

E-test determination of antifungal susceptibility of *Candida* species isolated from turkeys

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Abstract

Introduction: *Candida* species are a natural component of the intestinal tract microflora, but in favourable conditions they can cause superficial, mucosal, or even systemic candidiasis. Poultry production might be a source of human drug-resistant yeast infections, including *Candida* spp. The limited data concerning the antifungal susceptibility of poultry *Candida* isolates prompted us to carry out research to determine the susceptibility of isolates from turkey intestinal tracts. **Method and Materials:** The beak cavity, crop and cloaca were swabbed of 580 turkeys from 58 flocks in western Poland. The susceptibility tests were conducted using the E-test method with amphotericin B, fluconazole, itraconazole, and voriconazole on 52 isolates of *C. albicans*, *C. catenulata*, *C. glabrata*, *C. palmioleophila*, *C. rugosa*, *C. krusei* and *C. lusitaniae*. **Results:** All isolates were susceptible to voriconazole. According to the MIC values obtained for amphotericin B and fluconazole, all *Candida* spp. isolates were classified as susceptible according to the described breakpoints except for *C. krusei*, which was the only isolate that was amphotericin B-, fluconazole- and itraconazole-resistant. The susceptibility to itraconazole varied: 11 of the *Candida* isolates were susceptible (21.1%), 29 were dose-dependently susceptible (55.8%), and 12 isolates were resistant (23.1%). **Conclusion:** There are few resistant strains of *Candida* in turkeys, and the drug resistance varies. When *Candida* passes from turkeys to humans, there is a wide range of antifungal treatment options.

Keywords: antimicrobials, *Candida*, E-test, MIC, susceptibility, turkeys.

Introduction

Fungi are a natural component of the intestinal tract microflora and have great influence on gut health. Both filamentous fungi and yeasts were isolated from the poultry gastrointestinal tract with *Candida* species predominating, followed by *Trichosporon* spp., *Geotrichum* spp., *Rhodotorula* spp., and *Saccharomyces* spp. (18, 22, 23, 25). The best-known yeast species is *Candida albicans*, an opportunistic fungal pathogen responsible for superficial and mucosal or systemic infections. Other species such as *C. parapsilosis*, *C. rugosa*, *C. famata*, *C. tropicalis*, and *C. krusei* might also cause candidiasis in poultry (13, 19, 20). Lesions may be observed all along the length of the intestinal tract, but mostly occurs in the beak and crop, where during severe infections pseudomembrane or diphtheric membrane formations

are observed, sometimes with “cheese-like” material (7). Our previous research has shown that antimicrobial treatment of turkeys, especially with beta-lactams, predisposes them to fungal infestations of the gastrointestinal tract (23). The highest number of species isolated in that research belonged to the *Candida* genus, which is a potentially pathogenic group. Birds can be a reservoir of harmful fungi to humans, and direct transmission of dermatophytes from poultry to workers was confirmed (27). Avian faeces as a potential source of yeast exposure for bird owners and poultry house workers are often discussed (2, 25). Due to the increasing number of drug-resistant *Candida* isolates in humans (17, 26), and a lack of research into the antifungal susceptibility of *Candida* species isolated from turkeys’ intestinal tracts, we decided to address the lack of data. The aim of this study was to determine amphotericin B, fluconazole,

itraconazole and voriconazole susceptibility of *Candida* using the E-test method on isolates from turkeys' intestinal tracts. The E-test method was chosen because previous comparative analyses of susceptibility testing methods showed that it is a reliable alternative to the broth microdilution method (3, 11, 16). Moreover, this method is easier and less time-consuming thanks to its design as a plastic strip impregnated with a predefined concentration gradient of an antifungal drug.

