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Isolation and identification of *Cryptococcus neoformans* from avian droppings on Pal sunflower seed medium

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Abstract
Cryptococcus neoformans is a basidiomycetous yeast of medical and veterinary importance. The objective of this study was to assess the efficacy of Pal sunflower seed agar (Pal medium) for the rapid isolation and presumptive identification of *Cr. neoformans* from the droppings of birds, mainly the pigeons, and parrots collected from different areas in India. A total of 34 old and dried bird's faecal samples (17 from pigeons and 17 from parrots) were examined mycologically by employing dilution technique. All the 34 samples of bird's droppings were inoculated onto Sabouraud dextrose agar and Pal sunflower seed medium. *Cryptococcus neoformans* was isolated from 16 avian samples, which comprised of 11 from pigeons and 5 from parrots. However, there was no isolation of *Blastomyces dermatitidis*, and *Histoplasma capsulatum* from the bird's excreta. The microscopic morphology of all the 16 isolates of *Cryptococcus neoformans* when examined in "Narayan" stain revealed circular to oval, single or budding yeast cells with thin capsules. Interestingly, all the 16 isolations of *Cr. neoformans* were easily made only on Pal sunflower seed agar by observing light to dark brown pigmented colonies. There was no isolation on Sabouraud medium as all the inoculated plates were heavily contaminated by fast growing moulds. The high rate of isolations of *Cr. neoformans* on Pal sunflower seed agar clearly revealed the advantage of this medium for the selective isolation of *Cr. neoformans* from bird's droppings. As *Cr. neoformans* is a highly pathogenic fungus, the people with weak immune system are advised not to visit any such buildings where the pigeons roost, and also not to clean the pigeon/parrot droppings without wearing the face mask.

Keywords: *Cryptococcus neoformans*, Narayan stain, pal sunflower seed medium, parrot droppings, pigeon excreta, public health imlications, yeast

Introduction
Cryptococcus neoformans, the prime cause of cryptococcosis, is an important pathogenic yeast that produces serious disease in humans as well as in a wide variety of animals globally (Pal, 2007a; Pal *et al*., 2014) [20, 22]. It is reported that one million cases of cryptococcal meningitis occur every year in HIV/AIDS patients resulting in around 625,000 deaths worldwide (Pal, 2014) [22]. Although the yeast was isolated for the first time from the peach juice by Sanfelice in 1894 [32] (Sanfelice, 1894) [32], the ecological association of *C. neoformans* was not known until 1955, when Dr. C.W. Emmons, an American Scientist first isolated this fungus from the pigeon (*Columbia livia*) excreta (Emmons,1955) [6]. Later, this finding was authenticated by several investigators from various countries of the world including India (Pal and Baxter, 1985; Pal, 1986; Pal *et al*., 1990; Pal, 1997a; Sasaki *et al*., 1999; Cermeno *et al*., 2006; Pal, 2015) [26, 8, 30, 14, 33, 3, 23]. In addition to pigeon droppings, *Cr. neoformans* has been recovered from other natural sources, such as the soil, bat guano, parrot excreta, empty wooden canary cages, wooden nest boxes, perches, fruits, vegetables, and other avian droppings like budgerigar, lorikeet, and munia birds (Ajello,1958; Pal,1978; Pal, 1986; Pal, 1989; Pal *et al*., 1989; Pal *et al*., 1990; Pal, 1995; Pal, 2005a; Pal, 2007a; Pal, 2017) [1, 7, 8, 9, 30, 12, 17, 22, 24] However, the droppings of pigeon serve as the chief saprobic reservoir of *C. neoformans.* It is pertinent to mention that *Cr. neoformans* can remain viable in the dry and old pigeon droppings for about 20 years (Pal, 2007b) [40]. However, direct exposure of pigeon excreta to sunlight is detrimental to the survival of this yeast (Pal, 2007a) [20]. Experimental studies has proved that all the environmental strains of *Cr. neoformans* when inoculated intracerebrally into laboratory mice were found pathogenic as indicated by the death of mice (Pal, 2005b) [39]. The source of infection is exogenous, and the respiratory tract is recognized as main portal of entry for Cr. neoformans (Pal and Dave, 2006; Pal *et al*., 2014) [19, 22].

