

Experimental infection in mice with *Acremonium* spp. mold and *Rhodotorula* spp. yeast isolated from cow's milk

S.N. Yassein¹ and Z.R. Zghair²

¹Department of Microbiology, ²Zoonotic Diseases Unit, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq, Email: ¹ shaimaa_nabhan@yahoo.com, ² zghair@yahoo.com

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Abstract

This study was performed in isolation of some pathogenic fungi from milk of apparently healthy cows. Eighty milk samples were collected from four quarters of twenty cows in the Abu Ghraib. Each sample was cultured on Sabouraud dextrose Agar at 28±2 °C for 4-7 days. The most predominant mold and yeast were *Acremonium* spp. and *Rhodotorula* spp. that had used in the experimental infection. The number of experimental mice used in this study was 30 which divided into three equal groups. The 1st group was infected with *Acremonium* spp. by injection of 0.2 ml of 2*10⁷ conidia/ml intraperitoneally. The 2nd group was inoculated with same dose and route with *Rhodotorula* spp., while the 3rd group served as control group. All mice were sacrificed after 2 weeks of injection, Serum was obtained for biochemical analysis of hepatic and renal enzymes. Some of internal organs of infected groups were taken for histopathological study. The result recorded that the total percentage of fungal infection was 53 (66.3%) of these *Acremonium* spp. 9 (24.3%) and *Rhodotorula* spp. 7 (43.8%). Histopathological sections of the 1st group showed severe lesions in kidney than 2nd group although both groups showed lesions in most internal organs. Blood biochemical results showed the yeast has highest significant differences on ALT levels, while the mold has highest effect on serum creatinine, with insignificant difference on urea. In conclusion it could be said that in spite of *Acremonium* spp. and *Rhodotorula* spp. are considered as contaminant fungi, but they can cause disseminated mycosis in mice.

Keywords: Opportunistic fungi, Bovine mastitis, ALT, Creatinine

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الإصابة التجريبية في الفئران بعفن *Acremonium* spp. و خميرة *Rhodotorula* spp. المعزولتان من حليب الأبقار

شيماء نبهان ياسين^١ و زينب رزاق زغير^٢

^١ فرع الأحياء المجهرية، ^٢ وحدة الأمراض المشتركة، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

أجريت هذه الدراسة لعزل بعض اهم أنواع الفطريات الممرضة من حليب الأبقار السليمة ظاهرياً. جمعت ٨٠ عينة حليب من شطوط الضرع الأربعة الى ٢٠ بقرة من منطقة حزام أبو غريب وزرعت على وسط السابرويد دكستروز الصلب بدرجة حرارة ٢٨±٢ م° ولمدة ٤-٧ أيام. سجلت نسبة عزل فطرية عالية وصلت الى ٦٦,٢٥%. وكان من أكثر الاعفان والخمائر شيوعاً هو عفن *Acremonium* spp ٩,٩٨% وخميرة *Rhodotorula* spp ١٣,٢% اللذان تم استخدامهما في أحداث الإصابة التجريبية تم استخدام ٣٠ فأراً قسموا الى ٣ مجاميع بالتساوي. خمجت المجموعة الأولى بالعفن *Acremonium* spp ٠,٢ مل من ٢×١٠ سيور/مل بالحقن داخل الخلب، أما المجموعة الثانية فقد حقنت بالخميرة *Rhodotorula* spp وبنفس الجرعة والطريقة بينما أقيمت المجموعة الثالثة كمجموعة سيطرة. تمت التضحية بالحيوانات بعد أسبوعين من الحقن وتم الحصول على المصل وبعض الأعضاء الداخلية من المجاميع المخمجة لدراسة التغيرات المرضية النسجية. بينت المجموعة الأولى إصابة حادة اقوى في الكلية مقارنة بالمجموعة الثانية التي أظهرت إصابة اقوى في الكبد على الرغم من إن المجموعتين

أظهرتا آفات مرضية في بعض الأعضاء الداخلية، كذلك أظهرت الخميرة اختلاف معنوي كبير في فعالية ناقلة أمين الالانين، أما العفن فكان تأثيره الأعلى على الكرياتينين ولم يلاحظ أي اختلاف معنوي لليوريا. نستنتج من ذلك انه بالرغم من أن عفن *Acremonium* و خميرة *Rhodotorula* spp يعتبران من الملوثات الفطرية، إلا انه يمكنهما إحداث إصابات فطرية جهازية في الفئران المخمجة بهما.

