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Study on isolation of *Cryptococcus neoformans* from human and animals and its correlation with human infection with special reference to HIV/AIDS patients

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The study was done to isolate and identify *Cryptococcus neoformans* from human and animal sources. One hundred forty cerebrospinal (CSF) samples of clinically suspected patients with varying status of exposure to pigeon and/or pigeon excreta and 58 avian excreta and animal samples were studied for isolation of *Cryptococcus neoformans*. Human samples were studied by microscopic examination of India ink preparation and culture isolation was done using SDA. Staib"s bird seed agar & GCP/ GOP media. Culture isolation from the avian samples was done using SDA, Staib's bird seed agar and GCP/GOP media. Confirmations of the isolates were done by urease test. All animal samples were studied by microscopic examination of India ink and KOH preparations of scraping material from the skin.

Key words : *Cryptococcus neoformans,* India ink, AIDS

INTRODUCTION

Cryptococcus neoformans has been found in close association with human infections predominantly in immunocompromised individuals and is often isolated from avian excreta especially with pigeon faeces (Littman 1959). It is a gram positive yeast fungus and the cell varies from 4 to 20 μ in diameter surrounded with a polysaccharide capsule containing (glucuronoxylomannan (Cherniak *et al.*,1980). The genus Filobasidiella (Cryptococcus) has a total of thirty four species and the species which is pathogenic to both man and animals is the *Cryptococcus neoformans* and is divided into three varieties *(C. neoformans* var. *neoformans, C. neoformans var.grubii* and *C. neoformans* var. *gattii)* and five serotypes (A,B.C,D & AD serotypes). Man and animals contract the infection primarily by inhalation of the fungal particles (Fessel, 993). The human infection varies from a localized skin lesion or a primary pulmonary infection or it may infect all organs of the body .In animals it may follow implantation or ingestion (Rippon, 1988). In man Cryptococcosis is often seen in immunocompromised host predominantly and followed by prolonged treatment with corticosteroids, organ transplantation, malignancies and sarcoidoisis (Pappas *et al.*, 2001; Perfect and Casadeval, 2002) The Indian subcontinent has a tropical climate which offers a suitable environment for *Cryptococcus neoformans* and with the emergence of AIDS in the early 1990's, has led to a sharp increase in the number of reported cases of Cryptococcosis in the past two decades (Banerjee *et al.*, 2001).

The present study is aimed to isolate *Cryptococcus neoformans* from avian excreta and animal samples and also from CSF of human and to correlate with human infection with special reference to HIV/AIDS patients. The isolation has been done by direct microscopic examinations of India Ink preparation, culture on suitable media and urease test for the identification of the fungal isolates.

MATERIALS AND METHODS

The present study was undertaken at the Department of Microbiology (Mycology Division), Calcutta School of Tropical Medicine, Kolkata during the period from July 2008 to December 2008

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to isolate *Cryptococcus neoformans* from avian excreta and animal samples and also from CSF of clinically suspected human patients and to correlate it with human infection with special reference to HIV/ AIDS patients.

The study population included 58 pigeons and other species of birds and animal. For human cases the study population included the patients (especially immunocompromised patients) admitted to the hospital with a clinical presentation of Meningitis / of meningeal irritation. Cerebrospinal fluid (CSF) samples from the patients were collected aseptically by lumbar puncture technique and kept in sterile test tubes at room temperature till examined.

Processing of avian excreta

One g of the bird excreta was weighed and suspended in 10 ml of sterile physiological saline solution (0.85%) in a sterile test tube. The test tube containing the test excreta was vigorously shaken on a shaker for 3-5 minutes and the resulting suspension was allowed to stand for 30 minutes. With the help of sterile pipette about 1.25 ml of the supernatant was removed in a sterile test tube and 0.2 ml of Chloramphenicol (2 mg) and 0.5 ml of Penicillin (5 lakh units) added and incubated at 37° C for10 minutes. From this suspension with the help of a sterile loop streaks were made on Petri plates/ tubes of SDA, Staib's medium and GCP medium/ GOP medium.

The incubation for SDA was done at 37°C for two weeks; Staib's medium for four weeks and GCP/ GOP media at 27 °C in B.O.D incubator (ICT. Kolkata) for five days and checked each day for growth, if any.

Procedure of examination of animal skin scraping

In this direct examination was done. The area from where the material was to be collected was scrapped with a scalpel blade and the scraping was placed on a glass slide containing a drop of India ink. A cover glass was applied on the slide without pressure and the slides were examined under microscope.

