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Investigation of fungal isolates of chronic rhinitis in sheep

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Abstract Fungal rhinitis is an important infection in sheep characterized by its chronic status. The study was conducted to clinically and in vitro characterize fungal rhinitis in sheep. The current study was conducted to clinically examine chronic suspected fungal rhinitis in sheep and to isolate fungal species from the lesions and characterize these isolates using molecular techniques. Clinical examination and nasal endoscopy were used first to identify clinical status and nasal lesions in 80 sheep distributed over nine livestock farms in Babylon Province, Iraq. Then, a total of 160 lesion nasal swabs (80 for direct smear and 80 for cultivation) were collected from 80 sheep. The swabs were stained with Grocott's methenamine silver stain (GMS) to identify fungal hyphae. Furthermore, polymerase chain reaction (PCR) and partial gene sequencing methods were performed based on the 18S rRNA gene. The results of the clinical examination of each animal revealed slightly increases in the body temperature, emaciation, nasal congestion, nasal mucoid, mucopurulent, or blood-stained discharge, sneezing, nasal itching. Moreover, varied types of nasal lesions were visualized by endoscope. For the cultivation of the swabs, the findings demonstrated the isolation of 55/80(68.75%) isolates. The endoscopy showed various types of chronic lesions, such as nodules and ulcerations. Based on the molecular methods, the results revealed the presence of seven species of fungi; 13(16.25%) Aspergillus terrusre,10(12.5%) A. flavus,11 (13.75) A.niger,9(11.25%)Rhizopus arrhazus,7(8.75%)Alternaria alternate,3(3.75%)A.tenesumma, and 2(2.5%) Penicillumsoppi.The phylogenetic analysis recorded 20 distinct isolates that were similar in their sequence to global isolates from Saudi Arabia, India, Pakistan, and China. The impact of the study can be emphasized that chronic rhinitis in sheep was primarily caused by Aspergillus species. These isolates were sequencing-based similar to those isolated from different world regions indicating high evolution in the current isolates and more need for improvement of animal health and management. However, the study comes with limitation of the area size sampled, which can be solved with future studies.

Keywords: Fungal infection, molecular diagnosis, nasal infection, sheep rhinitis.

Introduction One of the most important diseases of sheep is rhinitis, Sheep in any production system and housing conditions can be affected by fungal rhinitis, depending on the environmental temperature and humidity. Sheep that stay in hot and humid conditions are more susceptible to this disease. Fungal rhinitis is characterized by the inflammation of nasal mucosa by some pathogenic fungi, such as Aspergillus spp (1-3). The nasal cavity is one of the most prevalent sites for fungal infections in small ruminants resting on the ground, and parts of the rumen or reticulum of dead sheep that serve as substrates (4,5). Normal nasal compartment may contain fungal species and phyla, such as Candida sp., Ascomycota, Basidiomycota fungi. Viral rhinitis may predispose the occurrence of mixed and bacterial infection (6,7).

The chronic fungal rhinitis in sheep has unspecific signs of a pale appearance, mucus flowing from the nostrils, nasal discharge, sneezing, coughing, head rubbing or shaking, and swollen eyes. The additional clinical signs may also be presented as fever, difficult breathing, poor appetite, weight loss, and sudden death. Taken together, the economic losses include the cost of treatment drugs, labor, and decreased production and reproduction. The decreased reproductive can be from temporary infertility in males and involvement of the laryngeal opening in females. Dara reported that sporadic and non-contagious, but significant economic losses are

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attributed clinically significant in ruminant rhinitis (8,9).

It was reported that the presence of fungal rhinitis allows bacterial proliferation, so it is a predisposing factor for secondary bacterial infection. Chronic fungal rhinitis results in bony erosion and pus formation and slowly destroys turbinate and nasal septal bones. The pus in the nasal cavity and dried nasal mucus accumulates, causing hollowing by nasal turbinate bone erosion and leading to a nasal mucus impaction. This disease results in airflow obstruction and endogenous inflammation. When the middle turbinate bone is damaged, mucus and necrotic tissue including food areal colonized, leading to a chronic disease when severe. In addition, inflammatory bacteria may carry the risk of leading to pneumonia. Therefore, fungal rhinitis has turned into a serious indirect economic loss with direct medical care after infection and increased mortality (10,11). Fungal rhinitis usually results from a rupture of the septa that separates the ethmoturbinates and frontal sinuses. The course of the septa's rupture is extended increasingly by aspergillosis, and the septa appear deformed. The deformed septa damage the osteomata complex, which leads to ostium obstruction that can be managed through surgical methods (12).