Introduction

On one hand, milk and its products are considered as one of the major important sources in the human diet because it contained numerous essential components like vitamins, calcium, minerals and fatty acids (1,2). On the other hand, milk may during udder infection plays an important role in transmission of the different microbes that cause the subclinical mastitis (SCM) in non-obvious changes occur in the manifestations of the milk and udder compared with clinical mastitis (3), And still represents a source of infection to healthy animals (4-7). In the dairy cattle and buffaloes, the prevalence of mastitis may exceed 50% to 15-40 more times the incidence of subclinical mastitis than clinical mastitis (8). Many previous studies performed for detecting of clinical mastitis with the efforts for treatment through performing antibiotic sensitivity test especially case of bacterial infection (9,10). Regarding mycotic mastitis, more attention was given to the pathogenic fungi causing mastitis, but lower researches are dealing with saprophytic or opportunistic fungi as probable causes of clinical or subclinical mastitis (11-13).

Therefore, the objective of the present study is to elucidate the possible role of saprophytic fungi in causing systemic fungal infection in mice through their effect on liver and kidney enzymes and through other histopathological changes in internal organs.

Materials and methods

Source of samples

The number of milk samples collected from four quarters of twenty cows which appeared healthy from Abu Ghraib zone was 80. The period of this study was starting from 5 November 2017 to 25 January 2018, by cleaning the teat end with 70% ethyl alcohol and discard of the first three streams of milk 5-10 ml in each test tube. The samples were kept at 4 °C during transportation to the laboratory during three hours after collection according to Coles (14). All sample was centrifuged at 3000 rpm/ minutes for 5-10 and the

The present study showed a high percentage of fungal contamination in the milk samples of apparently healthy cows. The total fungal isolates which gave the positive result was 53 (66.25%) that were tabulated into 2 types: 7 mold species 37 (69.8%) and 3 yeast species 16 (30.1%) (Table 1). The current study revealed that the fungal contamination of the milk samples was caused by molds more than yeasts (Tables 2 and 3). The result shown in tables 2 and 3 revealed that the most predominant mold and yeast were *Acremonium*

sediments were cultured on Sabouraud dextrose agar (Himedia, India) and incubated at 28±2 °C for 4-7 days then examined and identified macroscopically and microscopically according to Washington *et al.* (15). Conidia suspension was prepared from more prevalent mold and yeast according to Van der Velden *et al.* (16) and adjusted to 2x10⁷ conidia/ml according to (17) by hemocytometer chamber.

Experimental design

Thirty albino male mice at 12 weeks of age ranging between 20-25 g of weight were used in this study. All the mice were divided into three groups and each group was separated in the cages for preliminary period one week as the acclimatization period. The first group was infected with the more prevalent mold *Acremonium* spp by giving 0.2 ml of 2*10⁷ conidia/animal intraperitoneally. The second group was inoculated with the same dose as well as the same route with the more prevalent yeast *Rhodotorula* spp. While the third group was inoculated with normal saline which served as a control group according to Fernandez-Silva (18).

All the mice were monitored for 10 days after infection and then were sacrificed after two weeks of injection. Blood samples were collected in the vacutainer blood collection tubes by cardiac puncture, then the serum was obtained by centrifugation of the blood samples for 3000 rpm/minute for 10 minutes to study the biochemical analysis of liver and kidney enzymes. According to Bancroft and Stevens (19) some of the internal organs of the infected groups like liver, kidney, intestine, spleen, and heart were taken to demonstrate the histopathological changes.

