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Research Note—

Unexpected Diversity of Yeast Species in Esophageal Mycosis of Waterfowls

Marianna Domán,[AE] László Makrai,[B] Krisztina Bali,[A] György Lengyel,[C] Tibor Laukó,[D] Krisztián Bányai[A]

[A]Institute for Veterinary Medical Research, Centre for Agricultural Research, H-1143 Budapest, Hungary
[B]Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, H-1143 Budapest, Hungary
[C]Laboratory of Epidemiological Virology, Hungarian Defence Forces Military Medical Centre, H-1134 Budapest, Hungary
[D]Veterinary Diagnostic Laboratory for Animal Health Ltd., H-5600 Békéscsaba, Hungary

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SUMMARY. This study was performed to evaluate the diversity and prevalence of yeasts associated with esophageal mycosis in domestic ducks and geese. Fungi were isolated from esophageal lesions of dead animals sent for microbiologic laboratory diagnosis. Species identification using a culture-dependent method was carried out by sequencing of the internal transcribed spacer (ITS)1-5.8S rRNA-ITS2 region. The most frequently isolated yeast was *Candida albicans* (43.1%) followed by *Saccharomyces cerevisiae* (17.6%), *Candida kefyr* (11.7%), *Kazachstania bovina* (11.7%), *Candida lambica* (3.9%), and single isolates (1.9%) representing *Candida inconspicua*, *Candida rugosa*, *Candida pelliculosa*, *Candida krusei*, *Magnusiomyces capitatus*, and *Trichosporon asahii*. Our results indicate that a number of potentially pathogenic yeast species can be isolated from esophageal mycosis of waterfowls, but additional studies are needed to make conclusions regarding their possible etiologic role in disease.

RESUMEN. *Nota de Investigación*- Diversidad inesperada de especies de levaduras en la micosis esofágica de aves acuáticas.
Este estudio se realizó para evaluar la diversidad y prevalencia de levaduras asociadas con micosis esofágica en patos y gansos domésticos. Se aislaron hongos de lesiones esofágicas de animales muertos enviados para diagnóstico de laboratorio microbiológico. La identificación de especies utilizando un método dependiente del cultivo se llevó a cabo mediante la secuenciación de la región ITS1-5.8S rRNA-ITS2. La levadura aislada con mayor frecuencia fue *Candida albicans* (43.1%) seguida de *Saccharomyces cerevisiae* (17.6%), *Candida kefyr* (11.7%), *Kazachstania bovina* (11.7%), *Candida lambica* (3.9%) y aislados simples (1.9% a 1.9%) que representan a *Candida inconspicua*, *Candida rugosa*, *Candida pelliculosa*, *Candida krusei*, *Magnusiomyces capitatus* y *Trichosporon asahii*, respectivamente. Estos resultados indican que una serie de especies de levaduras potencialmente patógenas se pueden aislar de la micosis esofágica de las aves acuáticas, pero se necesitan estudios adicionales para obtener conclusiones sobre su posible papel etiológico en la enfermedad.

Key words: esophageal mycosis, yeast, *Candida*, *Magnusiomyces, Trichosporon,* waterfowls

Abbreviations: ITS = internal transcribed spacer; NCBI = National Center for Biotechnology Information

Fungal diseases affect the health and survival of domesticated and wild animals. The spread of mycoses is promoted by spillover events, where animals may transfer pathogenic fungal organisms to other animal hosts or humans and *vice versa* (1). *Candida* species are common commensal components of the normal microbiome in the oral cavity and the gastrointestinal tract of humans, livestock, and wild animals. However, the opportunistic pathogen fungi that readily colonize vertebrates may cause clinical illness when predisposing factors (e.g., impairments of host's immune system, prolonged use of antibacterial therapy, long-term use of indwelling devices or intravenous catheters) are present. In birds, the upper gastrointestinal tract is affected most frequently, but infections ranging from superficial mucosal infections to systemic candidiasis may also occur (2,3). Although the occurrence of candidiasis is sporadic, outbreaks can be costly. Crop mycosis mostly caused by *C. albicans* is characterized by whitish, thickened areas of the crop accompanied by depression, reduced feed intake, indigestion, and delayed growth (4,5). Even though candidiasis-associated mortality is negligible, the productivity of affected poultry farms may decrease while the production costs increase. A mycologic analysis of healthy broiler chickens revealed that the incidence of *Candida* species in the crops ranged from 17.4% to 51.5%, but less than 1% of the examined birds exhibited visible lesions attributable to *Candida* (6). Colonization and infection of turkeys, pigeons, ornamental birds, and avian scavengers with *Candida* spp. were also reported; nevertheless, data from duck and goose mycosis are scant (5,7,8). Therefore, the aim of our study was to identify fungal pathogens from domestic waterfowls diagnosed with esophageal mycosis.