Material and Methods

The isolates of fungi were obtained from British United Turkeys Big 6, from 58 commercial flocks in western Poland managed by one company in the years 2014–2017, as described previously (22, 23). The birds were monitored during the rearing period for gastrointestinal disorders, and there was no case of clinical mycosis of the gastrointestinal tract. Ten birds from each flock were sampled. The swabs taken from beak cavity, crop and cloaca were cultured on Sabouraud glucose agar with chloramphenicol (Emapol, Poland) and incubated at 37°C for 48 hours. The strains were isolated and identified using classical mycological diagnostic methods: microbiological cultures and microscopic examination. Identification of the genera of the fungi was based on the morphological characteristics of the isolates and their growth on CHROMagar media (Emapol, Poland). For the susceptibility study, only the cloacal isolates were chosen. For the species identification, a PCR with ITS1 (5'-TCCGTAGGTGAA CCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (Genomed, Poland) amplified the ITS1–5.8rRNA–ITS2 fragment (29) and the PCR products were sequenced. The genetic material was isolated using the Genomic Mini AX Yeast (A&A Biotechnology, Poland) according to the manufacturer's instructions. The PCR was performed in a 25 µL reaction mixture containing 50 ng of DNA in 2 µL, 0.25 µL of each primer at a concentration of 25 mM, 12.5 µL of PCR Mix Plus (A&A Biotechnology), and 10 µL of water. Amplification was carried out in a T100 PCR thermal cycler (Bio-Rad, USA). The PCR was run through initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and final elongation at 72°C for 10 min. The PCR products were subjected to electrophoresis in a 1.5% agarose gel stained with SYBR Green (Sigma Aldrich, USA) and visualised under ultraviolet light. They (a 900 bp product for *C. glabrata*, one of 550 bp for *C. albicans* and a 400 bp length for the rest of the examined species) were sequenced using the PCR primers detailed above designed by Genomed. Raw readings of respective sequences were edited, analysed, and assembled using Mega 6 software (28) and compared to other sequences in the GenBank database. For the susceptibility tests, 52 isolates originating from cloaca samples were selected which classified to 10 strains of *C. albicans*, *C. catenulata*, *C. glabrata*,

C. palmioleophila, and *C. rugosa*. Additionally, one cloacal isolate of *C. krusei* and one of *C. lusitaniae* were examined.

Inoculum suspensions. Yeast inoculum suspensions were prepared as described in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 (5). Briefly, inoculum suspensions of *Candida* spp. isolates were prepared in 0.9% saline solution and adjusted to a final concentration of 0.5 according to the McFarland standard with approximately $1-5 \times 10^6$ CFU/mL. This suspension was used directly to inoculate agar plates for the E-test (bioMérieux Polska sp. z o.o., Poland).

Antifungal agents. E-test strips for amphotericin B (AMB) 0.002–32 µg/mL, itraconazole (ICZ) 0.002–32 µg/mL, voriconazole (VCZ) 0.002–32 µg/mL and fluconazole (FCZ) 0.016 – (256 µg/mL) were purchased from bioMérieux Polska sp. z o.o.

E-test. The E-test was performed according to the instructions of the manufacturer. For the antifungal susceptibility testing, RPMI 1640 medium containing 2% glucose (Emapol, Poland) was used. Each solidified medium was inoculated with a sterile swab dipped into the respective inoculum suspension and then evenly smeared over the surface of the plate. The inoculated agar surface was allowed to dry for 15 minutes before the E-test strip was placed on it. The plates were then incubated at 35°C for 48 h. After the plates had been incubated for 24 and 48 hours, MICs were read as the lowest drug concentrations at which the border of the ellipse touched the scale on the strip. The final MIC values were based on the consensus between two readers. Guidelines for the in vitro susceptibility of *Candida* species to fluconazole (FCZ), itraconazole (ICZ), and voriconazole (VCZ) were adapted from the M27-A3 and M60-ED 1 documents from the CLSI (5, 6), and the interpretative criteria for amphotericin B (AMB), which were not defined in CLSI documents, were adopted from the literature (14, 24). The lowest concentrations at which 50% (MIC₅₀) and 90% (MIC₉₀) of the isolates were inhibited were determined for each group.

Results

The MIC ranges and values of MIC₅₀ and MIC₉₀ obtained for amphotericin B, fluconazole, itraconazole, and voriconazole in susceptibility testing determined by the CLSI method are summarized in Table 1.

According to the MIC values obtained for amphotericin B, fluconazole and voriconazole, *C. albicans*, *C. catenulata*, *C. glabrata*, *C. palmioleophila*, *C. rugosa*, and *C. lusitaniae* isolates were classified as susceptible according to the described breakpoints. *C. krusei* was the only isolate which was amphotericin B and itraconazole resistant. The MIC values for itraconazole were 0.125 to 8 µg/mL, indicating that 11 of the *Candida* isolates were susceptible (21.1%), 29 were dose-dependently susceptible (55.8%), and 12 isolates were resistant (23.1%) (Table 2).

