

4 **ISOLATION AND MOLECULAR CHARACTERIZATION OF *Candida* SPP FROM**  
5 **POULTRY BIRDS WITH SYMPTOMS OF CANDIDIASIS IN ADO EKITI, NIGERIA**  
6

7 **ABSTRACT**

8 **Aim:** The aim of this study is to isolate, identify and characterize *Candida* spp from poultry birds' secretion  
9 from the anus of the birds in Ekiti State University poultry farm, Ago-aduloju poultry farm and Federal  
10 Polytechnic of Ado Ekiti poultry farm using molecular method.

11 **Place and period of Study:** The study was carried out in the Department of Microbiology, Faculty of  
12 Science, Ekiti State University, Ado Ekiti, Nigeria in August 2016.

13 **Methodology:** Fifty samples of poultry droppings were collected from three farms within Ado Ekiti. The  
14 samples were inoculated on Sabourand dextrose agar amended with chloramphenicol. All the fungal  
15 isolates were isolated using pour plate method. The isolates were identified based on their morphological,  
16 cultural characteristics and molecular analysis.

17 **Result:** Eight isolates were obtained from a total of fifty samples. Four isolates were identified as *Candida*  
18 *albicans* strain E10-15 while the fifth isolates was *Candida zemplinina* strain MCR9. The result showed that  
19 three of the eight isolates had small amplicon which were not enough to give the sequence identity of the  
20 isolates while the remaining five isolates had large amplicon.

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

25 **Key words:** *Candida albicans*, *Candida zeylanoides*, Poultry, Candidiasis, Molecular  
26 Analysis, Isolates

## 27 **1.0 INTRODUCTION**

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

42 Chicken is a type domesticated fowl, which is a subspecies of the Red Jungle fowl. It is one of the  
43 most widespread and the most common domesticated birds. In 2003 the total population was more than  
44 24 billion worldwide and out of this population, chickens were the majority compared to any other species  
45 of birds [2] There are two major ways through which human beings can acquire diseases from domestic  
46 poultry birds. The first is getting in contact with sick Chicken or faeces of the sick Chicken, usually by a  
47 veterinarian or a caretaker. Another is ingestion of disease causing pathogens that colonized the sick  
48 Chicken/eggs. When an individual eats these eggs, she/he can also be infected. If a certain pathogen  
49 like fungi, bacteria, protozoa, chlamydial or viral agents are of great concern to human health.  
50 Fungal/mycotic infections are common in all kinds of poultry birds[2]. Fungal diseases of poultry include  
51 Aspergillosis, Candidiasis, Dactylariosis, Cryptococcosis, Favus, Rhodotorulosis, Torulopsis,  
52 Mucormycoses, Histoplasmosis and Cryptococcosis. Out of these, Aspergillosis and Candidiasis are  
53 having much medical importance. Candidiasis as a thrush is a fungal disease caused by yeasts of the

54 genus *Candida* having nearly 200 species [2] Among them, six are the most frequently isolated, while *C.*  
55 *albicans* is the most abundant and significant species.

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

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

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

65

## 66 2.0 Materials and methods

### 67 2.1 Clinical Examination of Birds

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

### 72 2.3 SAMPLES COLLECTION

73 The anus of each birds showing Candidiasis symptoms were first swab with cotton wool  
74 soaked with ethanol to avoid contamination during sample collection. Sterile swab sticks were  
75 used to swab the anus of each diseased birds in various farms after careful examination of the  
76 birds. Sample were collected in Ekiti State University poultry farm, where a total of fifteen  
77 samples were collected randomly from over 500 birds. In Ago Aduloju poultry farm, samples  
78 were also collected from five sick birds showing symptoms of candidiasis and fifteen samples  
79 were collected randomly from other birds which are over 1000 birds making a total of twenty

80 samples. Fifteen samples were also collected from Federal Polytechnic Ado Ekiti randomly from  
81 over 1000birds, making it a total of 50samples collected from the three poultry farms. The  
82 samples were then packed aseptically in ice packs and transported to the laboratory.

## 83 **2.4 ISOLATION OF FUNGI**

84 Each collected samples was immersed in 2ml of sterile peptone water in a test tube and incubated for  
85 two hours. After two hours of incubation, each swab stick in the peptone water in the test tube, was  
86 removed and discarded. The content of each test tube was poured into different petri dish and overlaid  
87 aseptically with Sabouraud Dextrose Agar (LAB009 UK). Each plate was then incubated at 37<sup>0</sup>c for 72  
88 hours. Subculture was made for each petri dish into new plates until pure cultures were obtained. Each  
89 isolates was transferred to Sabouraud Dextrose Agar slants and stored at 4<sup>0</sup>C.

