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Isolation and Molecular Characterization of *Candida* SPP from Poultry with Symptoms of Candidiasis in Ado Ekiti, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author EDF designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KJA and ATD managed the analyses of the study. Authors KJA and ATD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study is to isolate, identify and characterize *Candida* spp from cloacal swabs of poultry or birds in Ekiti State University poultry farm, Ago-aduloju poultry farm and Federal Polytechnic of Ado Ekiti poultry farm using molecular method.
Place and Period of Study: The study was carried out in the Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Nigeria in August 2016.
Methodology: Fifty samples of poultry droppings were collected from three farms within Ado Ekiti. The samples were inoculated on Sabourand dextrose agar amended with chloramphenicol. All the fungal isolates were isolated using pour plate method. The isolates were identified based on their morphological, cultural characteristics and molecular analysis.
Results: Eight isolates were obtained from a total of fifty samples. Four isolates were identified as *Candida albicans* strain E10-15 while the fifth isolates was *Candida zemplinina* strain MCR9. The result showed that three of the eight isolates had small amplicon which were not enough to give the sequence identity of the isolates while the remaining five isolates had large amplicon.

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Conclusion: The result of the work demonstrated that poultry birds harbor *Candida albicans* which is a potential pathogenic yeast. This study signifies the need to discover more environmental niches for yeast especially of *Candida* species and also recommends that poultry birds should always be treated with proper antibiotics to avoid candidiasis.

Keywords: Candida albicans; Candida zemplinina; poultry; candidiasis; molecular analysis; isolates.

1. INTRODUCTION

Poultry are a diverse group of species of birds that are raised majorly for meat and eggs but sometimes for feathers, skin and oil [1]. These species comprise of chickens, turkeys, ducks, geese, pheasants, quail, squabs (young pigeons), Guinea fowl, partridges and ratites (ostrich, rhea and emu). Knowledge about the type of birds, their anatomy and how they are managed helps one to understand the type and kind of diseases that can affect different birds. In some species of bird that are raised for egg production or meat, such as commercial poultry, infectious diseases can easily spread among birds housed in a confined space. Raring of poultry can also be carried out in small numbers as backyard flocks for eggs and meat, as hobby and pet birds. They are often exposed to adverse climatic conditions and often not vaccinated, some may lack proper nutrition and bio-security that can lead to frequent viral, bacterial, parasitic and nutritional diseases. Backyard poultry can also be a source of infectious diseases to the commercial poultry. In addition to the different management practices that are used for raising poultry birds, genetics and nutrition play a significant role in the initiation and outcome of a disease. There is also increased demand for poultry raised as antibiotic free and organic which can lead to unintended consequences [1].

Chicken is a type domesticated fowl, which is a subspecies of the Red Jungle fowl. It is one of the most widespread and the most common domesticated birds. In 2003 the total population was more than 24 billion worldwide and out of this population, chickens were the majority compared to any other species of birds [2]. There are two major ways through which human beings can acquire diseases from domestic poultry birds. The first is getting in contact with sick Chicken or faeces of the sick Chicken, usually by a veterinarian or a caretaker. Another is ingestion of disease causing pathogens that colonized the sick Chicken/eggs. When an individual eats these eggs, she/he can also be infected. If a certain pathogen like fungi, bacteria, protozoa, chlamydial or viral agents are of great concern to human health. Fungal/mycotic infections are common in all kinds of poultry birds [2]. Fungal diseases of poultry include Aspergillosis, Candidiasis, Dactylariosis, Cryptococcosis, Favus, Rhodotorulosis, Torulopsis, Mucormycoses, Histoplasmosis and Cryptococcosis.ref Out of these, Aspergillosis and Candidiasis are having much medical importance. Candidiasis as a thrush is a fungal disease caused by yeasts of the genus *Candida* having nearly 200 species [2] Among them, six are the most frequently isolated, while *C. albicans* is the most abundant and significant species.

Birds below 3 weeks of age are more susceptible to candidiasis. Affected poultry show symptoms ranged from poor and stunted growth, depression, diarrhea and dehydration which are responsible for direct mortality [3,4,5].

