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First report on the isolation of *Cryptococcus neoformans* from the dust of air conditioners in Bharuch, Gujarat, India

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Abstract
Cryptococcus neoformans is an important basidiomycetous fungus that produces high morbidity and mortality in humans and animals throughout the world including India. The present investigation was conducted to isolate and identify *Cryptococcus neoformans* from the dust of 25 air conditioners and also to assess the efficacy of Pal sunflower seed agar as a differential medium. A total 25 samples of dust collected from the same number of air conditioners were inoculated onto Sabouraud dextrose agar and Pal sunflower seed medium. *Cryptococcus neoformans* was isolated on Pal sunflower seed agar only from one sample of air conditioner dust that was admixed with pigeon excreta. The fungus produced light to dark brown colored colonies on Pal sunflower seed agar. However, the yeast could not be recovered onto Sabouraud dextrose medium from the dust samples of air conditioners. The microscopic morphology of brown pigmented colonies in PHOL stain and Narayan stain showed many circular, single or budding yeast cells with thin capsules. The high sensitivity and specificity of Pal sunflower seed agar makes it an excellent medium for the study of *Cr. neoformans*. Since *Cr. neoforams* is a highly infectious pathogenic fungus, the immunocompromised people are advised to use the face while cleaning the dust of the air conditioners in home.
To the author's knowledge, this seems to be first report of isolation of *Cr. neoformans* on Pal sunflower seed medium from air conditioner's dust from India and probably the second record from the world.

Keywords: Air conditioner dust, Basidiomycete yeast, *Cryptococcus neoformans*, Pal sunflower seed medium, Pathogen, Public health

Introduction
Cryptococcus neoformans, the prime cause of cryptococcosis, is an important capsulated pathogenic yeast that produces serious disease in humans as well as in a wide variety of animals globally (Pal, 2007a; Pal *et al*., 2014; Dave and Pal, 2015) [17, 25, 4]. The global burden of *Cr. neoformans* is estimated to affect around one million people with 700,000 deaths each year (Park *et al*., 2009) [26]. The infection due to *Cr. neoformans* is more commonly observed in the people who are suffering from AIDS/HIV, sarcoidosis, diabetes, and idiopathic CD4 or undergone organ transplantation or having prolonged treatment with corticosteroids (Kwon-Chung and Bennett, 1992; Casadevall and Perfect, 1998; Perfect and Casadevall, 2002; Pal,2007a; Chander,2009) [6, 17, 27, 2].
The fungus *Cr.neoformans* possesses several virulence factors including the melanin synthesis, capsule production and growth at 37 ^{0}C (Nosanchuk and Casadevall 2006; Chander,2009) [9, 2]. It is reported that melanin is present in the innermost layer of the cell wall, near the plasma membrane of this enigmatic pathogen (Nosanchuk and Casadevall 2003) [6].
Cryptococcus neoformans is an encapsulated, basidiomycete fungus pathogen that produces the enzyme phenoloxidase, which is necessary in melanin synthesis. Melanin production by *Cr.neoformans* on Pal sunflower seed agar is an important phenotypic character that helps in an early identification of this zoopathogenic basidiomycetes yeast from clinical as well as environmental sources. Furthermore, it has been studied that both species of *Cryptococcus* i.e. *neoformans* and *gattii* has produced melanin on Pal sunflower seed medium as evidenced by dark brown pigment (Pal, 1986) [11].
Sanfelice (1894) [29] is credited to describe the yeast for the first time from the peach juice. However, the ecological association of *C. neoformans* was reported by Emmons (1955) [5] who first time isolated this fungus from the pigeon excreta. Later, *Cr. neoformas* was recovered from the pigeon droppings and other avian excreta by many workers of the world

including India (Pal,1978; Staib and Schulz-Dieterich,1984; Pal and Baxter, 1985; Pal *et al*., 1990; Yildiram *et al*.,1998; Sasaki *et al*.,1999; Cermeno *et al*., 2006; Pal, 2015; Zainu *et al*., 2019) [10, 24, 22, 35, 30, 19, 36]. Natural infection due to C*r.neoformans* has been described in humans as well as in animals from various parts of the word (Wilson *et al*.,1970; Pal and Mehrotra, 1983; Pal and Mehrotra 1985; Dave and Pal, 2015) [4, 23, 24].

