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Diagnosis of Cryptococcosis in Dogs by Latex Agglutination Test and Enzyme Immunoassay

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ABSTRACT

Cryptococcus spp. is a pathogenic fungus which is an increasingly important cause of infection, particularly in the immunocompromised hosts. Diagnosis of cryptococcosis in animals can be carried out by isolation of the fungus but this requires several days to detect and identify the organism. Detection of cryptococcal antigen by latex agglutination test and enzyme immunoassay in serum is a rapid and easy method for diagnosis of cryptococcosis. In the present study, a total of 142 blood samples were collected from apparently healthy (n=89) and diseased dogs (n=53) for diagnosis of cryptococcosis. Latex agglutination test and enzyme immunosorbent assay (EIA) were carried out for the detection of cryptococcal antigen in serum. Of the 142 serum samples tested, six samples tested positive by Latex agglutination test while one sample tested positive by EIA. The sample which was positive by EIA was also positive by Latex agglutination test. The serum samples of dogs that tested positive for cryptococcal antigen were obtained from dogs suffering from symptoms like bloody faeces and vomit, emesis, chronic ear infection and discharge. Based on our findings, we conclude that the latex agglutination test in combination with the enzyme immunoassay can be used for the diagnosis of Cryptococcosis in dogs.

Keywords: *Cryptococcus*, blood, dogs, latex agglutination test, enzyme immunoassay

Fungi are parasitic, spore-producing organisms. Many species of fungus exist in the environment, but only a very few cause infection. A variety of fungal infections which cause serious diseases are soil related (Baumgardner, 2012). *Cryptococcus* spp. are distributed in nature and may be isolated from soil, bark scrapings, animal organic residues, leaves, flowers, tree trunks and other decaying woods (Cogliati *et al*., 2016; Gugnani *et al*., 2005). Fungal infections (yeast and mold infections) can be acquired by inhalation, ingestion, or through the abraded skin. Disease may result from the inhalation of the infectious propagules of *cryptococcus* from the environment (Springer *et al*., 2012). Some fungal infections can cause disease in otherwise healthy animals, while others require a host that is incapacitated or immunocompromised (for example, stresses such as captivity, poor nutrition, viral infections, cancer, or drugs like steroids) to establish infection (Merck's Veterinary Manual, 2011). Of all the fungal infections affecting dogs, the most common are dermatophyte infections followed by yeast infections like that of *Malassezia* spp., *Cryptococcus* spp. and *Candida* spp. Epidemiology *of C. neoformans, an increasingly important pathogen,* is well-characterized and this organism causes disease in immunocompromised individuals (Maziarz and Perfect, 2016; Poeta and Casadevall, 2012).

Cryptococcosis is of zoonotic importance and is an uncommon but important life threatening infectious

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disease of animals and humans throughout the world. The clinically important cryptococcal organisms are considered to be *Cryptococcus neoformans* var *neoformans* and *C. neoformans* var *gattii* (the latter also identified as the separate species *C. bacillisporus*) (Lester *et al*., 2004). Results of genetic studies shows that *C. neoformans* var *gattii* and the *C. neoformans* group are separate species, although taxonomic changes have yet to be finalized (Kwon-chung *et al*., 2002).

Various diagnostic tests, namely isolation of the organism from blood or from other samples from like biopsies, serum, impression smears, aspirates or swabs of affected sites and polymerase chain reaction (PCR) are carried out to diagnose a Cryptococcal infection. However, isolation is hazardous and time consuming. The detection of cryptococcal antigen by latex agglutination tests (LATs), enzyme linked immunosorbent assays (ELISA) or Lateral flow assays (LFA) is an important tool for the diagnosis of a Cryptococcus infection (Tintelnot, *et al.,* 2015). Also, latex agglutination test can be used for detection of cryptococcal antigen is serum and cerebro spinal fluid (CSF) and when used on CSF, could be taken as the early diagnostic method (Wang *et al*., 2015; Saha *et al*., 2009). The enzyme immunoassay is based on the principle of sandwich ELISA in which the microwells are coated with anticryptococcal polyclonal antibodies which capture the cryptococcal antigens from the suspected samples; the results of which are then calculated by the help of an ELISA reader. Limited studies have been carried out to study the prevalence of cryptococcosis in dogs in and around Ludhiana distt., Punjab. Therefore, the present study was carried out for the detection of Cryptococcal antigen in the serum of dogs by the use of latex agglutination kit and enzyme immunosorbent assay (EIA).

