farm, 112 fungal isolates were identified, while 132 isolates were identified in the broiler farm. In all the poultry farms, *Aspergillus* and *Candida* species had the highest occurrence of 32.4 and 24.6%, respectively while other fungi (*Dematiaceous*, *Rhizopus*, *Penicillium*, *Mucor* and *Rhodotorula*) had 43% occurrence. For the locations, poles and window nets had the highest isolation frequency of 15.2% each. The roofs, feeders and floors had 14.3 and 13.1%, while other locations had 27% isolation rate. Phylogenetic comparison of the isolates showed that closely related fungi from different countries formed separate clades. *Candida* species were sensitive to the three antifungal agents with the zone of inhibition diameter ranging from 19.08-25.36 mm. All the *Aspergillus* species were resistant to fluconazole but were sensitive to nystatin and voriconazole. **Conclusion:** Different fungi were identified in this study and they were all susceptible to nystatin antifungal agent.

Key words: Antifungal agents, fungi, layers farm, poultry, fungal contamination

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Corresponding Author: A.C. Mgbeahuruike, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, PMB 420001, Nsukka, Nigeria

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The poultry industry comprises different species of birds, chickens, turkeys, ducks, geese, swans, pigeons, peafowl, ostriches, pheasants and other game birds[1]. In Nigeria, the industry has a revenue base of over ₦80 billion ($600 million) and it produces 650 000 megatons of egg and 290 000 megatons of meat annually[2,3]. Although, poultry may be the most commercialized and fastest growing sector in animal husbandry, it is faced with numerous challenges[2,4]. Raising birds under intensive system of management where birds are fed, densely stocked and reared in confined structures is crucial in attaining utmost production[5]. However, this system of production has its problems as microorganisms may easily accumulate in such buildings, posing serious health hazard to the birds. Fungal diseases pose serious health challenges to the poultry industry and its workers [6-9]. Several species of fungi have been identified as disease agents and contaminants of feed and poultry environments[10,11]. Contamination of poultry farms by fungi could occur through the use of mouldy litters or by introduction of day-old chicks that are carrying conidia from hatchery facilities. Furthermore, inappropriate bedding management, poor quality feedstuffs, or admission of outside air loaded with conidia could result in heavy contamination of poultry houses[12]. Microbial agents from feed, litter, droppings and other materials are easily accumulated in confined animal houses and they form a key part of environmental contaminants in facilities where animals are housed[5,8]. Additionally, the presence of warmth and humid conditions in such buildings encourage the growth and dissemination of fungal spores[13,14]. Chronic exposure to fungal spores produces allergic responses in birds resulting in illness and decreased productivity.

Fungi in a poultry environment could produce diseases in different ways; by invading, harming and destroying body tissues of the birds and by producing mycotoxins which result in diseases, immunosuppressive conditions and decreased productivity[10,15]. These conditions could consequently result in big economic losses. Birds are susceptible to fungal infections due to the anatomy of their respiratory and nervous systems and poor serum antifungal action[16]. Also, the nature of the gastrointestinal tract of birds plays a vital role in fungal growth[17,18]. Fungal infections are triggered in birds by factors such as malnutrition, poor ventilation, vaccination, long term corticosteroid administration and immune status of birds[9,19]. In Africa, poultry business suffers annual losses to the tune of over 670 million USD because of the diseases caused by fungal contaminants in poultry environment[20]. It is therefore pertinent to identify the fungal agents that constitute health hazards in poultry houses and find ways of reducing their adverse impact in poultry production.

The objective of the present study was to determine the presence and type of fungal contaminants in different locations in a poultry environment, using morphological and molecular identification techniques. The susceptibility pattern of the identified fungi to some conventional antifungal drugs was also tested.