Statistical analysis

The statistical analysis system (SAS) program was used to detect the effect of different treatments (20). The P value of 0.01 was considered for statistical difference.

Results

spp. 9 (24.3%) and *Rhodotorula* spp. 7 (43.8%), respectively, those were used in the experimental animal to study their effects on the internal organs of mice and their biochemical alteration in the hepatic and renal enzymes.

Table 1: Prevalence of fungal contamination in cow's milk samples

Type	No. of spp.	No. of isolates	%	Total fungal isolates
Mold	7	37	69.8	53/80
Yeast	3	16	30.1	(66.25%)

Table 2: Type of molds that contaminated milk samples

Type of mold	No. of isolates	Percentage %
<i>A. flavus</i>	5	13.5%
<i>A. niger</i>	4	10.8%
<i>A. fumigatus</i>	3	8.1%
<i>Alternaria</i> spp.	3	8.1%
<i>Acremonium</i> spp.	9	24.3%
<i>Curvularia</i> spp.	3	8.1%
<i>Fusarium</i> spp.	2	5.5%
<i>Geotricum</i> spp.	4	10.8%
<i>Rhizopus</i> spp.	4	10.8%
Total	37	100%

Table 3: Type of yeasts contaminated milk samples

Type of yeast	No. of isolates	Percentage %
<i>Cryptococcus</i> spp.	4	25%
<i>C. albicans</i>	5	31.2%
<i>Rhodotorula</i> spp.	7	43.8%
Total	16	100%

Histopathology

Histological sections of the internal organs of inoculated group of mice with *Acremonium* spp. show sever histological changes than those inoculated with *Rhodotorula* spp. In respect to those changes in spleen of mice inoculated with *Acremonium* spp. for two weeks revealed lymphocytic depletion and infiltration of the inflammatory cells and macrophages with the presence of the hemosiderin pigment (Figure 1). In the intestine, there was hyperplasia of the goblet cells and infiltration of the inflammatory cells (Figure 2). Liver showed the necrosis in hepatocytes and infiltration of the inflammatory cells in the liver parenchyma (Figure 3), and there was an infiltration of the inflammatory cells between myocardial fibers of myocardium (Figure 4). The histopathological examination of the inoculated mice with *Rhodotorula* spp. after two weeks showed lesions multifocal lymphocytic aggregation and macrophages scattered throughout the parenchyma with vacuolation of hepatocytes cytoplasm of liver (Figure 5). In the myocardium, there was infiltration of the inflammatory cells between dilated myocardial fibers (Figure 6). The kidney showed periglomerular edema, acute cell swelling of the renal tubules and infiltration of the inflammatory cells in the renal parenchyma (Figure 7). Intestine showed infiltration of the inflammatory cells of mucosa and sub mucosa (Figure 8).

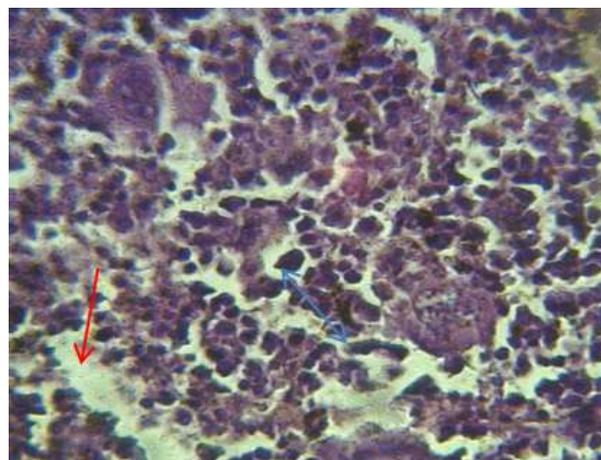


Figure 1: The histopathological changes in the spleen of the inoculated mice with *Acremonium* spp. after two weeks shows lymphocytic depletion (red arrow) and infiltration of the inflammatory cells and macrophage with the presence (red arrow). H&E stain, 400x.



