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Review Article

Recent concepts in diagnosis of Canine Ehrlichiosis

Bindu Lakshmanan[1] and Lalitha John[2]
1. Assistant Professor, Department of Parasitology,
College of Veterinary and Animal Sciences, Pookot, Wayanad-673576
2 .Dean, Madras Veterinary College, Chennai

Introduction

Ehrlichiae are one of the several kinds of obligate intracellular pathogens first described at the Pasteur Institute in Algeria by Donatien and Lestoquard in the year 1935. *Ehrlichia canis* causes canine monocytic ehrlichiosis, a potentially fatal tick borne disease. Besides *E.canis*, other Ehrlichiae have also been encountered in canines, *viz*., *E.ewingi* (Anderson *et al.,* 1992), *E.risticii* (Kakoma *et al.,* 1994) and *E.chaffeensis* (Dawson and Ewing, 1992).

In India, *E.canis* was first reported in Madras by Mudaliar (1944). Diagnosis of ehrlichiosis frequently seems difficult, as there are no pathognomonic signs for the disease. Perhaps the greatest challenge in battling ehrlichiosis is in detecting and accurately assessing the clinical signs. An early diagnosis of the disease is imperative to ensure successful treatment and good prognosis.

Currently definite diagnosis of ehrlichiosis is based mostly on haematological, biochemical and serological results. Microscopic demonstration of typical intracytoplasmic morulae of *E.canis* in leukocytes is not a very reliable diagnostic technique as the organism is not readily demonstrable in blood smears (Woldehivet and Ristic, 1993, Waner *et al*., 1999).

Acridine orange staining of peripheral blood smear allows the staining of organisms alone, thus differentiating from stain particles/ other inclusions. The methanol fixed smears should be flooded with 0.01 % AO stain, allow to act for two minutes and then wash slowly in tap water. The smears are mounted with a cover slip and examined, when moist under a fluorescent microscope.

Polymerase Chain Reaction (PCR)

PCR is a molecular diagnostic technique that enables the amplification of specific sequences of nucleic acids. PCR contributes significantly to the early detection of canine ehrlichiosis by increasing the speed of diagnosis, specificity, sensitivity, reproducibility and ease of interpretation Invented by K.B Mullis in 1985 and described originally by Saiki *et al.,* (1985), PCR has provided the basis for development of new generation molecular diagnostics.

Iqbal *et al.,* (1994) developed for the first time a PCR assay for the amplification of *E.canis* DNA from mononuclear fractions of whole blood. A 600 bp product was obtained using a **single PCR** targeting 16Sr RNA gene. PCR is a highly sensitive and specific test for the detection of very low levels of the *E.canis* in tissues of dogs. The PCR evidence of *E.canis* in dogs even after specific treatment with doxycycline could throw light into the carrier status of infection in dogs after antibiotic therapy (Iqbal and Rikihisa, 1994). PCR diagnosis of human ehrlichiosis using cerebrospinal fluid, bone marrow and blood samples (Dunn *et al*., 1992, Anderson *et al*., 1992) and ehrlichiosis in equines (Biswas *et al*., 1991) are also documented.

Recent methodological advances in PCR to improve sensitivity (e.g. Nested PCR) and specificity (e.g. Hot Start PCR) involve more manipulative steps, reduce the threshold target copy number for a positive result and/or increase the concentration and amount of DNA products.

A PCR assay with chemiluminiscent hybridization (CH) to detect 16Sr RNA gene of *E.canis* is *equally* or more sensitive to cell culture isolation (McBride *et al*., 1996). The specificity of this PCR

assay is based upon amplification of the 495bp product using specific primers, namely, EC1 and EC2.This PCR/CH detected as little as 30fg of *E.canis* genomic DNA, the equivalent of approximately 150 organisms.

Nested PCR is a modification where the first PCR product is reamplified with another set of primers. This assay is reported to be 20 fold more sensitive than the direct PCR for *E.canis* (Warner and Dawson, 1996). Nested PCR is a highly effective tool for assessing the clearance of organisms after antibiotic therapy (Wen *et al*., 1997). DNA extracted from splenic aspirates proved to be the best sample for PCR in order to diagnose the *E.canis* carrier status during sub clinical phase (Harrus *et al*., 1998). Many workers have reported the efficacy of nested PCR in diagnosis of ehrlichiosis (Barlough, 1996, Murphy et al., 1998, Breitschwerdt *et al*., 1998,Massung *et al.,* 1998). **Multiplex PCR** allows the detection of more than one species in a shorter span of time.

Quantitative Real Time PCR with TaqMan fluorogenic detection system uses a specific fluorogenic probe and two sets of primers. This method was used for detection of bovine ehrlichiosis (Pusterla, N *et al*., 1999). This technique enables to quantify the material for experimental infection or production of antigen.

