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A rare case of canine Neurocryptococcosis in a German Shepherd dog

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Abstract

An eleven months old male German shepherd dog was brought with an anamnesis of fever and anorexia. A preliminary diagnosis of haemoprotozoal disease was made and corresponding treatment protocol was initiated. The condition of the dog deteriorated and neurological deficits were observed. The cerebrospinal fluid (CSF) was collected and subjected to Giemsa, Lactophenol cotton blue and Albert's staining. Cytological analysis revealed the presence of round to oval capsulated yeast like organisms of around 4-6 microns in diameter consistent with the morphology of cryptococcal yeasts. The CSF was cultured in Sabouraud dextrose agar and the organisms were isolated and stained. Cryptococcosis was confirmed by PCR performed targeting the CAP59 gene. To our knowledge, the present work is the first report of CSF based diagnosis of canine neurocryptococcosis from India and it emphasizes the importance of routine cerebrospinal fluid evaluation under clinical setup in dogs with neurological deficits.

Keywords: Fungus, *Cryptococcus neoformans*, neurocryptococcosis, encephalitis, meningitis, mycoses

1. Introduction

Cryptococcosis is an opportunistic mycotic infection reported throughout the world affecting several species including canines, felines as well as humans. Around thirty-seven species have been recognised under the genus *Cryptococcus*. Among them, *C. neoformans* and *C. gattii* have been frequently found to infect dogs and cats ^[1, 2]. *Cryptococcus* is a facultative intracellular pathogen which is transmitted through inhalation or ingestion of the spores and through contaminated wounds. It is an encapsulated yeast and causes characteristic granulomas, i.e., aggregates of histiocytes containing encapsulated yeasts surrounded by fibrous tissue and mild inflammatory changes in multiple organs which can lead to systemic failure. In canines, it causes lesions primarily in the brain, eyes and lungs and usually causes disease in immune-competent animals ^[1, 2]. It is present frequently in soil, poultry manure and pigeon droppings. The route of transmission is believed to be through the ingestion of desiccated yeasts or inhalation of the basidiospores ^[3, 4]. It has been frequently reported in the western U.S., Canada and Australia and has been rarely reported from India. Here, we present a rare case of canine neurocryptococcosis in a German shepherd dog diagnosed through cerebrospinal fluid examination from Tamil Nadu, India.

2. Clinical History

An eleven-month-old male German shepherd dog weighing 23 kilograms was brought to the Veterinary Clinical Complex, Veterinary College & Research Institute, Orathanadu with a history of fever and anorexia since a week. General clinical examination was done including evaluation of capillary refill time, heart rate and rectal temperature. Neurological and radiological assessment were done. Cerebrospinal fluid was collected and cytology was performed using Giemsa stain, Lactophenol cotton blue stain and Albert's stain.

3. Material and Methods

3.1. Microbial culture

An aliquot from the cerebrospinal fluid collected was cultured on Sabouraud Dextrose Agar (SDA) containing 40 g/L dextrose and 10 g/L peptone, adjusted to pH 5.6 ± 0.2 at 25 °C. To inhibit bacterial growth, the medium was supplemented with gentamicin (0.1 g/L) and chloramphenicol (0.05 g/L) (Sigma-Aldrich, USA). The antibiotics were filter-sterilized (0.22 µm) and aseptically added to the autoclaved and cooled (45–50°C) medium before pouring into the plates. Samples were streaked onto the surface of the SDA plates under aseptic conditions and the inoculated plates were allowed to dry at room temperature for approximately 15 minutes before incubation. The plates were incubated aerobically at 25 ± 2 °C for 10 days with daily observation for visible yeast colony growth. Following incubation, plates were examined for colony morphology, pigmentation and texture under adequate lighting. Representative colonies were sub-cultured onto fresh SDA plates without antibiotics to obtain pure isolates for further identification [13].

