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Isolation and Molecular Identification of *Candida albicans* from the Oral cavity of Domestic Chickens using 28S rDNA in Diyala Governorate, Iraq

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ABSTRACT

Candida albicans (*C. albicans*) is an opportunistic fungal pathogen that affects humans, animals, and birds. It is one of the most prevalent microbes found in clinical specimens. Candidiasis refers to endogenous fungal overgrowth under conditions of microbiota disruption or other host impairments. The study aimed to detect and isolate *C. albicans* based on morphological, cultural, and biochemical analysis. The present study examined 36 oral cavity samples from domestic chickens suspected of having candidiasis across various regions of Diyala Governorate in Iraq from September to November 2023. A total of twenty *C. albicans* were isolated from collected samples. PCR amplification of the 28S rDNA fungal gene, yielding the expected 260bp products, confirmed the identification of the yeasts. Using the Vitek 2 automated system, minimum inhibitory concentrations (MICs) for 6 common antifungal drugs were determined to test the antifungal susceptibility of a *Candida* clinical isolate. No resistance was found to Amphotericin B, Fluconazole, Flucytosine, Voriconazole, Caspofungin, or Micafungin in isolates from the oral cavity. In conclusion, the current study identified twenty *C. albicans* strains from chicken oral cavities and found them to be susceptible to all major classes of antifungals, indicating a lack of antifungal resistance in these isolates

Keywords: *Candida albican*, demostic chicken, Vitek 2, 28S rDNA.

INTRODUCTION

Candida albicans (*C. albicans*) is a common fungal pathogen and opportunistic yeast that is present in the digestive, reproductive, and urinary tracts of birds (Osorio et al., 2007; Vieira and Coutinho, 2009). While over 200 *Candida species* exist, *C. albicans* is the primary causative agent of candidiasis (Dhama et al., 2013; Mugale et al., 2015). In birds, *Candida* infections can occur as either primary or secondary infections (Vieira and Coutinho, 2009). Under immunosuppressive conditions brought on by factors like viral infections, steroid administration, prolonged antibiotic therapy, and subclinical malnutrition, invasive infections are more likely to develop (Razmyar et al., 2014). *C. albicans* grows as oval budding yeast cells measuring 3.5-6.0 × 6.0-10.0 µm in size. When *C. albicans* is cultured on agar plates or in animal tissues, round colonies will appear (Samour and Naldo, 2002).

Sequence analysis of ribosomal DNA (rDNA) is a common molecular method for fungal identification. Previous studies have used PCR with primers targeting the 28S rRNA gene, which contains conserved sequences shared among all fungal pathogens, to effectively detect fungi in clinical samples (Anand et al., 2001; Ninet et al., 2003; Zunaina et al., 2008; Nayak et al., 2011). These prior investigations found that PCR amplification of DNA fragments less than 300 bp in length, such as the 250-280 bp 28S rDNA amplicons, results in greater detection sensitivity compared to larger fragments (Evertsson et al., 2000; Gade et al., 2017).

Antifungal susceptibility testing plays a vital role in resistance detection, epidemiological research, and comparing the *in vitro* efficacy of novel and existing antifungal medications (Cejudo et al., 2010). The Vitek 2 system by bioMérieux, Inc.is an automated commercial platform that uses spectrophotometry to determine yeast growth and enables combined fungal identification and minimum inhibitory concentration (MIC) determination (Cuenca-estrella et al., 2010). Candida spp. has unknown

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susceptibility patterns often and empirical therapy is often used to treat infections (Melhem et al., 2013). The susceptibility testing products available commercially play an important role in treating patients with invasive fungal infections by providing important patient data to guide suitable therapy (Cretella et al., 2016).

The aim of this study was to isolate and identify *C. albicans* from the oral cavity of local chickens utilizing a PCR assay targeting the 28S rDNA region, as well as evaluate the isolated strains for antifungal sensitivity profiles using the commercial Vitek 2 system.

MATERIALS AND METHODS

Ethical approval

The study received approval from the Scientific and Ethical Committee of the College of Veterinary Medicine, University of Diyala, Iraq, with approval number Vet Medicine (300), dated November 2023, signed by M, S, O, and A.