The source of infection is exogenous, and the respiratory tract is considered as chief portal of entry for *Cr. neoformans* (Pal *et al*., 2014) [25]. There are evidences to believe that humans and animals may acquire *Cr. neoformas* infections from saprobic reservoirs where the fungus grows luxuriously (Pal,2007a) [17]. Rarely, the infection can occur following the traumatic injury to the skin by the fungal contaminated object (Pal 2007b; Dave and Pal, 2015) [18, 4]. It is important to mention that *Cr. neoformans* can remain viable for about 20 years in the dry and old pigeon droppings in dark, and humid site, which is not directly exposed to sunlight (Pal,2007b) [18]. This is considered the longest survival time of this pathogenic yeast in environment. Cryptococcal infections have been reported in pet bird enthusiast and pigeon handler from Germany (Wagner and Satib, 1983) [34] and India (Pal,1993) [13], respectively. The pathogen can produce the infection in immunocompetent and immunocompromised subjects (Pal, 2007a; Dave and Pal,2015) [17, 4]. It is emphasized that an early diagnosis and immediate therapy is necessary to prevent the serious complications of disease (Pal *et al*.,2014) [25].

The literature scan revealed only one report from USA where Littman (1959) [7] the patient developed primary cryptococcal meningitis following exposure to a library air conditioner laden with pigeon droppings that contained high concentration of *Cr. neoformans* (Littman,1959) [7]. The great paucity of information on the prevalence of *Cr, neoformans* in the dust of air conditioners prompted the author to investigate the occurrence of this medically important pathogen in the air conditioner's dust by employing Pal sunflower seed medium as a selective medium for its isolation.

Materials and Methods

Sunflowers are grown in many countries of the world including India. Most sunflowers have rough-hairy oval to heart shaped leaves, large flowerheads with yellow ray florets (Fig-1).

Fig 1: Sunflower with bright yellow colour grown in Gujarat, India

Some varieties of sunflowers are grown for food, oil, and seeds. It is interesting to know that a sunflower head can produce up to 1,000 seeds. The dry seeds of sunflower (*Helianthus annus*) purchased from the local market were utilized to prepare the medium for this study (Fig.2).

Fig 2: Dry seeds of sunflower seed used to prepare Pal sunflower seed medium for the isolation of *Cryptococcus neoformans*

A total of 25 samples of dust collected with the help of sterilized moist cotton swabs from window air conditioners in a pose colony in Bharuch, Gujarat, India constituted the material for this investigation. Three swabs were taken from each air conditioner, and after collection, they were dipped in sterilized disposable tubes, which contained around 5 ml of sunflower seed broth. The composition of Pal sunflower seed is 45 g of sunflower seed, 20 g agar power, 50 mg chloramphenicol and 1000 ml distilled water (Pal,1997a) [14]. In order to prepare the medium, 45 g of sunflower seeds were pulverized in an electric blender and then boiled in 500 ml of distilled water for 20 min. The seed extract obtained was left at room temperature to cool and filtered through five layers of gauze and then distilled water was added to the sunflower seed extract to make the volume to one litre. Then, 20 g agar powder was added to the extract. After adjusting the pH to 5.6 using hydrochloric acid and NaOH, it was autoclaved at 110°C for 20 min. The antibiotic chloramphenicol was added after the autoclaving. The medium was cooled, and later dispensed in sterile glass Petri dishes and in glass tubes. The prepared media was checked for its sterility by keeping one Petri dish at 25 °C and another at 37 °C and later stored at a refrigerator temperature of 5 °C (Pal,2007a) [17].