Cleanliness, adequate hygienic/disinfection measures, proper care and vitamin A supplements are important for disease prevention. Indiscriminate use of antibiotics and other stressors should be avoided [5]. Addition of chlorohexidine in the drinking water helps to prevent overgrowth of *Candida*in poultry flocks or nurseries [6,7].

This study was designed to identify pathogenic *Candida albicans* harbored by Domestic Chicken secretion from the anus.

2. MATERIALS AND METHODS

2.1 Clinical Examination of Birds

Clinical signs of birds infected with *Candida albican*s depends on the site of infection and the crop is commonly the affected organ in young birds. The birds were examined for symptoms of candidiasis as described by Schmidt et al. [8] and Godoy [9]. The symptoms observed in the birds were depression, stunted appearance, weight loss, diarrhea, vomiting, roughness of feathers and loss of appetite.

2.2 Samples Collection

The anus of each birds showing Candidiasis symptoms were first swab with cotton wool

soaked with ethanol to avoid contamination during sample collection. Sterile swab sticks were used to swab the anus of each diseased birds in various farms after careful examination of the birds. Sample were collected in Ekiti State University poultry farm, where a total of fifteen samples were collected randomly from over 500 birds. In Ago Aduloju poultry farm, samples were also collected from five sick birds showing symptoms of candidiasis and fifteen samples were collected randomly from other birds which are over 1000 birds making a total of twenty samples. Fifteen samples were also collected from Federal Polytechnic Ado Ekiti randomly from over 1000 birds, making it a total of 50samples collected from the three poultry farms. The samples were then packed aseptically in ice packs and transported to the laboratory.

2.3 Isolation of Fungi

Each collected samples was immersed in 2ml of sterile peptone water in a test tube and incubated for two hours. After two hours of incubation, each swab stick in the peptone water in the test tube, was removed and discarded. The content of each test tube was poured into different petri dish and overlaid aseptically with Sabouraud Dextrose Agar. Each plate was then incubated at 37°C for 72 hours. Subculture was made for each petri dish into new platesuntil pure cultures were obtained. Each isolates was transferred to Sabouraud Dextrose Agar slants and stored at 4°C.

2.4 Identification of Fungal Culture

The pure culture of each isolates were examined using standard mycological techniques such as slide culture techniques and needle mount preparation as described.

2.5 Needle Mounts Preparation

Following the procedure of Fagbohun et al. [10], the spores' fragment of the original culture was taken from the center of the colony. This was teased out in drops of alcohol on a sterilized glass slide using botany needle. The fragments were stained by adding a drop of lacto phenol blue. The preparation was covered with cover slip and examined under x10 and x40 objective lens of the microscope respectively.

2.6 Slide Culture Techniques

From a plate 2 mm deep, 1 cm^2 solidified PDA was cut and placed on a sterile glass slide. Fungus isolate was inoculated into the four vertical sides using a sterile needle. A sterile cover slip was placed on it so that it over lapped the medium on all sides. The Fungus suspension was placed on a suitable support in a Petri dish containing blotting paper soaked in 20% glycerol in water. The preparation was kept moist at 28°C until adequate growth was observed. After removing the medium with scalpel, the fungus adhering to both cover slip and slide was examined [11]. A drop of alcohol was added, and a drop of lacto phenol blue. The preparation was covered with slip and examined under the low power objective of microscope.