Sample collection

From September 2023 to November 2023, 36 oral cavity samples were collected from domestic chickens in various regions (Muradiya, Razi, Asada , Canaan, and Al-Hadid) of the Diyala governorate in Iraq. The local chickens were mixed breed, different ages and unsex. Some local chickens had visible oral membrane infections at the time of sampling. Samples were collected by gently swabbing the oral membranes and cavities using sterile cotton swabs. Swabs were immediately placed in sterile tubes and transported beside an ice pack to the Microbiology Laboratory at Diyala University in Iraq within an hour after collection.

Sample processing

Upon receipt in the lab, cotton swabs were used to directly inoculate Sabouraud Dextrose Agar plates supplemented with chloramphenicol (SDA-C) to inhibit bacterial growth. Plates were streaked for isolation and incubated at 37°C for up to 72 hours. During this incubation period, the plates were periodically monitored for the development of fungal growth (Matare et al., 2017). Developing colonies were subcultured on fresh SDA-C plates to obtain pure cultures. Gram staining was performed to assess morphology.

Identification of *Candida albicans*

To identify *C. albicans* isolates, all Gram-positive yeast isolates were subjected to germ tube formation by inoculating isolates into serum and incubating at 35°C for up to 3 hours (Sheppard et al., 2008), then visually assessing for generation of germ tubes under a light microscope (Olympus, Japan). Additionally, chlamydospore production was evaluated by spot inoculating corn meal agar plates with isolates, incubating for 48-72 hours at 25°C, and examining under the microscope for distinctive chlamydospores produced by *C. albicans* (Navarathna et al., 2016). Isolates were also inoculated onto CHROMagar *Candida* medium to take advantage of the ability of this differential medium to identify *C. albicans* colonies by their distinctive green color (Sivakumar et al., 2009). Using these three methods, 20 isolates exhibited traits consistent with *C. albicans* identification.

Molecular diagnosis

Fungal genomic DNA was extracted from isolates using a commercial Nucleospin DNA extraction kit (Promega, USA) with the following modifications to the manufacturer's protocol. A single fungal colony was picked with a sterile loop and suspended in 300µl of lysis buffer (1mM EDTA pH8, 10mM Tris, 100mM NaCl, 2% Tween 80, 1% SDS) followed by the addition of 300µl phenol-chloroform (1:1). The mixture was shaken for 5 minutes and centrifuged at 10,000 rpm for 5 minutes. The aqueous supernatant was transferred into a new microcentrifuge tube and an equal volume of chloroform was added. After mixing thoroughly, the solution was centrifuged again at 10,000 rpm for 10 minutes. Next, 500 microliters of 100% ethanol was mixed with the supernatant to precipitate DNA. Tubes were inverted several times before centrifuging again at 10,000 rpm for 7 minutes. Finally, the DNA pellets were air-dried for 10 minutes and resuspended in 100 microliters of TE buffer at pH 8.0 (Mousavi et al., 2007). Extracted DNA was stored at -20°C prior to further analysis. Quality and yield of extracted fungal DNA was assessed by OD260/280 spectroscopic measurements using a NanoDrop. Extracted DNA had OD260/280 ratios ranging from 1.7 to 1.8, indicating high-purity DNA.

PCR

Amplification of the 28S rDNA gene was performed using the following primers that are mentioned in a previous study (Anand et al., 2001).

Forward: 5'-GTGAAATTGTTGAAAGGGAA-3'
Reverse: 5'-GAC TCCTTGGTCCGTGTT-3'

PCR reactions were carried out in a 25µl volume containing 1µl (10pmol) of each primer, 5µl DNA template, 12.5µl Green PCR master mix, and 5.5µl PCR-grade water. Thermal cycling was conducted using a PCR Thermal Cycler (TC-3000, PCR Thermal Cycler, USA) with the following protocol. Initial denaturation at 94°C for 15 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 15 minutes.

To analyze the polymerase chain reaction (PCR) products, a 2% agarose gel electrophoresis was performed using ethidium bromide staining. A 50-base pair DNA ladder was run alongside the PCR products as a molecular weight size marker. Gels were visualized under UV light and photographed.

Vitek 2 antifungal susceptibility testing

The Vitek 2 AST-YS07 card was utilized, which contains serial two-fold dilutions of the following antifungals included of Amphotericin B, Micafungin, Fluconazole, Flucytosine, Caspofungin, and Voriconazole. Inoculum suspensions were prepared from 24-hour Sabouraud dextrose agar cultures of *C. albicans* incubated at 37°C. Suspensions were adjusted to a 2.0 McFarland standard and loaded into Vitek 2 cassettes along with sterile polystyrene tubes according to manufacturer's instructions.