Dust and grains are significant in the feeding and mobilization of animals. These are also important factors causing diseases originating from the upper respiratory system of the animals. Aspergillus and Penicillium, known as fungi, are certain most important fungi causing diseases in animals in nature. Fungi, which develop easily with humidity and temperature values of over 60% and 20 respectively, cause the development of spore forms and their spread by air. The number of spores in the air inside and outside of the building is very important in being affected by disease. The animals are affected because the spores stick into turbinate bones in the nasal tract and infect them (13, 14).

For those fungi where studies have been conducted, the mechanism of infection used is the result of a combination of factors - including spore characteristics such as size, structure, and wall chemistry, animal behavior during feeding and grooming, olfactory or Schneiderian membrane organization, and external factors such as humidity and temperature in the animals' environment. For some fungi, infection occurs via adhesion to mucosa, mediated by tubular structures of the spores. Other spores are able to invade across physical barrier layers, and then spread into deeper tissues (15,16).

A variety of different factors, generally terms, influence the establishment of an infectious fungal disease including virulence factors of the pathogen, anatomical site of the proposed infection, underlying immune status and integrity of the physical barriers to infection. Host-fungal interactions, in particular regarding the establishment of an infection of the upper respiratory tract, are not well understood, and this lack of knowledge hampers the development of appropriate control strategies (17).

Because fungal rhinitis in sheep has no or limited studies in the area, the current study was conducted to clinically examine chronic suspected fungal rhinitis in sheep and to isolate fungal species from the lesions and characterize these isolates using molecular techniques.

Materials and methods

Ethical approval

The present study was approved by the Committee for Research Ethics in the College of Veterinary Medicine, University of Al-Qadisiyah.

Sample collection

During June to December 2023, clinical examination and nasal endoscopy were used first to identify clinical status and nasal lesions in 80 sheep distributed over nine randomly livestock farms in Babylon Province, Iraq. Then, a total of 160 lesion swabs (80 for direct smear and 80 for cultivation) were collected from 80 sheep.

The cultivation process included direct inoculation in 1% semi-sold Sabouraud Dextrose Agarwith chloramphenicol (SDAC) (Himedia, India), which was utilized as a transport medium. The inoculums were incubated at 25°C for 72 hrs. For the identification of fungi, SDAC-based sub-cultivation was performed and incubated at 37°C for 5-7 days (18).

Direct smearing and morphological examination of colonies

The swabs were stained with Grocott's methenamine silver stain (GMS) to identify fungal hyphae (19,20). It is important to use this stain because of its high ability to visualize fungi in tissues with high sensitivity and specificity. The colonies were characterized using color, shape, and consistency (21). For confirmation, the genus level was detected using conventional techniques, such as lactophenol cotton blue stain

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(LPCB) (Switzerland, Fluka) on a microscopic slide examinedat 10X and 40X for the detection of fungal structures, such hyphal and conidial elements. The identified fungus was placed in Eppendorf tubes for the molecular techniques (16).

Molecular identification

DNA Extraction

The DNA from 55 fungal isolates was extracted by employing Addbio mini kit(catalogue#:10023) and conducted according to the manufacturer. The DNA was kept at -20°C until use.

PCR Technique

The PCR included the use of the 18S rRNA gene primers; table (1) that targeted a piece with size at 555 bp. The master mix (AddBio, Korea) of the PCR contained 10 µl,1µl DNA, 2 µl of each primer, 5 µl nuclease free water in a total mixture volume of 20 µl and was done based on a method by stecheret al., (22). The thermocycler conditions were set to a 95 ºC for 3 min-initial denaturation for one cycle, followed by a 95 ºC for 35 s-denaturation, 50-60 ºC for35 s-annealing, and a72 ºC for 35s-extension for 35 cycles, and a 72 ºC for 5 min-final extension. The PCR reaction was tested using 1.5 agarose gel electrophoresis stained by ethidium bromide at 100 volt and 80 AM for 1 hour and visualized using documentation system (Syngene,Taiwan).

Sequencing AND Phylogenetic Analysis

Here, 20 µl of each of 20 purified positive PCR products were sent out to sequencing using Sanger method at Macrogen Company (Korea). The received sequences were noise-trimmed-processed. Then, accession numbers were obtained for each isolate sequenced after been deposited in the GenBank. The phylogenic tree analysis was constructed by comparing differences and similarities with the global isolates from NCBI sequences and was based on Mega x and Clustal W alignment analysis (22,23).

