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Research Article

Morphological and Molecular Characterization of Mucormycosis and Other Fungal Agents in Cattle

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Abstract

Mucormycosis is a type of opportunistic fungal infection caused by the Mucorales order of Zygomycetes. The study's goal was to characterize *Lichtheimia* and other fungal agents in Tekirdağ province of Türkiye by morphological and molecular methods. Head hair and skin scrapings of 13 cattle with mucormycosis lesions inoculated onto Rose Bengal Agar, Potato Dextrose, and Malt Extract Agar. After the incubation at 25°C and 27°C, pure colonies were evaluated morphologically and microscopically. For molecular identification, DNA isolation and PCR studies were followed by sequence analysis and the results were compared with the data in GeneBank using the nBLAST tool. ITS1/ITS4 primers used in PCR study. Fungal species were identified with data verified after morphological and molecular identification. The sequence analyses revealed that 12 samples had *L. ramosa* HBF570, 7 samples contained *A. niger* HBF572 and *P. crustosum* HBF571, 2 samples contained *A. chevalieri* HBF573 and *A. flavus* HBF576, and one sample contained *A. pseudoglaucus* HBF577 and *Aspergillus* sp. HBF570. The study's causative agents emerged were environmental fungus species. In conclusion, because of the fungal diversity in the environment, hygiene investigations must be conducted and implemented for the protection of mucormycosis.

Keywords: Cattle, *Lichthemia ramose*, Mucormycosis, Sequencing

Introduction

Mucormycosis is a major cause of death in humans and animals caused by opportunistic infections present in the environment. Mucormycosis is a dangerous fungal infection that primarily affects the immunocompromised. Infections can be distinguished by widespread angioinvasion and necrosis. Mucor pathogens are zoonotic pathogens found in soil, decaying debris and cow feed, such as straw [1]. Mucormycosis is caused mostly by Mucorales (Mucoromycotina), which includes *Mucor, Rhizomucor, Rhizopus*, and *Lichtheimia* (previously *Absidia),* and Mortierellales *(Mortirellomycotina),* which includes *Mortierella* [2].

Fungi from the order Mucorales cause zygomycosis more frequently in people than fungi from the order Entomophthorales. Zygomycosis is classified into several infection kinds based on the location of the lesions. Zygomycosis, regardless of the kind of fungal infection, is a deadly threat which demands prompt, correct diagnosis and treatment. Mucormycosis is the most common cause of zygomycosis in humans and cattle. Bovine zygomycosis typically causes isolated lesions (most commonly lymphadenitis or stomach zygomycosis) and is usually discovered inadvertently during a necropsy or slaughterhouse check. Cattle deaths from zygomycosis are uncommon [3]. Mucormycoses are divided into rhinocerebral, pulmonary, cutaneous, gastrointestinal, and

disseminated forms based on the location of the lesions. In cattle, these infections have been linked to gastroenteritis, meningoencephalitis, and endometritis [4]. Mucormycosis spreads quickly. It causes fungal emboli and necrotic suppurative lesions. *Mucoromycotina* is extremely invasive in the circulatory system. The agent is rarely discovered in suspect material for fungal isolation during diagnosis, and the fungus required for identification cannot be produced in the majority of cases. Due to the lack of clinical signs in cows and the complexity of the diagnostic process, diagnostic examination for fungal agents is frequently overlooked [5].

