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Original article

Investigation of the correlation between virulence factors and genotypic profiles of *Candida albicans* isolated from turkeys

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Abstract

Candida albicans exists as an element of the normal flora in the skin, mucosa, and gastrointestinal tract, which is the principal reservoir for this organism. When the delicate balance in the host-yeast relationship is tipped in favour of the microorganism by antimicrobial treatment, illness, or other debilities of the host, fungi may overgrow and cause disease, such as candidiasis. The aim of this study was to analyse three virulence factors, haemolysin, proteinase, and phospholipase, with the genotypic profiles of *C. albicans* isolated from turkeys and to investigate if any correlation between these features exists. The genotypic profiles were generated using the polymerase chain reaction (PCR) with the RDS6 primer, and the enzymatic activity was evaluated in culture. Among the *C. albicans* isolates, four genotypic profiles using random amplified polymorphic DNA (RAPD) and three enzymatic profiles were defined. Genotypic profile A was characterised by seven bands, genotypic profile B was characterised by nine bands, genotypic profile C was characterised by nine bands, and genotypic profile D was characterised by four bands. Enzymatic profile I demonstrated no haemolysin or proteinase activity, but phospholipase activity was present; enzymatic profile II displayed no proteinase activity, but haemolysin and phospholipase activity were produced; and enzymatic profile III revealed all three types of enzymatic activity. The genotypic profiles were strictly correlated with the enzymatic profiles among the *C. albicans* isolates from poultry.

Key words: *Candida albicans*, turkeys, genotypic profile, RAPD, proteinase, haemolysin, phospholipase

Introduction

Candida albicans is the most common fungal pathogen isolated from clinical samples and is also the most common yeast species carried by healthy individuals. *C. albicans* exists as an element of the normal flora of the skin, mucosa, and gastrointestinal tract, which is the principal reservoir for this organism. When the delicate balance in the host-yeast relationship is tipped in favour of the microorganism by anti-

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microbial treatment, illness, or other debilities of the host, fungi may overgrow and cause disease, such as candidiasis (Dharma et al. 2013, Sokół et al. 2015).

C. albicans produces some virulence factors that facilitate its proliferation. These virulence factors aid in adhesion to the epithelium and invasion of the host tissue. For example, extracellular hydrolytic enzymes, including secreted aspartyl proteinases and phospholipases, degrade immunoglobulins and proteins of the extracellular matrix; they also inhibit the phagocytosis of polymorphonuclear neutrophils and induce inflammatory reactions (Calderone and Fonzi 2001, Hube and Naglik 2001, Inci et al. 2012). Haemolysin is another putative virulence factor thought to contribute to the pathogenesis of *Candida* infections. In particular, the secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis (Tsang et al. 2007).

Genetic analysis of *Candida* isolates has revealed that virulence factors may be intimately related to the genotypic profiles. Molecular methods have been used for such analysis because of their sensitivity and specificity. Random amplified polymorphic DNA (RAPD) analysis is commonly used for characterisation of *C. albicans* in humans (Costa et al. 2011). Therefore, the aim of this study was to analyse the virulence factors and genotypes of *C. albicans* isolated from poultry and to investigate if any correlation between these features exists.

Materials and Methods

Fungal strains and polymerase chain reaction (PCR) identification of C. albicans

The fungal strain collection consists of the strains isolated from turkeys (BIG 6) intestinal tract (Sokół et al 2017). DNA was extracted from the 38 strains identified as *C. albicans* using CHROMagar & Candida medium plates, according to the instructions of the Genomic DNA Extraction Kit (A&A Biotechnology, Gdynia, Poland). A spectrophotometric method was used to test the DNA purity and content, and the DNA samples were preserved at -20°C until use.

PCR amplification and electrophoresis

The general primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for the PCR (White 1990). 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 performed in a Bio-Rad T100 PCR Thermal Cycler. The PCR was run at 95°C for 5 min, 95°C for 1 min, 55°C for 1min, and annealing at 72°C for 2 min for 35 cycles, and then a final extension at 72°C for 10 min. The PCR products were subjected to electrophoresis in a 1.5% agarose gel stained with SybrGreen (Sigma Aldrich, Poland) and visualised under ultraviolet light. The size of the respective PCR products was determined using molecular mass marker DNA Marker 1 (A&A Biotechnology). For future analysis, the isolates with 550 bp product size, typical for *C. albicans* were used.

RAPD analysis

For the genetic analyses, the primer RSD6 was selected for DNA amplification because it produced the largest number of bands with the clearest patterns. The amplification reactions using primer RSD6 (5'-GCGATCCCCA-3') were performed as described by Dassanayake et al. (2002). The reactions were performed in a volume of 25 µL, consisting of approximately 50 ng of DNA template in 2 µL, 12.5 µL of PCR Mix Plus (A&A Biotechnology), 1.25 µL of 1.5 µM RSD6, and 9.75 µL of water. Amplification was performed in a Bio-Rad T100 PCR Thermal Cycler programmed as follows: five cycles including denaturation at 94°C for 30 s, annealing at 27°C for 2 min, and primer extension at 72°C for 2 min, followed by 45 denaturation cycles at 94°C for 30 s, annealing at 32°C for 2 min, and a final extension at 72°C for 2 min, held at this temperature for 15 min. The PCR products were subjected to electrophoresis in a 1.5% agarose gel stained with SybrGreen (Sigma Aldrich, Poland) and visualised under ultraviolet light.