Loaded cassettes were placed into the Vitek 2 instrument and ran automatically. The yeast suspensions were diluted appropriately prior to card filling and incubated for 5-17 hours depending on the growth rate of the drug-free control well. Results were reported as minimum inhibitory concentrations (MICs) in µg/mL for each antifungal agent. Interpretive criteria were based on EUCAST 2013 & CLSI 2013 clinical breakpoints used for routine diagnostics as follows: Amphotericin B - Susceptible (S) $\leq$ 1 µg/mL, Resistant (R) $\geq$ 2 µg/mL; Voriconazole - S $\leq$ 0.125 µg/mL, R $\geq$ 0.25 µg/mL; Fluconazole - S $\leq$ 2 µg/mL, Intermediate (I) = 4 µg/mL, R $\geq$ 8 µg/mL; Micafungin - S $\leq$ 0.25 µg/mL, I = 0.5 µg/mL, R $\geq$ 1 µg/mL; Flucytosine - S $\leq$ 4 µg/mL, 8 µg/mL $\leq$ I $\leq$ 16 µg/mL, R $\geq$ 32 µg/mL; Caspofungin - S $\leq$ 2 µg/mL.

RESULTS

Isolation of *Candida albicans*

Of the 36 oral swabs collected from local chickens, 20 (55.6%) yielded isolates identified as *C. albicans* using morphological, cultural, and biochemical characteristics.

Microscopic morphology and cultural characteristics

Gram staining of isolates revealed large, oval purple cells occurring singly or in budding yeast chains. Additionally, wider, tube-like pseudo-hyphae structures were observed in some preparations. When grown on Sabouraud Dextrose agar at 30°C for 48 hours, isolates formed white, smooth, creamy colonies that were circular and concave with entire margins. On corn meal agar incubated at 30°C for 72 hours, distinctive round chlamydospores were visualized terminally along hyphal elements and at intercalary, immediately subtending septa of the hyphae. Chlamydospores were large, thick-walled, refractile structures. Blastoconidia were also observed adjacent to hyphae. Growth on CHROMagar *Candida* medium yielded forest green colonies, consistent with *C. albicans* species identification. Inoculation into serum followed by incubation at 35°C for 3 hours induced the formation of germ tubes, microscopic tubular extensions characteristic of *C. albicans*. This confirmed the identification of this fungal species (Figure 1).

Molecular identification via PCR

The extracted DNA from all isolates was amplified via polymerase chain reaction (PCR) targeting the 28S ribosomal DNA (28S rDNA) region. PCR amplification products obtained using 28S rDNA primers were analyzed by agarose gel electrophoresis and visualized under UV light. Gel electrophoresis and comparison to a size standard were utilized to determine that the DNA isolated from the microbial isolates contained discrete fragments of approximately 260 base pairs. The technique confirms the extracted DNA was of the expected size and has been successfully isolated from the organism samples for further analysis (Figure 2).

Antifungal susceptibility profile of *Candida albicans*

Use a device Vitek 2 Compact system to determine MICs for 6 different antifungal drugs against the yeast that were being tested as shown in Table 1.

As can be seen in Table 1 the results of antifungal susceptibility testing against 20 isolates of the *C. albicans* fungus is presented. The results indicated that all isolates were fully sensitive to the used antifungal agents including Fluconazole, Voriconazole, Caspofungin, Micafungin, and Flucytosine. Regarding Amphotericin B, 19 isolates were sensitive (95%) while one isolate had intermediate sensitivity (5%). There were no resistant isolates to any of the chosen antifungal agents. The results revealed the possible efficacy of all the antifungal agents tested during this study against *C. albicans*.