3.2. Molecular Identification of *Cryptococcus* by Gradient PCR Targeting the CAP59 Gene

Genomic DNA was extracted from purified yeast cultures grown on Sabouraud Dextrose agar and a loopful of yeast biomass was suspended in 200 µL of sterile distilled water and subjected to boiling at 95 °C for 10 min, followed by centrifugation at $12,000 \times g$ for 5 min. The supernatant containing crude DNA was used as the template for PCR amplification [5]. The CAP59 gene, a species-specific capsule-associated gene of *Cryptococcus neoformans*, was selected as the molecular marker for identification. Amplification was performed using specific primers targeting a 229 base pair fragment of the CAP59 gene [6]. PCR reactions were performed in a total volume of 25 µL, containing 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.0 mM MgCl₂, 200 µM each dNTP, 0.4 µM of each primer, 1 U of Taq DNA polymerase (Thermo Fisher Scientific, USA) and 2 µL of template DNA. Thermal cycling was carried out using a gradient PCR thermocycler to optimize annealing temperature across the range 50–65°C. The final optimized PCR program consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s and extension at 72°C for 45s with a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel prepared in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (0.5 µg/mL). A 100 bp DNA ladder (Thermo Fisher Scientific, USA) was used as the molecular size marker. Electrophoresis was performed at 100 V for 45 min, and the amplified products were visualized under UV illumination using a gel documentation system [6].

4. Results

4.1. General clinical examination and course of the case

General clinical examination of the dog suggested apparently normal activity, alert behaviour and cachectic appearance with a poor body condition score. The conjunctival mucous membranes were congested and moist and the lymph nodes were swollen. The capillary refill time was less than 3 seconds; heart rate was greater than 170 beats per minute (tachycardia) and the rectal temperature was around 106.1°F. A presumptive diagnosis of haemoprotozoal disease was made and a symptomatic treatment regime was initiated. The treatment included oxytetracycline @10 mg/kg, I/V, pantoprazole @ 1 mg/kg, I/V, meloxicam @ 0.2 mg/kg, I/V and Vitamin B complex, I/M. The patient was presented on the next day with a normal alertness, heart rate of 130 bpm, anuria and constipation since the previous day. The temperature decreased to 104.7° F and the dog was still anorectic and the same treatment was repeated. In addition, the owner was advised to give Tab. Doxycycline @ 10 mg/kg, s.i.d, Tab. Ranitidine @ 2 mg/kg b.i.d, Tab. Cyproheptadine @ 0.2 mg/kg, s.i.d and hematinic syrup (Sharkoferol Pet) 3 ml, t.i.d for 3 days.

After 6 days, the dog was presented in a deteriorated condition. The owner reported that the dog had become laterally recumbent since the previous day (Figure 1). The dog was brought in a state of non-ambulatory tetraplegia and showed severe neurological deficits. Neurological examination revealed negative pupillary light reflex, negative menace reflex, negative cutaneous trunci reflex, negative conscious proprioception in all 4 limbs, sluggish deep pain reflex, positive palpebral reflex, positive swallowing reflex and a good jaw tone. The conjunctival mucous membranes were congested; the temperature was 103.4°F and capillary refill time was greater than 3 seconds. The animal had not voided urine or motion suggestive of neurological atony of the bladder and rectum. Electrocardiographical evaluation revealed electric alterans and mildly depressed QRS complex amplitudes suggestive of pulmonary infiltration (Figure 2). A radiological assessment revealed upper motor neuronal signs of bladder and faecal stasis indicating midbrain / brainstem lesions (Figure 3). An alveolar development in its early stages was noticeable and vertebral affections were ruled out. The dog collapsed the very next day in spite of the treatment.

4.2. Cerebrospinal fluid evaluation

The CSF collected was blood-tinged in nature and mildly translucent. Microscopic examination of CSF stained with giemsa stain revealed neutrophilic pleocytosis and presence of large number of RBCs along with clusters of certain rounded/oval cells with a halo surrounding them which corresponded to capsulated yeast-like organisms (Figure 4). Staining of the CSF by Lactophenol cotton blue (Figure 6) and albert staining (Figure 7) showed the presence of numerous yeast organisms appearing as bluish, round to oval structures along with budding of small daughter cells adhering to their sides consistent with the morphology of cryptococcus.