Processing of human sample

After physical examination of CSF regarding its turbidity, presence of blood or coagulum, it was centrifuged at about 3000 rpm for 10 minutes. After discarding the supernatant, the centrifuged deposit was used for India ink preparation for the direct detection of capsulated budding yeast cell. The centrifuged deposits were inoculated into SDA. Staib"s medium and GCP/GOP media. The incubation for the SDA was done at 37°C for two weeks, Staib*s medium at 25°C for a maximum of two weeks and GCP/GOP at 25"C and examined each day for five days.

The media used for isolation of Cryptococcus neoformans

(i) Sabouraud's Dextrose Agar (SDA) (HI MEDIA),
(ii) Staib's Bird Seed Agar (HI MEDIA),
(iii) Glycine Cycloheximide Phenol Red (GCP), and
(iv) GCP without Cycloheximide (GOP)

Growth on the SDA medium if any was identified at the earliest by performing some biochemical test like urease test.

Growth on the Staib's medium can be identified outright by the development of brown coloured colony by the *Cryptococcus* species.

The variants of the *Cryptococcus* species were identified by the use of GCP/GOP media.

Urease test

This test detects the ability of *Cryptococcus* to convert urea to ammonia by the enzyme urease which is responsible for the colour change. This test was done in Christensen's Urea medium. The test tube containing the sloped medium was inoculated heavily with the growth and incubated at 37°C overnight. Urease positive cultures produce a purple pink colour.

India ink preperation

A drop of centrifuged deposit of CSF was put on a glass slide and mixed with a drop of India ink (HI MEDIA). Then it was covered with a cover slip and examined under the microscope (first using 10X power followed by 40X objective). The cell outline of *C. neoformans* (with or without budding) can be seen with a clear halo of varying width around it.

Serology

The supernatant of the CSF samples were tested for the detection of Cryptococcal antigen by the Latex particle agglutination test (CALAS, Meridian Bioscience, Inc. USA) for confirmation and evaluation of severity as well as prognosis.

RESULTS

Out of 58 avian samples only 23 isolates showed growth at 37°C on SDA and 23 showed growth on Staib's bird seed agar at 27°C. Among these 23 samples, only 14 belonged to pigeon, 1 Kalij pheasant, 1 Burmese fowl, 1 Crow pheasant, 1 parrot, 1 sparrow, 2 cocktail and 2 canaries. All the isolates were urease positive. Out of the 23 avian isolates five(5) showed colour change on GCP medium (yellow-orange to bright pink colour) which was suggestive of Serotype B & C i.e. *C'.neoformans* var *gatti*. But the species status of these isolates could not be verified as the serotype specific antisera were not available during this study. These findings were also seen with another differential medium like GOP medium.

For human CSF samples microscopic examination was done using India ink preparation method and culture was done on SDA, Staib's and GCP/GOP media. Out of the total 16 positive India ink preparations, 14(10%) belonged to male patients and 2(1.4%) to female patients and the majority of positive male patients (9 of 14) belonged to the age group of 21-40 years. Out of the total 28 culture positive samples. 25(17.8%) belonged to male patients and 3(2.1%) belonged to female patients and the majority of the culture positive male patients (15 of 25) belonged to the age group of 21-40 years. There was no patient in the age group of above 60 years in both sexes. All the isolates showed positive urease test and grown at 37°C. None of the isolates showed any colour change on GCP and GOP media (another differential medium) i.e all isolates from human samples belonged to *C.neoformans* var. *neoformans* (Serotype D).

Table 1 : Isolation pattern from Avian samples (n = 58)

	Kolkata				Imphal		
Source	SDA	Staib's	GCP	Source	SDA	Staib's Medium	GCP
Pigeon	12	12	9 No change	Pigeon	2	2	1-colour change
				Kalij pheasant	1	1	No change
				Burmese fowl	1	1	No change
				Crow pheasant	1	1	No change
				Parrot	1	1	No change
				Sparrow	1	1	No change
				Cocktail	2	2	1 - color change
				Canaries	2	2	No change

Table 2 : Age & Sex-wise isolation pattern from Human samples in Kolkata (n=140)