Table 1. MICs ($\mu\text{g/mL}$) of antifungal agents for *Candida* isolates determined by E-test

<i>Candida</i> species (number of strains)	Antifungal agent	MICs $\mu\text{g/mL}$ determined by E-test	MIC ₅₀	MIC ₉₀	Susceptibility breakpoints	Resistance breakpoints
<i>C. albicans</i> (10)	Amphotericin B	0.125–1	0.38	1	≤ 1	> 1
	Fluconazole	0.125–0.75	0.19	0.75	≤ 2	≥ 8
	Itraconazole	0.125–1	0.38	1	≤ 0.125	≥ 1
	Voriconazole	0.002–0.094	0.012	0.094	≤ 0.12	≥ 1
<i>C. catenulata</i> (10)	Amphotericin B	0.094–1	0.5	1	≤ 1	> 1
	Fluconazole	0.064–16	0.5	16	≤ 8	≥ 64
	Itraconazole	0.032–3	0.075	3	≤ 0.125	≥ 1
	Voriconazole	0.004–0.19	0.094	0.19	≤ 1	≥ 4
<i>C. glabrata</i> (10)	Amphotericin B	0.125–1	0.19	1	≤ 1	> 1
	Fluconazole	0.75–4	2	4	≤ 8	≥ 64
	Itraconazole	0.125–0.38	0.125	0.38	≤ 0.125	≥ 1
	Voriconazole	0.012–0.38	0.064	0.38	≤ 1	≥ 4
<i>C. palmiroleophila</i> (10)	Amphotericin B	0.125–0.75	0.19	0.75	≤ 1	> 1
	Fluconazole	0.5–2	1	2	≤ 8	≥ 64
	Itraconazole	0.125–8	0.5	8	≤ 0.125	≥ 1
	Voriconazole	0.023–0.125	0.064	0.125	≤ 1	≥ 4
<i>C. rugosa</i> (10)	Amphotericin B	0.19–0.5	0.25	0.5	≤ 1	> 1
	Fluconazole	0.25–1	0.75	1	≤ 8	≥ 64
	Itraconazole	0.125–1	0.38	1	≤ 0.125	≥ 1
	Voriconazole	0.006–0.047	0.012	0.047	≤ 1	≥ 4
<i>C. krusei</i>	Amphotericin B	1.5	-	-	≤ 1	> 1
	Fluconazole	24	-	-	≤ 8	≥ 64
	Itraconazole	1	-	-	≤ 0.125	≥ 1
	Voriconazole	0.38	-	-	≤ 0.5	≥ 2
<i>C. lusitaniae</i>	Amphotericin B	0.5	-	-	≤ 1	> 1
	Fluconazole	0.25	-	-	≤ 8	≥ 64
	Itraconazole	0.19	-	-	≤ 0.125	≥ 1
	Voriconazole	0.02	-	-	≤ 1	≥ 4

Fluconazole, itraconazole and voriconazole range values according to Clinical and Laboratory Standards Institute (CLSI) literature guidelines for amphotericin B were adapted from references (11, 23). Ranges: FCZ (0.016–256 $\mu\text{g/mL}$), ICZ (0.002–32 $\mu\text{g/mL}$), VCZ (0.002–32 $\mu\text{g/mL}$), and AMB (0.002–32 $\mu\text{g/mL}$)

Table 2. Susceptibility patterns of *Candida* species isolated from turkeys' intestinal tracts based on MIC values

<i>Candida</i> species (number of strains)	Antifungal agent	Susceptible	Dose-dependently susceptible	Resistant
<i>C. albicans</i> (10)	Amphotericin B	10 (100%)	-	-
	Fluconazole	10 (100%)	-	-
	Itraconazole	1 (10%)	7 (70%)	2 (20%)
	Voriconazole	10 (100%)	-	-
<i>C. catenulata</i> (10)	Amphotericin B	10 (100%)	-	-
	Fluconazole	10 (100%)	-	-
	Itraconazole	1 (10%)	7 (70%)	2 (20%)
	Voriconazole	10 (100%)	-	-
<i>C. glabrata</i> (10)	Amphotericin B	10 (100%)	-	-
	Fluconazole	10 (100%)	-	-
	Itraconazole	5 (50%)	5 (50%)	-
	Voriconazole	10 (100%)	-	-
<i>C. palmiroleophila</i> (10)	Amphotericin B	10 (100%)	-	-
	Fluconazole	10 (100%)	-	-
	Itraconazole	2 (20%)	4 (40%)	4 (40%)
	Voriconazole	10 (100%)	-	-
<i>C. rugosa</i> (10)	Amphotericin B	10 (100%)	-	-
	Fluconazole	10 (100%)	-	-
	Itraconazole	2 (20%)	5 (50%)	3 (30%)
	Voriconazole	10 (100%)	-	-
<i>C. lusitaniae</i> (1)	Amphotericin B	-	1 (100%)	-
	Fluconazole	1 (100%)	-	-
	Itraconazole	-	1 (100%)	-
	Voriconazole	1 (100%)	-	-
<i>C. krusei</i> (1)	Amphotericin B	-	-	1 (100%)
	Fluconazole	-	1 (100%)	-
	Itraconazole	-	-	1 (100%)
	Voriconazole	1 (100%)	-	-