Although *Lichtheimia* species (particularly *L. ramosa* and *L. corymbifera*) are prominent mucormycosis causes, it is uncertain whether the same risk factors underpin fungal infection caused by different mold genera and species [6]. *L. ramosa* is a thermotolerant soil fungus commonly found near cattle in hay, grain, bedding, and silage. *L. ramosa* is also a common part of the stomach fungal flora [7]. The genus *Lichtheimia* (*Mucorales, Lichtheimiaceae*) is made up of saprotrophic fungi that live in soil, plants, indoor air, food products, and excrement, and it contains major mucormycosis causal agents in humans and animals. *Lichtheimia* species are found on all continents, with isolates from both environmental and clinical sources [8]. Fungal spores and hyphae are abundant in cattle feed, and oral intake appears to be the most likely route of infection. Endogenous infections may also occur since mucoralean species, including *L. corymbifera* and *L. ramosa*, are among the filamentous fungi found in healthy bovine rumen fluid [6]. Rhinocerebral mucormycosis has been observed as a result of a possible postnatal infection with *L. ramosa* [9]. Striatal necrosis caused by *L. ramosa* was reported in a newborn calf [7]. In humans, nasal *L. ramosa* infection arises as a result of inhaling asexual spores and leads to cerebral lesions [6].

Morphological, microscopic and molecular methods need to be evaluated together in the identification of fungi. Nowadays, molecular methods with the support of universal databases are quite successful in obtaining accurate results. Specialized regions of Fungal nuclear ribosomal DNA are used in the molecular identification of fungi. Fungal nuclear ribosomal DNA (rDNA) consists of three parts: the large subunit gene (25S), the small subunit gene (18S) and the 5.8S gene, separated by internal transcribed spacer (ITS) regions. ITS regions play an important role in the molecular identification of fungi. Primers targeting the amplification of these ITS regions are generally used to identify fungi, and these primers are universally used to identify many fungal species [10,11].

This study aimed to determine the morphological and molecular characteristics of fungal agents that induce keratinization lesions of varying sizes and numbers on the skin of cattle bred in Tekirdağ province, Türkiye.

Material and Methods

Ethical Statement

"Informed Consent Form" was obtained from animal owners for sampling the material of the study, and "Animal Experiments Local Ethics Committee Approval" is not required for the study.

Hair and Skin Rash (Scraping) Samples

The material of the study was selected from a private enterprise engaged in mixed cattle and dairy breeding in Kırkkepenek village of Tekirdağ province (Türkiye), which had a total of 35 cattle, including 22 cattle with keratinization lesions of various sizes and numbers on skin. Hair and skin scraping samples were taken from 13 cattle with at least five lesions of similar size *(Fig. 1)*. Before sampling, the areas around the lesions were cleaned with 70% alcohol, the skin debris was scraped with a sterile scalpel, and the hairs were pulled with a sterile forceps and placed in sterile containers. The samples taken were stored at +4°C until the laboratory studies [12].

Fungus Isolation

From the samples brought to the laboratory, a certain amount of hair and skin scrapings were taken with sterile forceps under aseptic conditions and inoculated onto Rose Bengal Agar and incubated at 25°C and 27°C for 30 days. During the incubation, the samples were checked at certain periods (every 7 days) and their growth was observed. Different colonies or mycelia were planted on Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) and pure colonies were obtained by incubating the petri dishes at 25°C and 27°C.

Morphological and Microscopic Identification

Morphological characteristics of pure colonies obtained in PDA and MEA were evaluated. For the morphological evaluation, visual characteristics of their colonies, such

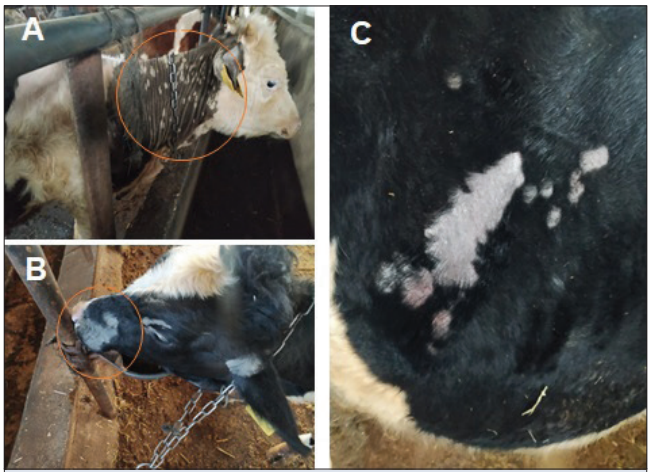

Fig 1. Macroscopic view of the sampled lesions **(A-C)**

as color, shape, size, and surface texture, were taken into account [14]. Fungal samples were stained with lactophenol cotton blue. The cellular structures, hyphae and spores were measured under the microscope.