2.7 Extraction of Fungal DNA

Genomic DNA was prepared from a loopful of cells grown in Nutrient Broth for 24 h. The cell pellet was re-suspended in 250 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, and 10 mM EDTA). To lysethe cells adding 25 µl of solution II [200 mM NaOH and 1% (w/v) SDS] were added and mixed for 5 min. Then, 500 µl of solution I and 2.5 µl of RNAse A (10 mg/ml) was added and incubated for 2 h at 37°C. This methodology was adapted from alkaline lysis first described by Vuong et al. (2000). DNA was then purified with phenol-chloroform using a standard laboratory protocol and after precipitation, DNA was re-suspended in 30 µl of TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

2.8 Polymerase Chain Reaction (PCR)

About 2.5 g of fungal genomic DNA was added to a 50 µl PCR mix which contained 1 X Hot start reaction buffer, 0.25 mM dNTPs, 0.01 M (each), and 2.5 U Hot start polymerase (Jena bioscience). Thermal cycling was done in a veriti thermal cycler (Applied Biosystems, USA) and cycling conditions were 95°C for 3 min followed 45°C cycles of 95°C for 1min, by 45°C for 1 min, 72°C for 1 min 45secs with ramp from 45°C to 72°C set at 40%. Subsequently, the reaction was held at 72°C for 10min after which it was held at 4°C till terminated. PCR products were resolved on 1% (w/v) agarose gel stained with ethidium bromide and viewed on a transilluminator [12].

2.9 Sequencing of Amplified 23S RRNA Gene

The PCR products were purified using Montage PCR Clean up kit (Millipore). The purified PCR products of approximately 1,500 bp and the fungal sequencing and identification were

performed as described by Lachance et al. [13] sequencing sequenced using two primers ITS4 (TCCTCCGCTTATTATTGACATG) and ITS 1 (TCCGTAGGTGAACCTGCGG). The sequences of PCR products were analyzed using standard protocols with a dideoxy nucleotide dye terminator (Big Dye vs. 3.1—Applied Biosystems, USA) and Genetic Analyzer 3130 (Applied Biosystems, CA, USA). All 23S rRNA gene sequences were checked for quality, aligned, and analyzed with Codon-Code Aligner v.3.7.1 (CodonCode Corp., Centerville, MA, USA).

All the sequences were compared with reference sequences in the Ribosomal Database Project (RDP) using sequence Match and the sequence were analyzed in GenBank using the BLAST (Basic Local Alignment Search Tool) bioinformatics program on the NCBI (National Center for Biotechnology Information) website. BLAST was done to identify 16S rRNA sequences in Genbank most similar to the query sequence sent.

3. RESULTS

In this study, a total of fifty samples were collected in three poultry farms from birds showing symptoms of candidiasis in Ado Ekiti. Eight different fungal isolates were isolated from fifty samples collected. The isolates were coded as CAN 1, CAN 2, CAN 3, CAN 4, CAN 5, CAN 6, CAN 7 and CAN 8 The cultural, morphological characteristics and molecular analysis was studied. The genomic DNA was extracted from all isolated fungi. The entire 16S RRNA gene was amplified and sequenced, the PCR result of the amplified 16S rRNA of the isolates is displayed in plate showing different bands of the DNA.

3.1 Molecular Identification of the Isolates with 16S Ribosomal RNA Gene and Partial Sequence

In Fig. 1 out of eight organisms isolated, five of them showed large amplicon of which the first four were identified as *Candida albicans* strains and the fifth isolate was identified as *Candida zemplinina.* The polymerase chain reaction amplification result showed a clear band with large amplicon while the fifth isolates did not have a clear band. The DNA Extracted and Amplified showed different band width. Three of the isolates had small amplicon which were not enough to give the identity of the isolates.

