

Disseminated Cryptococcosis in a dog due to *Cryptococcus neoformans* var. *neoformans*

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한 마리의 개에 있어서 *Cryptococcus neoformans* var. *neoformans*에 의한 파종성 크립토콕스병

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요 약 : 일반적인 치료에 반응을 나타내지 않는 호흡곤란증에 걸린 4년령의 Shepherd개에서 피부, 눈, 림프절의 병변을 동반한 치명적인 파종성 크립토콕스병이 진단되었다. 세포학적 검사에 의해 난원형 내지 구형이고, 두꺼운 껍막을 갖고 있는, 형태학적으로 *Cryptococcus neoformans*와 일치하는 효모균이 증명되었다. 이 병원체는 혈액, 오줌, 콧물, 건갑전립프절 흡인물, 피부생검 시료, 피부 면봉 시료 등을 25°C의 Pal씨 배지에 접종하여 용이하게 분리되었다. 배양물을 PHOL염색액으로 염색하여 현미경으로 검사한 결과 얇은 막에 싸인 발아를 나타내거나, 나타내지 않는 구형 내지 난원형의 효모균이 증명되었다. 이 개는 ketoconazole로 치료를 시작한 후 6일만에 폐사하였다. 공기, 흙, 비둘기 배설물, 톱밥 등을 Pal씨 배지에 접종하여 *C. neoformans*가 배양됨으로써 역학적으로 환경이 병원소 역할을 한 것으로 판단되었다. 환측과 환경으로부터 분리된 균주는 세밀한 동정 결과 *neoformans* (serotype AD)에 속하며, *Filobasidiella neoformans* "alpha" mating type을 나타내었다. 이 연구 결과, Pal씨 배지가 크립토콕스병의 조기진단과 역학적 조사에 훌륭한 감별배지라는 것이 입증되었다. 개량된 Pal씨 배지는 *C. neoformans*의 genetic crossing을 판단하는데 성공적으로 사용할 수 있었다.

Key words : Cryptococcosis, *Cryptococcus neoformans*, dog, dissemination, environment, Pal's medium

Introduction

Cryptococcosis, primarily caused by the amorph fungus *Cryptococcus neoformans*, is a cosmopolitan, infectious mycotic disease of humans and animals^{10,21,23,24}. The disease produces con-

siderable morbidity and mortality in immunocompetent and immunosuppressed hosts^{10,18,21}. Though the yeast has a predilection for the central nervous system, it can also infect other tissues of the body such as skin, eye, ear, nose, bone, muscle, joint, adrenal gland, mammary gland, kidney, liver, spleen, heart and lung etc.^{10,17,21,25}. The present communication describes a fatal case of disseminated

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cryptococcosis in a large breed of dog due to *Cryptococcus neoformans* var. *neoformans*. In addition, the efficacy of Pal's medium as an excellent selective medium for the rapid isolation of *C. neoformans* from clinical and environmental samples is also confirmed.

Material and Methods

A four-year-old male German shepherd with a 25 days history of respiratory disorder constituted the material for this investigation. The dog belonged to a business man who kept the animal in his poultry farm. The dog was treated by a local veterinarian with broad-spectrum antibiotics (ampicillin, tetracycline, gentamicin), corticosteroids (prednisolone, dexamethasone), antihistamines (chlorpheniramine, diphenhydramine) and other supportive drugs like vitamins and liver extract preparations but clinical response was very poor. Later the animal developed cutaneous mass on the nose and showed conjunctivitis and superficial lymphadenopathy. Blood was collected by venipuncture for haematology and cultural examination. The punch biopsy and swabs from cutaneous mass, fine needle aspirate from swollen preapical lymph node, urine and nasal exudate were submitted to the laboratory of Veterinary Public Health. The urine sediment, lymph node aspirate and skin swab were directly examined under light microscope by 'PHOL' technique¹². The smears prepared from biopsy and swab were stained with periodic acid-Schiff (PAS) for the demonstration for fungus. All the clinical specimens were cultured onto the slants and plates of nutrient agar, Sabouraud dextrose agar with chloramphenicol (0.05 mg/ml) and cycloheximide (0.5 mg/ml) and Pal's medium (pulverized sunflower seed 45 g, agar 20 g, chloramphenicol 500 mg, distilled water 1000 ml)¹⁰. The former three media were kept at 37°C while the later was incubated at 25°C. The inoculated media were daily examined for microbial growth. The microscopic morphology of the isolate was studied in 'PHOL' which contained 3 ml of glycerol, 0.3 ml of 3% aqueous solution of methylene blue and 5 ml 4% solution of 35% formaldehyde. The variety dif-