MATERIALS AND METHODS

Detection of *cryptococcus* spp. antigen in serum samples of dogs was carried out by cryptococcal antigen latex agglutination system (CALAS® kit) [Meridian Bioscience Inc., Cincinnati, USA] and Enzyme immunoassay (EIA) (Premier® kit) Premier® Cryptococcal Antigen kit [Meridian Bioscience Inc., Cincinnati, USA].

Sample collection

A total of 142 blood samples were collected from apparently healthy (n=89) and diseased dogs (n=53) from Veterinary Clinics (Guru Angad Dev Veterinary and Animal Sciences University) Ludhiana (Punjab). Some samples were also collected from small animal camps conducted by a Non Government Organisation, Punjab.

In the present study, diseased dogs were the ones suffering from non-neurological signs such as inappetence and vomiting, upper respiratory tract signs, gastro-intestinal tract infections, ear infections, skin infections and neurological infections (Sykes *et al*., 2010; Vorathavorn, *et al*., 2013) while the apparently healthy dogs were free from any aforementioned signs, though they were suffering from other ailments like gynaecological problems, urinary incontinence, partial anorexia, fever, diarrhoea and abdominal pain etc.

Cryptococcal antigen latex agglutination system (CALAS)

CALAS® is a qualitative and semiquantitative test system for the detection of capsular polysaccharide antigens of *Cryptococcus neoformans* in serum and CSF. However, in this study CALAS® was used only as a qualitative system for detection of *Cryptococcus neoformans* in the test samples. The test was carried out as per the manufacturer's instructions. In brief, the serum samples were treated with pronase enzyme provided with the kit before testing it for the presence of Cryptococcal antigen. Pre-treatment of serum samples with pronase, a proteolytic enzyme, reduces the number of false-positive test results by eliminating nonspecific interference with macroglobulins. The pronase treatment is important for the serum samples as the results may show discrepancy if the pronase treatment is absent or the enzyme is faulty (Stoeckli *et al*., 2001).

The serum samples and controls were mixed with the detection latex and control latex reagents in separate rings inscribed on the cards supplied along with the kit. After gently shaking the cards for five minutes, the readings were noted, and the samples were analysed in the relation to the controls provided in the kit.

The following test cut-offs were applied as per the instructions in the package insert: negative result, a homogeneous suspension of particles with no visible

clumping; one plus (1+), fine granulation against a milky background; two plus (2+), small but definite clumps against a slightly cloudy background; three plus (3+), large and small clumps against a clear background; four plus (4+), large clumps against a very clear background. In this study, the samples which tested positive showed 2+ or more intensity of clumping.

Enzyme immunosorbent assay (Premier Cryptococcal Antigen kit)

The Premier® Enzyme Immunosorbent Assay (EIA) utilizes an anti-Cryptococcus polyclonal capture antibody adsorbed to microwell plates in combination with a MAb-peroxidase conjugate. If cryptococcal antigens are present in the sample, a complex is formed between the antigens, enzyme conjugate and the adsorbed antibody. After washing to remove unbound conjugate, a substrate solution is added. Colour develops in the presence of bound enzyme. The test was carried out as per the instructions given in the kit. In brief, in the first step 50µl of each serum sample was put in the microwells and incubated at room temperature for 10 minutes. This step was followed by washing, then enzyme conjugate was added to the wells and the incubation step was repeated. After further washing, substrate was added to the wells and incubated at room temperature for 10 minutes. Stop solution was added and reading was noted after 15 minutes. Qualitative results were read on a plate reader set at 450nm. The following test cut-offs were applied as per the instructions in the package insert: negative result, OD_{450} of < 0.100; indeterminant result, OD_{450} of ≥ 0.100 and < 0.150; and positive result, OD_{450} of ≥ 0.150. In accordance to the kit instructions, the sample which was positive showed a definite yellow colour after adding stop solution while the samples which were negative did not display any colour. EIA was carried out on the same batch of serum samples used for CALAS® testing.