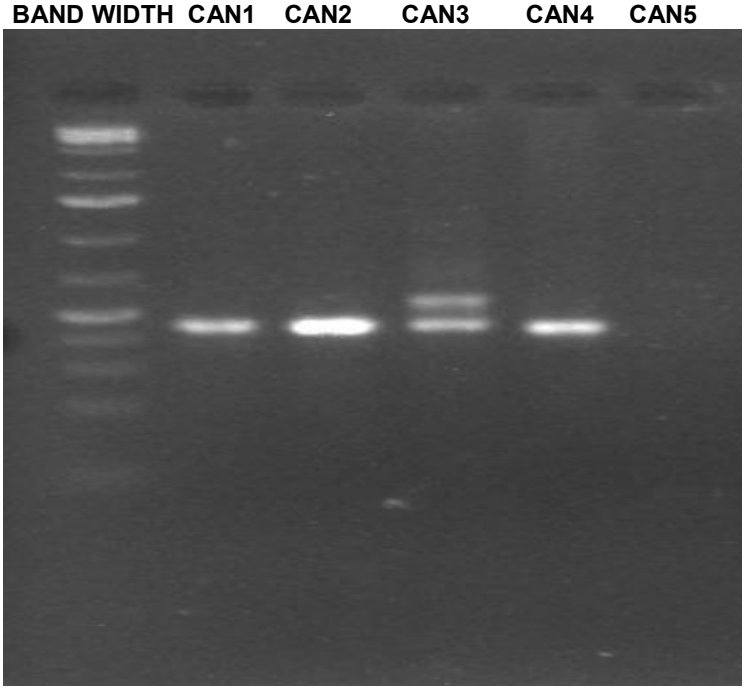

Fig. 1. Amplicon of isolated fungi

Table 1. Cultural and morphological characteristics of the isolates

Isolates	Temperature	Texture	Colonies colour	Edge/appearance	Growth rate
CAN1	37°C	Texture of the colony were pasty, glistening and butyrous	Cream coloured	Smooth	Growth rapidly and mature within 3days
CAN2	25°C	Colonies at 25°C are soft to touch.	White to cream,	Smooth to wrinkle. blastoconidia are formed in grape-like clusters along the length of the hyphae	Abundant branched pseudohyphae and true hyphae with blastoconidia are present
CAN3	37°C	The Colonies were creamy in colour, smooth and butyrous	The appearance was soft and the surface was smooth.	The texture of the colony were pasty, smooth, glistening and butyrous at a temperature of 37°C	They grow rapidly and mature in 3days,
CAN4	25°C	The colonies are cream in colour	The texture of the colony were pasty, smooth, glistening then developed to dry, wrinkled and dull	They produce blastoconidia singly or in small cluster. blastoconidia may be round or elongated	They grow rapidly and mature in 3days. blastoconidia singly or in small cluster. blastoconidia may be round or elongated. Abundant branched pseudohyphae and true hyphae with blastoconidia were present. The blastoconidia are formed in grape-like clusters along the length of the hyphae
CAN5	25°C	The cultural colonies appeared as white to ivory colour	Smooth having a yeasty smell it develops as cream,	Convex colonies	Moderately grow
CAN6	37°C	The texture of the colony were pasty, smooth, glistening and butyrous at a temperature of 37°C	The colonies were creamy in colour smooth	The appearance was soft and the surface was smooth	They grow rapidly and mature in 3 days
CAN7	25°c	Soft and smooth to wrinkle	Colonies are white to cream,	The blastoconidia are formed in grape-like clusters along the length of the hyphae	Abundant branched pseudohyphae and true hyphae with blastoconidia are present
CAN8	37°C	Smooth having a yeasty smell and it develops as cream	The colonies appeared as white to ivory colour	Pasty and convex colonies	Moderate

Isolates CAN 1,2,3, and isolate CAN4 Sequences

Sequence ID: gb|KF030773.1|Length: 1542Number of Matches: 1

Related Information

Range 1: 590 to 1253GenBankGraphics Next Match Previous Match First Match

Alignment statistics for match #1					
Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
695 bits(376)	0.0	576/666(86%)	39/666(5%)	Plus/Plus	

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Query 255 TA-TCCATGCC-TAAACGATGAGCGCTAGGTG-TGGAGGATTTCCGCC-TTCA-TGCCGG 309
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Sbjct 828 TAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGG  887

Query 310 AGCTAACGCATTAAGCACTCCGCCCGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAT  369
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Query 598 CGGAGACAG-CCGCTTGACG-TGGGGACTATCCCATATC-TCACG-CCCTTACGACCAGG  653
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Identification: *Candida albicans* E10-15

Isolates CAN 5

Sequence ID: gb|KF030773.1|Length: 1542 Number of Matches: 1

Related Information

Range 1: 590 to 1253 GenBank Graphics Next Match Previous Match First Match

Alignment statistics for match #1					
Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
695 bits(376)	0.0	576/666(86%)	39/666(5%)	Plus/Plus	

