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# First isolation and molecular characterization of *Ehrlichia canis* in Spain

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**Abstract**

This paper reports the first isolation and culture of *Ehrlichia canis* in Spain from a naturally infected dog using the DH82 cell line. After DNA extraction and PCR amplification, a nearly complete (1412 bp) sequence of the 16S rRNA gene of the new *E. canis* strain was obtained. The GenBank accession number for the nucleotide sequence of this strain is AY394465. This sequence was aligned with the 16S rRNA gene sequences of other *Ehrlichia* strains accessible in GenBank. The 16S rRNA gene sequence of the *E. canis* strain reported here showed a high percentage of similarity with the 16S rRNA gene sequence of *E. canis* from different geographic areas including Japan, Venezuela and Israel. These data confirm the presence of *E. canis* in Spain.


*Keywords:* *Ehrlichia canis*; Dog; Spain; Isolation; Sequence

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## 1. Introduction

*Ehrlichia canis* infection is a tick-borne disease of dogs. This agent is a Gram-negative obligatory intracellular bacterium with a tropism for monocytes and macrophages. It was first described in 1935 in Algeria, associated with acute cases of fever and anemia in several dogs (Donatien and Lestoquard, 1935). *E. canis* is transmitted by the brown dog tick *Rhipicephalus sanguineus* (Groves et al., 1975; Lewis et al., 1977). This tick is worldwide distributed but particularly frequent in tropical and subtropical regions (Ewing, 1972; Keefe et al., 1982; Ristic and Holland, 1993; Hua et al., 2000; Suksawat et al., 2001; Suto et al., 2001). The distribution of this vector is likely related to the prevalence of the disease caused by *E. canis*: the canine monocytic ehrlichiosis (CME) (Neer et al., 2002). Classically, three clinical stages have been differentiated: acute, subclinical, and chronic stage (Harrus et al., 1997). Clinical signs usually described in canine ehrlichiosis are fever, depression, anorexia, weight loss, hemorrhages, epistaxis, gastrointestinal signs like vomiting or diarrhea, respiratory disorders and ocular signs. Laboratory findings most frequently determined are thrombocytopenia, leukopenia, anemia and hipergammaglobulinemia (Woody and Hoskins, 1991).

In Spain, the first reference about canine ehrlichiosis is from the northeast of the country, in 1988 (Font et al., 1988). Since then, different cases of this disease have been reported in almost all regions of the country. In fact, in our laboratory we have detected antibody titers against *E. canis* in canine serum samples from every province of Spain. Several epidemiological studies have shown different prevalence rates depending on the areas. Seroprevalence rate varies from 1.98% in the Canary Islands to 19.2% in Castilla-León (Sainz et al., 1996). In Madrid (in the center of the country), the prevalence rate of *E. canis* has been established in 6.5% (Sainz et al., 1998). All these prevalence values have been determined by IFA test, considered the gold standard technique for diagnosis of canine ehrlichiosis (Neer et al., 2002). Nevertheless, it is important to consider the existence of cross-reactions between different species of *Ehrlichia*, when using this technique. Although *E. canis* has been suspected to be the mayor agent implicated in canine ehrlichiosis in Spain for many years, this could not be established definitely until its isolation and sequentiation.

In the present work it is reported the first isolation of *Ehrlichia* species in Spain, its culture and its genetic characterization. These data confirm the presence of *E. canis* in Spain.

## 2. Materials and methods

### 2.1. Case report

A 2-year-old female dog was admitted to the Veterinary Clinic Hospital of Madrid in April 2002. The animal had a massive tick infestation 3 weeks prior, and presented vomiting, cough and hematuria for 4 days. Physical examination revealed apathy, fever (39.4 °C), pale mucous membranes, and petechiae in conjunctive, oral and vulvar mucous membranes. In addition, the dog presented generalized lymphadenopathy. Complete blood

cell counts revealed thrombocytopenia (platelet count: $15 \times 10^3$ $\mu l^{-1}$) and anemia (PCV: 20.0%, hemoglobin: 6.2 g/dl, red blood cell count: $2.92 \times 10^6$ $\mu l^{-1}$). The observation of 17 erythroblasts/100 white blood cells in a blood smear suggested the presence of a regenerative anemia. White blood cell count was $13.3 \times 10^3$ $\mu l^{-1}$. Blood biochemistry revealed hypoproteinemia (5.4 g/dl). The rest of the hematological and blood chemistry parameters remained within the physiological range.