MATERIALS AND METHODS

Study area and sample collection: The study was conducted in Nsukka local government of Enugu State, South East Nigeria. It is located on latitude 6°51'21°N and longitude 7°51'24°N. The total land area is 1,810km^2 and it has a population of 309,633[21]. It lies between the Savannah vegetation and the Rainforest belt of the South. Rainfall spans over a period of 7months annually from April to October and dry season from November to March. Ten poultry farms were randomly selected for the study. Four were layer farms and six were broiler farms. Samples were collected following the procedure described by Kwanashie *et al.*[9]. Briefly , samples were collected from eight locations. The total samples collected in each location in all the farms sampled were; doors (28), drinkers (22), feeders (32), floors (25), poles (37), roofs (34), walls (28) and window nets (37). Sterile cotton swabs were moistened in sterile distilled water. The swabs were used to rub and roll severally over the sampling area of about 10 cm^2 to obtain the fungal biomass. The swabs were carefully placed into the tubes, labeled and stored in ice packs in a cooler and transported within 24 h to the Microbiology Laboratory of the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria Nsukka for culture and fungal isolation.

Fungal Isolation and Identification: Fungal isolation was done on Sabouraud dextrose agar (SDA) supplemented with 0.05 mg mL^{-1} of chloramphenicol. The prepared media were incubated overnight at 37°C for sterility check before swab inoculation. The plates were incubated at room temperature in the dark for 3-7 days[9,22,23]. Mixed cultures were further purified by sub-culturing to obtain a pure culture. Morphological identification to genus and species level was carried out with the aid of the color, size, topography, consistency and aerial growth of the fungal cultures[9,24,25]. Microscopic view was done by staining the fungal colonies with Lactophenol Cotton Blue and viewed under ×40

magnification to identify the arrangement of different fungal features. Gram staining was also done on yeast cells to aid proper identification[9,26]. The microscopic features were compared with those in fungal atlas[19,27].

Molecular identification and phylogenetic analysis: To confirm the data for the morphological and microscopic identification, we randomly selected one isolate from the unidentified group, two from the group identified as *Penicillium*, two from those identified as *Aspergillus* and one from those identified as *Candida* for molecular identification. Mycelia was harvested from pure cultures of these selected fungi and genomic DNA was extracted from their pure cultures using the hexadecyltrimethylammonium bromide (CTAB)-based protocol[28]. This was followed by PCR amplification of the fungal internal transcribed spacer (ITS) region using primers ITS1F and ITS4[29,30] as described in other studies[31]. Amplified amplicons were cleaned and sequenced at Macrogen Inc. (Amsterdam, The Netherlands). The sequences were used for BLAST searches at the National Centre for Biotechnology Information (NCBI). Obtained sequences were deposited in GenBank at NCBI. The sequence of each of the identified fungi was used for further blast searches using BlastN algorithm. The first 5 hits having different countries of origin with 99.8% sequence identity with the query sequence were selected for phylogenetic analysis. The obtained sequences were aligned using MUSCLE algorithm[32] and phylogenetic analysis was performed using maximum likelihood methods implemented in MEGA7.0[33]. Statistical support for branches was assessed by 1000-iteration bootstrap resampling.

Antifungal susceptibility testing: Disc diffusion technique of the Clinical Laboratory Standard Institute was used for the assay[34]. Stock inocula from 3-5 days old cultures as demonstrated by Petrikkou *et al*.[35] was used for the assay. A portion of each test colony was transferred into a sterile test tube and covered with 5 mL of sterile distilled water, the mixture was vortexed for 15 sec. The homogenized mixture was allowed to settle for 15 min at room temperature. The supernatant containing fungal spores were harvested and adjusted to 0.5 McFarland's standards (approximately 1.5×10^8 spores per ml) with normal saline[36]. The prepared inocula were seeded over the surface of freshly prepared SDA medium and allowed to dry. The antifungal discs containing nystatin (100 µg mL^{-1}), fluconazole (25 µg mL^{-1}) and voriconazole (1 µg mL^{-1}) were placed on the surface of the inoculated plates and incubated at room temperature for 3-10 days. The zone of inhibition around each disc was observed and recorded in millimeters. The susceptibility test for each fungus was done in triplicate and the mean zone of inhibition was determined.

Statistical analysis: Data was analyzed using descriptive statistics and reported as percentages. Phylogenetic analysis was done using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together were determined using bootstrap test (1000 replicates). The analysis involved 28 nucleotide sequences and was conducted using MEGA 7.0 software.