Molecular Identification

Each of the pure fungal colonies was incubated at 25°C and 27°C for 5-7 days, and after mycelial development was achieved, the micelles were used in DNA isolation. For DNA isolation, mycelial samples were frozen at -20°C and then the cell walls were disrupted by applying liquid nitrogen. Following this physical fragmentation, the Phenol-Chloroform Isoamyl Alcohol method was used for DNA isolation [12]. After DNA isolation, the total DNA amount of the isolates was measured at 260-280 nm absorbance with a nanodrop spectrophotometer. DNAs were stored at -20°C until used in the PCR.

For the fungal isolates, the PCR method was performed as described by Bıyık et al.[15] and White [14]. For this purpose, 25 µL PCR buffer was prepared for each 35-cycle PCR reaction. Mastermix (AMPLIQON) was used for the PCR reaction. ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers were used in the PCR mix was adjusted to 25 µL with containing 12.5 µL of master mix, 9.5 µL of dH2O, 0.5 µL of each primers and 2 µL of DNA. PCR conditions were denaturation at 95°C for 2 min, annealing at 56.9°C for 30 sec, and extension at 72°C for 1 min. Sequence analysis of the amplicons whose PCR studies were completed was outsourced to Innopenta Biotechnology company. The analyzed DNA sequence results were compared with the data in GeneBank using the nBLAST program *(https://blast.ncbi.nlm.nih.gov/)* and molecular identifications were made.

The analyzed DNA sequence results were compared with the data in GenBank using the nBLAST program *(https://blast.ncbi.nlm.nih.gov/)* and molecular identification was performed. The identified isolates were registered in GenBank. A phylogenetic tree was created for the evolutionary relationships of the isolates identified using the MEGA X program. Phylogenetic tree created with the maximum likelihood method. The percentage of trees in which related taxa were brought together was determined using the bootstrap (1000 repetitions) test. Evolutionary history was evaluated using the Tamura-3 parameter model and the Maximum likelihood method. Gamma distribution (G+ parameter 1) was used to model the difference in evolutionary rates between regions.

Results

Isolation Results

Images of hair and skin rash (scraping) samples taken from 13 animals with at least five lesions after Rose Bengal

Fig 2. Images of the samples after 7 and 30 days of incubation on Rose Bengal Agar Chloramphenicol Agar, respectively

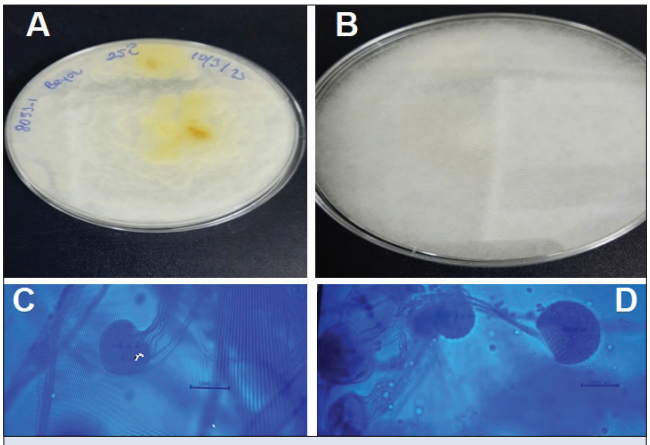

Fig 3. Morphological and microscopic images of *L. ramosa*. **A, B**- lower and upper surface of the colony; **C, D**- columella structures

Agar Chloramphenicol Agar incubation are presented in *Fig. 2*. Samples with different morphologies developed in petri dishes were purified. Purified samples were identified as *Lichthemia ramosa, Penicillium crustosum, Aspergillus niger, Aspergillus chevalieri, Aspergillus cristatus, Aspergillus flavus, Aspergillus pseudoglaucus* and *Aspergillus* sp. according to their morphological and microscopic examinations.

