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Comparison of a clinic-based ELISA test kit with the immunofluorescence test for the assay of *Ehrlichia canis* antibodies in dogs

Trevor Waner, Carmella Strenger, Avi Keysary

Abstract. The ''gold standard'' for the detection of antibodies to *Ehrlichia canis,* the cause of canine monocytic ehrlichiosis (CME), is the indirect immunofluorescence antibody (IFA) test. The IFA test however is generally available only in selected laboratories and requires extensive equipment and trained personnel. A double-blind study was conducted to compare the ability of an in-clinic standardized enzyme-linked immunosorbent assay (ELISA) test kit to measure *E. canis* IgG antibodies in dogs compared with the standard IFA technique. A good correlation was found between the 2 techniques ($r^2 = 0.8793$; $P < 0.0001$). Evidence for the sensitivity of the ELISA technique for the early detection of *E. canis* IgG antibodies was demonstrated by comparing the appearance of *E. canis* antibody titers by the IFA and ELISA techniques after artificial infection of 2 sets of dogs. In both experimental infections, both tests were equally sensitive for the early detection of IgG antibodies against *E. canis,* and the results correlated well with the appearance of fever and clinical signs. Proposed application of the in-clinic ELISA test is to aid in the diagnosis of CME.

Canine monocytic ehrlichiosis (CME) is a tick-borne disease of dogs caused by *Ehrlichia canis,* an obligate intracytoplasmic rickettsia localizing in the reticuloendothelial cells of the liver, spleen, and lymph nodes and replicating primarily in mononuclear macrophages.[14] The disease is diagnosed by clinical signs, hematologic abnormalities, demonstration of morulae in peripheral monocytes, and detection of serum antibodies to *E. canis* by the indirect immunofluorescence antibody (IFA) test.[11] However, clinical signs may be variable throughout all stages of the natural disease and may be nonexistent during the subclinical stage.[2,15] Only about 4% of blood smears evaluated for dogs with acute *E. canis* infection may be positive for morulae.[20] The IFA test is considered the most reliable and sensitive method for detection of antibodies during all stages of the disease;[11] however, the technique is generally available only in selected laboratories and requires specialized equipment and trained personnel.

Enzyme-linked immunosorbent assay (ELISA) technology has been used to produce a semiquantitative test where small quantities of antigen were applied to nitrocellulose surfaces. In the field of canine medicine, this technique has recently been successfully used for the detection of serum immunoglobulin G (IgG) antibodies to canine parvo virus and distemper virus.[17,18] The technique requires a minimum of equipment, is easy and quick to perform, and involves a single-step dilution.

A comparative study was conducted for the detection of *E. canis* antibody titers using a standardized ELISA test kit with the ''gold standard'' IFA test.[12] In addition, the kinetics of antibody production was studied in artificially infected dogs using both diagnostic methods. The aim of these studies was to investigate the diagnostic value of the ELISA kit for the detection of IgG antibodies to *E. canis.*

Materials and methods

Animals. Three separate study groups of dogs were used. In the first study, 148 coded canine serum samples from veterinary clinics in Israel were used in a double-blind procedure to evaluate the correlation between the IFA and ELISA techniques. For experimental infection study 1, 6 beagle dogs about 8 mo of age were infected intravenously with 5 ml of blood from a single *E. canis*-infected beagle dog. For experimental infection study 2, another group of 6 beagle dogs were infected intravenously with 5 ml of tissue culture growth medium containing 10^6 DH82 cells heavily infected (≥80%) with *E. canis.* In both experiments, Israeli isolate 611 of *E. canis* was used.[7]

For both experimental infection studies, all dogs were antibody negative for *E. canis* prior to infection, and their hematologic and clinical chemistry parameters were within reference ranges. Blood was collected at least 3 times weekly after infection to evaluate hematologic parameters and antibody levels. Hematologic analyses were carried out using a semiautomatic impedance cell counter.[a]

E. canis antibody IFA test. The IFA test was carried out as described previously.[7,12] Five microliters of 2-fold serum dilutions were applied onto wells of slides prepared with acetone-fixed heavily infected DH82 cells. The slides were

From the Israel Institute for Biological Research, PO Box 19, Ness Ziona 70450, Israel (Waner, Keysary), and Life Science Research Israel, PO Box 139, Ness Ziona 70400, Israel (Strenger).