There are evidences to believe that humans as well as animals may acquire the infection from saprobic reservoirs where the fungus grows luxuriously. In this context, Dave and Pal (2015) [42] reported primary cutaneous mycosis in an immunocompetent parrot keeper due to *Cr. neoformans*. Cases of cryptococcosis are described in pet bird enthusiast and pigeon handler are reported from Germany (Wagner and Satib, 1983) [41] and India (Pal, 1993) [11]. The yeast has the potential to infect many organs of the body, such as lungs, brain, skin, bone, eye, adrenal gland, lymph node and others; and can produce the infection in immunocompetent and immunocompromised subjects (Pal and Dave, 2006) [19]. Mycological, immunological and molecular techniques are employed to make an unequivocal diagnosis of disease (Pal, 2007b) [21]. A number of antifungal drugs, such as amphotericin B, fluctocine, fluconazole, itraconazole, and liposomal AMB are available to treat the cases of cryptococcal infection (Pal and Dave, 2006; Dave and Pal, 2015a) [19, 4]. It is advised that an early diagnosis and prompt treatment is imperative to prevent the fatal consequences of cryptococcosis (Pal *et al*., 2014) [19].

The natural infection has been described in humans, and also in many species of animals, such as cattle, cat, dog, goat, horse, buffalo, monkey, sheep, baboon, mink, ferret, bear, cheetah, rat, guinea pig, koala, gazelle, wallaby, parrot, cockatoo, palm civet, shrew (Pal and Mehrota, 1983; Pal, 1986; Pal, 1991 Pal, 2007b; Pal *et al*., 2014) [25, 8, 10, 21, 22]. Pal is credited to elucidate the etiologic significance of *Cr, neoformans* with mastitis in goat and buffalo in 1975 and 1980, respectively (Pal, 1996) [13]. In India, the first report of cryptococcal infection in cat, monkey and sheep was published by Pal and co-workers (Pal and Mehrotra, 1983b; Pal *et al*., 1984; Pal and Mehrotra, 1985; Pal, 1989; Pal, 1991b) [25, 27, 26, 9, 10].

The paucity of information on the environmental prevalence of *Cr,neoformans* in three different regions of India *viz*: Gujarat, Uttar Pradesh and Haryana prompted the author to undertake this study to investigate the natural occurrence of this medically important yeast in the birds droppings by employing Pal sunflower seed medium as a selective medium for its isolation.

Materials and Methods

A total of 34 samples of old and dried pigeon (17) and parrot (17) droppings were collected with the help of wooden spatulas and kept in a clean polythene bag for mycological investigation of *Cr. neoformans*. All the avian excreta were taken from the dark and humid sheltered sites that were protected from direct sunrays. Approximately, 5 grams of dry and old bird's faeces were obtained from different places namely Bharuch (Gujarat), Ukhlina (Uttar Pradesh), and Gurugram (Haryana). All the 16 samples of parrot excreta were originated from Gujarat whereas the pigeon droppings were taken from three States of India that included Gujarat (8), (Uttar Pradesh (4), and Haryana (16). During the collection of the samples, a facemask was applied to prevent the environmental exposure of *C. neoformans*. One gram of bird excreta was suspended in sterilized glass bottle that contained 9 ml of sterile physiological saline (0.85% NaCl) supplemented with chloramphenicol (10 mg/ml). This mixture was left at room temperature for 15 min, then shaken manually for 5 min, and later kept in incubator at 37 ℃ for 30 minutes (Pal, 2015). An aliquot of 0.1 ml of supernatant from the suspension was inoculated onto duplicate plates of Sabouraud dextrose agar with chloramphenicol (0.05 mg/ml) (Pal, 2007a) [2] and Pal's sunflower seed medium that contained pulverized sunflower seed 4.5 g, agar 2.0 g, chloramphenicol 50 mg, and distilled water 100 ml. (Pal, 1997b) [15]. The inoculated plates were incubated at 30^0C, and examined daily for the growth of yeast, and the number of colonies showing brown pigment were counted. The single colony was sub-cultured on APRM (Anubha, Pratibha, Raj and Mahendra) medium (Dave and Pal, 2015b) [5] for further confirmation. The new APRM medium contained 4.0 g of marigold dried flower, 2.0 g agar, 50 mg chloramphenicol and 100 ml distilled water. All the yeast isolates that grew well at 37 ℃ with capsule, urease positive, and negative for potassium nitrate and lactose were confirmed as *C. neoformans*. The detailed microscopic morphology of the yeast was done in Narayan stain, which contained 4 ml of glycerin, 0.5 ml of 3% aqueous solution of methylene blue and 6 ml of dimethyl sulfoxide (Pal, 2004) [40].