RESULTS

Fungal distribution in the poultry houses: A total of 244 fungal isolates were collected from the ten poultry farms and identified. Out of this, 112 fungal isolates were from the layers farm and 132 were from the broilers farm. The morphological and microscopic characteristics of selected fungi identified in this study is shown in Fig. 1. Based on morphological identification, the percentage distribution of each fungal species in the layers farm was; *Aspergillus* 43.75%, *Candida* 18.75%, *Penicillium* 7.14%. *Mucor*, *Rhizopus*, *Rhodotorula* and *Dematiaceous* fungi had percentages of 3.57, 5.36, 2.68 and 9.82% respectively. Unidentified fungi had a frequency of 8.93% (Table 1). For the broiler farms, *Candida* and *Aspergillus* had isolation rates of 29.55 and 22.73% respectively while *Rhizopus*, *Mucor*, *Penicillium* and *Dematiaceous* fungi had isolation rates of 12.88, 8.33, 7.58 and 9.90%, respectively. *Rhodotorula* and unidentified fungi had isolation rates of 6.06 and 3.03% respectively (Table 2). From the locations sampled in both the layers and broiler farms, the poles and window nets had the highest isolation rates of 15.2% each. This was followed by the roofs, feeders, floors and drinkers while the walls and doors had 11.5% isolation rates each (Fig. 2). Three most represented species of *Aspergillus* identified in both the broiler and layer farms were *A. niger*, *A. fumigatus* and *A. flavus* with isolation rates of 42.86, 34.69 and 22.45% respectively in the layers farm and 60.00, 10.00 and 30.00% isolation rates respectively in the broiler farm.

ITS sequencing and molecular identification: A randomly selected group of fungi from the unidentified and the identified groups, (*Penicillium*, *Aspergillus* and Candida) were sequenced. The sequenced ITS regions of the fungi confirmed the results from the morphological and microscopic studies. The isolate from the unidentified group was identified as *Trichoderma harzianum*. From the *Penicillium* group, the

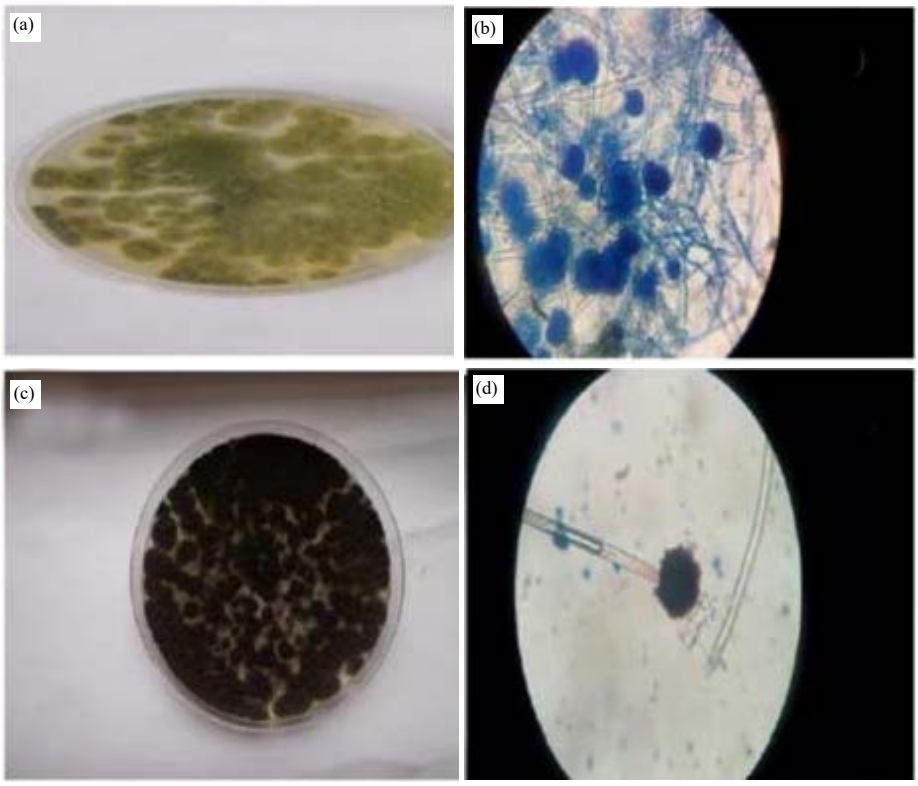

Fig. 1(a-d): The morphological and microscopic appearance of some fungi isolated in this study, *A. flavus*, morphological appearance (b) *A. flavus*, microscopic appearance (c) *A. niger*, morphological appearance (d) *A. niger*, microscopic appearance