The fungi identified as *L. ramosa* (Zopf) Vuill. (1903) grow slowly on PDA medium in the first 5 days and in the 7^{th}-10^{th} days. It grew to cover the entire petri dish within days. The hyphae covered the entire petri dish and reached the petri lid and were white in color. The bottom surface of the colony contained yellow color in certain areas *(Fig. 3-A,B)*. The columella was spherical and measured 1000 µm *(Fig. 3-C,D,* 40X). Sporangiospores were observed as flat spheroids and mostly hemispherical.

Colonies of the fungi identified as *A. cristatus* (Raper & Fennell) formed after 7 days of incubation in MEA at 25°C were examined. The lower part of the colony was yellow, fragmented, and the edges on the colony surface were surrounded by bright yellow color, and the middle part was brown *(Fig. 4-A,B)*. Colony diameter was measured as 15-18 mm. The cleistothecium structure and the ascus structures within it were seen *(Fig. 4-C)*. Ascus and ascospores are seen in *Fig. 4-D*, and conidiaspore and conidiophore structures are seen in *Fig. 4-E*.

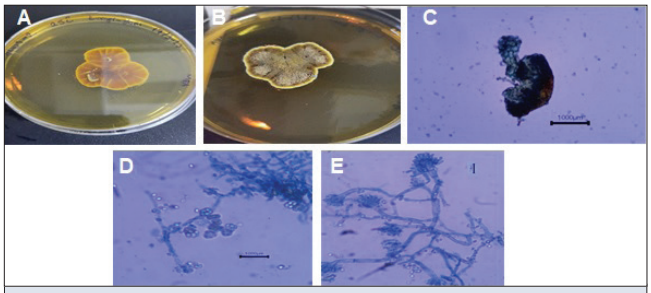

Fig 4. Morphological and microscopic images of *A. cristatus*. **A,B** - bottom of colony surface; **C**- cleistothecium and ascus structures within it; **D**- ascospores with ascus; **E**- conidiaspore and conidiophore structures

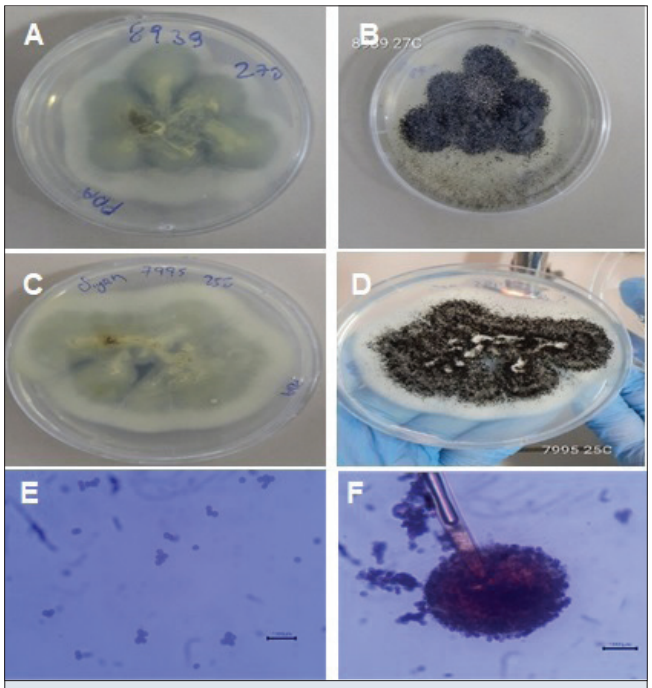

Fig 5. Morphological (A-D) and microscopic **(E, F)** images of *A. niger*

The fungi identified as *A. niger* (sensu auct. pro parte, pre 2007) spread and formed colonies on PDA medium during an incubation period of 7 days at 27°C and 25°C. Colony sizes varied between 15-25 mm in MEA and 50-60 mm in PDA medium. The lower surface of the colony was flat, surrounded by a yellow-white zone *(Fig. 5-A,C)*, and the colony surface had a black wooly structure *(Fig. 5-B,D)*. Conidia and conidiaspores were brown-black in color *(Fig. 5-E,F)*. Conidiaspores were 3-4 µm in diameter and had a rough-spiny surface.