Table 1. The minimum inhibitory concentration values for antifungals against *Candida albicans*

Antifungal against *C. albicans*	Sensitive		Intermediate		Resistance		MIC (µg/ml) Range
	No. of isolate	S (%)	No. of isolate	I (%)	No. of isolate	R (%)	
Fluconazole	20	100	0	0	0	0	≤ 1
Voriconazole	20	100	0	0	0	0	≤ 0.12
Caspofungin	20	100	0	0	0	0	≤ 0.25
Micafungin	20	100	0	0	0	0	≤ 0.06
Amphotericin B	19	95	1	5	0	0	≤ 0.5
Flucytosine	20	100	0	0	0	0	≤ 1

S: sensitive, *R:resistant; I: Intermediate, No: Number, MIC: minimum inhibitory concentration

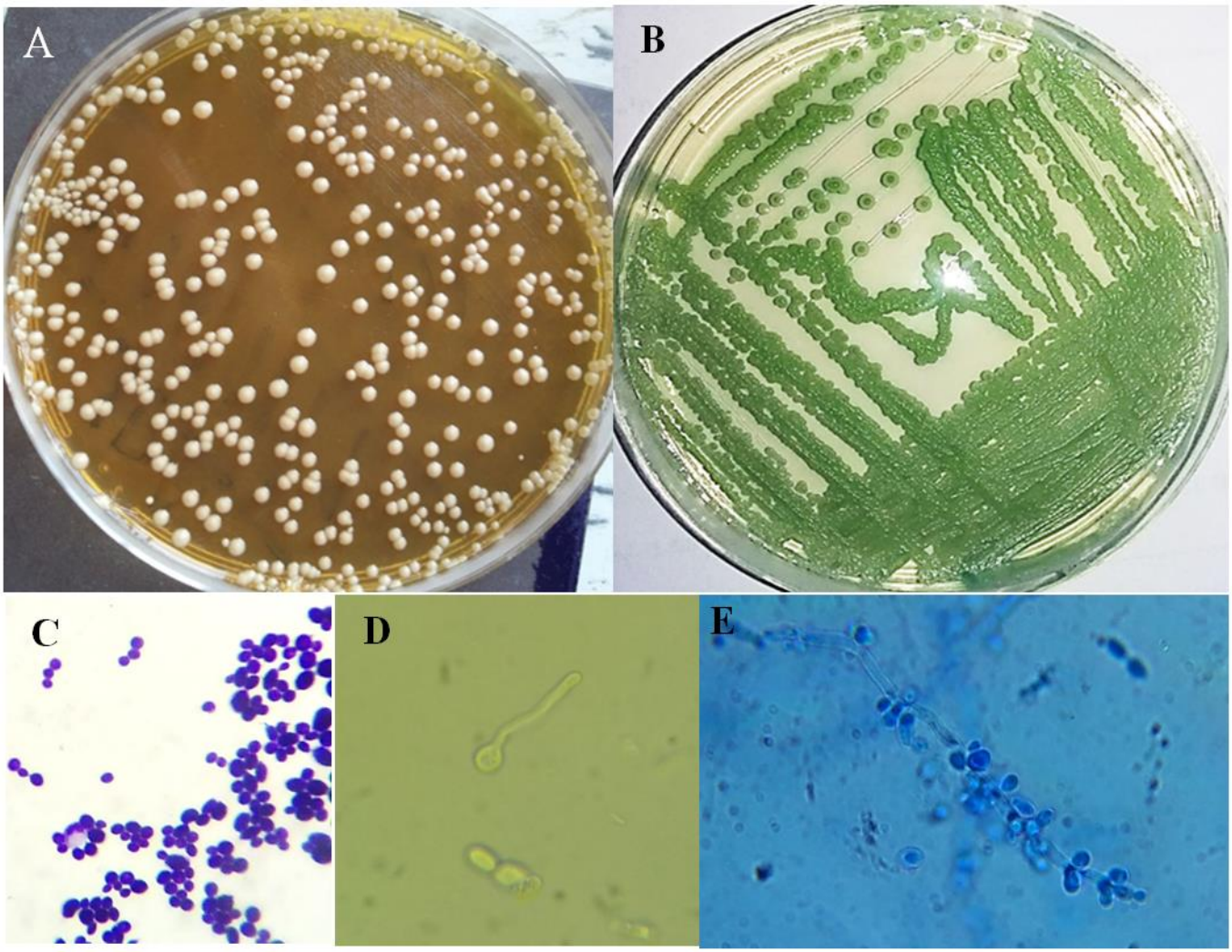

Figure 1. Identification and characterization of *Candida albicans* isolated from local chicken in Diyala Province, Iraq. Growth of *Candida albicans* on Sabouraud Dextrose agar (**A**), *Candida albicans* green colonies on CHROM agar (**B**), Blastoconidia and pseudohyphae of *Candida albicans* stained with gram stain (**C,** 100X), *Candida albicans* germ tube formation (**D**, 100X), *Candida albicans* in corn meal agar showing chlamydospores (**E**).