RESULTS AND DISCUSSION

Cryptococcus neoformans is widespread in the environment and can infect dogs, cats and a wide variety of mammals, including humans, with occasional cases also reported in birds, reptiles and amphibians (Cfsph, 2013). Cryptococcosis occurs primarily in animals that have a suppressed or deficient immune system. Diagnosis of cryptococcosis can be done by isolation of the organism from various samples like blood, aspirates, swabs from affected sites but this requires several days to detect and identify the organism. Although direct microscopic examination is rapid, this method is relatively insensitive. Detection of cryptococcal antigen by latex agglutination test and enzyme immunoassay in serum is a rapid and easy method for diagnosis of cryptococcosis on a routine basis (Rivera *et al.,* 2015). Cryptococcal antigen in serum and CSF can be detected by latex agglutination test and when this test is used on CSF, could be taken as the early diagnostic method (Wang *et al*., 2015; Saha *et al*., 2009). In the present study, the serum samples were tested using CALAS® Cryptococcal latex agglutination kit and Premier® enzyme immunoassay kit.

Of the 142 serum samples tested for the presence of cryptococcal antigen, six samples tested positive by CALAS® test while one sample tested positive by Premier® test (Table 1) (Fig. 1 and Fig. 2).

Table 1: Result of serum samples of dogs tested for cryptococcal antigen by latex agglutination test (CALAS®) and Premier®Enzyme immunoassay

CALAS	Enzyme Immunoassay					
Sample type	**+ve**	**–ve**	**Total**	**+ve**	**–ve**	**Total**
Apparently Healthy	2	87	89	0	89	89
Diseased	4	49	53	1	52	53
Total	**6**	**136**	**142**	**1**	**141**	**142**

However, one sample tested positive by both tests. The sample which was found to be positive in enzyme immunoassay produced yellow colour after adding stop solution while the negative samples did not display any colour (Fig. 2). This sample was collected from a diseased Labrador dog suffering from blood in vomit and recumbency. The serum samples from five dogs tested positive only with CALAS® test. One of the dogs was a Pomeranian suffering from bloody faeces and emesis, suggesting some viral infection leading to decrease in defence mechanism of the body. The other sample was of a German shepherd dog with a history of chronic ear infection and discharge. Cryptococcosis has been observed in large breed dogs like German Shepherds (O'Brien *et al.,* 2004). Cryptococcosis in German Shepherds has also been reported by O'Toole *et al*. (2003). One of the samples

was of a Pug dog suffering from anorexia with no previous history of vaccination against the major canine diseases. One of the dog that tested positive was of 4.5 years of age suffering from occasional itching in ears for two months. However, the details regarding the breed and history of one dog were not available. The samples which were found to be positive by latex agglutination test produced an agglutination on a scale of +2 or more while the samples which were found to be negative produced an agglutination of +1 or less (Fig. 1). Cryptococcosis in pure bred dogs less than six years of age has been observed but the disease can occur at any age in dogs and there is no sex predisposition (Newman *et al.,* 2003; Lester *et al*., 2011; Trivedi *et al.,* 2011). Though neurologic signs were observed in dogs suffering from cryptococcosis but non-neurologic signs such as inappetence, vomiting, weight loss, and upper respiratory tract signs have also been reported (Sykes *et al*., 2010). History of immunosuppressive illness or drugs may be seen in dogs suffering from cryptococcosis but is often not recognised (Trivedi *et al.,* 2011).