Results

The results of the clinical examination of each animal revealed slight increases in the body temperature, emaciation, nasal congestion, nasal mucoid, mucopurulent, or blood-stained discharge, sneezing, nasal itching (Figure1). Moreover, varied types of nasal lesions were visualized by endoscope.

For the cultivation of the swabs, the findings demonstrated the isolation of 55/80(68.75%) isolates. The morphological appearance of fungal hyphae from direct smears is presented in Figure (2). The prevalence of Aspergillus spp is important due to zoonosis, abundance, and the variety of diseases that it may cause.

The fungal structure is stained with Grocott's methenamine silver stain based on the molecular methods, the results revealed the presence of eight species of fungi; listed in table (1)

The cultivations revealed different colonies on the cultivation media with various hyphal and conidial structures, then we took from any colony of an isolate (Figure 3).

The amplification of the 18S rRNA gene using the PCR methods with gradient annealing temperatures are shown in Figure (3). This depicts that 50 C was an optimal annealing temperature. M is molecular marker (3000-100 bp) from Gene Direx (South Korea). NC is negative control in which similar PCR components were used except H2O was added instead of DNA. M is molecular marker (3000-100 bp) from GeneDirex (South Korea). Stain it with lactophenole cotton blue to primary diagnosis of fungi (Figure 5)

The phylogenetic analysis recorded 20 distinct isolates that were closely related to global isolates from Saudi Arabia, India, Pakistan, and China. These were compared via short branches, which indicates high similarities due to evolutionary pressure of same ancestor. These alignments and isolates are presented in (Figure 6)

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Figure 1: Ewe suffers A. Rhinitis and B. Nasal lesions (endoscope).

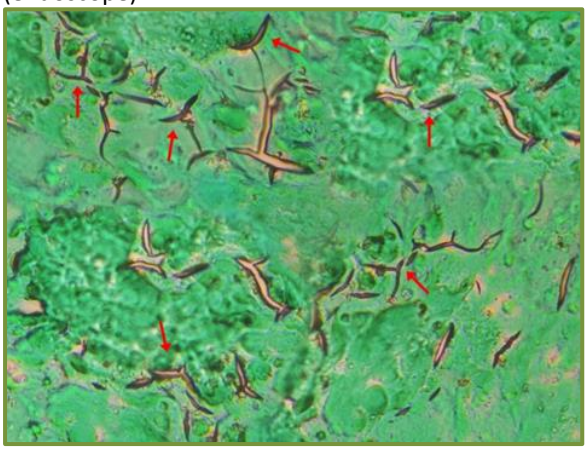

Figure 2: Fungal hyphae of sheep chronic rhinitis. The fungal structure is stained with Gromori's methenamine silver stain.

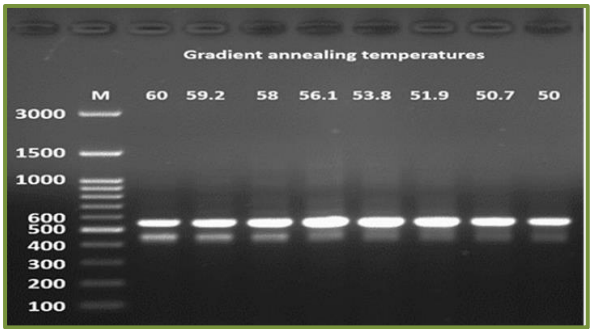

Figure 3: Agarose gel electrophoresis image (1.5 % agarose) shows the amplicons by gradient PCR (60-50 C) targeting partial region of 18S rRNA gene (size= 555 bp). This depicts that 50 C was an optimal annealing temperature. M is molecular marker (3000-100 bp) from GeneDirex (South Korea).

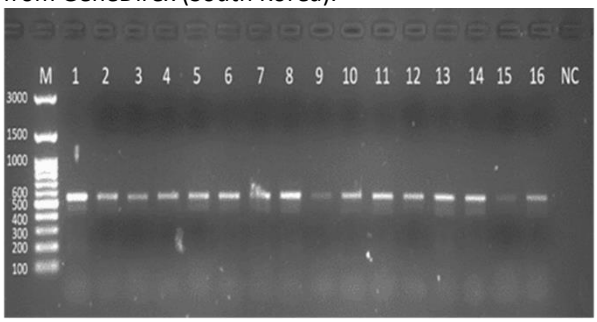

Figure 4: Agarose gel electrophoresis image (1.5 % agarose) shows the amplicons of PCR (1-16) targeting partial region of 18S rRNA gene (size= 555 bp). NC is negative control in which similar PCR components were used except H2O was added instead of DNA. M is molecular marker (3000-100 bp) from GeneDirex (South Korea).

