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ISOLATION OF *MUCOR*SPP FROM PATIENTS WITH OTITIS MEDIA AND ITS SENSITIVITY TO ANTIFUNGALS

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Abstract

The current study dealt with patients with otitis media, where the fungus was isolated from different pathological conditions, and the patients were distributed according to age, sex, residence, and chronic diseases. The fungal species isolated from these patients were diagnosed, and the focus was on *Mucor* as it is dangerous and may cause black fungus disease, as it was diagnosed phenotypically and molecularly by polymerase chain reaction (PCR) and a drug sensitivity test was conducted. The results showed that men are more susceptible to infection than women, and adults are more susceptible than adolescents and children, and chronic diseases increased the incidence. The study showed an average ability of *Mucor* spp to produce hydrolytic enzymes, which makes it dangerous only for patients with low immunity. The study also showed that the fungus is resistant to antifungals except for fluconazole, amphotericin B, and ketoconazole, where the diameter of inhibition was 32, 14, and 10 mm, respectively.

Keyword: *Mucor*, otitis media, antifungals, *Mucormycosis* and virulence factors.

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Introduction

Mucor is one of the most important genera belonging to the Phylum Zygomycota. It is characterized by its transparent, wide, branched filaments without septa. Therefore, it is called coenocytes [1]. It reproduces asexually by fragmentation or the formation of asexual spores that arise within spherical sporangium. It is fast growing and resistant to difficult conditions, pesticides, and antifungals. Its colonies on the culture media are white or light gray in color quickly turns dark. There are no rhizoids beneath the sporangiophores, this character differentiates it about another genus which it like it called *Rhizopus* which processes rhizoids [2].

Mucor is a weak pathogen that does not attack healthy people due to its weak virulence factors, but it can turn into a great danger when it attacks people with low immunity, especially those with uncontrolled diabetes, as it causes a disease called *Mucormycosis* or black fungus [3]. This disease became famous during the spread of the Corona pandemic, and it became the talk of the

world when it struck Corona patients, distorting their forms, and causing them death in a large proportion [4]. The reason for the spread of this disease with COVID 19 patients is because the patients take bronchodilator treatments, and these treatments cause immunosuppression, which facilitates the entry of the pathogen through the nose, mouth, and ear, and begins to attack the internal tissues and causes blood to dissolve, which provides the nutrients necessary for its growth and spread and may cause loss of parts of the body. Eyes, jaw, ear, etc. This is accompanied by severe pain, blackening of the affected area, and temporary or permanent loss of vision or hearing. The common infection is in the oral cavity, nose, ear, eye, and lungs, but the infection may reach the brain, heart, kidney, bones, skin, and all areas of the body [5, 6].

This fungus is known for its antifungal resistance, its enzymatic ability, and its simple nutritional needs. It spreads its spores in the air, and when people with low immunity inhale them, they become infected with them. Sometimes the spores reach the ear where the moisture and temperature are appropriate and where there are nutrients, so it begins to grow in the middle ear and when the infected person has a low immunity [7]. The danger becomes double, as the fungus colonies grow and attack to reach the inner ear, break the bones, cause hearing loss,

and may reach the skull and brain, which puts the infected person at risk of death if he does not receive appropriate health care and at the appropriate speed [8].

Immunodeficiency is one of the most important risk factors for infection with this fungus, as well as chronic diseases, previous ear infections, poor personal hygiene, and leaving waste in the house for a long time, especially organic waste, leftovers, and rotten fruits. The fungus is considered one of the approved fungi in the production of organic acids. It may be used as food in Southeast Asia. It is also considered one of the factors that rotate the element in nature [9].

The aim of this study is to for the purpose of shedding light on the importance of mycorrhizal fungus in ear infections and the possibility of its treatment by antifungals.