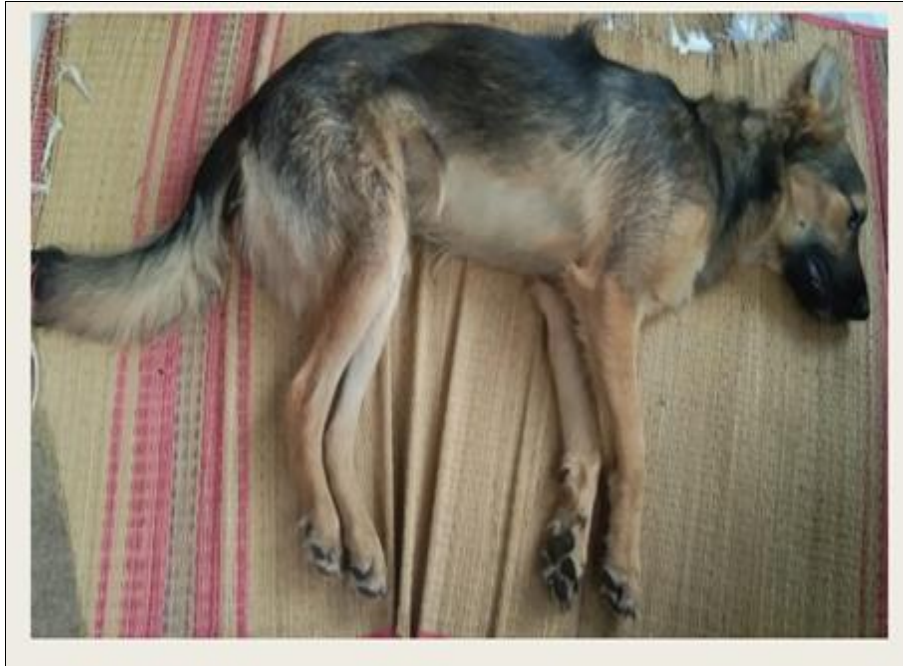


Fig 1: An eleven-month-old male German Shepherd brought in lateral recumbency with dullness, stupor and absence of conscious proprioception.

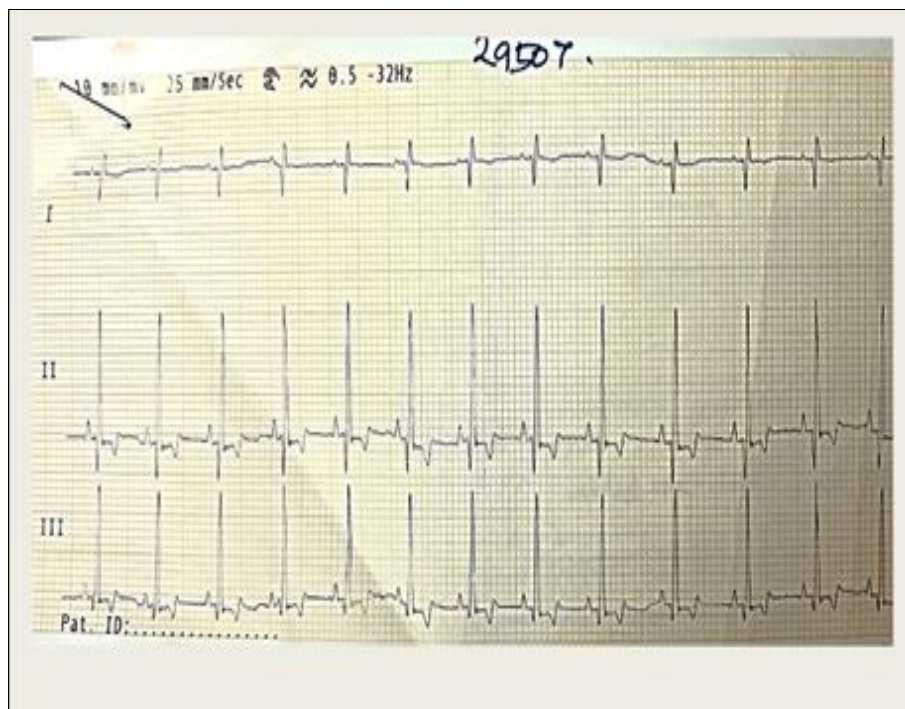


Fig 2: Electrocardiogram showing slightly depressed QRS, electric alternans, HR = 140bpm, P = 0.2mV, 0.04s; PR = 0.08s, QRS = 1.5mV, 0.04s, T = 0.12mV, 0.04s; QT = 0.12s.