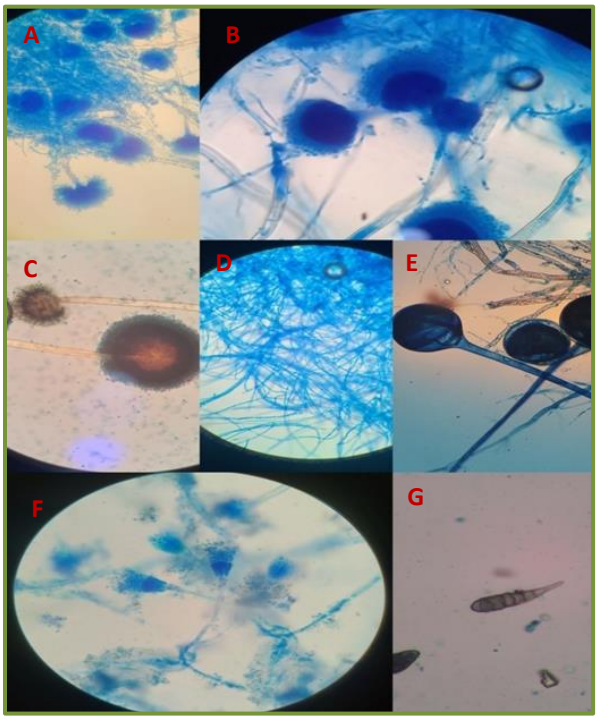

Figure 5: Microscopic examination of different types of fungi under light microscope A)Aspergillus terrus, B) Aspergillus flavus, C) Aspergillus niger, D) Aspergillus Flavips, E) Rhizopus arrhizus, F) Pencillium soppi,G) Alternaria Species. The power 40X used of all examinations.

Table 1: Primer of the *18S rRNA* gene used.

Sequence	size
F: GTAGTCATATGCTTGTCTC	555 bp
R: GGCTGCTGGCACCAGACTTGC	

Discussion

Nasal endoscopic, isolation and molecular study of fungi species from nasal lesions of sheep in Babylon Province addresses an important animal health problem related to livestock management. Chronic fungal rhinitis is one of the most common infections affecting young sheep. These infections negatively affect animal health and productivity and reduce farm earnings. The utilization of clinical examination, endoscopy and advanced molecular techniques in this study is an important step towards developing diagnostic techniques for these infections in sheep (24). More specifically, the sheep affected by fungal rhinitis in the study had slight increases in body temperature, emaciation and nasal congestion

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associated with variable nasal discharge. These symptoms strongly suggest a diagnosis of chronic rhinitis.

Table 2: Fungal species isolated from nasal lesion of sheep

Mold	Positive	%
Aspergillus terrus	13	16.25
Aspergillus niger	11	13.75
Aspergillus flavus	7	8.75
***Aspergillus* Flavips**	3	3.75
Rhizopus arrhazus	9	11.25
Alternaria alternata	7	8.75
Alternaria tineussoma	3	3.75
Pencillium soppi	2	2.5
Total	55	68.75

Figure 6: Phylogenetic tree of fungi species isolated from sheep rhinitis and is based on the sequencing of the 18S rRNA gene.

Similar clinical presentations were reported in a number of veterinary studies. For instance, Steyl (23). described clinical presentations consistent with chronic mycotic rhinitis, characterized by poor health status and a chronic debilitating disease, in ruminants affected by mycotic rhinitis (24). investigated three biopsy samples taken from deviated nares from Persian lambs with thick nasal discharges, and described a chronic, vasoactive, granulomatous dermatitis in one fetus associated with Mucor ramsayi.

The present of nasal itch and sneezing is suggestive of an allergic or non-specific irritative response to the fungal pathogens, consistent with findings in human medicine where these are the common clinical symptoms in patients with fungal sinusitis (24). Nasal endoscopy provided an excellent means to visualize the nasal lesions which is an integral part of diagnosis and treatment planning. The diversity of the lesions observed in this study further highlights the complexity of fungal infections. In human medicine, endoscopy has been used as a diagnostic tool for years and veterinarians have also utilized nasal endoscopy to diagnose nasal and sinus diseases in their patients. (24). demonstrated endoscopy to be a good method to diagnose nasal pathologies in sheep and was used in this study.

