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Research Article

Proposal for a Conventional Polymerase Chain Reaction Protocol for the Detection of *Ehrlichia Canis*

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Abstract

Canine Ehrlichiosis is an infectious disease with high morbidity and mortality among domestic dogs and other members of the *Canidae* family. It is found in countries throughout the world. It is transmitted by ticks and is caused by *Ehrlichia spp.,* an obligate intracellular Gram-negative bacterium. It has tropism for lymphocytes, monocytes, and granulocytes. It is endemic in tropical and subtropical regions and is increasingly spreading to areas with temperate climates. This may be due to both climatic and environmental changes that affect the distribution of ticks and travel made with pets, allowing the arrival of this disease to non-endemic areas. However, an increase in diagnosis is also contemplated due to an improvement in diagnostic techniques.

Depending on the phase of the disease (acute, subclinical or chronic), it can produce signs such as high fever, anorexia, corneal opacity, weight loss, epistaxis, vomiting, diarrhea, anemia, thrombocytopenia.

Diagnostic techniques include hematology, serology, bacterial isolation, and molecular techniques. Among these, the polymerase chain reaction (PCR) method offers a definitive diagnosis due to its greater sensitivity and specificity, as it uses genus-specific and species-specific primers.

Diagnosis is often difficult because coinfection with other tick-borne pathogens is common, complicating pathogenesis, clinical manifestations, diagnosis, and treatment. Therefore, it is important to choose a diagnostic technique with greater specificity and sensitivity.

This disease is a problem in veterinary medicine for the reasons mentioned above, as well as for the zoonotic potential of these agents and their potential impact on human health.

Thus, this work proposes a molecular protocol with *in silico* designed primers for the trp36 gene of *Erlichia canis* as a diagnostic method.

Background

Pathogenesis and Pathology

Ehrlichia spp. are obligate intracellular Gram-negative bacteria that infect monocytes, granulocytes, and platelets [1]. They do not synthesize Lipopolysaccharides (LPS) and produce little peptidoglycan, which diminishes the host's immune response and provides flexibility and plasticity, facilitating intravascular circulation in infected leukocytes [2]. In its infective form, it enters the cell by endocytosis, where it matures and multiplies, forming morulae with 20–40 infective cells. These morulae are released after a few days, initiating a new infectious cycle [2,3]. It spreads via the blood or lymphatic system within infected cells, reaching organs [4], such as the liver, spleen, bone marrow and lymph nodes where they multiply (incubation period of 8-20 days) [3,5].

Transmission

Rhipicephalus sanguineus ticks (*R. sanguineus*) are the main vector of *E. canis* transmission (hematophagous arthropods of the *Ixodidae* family, *Arachnida* class). *R. sanguineus* is the most widely distributed tick species in both tropical and subtropical areas [1], which are most susceptible to *E. canis* infestation [6,7]. Its prevalence is high year-round, while in temperate climates it only increases in summer [8]. There is no assessment of infestation by age, sex, or breed [1]. The tick becomes infected from larvae to nymphs

and from nymphs to adults, or during the infection, after ingesting blood from an infected dog. Once infected, they can inoculate the bacteria into a new host [3]. *E. canis* DNA has been detected in fox fleas (*Vulpes vulpes*) and in small mammals that do not belong to the Canidae family, suggesting another transmission cycle that may include other arthropods [3], blood transfusions and accidents with objects contaminated with infected blood as serious potential forms of transmission [3,4,9].

Clinical Signs

They vary depending on the phase: acute, subclinical, or chronic [2,10], the level of virulence of the strain, the pathogen dose, the host immune system, and breed (German Shepherds have higher morbidity and mortality) [3,4,9], and coinfection with other pathogens [3].

Common symptoms at different stages are nonspecific, the most common being fever, anorexia, corneal opacity, weight loss, epistaxis, vomiting, diarrhea, anemia, and thrombocytopenia (occurring in more than 90% of infected individuals). In severe cases, hepatomegaly, splenomegaly, nervous system damage (meningitis, ataxia, and seizure symptoms), hemorrhages, and significant weight loss may occur [2,3,9]. Respiratory signs such as dyspnea, seropurulent secretions in the nasal passages and conjunctival sacs, and even interstitial pneumonia are also present. In addition to lymphadenomegaly, there is a tendency to bleed (dermal petechiae and/or ecchymosis) due to thrombocytopenia, which is attributed to increased platelet consumption due to vasculitis, increased splenic sequestration and immunological destruction or decreased platelet half-life [3,9].