MATERIALS AND METHODS

Mortality associated with esophageal mycosis seen in duck and goose flocks prompted owners to send samples for laboratory diagnosis. Upon gross pathologic investigation, samples were collected from the whitish area of the esophageal mucosa of dead geese and ducks using sterile cotton swabs after necropsy. No histopathology was performed. A total of 94 isolates were received over a 14-yr period.

The swabs were cultured on Sabouraud dextrose agar supplemented with chloramphenicol and incubated at 35 C for 48 hr. All colonies with a yeast-like morphology were isolated and identified by the REMEL Yeast Plus kit (Biomedica, Vienna, Austria). The species level identification was confirmed by molecular analysis. In brief, DNA extraction was performed using the FavorPrep Fungi/Yeast Genomic

[E]Corresponding author. Institute for Veterinary Medical Research, Centre for Agricultural Research, H-1581 Budapest, P.O. Box 18, Hungary. E-mail: doman.marianna@agrar.mta.hu

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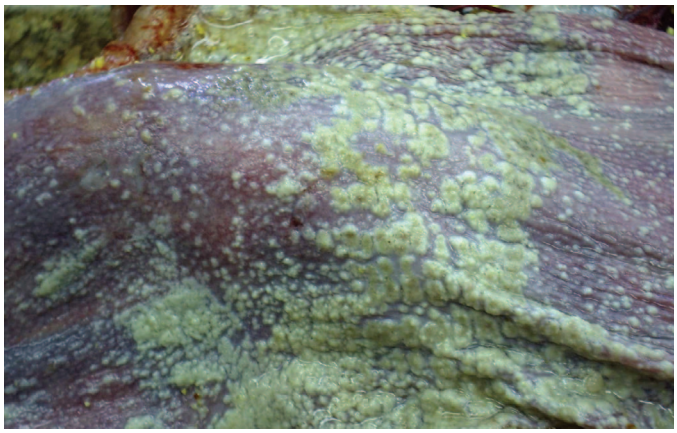

Fig. 1. Whitish, thickened areas on the esophageal mucosa caused by yeasts.

DNA Extraction Mini Kit (Favorgen, Ping-Tung, Taiwan) according to the manufacturer's instructions. A single colony of each isolate (containing $\sim 1 \times 10^6$ to 5×10^6 yeast cells) was suspended in 2 ml phosphate-buffered saline and 300 µl of suspension was used for DNA extraction. The extracted genomic DNA was used for PCR. PCR amplification targeting the entire internal transcribed spacer (ITS) rRNA region was performed with universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (5,9,10). The reaction mixture was prepared in a final volume of 15 µl that contained 1 µl fungal DNA, 2 µl 10× DreamTaq™ buffer, 0.5 µl dNTP (10 mM), 0.5 µl forward and reverse primers (10 µM each), 0.1 µl DreamTaq DNA polymerase (5 U/µl; Thermo Fisher Scientific, Waltham, Massachusetts) and 10.4 µl distilled water. Amplification consisted of an initial denaturation at 95 C for 3 min followed by 40 cycles of denaturation at 95 C for 30 sec, annealing at 50 C for 30 sec, and extension at 72 C for 1 min; and a final extension at 72 C for 10 min. After the electrophoresis in 1% agarose gel stained with GelRed (Biotium, Fremont, California), the PCR products were purified using Gel/PCR DNA fragments kit (Geneaid, New Taipei, Taiwan). Bidirectional sequencing of purified PCR products was carried out using the abovementioned primers with BigDye™ Terminator v3.1. cycle sequencing kit (Thermo Fisher Scientific) on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). Sequences were edited and assembled using the Mega 6 software (11) and evaluated with the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (12) using standard parameters. Each result was verified by comparing sequences against the NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/). Generated nucleotide sequences were deposited in GenBank under the following accession numbers: MT136511–MT136561.