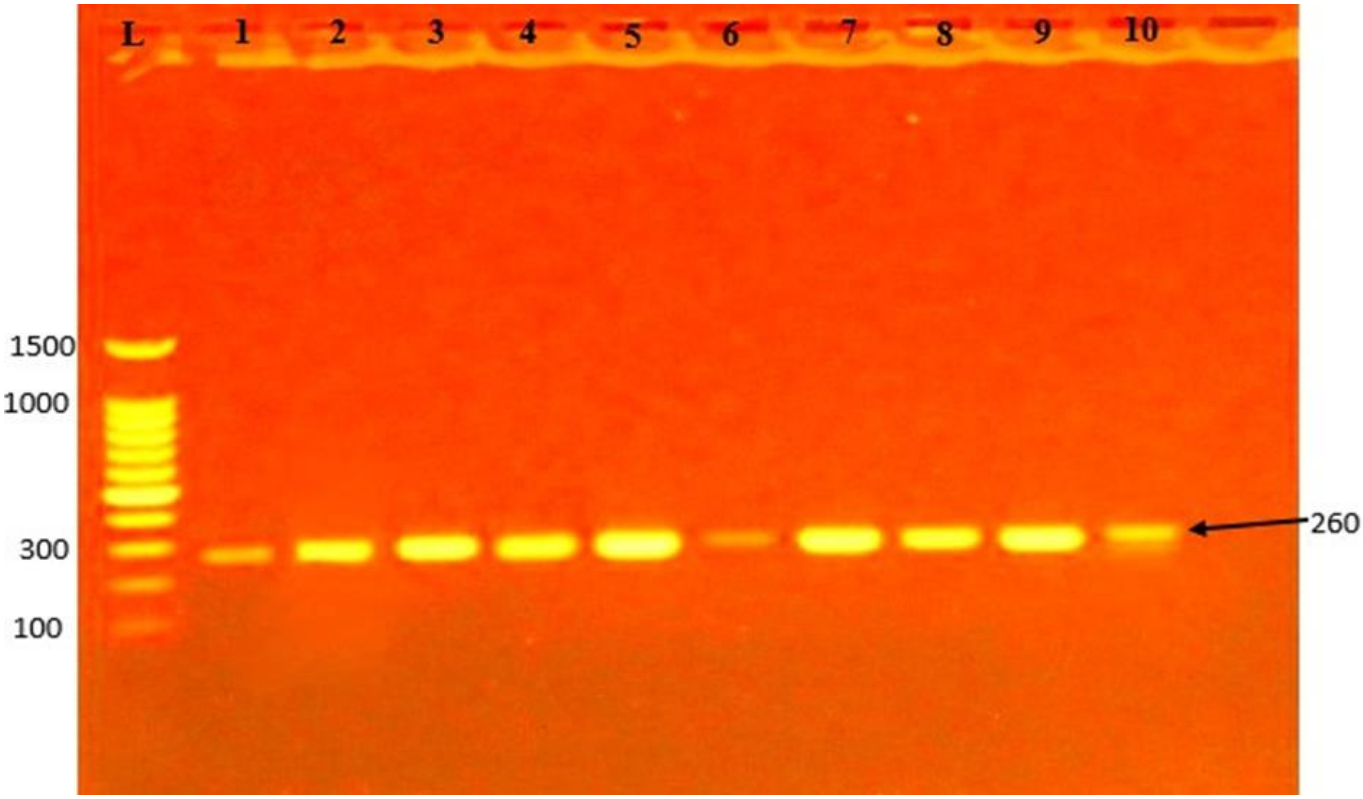

Figure 2. Agarose gel electrophoresis of 28S rDNA PCR products for *Candida albicans* isolated from local chickens under UV transilluminator. **M**: DNA ladder (100 bp – 1500bp). **Lanes**: 28S rDNA bands (260bp) for *Candida albicans*.

DISCUSSION

The study used a comprehensive approach to accurately identify *C. albicans* isolates from chicken oral cavities. The characteristic morphological signs of *C. albicans* were identified during primary examination using the Gram staining method; its cells were large, oval, purple-stained, and occurred singly or in budding yeast chains. This characteristic morphology was also mentioned by Chaffin et al. (1998). The colonies obtained on Sabouraud Dextrose agar, which was the appropriate medium for *C. albicans* and other fungi from the genus *Candida*, also pointed to this species. The culture was white with a smooth, shiny-looking growth. It was creamy and had a circular shape of entire margins; these characteristics were also reported by Hospenthal et al. (2006).