Fig 3: Radiograph showing upper motor neuronal signs of bladder and faecal stasis suggesting midbrain / brainstem lesions.

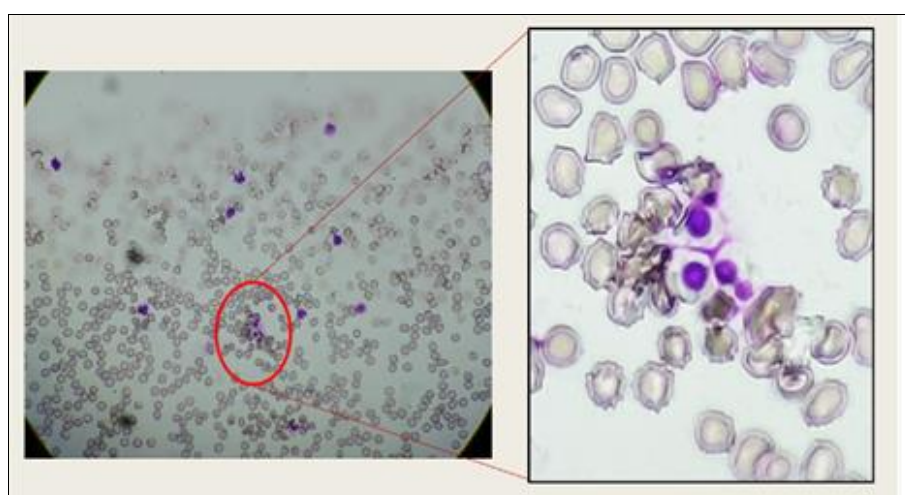


Fig 4: Cytological examination of CSF revealing clusters of round, encapsulated organisms with a halo (unstained capsule) consistent with *Cryptococcus* &, Giemsa stain, 40x

4.3. Cultural examination and RT PCR confirmation

The CSF was cultured on Sabouraud Dextrose Agar plates which showed white coloured round colonies with a powdery texture (Figure 5). CAP59 gene, used commonly as the molecular marker for identification of *Cryptococcus*, was

present in the cultured organism, was amplified and visualized under gel electrophoresis. A distinct 229 bp band indicated the presence of the CAP59 gene specific to *Cryptococcus* sp. confirming the yeast to be *Cryptococcus* (Figure 8).



Fig 5: Cryptococcal yeasts appearing like whitish round colonies

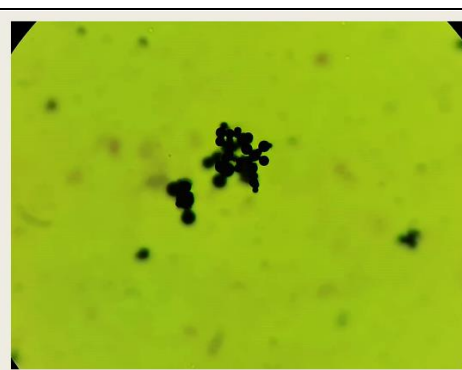
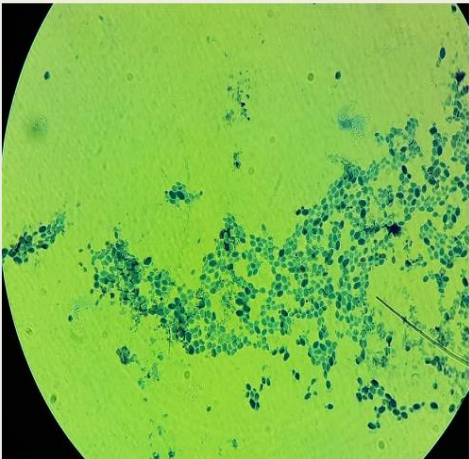
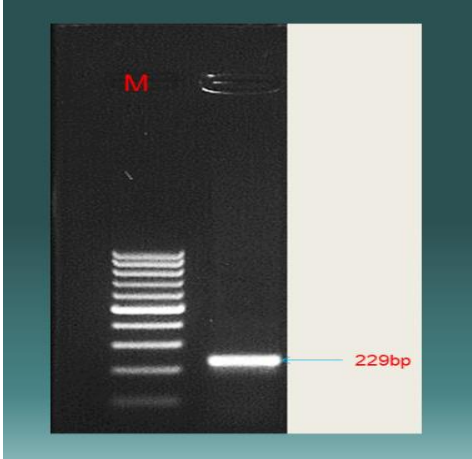