The fungi identified as *A. chevalieri* (L. Mangin, Thom & Church 1926) formed a colony on PDA medium during 7 days of incubation at 25°C, with a yellow, shiny appearance on the lower surface and a light yellow middle part on the upper surface, surrounded by a light brown line *(Fig. 6-A,B)*. Colony sizes in MEA was measured between 15-20 mm. The cleistothecium structure was measured as 520 µm *(Fig. 6-C)*.

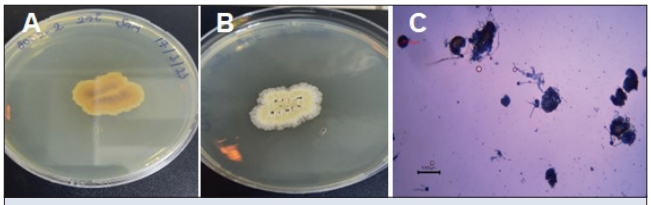

Fig 6. Morphological **(A, B)** and microscopic **(C)** images of *A. chevalieri*

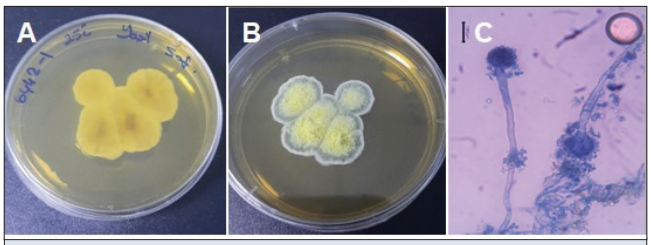

Fig 7. Morphological **(A, B)** and microscopic **(C)** images of *Aspergillus* sp.

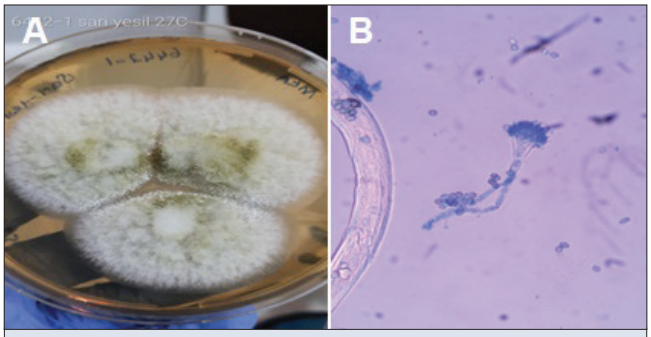

Fig 8. Morphological **(A)** and microscopic images **(B)** of *A. flavus*

The fungi identified as *Aspergillus* sp. formed a colony of 15 mm with yellow, slightly fragmented lower surface in the MEA. The upper surface of the colony was surrounded by green lines and the middle was yellow *(Fig.7-A,B)*. As a result of microscopic examination, hyphae, conidia and vesicle structures were seen, with septa and spores in a circular structure *(Fig.7-C)*.

Fungi identified as *A. flavus* (Link, 1809) formed green-white colonies with a diameter of 30-35 mm on a 60 mm petri dish in MEA *(Fig. 8-A)*. The stem of the vesicle, which had a spherical structure and a diameter of 20-30 µm, was seen to be long and the conidia had rough surfaces with a diameter of 3-4 µm *(Fig. 8-B)*.