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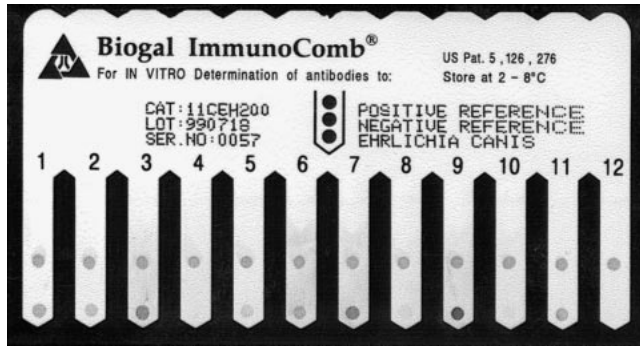

Figure 1. Developed immunocomb from *E. canis* antibody ELISA. The developed spots represent different concentrations of serum antibodies in 12 dogs. The upper spot represents a known positive control indirect immunofluorescent antibody test (IFA) pretititrated serum sample of 1:80. Comb numbers 4, 8, and 12 are from dogs without detectable serum levels for *E. canis* antibodies. Comb 9 represents the dog with the highest antibody level.

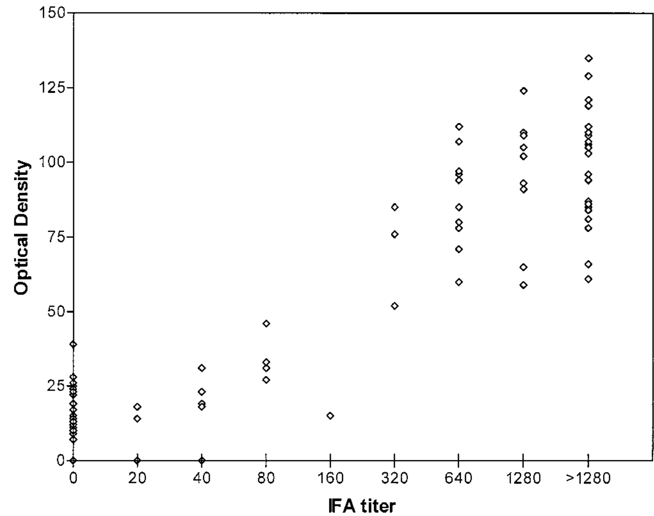

Figure 2. Correlation between serum indirect immunofluorescence (IFA) *E. canis* antibody titers and serum *E. canis* antibody ELISA optical density results for 148 canine sera. (r^2 = 0.8793; $P < 0.0001$).

incubated in a humidified chamber at 37 C for 30 min, washed gently with water, and air dried, and 5 μl of anti-dog IgG–fluorescein isothiocyanate conjugate[b] was added to each well. After a similar incubation for 30 min, the slides were washed, dried, and examined under a fluorescent microscope. A titer of 1:64 was considered serologic evidence of exposure to *E. canis.*

E. canis antibody ELISA. Ehrlichia canis IgG antibody titers of serum samples were determined using a commercial ELISA test kit[c] containing plastic combs sensitized with *E. canis* antigen derived from mouse J774.A1-infected cells.[13] Sera were diluted 1:36 in buffer and incubated with the antigen spots for 5 min. After washing to displace unbound antibodies, the combs were allowed to react for 5 min with whole molecule goat anti-dog IgG–alkaline phosphatase conjugate.[d] After 2 successive washing steps, bound antibodies were detected with a precipitating chromogen, 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium[e] (Fig. 1). The concentration of *E. canis* antibodies for each sample was reported from a scanning device designed for automatic reading of the color intensity of the reaction spots on the comb.[f] The results were recorded as optical density (OD) units.