Figure 2: The histopathological changes in the intestine of the inoculated mice with *Acremonium* spp. after two weeks shows hyperplasia of the goblet cells and infiltration of the inflammatory cells of hemosiderin pigment (blue arrow). H&E stain, 400x.

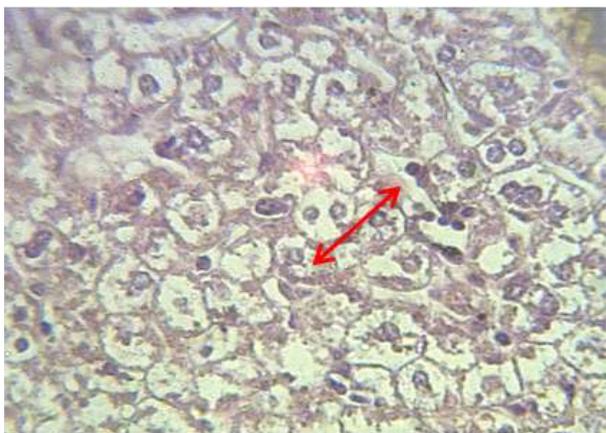


Figure 3: The histopathological changes in the liver of the inoculated mice with *Acremonium* spp. after two weeks shows necrosis in the hepatocytes and infiltration of the inflammatory cells in the liver parenchyma (red arrow). H&E stain, 400x.

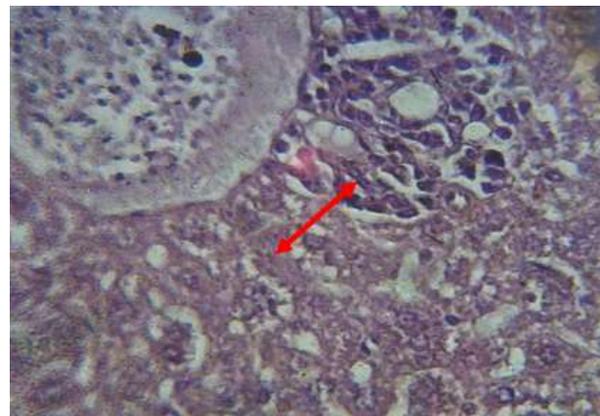


Figure 5: The histopathological changes in the liver of the inoculated mice with *Rhodotorula* spp. after two weeks shows inflammatory infiltrations formed from lymphocytes and macrophages scattered throughout the parenchyma with vacuolation of the hepatocyte's cytoplasm (red arrow). H&E stain, 400x.

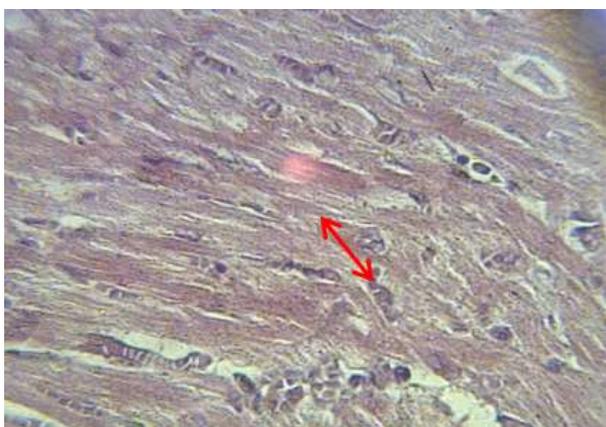


Figure 4: The histopathological changes in the myocardium of the inoculated mice with *Acremonium* spp. after two weeks shows infiltration of the inflammatory cells between the myocardial fibers (red arrow). H&E stain, 400x.