Material and methods

Isolation and purification

The current study included 100 patients from ear, nose, and throat clinics in the city of Diwaniyah, 50 females and 50 males. Information about their residence, the nature of their work and other information was collected and recorded in a special form created specifically for this purpose [10]. Cotton swabs were taken for both ears by inserting them with caution to the ear and gently shoved it, then it was taken out and placed in a dark envelope and quickly transferred to the laboratory, where it was quickly planted on the nutrient medium Sabouraud dextrose agar in plastic petri dishes in the planning manner and the dishes were incubated in the incubator at 27 °C until colonies appeared, where they were purified by taking parts of the fungus colony and planting them on new plates containing SDA medium [11].

Identification

Pure cultures were diagnosed by the shape of the colonies, the nature of their growth, its speed of growth, texture, color, and height, as well as it was examined under the microscope to note the shape of the filaments, their branches, and the reproductive structures of pods, capsules, and spores [12, 13], while the diagnosis was confirmed by following molecular biological methods [14] by PCR using the primers 18S rRNA gene, and then gel migration and matching the results with the source or ladder isolates, where a special kit for the molecular study was brought from the Pioneer company, according to the instruction acquired from the company and as mentioned in [15].

Antifungal sensitivity

Antifungal sensitivity was tested by using SDA medium, where the medium was poisoned with different concentrations of these antifungals, which included Nystatin, ergosterol, amphotericin B, ketoconazole, and fluconazole, where these antifungals were brought from stockpiles in the city of Diwaniyah, and three concentrations of each antifungal were used [16]. The control treatment consisted of dishes without antifungal,

all dishes were inoculated by taking a disc of diameter 5 mm from a young fungal culture that was seven days old, after that the disc was placed in the middle of the dish, and all dishes were incubated at a temperature of 27 °C for a week, after which the diameter of the growing colonies was measured [17].

Virulence factors

Regarding protease production medium was prepared by dissolving 20 g of agarin in 900 ml of distilled water in the beaker. distilled in another beaker, then the two solutions were sterilized by autoclave each separately, and then they were mixed after cooling to a temperature of 45 °C, after that the antibacterial Chloramphenicol 250 mg / l was added, then poured into plates and left to solidify [17, 18].

As lipolytic activity test the medium was prepared by dissolving 20 gm of agar, 10 gm of peptone, 1.0 gm of aqueous calcium chloride, and 5 gm of sodium chloride in 1000 ml of distilled water, mixing it well with heating, and adding 5 ml of Tween 80 per 100 ml of the mixture, then adding the antibacterial Chloramphenicol 250 mg after That is, pouring the medium into petri dishes until it hardens, and inoculating the medium with a 5 mm disc of a pure colony, and it was incubated at a temperature of 27° C for a period of 3 days [19].

Phospholipase production test was done using yolk of eggs are used to obtain egg yolk, and they are placed in sterile test tubes with tight covers, and then they are placed in the centrifuge at gravity force 500 for 15 minutes, then the supernatant is taken from the emulsion, and then it is completed to its original size. With sterile distilled water. This emulsion is used immediately in the preparation of the egg yolk medium [20].

This medium is prepared according to [21] by adding 1 M (58.44) grams of NaCl and 0.005 M (0.55) grams of Calcium Chloride (CaCl₂) to a liter of Sabouraud Dextrose agar SDA food medium prepared and the pH is adjusted to the medium at 4.3 and sterilized In an autoclave, then the medium is left until it cools down to a temperature of 45_50°C, after which the egg emulsion is added under sterile conditions at an amount of 8% (volume / volume), then mixed and distributed in Petri dishes, and this medium was used to measure the activity of fungus in the production of phospholipase [22].

Hemolysis activity test, the medium was prepared by dissolving 40 gm in 1000 ml of distilled water, then autoclaving it, then cooling it to a temperature of 45 °C, then adding 50 mm of sheep blood to it, then pouring it into dishes and leaving it to solidify, for the purpose of detecting blood decomposition [23].

Results and Discussions

Fungal isolate according to sex, age, living region and chronic diseases.