Fungi were inoculated on SDA plates and incubated at room temperature for 3-7 days. Mycelia were scraped from the culture and placed on microscopic slides. The mycelia were macerated and stained with lacto-phenol cotton blue and examined under x40 eye piece of a microscope

Table 1: Fungal isolation frequency in layer farms

Fungal species	Frequency of isolation	% age
Candida species	21	18.75
Aspergillus spp.	49	43.75
Penicillium spp.	8	7.14
Mucor spp.	4	3.57
Rhizopus spp.	6	5.36
Rhodotorula spp.	3	2.68
Dematiaceous spp.	11	9.82
Unidentified	10	8.93

% age: Percentage of total fungal species isolated from the layer farms

Table 2: Fungal isolation frequency in broiler farms

Fungal species	Frequency of isolation	% age
Candida species	39	29.55
Aspergillus spp.	30	22.73
Penicillium spp.	10	7.58
Mucor spp.	11	8.33
Rhizopus spp.	17	12.88
Rhodotorula spp.	8	6.06
Dematiaceous spp.	13	9.85
Unidentified	4	3.03

%age: Percentage of total fungi species isolated the from broiler farms

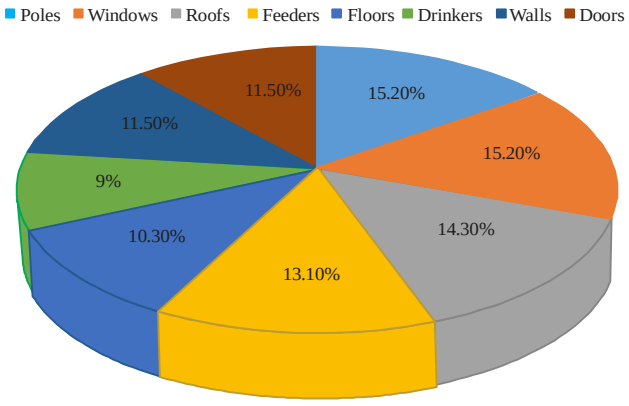

Fig. 2: Percentage distribution of fungi isolated from different locations (poles, roofs, windows, drinkers, feeders, walls and doors) in both the broiler and layer farms

two isolates selected were both confirmed to be *Penicillium rolfsii* while the two from *Aspergillus* were *A. niger* and *A. violaceofuscus*. The representative isolate from *Candida*