Additionally, the present study noticed that all C. albicans isolates presented distinct features which were separated them from other *Candida* species. Firstly, a positive germ tube test was evident in 2 hours when they were incubated in serum (Sharma et al., 2017). Another unique feature is the presence of chlamydospores when they were cultured on Corn meal agar, the medium also referred to as cell starvation medium (Pincus et al., 2007). In addition, CHROMagar Candida CAC medium was used and all presumptive *C. albicans* isolates yielded typical green colonies (Khadka et al., 2016). The presence of green colonies on CAC, along with the capacity to germinate tubes and chlamydospores formation, are of notable properties for *C. albicans* identification among others *Candida*, in agreement with published work (Souza et al., 2015; Kidd et al., 2016).

The identity of the isolates was molecularly confirmed by a species-specific PCR targeting the 28S rRNA gene, performed with published primers UI and U2 (Sandhu et al., 1995). Owing to the multicopy nature and conservation of fungal ribosomal RNA genes, 28S rRNA PCR effectively and sensitively detects major human and animal fungal pathogens, including *C. albicans* (Ogawa et al., 2012). In this study, all green CAC isolates that tested positive for chlamydospore and germ tube formation contained the 260-bp PCR amplicon specific for *C. albicans*.

The purpose of the current study was to determine whether the local chickens in Diyala, Iraq, were suspected of having *Candida albicans* in their mouths. The majority

of yeast isolates obtained were identified as *C. albicans*, aligning with previous findings by Samaka et al. (2011), Kaab (2013), and Razmyar et al. (2014) that *C. albicans* is the predominant *Candida* species found in avian oral cavity and digestive tract infections (Vieira and Coutinho, 2009). These results are consistent with other studies conducted in different regions, such as the study of Cafarchia et al. (2006), who isolated *C. albicans* from the crop and intestinal contents of poultry in Italy, and Talazadeh et al. (2022), who identified *C. albicans* in the pharyngeal swab and cloacal swab from birds in Ahvaz, Iran.

Antifungal susceptibility profiles of *C. albicans* vet isolates were determined using the VITEK 2 automated system. A study indicated VITEK 2 is an accurate and rapid tool for the characterization of fungal drug resistance and guiding antifungal therapy selection (Mendes et al., 2018). Here, nearly all *C. albicans* isolates exhibited sensitivity to the panel of antifungals tested, contrasting with a previous study conducted on poultry in Turkey, which reported 3 fluconazole-resistant isolates (Samaka et al., 2011) but concurring with broader animal resistance trends (Dalvand et al., 2018). Further accumulation of susceptibility data through wider geographic surveillance studies will help establish appropriate treatment guidelines for infections of animal candidiasis.

CONCLUSION

The combined use of selective culture, species-specific PCR, and antifungal sensitivity profiling on Vitek 2 offers a robust methodology to examine *C. albicans* carriage and drug susceptibility patterns in avian populations. However, further investigation is warranted to identify risk factors driving carriage in poultry flocks and develop evidence-based interventions to mitigate dissemination. A priority for future research should be multidisciplinary studies that elucidate modifiable risks, transmission dynamics, and genetic determinants of drug resistance. Implementing standardized surveillance and biosecurity protocols based on these findings will be paramount to containing outbreaks. With vigilance and proactive management, the threat posed by antifungal-resistant *C. albicans* strains in poultry can be curtailed.

DECLARATIONS

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Competing interests

The authors have no conflicts of interest to disclose related to this work.

Authors' contributions

Mahmood Ahmed Kadhim was responsible for conceptualizing and designing the study, as well as collecting local chicken samples. Sarah Jasim Abdul-Ameer, Osama Ghazi Jalil Al-Dulaimi, and Amer Khazaal Al-Azawy contributed to the manuscript by editing and analyzing data. All authors checked the analyzed data, presented findings, and the final draft of the manuscript before submission and publication.

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Ethical considerations

This study was conducted ethically and in accordance with guidelines for research integrity. Informed consent for participation was obtained from all subjects. The authors adhered to ethical principles related to plagiarism, fabrication, falsification, duplicate publication, and redundancy. All authors have reviewed the final manuscript and confirmed that any potential ethical issues have been avoided.

Availability of data and materials

The data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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