The results of our study showed that there is a discrepancy in infection rates between different races, ages, residential areas, and chronic diseases, where males

were the most susceptible to infection, as they constituted the number of fungal isolates amounting to 66 isolates, 55%, compared to 54 isolates for females, 45%. As for age, adults were the most. Affected by the infection, the number of fungal isolates reached 66 isolates 55%, then adolescents 40 isolates 33%, then children at 14 isolates 12%. Regarding chronic diseases, those who suffer from them were the most infected compared to healthy people with 86 isolates 72% and 34 isolates 28%. As for housing, it was the urban population was 75 seclusions, 63%, and the rural population, 45 seclusions, 37%. It can be explained that men are more likely to be infected with otitis media because they engage in hard work such as farming, blacksmithing, construction work, drilling, dyeing, etc., which makes them vulnerable to pathogens entering the ear and colonizing it, especially if there is a predisposing factor such as previous injuries, wounds, secretions, etc., unlike women, who are less susceptible to this [24]. The type of work, but they are also infected, but in proportions. With regard to ages, adults are the most susceptible to infection, and the reason for that is due to the fact that they go out a lot from their homes intending to work and spend other concerns such as shopping and others, which puts them in more contact with pathogens and they may get them from contact with Affected people, while adolescents are less likely, may be at risk in schools only when they play violent games and are exposed to pollution with dust and moldy materials. This applies to children as well, but in lower proportions because they do not go out much from their homes [25]. Chronic diseases are among the most dangerous risk factors, as immunity is the decisive factor in the occurrence of infection, as well as in its later development, as the more a person is healthy, the more he defends himself well, and vice versa [26].

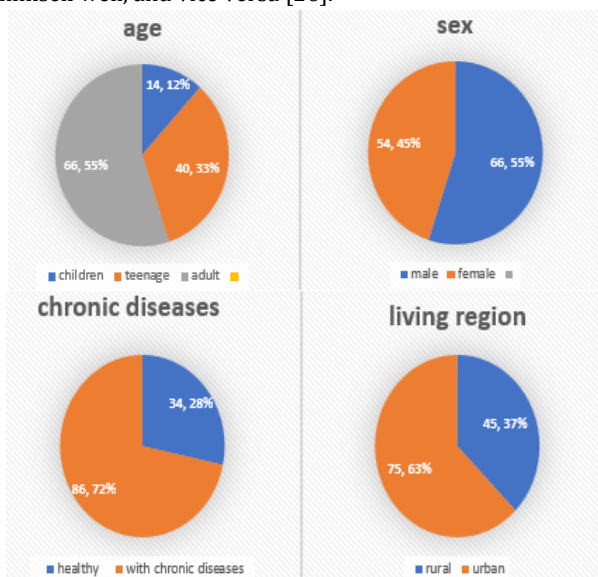


Figure (1) Otitis media infection distributed according to sex age chronic disease and living region.

The weaker the immune system, the more the infection is imminent and develops into a widespread and invasive one, as diabetes, AIDS, kidney and liver failure, thalassemia, and cancers are accompanied by otitis media infections and can even develop and become fatal [27]. As for housing, it is also influential, as the city environment is always crowded and contaminated with opportunistic fungi that come from rubbish or rotten food, or from contact with infected people in transportation, the army, prisons, markets, public swimming pools, etc. Usually, rural people rarely crowd because of the vast distances that separate them, and because they get clean and unpolluted air, but they also get infected sometimes because they work in the fields and graze animals, which exposes them to infection but at lower rates than in the city [27].

Fungi isolated from otitis media infection

Figure 2 shows the numbers and percentages of fungi isolated from Otitis media patients, where it appears that *Aspergillus niger* was the most present with 37 isolates 31%, followed by *Candida albicans* 27 isolates 18%, then *Aspergillus flavus* 22 isolates 18%, then *Aspergillus terreus*, *Rhizopus*, *Mucor*, *Penicillium*, *Fusarium*, and *Alternaria alternata* by . 12, 7, 6, 5, 2 and 2 isolates, respectively.

