

FREQUENCY OF YEASTS AND FILAMENTOUS FUNGI IN THE EXTERNAL EAR CANALS OF CATTLE IN IRAN

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Summary: Fungal microbiotas are saprophytic microorganisms that can act as opportunistic pathogens in animals. This study was carried out in order to isolate and identify the ear fungal biota from healthy cattle. The samples were taken using premoistened swabs from the right and/or left external ear canals of 32 healthy cattle and cultured onto Sabouraud glucose agar and modified Dixon's agar media. A total of eight different fungal genera were isolated from 29 (90.6%) of 32 healthy cattle. Both filamentous fungi and yeasts were isolated with the predominance of *Aspergillus* spp. (35.6%), *Candida* spp. (18.9%) and *Malassezia* spp. (16.8%). The most frequent *Aspergillus* spp. were *A. fumigatus* (16.8%), *A. glaucus* (14.9%) and *A. flavus* (4%). Among the fungal isolates, 46.5% and 17.8% colonies were associated with hyaline and dematiaceous fungi, respectively ($p = 0.003$). The recognized fungi, especially *Aspergillus* spp. and *Candida* spp., were colonized as saprophytic fungal contaminants in the external ear canals of healthy cattle.

Keywords: cattle; external ear canal; mycobiota; *Aspergillus*; *Candida*

Introduction

Fungi are part of the normal microbial biota in the external ear canals of animals (1,2). The normal microbiota could benefit the host by avoiding excessive growth of pathogenic microorganisms through a competitive process. Factors associated with the increase of fungal population in the ear canals of animals include increased humidity, long and pendant ears, the presence of hair in the ear,

immune system deficiency, poor nutrition and abnormal hormonal status (3,4). The most common causes of hearing impairment in animals are otitis media, inflammatory processes or infections resulting in accumulation of fluid in the middle ear, which interferes with the tympanic vibrations (5).

Yeasts and filamentous fungi are frequently associated with otitis externa in human (6) and different animals such as horse (7), camel (8), sheep (9), dog and cat (10), rabbit (11), monkey (12) and elephant (13). The study of external otitis in the cattle is almost exclusively limited to parasitic otitis (14). However, large animals like cattle can

be seen for non-parasitic external otitis (allergic, fungal, or bacterial). Without cytological data on external ear canals, it is almost impossible to study such conditions. Cytological examination of external ear canals is the key point of external otitis diagnosis and treatment. In different animals, the results of cytological examination of ear swab are used to choose the proper topical treatment, based on the presence and concentration of bacteria, fungi and inflammatory cells. It is a simple, practical and inexpensive diagnostic test, which gives immediate results. Interpretation of such a test is impossible without any data in healthy animals. There is little data regarding to the cytological examination of the external ear canals of healthy cattle. Many studies have used samples collected from only one ear per animal (15), others have used samples collected from one or two ears and considered them as different samples (16).

Despite many the published information on bacterial biota of ear canals, there is still an extraordinary lack of specific well-organized, comprehensive information on ear mycobiota in cattle. The aim of this study was to isolate and identify the fungal biota from the external ear canals of healthy cattle.

Materials and methods

Animals

A total of 32 healthy cattle were selected in this study from different locations in Tehran province, Iran. Animals of any age, breed or sex were eligible for enrollment. Cattle were included in the normal group if there was no previous history of skin or ear disease and no history of underlying metabolic disease. Animals in the normal group were not currently on medications other than preventive parasitic or flea and tick treatments. In addition, cattle were free of clinical signs of skin or ear disorders, with no evidence of inflammation or infection on cytological analysis of ear specimens. Cattle were not included if any ototopical medications or flushes were used in the previous two weeks or if the animal was administered any systemic antifungal medication in the previous four weeks. In this study, all conducted experiments on cattle were in accordance with the guidance of ethical committee for research on animals of University of Tehran, Iran.

Clinical examination and sample collection

Complete physical and dermatological examinations were performed prior to collection of ear samples in each cattle. Each animal had a fungal culture obtained from the right and/or left external ear canals at the furthest accessible level of the auditory canal. Collection of samples was performed by passing a sterile culture swab into the ear canal. Samples were transferred overnight to the Mycology Center, Amol University of Special Modern Technologies, Amol, Iran according to the submission protocol.

Laboratory methods

Direct microscopic examination was carried out on the samples mounted in 10% potassium hydroxide (KOH)/dimethyl sulfoxide (DMSO) (Merck Co., Darmstadt, Germany). In addition, each cotton swab was slowly rolled once onto a glass slide. The glass slides were air-dried for 10 min, fixed and stained with a modified Wright's stain kit (RAL555, RAL Diagnostics France) as prescribed by manufacturer. Using this staining technique, fungal elements were stained in blue (17).