## 90 **2.5 IDENTIFICATION OF FUNGAL CULTURE**

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

## 93 **2.6 NEEDLE MOUNTS PREPARATION:**

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

## 99 **2.7 SLIDE CULTURE TECHNIQUES:**

100 From a plate 2mm deep, 1cm<sup>2</sup> solidified PDA was cut and placed on a sterile glass slide. Fungus  
101 isolate was inoculated into the four vertical sides using a sterile needle. A sterile cover slip was placed on  
102 it so that it over lapped the medium on all sides. The Fungus suspension was placed on a suitable  
103 support in a Petri dish containing blotting paper soaked in 20% glycerol in water. The preparation was  
104 kept moist at 28<sup>0</sup>C until adequate growth was observed. After removing the medium with scalpel, the

105 fungus adhering to both cover slip and slide was examined [11] A drop of alcohol was added, and a drop  
106 of lacto phenol blue. The preparation was covered with slip and examined under the low power objective  
107 of microscope.

108

## 109 **2.8 EXTRACTION OF FUNGAL DNA**

110 Genomic DNA was prepared from a loopful of cells grown in Nutrient Broth for 24 h. The cell pellet was  
111 resuspended in 250 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, and 10 mM EDTA). To  
112 lysethe cells adding 25 µl of solution II [200 mM NaOH and 1% (w/v) SDS] were added and mixed for 5  
113 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  
114 37°C. This methodology was adapted from alkaline lysis first described by Vuong *et al.* (2000). DNA was  
115 then purified with phenol-chloroform using a standard laboratory protocol and after precipitation, DNA was  
116 resuspended in 30 µl of TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

117

## 118 **2.9 POLYMERASE CHAIN REACTION (PCR)**

119 About 2.5 g of fungal genomic DNA was added to a 50 µl PCR mix which contained 1 X Hot start  
120 reaction buffer, 0.25 mM dNTPs, 0.01 M (each), and 2.5 U Hot start polymerase (Jenabioscience).  
121 Thermal cycling was done in a veriti thermal cycler (Applied Biosystems, USA) and cycling conditions  
122 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  
123 ramp from 45°C to 72°C set at 40%. Subsequently, the reaction was held at 72°C for 10min after which it  
124 was held at 4°C till terminated. PCR products were resolved on 1% (w/v) agarose gel stained with  
125 ethidium bromide and viewed on a transilluminator [12]

## 126 **2.10 SEQUENCING OF AMPLIFIED 23S RRNA GENE**

127 The PCR products were purified using Montage PCR Clean up kit (Millipore). The purified PCR  
128 products of approximately 1,500 bp and the fungal sequencing and identification were performed as  
129 described by [13] sequencing sequenced using two primers ITS4 (TCCTCCGCTTATTATTGACATG)  
130 and ITS 1 (TCCGTAGGTGAACCTGCGG). The sequences of PCR products were analyzed using  
131 standard protocols with a dideoxy nucleotide dye terminator (Big Dye vs. 3.1—Applied Biosystems, USA)  
132 and Genetic Analyzer 3130 (Applied Biosystems, CA, USA). All 23S rRNA gene sequences were

133 checked for quality, aligned, and analyzed with Codon-Code Aligner v.3.7.1 (CodonCode Corp.,  
134 Centerville, MA, USA).

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

### 140 3.0 RESULTS

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

147 **TABLE1: CULTURAL AND MORPHOLOGICAL CHARACTERISTICS OF THE ISOLATES**

Isolates	Temperature	Texture	Colonies Colour	Edge/appearance	Growth rate
CAN1	37 <sup>0</sup> C.	texture of the colony were pasty, glistening and butyrous	cream coloured	Smooth	Growth rapidly and mature within 3days
CAN2	25 <sup>0</sup> C	Colonies at 25 <sup>0</sup> C are soft to touch.	white to cream,	Smooth to wrinkle. blastoconidia are formed in grape-like clusters along the	Abundant branched pseudohyphae and true hyphae with blastoconidia

				length of the hyphae	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	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,	The Colonies were creamy in colour smooth	The appearance was soft and the surface was smooth	They grow rapidly and mature in 3days

		smooth, glistening and butyrous at a temperature of 37°C			
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