We have previously explained that the ear is a suitable place for the growth of many fungi, being moist and warm and containing nutrients. Therefore, it is not surprising that fungi are present in them, especially since some of them do not need many nutritional requirements. The mere presence of moisture is sufficient for its growth, especially the *Aspergillus*, *Penicillium*, and *Mucor*. These fungi release their spores in huge numbers into the atmosphere. It may reach the ear and begin to germinate, grow, and colonize in it, which causes the Otitis media. It may be a mixed infection from different groups of fungi, and it may be accompanied by bacteria. The ear may fester and swell, and the fungal growths become a plug that blocks the ear and prevents hearing, which requires a quick medical intervention, as the ear must be cleaned. It also helps to use topical and oral antifungals [28].

One of the dangerous things that deserves attention is that the *Mucor* fungus is the main cause of the black fungus disease, which spread in conjunction with the Corona pandemic, as the infection may come from the mouth, ear, or eye, then the disease develops in people with weak immunity to become frightening and deadly, destroying a part of the face, and people lose their hearing and sight, and perhaps Therefore, we focused here on this fungus, although it was isolated from the ear in smaller numbers, but it remains a source of latent and terrifying danger [29].

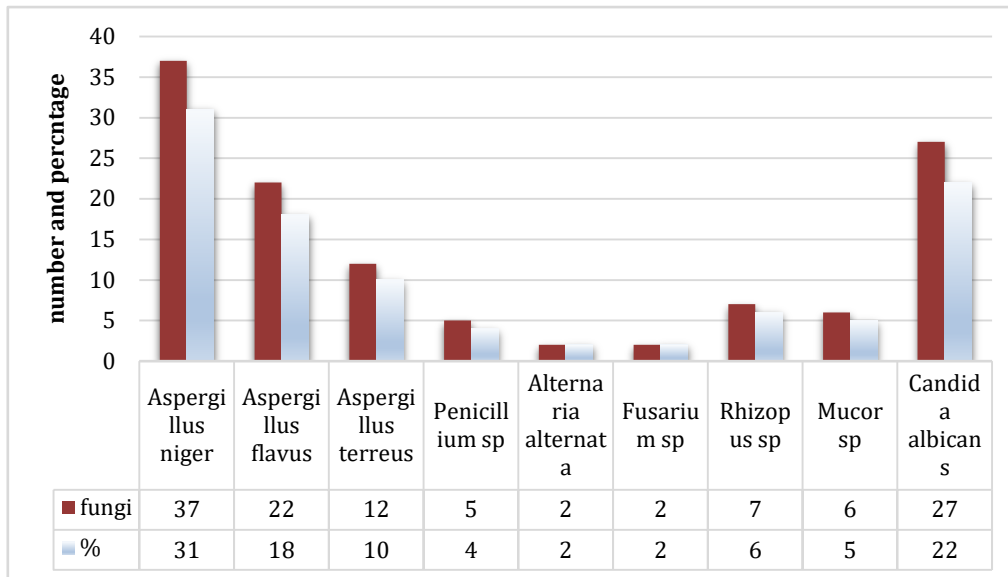


Figure (2) fungi isolated from otitis media

Morphological and Molecular Diagnostics of *Mucor* sp

The current study showed that the fungus has broad, undivided, unsegmented, translucent, branched hyphae that do not form rhizoids, producing sporangiophore that bear relatively large spherical sporangium at the end and contain many non-flagellated spores that come out at maturity after the destruction of the peridium [30].

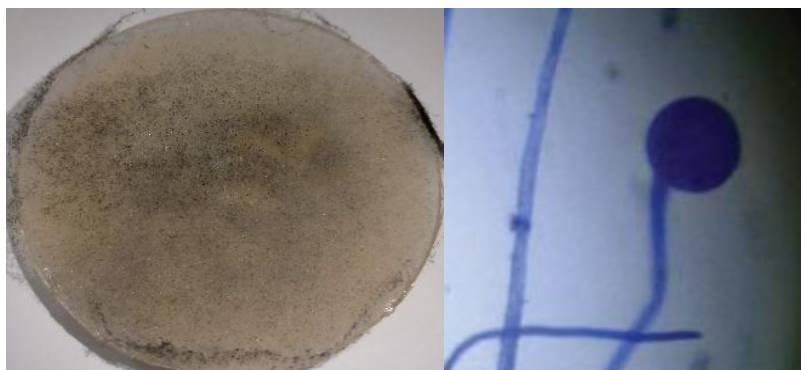


Figure (3) morphology of *Mucor*

In a study by [31] one of the important factors in the molecular diagnosis of filamentous fungi is the use of the 18S rRNA primer, which is considered one of the best primers used.