The fungi identified as *A. pseudoglaucus* (Blochwitz) were observed to form yellow-light brown colored colonies on

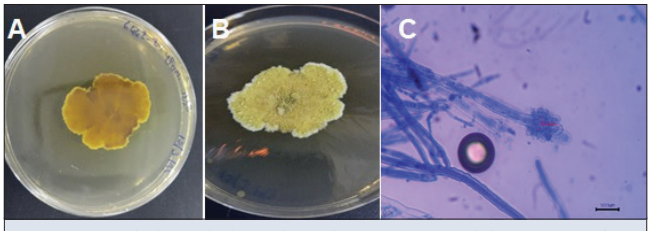

Fig 9. Morphological **(A, B)** and microscopic **(C)** images of *A. pseudoglaucus*

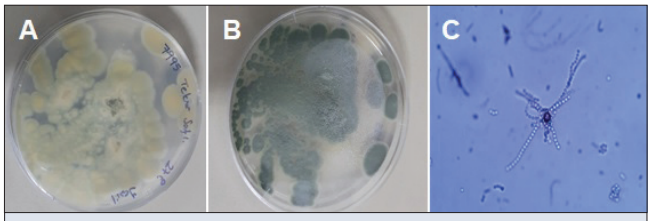

Fig 10. Morphological **(A, B)** and microscopic **(C)** images of *P. crustosum*

the PDA medium with dull yellow and short mycelium on the lower surface *(Fig. 9-A,B)*. The colony diameter was measured as 30 mm and the vesicle section at the end of the long stalk was measured as 700 µm with a 40X objective *(Fig. 9-C)*. The conidia were 4-7 µm long, had an ellipsoid structure and a rough surface.

The fungi identified as *P. crustosum* (Thom, 1930) formed colonies with a yellow-cream colored lower surface and a green powdery structure on the upper surface of the colonies growing on PDA medium *(Fig.10-A,B)*. A colony surrounded by a white thin structure and forming a light yellow pigmentation was observed. Conidia were spherical and in long chains *(Fig.10-C)*.

Molecular Identification Findings

Thirteen fungus samples, which were predicted to be morphologically different from the fungus samples growing in petri dishes, were sent to the sequencing service. As a result of the sequencing, 8 different species, some of which were the same species, were identified from these 13 fungal samples. As a result of the identification, of the 13 samples examined in the study (hair and skin rash/scratching), 12 samples contained *L. ramosa* HBF570, 7 samples contained *A. niger* HBF572 and *P. crustosum* HBF571, two samples each contained *A. chevalieri* HBF573 and *A. flavus* HBF576, and one sample each contained *A. pseudoglaucus* HBF577 and *Aspergillus* sp. HBF570 was determined.

The species obtained as a result of the sequence were recorded in GeneBank. GeneBank registration codes and accession numbers of the species are given in *Table 1*. The

Table 1. *Data obtained as a result of the sequence*

Species Name	Accession Number	Blast Similarity (%)
Lichthemia ramosa HBF570	OR588056	98
Penicillium crustosum HBF571	OR588057	99
Aspergillus niger HBF572	OR588058	99
Aspergillus chevalieri HBF573	OR588059	97
Aspergillus cristatus HBF574	OR588060	99
Aspergillus sp. HBF575	OR588061	96
Aspergillus flavus HBF576	OR588062	98
Aspergillus pseudoglaucus HBF577	OR588063	100