Similarly, endoscopic nasal findings associate with histopathological changes nested within the broader scientific literature, including the one summarized by Enache et al., (25). who described endoscopic and histopathological assessment of chronic rhinosinusitis in goats, with the chronology and pathology of fungal colonization and rhinosinusitis showing broadly similar patterns.

Fungal species isolated from nasal swabs showed a high prevalence (68.75%) of fungal infection among sheep. Aspergillus species such as A. terrusre, A. flavus and A. niger were the predominant species infecting the animals. Aspergillus species are notorious for their environmental persistence and ubiquity. They are major pathogens of animals and men, causing both superficial infections (such as onychomycosis) to life-threatening diseases (such as invasive aspergillosis) (24). Aspergillus species produce several toxins and enzymes that facilitate tissue invasion and immune escape, which was reported by (26). on the pathogenicity of Aspergillus species. Latgé study showed an immunologic function of the toxins and enzymes produced by species of this

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genus. Aspergillus species secrete proteases and lipases that help the fungus to degrade tissue, in addition to low molecular weight molecules which downregulate key immune functions. Another major group of toxins secreted by Aspergillus are mycotoxins, which kill wide range of cell types such as aerobic bacteria, yeasts, fungi and protozoa. The study results corroborate existing reports that suggest Aspergillus sp. as one of the potential pathogens causing chronic rhinitis.

The other two fungi add to the complexity of fungal infections in sheep. Both Rhizopus arrhazus and Alternaria alternate are opportunistic pathogens in immunocompromised hosts that can cause severe systemic infections. For example, Ribes et al (27) studied mucormycosis; such infection results from fungi in the Rhizopus species. They write that such infections evolve aggressively, leading to devastating tissue loss (27).

The use of PCR and partial gene sequencing at the 18S rRNA gene for fungal identification is a ground-breaking advancement in veterinary diagnostics. The molecular approaches offer high sensitivity and specificity required for precise fingerprinting of pathogens. The molecular approach by the study correlates with findings of others, such as (28), who have shown that PCR could detect fungal-derived DNA from clinical samples.

Phylogenetic analysis showed that the isolates in this case are the nearest phylogenetic relatives to isolates from worldwide locations including Saudi Arabia, India, Pakistan and China. An extremely high level of evolutionary conservation and potential global dissemination of these filamentous fungi might be implied by this genetic similarity. A similar finding was identified by Richardson & Rautemaa-Richardson, (29), who reported the distribution of Aspergillus species in diverse geographical areas, pointing to their successful adaptation and environmental resilience.The molecular techniques confirmed those findings by clinical and traditional laboratory examinations.The Aspergillus findings aligned with those by Lahouar et al., (18).

More importantly, the results provide some clear directions on the best ways to manage fungal infections in livestock. Firstly, all the Aspergillus species are so prevalent that biosecurity measures and environmental control on farms must be greatly improved. Secondly, fungal infections should be monitored and detected early to reduce their effects on the health and welfare of the animals. Animal health management is important to prevent and control fungal rhinitis, such as proper ventilation, sanitization, regular health examination, proper food storage, etc. Expanding the research geographical areas and more sample size could bring more data for better understanding fungal rhinitis in sheep.

Conclusion

The study finds that chronic rhinitis is caused by fungi, in which Aspergillus species were the most prevalent. This is important because of the connection of this fungus with zoonotic diseases in both humans and animals. These isolates were sequencing-based similar to isolated from different world regions indicating high evolution in the current isolates. The findings clear the road for veterinarians and farmers to use better animal management methods to prevent fungal rhinitis. Future research should identify factors specific to the environment that explain the increased prevalence of fungal infection. Research is needed on farm management practices leading to infection, how changes in climate may affect the risk of infection, and what host genetic factors may make animals more susceptible to infection. Moreover, we need research on possible antifungal treatments and how they might work in livestock to develop effective antifungal therapeutics. The impact of the study can be emphasized that chronic rhinitis in sheep was primarily caused by Aspergillus species. These isolates were sequencing-based similar to those isolated from different world regions indicating high evolution in the current isolates and more need for improvement of animal health and management. However, the study comes with limitation of the area size sampled, which can be solved with future studies.

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Conflicts of Interest

The authors declare there is no conflict of interest.

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