Fig 6: Cryptococcal yeasts isolated from the culture appearing as round

on Sabouraud Dextrose Agar.	bluish yeast-like structures with narrow budding of daughter cells adhering to the sides. Lactophenol cotton blue staining. 40x.
	
Fig 7: Cryptococcal yeasts isolated from the culture revealing numerous yeast cells (4-6 μ m). Albert's stain. 40x.	Fig 8: Agarose gel electrophoresis showing amplicons of CAP59 gene fragments, Lane 1M - Molecular marker, Lane 2- 229bp amplicon (CAP 59).

5. Discussion

Cryptococcal infection is considered to be one of the most frequent systemic fungal infection of domestic animals. *Cryptococcus* are encapsulated basidiomycete yeasts that grow at 37 °C and produce melanin. Great Danes, German Shepherds and Doberman Pinchers are reported to have greater susceptibility towards cryptococcal infection [1,3]. Larger breed dogs, especially young adults of both the sexes have been reported to be frequently affected. Immune-competent dogs are frequently affected and the organisms get into systemic circulation causing damage to multiple organs [1,2]. In agreement with those previously published case reports, the present case was in a young adult and apparently healthy animal, with no previously reported signs of immunodeficiency. The transmission of cryptococcal infection usually occurs following ingestion of desiccated yeasts or inhalation of basidiospores from the environment. The reason for larger breeds of dogs being more susceptible is believed to be because of their greater active outdoor lifestyle coupled with their frequent sniffing of soil and dirt possibly contaminated with cryptococcal spores [7].

Soil contaminated with bird droppings, especially of pigeons, has been implicated in creating an ideal ecological niche for the growth of *Cryptococcus*. *Cryptococcus* yeasts are reported to be frequently found in pigeon guano as well as soil contaminated with bird droppings [8]. Due to higher body temperature, birds rarely develop disease, but can shed *Cryptococcus* in faeces, which can survive for years in the environment [9]. Fungal spores present in the fowl fecal and litter material have been reported to get dispersed in the air and get inhaled where the spores may directly get lodged in the bronchi leading to respiratory infection. The spores get deposited over the cribriform plate of the ethmoid bone and enter brain causing nervous system lesions. In the present case, history indicated that the dog was in close proximity with backyard chicken and their litter material, a possible source of infection to this animal.

Cryptococcus in the environment is found in many different forms and it converts to a yeast form in the body, generally with a large polysaccharide capsule. These yeasts reproduce by budding and the characteristic narrow budding of yeasts found in tissue or body fluid samples actually aids in diagnosis. *Cryptococcus* is relatively easy to culture and most studies report a relatively high positive culture rate when known infected tissues or body fluids are sampled [18, 11, 10].

They can be found in tissue aspirates, urine, CSF, cavitory effusions, skin swabs, nasal swabs, sub-conjunctival aspirates, etc. In case of canines, the CSF should be clear, with a WBC count of less than 5/ μ L and a total protein of <0.25g/L (cisternal) or <0.5g/L (lumbar). In our case, it was blood-tinged in nature and mildly translucent along with neutrophilic pleocytosis. Cryptococcal organisms are usually round or oval shaped and are around 4 to 6 μ m in diameter with a narrow neck budding and differ from blastomyces by the presence of thick capsule and a thin cell wall and from *Coccidioides* by its budding and absence of endospore formation. The best stains to demonstrate these organisms include Periodic Acid Schiff (PAS) stain and Gomori methanamine silver stain. In our case, we demonstrated round to oval shaped cryptococcal organisms with a thin capsule along with narrow neck using lactophenol cotton blue and Albert's staining.