Statistical analysis. Correlation between the IFA IgG antibody titers and the ELISA OD results were assessed using Pearson's correlation coefficient (r^2).

Results

IFA E. canis antibody titers versus ELISA OD units. Figure 2 represents the correlation between the IFA *E. canis* antibody titers and the ELISA *E. canis* antibody OD values for 148 canine serum samples. A close correlation was found (r^2 = 0.8793) between the 2 assays ($P < 0.0001$).

Experimental infection studies. All beagle dogs from both experimental studies developed typical clinical signs of acute ehrlichiosis. In both groups, pyrexia was accompanied by anorexia and generalized lymphadenomegaly. Dogs injected with infected blood developed fever and clinical signs 15 days postinfection (PI), whereas those injected with *E. canis*-infected DH82 cells developed similar signs on day 10 PI. Thrombocytopenia was a consistent finding in all dogs from both groups accompanied by a concomitant increase in mean platelet volumes. A transient mild decrease in leukocyte count was accompanied by a mild nonregenerative anemia.

Using the IFA assay, *E. canis* antibody serum titers for dogs injected with infected blood were first detected on day 15 PI, when their IFA antibody titers ranged from 1:160 to 1:640 (Fig. 3). On day 17 PI, antibody titers in all dogs rose to 1:320–1:1,280. Three days later, on day 20 PI, the titers had reached levels

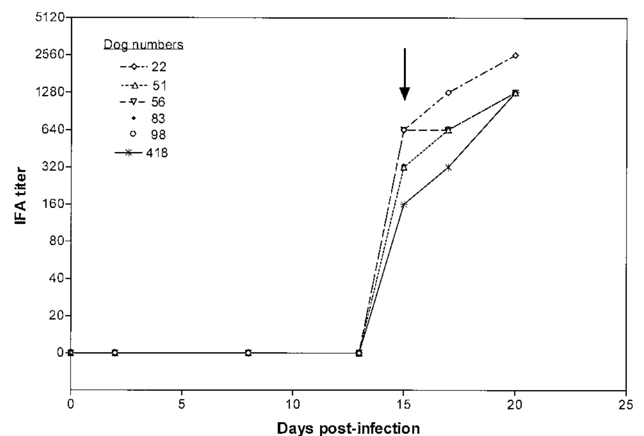

Figure 3. *Ehrlichia canis* antibody titers as detected by the indirect immunofluorescence assay (IFA) after artificial infection of 6 beagle dogs by intravenous injection of infected blood. The arrow indicates the first appearance of fever and clinical signs.

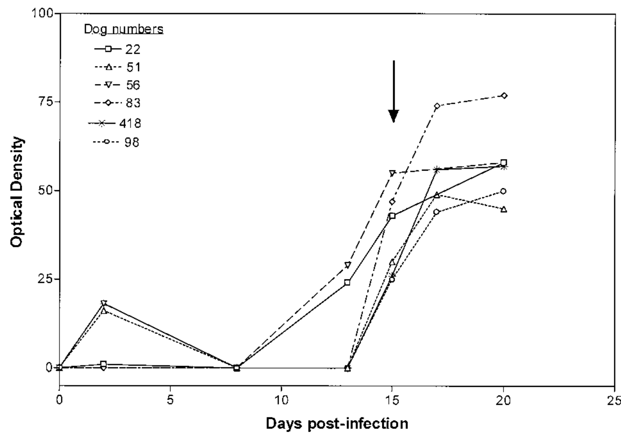

Figure 4. *Ehrlichia canis* antibody levels as measured by the ELISA technique after artificial infection of 6 beagle dogs by intravenous injection of infected blood. The results are expressed as optical density units. The arrow indicates the first appearance fever and clinical signs.