Determination of enzymatic activity

To prepare the inocula, yeast cell suspensions were adjusted to match the turbidity of a 0.5 McFarland standard, corresponding to 1~5 × 10^6 colony forming units (CFU)/mL.

Phospholipase activity

To determine the phospholipase activity, the egg yolk agar method of Price et al. (1984), modified by Samaranayake et al. (1984), was employed. The culture medium consisted of 1 L of Sabouraud Dextrose Agar (Oxoid) containing 1 M NaCl, 0.005 M $CaCl_2$, and 10% sterile egg yolk. Ten microliters of previously

Table 1. The enzymatic activity and genotypic profiles of the isolates.

Enzymatic profile	Hemolysin	Proteinase	Phospholypase	Number of strains	RAPD profile
I			+	6	D
II	+		+	12	A
II	+		+	10	C
III	+	+	+	8	B

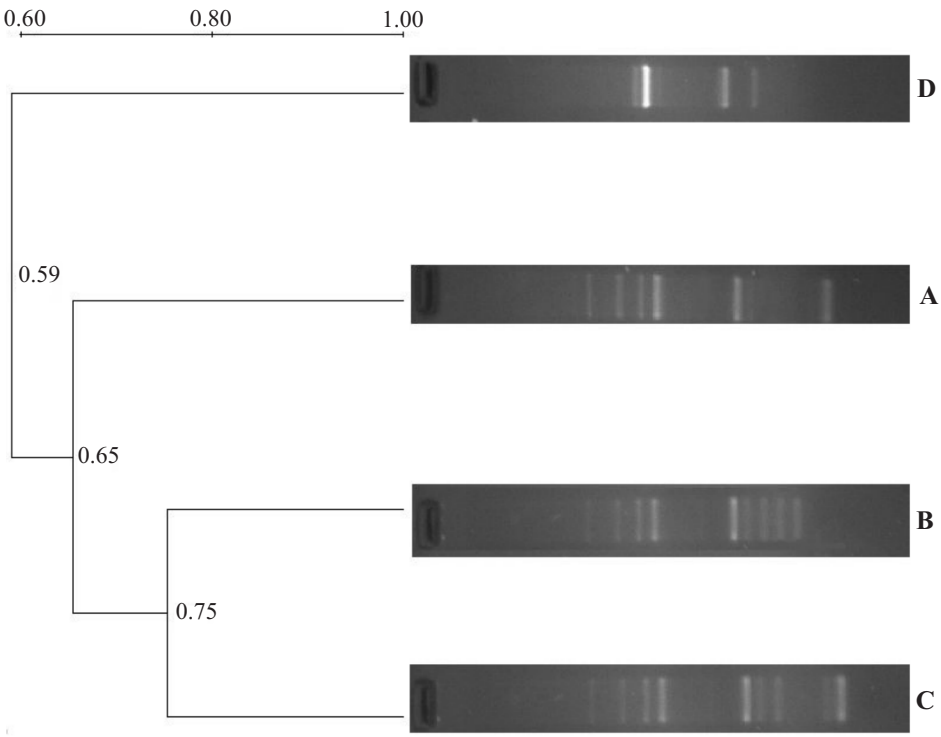

Fig. 1. Dendrogram showing the relationship between *C.albicans* genetic profiles on the basis of RAPD patterns with similarity percentages (UPGMA).

prepared yeast suspension was inoculated onto plates; these plates were then incubated at 37°C for 5 days under aerobic conditions. The presence of enzymatic activity was determined by the formation of a precipitation zone around the yeast colonies.

Proteinase activity

To determine proteinase activity, bovine serum albumin agar, defined by Staib (1965), was employed. The agar contained 0.1% KH_2PO_4, 0.05% $MgSO_4$, 2% agar, and 1% bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA). The final pH was adjusted to 4.5. Ten microliters of previously prepared yeast suspension was inoculated onto plates; these plates were then incubated at 37°C for 10 days under aerobic conditions. The presence of proteinase activity was determined by the formation of a transparent halo around the yeast colonies.

Haemolysin activity

To determine haemolysin activity, Sabouraud Dextrose Agar (Oxoid) containing 7% sheep blood and 3% glucose at a final pH adjusted to 5.6 ± 0.2 was employed. Ten microliters of previously prepared yeast suspension was inoculated onto plates; these plates were then incubated at 37°C for 48 h under aerobic conditions. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive haemolysin activity.

Results

Among the 38 isolates presumptively identified as *C. albicans*, only 36 isolates gave the PCR product that was 550 bp typical for *C. albicans* and were used for the enzymatic and genetic analyses. Among the 36 strains of *C. albicans*, 4 genotypic profiles and 3 enzymatic profiles were defined (Table 1).