RESULTS

Based on gross pathologic findings (Fig. 1), a total of 94 isolates was collected between 2005 and 2019 from commercial moulard and Barbary ducks and geese (Anatidae) held in distinct flocks in the south-eastern region of Hungary. Fifty-one isolates from this collection were selected for molecular analysis (Table 1). Amplification of the entire ITS region yielded fragments 400–770 bp in length. By sequence analysis, 11 different yeast species were identified among the isolates. The most frequently isolated yeast was *C. albicans* ($n = 22$; 43.1%) followed by *Saccharomyces cerevisiae* ($n = 9$; 17.6%), *C. kefyr* ($n = 6$; 11.7%) and *Kazachstania bovina* ($n = 6$; 11.7%), *C. lambica* ($n = 2$; 3.9%), and finally one isolate each (1.9%) was identified as *C. inconspicua*, *C. rugosa*, *C. pelliculosa*, *C. krusei*, *Magnusiomyces capitatus*, and *Trichosporon asahii*. The sizes of PCR products were almost equal for some species (*K. bovina* and *S.*

Table 1. Summary of investigated avian-associated yeast isolates originated from Hungary.

Isolate identifier	Host	Year of sample collection	Region or town	Yeast species
OM-1	Goose	2005	Békéscsaba	*Candida albicans*
OM-2	Goose	2005	Békéscsaba	*C. albicans*
OM-3	Goose	2005	Békéscsaba	*Kluyveromyces marxianus*
OM-5	Goose	2005	Békéscsaba	*K. marxianus*
OM-6	Goose	2005	Békéscsaba	*Candida inconspicua*
OM-7	Duck	2006	Petőfiszállás	*C. albicans*
OM-8	Duck	2006	Orosháza	*C. albicans*
OM-9	Duck	2006	Orosháza	*K. marxianus*
OM-10	Duck	2006	Orosháza	*Pichia kudriavzevii*
OM-11	Goose	2008	Békéscsaba	*C. albicans*
OM-12	Goose	2008	Békéscsaba	*C. albicans*
OM-13	Goose	2008	Gádoros	*C. albicans*
OM-14	Goose	2008	Gádoros	*C. albicans*
OM-15	Duck	2009	Orosháza	*Trichosporon asahii*
OM-16	Duck	2009	Petőfiszállás	*Candida albicans*
OM-17	Duck	2009	Petőfiszállás	*C. albicans*
OM-18	Duck	2009	Orosháza	*C. albicans*
OM-19	Goose	2011	Csanádapáca	*C. albicans*
OM-20	Duck	2011	Orosháza	*Saccharomyces cerevisiae*
OM-21	Goose	2011	Gádoros	*Kazachstania bovina*
OM-22	Duck	2012	Petőfiszállás	*S. cerevisiae*
OM-23	Duck	2012	Petőfiszállás	*S. cerevisiae*
OM-24	Duck	2012	Orosháza	*S. cerevisiae*
OM-25	Duck	2012	Orosháza	*S. cerevisiae*
OM-26	Goose	2014	Gádoros	*K. bovina*
OM-27	Duck	2014	Orosháza	*S. cerevisiae*
OM-28	Goose	2014	Gádoros	*K. bovina*
OM-29	Goose	2014	Békéscsaba	*C. albicans*
OM-30	Duck	2015	Orosháza	*Pichia fermentans*
OM-31	Goose	2015	Csanádapáca	*C. albicans*
OM-32	Duck	2015	Orosháza	*S. cerevisiae*
OM-33	Duck	2015	Orosháza	*P. fermentans*
OM-34	Duck	2016	Petőfiszállás	*C. albicans*
OM-35	Duck	2016	Petőfiszállás	*K. bovina*
OM-36	Duck	2016	Petőfiszállás	*C. albicans*
OM-37	Duck	2016	Petőfiszállás	*K. bovina*
OM-38	Duck	2017	Orosháza	*S. cerevisiae*
OM-39	Duck	2017	Orosháza	*K. bovina*
OM-40	Duck	2017	Orosháza	*S. cerevisiae*
OM-41	Goose	2017	Gádoros	*Diutina rugosa*
OM-42	Duck	2017	Orosháza	*C. albicans*
OM-43	Goose	2017	Gádoros	*Wickerhamomyces anomalus*
ML-1	Goose	2019	Mezőberény	*Candida albicans*
ML-2	Goose	2019	Csanádapáca	*C. albicans*
ML-3	Duck	2019	Gerendás	*C. albicans*
ML-4	Duck	2019	Kaszaper	*C. albicans*
ML-5	Goose	2019	Mezőberény	*C. albicans*
ML-6	Duck	2019	Battonya	*Magnusiomyces capitatus*
ML-7	Goose	2019	Gádoros	*K. marxianus*
ML-8	Goose	2019	Elek	*K. marxianus*
ML-9	Duck	2019	Magyarbánhegyes	*K. marxianus*