For initial fungal cultures, the samples were inoculated onto Sabouraud glucose agar (Merck Co., Darmstadt, Germany) supplemented with chloramphenicol (0.005%), Mycosel agar (Merck Co., Darmstadt, Germany) and modified Dixon's agar for identification of saprophytes, dermatophytes and *Malassezia* spp., respectively. The cultures were incubated at both 25°C and 32°C and examined daily for two to four weeks. Homogenized mixtures were prepared from the hairs, which had been collected by pincetle and inoculated onto the media as well. Saprophytic colonies were inoculated onto Malt extract agar (Merck Co., Darmstadt, Germany), Czapek-dox agar (Merck Co., Darmstadt, Germany), Potato dextrose agar (Merck Co., Darmstadt, Germany) and Cornmeal agar containing Tween-80 (Sigma Chemical Co., St Louis, MO, USA) media for identification at the genus level (18). Subsequently, fungal genera were identified based on micro- and macromorphology, reverse and surface coloration and size of colonies grown on the above-mentioned media (19,20).

Candida spp. were also identified by germ tube production, micromorphology and chlamydospore production on Tween 80-corn meal agar and by Rap ID™ yeast identification system (Remel, USA) (21). The identification of *Malassezia* yeasts was based on the ability to use certain polyoxyethylene sorbitan esters (Tweens 20, 40, 60 and 80), catalase reaction, cremophor EL assimilation test, splitting of esculin and precipitate production on modified Dixon agar (22).

Real-time PCR assay for identification of Aspergillus species

Spore suspensions from 7-day cultures on Czapek-dox media were inoculated into 15 ml of yeast peptone dextrose broth (Merck Co., Darmstadt, Germany) media and incubated at 32°C for 48 to 72 h. Then, fungal DNA was extracted and purified by MagNA Pure LC DNA I isolation kit (Roche, Mannheim, Germany). The preparation and settings of the instrument were according to the manufacturer's instructions.

The LightCycler system (Roche, Mannheim, Germany) was used for amplification of *Aspergillus* DNA. LightCycler hot-start PCR was performed in glass capillaries with a LightCycler Fast Start DNA Master Hybridization Probes kit (Roche, Mannheim, Germany) as specified by the manufacturer. The primers and hybridization probes for *Aspergillus* species were those described by Loeffler *et al.* (23). The PCR master mix (10 µl) contained 1× Fast Start reaction mixture with Fast Start Taq DNA polymerase, reaction buffer, dNTPs, 1.6 µl of 25 mM MgCl₂, 1 µl of each primer (3 µM), and 1 µl (2 µM) of each hybridization probe. PCR was performed in a final volume of 20 µl (10 µl of master mix + 10 µl of DNA extract) with 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 10 s at 58°C and 20 s at 72°C, with a temperature transition rate (TTR) of 20°C / s. The PCR was followed by a melting temperature analysis cycle comprising 95°C for 10 s (TTR of 20°C/s), 50°C for 60 s (TTR of 20°C/s) and 75°C for 0 s (TTR of 0.1°C/s) to check the specificity of the PCR product. DNA extracts from the samples were analyzed in parallel with an extraction control and a PCR control containing fungal DNA.

Statistical analysis

Student's t-test was used to compare the differences among different fungi using SPSS software (Version 15). A *P*-value less than 0.05 was considered to be statistically significant.

Results

Of 32 examined cattle, different fungal genera were recovered from 29 healthy animals (90.6%), whereas three cattle (9.4%) did not have positive cultures. A total of 101 fungal colonies were isolated from animals, 53 colonies from right ears and 48 colonies from left ears. There was no statistically significant difference between right and left ears. The following fungal genera (no. 8) were recovered: *Aspergillus* spp. (35.6% of the total examined cattle), *Candida* spp. (18.9%), *Malassezia* spp. (16.8%), *Cladosporium* spp. (10.9%), *Mucor* spp. (9.9%), *Alternaria* spp. (5.9%), *Ulocladium* spp. (1%) and *Fusarium* spp. (1%) (Table 1). There were statistically significant differences between *Aspergillus* spp. and *Malassezia* spp. ($p = 0.007$), *Cladosporium* spp. ($p = 0.000$), *Mucor* spp. ($p = 0.000$), *Alternaria* spp. ($p = 0.000$), *Ulocladium* spp. ($p = 0.000$) and *Fusarium* spp. ($p = 0.000$). In addition, there were statistically significant differences between *Ulocladium* spp. and *Fusarium* spp. and *Candida* spp. ($p = 0.000$), *Cladosporium* spp. ($p = 0.003$), *Mucor* spp. ($p = 0.008$) and *Alternaria* spp. ($p = 0.025$).