Values are presented as number of isolates (%)

Discussion

Infections of the intestinal tract, caused by *Candida* species, are common in birds. Usually, candidiasis in poultry does not present in its severe form, and many cases might not be diagnosed. The outbreaks reported in the literature are mostly related to a combination of alterations in the host's immune system defences, such as the integrity of the mucocutaneous barrier, an imbalance in the normal microflora, failure of proper immune response or an immunocompromised condition through stress, and the virulence factors of the *Candida* strain (13, 21). In poultry production, healthy intestinal tracts in reared birds are crucial to realising the expected profit, and consequently, *Candida* infections should be documented and widely analysed. The only analysis of antifungal susceptibility of *Candida* spp. in poultry isolates was conducted by Subramanya *et al.* (25) in chicken flocks. In previous research on yeast occurrence in turkey intestinal tracts, *Candida* was the most frequently isolated genus (22, 23). Among *Candida* species, *C. albicans*, *C. rugosa*, *C. catenulata*, *C. glabrata*, *C. palmiophila*, and *C. krusei* were isolated in the present study, which were previously noted as constituents of avian microbiota (4, 8, 10, 12, 13, 25). The results of antifungal susceptibility of *Candida* species isolated from turkeys' intestinal tracts showed that all isolates were susceptible to voriconazole ($\leq 1 \mu\text{g/mL}$), whereas in contrasting findings the chicken *Candida* isolates susceptibility interval was 0.06–8 $\mu\text{g/mL}$ and *C. albicans*, *C. tropicalis*, and *C. krusei* were among the resistant strains (25). In our research the *Candida* spp. susceptibility to amphotericin B and fluconazole was high, and among 52 *Candida* isolates, only one, the *C. krusei* strain, was amphotericin B resistant. The susceptibility to amphotericin B of chicken *Candida* isolates varied, and the chicken *C. krusei* strains were susceptible to amphotericin B, and resistant to fluconazole (25). Comparison of our results to Subramanya *et al.* (25) data shows the need for further research on antifungal susceptibility of *Candida* spp. in poultry because of the variability of susceptibility among the *Candida* species isolated from turkeys and chickens. The lack of data is not the only reason why the investigation should be conducted. Birds and their faeces are a source of many fungi which can threaten human health (2, 30). The frequency of isolation of *Candida* in poultry confinements (in litter and settled dust) was approximately 50% (15), which can be dangerous for poultry house workers, especially those with immunosuppressive diseases. The knowledge deficiency about the antimicrobial susceptibility of poultry *Candida* species might engender a human health risk, and the antifungal resistance of these microorganisms should be monitored, due to the risk of zoonotic infections. The common form of invasive candidiasis in humans is candidaemia, a blood stream infection which often results in long-term hospitalisation and death (1). For years, the most common species isolated from blood

was *C. albicans* (8), but the latest data show an increasing proportion of non-*C. albicans* infections caused by *Candida* species which were also isolated from poultry (24). Falagas *et al.* (8) noted that 21% of *Candida* isolates obtained from patients' blood were resistant to one or more antifungal agents. Among turkey isolates, 23% of the strains were resistant to one antifungal, and multi-drug resistance was observed in one isolate. *Candida* species isolated from humans are mostly resistant to fluconazole and voriconazole (8, 9). In contrast to human isolates, turkey isolates are resistant to itraconazole. The obtained data shows that there are few resistant strains of *Candida* in turkeys, and the drug resistance varies. In the case of transmission of *Candida* from turkeys to humans, there is a wide range of antifungal treatment options.

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