The PCR assay was performed for *Mucor* sp fungus using the primer 18S rRNA, if the DNA was extracted and amplified and then transferred on an agarose gel and detected using the ethidium bromide dye and examined with ultraviolet light, where one isolate was tested by relying on specialized primers for diagnosis and it appeared The result is a band with a length of 430 bp after being transferred on a 1.5% agarose gel [32].

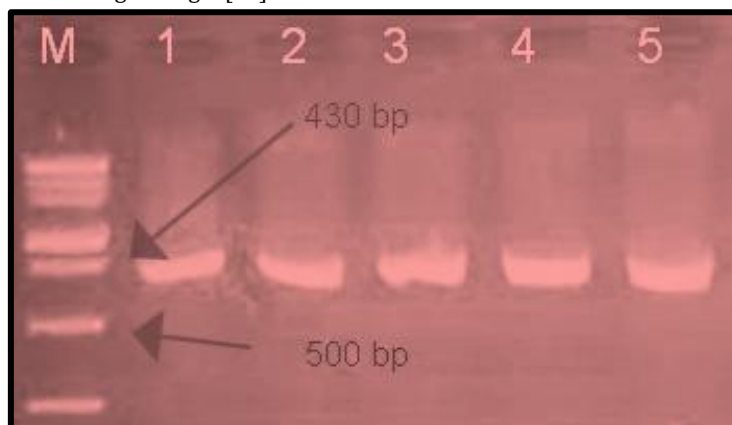


Figure (4) Electrophoresis results for the polymerization of the 18S rRNA gene of *Mucor* sp, 1.5% agarose gel concentration for an hour and a half, using 1x TBE buffer and the size scale of the DNA bundle (100bp-5000bp))

Virulence factors

Regarding *Mucor* sp virulence factors including hydrolytic enzymes the results in table 1 showed that there are mediate activity of protease and low activity of lipase and Phospholipase and high activity of hemolysin.

The hydrolytic enzymes indicate the pathogenicity of the pathogen because they are main weapon to invade host body and to possess nutrients for the pathogen and make the host immunity weak so prespring infection and invasion [33-40].

Table (1) virulence factors of *Mucor* sp isolated from Otitis media patients

ENZYMES	INHIBITION ZONE (MM)
PROTEASE	++
LIPASE	+
PHOSPHOLIPASE	+
HEMOLYCIN	+++

Antifungal sensitivity

Regarding the pharmacological sensitivity of the *Mucor* sp fungus towards antifungals, the results showed that the fungus had complete resistance to both nystatin and ergosterol, as it did not affect it at all, while the antifungal fluconazole gave the best results, followed by amphotericin B and ketoconazole, with an inhibition area of 32, 14, and 10 mm, respectively, because antifungals are substances Chemical kills or inhibits the growth of fungi, and they differ in the way they affect and the goal that they work on. Fluconazole works on the metabolism of building the cell membrane, affecting the enzymes responsible for building, and partially inhibiting the building process, which leaves the cell membrane of the pathogenic fungus perforated and distorted and makes the cell membrane completely permeable, which kills the cell [34].

Table (2) antifungal sensitivity (disk diffusion method)

ANTIFUNGALS	INHIBITION ZONE (MM)	%
NYSTATIN	0	0
ERGOSTEROL	0	0
AMPHOTERICIN B	14	22
KETOCONAZOLE	10	20
FLUCONAZOLE	32	65

Conclusion

- 1- There are many fungi located in otitis media patients one or gather of them can cause infection.
- 2- *Mucor* sp is included in otitis media and this matter is dangerous because it may lead to black fungus case infection.
- 3- Adult men and chronic disease were they were abundant in otitis media infection.
- 4- Fluconazole was the best in treatment of *Mucor* sp.

Conflict of Interest

Authors are declared that no conflict of Interest

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