The most frequent *Aspergillus* species was *A. fumigatus* (16.8%), followed by *A. glaucus* (14.9%) and *A. flavus* (4%). There were no statistically significant differences among various *Aspergillus* species.

Of 65 filamentous fungi detected, 47 (46.5%) and 18 (17.8%) colonies were associated with hyaline and dematiaceous fungi, respectively (Table 1). There was statistically significant difference between hyaline and dematiaceous fungi ($p = 0.003$). As shown in Table 1, the frequency of filamentous fungi (64.4%) was higher than yeasts (35.6%). There was no statistically significant difference between filamentous fungi and yeasts ($p = 0.125$). Of 29 positive samples from healthy cattle, 16.8% yielded positive yeast cultures. The frequency of *Malassezia* spp. (16.8%) was lower than *Candida* spp. (18.8%). There was no statistically significant difference between *Malassezia* spp. and *Candida* spp.

Table 1: Frequency of different fungi isolated from the external ear canals of healthy cattle

Cattle no.	Mycobiota of external ear canal								Total
	Filamentous fungi						yeasts		
	Hyaline (no., %)			Dematiaceous (no., %)			Candida (no., %)	Malassezia (no., %)	
	Aspergillus	Mucor	Fusarium	Cladosporium	Alternaria	Ulocladium			
1	2 (1.9)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	4 (4)
2	1 (1)	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)	5 (5)
3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)	2 (1.9)
5	2 (1.9)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	4 (4)
6	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	3 (3)
7	0 (0)	2 (1.9)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	1 (1)	5 (5)
8	3 (2.8)	0 (0)	0 (0)	2 (1.9)	0 (0)	0 (0)	0 (0)	1 (1)	6 (5.9)
9	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	2 (1.9)
10	2 (1.9)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	0 (0)	4 (4)
11	1 (1)	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	4 (4)
12	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
13	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	3 (2.8)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	5 (5)
15	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	1 (1)	4 (4)
16	2 (1.9)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	4 (4)
17	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)
18	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)
19	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
20	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
21	2 (1.9)	0 (0)	0 (0)	0 (0)	2 (1.9)	0 (0)	2 (1.9)	0 (0)	6 (5.9)
22	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
23	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)
24	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
25	2 (1.9)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)	6 (5.9)
26	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	2 (1.9)
27	3 (2.8)	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	6 (5.9)
28	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
29	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	2 (1.9)
30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31	2 (1.9)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)	6 (5.9)
32	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	2 (1.9)
Total	36 (35.6)	10 (9.9)	1 (1)	11 (10.9)	6 (5.9)	1 (1)	19 (18.9)	17 (16.8)	101 (100)

Discussion

Domestic animals are often affected with external ear injuries followed by secondary infections. Little is known of the significance of resident microbiota in cattle ears and this knowledge would be very useful in assessing the accuracy of treatments. The mycological examination of the external ear canals of healthy cattle showed the presence of different fungi in 90.6% of the animals. The most common fungal isolate was *Aspergillus* spp. (35.6%), followed by *Candida* spp. (18.9%), *Malassezia* spp. (16.8%), *Cladosporium* spp. (10.9%), *Mucor* spp. (9.9%), *Alternaria* spp. (5.9%), *Ulocladium* spp. (1%) and *Fusarium* spp. (1%). In a study conducted by Duarte *et al.* (23), the most frequent fungus was *Malassezia* spp. (68.9%), followed by 'Micelia sterilia' (17.8%), *Candida* spp. (15.5%), *Rhodotorula* spp. (11.1%) and *Aspergillus* spp. (4.4%). Saprophytic fungal organisms are ubiquitous in nature and are normal contaminants of body and mucosal surfaces; thus, it is not surprising that these organisms could be found transiently in the ear canals of cattle. Our results are also consistent with similar studies on saprophytic fungal contaminants from the external ear canals of other animals (8,12,17).

In this study, the most predominant *Aspergillus* species was *A. fumigatus* (16.8%), followed by *A. glaucus* (14.9%) and *A. flavus* (4%). In accordance with our results, previous studies indicated that different *Aspergillus* species were isolated from the external ear canals of various animals, especially cattle (7-9,24).

Of 65 filamentous fungi detected, 47 (46.5%) and 18 (17.8%) colonies were associated with hyaline and dematiaceous fungi, respectively ($p = 0.003$). There are no indications in literature for difference in the occurrence of hyaline and dematiaceous fungi from the ears of healthy cattle. From culture-positive samples, the occurrence of filamentous fungi (64.4%) was higher than yeasts (35.6%). According to our best knowledge, there are no previous reports concerning the saprophytic fungal contaminants, especially filamentous fungi, from the external ear canals of healthy cattle. Duarte *et al.* (25) showed that the positive cultures were 28% for filamentous fungi and 34.6% for yeasts in cattle. In this study, the frequency of filamentous fungi recovered from cattle ears were considerably greater than those

reported in other studies (25,26). One possible reason for this difference may be related to the long incubation period of fungal cultures. In the present study, fungal cultures were monitored for four weeks, in contrast to other studies, in which fungal cultures were kept from two to 15 days.