Results

In the present study, *C. neoformans* was isolated from 16 of 34 samples of the bird's droppings giving a prevalence of 48.0%. Out of 16 positive sample, 9 originated from pigeon droppings (5 from Bharuch, 2 from Ukhlina, and 2 from Gurugram), and 7 from parrot excreta (Bharuch). All the 16 positive samples of bird's droppings were dry, and old, and originated from the sites, which were not exposed to direct sunlight. All the isolations were achieved only on Pal sunflower seed medium by observing light brown to dark brown pigmented colonies of *C. neoformans*. In contrast, *C. neoformans* was not recovered from any of the 34 avian droppings on Sabouraud medium, as all of the inoculated plates were badly contaminated with fast growing filamentous fungi masking the growth of the yeast. The number of colonies, which grew from bird droppings on Pal's sunflower seed medium varied from 9 to 25. All the isolates grew well on Sabouraud medium at 37 ℃, hydrolyzed urea, but failed to utilize lactose, and potassium nitrate. The sub-cultures of yeast isolates from APRM medium, when examined for microscopic morphology in Narayan stain under revealed numerous spherical to few oval shaped thinly encapsulated yeast cells, with and without budding. All the 16 isolates were identified as *Cr. neoformans* (Pal, 2007a) [20].

Discussion

A number of cultural media, such as nutrient agar, blood agar, brain heart infusion agar, Mac-Conkey medium, eosin methylene blue agar, brilliant agar, Sabouraud agar, Pal sunflower seed medium, Lowenstein Jensen medium, and others are used in the public health and microbiology laboratories for the cultivation of bacteria and fungi worldwide (Pal, 2007b) [21]. Selective medium plays a pivotal role in the isolation and identification of microorganisms from a variety of clinical samples and thereby, helps in establishing an early diagnosis of the disease that assists the physicians to institute specific treatment and thus save the life of the patient (Pal, 2007a) [2]. Sabouraud medium is commonly employed in the diagnostic microbiology laboratories for the isolation of fungi from the variety of clinical specimens, such as sputum, bronchioalveolar lavage (BAL) cerebrospinal fluid

(CSF) sputum, ear wax, corneal scrapings, nail clippings, skin biopsies, and others (Pal, 2007a) [2]. The growth of *Cr. neoformans* on Sabouraud agar simulate to *Candida*, *Trichosporon*, and other yeasts; and therefore, creates problem in its differentiation. Sunflower seed medium was first time developed by Pal in 1980 as a selective medium for the raid isolation and presumptive identification of *Cr. neoformans* (Pal, 1997) [14]. The development of brown color colonies of *Cr. neoformans* on Pal sunflower seed medium helps in early recognition of this pathogenic yeast (Pal, 1997; Pal, 2007a) [14, 2]. The isolation of *Cr. neoformans* from avian excreta on Pal sunflower seed medium in the current study corroborates with the findings of previous researchers from other nations who also employed Pal sunflower seed medium for the recovery of this yeast from the excreta of birds (Sasaki *et al*., 1999; Cermeno *et al*., 2006) [33, 3].

The results of this study unequivocally established the high specificity and sensitivity of Pal sunflower seed agar as an excellent selective medium for the early recognition of *Cr. neoformans* from environmental materials. The usefulness of Pal sunflower seed medium for the study of *Cr, neoformans* has been reported by several investigators (Pal and Baxter, 1985; Baro *et al*., 1998; Sasaki *et al*., 1999; Cermeno *et al*., 2006; Dave and Pal, 2015a) [26, 2, 33, 6, 4]. The routine application of Pal sunflower seed medium in the clinical microbiology, and public health laboratories would certainly help to diagnose more cases of cryptococcosis both in humans as well as animals.

As far as it is ascertained, the present study records the first isolation of *Cr. neoformans* from the faecal matter of birds from three different areas, namely Bharuch, Ukhlina, and Gurugram of India. It is hoped that further research by using Pal sunflower seed medium may establish the new ecologic niche for *Cr. neoformans*, an emerging highly infectious pathogen of global distribution.

Conclusion

Cryptococcus neoformans is a known zoo pathogenic organism that produce life threatening disease in humans and animals. The excreta of *Columbia livia* serves as an important environmental niche for *Cryptococcus neoformans*. All the 16 isolations of *Cr. neoformans* were obtained on Pal sunflower seed medium. There was no isolation of *Cr. neoformans* on Sabouraud dextrose agar. This indicated the overwhelming superiority of Pal sunflower seed medium to Sabouraud dextrose agar. The development of brown colored colonies on Pal sunflower seed medium helped in an early recognition of *Cr. neoformans*. As sunflower seed medium is simple to prepare and less expensive than Sabouraud agar for the rapid isolation and presumptive identification of *Cr. neoformans* from environmental and clinical materials, thereby, it can be widely employed in the clinical microbiology and public health laboratories for the study of *Cr. neoformans* and also other yeasts like *Candida*, *Rhodotorula*, and *Trichosporon* particularly in the poor resource nations of the world where facilities for immunological and molecular techniques are not easily available.

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