Table 2. The characteristics of yeasts isolated from the esophagus of ducks and geese.

Species anamorph stage (teleomorph stage)	Esophagus, $n = 51$ (%)	ITS1–ITS4 product (bp)	Percent sequence identity with reference strain (GenBank no.)
Candida albicans	22 (43.1)	540	98.8–100 (NR_125332.1)
Candida krusei (*Pichia kudriavzevii*)	1 (1.9)	510	99.58 (NR_131315.1)
Candida inconspicua (*Pichia cactophila*)	1 (1.9)	420	97.12 (NR_111116.1)
Saprochaete capitata (*Magnusiomyces capitatus*)	1 (1.9)	460	100 (NR_164086.1)
Trichosporon asahii	1 (1.9)	540	100 (NR_073341.1)
Candida kefyr (*Kluyveromyces marxianus*)	6 (11.7)	700	99.83–100 (NR_111251.1)
Candida pelliculosa (*Wickerhamomyces anomalus*)	1 (1.9)	620	99.48 (NR_111210.1)
Diutina rugosa / *Candida rugosa*	1 (1.9)	400	93.84 (NR_111249.1)
Candida lambica (*Pichia fermentans*)	2 (3.9)	450	98.88–99.78 (NR_130688.1)
Saccharomyces cerevisiae	9 (17.6)	750	94.76–98.49 (NR_111007.1)
Candida bovina (*Kazachstania bovina*)	6 (11.7)	770	99.03–99.59 (NR_155228.1)

cerevisiae, ~750 bp; *M. capitatus* and *C. lambica*, ~450 bp; *C. rugosa* and *C. inconspicua*, ~400 bp) and were identical in the case of *C. albicans* and *T. asahii* (540 bp; Table 2).

DISCUSSION

The diagnosis of esophageal mycosis in waterfowls is generally postmortem due to the lack of unequivocal clinical signs. In the course of force-feeding, microlesions arise on the mucosal surface of esophagus through direct mechanical damage caused by a feeding-tube and extreme dilatation caused by maize. Predisposing factors may alter the microbial balance and promote infection by rapidly proliferating members of the normal flora (13). The infection results in an inflexible esophagus that may rupture during the force-feeding process and ultimately may lead to death. Because the length of force-feeding is ~2 wk, and afterwards the birds are slaughtered, timely diagnosis of disease and choosing adequate treatment is difficult. Importantly, due to food safety issues, antifungal drugs to treat yeast origin mycoses in waterfowls have not been licensed in Hungary.