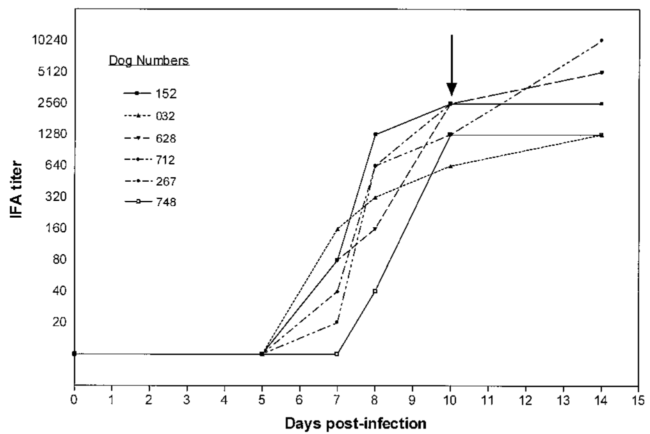

Figure 5. *Ehrlichia canis* antibody titers as detected by the indirect immunofluorescence assay (IFA) after artificial infection of beagle dogs by intravenous injection of DH82 *E. canis*-infected cells. The arrow indicates the first appearance of fever and clinical signs.

of 1:1,280–1:2,560. Employing the ELISA test kit on these same sera, all preinfection sera gave 0 OD results (Fig. 4). On day 13 PI, 2 samples (22, 56) showed low to moderate OD results, increasing to high OD values on day 15 PI, with 3 dogs in the high OD range (22, 56, 83). On days 17 and 20 PI, all dogs had high OD results.

Five of the 6 dogs injected with *E. canis*-infected DH82 cells were IFA positive on day 7 PI, with IgG antibody titers ranging from 1:20 to 1:160 (Fig. 5). On day 8 PI, all dogs were seropositive (range, 1:40–1:1,280), and their IFA *E. canis* antibody titers continued to rise on days 10 and 14 PI (Fig. 5). Evaluating the same sera by the ELISA test, all preinfection samples had 0 OD (Fig. 6). On day 5 PI, 3 dogs had low OD results, and on day 7 PI, 4 of the 6 dogs had high OD values. On day 8 PI, all dogs had high OD values, which were also detected on days 10 and 14 PI.

Discussion

The results of this study demonstrated that canine serum IgG antibodies to *E. canis* can be semiquantitatively measured with a minimum of laboratory equipment using a standardized clinic-based ELISA test kit. The ELISA OD units and the IFA titers were closely correlated. Taking into account that 2–4-fold variations in IFA titers are usually expected, the high correlation coefficient should be regarded as evidence of the close relationship between the 2 techniques.

The ELISA method was clearly able to differentiate *E. canis* seronegative samples from those with titers of ≥1:320. It was not able to distinguish seronegative titers from those of 1:20–1:40. Titers in the range of 1:80–1:160 were not adequately tested in this study because of the lack of samples in this range. However, from the results of the IFA range of 1:80–1:320 and the general positive trend of the OD in relation to the IFA results in this range, it appears that that the ELISA kit would be able to differentiate seropositive titers of 1:80 from seronegative titers. Titers of 1:64 are generally considered as evidence of exposure.[3] In the case of an early acute infection with relatively low antibody titers, a follow-up examination 1–2 days later would confirm or reject a diagnosis of CME, as was shown adequately in the experimental infections. Furthermore, in contrast to the IFA method, the ELISA technology was not able to differentiate between IFA titers ≥1:640. This limitation is not considered crucial because IFA titers ≥1:640 are considered unequivocal evidence of exposure to *E. canis.*