Features:

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Identification: *Candida zemplinina* MCR9

4. DISCUSSION

Candida spp is the causative agent of an infection termed candidiasis or candidosis. Infection caused by these fungi show a wide range of clinical presentations and can be classified as superficial, cutaneous and mucosal infections, to deep, widespread and very sever, as is the case with invasive candidiasis. *Candida* species have been isolated from the air and soil coming from poultry breeding and rearing houses, old litter and litter-containing water, wet feed and bird droppings [14]. However, in this research efforts has been put in place to isolate directly from birds majorly those that show the symptoms. This study found out that poultry birds are reservoir of *C. albicans* causing candidiasis in them. However the result shows that *C. albican* are predominate in poultry birds. The present result shows that *C. albicans* is the most common *Candida* species isolated from the anus of birds showing symptoms such as depression,

stunted appearance, weight loss, diarrhea, vomiting, roughness of feathers and loss of appetite as earlier reported by Speer [15].

In this study higher percentage of isolated candida belong to *Candida albicans.* This is in agreement with Caldrone and Clancy [16] who stated that *Candida albicans*is commensal and a part of the normal gut microfloral that live in the gastrointestinal tract. *C. albicans*lives in 70% of the human population without any harmful effects, although overgrowth of the fungus results in candidiasis (candidosis). The genus *Candida* have nearly 200 species and among them, six are most frequently isolated, out of which *C. albicans*is the most abundant and significant species. Susceptible hosts for *C. albicans* include domestic poultry, water fowls and wild birds [17]. Involvement of the digestive tract is common in young birds as compared to older birds and this could be as a result of undeveloped immunes system. Increased virulence of the fungus plays a vital role in establishing the disease [18].

Apart from *C. albicans* which is the major isolated fungus (80%) in this study, it's also interesting that a yeast strain discovered just of recent by Sipiczki [19] and recognized as a distinct new species and named it *C. zemplinina* in 2003 was also isolated in this study alongside *C. albicans*. This strain (*Candida zemplinina* strain MCR9) is newly discovered and first reported in Nigeria ever since its first isolation in 2003. The most commonly isolated yeast in Nigeria and Ekiti region in particular has been *Candidaalbicans* and *Saccharomyces cerevisiae* upon which most research and publications had been centered on. Therefore this type of yeast (*C. zemplinina*) has not been reported in this state and this report is emphasizing that the fermentation ability of this yeast has not been ascertained in this region as well though it's gaining global recognition as result of its valuable contribution to good wine production. As a non-saccharomyces yeast, it has been reported that it has enormous significant in wine production owing to its fermentative potential [18].

Although *C. zemplinina*was isolated in the poultry, we are trying to link its existence in this environment to the previous study and see the relationships. The isolation of the *C. zemplinina* had been linked with the wine environment being fructophilic, enologically important yeast. Sipiczki [19] described the *Candida zemplinina* as a novel, osmo- and psychrotolerant, fructophilic and acidogenic enamor -phous yeast species that shared some characteristics with *Candida stellata* [17]. The fact that *C. zemplinina*was isolated from poultry is not evident enough to link it to diseased condition of the fowls. Going through the reported literature, the pathogenicity of the *C. zemplinina* has not been reported, though we are not saying it cannot be opportunistic organism. Further studies areneeded to prove its pathogenicity either in man or poultry as many research on it were focused on its positive aspect of its character majorly in wine fermentation and production.

5. CONCLUSION

The result of this research showed that poultry birds in the area of this study harboured *Candida* species like *Candida albicans* and *Candida zemplinina* thereby causing increase in the death rate of poultry birds, and humans cohabiting with Chicken are at a risk of contracting Candidiasis infections, especially immune compromised individuals. This study signifies the need to discover more environmental niches for yeast especially of *Candida* species and recommends that poultry birds should always be treated with appropriate antibiotics to avoid candidiasis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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