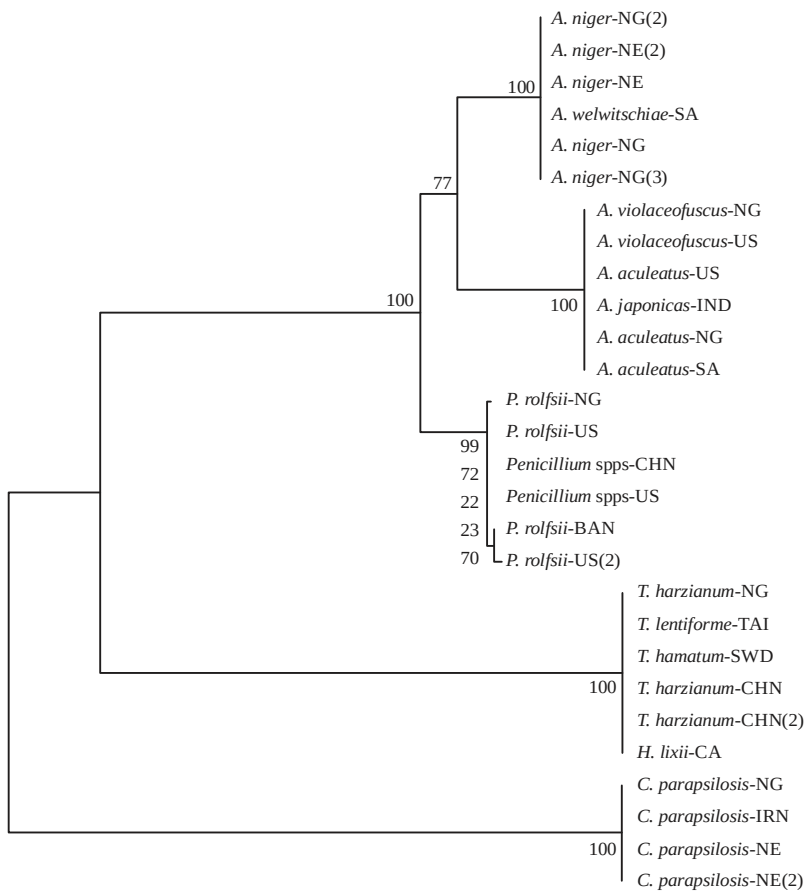

Fig. 3: The evolutionary history of the isolated fungi inferred by Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA. All the clades had 100% branch support for all the branches except the clade containing *Penicillium* isolates

Abbreviations on the taxon names represent the countries where the isolates were obtained: NG: Nigeria, NE: Netherlands, US: United States of America, CHN: China, TAI: Taiwan, SA: South Africa, SWD: Sweden, IRN: Iran, BAN: Bangladesh, IND: India

Table 3: Fungal species identified through m sequencing of the ITS region

Organism	Query cover	Percentage identity[1]	Accession
Trichoderma harzianum	100%	100%	KR868358.1
Penicillium rolfsii	100%	100%	LT558938.1
Penicillium rolfsii	100%	100%	LT558938.2
Aspergillus niger	100%	100%	MN788116.1
Aspergillus violaceofuscus	100%	100%	MG682503.1
Candida parapsilosis	91%	99.78%	MK394125.1

Fungal internal transcribed spacer regions were sequenced and blasted against the NCBI database. Homologous sequences sharing high sequence identity as the query sequence were selected as the fungal species. [1]Percentage identity is the percentage of the coverage of the query sequence to the output sequence in the database

group was found to be *Candida parapsilosis.* The sequences of the isolates were deposited in GenBank with the accession numbers KR868358.1-MK394125.1 (Table 3).

Phylogenetic analysis: The sequence alignment created room for more conserved regions of the genes after cutting off areas with so many gaps. From the phylogenetic analysis, five

Table 4: Antifungal susceptibility profile of the isolated fungal species

Fungal agents	NS (100 μg mL^{-1})	FCA (25 μg mL^{-1})	VOR (1 μg mL^{-1})
Candida parapsilosis	19.41±1.57	19.08±7.69	25.36±4.55
A. niger	20.55±4.37	R	21.61±2.96
A. flavus	20.97±2.44	R	25.10±7.49
A. fumigatus	21.26±2.92	R	28.32±3.81
Penicillium	18.71±2.92	R	R
Mucor	21.77±1.38	R	R
Rhizopus	18.42±2.98	R	R
Rhodotorula	23.00±2.34	R	R
Dematiaceous	20.03±4.05	R	20.67±1.51

Antifungal discs containing nystatin (NS, 100 μg mL^{-1}), fluconazole (FCA, 25 μg mL^{-1}) and voriconazole (VOR, 1 μg mL^{-1}) were placed on the surface of the inoculated plates and incubated at room temperature for 3-10 days. The zones of inhibition were recorded in millimeters. R: Resistant. NS: Nystatin, FCA: Fluconazole, VOR: Voriconazole

different clusters were observed, each of the cluster representing related fungal species which grouped together irrespective of their country of isolation. However, *Aspergillus* species formed two separate clusters (Fig. 3). The first cluster included a group of *A. niger* from Nigeria, Netherlands and one species of *A. welwitschiae* from South Africa, while the second cluster of *Aspergillus* included *A. violaceofuscus* from Nigeria and United States, *A. aculeatus* from Nigeria, US and South Africa and *A. japonica* from India. All the branches for the different clusters had 100% branch support except the cluster for *Penicillium* species which had 99% bootstrap support (Fig. 3).