In the acute phase, unilateral or bilateral epistaxis, extravasculations at injection sites and in the anterior chamber of the eyes, blood in urine and feces have been described. These signs remit in 1-4 weeks, but if not treated, they can enter a subclinical phase, for months [10] and even up to 10 years without eliminating it and without clinical signs (asymptomatic) with mild thrombocytopenia. If the infection passes to the subclinical phase, it becomes chronic [3,9], a phase that can present in a mild or severe form, where we find pancytopenia, hemorrhagic diathesis, bone marrow aplasia (myelosuppression) [3], marked splenomegaly, glomerulonephritis, kidney failure, interstitial pneumonitis, anterior uveitis and meningitis [9]. Death is usually due to extensive hemorrhages or secondary infections [3].

Other alterations are: hypoalbuminemia, hyperglobulinemia and hypergammaglobulinemia, increased alkaline phosphatase, increased alanine aminotransferase and increased urea and creatinine concentrations, non-regenerative normocytic anemia and a level of leukopenia with monocytosis [3].

Diagnosis

Microscopic Visualization

Giemsa staining of peripheral blood smears can visualize morulae in monocytes using microscopy during the acute phase. It is inexpensive and rapid, but has low sensitivity [2]. White-cap smears, made from concentrated leukocytes, are also available to detect morulae, which has greater sensitivity. False negatives can occur, resulting in a diagnostic disadvantage and requiring highly trained personnel [2,3].

Serology

Serology involves the search for anti-*Ehrlichia* antibodies (ABs) using methods such as Enzyme-Linked Immunosorbent Assay (ELISA) and Indirect Immunofluorescence (IIF). The latter is considered the gold standard, but the ABs detected are not specific and cross-react with other Ehrlichia spp. agents [2,3,10]. *Anti-E. canis* IgG antibody detection tests are available (SNAP 3Dx® and SNAP 4Dx® from IDEXX laboratories) [2,3] with a sensitivity and specificity of 97.8% and 92.3%, respectively [2].

The IgG antibody response can persist for weeks or even months after infection, so we can have false positives [9].

Molecular Diagnosis

Polymerase Chain Reaction (PCR) has proven to be an effective tool for the molecular detection of *E. canis* [1]. It is genus and species-specific and allows for diagnosis in acute and chronic phases [2], confirming active *E. canis* infection before seroconversion by AC occurs [3]. There are methods based on the amplification of coding genes with highly conserved regions, achieving 100% specificity [2]. For *E. canis*, the 16S rRNA gene and the genes encoding the p28 and p30 proteins have been used as target genes, the latter being the most sensitive [2,3]. PCR samples can be blood, aspirates, or biopsies from organs such as lymph nodes, liver, bone marrow, or spleen [9]. The latter is more sensitive for assessing *E. canis* clearance. The disadvantage of PCR is that it is easily contaminated, so the use of controls at each step is recommended [3].

Treatment

Doxycycline is the antibiotic of choice and should be administered at a dose of 10 mg/kg every 24 hours for 3–4 weeks [3,9,11], as it eliminated *E. canis* infection in most dogs in studies. In chronic cases, supportive therapy may be required [9].

The phase of the disease during which treatment is initiated influences the final results; patients with acute or subclinical phases maintained negative PCR results when clinical parameters improved, but those treated in the chronic phase remained intermittently positive for *E. canis* [3].

Prevention

Prevention of ehrlichiosis and other tick-borne diseases is achieved by eliminating ticks from the environment and preventing re-infestation. This includes prophylactic management of the pet's environment [3,4]. Chemical control and infestation prevention with acaricides, collars, sprays, oral products, injectables, and chewable tablets are available [12].

If you live in areas with a high tick population, pets should avoid contact with wildlife. Environmental care should be provided in ar-

eas where pets are kept, such as areas surrounding the home, generally through the application of tick-repellent solutions [3]. Maintaining a balanced diet produces a better immune response. It does not prevent *E. canis* infection, but it does prevent complications [4].