The capsule of *Cryptococcus* makes the organism resistant to cell-mediated immunity and phagocytosis by macrophages and contains polysaccharides such as glucuronoxylomannan and galactoxylomannan. The synthesis of this capsule is mediated by the capsular associated protein (CAP) genes namely, CAP10, CAP 59, CAP60 and CAP64. Among them, CAP59 gene was used as the molecular marker for identification of cryptococcus to confirm *Cryptococcus* species. Serological testing using Latex agglutination test is frequently done for confirmation of the organism as well as for evaluation of treatment response in dogs and cats [10, 12].

Canine cryptococcosis has been found to get disseminated during the early course of the disease itself to multiple organs causing a greater percentage of cases showing neurological, ocular or multiorgan involvement [3, 13, 14]. The ingested or inhaled spores reach the brain either through the cribriform plate or through hematogenous route or through arachnoid villi infiltration [1] resulting in nervous signs due to meningitis or meningoencephalomyelitis [1, 15]. In our case also nervous signs were observed suggesting meningoencephalitis. The clinical signs observed suggested the presence of possible lesions in the midbrain, the regions associated with cranial nerves 1 to 7. Similar nervous signs have been reported in more than eighty per cent of cases [18, 14]. Cerebellum, cerebrum and spinal cord are the frequently involved regions [1, 15]. It is very important that these lesions have to be properly evaluated as they can be misdiagnosed as tumours, especially in countries like UK, India where cryptococcus is

not frequently reported [16].

Auscultation of the thorax revealed exaggerated heart sounds over the thorax indicating pulmonary infiltration and the possibility of a respiratory involvement along with the nervous involvement. The reduced QRS amplitudes in the ECG and alveolar pattern in the X ray were indicative of the respiratory involvement. Electrocardiographical evaluation revealed electric alterans and mildly depressed QRS complex amplitudes suggestive of pulmonary infiltration. A radiological assessment revealed upper motor neuronal signs of bladder and faecal stasis suggestive of midbrain / brainstem lesions. In the present case, the involvement of the CNS, the cachectic body condition along with the negative response to previously administered antibiotics might have flared up the severity of the disease. Cryptococcosis diagnosed at an earlier stage may be treated with the appropriate antifungal drugs, based on sensitivity testing, such as azole antifungals (fluconazole, itraconazole, voriconazole) and amphotericin B. However, early diagnosis is a challenging task as the disease becomes chronic and gets disseminated to different organs. Most cases of cryptococcal infection with nervous involvement have been reported to have very poor prognosis with no success rates so far. As frequently reported in literature, the prognosis of the present case was poor and survived just five days after diagnosis. The outcome of neurocryptococcosis is considered to be very poor and a very few dogs have been reported to survive (2/7 (28%) post-diagnosis [3]. It has been reported that the dogs showing nervous signs were 4.54 times more likely to succumb as compared to those dogs showing no nervous signs [2]. In another study, it has been observed that all dogs showing multiple organ involvement or nervous signs died in spite of treatment [4]. In one of the largest study of cryptococcal infections in dogs, the mortality rate was observed to be 94% and the median survival time of the dogs after the onset of nervous signs was observed to be 7 days [15].

6. Conclusion

The present case is the first and rare documented case of canine neurocryptococcosis reported from India diagnosed using cerebrospinal fluid cytology, with another case of cryptococcuria being reported earlier [17]. This case study hopes to enlighten the veterinary community of the need to include fungal infections as a part of the differentials when dealing with diseases of the CNS and the possibility of diagnosis using cerebrospinal fluid analysis. It also warns us against indiscriminate use of antibiotics prior to ruling out of fungal infections which might actually aggravate the condition. When compared with the other assays available for the diagnosis of cryptococcus, CSF cytology offers a confirmative diagnosis, as in our case, through the direct observation of the fungal spores. It is essential to understand that the granulomas caused by cryptococcus may appear like neoplasms during non-invasive evaluations. Intensive supportive therapy along with administration of antifungal drugs is extremely important to improve the prognosis. The present study highlights the importance of including cryptococcus in the differential diagnosis of dogs showing nervous signs and presented to our clinicians in India.

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