Isolation and Identification of *Cryptococcus neoformans* from Human Skin Lesions and Application of Animal Experiment (in vivo)

Mahmood F.A and Alwan M.J
Zoonosis unit, College of Veterinary Medicine, Baghdad University, Iraq.
E-mail: alwanmohammed44@yahoo.com
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Summary

The study aimed at determining the percentage of *Cryptococcus neoformance* isolated from skin lesions in human in order to find the effect of heat killed cryptococcal antigen on infection with this fungus in mice and efficiency of chitosan in protecting the mice against the infection with the fungus *Cryptococcus neoformance*. In order to achieve the first aim, 100 cutaneous samples were collected from variable skin regions in humans suffering from cutaneous lesions from both sexes and with variable ages. The samples were collected from Abu-Ghraib Hospital,Al-Yarmook Teaching hospital and Al-karama Hospital during a period from December 2012 untill March 2013. The results showed that 3 (3%) isolates out of 100 skin samples were the fungus *C.neoformans*. The fungal isolates were from patients with average age 1-25 years they did not have any systemic clinical signs. Also the results showed that the ratio of fungal isolates from females was higher than those isolated from males. In order to achieve the second aim ,Sixty white mice from both sexes and with average age 8-10 weeks were divided randomly into 4 groups equally and treated as follows 1st group was immunized with 0.2 ml of heat killed C.*neoformans* antigens (HKCAgs) in a dose of ($1X10^7$) fungal cells,subcutanously at two doses with two weeks interval. The 2nd group was immunized similar to 1st group and at the same time fed diet supplemented with 1g\kg diet of chitosan for 8 weeks. While the 3rd group served as control positive group, 4th group served as control negative group.Cell mediated and antibody mediated immune responses were determined at day 27-30 post-immunization. Immunized animals fed diet supplemented with chitosan showed higher values of delayed type hypersensitivity reaction and Abs titer compared with those values of immunized animals only.

Keywords: *Cryptococcus neoformance*, skin lesions, killed Cryptococcal antigen, Chitosan.

Introduction

Cryptococcosis, or cryptococcal disease, is an important zoonotic fungal disease worldwide in distribution, caused by *Crptococcus neoformans* or *Cryptococcus gattii*. These fungi can infect both immunocompetent and immunocomprised individual (1). During the past two decades, the prevalence of C.neoformans as emerging fungal pathogen is widely recognized throughout the world (2). Perfect and Casadevall (3) recorded that asymptomatic or mild infection with C.neoformans in the healthy individuals while severe infection with significant mortality in the immunocompromised patients in which the disease progress to pneumonia and fatal meningoencephalitis, with prevalence ranges from 8% in the United states to 30% in Africa (4). However, these fungi can infect most internal organs in addition to skin, bone and joints (5) but the main target organs are brain and lungs (6) .It was found that the antifungal treatment of the cryptococcosis has major limitations (1), therefore new strategies to control cryptococcosis were required.

Casadevall and Pirofski, (7) reported that immunomodulatory therapy and vaccine are effective programs in the control of this disease. Chitosan is a good immunomodulated that improve immune responses against certain pathogens such as E.coli infection (8 and 9). In Iraq, there are few research about the percentage of C. neoformans associated with skin wound and using chitosan as immunomodulater factors against fungal infection. Therefore the aims of the present study is investigate the incidence of primary cutaneous cryptococcosis in humans and the evaluation of the efficacy of chitosan for protection the mice against C. neoformans infection.

Materials and Methods

In this study the primitive media used was sabouraud dextrose agar for primary isolation, sunflour seed agar was used as selective media, urease test as biochemical test and Rapid Yeast Plus Panal biochemical kit for confirematory diagnosis of the isolate. Also, 40 white mice were used in the immunological study of this fungus. For detection fungal pathogenicity**,** 4 mice were inoculated 1\P with 0.3ml of fungal suspension containing $1X10^7$ of viable C. neoformans, this process was repeated until the inoculated mice were died and C. neoformans that isolated from these dead mices was used in preparation of heat killed C. neoformans antigens (HKCAgs) according to (10). Also statistical analysis was conducted by using ready-made statistical design: statistical package for social science (SPSS). Sample collection: A total 100 swabs from skin lesions were collected from the patients from both sexes and at different ages, Swabs were obtained using transport media and then transmitted to the Zoonotic Unit laboratory where they were cultured on Sabouraud dextrose agar and then incubated in 30˚C for (2-7) days.