According to clinical signs, a tentative diagnosis of canine ehrlichiosis was performed. Empirical treatment with imidocarb dipropionate (5 mg/kg SQ) was initially administered until the diagnosis was confirmed. In order to avoid side effects of this drug, atropine was also administered. Support therapy was started, but unfortunately the dog died 24 h later. Necropsy could not be performed.

Serology for *E. canis* and *Leishmania infantum* was performed. IFAT for *L. infantum* was negative. IFAT for *E. canis* was positive (1:160). Blood was also collected for PCR and culture.

### 2.2. Isolation and culture of E. canis in a continuous cell line

A heparinized 5 ml blood sample was collected aseptically from the jugular vein of the dog reported on here. The sample was stored at 4 °C until it was processed on the same day of its extraction. The blood was centrifuged at $1000 \times g$ for 10 min. The buffy coat obtained was harvested and overlaid directly onto monolayers of the continuous canine cell line DH82 (ATCC number: CRL-10389) in a 25 $cm^2$ tissue culture flask. It was grown and maintained according to methods previously described (Dawson et al., 1991).

The culture medium was changed once weekly, and the supernatant was collected in order to detect infectivity by examination of a Giemsa-stained cytocentrifuged preparation, indirect immunofluorescence antibody (IFA) test or polymerase chain reaction (PCR).

### 2.3. DNA extraction and PCR

DNA was extracted from 200 μl of EDTA-anticoagulated peripheral blood and from 200 μl of culture medium (after supernatant centrifugation and concentration of all the cells). DNA was isolated with a QIAamp DNA Blood KIT (Qiagen) according to the manufacturer's instructions. Additionally, DNA was extracted from the culture cell line DH82 infected with *E. canis* (Oklahoma isolate, ATCC number: CRL-10390) and from a non-infected DH82 cell line culture, to serve as positive and negative controls, respectively.

Extracted DNA (5 μl) was used as a template to amplify a fragment of the 16S rRNA gene of *E. canis* by nested PCR as previously described (Wen et al., 1997) with some modifications. Briefly, in a 50 μl reaction mixture containing 5 μl of 10× PCR buffer, 2 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphate mixture, 1.2 U of *Taq* polymerase (Biotools), 0.5 μmol of primer ECC (Dawson et al., 1994), and 0.5 μmol of primer ECB (Dawson et al., 1994). PCR was performed at 94 °C for 4 min and then for 40 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min.

The conditions were the same in the second PCR, except for the DNA templates (1 μl of the product of the first PCR) and the primers: 0.5 μmol of primer HE-3 (Dawson et al., 1994) and 0.5 μmol of primer ECA which is the sequence of the *E. canis* gene corresponding to *E. chaffeensis* primer HE-1 (Anderson et al., 1992).

### 2.4. Sequence analysis of the 16S rRNA Ehrlichia gene

Universal primer pairs EC9 and EC12 were used to amplify nearly the entire 16S rRNA gene of eubacterias (Anderson et al., 1991). In order to sequence the 1400 bp of the corresponding fragment, an internal primer was used. One minute at 94 °C, 2 min at 48 °C, and 1 min 30 s at 66 °C, followed by 27 cycles at 88 ° (1 min), 52 ° (2 min), and 68 °C (1 min and 30 s), ending with an hour at 10 °C were used as cycling conditions.

The sequencing reaction of the PCR product (1412 pb) obtained was carried out in an automatic DNA sequencer ABI 3100, using the Big Dye sequencer kit version 3.1 (Applied Biosystems) and following manufacturer's instructions.

DNA sequences for the 16S rRNA of all the strains tested were processed to yield a Jukes-Cantor distance matrix by using the Molecular Evolutionary Genetics Analysis (MEGA program version 2.1). This distance measure gives the maximum likelihood estimate of the number of nucleotide substitutions between two sequences. The topology of the dendrogram was performed using Neighbour joining method and a bootstrap test was conducted to assess the reliability of the nodes of the tree.

### 2.5. Nucleotide sequence accession numbers

The GenBank database accession numbers for the 16S rRNA nucleotide sequences of organisms used for comparison in this study are as follows: *E. canis* Oklahoma, M73221; *E. canis* Florida, M73226; *E. canis* Israel, U26740; *E. canis* Germishuys, U54805; *E. canis* Gzh982, AF162860; *E. canis* Gxht67, AF156786; *E. canis* Gdt3, AF156785; *E. canis* Okinawa, AF308455; *E. canis* VHE, AF373612; *E. canis* VDE, AF373613; *E. canis* from ticks, AF373614; *E. chaffeensis*, M73222; *E. ewingii*, M73227; *E. muris*, U15527; *E. ruminantium*, AF069758; *Anaplasma platys*, AF156784; *A. platy*s Venezuela, AF287154; and *N. helminthoeca*, U12457. The nucleotide sequence reported here has been assigned GenBank accession number: AY394465.