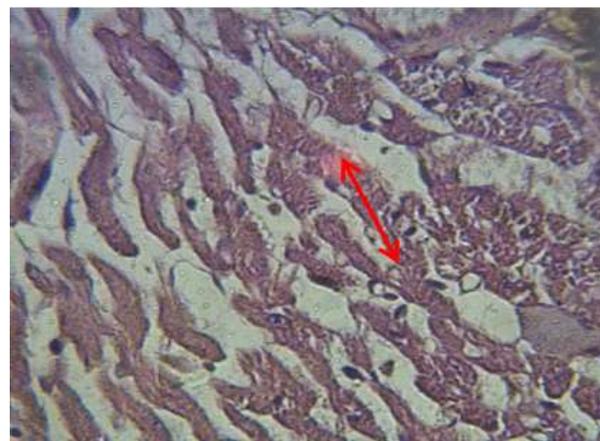


Figure 6: The histopathological changes in the myocardium of the inoculated mice with *Rhodotorula* spp. after two weeks shows infiltration of the inflammatory cells between the dilated myocardial fibers (red arrow). H&E stain, 400x.

Biochemical tests

The effect of *Acremonium* and *Rhodotorula* spp. inoculation in mice on liver and kidney enzymes showed highly significant differences compared with control group. Based on LSD analysis the *Rhodotorula* spp. has the highest effect on ALT levels ($P=0.0001$), while the mold has the highest effect on serum creatinine and most significant statistical difference ($P=0.0038$), with insignificant difference on serum urea ($P=0.0462$) (Table 4).

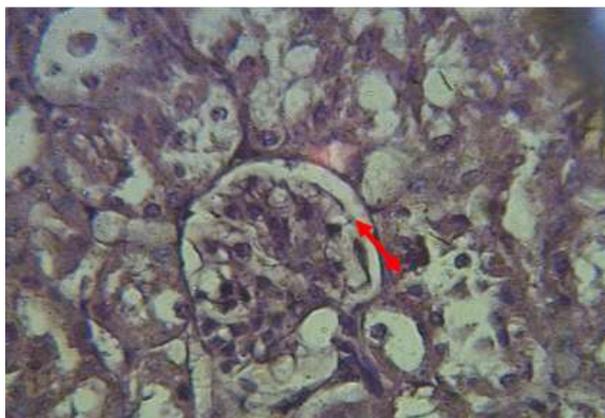


Figure 7: The histopathological changes in the kidney of the inoculated mice with *Rhodotorula* spp. after two weeks shows periglomerular edema, acute cell swelling of the renal tubule's infiltration of the inflammatory cells in the renal parenchyma (red arrow). H&E stain, 400x.



Figure 8: The histopathological changes in the intestine of the inoculated mice with *Rhodotorula* spp. after two weeks shows mild enteritis and manifested by infiltration of the inflammatory cells of mucosa and sub mucosa (red arrow). H&E stain, 100x.

Table 4: Effect of *Acremonium* and *Rhodoterella* spp. inoculation on enzymes of liver and kidney in mice after two weeks of inoculation intraperitoneally

Groups	Mean ± SE		
	ALT (U/L)	Creatinine (µmol/L)	Urea (µmol/L)
G1 <i>Acremonium</i> spp.	24.80 ± 2.47 b	0.744 ± 0.07 a	33.58 ± 1.76 a
G2 <i>Rhodotorula</i> spp.	11.73 ± 1.24 c	0.540 ± 0.03 b	28.55 ± 1.90 b
G3 (Control)	40.20 ± 1.33 a	0.514 ± 0.02 b	30.68 ± 1.75 ab
LSD value	5.142 *	0.139 *	4.250
P value	0.0001	0.0038	0.0462

Different letters in the same column are significantly different, $P < 0.05$.

Discussion

The present study appeared that the percentage of the mold contamination of milk samples was 69.8% taken from a healthy cow. The high percent of mold in milk may be due to bad management or even lack of hygiene in that rural area. This opinion coincides with the Luaibi *et al.* (21), most of the contaminated fungi are considered as saprophytic and habitat in soil and vegetation but when there is stress or defect in the defense mechanism of the udder, the isolation of these fungi can be discovered, this fact was highlighted by Zhou *et al.* (22). The type of milking using in this area is the manual milking which participates in introducing of the conidia into the udder through the contaminated teat and the teat canal by the workers contaminated hands. The previous study found that the fungal contamination of milk could reach to 46.15% with the manual milking with partially lower 44.70% percentage by machine milking (23).