ferentiation was done with D-proline assimilation test¹.

Modified Pal's medium which contained 22.5 g pulverized sunflower seed, 1.0 g KH_2PO_4 , 0.5 g MgSO_4 , 20 g agar and 1000 ml distilled water, was used to know the mating behaviour of the yeast⁹.

The plates of Pal's medium were exposed inside (5) and outside (5) of the animal pen to find out the concentration of organisms in the air. Twenty environmental, four each of soil samples, poultry litter, pigeon droppings, saw dust and rice husk were tested for *C. neoformans*^{14,13}.

Therapy with ketoconazole (15 mg/kg body weight, PO, bid) was instituted but the animal died after six days of the treatment. However, the dog was not available for post-mortem examination.

Results

The dog appeared lethargic, dull and showed fever (39.2°C), anorexia, mucoid cough, dyspnea, nasal discharge, keratoconjunctivitis, enlargement of preapical and submandibular lymph nodes besides granulomatous lesion on the nose. The haematological examination indicated Hb 8.0 g/dl, total leukocyte count 5700/ μl , neutrophils 71%, lymphocytes 26%, monocytes 2% and eosinophil 1%.

The fine-needle aspirate of swollen preapical lymph node revealed numerous circular to oval thick wide capsulated yeast cell with and without budding morphologically indistinguishable from *C. neoformans*. Similar fungal bodies were demonstrated in the PAS stained impression smears of skin biopsy and cutaneous lesions swab (Fig. 1).

Many light to dark pigmented smooth colonies of *C. neoformans* were observed on Pal's medium after 72 hr of incubation at 25°C (Fig. 2). The pathogen was easily isolated from all the clinical specimens on Pal's medium (Table 1).

On the contrary, Sabouraud dextrose agar with chloramphenicol gave positive isolations only from the blood, aspirate and urine. No growth of the organism occurred on nutrient agar and Sabouraud medium with chloramphenicol and cycloheximide. Cultures recovered from all clinical specimens when

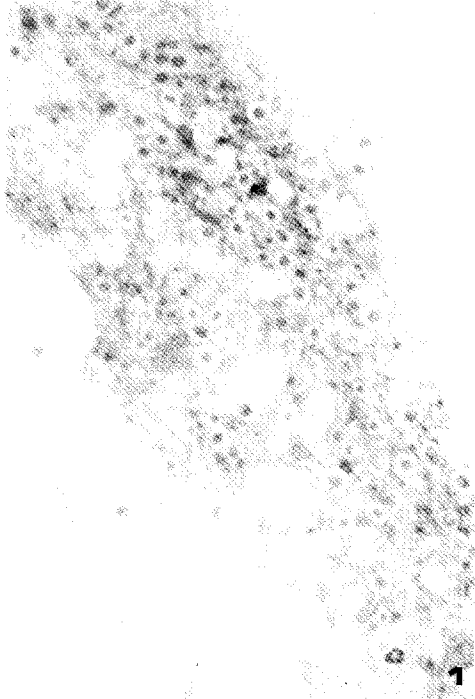


Fig. 1. Microphotograph showing numerous spherical to oval, thick, wide encapsulated yeast cells of *Cryptococcus neoformans* with and without budding in the impression smear of cutaneous granuloma. Periodic-acid Schiff X200.