Fig. 1: Positive samples detected by CALAS (latex agglutination)

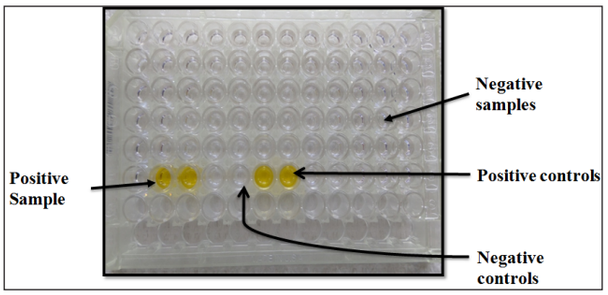

Fig. 2: Positive samples detected by Premier® enzyme immunoassay

Latex agglutination test and EIA for the detection of cryptococcal antigen has been demonstrated by various other researchers (Illnait *et al*., 2001; O'Toole *et al*., 2003; Babady *et al.,* 2009). Illnait *et al*. (2001) evaluated ELISA in clinical samples from patients with and without previous cryptococcosis diagnosis. They found ELISA to be highly sensitive and specific, and no significant differences were observed when it was compared with latex agglutination test. Hansen *et al*. (2013) tested 589 serum samples and 411 CSF samples with lateral flow assay, EIA and CrAg test. In all, 56 (41 serum and 15 CSF) samples were positive and 921 (527 serum and 394 CSF) samples were negative by all three methods. Binnicker *et al*. (2012) tested 634 serum samples for the presence of cryptococcal antigen by the use of Premier EIA, CALAS, CrAg lateral flow assay and Alpha CrAg EIA. Out of these, Alpha CrAg EIA had the maximum sensitivity while the specificity of Premier EIA was maximum. McMullan *et al*. (2012) tested 106 serum samples out of which 51 tested positive by latex agglutination test (LAT). 50 sera from patients without cryptococcosis yielded negative results by LAT. They compared these results with lateral flow assay and found that LAT yielded more false negative results. Duncan *et al*. (2005) screened a total of 268 canine serum samples by CALAS, out of which two samples tested positive while 266 samples tested negative by the test. Saha *et al*. (2009) compared latex agglutination test (LAT), enzyme immunoassay (EIA) and PCR on a total of 359 CSF and urine samples from 82 patients for detection of cryptococcosis. Of these, 269 CSF samples and 52 urine samples were detected positive for *Cryptococcus* spp. They found that the sensitivity of LAT, EIA and PCR was similar; however, the specificity of LAT decreased due to false-positivity in two samples.

In order to limit cost and mortality associated with invasive diagnostic procedures requiring anaesthesia, when possible, a diagnosis of cryptococcosis should be made on the basis of serum antigen testing or aspiration cytology of other organs. Negative results using these methods do not rule out cryptococcosis, so when progressive central nervous system (CNS) disease is present and a diagnosis of cryptococcosis cannot be made by serum antigen testing or aspiration cytology of other organs, CSF collection should be considered for diagnosis. However, detection of cryptococcal capsular antigen in serum, urine, or CSF is a useful, rapid method for diagnosis of cryptococcosis especially in those suspected cases in which the organism is not identified (Merck's Veterinary Manual, 2011). In

addition, monitoring the serum latex agglutination test results may provide a safe and less invasive means of monitoring response to treatment (O'Toole *et al*., 2003). In the present study, Latex agglutination for detection of cryptococcal antigen in the serum was found to detect more positives in comparison to EIA. Prevalence of cryptococcosis in dogs in the area of study based on the results of our tests was considerably low.

CONCLUSION

Based on our results, it was found that the latex agglutination test was able to detect more number of positives than the enzyme immunoassay. However, due to differences in the sensitivity and specificity of tests, a combination of these two tests can be used for the diagnosis of cryptococcosis in dogs.

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