Age Group	India Ink Prep.		SDA	
Kolkata samples	Male	Female	Male	Female
< 20 years	1(0.7%)	1(0.75)	4(2.8%)	1(0.7%)
21-40 years	9(6.4%)	1(0.7%)	15(10.75%)	2(1.4%)
41-60 years	4(2.8%)	Nil	6(4.2%)	Nil
> 60 years	Nil	Nil	Nil	Nil
Grand Total	14(10%)	2(1.4%)	25(17.8%)	3(2.1%)

DISCUSSION

The present study carried out in the Department of Microbiology, Mycology unit of School of Tropical Medicine, included 187 CSF samples collected from

Table 3 : Distribution of antigen titre among human cases in Kolkata (n = 30)

Antigen titre	Male	Female	Total
> 1:2- 1:64	2 (6.67%)	1(3.33%)	3 (10.0%)
>1:64- 1:128	11 (36.67%)	2 (6.67%)	13(43.34%)
> 1:128 - 1:256	6 (20.0%)	3 (10.0%)	9 (30.0%)
>1:256- 1:512	4(13.33%)	0	4(13.33%)
>1:512	1(3.33%)	0	1(3.33%)
GRAND TOTAL	24 (80%)	6 (20%)	30(100%)

Table 4 : Correlation among India ink preparation, culture isolation and antiger detection in cases from Kolkata (n = 30)

	India ink prep.(%)	Culture (%)	Ag detection
Male	14(46.6%)	25 (83.3%)	24 (80%)
Female	2 (6.6%)	3(10%)	6 (20%)
Total	16(53.3%)	28 (93.3%)	30 (100%)

the clinically suspected human patients from Kolkata (140) and Imphal (47), Manipur. The avian excreta consisted of 58 samples collected from different parts of Kolkata (40) and Manipur (18).

Our study showed that out of the 23 isolates from a total of 58 avian samples, 14 belonged to pigeon. 1 parrot, 1 crow pheasant. 1 sparrow and 2 canaries. This finding co-related well with the studies carried out by Emmons (1955). Bauwens *et al* (1986), Hajsid & Curja (1965), Muchmore *et.al.* (1980) and Staib (1963). In addition, isolations could also be done from 1 Khalij pheasant, 1 Burmese fowl and 2 cocktail.

According to Dolan & Woodward (1971), non-pigmented *Cryptococcus* sp. can be identified by their growth at 37°C and positive urease test. In our study also we detected some strains showing no pigmentation. These were then confirmed by the methods of growth at 37 ^{0}C and urease positivity.

When 23 avian culture isolates were identified and differentiated with respect to their growth on GCP/ GOP media, only 5 showed change in colour and was suggestive of the presence of Serotype B & C (C *neoformans* var *gatti)*.This observation was quite similar to the results observed by Salkin *et al.*(1982).

Considering a total of 187 suspected human samples in this study, 146 were found to be male and 41 were female. Hence, there was a predominance of male over female by a ratio of 3.6:1. Similar result was observed in the study done by Sow *et al* (1988). They showed that out of 7 suspected patients, 6 were male and only 1 was female.

Though the total patients belonged to various age groups, maximum numbers belonged to the age group of 21-40 years (70.7%), very few were in the below 21 years group (-10%) and no patient was found in the group above 60 years. Our study showed similar results as the studies done by Mohr *et al.* (1972), Chen *et al.* (1980). Goldman *et al* (2001) and Jeeten *et al* (2001). From these findings it may be concluded that subjects were mostly found in the sexually active group.

Analysis of 140 human patients based on HIV serology showed that 30 were positive for *Cryptococcus* sp. by laboratory investigations and microscopy. Similar findings were documented in the studies by Perfect and Casadevall (1998).

Our study also showed that in 43.34 % of positive cases, the antigen titre was in the range of >1:64 to 1:128. This titre has good co-relation with the severity of infection and prognosis of the patient as was observed by Sow *et al.* (1988). Roux *et. al.* (1986) and Kaufman (1984). We also found that patients belonged to mild to moderate infection groups and none showed antigen titre more than 1:512. Finally, all the patients recovered well after specific treatment, as was found from follow up study done by the department.

Findings related to India ink test, cultural isolation and antigen detection methods for diagnosing Cryptococcal infection clearly revealed that antigen detection even in serum samples is very much useful for early detection of infection among immunocompromised patients.

Thus it can be concluded that, in our study some important aspects came out which could have relevance in the proper understanding of the pathophysiology and outcome of Cryptococcal infection, especially in the immunocompromised individuals like HIV infected patients.

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