The current study revealed that the most predominant mold was *Acremonium* spp. constitute large genera of

polyphyletic fungi due to its containing about 150 species, most of them habitat and isolated from soil and plant debris and could cause pathogenic infection to plant (18). The food-borne pathogens play an important role to infect human and animals (24). On the other hand, it can result in localized infection through traumatic contamination such as mycetoma, onychomycosis, keratitis (25) and in rare cases disseminated infection in immunosuppressed host (26-28). The most organs affected were spleen, liver and kidney. which coincide with the finding of Fernandez Silva *et al.* (18) how described the lesion in spleen, kidney and liver while the least organ affected was the brain. There are few reports about the systemic infection with *Acremonium* spp. But Nedret Koç (29) could isolate *Acremonium strictum* with *S. aureus* from the pleural fluid in case of colon adenocarcinoma. However, most these researches pointed to this type of fungi associated with the immunocompromised hosts particularly after chemotherapy, in post-transplantation period and in the patients with primary immunodeficiency. Fakharian *et al.* (30) and even Fernandez

Silva *et al.* (16) revealed to the histopathological changes of liver and kidney in immunosuppressed mice, interestingly, the finding of the current study appeared in immunosuppressed mice, this indicate that the fungus has different types of the virulence factors that could lead to induction of the pathological alteration in some of the internal organs.

In concerning of contaminated milk with yeasts, the present study recorded the rate of yeast isolated from cow's milk was 30.1%. This result could be attributed to multifactor such as the presence of irritation in milk ducts and alveoli, injury in milk sinus, intramammary administration of antibiotic particularly for long period in case of bacterial infection and the virulence factors of the pathogen. All these factors are related to yeast infections of the udder, the previously study revealed that the incidence of bovine mycotic mastitis caused by yeast in Poland and found the *Rhodotorula* occupy the third position after *Candida* and *Trichosporon* spp. (31). In addition, the investigation of the current study disagree with Akdouche *et al.* (23) who recorded the highest incidence of bovine mycotic mastitis caused by yeast and this difference may be due to the season of the sample collection especially during winter and spring when the yeast isolation is abundant compared to summer the samples of the present study were collected are least.

This study had been recorded a high ratio of *Rhodotorula* spp. isolation 7 (43.8%) when compared with other yeasts as *Candida* and *Cryptococcus* spp. and this percentage was higher than reported by Wawron *et al.* (31) and Hasan and Yassein (32) 9.3% and 7.5%, respectively. *Rhodotorula* spp. are common airborne contaminant fungi and can be isolated from the soil, and in spite of consideration as normal habitats of the skin, urine and feces in human, It associated with invasive mycosis through the catheter, endocarditis, keratomycosis, peritonitis and meningitis among the immunocompromised hosts because these species have emerged as opportunistic fungi in those hosts (33,34).

In the past, *Rhodotorula* spp. considered as one of non-virulent yeast whereas at the last two decades, it was found there were increasing in the number of infections that caused by *Rhodotorula* spp. (35). So, the present study is considered as the first one that described the experimental model of disseminated Rhodotulosis in mice liver when compared with other organs. This evidence is in consistence with Wirth and Goldani (35) who studied the infection of this yeast in rats and found that the liver showed sever degree of infection. Also, this evidence was corresponding with the abnormalities of chemistry analysis represented by significant differences in ALT associated with the organ damage.

Conclusion

Based on current findings, it can be concluded that in spite of *Acremonium* spp. and *Rhodotorula* spp. are considered as saprophytic fungi, but they can result in disseminated mycosis in mice after intraperitoneal inoculation of these pathogens.

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