Table 1. Isolation of *Cryptococcus neoformans* from clinical specimens

Source of clinical specimen	Number examined	Number yielded cultures on:	
		Pal's medium	SDA with chloramphenicol
Blood	1	1	1
Urine	1	1	0
Lymph node aspirate	1	1	1
Skin biopsy	1	1	1
Nasal exudate	1	1	0

examined by 'PHOL' technique showed single round to oval or budding yeast cells with thin capsules. The isolates grow well on plain Sabouraud medium at 37°C, hydrolyzed urea but failed to assimilate potassium nitrate and lactose. D-proline assimilation test was negative. *In vitro* sexual reproduction of the isolates with *Filobasidiella neoformans* mating type

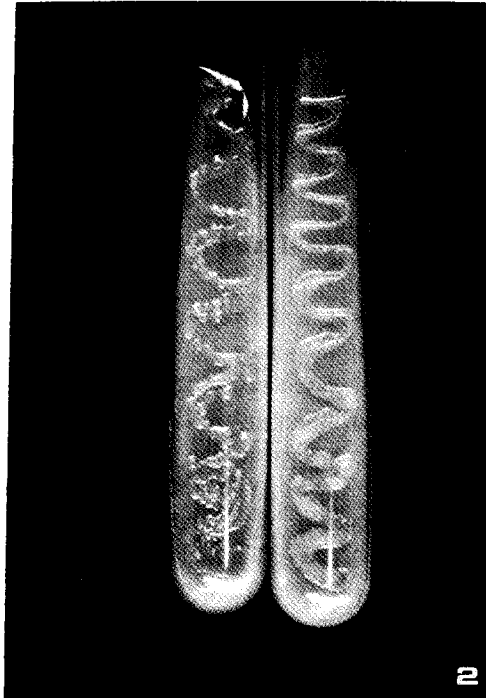


Fig. 2. Light to dark brown coloured, smooth colonies of *Cryptococcus neoformans* on Pal's medium as isolated directly from the blood and lymph node aspirate.

Table 2. Recovery of *Cryptococcus neoformans* from environmental samples

Type of material	Number tested	Number positive for <i>C. neoformans</i> on:	
		Pal's medium	SDA with chloramphenicol
Pigeon dropping	4	3	0
Poultry litter	4	0	0
Rice husk	4	0	0
Saw dust	4	1	0
Soil	4	1	0
Total	20	5	0

'a' on modified Pal's medium showed white mycelial growth over the margin of the culture streak after 10 days of incubation at 20°C. This indicated that all the isolates were of variety *neoformans* (serotype AD) and 'α mating of *F. neoformans*. Aeromycological survey revealed high concentration of the pathogen in the immediate environment of the dog. The number of colonies were more in in-

side (24-36) than outside (13-20) of the dog pen. The organism was recovered on Pal's medium from 5 of the 20 environmental materials screened (Table 2). No isolations could be achieved on Sabouraud medium with chloramphenicol as all the plates were badly contaminated with rapidly growing moulds.

Discussion

Spontaneous cryptococcosis has been recorded in the cat, dog, cattle, pig, horse, goat, sheep, buffalo, monkey, baboon, koala, mink, ferret, cheetah, civet besides man^{4,6,8,10,11,15}. The disease in lower vertebrate animal mostly occurs in sporadic form, however, epidemics have been detected in dairy cattle who suffered from severe mastitis^{10,15,30}. Maximum cases have been diagnosed in the cats and dogs^{7,16,25}. The prognosis is often grave when the animal either develop meningoencephalitis or shows dissemination. In the present case, the dog died due to dissemination of infection. The early diagnosis and chemotherapy may prevent the fatal outcome of this enigmatic mycosis.