In this study, the results were positive for yeasts of *Candida* spp. and *Malassezia* spp. in 18.9% and 16.8% of the samples from the external ear canals of cattle, respectively. In a previous study by Duarte *et al.* (24), the presence of seven yeasts of the genus *Candida* (15.5%) was confirmed in the ears of cattle. In addition, the frequency of *Malassezia* spp. was value near to that found by Gustafson study with positivity of 16% from the external ear canals of 50 healthy cattle (27). Duarte *et al.* (25) exhibited that 34.6% of healthy cattle were positive for *Malassezia* spp. In another study by Duarte *et al.* (28), 39.6% of isolates from healthy cattle were positive for *Malassezia* spp. They showed a relatively high frequency of *Malassezia* spp. in Holstein cattle, especially in the summer months, indicating a correlation between the open and air-exposed ears of this breed and *Malassezia* number. Pendulous-eared zebu breeds and hybrids had higher levels of colonization, although this effect was more pronounced in humid regions. Among other reports on *Malassezia* occurrence in healthy cattle, Dufait (29) obtained two positive cultures of *Malassezia* spp. from the ear of six sampled cattle (33.3%). In a study based on direct microscopic examination, the frequency of *Malassezia* species was found to be 29% in samples collected from the external ear canals of 55 healthy cattle (30). The significant frequency of *Malassezia* spp. in healthy cattle may indicate that it is a member of the normal microbiota of the ears in these animals (25).

The ear microenvironment consists of resident organisms that are believed to live and multiply on the skin and transient organisms that are acquired from the environment. Currently, it is believed that saprophytic fungi are transient contaminants by airborne fungi or fungi in soil. Saprophytic fungal organisms transiently found on the skin can take advantage of changes in microenvironment or host defenses to establish infection (31). It is unlikely that these fungal saprophytes could be the primary cause of otitis externa; however, they could complicate cases of prolonged bacterial otitis treated with topical

antibiotics and corticosteroids (32). Since the introduction of antibiotic eardrops containing corticosteroids, there has been an increasing prevalence of otomycosis most commonly due to *Aspergillus* spp. and *Candida* spp., which were isolated in our study. The pathogenesis of ear infection is not clear, although in calves extension of infection from the pharynx via the Eustachian tube is the most common means of entry into the middle ear (33). In cattle, exudate fills the cavity, increases pressure, ruptures the tympanic membrane and discharges into the external acoustic meatus (34).

In conclusion, this study demonstrated that filamentous fungi were more common than yeasts from the external ear canals of healthy cattle. Future studies may contribute to clarify the importance of their possible participation in the aetiology of otitis externa.

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POGOSTOST KVASOVK IN NITASTIH GLIV V ZUNANJEM UŠESNEM KANALU PRI GOVEDU V IRANU

H. Shokri

Povzetek: Glivično mikrobioto predstavljajo saprofitski mikroorganizmi, ki lahko delujejo kot oportunistični patogeni pri živalih. Raziskava je bila izvedena z namenom izolacije in identifikacije glivične mikrobiote pri zdravem govedu. Vzorci so bili vzeti s pomočjo vlažnih tamponov iz desnega in/ali levega zunanjšega ušesnega kanala pri 32 zdravih govedih in kultivirani na glukoznem agarju po Sabouraudu ter modificiranem agarskem mediju po Dixonu. Izolirali smo skupno osem različnih glivičnih rodov iz 29 (90,6 %) od 32 zdravih goved. Med nitastimi glivami in kvasovkami, ki smo jih izolirali, so prevladovali *Aspergillus* spp. (35,6 %), *Candida* spp. (18,9 %) in *Malassezia* spp. (16,8 %). Najpogostejši predstavniki rodu *Aspergillus* spp. so bili *A. fumigatus* (16,8 %), *A. glaucus* (14,9 %) in *A. flavus* (4 %). Med glivičnimi izolati je bilo 46,5 % in 17,8 % kolonij povezanih s hialinskimi oziroma pigmentiranimi glivami ($p=0,003$). Prepoznane glive, zlasti *Aspergillus* spp. in *Candida* spp., so kolonizirale kot saprofitski glivični onesnaževalci zunanje ušesne kanale zdravih govedi.

Ključne besede: govedo; zunanji sluhovod; mikrobiota; *Aspergillus*; *Candida*