All the 25 samples of dust were inoculated onto the plates of Pal sunflower seed medium and Sabouraud dextrose agar with chloramphenicol (Pal,2007a) [17] and incubated at 30°C.The inoculated media were daily observed for the fungal growth. The colonies showing light to dark brown colour on Pal sunflower seed medium were subjected to PHOL (Pal *et al*.,1990) [22] and Narayan stain (Pal, 2004) [16] wet mounts for morphological studies. Yeast cells showing characteristics capsule in wet mount preparation gives presumptive evidence of *Cryptococcus* species (Pal, 2007a) [17]. The pure growth of the yeast was inoculated onto Sabouraud medium and kept at 37 °C to see its temperature effect. The biochemical test that includes urease activity was observed by inoculating the brown pigmented colony on

urea agar. The known culture of *Cr. neoformans* was inoculated along with several other fungal starins on Pal sunflower seed medium just to confirm the brown colour effect of *Cr. neoformas* on Pal sunflower seed agar at 25 °C.

Results

Out of 25 dust samples examined, only one specimen yielded several brown, mucoid pigmented colonies on Pal sunflower seed medium. The brown coloured growth on Pal sunflower seed agar was considered as a presumptive evidence of *Cryptococcus* spp. The known culture of *Cr. neoformans* produced brown pigment in 24 hours on Pal sunflower seed agar thus confirming its sensitivity. The brown colour effect was observed only in *Cr. neoformans* colony at the centre whereas the growth of other known fungal cultures inoculated at the periphery failed to develop brown pigment indicating the specificity of Pal sunflower seed medium for *Cr. neoformans* (Fig.3).

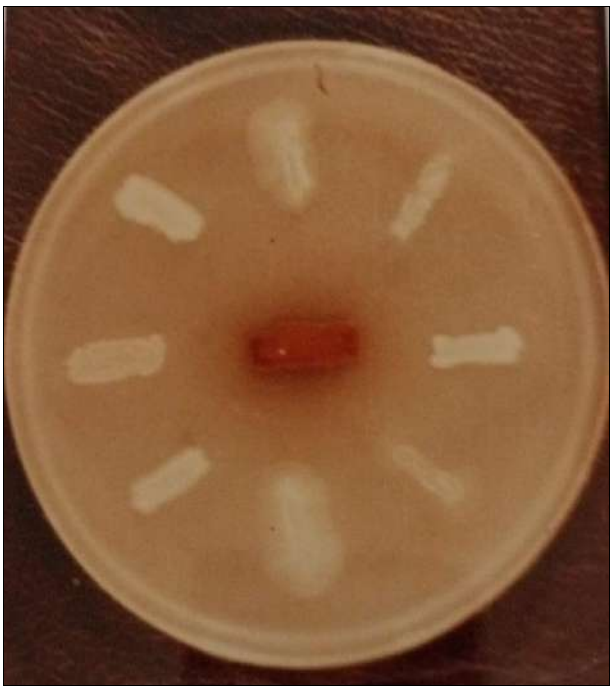

Fig 3: *Cryptoccus neoformans* grown in the centre of Pal sunflower seed medium showed brown coloured growth where as several other fungal cultures inoculated at the periphery of medium failed to reveal brown colour effect.

There was no isolation of *Cr.neoformans* on Sabouraud medium as the plates were overgrown with fast growing moulds. The positive specimen of dust was found admixed with pigeon droppings, and originated from the window air conditioner of a house that was protected from direct sunlight. The dust collected from the remaining window air conditioners were not mixed with any other bird excreta, and also were directly exposed to the sunrays.

The growth from the brown coloured colonies on Pal sunflower seed when examined in PHOL stain and Narayan stain wet mount revealed many circular shaped, thinly encapsulated yeast cells with and without budding morphologically simulating to *Cryptococcus* species

Luxuriant growth of yeast on Sabouraud medium was noticed at 37 °C. The culture straked on urease slant showed characteristic pinkish colour changes indicating that the isolate of yeast was urease positive.