148

149 **3.1 MOLECULAR IDENTIFICATION OF THE ISOLATES WITH 16S RIBOSOMAL RNA GENE AND**  
150 **PARTIAL SEQUENCE**

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

156

157 **BAND WIDTH      CAN1      CAN2      CAN3      CAN4      CAN5**







237 **Identification:** *Candida albicans*E10-15

238 **Isolates CAN 5**

239 Sequence ID: [gb|KF030773.1](#)|Length: 1542Number of Matches: 1

240 Related Information

241 Range 1: 590 to 1253[GenBankGraphics](#) 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	
242	Features:					
243	Query 353					
244	GCGAATCTTACCCGTACGGTTGCCTCGGCGCTGGCGGTCCGGAAAGGCCCTCGGGTCCTC 412					
245						
246	Sbjct 61					
247	GCGAATCTTACCCGTACGGTTGCCTCGGCGCTGGCGGTCCGGAAAGGCCCTCGGGTCCTC 120					
248						
249	Query 413					
250	CCGATCCTCGGGTCTCCCGCTCGCGGGAGGCTGCCCGCCGGAGTGCCGAAACTAAACTC 472					
251						
252	Sbjct 121					
253	CCGATCCTCGGGTCTCCCGCTCGCGGGAGGCTGCCCGCCGGAGTGCCGAAACTAAACTC 180					
254						
255	Query 473 TTGATATTTTATGTCTCTCTGAGTAAACTTTTAAATAAGTCAAACCTTTCAACAACGGAT					
256	532					
257						
258	Sbjct 181 TTGATATTTTATGTCTCTCTGAGTAAACTTTTAAATAAGTCAAACCTTTCAACAACGGAT					
259	240					
260						
261	Query 533					
262	CTCTTGGTTCTGGCATCGATGAAGAACGCARCGAAATGCGATAAGTAATGTGAATTGCAG 592					
263						
264	Sbjct 241 CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG					
265	300					
266						
267	Query 593					
268	AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGTATTCTGGCGAGCA 652					
269						
270	Sbjct 301 AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGTATTCTGGCGAGCA					
271	360					
272						
273	Query 653					
274	TGCCTGTTTCGAGCGTCATTTCAACCATCAAGCTCTGCTTGCCTTGGGGATCCGCGGCTGC 712					
275						
276	Sbjct 361					
277	TGCCTGTTTCGAGCGTCATTTCAACCATCAAGCTCTGCTTGCCTTGGGGATCCGCGGCTGC 420					
278						
279	Query 713					
280	CCGCGGTCCCTCAAATCAGTGGCGGGCTCGCTAGTCACACCGAGCGTAGTAACTCTACA 772					
281						
282	Sbjct 421					
283	CCGCGGTCCCTCAAATCAGTGGCGGGCTCGCTAGTCACACCGAGCGTAGTAACTCTACA 480					
284						

285 Query 773  
286 TCGCTATGGTCGTGCGGCGGGTTCTTGCCGTAAAACCCCCATTTCTAAGGTTGACCTCG 832  
287 |||  
288 Sbjct 481  
289 TCGCTATGGTCGTGCGGCGGGTTCTTGCCGTAAAACCCCCATTTCTAAGGTTGACCTCG 540  
290  
291 Query 833 GATCAAGGTWSGAMTAAMCSGCATGAAYTTAAGCATATCAATAAGCCGGA 882  
292 ||| ||| || | || ||| |||  
293 Sbjct 541 GATC-AGGTAGGAATACCC-GC-TGAACTTAAGCATATCAATAAGC-GGA 586

294 **Identification:** *Candida zemplinina* MCR9

295

## 296 **DISCUSSION**

297 *Candida* spp is the causative agent of an infection termed candidiasis or candidosis. Infection  
298 caused by these fungi show a wide range of clinical presentations and can be classified as superficial,  
299 cutaneous and mucosal infections, to deep, widespread and very sever, as is the case with invasive  
300 candidiasis.[ref]*Candida* species have been isolated from the air and soil coming from poultry breeding  
301 and rearing houses, old litter and litter-containing water, wet feed and bird droppings[14].However, in this  
302 research efforts has been put in place to isolate directly from birds majorly those that show the symptoms.  
303 This study found out that poultry birds are reservoir of *C. albicans* causing candidiasis in them. However  
304 the result shows that *C. albican* are predominate in poultry birds. The present result shows that *C.*  
305 *albicans* is the most common *Candida* species isolated from the anus of birds showing symptoms such as  
306 depression, stunted appearance, weight loss, diarrhea, vomiting, roughness of feathers and loss of  
307 appetite as earlier reported by Speer[15]

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

317 Apart from *C. albicans* which is the major isolated fungus (80%) in this study, it's also interesting  
318 that a yeast strain discovered just of recent by Sipiczki[19] and recognized as a distinct new species and  
319 named it *C. zemplinina* in 2003 was also isolated in this study alongside *C. albicans*. This strain (*Candida*  
320 *zemplinina* strain MCR9) is newly discovered and first reported in Nigeria ever since its first isolation in  
321 2003. The most commonly isolated yeast in Nigeria and Ekiti region in particular has been  
322 *Candida albicans* and *Saccharomyces cerevisiae* upon which most research and publications had been  
323 centered on. Therefore this type of yeast (*C. zemplinina*) has not been reported in this state and this  
324 report is emphasizing that the fermentation ability of this yeast has not been ascertained in this region as

325 well though it's gaining global recognition as result of its valuable contribution to good wine production. As  
326 a non-saccharomyces yeast, it has been reported that it has enormous significant in wine production  
327 owing to its fermentative potential [18]

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

338  
339

## 340 CONCLUSION

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

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