It has been mentioned that isolation of *C. neoformans* should always be attempted on the media which do not contain cycloheximide as it inhibits the growth of the yeast^{2,24}. A recent report of Kamboj and co-workers³ from Punjab, India reported the luxuriant and pure growth of *C. neoformans* from clinical specimens of dog on Sabouraud dextrose agar with chloramphenicol and cycloheximide. This indicated that their isolates were resistance to cycloheximide. However, all our clinical and environmental isolates failed to grow in the presence of cycloheximide. The same opinion is expressed by other scientists that *C. neoformans* is highly sensitive to cycloheximide and therefore, it should not be incorporated in the media employed for the isolation of this opportunistic pathogen^{2,24}.

Numerous drugs such as amphotericin B, 5-fluorocytosine, ketoconazole, fluconazole and itraconazole have been used both in human and veterinary practice^{2,4,5,10,19,22}. Despite itraconazole therapy, our patient died six days after the initiation of treatment. It is therefore, advised that a dog with cu-

taneous granuloma and superficial lymphadenopathy should be suspected for cryptococcal infection and therefore, sincere attempts should be made to confirm an early diagnosis to start antifungal therapy which is mandatory to prevent dissemination of disease.

The causative agent of cryptococcosis exists in nature as a saprobe^{2,10,23,25}. It is believed that environment acts as reservoir of infection to man and animals^{10,13}. The epidemiological investigation seems imperative to establish the source of infection^{11,16}. The results of our study revealed the presence of *C. neoformans* in the immediate surrounding of sick dog. These observations indicated that the dog would have acquired the highly infectious yeast cells of *C. neoformans* from the saprobic reservoirs through the respiratory tract.

The development of brown colour to the colonies of *C. neoformans* on Pal's medium is a useful marker in the rapid diagnosis of cryptococcosis¹⁴. The results of this study confirms the observations of earlier workers that Pal's medium is an excellent selective medium for the diagnosis of the disease both in man and animals^{6,7,8,10,16,17}. This will help the physician to start the early chemotherapy of the patients to prevent the serious complications of cryptococcosis. It is therefore, emphasized that routine application of Pal's medium would certainly help the microbiologists in establishing quick diagnosis of this systemic mycosis in immunocompetent as well as immunocompromised hosts.

Conclusion

Fatal disseminated cryptococcosis with involvement of skin, eye and lymph nodes has been diagnosed in a 4-year-old German shepherd dog who was unresponsive to conventional treatment of respiratory distress. Cytological examination revealed numerous oval to spherical, thick encapsulated yeast cells morphologically consistent with *C. neoformans*. The pathogen was easily isolated from the blood, urine, nasal exudate, aspirate from prescapular lymph node, skin biopsy and swab from cutaneous lesion on "Pal's medium" at 25°C.

The microscopic morphology of the cultures in 'PHOL' stain showed the presence of many round to oval yeast cells with and without budding having thin capsules. The dog died six days after starting the ketoconazole therapy. The environment acted as the reservoir of infection as epidemiological investigation yielded cultures of *C. neoformans* from the air, soil, pigeon droppings and saw dust on Pal's medium only. The detailed typing of the isolates of clinical and environmental isolates indicated that they belonged to variety *neoformans* (serotype AD) and "alpha" mating typing of *Filobasidiella neoformans*. The results of this study confirmed that Pal's medium is an excellent differential medium for the early laboratory diagnosis and epidemiological investigation of cryptococcosis. The modified Pal's medium can be successfully used to determine the genetic crossing of the *C. neoformans*. The wider use of Pal's medium would help the microbiologists to diagnose more cases of cryptococcosis in man and a wide variety of animals.

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Reference

1. Dufait R, Vello R, De Vroey C. Rapid identification of the two varieties of *Cryptococcus neoformans* by D-proline assimilation. *Mykosen* 1987;