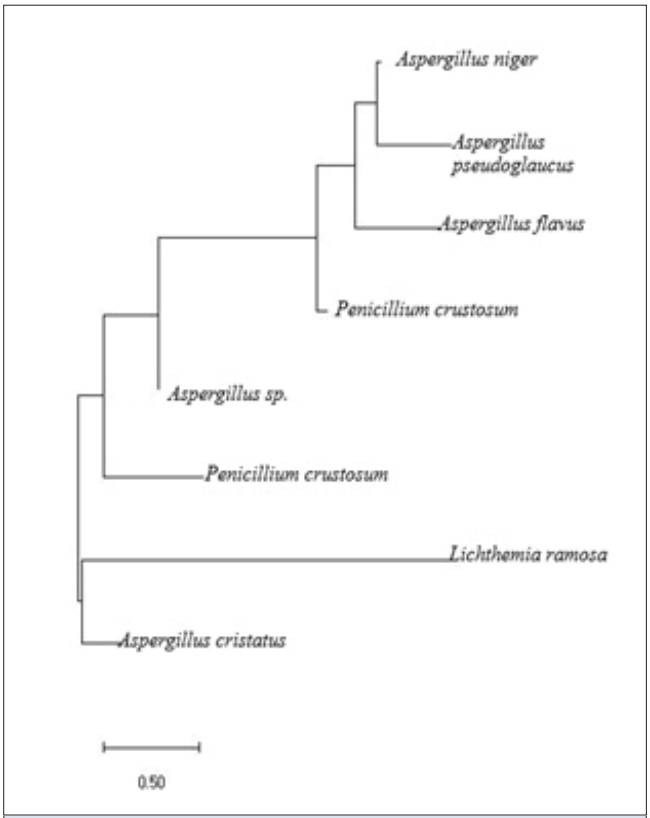

Fig 11. Phylogenetic tree model of isolated species

data obtained after molecular identification was analyzed in the MEGA X program and a phylogenetic tree was created *(Fig. 11)*.

After inoculation and incubation of the study samples by serial dilution, colonies with different morphological characteristics developed in the petri dishes. Considering the development density of these colonies in the petri dish, the development density of the species identified in each sample is expressed in *Fig. 12*. In the figure, the different species isolated and identified from each sample are given in their own column. The isolated species were expressed by dividing them according to a numerical ratio within the column, depending on the number of colonies in the petri dishes.

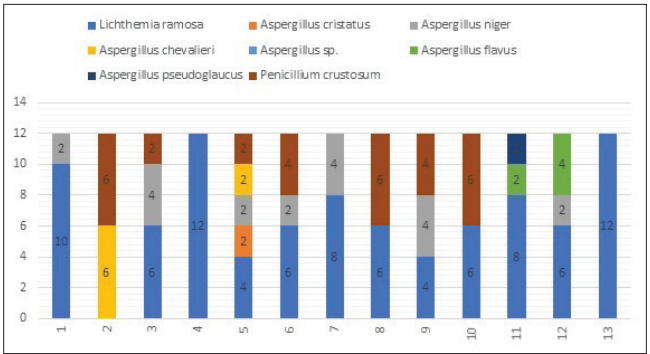

Fig 12. Number of colonies (density/distribution) of species isolated from hair and skin samples

Discussion

Mucormycosis (zygomycosis) is a dangerous but uncommon fungal infection caused by the Mucormycetes mold class. These fungi can be found all over the place. They are found in soil and decaying organic materials such as leaves, compost piles, and rotten wood [15]. Mucormycosis is caused by *Rhizopus, Mucor, Rhizomucor, Syncephalastrum, Cunninghamella Bertholletiae, Apophysomyces*, and *Lichtheimia* species [18]. The main source of infection is direct contact with fungus spores in the environment. *L. ramosa* is a fungus species that has been discovered as the dominating (dense) species in hair and skin scraping samples in this investigation. It is an opportunistic pathogen in both animals and humans. This type of fungus produces mucormycosis, which damages several organs. The only report on *L. ramosa* in our nation was of pulmonary mucormycosis in a human with HIV infection [19], but no reports on animals were discovered. Many diseases caused by *L. ramosa* are being studied around the world. *L. ramosa* was found in the internal organ tissues of a pregnant cow in a study conducted in Korea when different tissue samples were evaluated after death. This study documented the case of angioinvasive mucormycosis in cattle [3]. Tanaka et al.[9] investigated a case of rhinocerebral zygomycosis in calves caused by *L. ramosa* infection. The fungus infiltrated the brain via the olfactory nerves, according to histopathological analysis. This study was reported as the case of rhinocerebral zygomycosis affecting cattle. A recent study on the identification of *L. ramosa* in tissues was conducted and presented by Iwanaga et al.[7]. The researchers claimed that the histological study performed after the necropsy of a 12-day-old male calf that died as a result of different neurological abnormalities was the report of striatal necrosis induced by *L. ramosa* in a newborn calf. Furthermore, mucoral species such as *L. corymbifera* and *L. ramosa* have been detected in the rumen fluid of healthy calves and may cause endogenous infections. *L. ramosa* was found in samples taken from the calf neck area of a study in which dermophyte and non-dermophyte fungi isolated from lesional areas of different animals were identified. ITS regions were used in fungus molecular identification investigations [20]. This study's technique and findings sections overlap with ours. According to the research, *L. ramosa* has been detected in several cases in animals. *L. ramosa* was found in all but one of the hair follicles and skin scrapings/rash collected from lesional regions in cattle in our investigation.