In the literature, only few data are available concerning the microbial causes of mycoses in the upper digestive tract in poultry (14,15). This survey presents the occurrence and possible etiologic role of various yeast species isolated from esophageal mycosis of ducks and geese. It is of note that the progress of fungal diseases in animals caused by opportunistic pathogens is not well understood (16,17). Consistent with gross pathologic findings, we anticipated yeast origin of esophageal lesions and focused on the mycologic aspects when laboratory diagnosis was performed. The biochemical and genetic identification of isolated yeasts, in brief, showed that the majority of species belonged to the genus *Candida* (78.4%) followed by *Saccharomyces* (17.6%), *Magnusiomyces* (1.9%), and *Trichosporon* (1.9%). At the species level, the most prevalent pathogen was *C. albicans*; but from >10% of esophageal samples *S. cerevisiae*, *C. kefyr*, and *K. bovina* were isolated, whereas other yeasts such as *C. lambica*, *C. inconspicua*, *C. rugosa*, *C. pelliculosa*, *C. krusei*, *M. capitatus*, and *T. asahii* were rarely isolated species. Historic data indicate *C. albicans* takes the primary role in the etiology of crop mycosis of poultry (6), but non-*albicans Candida* species and other yeast-like species have not been mentioned in the literature. Of interest, all of the isolated yeast species either frequently cause human infection (*C. albicans* and *C. krusei*) or rarely (*C. kefyr*, *C. lambica*, *C. inconspicua*, *C. rugosa*, *C. pelliculosa*, *K. bovina*, and *S. cerevisiae*) (18,19). Moreover, *M. capitatus* and *T. asahii* are considered newly emerging human pathogens characterized by high rates of mortality (50%–90%) and resistance to antifungal drugs (20,21,22). In the poultry industry, yeast strains are more well-known in their use as probiotics to improve feed utilization, enhance feed digestibility, and reduce the rate of colonization by pathogenic microorganisms. The most commonly utilized yeast as feed additive in livestock is *S. cerevisiae*; thus, the routine use of *S. cerevisiae* may open the door to the colonization of the gastrointestinal tract in poultry (23).

In this study we demonstrate that laboratory diagnosis of esophageal mycosis can be complex and problematic due to the inadequate knowledge on the prevalence of yeasts in waterfowls and to the growing number of possible agents implicated in disease etiology. Because closely related *Candida* species might be misidentified due to the great similarity in their morphologic and biochemical characteristics, in our laboratory we applied genetic markers for species identification. The target region we selected has been used in species identification in numerous studies (5,10,24,25). The length of amplicons produced for the ITS1-5.8S rRNA-ITS2 region differs among *Candida* species; however, some species have near identical fragment length by electrophoresis, and we could not make a distinction between *C. albicans* and *T. asahii*. Previous ITS region amplification of different *Candida* species indicated that, similar to *C. albicans*, *C. tropicalis*, and *C. parapsilosis* had an ~540 bp amplicon length while an identical PCR product (400 bp) size was noted for *C. catenulata* and *C. rugosa* (9). According to these observations, sequencing of the ITS1-5.8S rRNA-ITS2 region is indispensable to confirm species identification.

In conclusion, investigating the prevalence of yeasts of the gastrointestinal tract is substantial to determine the disease process of esophageal mycosis in poultry, as non-*albicans* yeast species might contribute to the development of infection as well. The hypothesis of the etiology of non-*albicans* yeasts in esophageal disease, however, needs formal demonstration, given that the pathologic role of many of these fungi is currently unclear, and histopathologic investigations that could have confirmed macroscopic observations and genetic analysis of isolated yeasts were not included in this study. Furthermore, given that our study was performed on isolates having originated from targeted mycologic laboratory diagnosis of diseased animals, neither control nor healthy animals without lesions were available for comparison of colonization. Nonetheless, our findings have clinical and epidemiologic implications and warrants that further studies be completed. Sequencing the ITS region could be a widely available alternative method for species identification for veterinary diagnostic laboratories because the accuracy of biochemical

tests that are based on fermentation of chromogenic substrates are limited. The treatment of esophageal mycosis is not effective; therefore, adequate feed storage, handling practices, regular cleaning, and sanitizing of watering systems are essential for disease prevention.

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