## 3. Results and discussion

Nested PCR from the dog's blood using *E. canis*-specific primers was positive. Blood aseptically collected from the case report was inoculated into DH82 cells for culture isolation. One month later, cytoplasmic inclusions compatible with *Ehrlichia* were detected in the supernatant of culture. When 8% of the cells were infected, the monolayer was removed and its cells were divided into two new flasks. Until now, the isolate has been successfully subcultured 36 times in this cell line.

A nearly complete (1412 bp) sequence of the 16S rRNA gene of the new *E. canis* strain was obtained. This sequence was aligned with the 16S rRNA gene sequences of other *Ehrlichia* strains accessible in GenBank.

The schematic representation of the nucleotide sequence differences between 16S rRNA genes of Spanish ehrlichial agent and other ehrlichial agents is showed in a dendrogram (Fig. 1). The 16S rRNA gene sequence of the *E. canis* strain reported in the present study has shown the highest percentage of similarity with the 16S rRNA gene

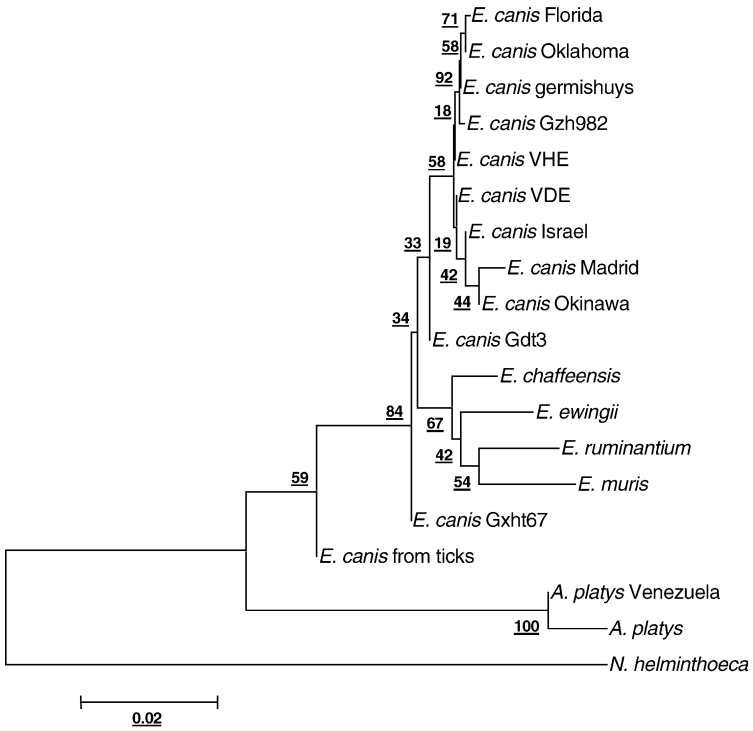


Fig. 1. Dendrogram showing the genetic relationship between the 16S rRNA sequences of different *Ehrlichia* spp. isolates. The legend bar represents the substitution rates of 2 per each 100 nucleotides. Values above branches indicate bootstrap percentages from 5000 iterations.

sequence of *E. canis* Okinawa, reported as the first confirmed case of *E. canis* infection in Japan (Suto et al., 2001). However, it must be taken into account that the sequence available for this Japanese isolate is only 358 pb, and the possibility that other nucleotide sequence differences occur in the rest of the sequence cannot be ruled out.

Apart from this isolate, the 16S rRNA gene sequence of the two Venezuelan isolates AF373612 (Unver et al., 2001) and AF373613 (Unver et al., 2001), and the 16S rRNA gene sequence of the *E. canis* strain U26740 isolated in Israel (Keysary et al., 1996) have appeared as the most similar to the sequence of the Spanish *E. canis* isolate. It should be noted that there are few but significant differences among the *E. canis* strains. The sequences of Spanish isolate differ in one nucleotide from the Venezuelan strains and in three nucleotides from the Israel strain. Furthermore, *E. canis* Madrid has a unique polymorphism at position 739 (AC) when comparing the 1412 bp fragment corresponding to the 16S rRNA gene sequence and the sequence of the other *E. canis* isolates. Other strains also show unique polymorphisms: *E. canis* Israel has AC at position 968 and GA at position 1296; the strain Gzh982 differs at position 1227 (CT), and Florida at position 1253 (CT).