Aspergillus species produce diseases known as "aspergillosis" in mammals. In pregnant animals, aspergillosis causes gastrointestinal disorders, lung tissue damage, and waste [21]. When *Aspergillus* instances in cattle were evaluated, mycotic abortion was frequently seen. *A. flavus, A. fumigatus, A. nidulans, A. terreus, A. niger*, and *A. versicolar* are the species that induce waste in cattle [22]. *A. cristatus* is a species that has been isolated from many different parts of the world. This fungus, known as golden blossom in China, is utilized as a flavour during the fermentation stage of a local tea [23]. It's been isolated from strawberry puree and Philippine peanuts as well [11]. These fungi have been found in the Kocaeli-Çatalca region of Türkiye [24]. *A. niger* is a fungus that has been thoroughly studied and has numerous features defined among all fungal species. It produces mycotoxin and has a classic mold appearance with its black conidia. Although this species is common in fruits, vegetables, cereals, and nuts around the world, it has also been detected in a variety of herbal items in our country. *A. chevalieri* is a common fungus species that causes spoiling in various plant products and foods (cereals, chocolate, fatty nuts, meat, and fruits) due to its enzymatic activities. *A. pseudoglaucus* is another species that causes food deterioration. This strain was identified from cheese, processed meat, and grains. It has been observed in our country from the Aegean, the Çatalca-Kocaeli, and Sakarya. Previously, *Penicillium crustosum* was isolated from animal feed and has been linked to crop losses in cereals [11]. In Türkiye, *P. crustosum* has also been isolated from rice and wheat [25].

It is thought that the *Aspergillus* species identified in the study contaminated calf hair and skin from their spores found in the barn environment. Likewise, it is predicted that *L. ramosa* HBF570 may be present in the barn environment and cause lesions by contaminating calf hair and skin. The pathogenicity of *Aspergillus* and *L. ramosa* species in animals and humans may have been learned earlier. These species include lung, nasal sinus, brain, eye, skin, gastrointestinal tract and multiple causes systemic ethnicity [26]. Fungi belonging to the order *Mucorales* cause saprophytic opportunistic infections [27]. In the study, the sequences were documented in GeneBank as a report on the isolation and identification of *L. ramosa* HBF570 from cow hair and skin scrapings. Other species isolated in the study were molecularly identified from bovine lesional skin by comparison with the updated data of Asan [28] and entered into the gene bank records. Control of these pathogenic agents is important for animal health. These agents can enter the body system through the air and skin and cause a systematic disease. For this reason, hygiene studies should be carried out by taking into account the richness of fungi in the barn environment, and diseases should be prevented from infecting the internal organs of the cow through the hair-skin surface and air.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (A. Çiftçi).

Funding Support: There is no funding support for the study.

Ethical Statement: Animal Experiments Local Ethics Committee Approval is not required for the study.

Competing Interest: The authors declared that there is no competing interest.

Author Contributions: Experimental design was performed by BB, TG, and AÇ; material preparation and analysis were performed by HHB, SA and SÖK; results were interpreted by AÇ, TG, BB and HHB; the first draft of the manuscript was written by AÇ, BB and HHB; all authors contributed to the final version of the manuscript.

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