Results and Discussion

Results of isolation and identification: The yeast can be grown on the Sabouraud dextrose agar and develops white, mucoid, convex and glistening colonies within 2-3 days of incubation the confirmative diagnosis was done by culturing it on sunflower seed agar, there was development of mucoid appearance with light-dark brown pigmentation which develop within 3-5 days (Fig. 1) then smear was made on slide and it was stained with Nigrosen stain. The cells appeared oval round shape surrounded by transperant hallow zome from its capsule. Urease test was done as biochemical test and the yeast gave positive result. And finally the Rapid Yeast plus Panal biochemical kit gives confirmatory diagnosis of the isolates.

The present finding demonstrated that 3(3%) out of 100 skin lesion samples expressed *C. neoformans* positive isolates, and the fungus was isolated from patients with average age 1-25 years and they were not suffering from systemic clinical signs, the ratio of fungal isolates in females (3.63%) was higher than those males (2.22%) (Table, 1).

Figure, 1: Mucoid light dark brown fungal colonies on Sunflower seeds agar.

Table, 1: Prevalence of fungal isolates according to age and gender.

Age	N.S	+	%	Male	+	%	Female	+	%
1-15	21	2	9.52	11	1	9.09	10	1	10
16-25	35	1	2.85	17	-	-	18	1	5.55
26-40	26	-		12	-		14	-	-
41-60	28	-		15	-		13	-	-
Total	100	3	3	45	1	2.22	55	2	3.63

(+) = Positive culture, (-) = Negative culture

The current study showed that *C. neoformans* was isolated in pure culture from (3%) of patients suffering from skin lesions without systemic clinical signs, This result may indicate that the fungal infection is primary cutaneous Cryptococcosis (PCC), this evidence agreed with definition PCC in the literature by the identification of *C. neoformans* in the skin lesion biopsy specimen or by culture (11)**,** With the absence of dissemination (12)**.** Also the present study revealed that skin lesions were localized region of the skin these observation was also supported by the idea that the skin lesions in the present study were PCC. These observations were consistent with (13) who found that primary cutaneous cryptococcosis confined to one body region and it was not associated with dissemination, also (14) explained that PCC is characterized by skin lesions confined to one body region without evidence of simultaneous dissemination and that it also occurs in immunocompromised and immunocompetent patients.The fungal isolates from females were more than that isolated from males and this is consistent with (13) who reported rare case of male with

normal cell mediated immunity infected with PCC.

Immunological finding: DTH reaction:The current finding showed, at 24 hours post test, the mean values of the skin thickness against soluble sonicated antigens of *C.neoformans* in the 2nd group was higher (1.68+0.03) than those values in 1st group (1.06±0.05), and at 48hrs post-examination these values were arise in 2nd group (1.76+0.03) and 1st group (1.39+0.04) (Table, 2).

Table, 2: Shows mean thickness of skin against SSCAgs in immunized animals at 24-48hrs posttest.

Groups	after 24 hrs	after 48 hrs
Control	0	0
1	1.06 ±0.05	1.39 ±0.04
2	1.68 ±0.03	1.76 ±0.03

The results of skin test may indicate that HKCAgs used in the present study stimulated the cell mediated immune response,since DTH reaction was considered one arm of CMI and this reaction control by activity of CD4+ and CD8+ T lymphocytes (15). DTH reaction have been classically used to detect cell-mediated responses to cryptococcal antigens and these reactions can be elicited by different type cryptococcal Ags such as heat – killed Cryptococci (HKC), supernatants Ags (16).