Further evidence for a close correlation between the

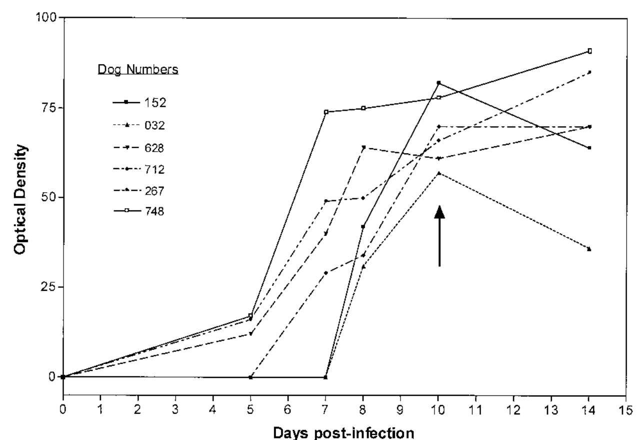

Figure 6. *Ehrlichia canis* antibody levels as measured by the ELISA technique after artificial infection of beagle dogs by intravenous injection of DH82 *E. canis*-infected cells. The results are expressed as optical density units. The arrow indicates the first appearance of fever and clinical signs.

2 techniques was demonstrated in the 2 experimental infection studies. In both studies, the appearance of antibodies to *E. canis* was closely correlated for the 2 techniques. Furthermore, from a diagnostic point of view, at the time of the appearance of fever and clinical signs, significant antibody titers were detected by both the IFA and ELISA techniques. Both assay systems were equally sensitive for the early detection of IgG antibodies against *E. canis* in artificially infected beagle dogs.

Similar results for *E. canis* antibody titers have been obtained with canine serum samples comparing a dot-ELISA method with the standard IFA test.[1] As in the present study, the correlation between the IFA and ELISA techniques was close. However, the immunocomb technology is advantageous because it involves a single-step dilution and is semiquantitative, whereas the dot-ELISA used in previous studies is only qualitative.[1]

For use in the clinic or laboratory, ELISA units can be translated to IgG *E. canis* antibody titers by using the color scale provided in the kit. This method is based on comparison of the reaction spot of the serum sample being tested with the color reaction of a spot from a known positive control IFA pretititrated serum sample of 1:80 (Fig. 1). This color scale technique for estimating IgG titers was not used in the present study because interpretation of the results required numerical values to be used for statistical testing. The results of the color scale method are closely correlated with those of the immunoscanning device.[18]

The ELISA kit can be used efficaciously during all phases of CME. During the acute stage of the disease, clinical signs may be confusing because of variations in ehrlichial strains, breed of dog, immunologic status of the host, and concurrent infection with other tick-borne parasites.[5] The most important hematologic finding during the acute phase is thrombocytopenia;[6,16] however, this sign should not be regarded as pathognomonic. The presence or absence of antibody titers to *E. canis* will therefore confirm or refute the diagnosis of CME. Throughout the subclinical phase of CME, clinical signs are nonexistent and hematologic signs of the disease are difficult to assess.[2,15] However, during this phase, antibody titers to *E. canis* are usually high and their detection is the only method of confirming that a dog has CME. The importance of identifying dogs in this relatively early stage lies in the prognosis for treatment, which is considered good. This aspect is particularly relevant to breeds that are considered more sensitive to the development of severe chronic CME, such as German shepherd dogs.[8] The chronic phase of the disease is accompanied by pancytopenia, and the prognosis is usually grave. Antibody titers are usually high at this stage,[4] allowing for easy diagnosis of the disease.

The specificity of both the IFA and ELISA technologies is similar, which is important when considering possible cross-reactions with other rickettsial species. Cross-reactivity between *E. canis* and *E. ewingii, E. equi, E. risticii,* and *Neorickettsia helminthoeca* have been documented.[9,10,19]

The results of this study verify the ability of an ELISA test kit to measure *E. canis* IgG antibodies in dogs with a minimum of laboratory equipment and without specially trained personnel. The ELISA results were compared with those from the standard IFA technique for detection of *E. canis* antibodies; results from both tests were closely correlated. This ELISA could be used to aid in the diagnosis of CME.

Sources and manufacturers

a. Cellanalyzer CA 580 A, Medonic, Solna, Sweden.
b. Sigma, Ness Ziona, Israel.
c. Immunocomb, Biogal, Kibbutz Gal'ed, Israel.
d. Jackson Immunosearch Laboratories, Baltimore, MD.
e. Biosynth International, Skokie, IL.
f. Immunoscan, Orgenics, Yavneh, Israel.

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