Progress has been made in developing a vaccine based on an attenuated strain of *E. canis*, but it has not yet been successful [13].

Materials and Methods

For primer design, the Genbank database will be used to search for the trp36 gene sequence, and the Invitrogen Oligoperfect Design program will be used to select the primer.

Design Of Primers from the *Ehrlichia Canis Trp*36 Gene.

From the Genbank® database, we will access the *E. canis* trp36 gene sequences, with a total of 65 sequences. From these, we will select those containing at least 700 base pairs. Within these, we will search for a consensus sequence using the Oligoperfect Design® program, where a ranking of optimal matches will be provided.

For primer selection, the following criteria will be used:

a. Primer length: between 18-25 nucleotides

b. GC content (%GC): 50%

c. Melting temperature (Tm): Between 50-60°

d. Absence of secondary structures: the primers must not form Dimers (self- or heterodimers) or hairpin structures.

e. Specificity: region of the trp36 gene

Propose a Protocol for Conventional PCR Detection of *E. Canis*.

The proposal for specific conditions for molecular detection considers the use of the assays designed in the previous point, in conjunction with the generic conditions for any PCR reaction, such as:

PCR Reaction Mix: The mixture to be formed in each DNA-like PCR tube (from a suspect sample, the vaccine, a positive control, a negative control), the designed primers, and a Master Mix solution (containing Taq Polymerase, nucleotides ([A, T, C, G]), and Mg^{+2}). The PCR tube, referred to as the reagent control, will not contain DNA but nuclease-free water and all the other reagents already mentioned.

PCR Reaction: The PCR reaction uses a protocol compatible with conventional PCR, which generally includes three steps: denaturation at 94°C for 30 seconds; annealing at a temperature defined by the primer design, initially (Tm-5)°C for 30 seconds; and the polymerization process at 72°C for 1 minute. The above is repeated 35 times, followed by a final extension step at 72°C for 10 minutes.

Visualization of Amplified Products: Once the PCR reaction is complete, the product must be visualized using 2% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) buffer. This electrophoresis can be performed at 90 volts for 40 minutes, using a molecular size marker.

The agarose gel is then incubated in a GelRed solution for 30 minutes at room temperature.

Finally, the gel is observed under an ultraviolet light transilluminator and a photographic record is obtained. The positivity criterion includes the visualization of an expected DNA fragment (e.g., 400bp) compared to the migration of the marker used.

The resulting nucleotide sequences can be aligned using the Clustal Omega program, yielding a consensus sequence for each fragment. These consensus sequences can then be entered into the BLAST program to determine the percentage of nucleotide identity with respect to the Genbank® database.

Biosafety Measures: Laboratory procedures will be carried out in accordance with the biosafety standards established for the FAVET Microbiology and Virology laboratories, which include the use of clean materials, proper waste disposal, and the use of a closed apron and gloves during laboratory work. Visualization of the magnified product will involve the use of red gel and a UV light transilluminator. Finally, an acrylic plate and UV-filtering glasses will be used when viewing the gel.

Results

We expect to obtain specific primers based on the identification of a consensus sequence of the *E. canis* trp36 gene that meets the aforementioned selection requirements and, using conventional PCR, produces DNA fragments of the size indicated in the match ranking provided by the Invitrogen® Oligoperfect Design program and the procedure of electrophoresis.

The identity of these DNA fragments can be corroborated using the BLAST program, with prior knowledge of their nucleotide sequences.

Discussion

The methodology outlined above undoubtedly allows for the detection of *E. canis* or any other pathogen of veterinary or non-veterinary interest in any basic laboratory in the developing world. Currently, the use of programs for obtaining optimal primers is within reach. Furthermore, the use of databases such as Genbank® and the BLAST program undoubtedly aid in the final identification of the amplified product obtained. Thus, the fantastic or perhaps it happens brilliance of Kary Mullis [14] once again makes it so that there is currently no excuse for the challenge of identifying any pathogen.

Conclusion

This example of detection of a veterinary pathogen serves to exemplify the use of molecular biotools currently still within reach and unquestionably makes clear the legacy of Kary Mullis [15].

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Conflict of Interest

None.

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