- 30: 483.
2. Emmons CW, Binford CH, Utz JP, Kwon-Chung KJ. *Medical Mycology*. Philadelphia: Lea and Febiger, 1977, pp 206-229.
 3. Kamboj DS, Sood N, Jand SK, Singh KB, Nauriyal DC. Cryptococcal infection in dog-a case report. *Ind J Vet Med* 1993; 13: 92-94.
 4. Malik R, Dill-Macky E, Martin P, Wigney DI, Muir DB, Love DN. Cryptococcosis in dogs; a retrospective study of 20 consecutive cases. *J Med Vet Mycol* 1996; 33: 291-297.
 5. Noxon JO, Monroe WE, Chinn DR. Ketoconazole therapy in canine and feline cryptococcosis. *J Am Anim Hosp Assoc* 1986; 22:179-183.
 6. Pal M. Studies on the prevalence of cryptococcosis in respiratory disorders of domestic animals. *Revista Iberica De Micol* 1989; 6:29-33.
 7. Pal M. Feline meningitis due to *Cryptococcus neoformans* var. *neoformans* and review of feline cryptococcosis. *Mycoses* 1991; 34: 313-316.
 8. Pal M. Pulmonary mycosis in a pigeon handler due to *Cryptococcus neoformans* var *neoformans*. 2nd Int. Conf. on Cryptococcus and Cryptococcosis. Milano, Italy, Sept. 19-23, 1993; p.119.
 9. Pal M. Natural occurrence of *Cryptococcus neoformans* var *neoformans* in wooden canary cages. *Revista Ibero Am De Micol* 1995; 12: 93-94.
 10. Pal M. Recent advances in cryptococcosis. In "Fungal Infections - An Update" published by Indian Association of Pathologists and Microbiologists Hyderabad, India. 1996; pp.2-11.
 11. Pal M, Dube GD, Mehrotra BS. Pulmonary cryptococcosis in a rhesus monkey (*Macaca mulatta*). *Mykosen* 1984; 27: 309-312.
 12. Pal M, Hasegawa A, Ono K and Lee CW. A staining solution for the morphological studies of fungi and Prototheca. *Jpn J Vet Sci* 1990; 51: 527-531.
 13. Pal M, Matsusaka N. Studies into prevalence of pathogenic fungi in wildlife environment. *Verh ber Erkrgr Zootiere* 1991; 33: 261-264.
 14. Pal M, Mehrotra BS. Studies on the efficacy of sunflower seed agar medium for the isolation and identification of *Cryptococcus neoformans*. *Arogya-J Hlth Sci* 1982; 8: 74-79.
 15. Pal M, Mehrotra BS. Cryptococcal mastitis in dairy animals. *Mykosen* 1983; 26: 615-616.
 16. Pal M, Mehrotra BS. Studies on the occurrence of cryptococcal meningitis in small animals. *Mykosen* 1985; 28: 607-611.
 17. Pal M, Ono K, Goitsuka R, Hasegawa A. Isolation of *Cryptococcus neoformans* var *neoformans* from canine otitis. *Mycoses* 1990; 33: 465-467.

18. Pal M, Yang MP, Hasegawa A. A review of mycoses in AIDS. *Teikyo Med J* 1990; 1: 183-192.
19. Richardson RC, Jaeger LA, Wigle W. Treatment of systemic mycoses in dogs. *J Am Vet Med Assoc* 1983; 183: 335-336.
20. Simons J, Nichols RE, Morse EV. An outbreak of bovine cryptococcosis. *J Am Vet Med Assoc* 1953; 122: 31-35.
21. Smith JMB. *Opportunistic Mycoses of Man and Other Animals*. Wallingford, UK, C.A.B. International 1989; pp.53-80.
22. Stampley AR, Barsanti JA. Disseminated cryptococcosis in a dog. *J Am Anim Hos Assoc* 1988; 24: 17-21.
23. Swine D. *Cryptococcus neoformans* and the epidemiology of cryptococcosis. *Ann Soc Belge Med Trop* 1979; 59: 285-299.
24. Van Cutsem J, Rochette F. *Mycoses in Domestic Animals*. Berse, Belgium. Janssen Research Foundation 1991; pp.100-101.
25. Wolf AM, Troy GC. Deep mycotic disease. In *Textbook of Veterinary Internal Medicine*. edited by S J Ettinger, Philadelphia, WB Saunders Co 1989; pp.341-372.