The present study showed that immunized animals with heat killed Ags showed low values of DTH reaction compared with those values in immunized animals with this Ags and fed diet supplemented with chitosan. This may be due to influence of temperature on protein Ags containing wall of the fungi practically mannoproteins (MPs) which play essential role in eliciting DTH reaction, these evidence was agreed with (16). Mansour *et al*., (17) who showed that the polysaccharide cell wall of *C.neoformans* contain GXM,GaIXM and MIPS but they found only MPs induces DTH reactions also (16 and 17) reported that mannoprotein play crucial role in the activated of T. cells. The high values of DTH in immunized –chitosan supplement Group may indicate that the chitosan augment the CMI response initiates by Cryptococcal Ags or directly stimulated the lymphocytes population, (18) also (19) reported that chitosan stimulated macrophage through activated NC cells and T.lymphocytes to produce IFN-ɤ that play essential role in DTH reaction. Serum Antibodies titers was showed that HKCAgs used in immunized animals in the current study stimulated humoral immune response this result was in consistent with (20) who reported that vaccinated mice with peptide mimetic of GXM of *C. neoformans* activated Abs response against these Ags. However, the Abs titers that elicited by immunized Ags with chitosan supplemented diet were higher than those values in immunized animals alone ,these results may be indicated that chitosan activated B cells to proliferate and differentiate into plasma cells producing Abs. (Table, 3).

Table, 3: Shows mean values of antibodies titers against SSCAgs in immunized animals.

Groups	Means of Abs titers against *C.neoformans* ± SE
Control	0
1	26±2.92
2	38±7.96

In conclusion of this study revealed that *Cryptococcus neoformans* induced Primary cutaneous cryptococcosis (PCC) in immunocompetent individuals. Primary cutineous cryptococcosis was associated with skin injury. Primary cutineous cryptococcosis is localized lesion in one region of the body. Chitosan with HKCAgs is a good stimulator of both arms of immune response compared with HKCAgs only.

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عزل وتشخيص فطر المكورات الخبيئة من الافات الجلدية في الانسان مع تطبيق تجربة حيوانية

محمد جويد علوان و فضاء عبد الله محمود
وحدة الامراض المشتركة، كلية الطب البيطري، جامعة بغداد، العراق.
E-mail: alwanmohammed44@yahoo.com

الخلاصة

هدفت الدراسة الحالية الى تحديد نسبة حدوث المكورات الخبيئة المعزول من الافات الجلدية في الانسان، بالاضافة الى ايجاد تاثير الانتجين المقتول بالحرارة للفطر على الاصابة بهذا الفطر في الفئران فضلا عن تقييم كفاءة الكيتوسان في حماية هذه الفئران ضد الاصابة بفطر المكورات الخبيئة. لتحقيق الهدف الاول، 100عينة من الاصابات جلدية تم جمعها من مناطق جلدية متنوعة في ناس يعانون من افات جلدية ومن كلا الجنسين وباعمار مختلفة. العينات تم جمعها من مستشفى ابو غريب، مستشفى اليرموك التعليمي ومستشفى الكرامة خلال فترة من شهر كانون الاول 2012 الى شهر اذار2013. اوضحت النتائج ان هناك عزلات من اصل 100 عينة جلدية (3%) كانت فطر المكورات الخبيئة. هذه العزلات كانت من اشخاص بمعدل عمر من 1-25 سنة وبدون ظهور علامات سريرية، ايضا اوضحت النتائج ان نسبة عزل الفطر من الاناث كانت اكثر من الذكور. لتحقيق الهدف الثاني 60 من الفئران البيض بمعدل عمر 8-10 اسابيع قسمت بصورةعشوائية الى 4 مجاميع متساوية وتم معاملتها كالاتي: المجموعة الاولى لقحت بـ 0.2 مل من الانتجين المقتول بالحرارة للفطر (1×10^{7}) خلية فطرية، تحت الجلد وبجرعتين وبمعدل اسبوعين بين الجرعتين، المجموعة الثانية لقحت مثل المجموعة الاولى وتم تغذيتها على عليقة مجهزة بالكيتوسان ولمدة 8 اسابيع، المجموعة الثالثة كانت مجموعة سيطرة موجبة والمجموعة الرابعة مجموعة سيطرة سالبة. تم قياس المناعة الخلوية والمناعة الخلطية بعد 27-30 من التمنيع. تستنتج الدراسة اظهار الحيوانات الممنعة والمتغذية على عليقة تحوي الكيتوسان معدلات من فحص الحساسية التاخر ومعيار الاجسام المضادة اعلى مما في الحيوانات الممنعة فقط.

الكلمات المفتاحية: فطر المكورات الخبيئة، الافات الجلدية، تأثير الانتجين المقتول، الكيتوسان.