Based on the melanin production on Pal sunflower seed medium, luxuriant growth at 37 °C, capsule demonstration in wet mounts and urease positivity clearly indicated that the isolate recovered from a sample of dust admixed with pigeon droppings obtained from the sunlight protected window air conditioner was *Cr.neoformans.*

Discussion

Cryptococcus neoformans is a basidiomycete yeast that produces life threatening infections in humans as well as animals and is reported from developing and developed nations of the world (Pal,2007a; Pal *et al*.,2014) [17, 25]. In the present investigation, *Cr.neoformans* was isolated on Pal sunflower seed medium from a solitary sample of dust admixed with avian droppings that was obtained from a window air conditioner that was protected from direct sunrays. The direct exposure of pigeon excreta to sunlight is detrimental to the survival of this yeast (Pal, 2007a) [17]. However, the remaining 24 samples of air conditioner dust failed to reveal the presence of *Cr. neoformans*. There was no isolation of *Cr. neoformans* on Sabouraud agar. This finding clearly indicated the superiority of Pal sunflower seed agar as a differential medium and hence, it should be widely employed as a good routine conventional culture medium the for the detection of *Cr. neoformans* from clinical as well as environmental materials of humans and animals.

The public health implications of environmental exposure to saprobic reservoirs are well documented in the literature (Muchmore *et al*., 1963; Pal,1986; Pal, 2007a; Pal *et al*.,2014; Dave and Pal,2015) [17, 11, 25, 4]. In this context, Littman (1959) [7] was the first to describe cryptococcosis in a man directly attributed to the infection from the pigeon excreta. The patient who was a physician who developed primary cryptococcal meningitis following exposure to a library air conditioner laden with pigeon droppings containing heavy concentration of this pathogenic yeast. Likewise, Procknow and co-investigators (1965) reported cryptococcal infection in a patient who was exposed to *Cr. neoformans* contaminated avian excreta. In one study, Walter and Atchinson (1966) [32] reported that pigeon keepers who are more frequently exposed to *Cr. neoformans*, showed a significantly higher incidence of serum antibodies to this zoopathogenic fungus than a control group that had no contact with pigeon. Fatal cryptococcosis of central nervous system in a bird enthusiast was recorded by Wegner and Staib (1983) [34] from Germany. The patient had a pet budgerigar (*Melopsittacus undulatus*) and examination of this bird droppings revealed high concentration of *Cr. neoformans.* It is suggested that epidemiological investigation should be conducted in all cases of cryptococcal infections to establish the source of infection. It will help to decontaminate the avian excreta. In this context, Walter and Coffee (1968) [33] mentioned that *Cryptococcus neoformans* can be controlled by alkalinization in the pigeon coops.

In the present study, the isolation of *Cr.neoformas* from avian excreta on Pal sunflower seed medium goes parallel with the observations of earlier investigators who successfully reported the isolation of this pathogenic basidiomycete yeast on sunflower seed medium yeast from the avian droppings examined from different countries of the world, such as New Zealand (Pal and Baxter,1985) [24], Belgium (Pal, 1986) [11], Nepal (Pal,1997b) [15], Colombia (Caicedo *et al*., 1999), Japan (Sasaki *et al*.,1999) [30],

Djibouti (Pal,2015) [19], India (Pal Pal,2017) [20], and Nigeria (Zainu *et al*.,2019) [36]. It is, therefore, advised that selective medium like Pal sunflower seed medium that is cheap, easily available and simple to prepare should be routinely used for the epidemiological investigation and laboratory diagnosis of cryptococcal infections in both in humans and animals.

As far as it could ascertained, the current study describes the first report on the isolation of *Cr. neoformans* from the dust of an air conditioner on Pal sunflower seed medium from India and probably the second report from world.

Conclusion

Cryptococcus neoformans is highly pathogenic fungus that is widely prevalent in the environment. The isolation of this basidiomycete yeast from environmental materials on conventional medium like Sabouraud agar is often difficult. This is evident in the present study that *Cr. neoformans* could not recovered on Sabouraud agar. The pathogen was easily isolated only on Pal sunflower seed medium. The use of selective medium like Pal sunflower seed medium is highly imperative for the epidemiological investigation. The development of brown colour pigmented colonies on Pal sunflower seed medium helps in rapid isolation and presumptive identification of *Cr. neoformans*. Therefore, melanin production by *Cr. neoformans* on Pal sunflower seed agar makes it as an excellent chromogenic medium for routine application in the microbiology and public health laboratories, particularly in low income nations where commercially available media due to high cost are not easily available for the laboratory diagnosis of cryptococcosis, a life-threatening mycosis of global importance.

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Conflict of Interest

Not available

Financial Support

Not available

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