Antifungal susceptibility pattern: *Candida parapsilosis* was sensitive to the three antifungal drugs used in the study with the zone of inhibition ranging from 19.08±7.69-25.36±4.55 (Table 4). All the *Aspergillus* species, *A. niger*, *A. flavus* and *A. fumigatus*, were sensitive to only nystatin and voriconazole but resistant to fluconazole. The other fungal isolates representing *Penicillium*, *Mucor*, *Rhizopus* and *Rhodotorula* were resistant to fluconazole and voriconazole but were sensitive to nystatin. The *Dematiaceous* fungi were sensitive to both nystatin and voriconazole (Table 4).

DISCUSSION

In the present study, all the poultry farms sampled showed high presence of fungi, indicating that fungi are common microbial agents in poultry houses in Nsukka. A total of six fungal genera were identified and all have been previously reported to be common disease-causing agents in poultry[8,9,14,22]. Morphological and molecular identification techniques agreed. Some of the identified fungi such as *Candida*, *Aspergillus*, *Penicillium* and *Rhodotorula* also cause human infections especially in immune-compromised individuals, thereby posing health risk to poultry workers[37,38]. In the broiler farms, high numbers of *Candida* species were isolated. Other researchers have documented the occurrence of *Candida* in poultry droppings[22,39], poultry materials and locations[9] and poultry slaughterhouses and workers[23]. However, *Aspergillus* species showed a very high isolation rates in the layers farms. This is in agreement with the results of previous studies[9,22]. According to Abbas *et al*.,[22] *Aspergillus* species were the most common fungal contaminants of bird droppings from poultry houses in Baghdad. *Aspergillus* species, especially *A. fumigatus* are known to cause *Aspergillosis* in birds, although *A. flavus* has also been incriminated in *Aspergillosis* in most poultry houses[10,40]. *Aspergillosis* affects all ages and types of poultry including turkey, especially the immune-compromised, stressed and mal-nourished birds[10,41]. Furthermore, the spores of *A. fumigatus* cause *Aspergillus* granuloma when inhaled by birds, because; they are resistant to avian alveolar macrophages[42]. *A. flavus* is reported to be more significant in mycotoxicosis as it contaminates poultry feeds to produce mycotoxin (aflatoxins) resulting in high mortality in poultry[43,44]. *Penicillium*, another prominent fungal species isolated in this study has been reported to be a common fungal species in poultry farms in Kaduna State, Nigeria and most hatcheries in Mazandaran provinc[9,45]. *Penicillium* also produces ochratoxin which is reported to be hepatotoxic in domestic animals[45]. Other fungi isolated in this study like the *Dematiaceous* fungi are known to be airborne fungi of mild climate zones and their potential pathogenicity in poultry is exactly not known but have been associated with dermatitis in chicken[45,46].

It was not surprising to observe that the window nets, poles, doors and roofs had the highest fungal isolation rates in this study. This is because, these locations are permanently fixed within the poultry farms and had not been cleaned or changed throughout the years of operations in the farm. Most of these locations have been in place since the existence of these poultry farms unlike the floor (litter and feces) which are changed on regular basis while drinkers were said to be washed on daily basis in most poultry farms. These

contaminated locations have been proved to be sources of contamination and infections to birds and poultry workers in previous studies[9,14,23,26,47].

The isolated fungal species showed varying degrees of resistance to the antifungal drugs used in this study. *Candida* species were sensitive to the three antifungal agents used in the screening. Most *Candida* species are naturally susceptible to available antifungal drugs, especially the azoles[48,49], although acquired resistance following prolong administration have been documented[50,51]. However, the three *Aspergillus* species showed resistance to fluconazole but were sensitive to nystatin and voriconazole at 100 and 1 μg mL^{-1} respectively. Voriconazole and fluconazole are approved drugs for invasive *Aspergillosis* and *Candidiasis*[48]. The observed resistance against fluconazole by the *Aspergillus* species could be attributed to the concentration of the drug used. It is possible that higher concentrations or higher doses of the drug may be required for effective results against the fungus. It is also possible that the strains of the three species of *Aspergillus* used in this study may have acquired some resistant traits against the drug.

CONCLUSION

Aspergillus and *Candida* are the most common fungi isolated in this study and are both of public health importance. This study has shown that apart from fluconazole which is a routine drug of choice for treatment of *Candidiasis* and voriconazole which is used in the treatment of *Aspergillosis*, nystatin can also be a drug of choice. The study has also shown that poultry materials can serve as fungal carriers for infection in poultry houses.

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