PCR assay of acute phase serum in the absence of whole blood specimens is a useful method of early detection of human ehrlichiosis (Comer *et al.,* 1999).

Recently, immunomagnetic separation techniques have been employed for separating the *E.risticii* organisms from the faeces and detecting them by PCR (Biswas et *al.,* 1994).

The authenticity of PCR products need to be confirmed either by Southern hybridization, by DNA sequencing or by restriction endonuclease cleaving patterns.

Indirect fluorescent antibody test (IFAT)

Immunodiagnosis of *Ehrlichia* is usually based on positive results of IFAT. This test detects serum antibodies as early as 7days after initial infection, although some dogs may not become seropositive until 28days after infection begin

Indirect fluorescent antibody test IFAT was used for the analysis of *E.canis* and *E.ewingii* infection (Rikihisa, Y., 1992). Tresamol *et al.,* (1998) had used IFAT to study the seroprevalence of canine ehrlichiosis in Chennai city.

A definite case of canine monocytic ehrlichiosis (CME) can be defined as one with clinical and haematological parameters suggestive of the infection with a single serum Ig G titre of $\geq$1:256. On the other hand a probable case of CME would be a case where the clinical signs and symptoms are suggestive of the disease and with a single IFA IgG titre of 1:64 to 1:128 (Waner *et al*., 2001). When assessing the antibody titres to *E.canis* it is essential to account the range of cross- reactivities that might confound the diagnosis. Infections with *E.canis, E.chaffeensis, E.ewingii, E.platys E.equi or Babesia canis* may cause disease manifestations that may be clinically, haematologically and serologically in-distinguishable from those of other infecting species (Baneth *et al.,* 1998). Serological cross reactivity between various ehrichial species may pose a serious problem in interpretation of results (Neer, 1998). However, IFAT for diagnosis of CME is an important aid in confirming the exposure to *E.canis* where more sophisticated techniques like tissue culture and PCR are not routinely used.

Enzyme Linked Immunosorbent Assay (ELISA)

Rikihisa *et al*., (1992) reported an indirect Enzyme Linked Immunosorbent Assay (ELISA) to detect and quantitate the IgG to *E.canis* test sera. They also reported that *E.canis* IgG ELISA would show the least cross-reaction with antisera to other ehrlichial species.

An ELISA test used for the early diagnosis of infection by the detection of plasma ehrlichial soluble antigen has also been reported (Waner *et al.,* 1996).

A dot blot immunoassay using recombinant protein of *E.canis* is a recent serodiagnostic test. The rp30 antigen was evaluated for serodiagnosis of CME and was found to react consistently (Ohashi *et al*., 1998). The specificity of dot –ELISA is similar to that of IFA and cross-reactivities hamper the final diagnosis. To overcome this problem, addition of a number of antigens to the

same stick or plate of dot-ELISA allows a real time simultaneous comparison between different ehrlichial species.

Western Immunoblotting

Western immunoblotting was applied to demonstrate cross-reacting antigens between Neorickettsia sp. and Ehrlichia sp. by Rikihisa (1991). Iqbal *et al.,* (1994) reported that WI was as sensitive as IFA in diagnosis of *E.canis* infection. Immunoblots for *E.canis* shows a prominent broad band at 27 kd (Hegarty *et al.,* 1997).

IgG ELISA and WI with purified *E.canis* antigen has been suggested as useful techniques in distinguishing between *E.canis* and *E.ewingii* (Rikihisa *et al*., 1992). The WI will detect *E.canis* antibodies as early as 2 to 8 days after exposure.

***In vitro* cultivation**

E.canis can be cultured in primary canine blood monocytes (Ristic *et al*., 1972). DH82 cell lines, spontaneously immortalized mouse peritoneal macrophage-dog monocyte hybrid cells or in canine macrophage cell lines. Cell culture isolation is the most specific test (Goodman, 1996) but this depends on the presence of ehrlichiaemia and takes about 1-4 weeks to give results, thus limiting its use as a rapid diagnostic tool.

Conclusion

While microscopy remains the 'gold' standard for the routine diagnosis of ehrlichial infections, the demonstration of morulae is of limited value because of the low level of parasitaemia in peripheral blood. Immunodiagnostic tests offer advantages over microscopy but these can be problematic due to the cross reactions between the related species and the inability to differentiate current infection from previous infection or exposure without establishment of infection. The development of molecular detection assays based on PCR has facilitated a sensitive, specific and quick diagnosis of ehrlichiosis in animals and humans. However, PCR is not without problems. Widely recognized limitations of amplification technology include those of false positive and false negative results. This can be kept to a minimum by adhering to the standard procedures for avoiding contamination.

A number of new technologies such as electro rotation assay (ERA) and fluorescent-in-situ hybridization (FISH) have been developed which may assist in diagnosis of ehrlichial organisms.

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