The very low polymorphism shown among the *E. canis* strains, also referred by other authors (Unver et al., 2003) suggests a common origin with a non very distant divergence in

time as shown in Fig. 1, where bootstrap value for the node separating most of the *E. canis* strains is 84.

Even though many of the isolates of *E. canis* currently described have many different geographic origins, most of them are very closely related on the basis of their 16S rRNA gene sequence. It has been recently proposed a cautious and balanced approach to taxonomy, considering not only molecular information but also phenotypical characteristics (Uilenberg et al., 2004). In fact, when comparing strains from different geographic origins as antigen for IFA test, slightly higher titers have been found using local isolates (Keysary et al., 1996). This may be related to the existence of subtle antigenic differences among *E. canis* organisms in different regions of the world (Hegarty et al., 1997) and to the greater specificity for the autochthonous strains (Keysary et al., 1996).

*E. canis* has traditionally been accepted as the major etiological agent of canine ehrlichiosis in Spain. Nevertheless, until now it had not been possible to isolate, culture and identify by genetic characterization any *Ehrlichia* species in Spain. In fact, we have not been successful in other attempts to obtain an isolate from several suspected clinical cases of canine ehrlichiosis. The Spanish isolate described here may be used for the development of different diagnostic techniques in our country.

Canine ehrlichiosis is a common disease in Spain. The disease was first described in the northeast of the country in 1988 (Font et al., 1988). The chronic evolution and the long periods of subclinical phase of this disease allow the detection of cases of ehrlichiosis throughout the year. However, acute cases are especially common in summer, spring and autumn, the seasons during which *R. sanguineus* is mainly active in our latitude.

The clinical case included in this study was treated in April at the Veterinary Hospital of Madrid, and its clinical and laboratory findings showed it was very likely suffering an acute phase of the disease. In our geographic area, the high prevalence of canine leishmaniasis, as well as the similarity of a great number of their clinical and laboratory signs obliges to consider leishmaniasis as the main process in the differential diagnosis of canine ehrlichiosis.

Until now, evidence of *E. canis* infection in Spain was based on the detection of antibodies against *E. canis* or the detection of DNA of this agent by biomolecular techniques. Although the IFA test is considered the gold standard technique in the diagnosis of canine ehrlichiosis (Neer et al., 2002), it has the disadvantage of presenting cross-reactions with other *Ehrlichia* species like *E. chaffeensis* or *E. ewingii*.

Although PCR makes it possible to detect all sequenced *Ehrlichia* spp. and can be used to identify individual species of *Ehrlichia* by designing species-specific primers, sensitivity is influenced by the sort of sample (Harrus et al., 1998). During the chronic phase of the disease, the sensitivity of PCR in blood samples may decrease because of the low presence of the agent in the sample. Additionally, there is no standardization among laboratories and insufficient quality controls can result in both false-positive and false-negative results (Neer et al., 2002). For the time being, PCR should be used in conjunction with serology for the initial diagnosis of canine ehrlichiosis (Neer et al., 2002).

With the isolation and DNA sequencing of the Spanish strain described in this work, *E. canis* can be confirmed as a causative agent of canine ehrlichiosis in Spain. Nevertheless, the presence of other species of *Ehrlichia* or other closely related genera in the dog in our country cannot be ruled out. It also has been suggested, based on clinical and serological criteria, that *A. platys* (formerly *Ehrlichia platys*) infection occurs in dogs in Spain (Sainz

et al., 1999). In the same way, different serologic studies have shown the presence of seropositive dogs for *Neorickettsia risticii* (formerly *Ehrlichia risticii*) and *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) (Sainz et al., 2000). However, the existence of cross-reactions among different species of *Ehrlichia* and other close genera, as well as the similar clinical signs caused by those agents, leads to the necessity of using more specific diagnostic techniques.

In our country, some studies have been performed on ehrlichiosis in other species apart from dogs. Although the agent has not been successfully isolated, different studies have described the presence of *A. phagocytophilum* in ruminants, ticks and humans in the north of Spain (Juste et al., 1989; García Pérez et al., 2000; Oteo et al., 2000). Further researches are required to determine the presence of different species of *Ehrlichia* or other closely related genera in Spain.

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