RAPD

The RAPD profiles are shown as a dendrogram in Fig. 1. The amplified fragments ranged from 400 to 1900 bp, and the number of bands for each primer varied from 4 to 9. The genotype interpretation criteria were related to differences in the position of the RAPD bands. The generated profile of the bands allowed the identification of four different genotypic profiles. Twelve strains (33%) presented genotypic profile A, which was characterised by seven bands; eight strains (22%) presented genotypic profile B, which was characterised by nine bands; ten strains (28%) presented genotypic profile C, which was characterised by nine bands; and eight strains (17%) presented genotypic profile D, which was characterised by four bands. The genotypes shared a similarity from 0.59 to 0.75.

Enzymatic activity

All 36 isolates expressed phospholipase activity, 8 (17%) expressed proteinase activity, and 30 (83%) expressed haemolysin activity. According to the enzymatic activity, three enzymatic profiles were noted. Enzymatic profile I demonstrated no haemolysin or proteinase activity, but phospholipase activity was produced; profile II had no proteinase activity, but haemolysin and phospholipase activity were produced; and profile III revealed all three types of enzymatic activity.

Genotypic profile and enzymatic profile correlation

A correlation between the genotypic and enzymatic profiles between the analysed isolates was observed. The three enzymatic profiles corresponded with four genotypic profiles: six isolates showed enzymatic profile I, which corresponded with genetic profile D; twenty two isolates showed enzymatic profile II, which corresponded with genetic profiles A and C; and eight isolates showed enzymatic profile III, which corresponded with genetic profile B. Bands at 1000 bp, 950 bp, and 550 bp were noted in all genetic profiles with haemolysin activity; bands at 1900 bp, 1300 bp, 620 bp and 580 bp were observed in profiles with phospholipase activity; and a band at 520 bp was unique in profiles with proteinase activity.

Discussion

It has been reported that the enzymatic activity of *Candida spp.* may vary depending on the species and the source of the isolates. In our study, 17% of the isolates from turkeys showed proteinase activity, 83% of the isolates showed haemolysin activity, and all strains showed phospholipase activity. The features are similar to the *C. albicans* isolated from humans. A total of 85% of the *C. albicans* strains isolated from human blood, 94.5% of the *C. albicans* strains isolated from subgingival biofilms of diabetic patients with chronic periodontitis, and 100% of the *C. albicans* strains from the oral rinse of diabetes patients have been shown to produce phospholipase (Tsang et al. 2007, Mohan das and Ballal 2008, Sardi et al. 2011). The correlation between the level of proteinase production and some genotypes has been noted by Tavanti et al. (2007). In our study, proteinases were produced by 17% of the turkey isolates, compared to 82% of *C. albicans* isolates from humans (Tsang et al. 2007). As for haemolysin activity, Sachin et al. (2012) noted that almost 95% of human isolates showed this type of activity, compared to 83% in our study of turkey isolates.

It is known that the genotype is strictly connected with the phenotypic features. However, this is the first report to analyse the genetic and virulence factors of *C. albicans* isolates from poultry. Moreover, a possible correlation between the enzymatic activity and RAPD profiles was demonstrated. It was noted that enzymatic profile I correlated with genotype D, profile II correlated with genotypes A and C, and profile III correlated with genotype B.

Furthermore, it was observed that the number and presence of specific bands might be correlated with specific enzymatic activity. The genetic profiles of the strains with the widest enzymatic activity (haemolysin, phospholipase, and proteinase) showed more bands (9 bands), compared to strains with phospholipase and haemolysin activity (7 bands) or only phospholipase activity (4 bands). Three of the four bands of genotype D, which showed only phospholipase activity, were also present in the remaining genotypes that showed phospholipase activity. The isolates that showed proteinase activity had one unique band, which was absent in the other strains. The genotypic profiles that showed haemolysin activity had four common bands.

Previous research conducted by others has analysed the correlation between the RAPD profile with the primer RSD6 and azole susceptibility. In the study by Costa et al. (2011), the generated profile of the bands showed 8 different genotypes among the 13 *C. albicans* isolates from humans infected with human immunodeficiency virus. In relation to RAPD-PCR, most of resistant isolates were classified into one group, suggesting that a correlation between genotypes and fluconazole-resistance may be present. In

contrast, Sun et al. (2009) did not observe a correlation between the genotype and fluconazole susceptibility. However, they noted that the RSD6 primer was successful in eliciting 17 genotypes among 205 isolates.

Our analysis of *C. albicans* isolated from turkeys suggests that the RAPD genetic profiles with the RDS6 primer may be correlated with enzymatic activity. The genetic profile may be useful for isolates virulence predicting and showing the need for *C.albicans* treatment in poultry flock.

Further comprehensive studies with a larger sample size of isolates from different hosts are needed to identify the universal relationship between *Candida* genotypic profiles and the virulence factors investigated.

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