Molecular detection and characterization of *Ehrlichia canis* from dogs in Turkey

**Molekularer Nachweis und Charakterisierung von *Ehrlichia canis* bei Hunden in der Türkei**

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Summary

Seroprevalence of *Ehrlichia canis* antibodies among dogs in Turkey were previously reported, however, the ehrlichial organism has never been characterized in this region. The current study examined dogs from Ankara with febrile illness for *E. canis* infection with *E. canis*-specific PCR. Three of the 12 blood specimens from dogs showing clinical signs compatible with canine ehrlichiosis were found to be positive by PCR using *E. canis*-specific primers. *E. canis* detected in one of the blood specimens was designated as Kutahya strain. The representative *E. canis* strain was characterized by 16S rRNA gene sequencing and Western blot analysis of the plasma sample from the dog infected with *E. canis*. The 16S rRNA sequence (1,388 bp) of the *E. canis* Kutahya was identical to that of *Ehrlichia ovina* from a sheep in Turkey and Venezuelan Dog Ehrlichia (VDE) and was closely related (99.9 %) to that of type strain of *E. canis*, Oklahoma. The plasma of the dog infected with *E. canis* Kutahya was analyzed by Western blotting using the purified *E. canis* Oklahoma strain as antigen. The reactive antibody profiles of the dog infected with *E. canis* Kutahya was found to be similar to those of dogs infected with *E. canis* Oklahoma and VDE, suggesting the antigenic similarities among these strains. The findings in this study would help for a better understanding of epidemiology of canine ehrlichiosis. This is the first report of molecular detection and characterization of an ehrlichial agent in Turkey.

**Keywords:** *Ehrlichia canis*, dogs, 16S rRNA gene, Western blot, Turkey

Zusammenfassung


**Schlüsselwörter:** *Ehrlichia canis*, Hunde, 16S rRNA Gen, Westernblot, Türkei
Introduction

_Ehrlichia canis_, a tick-borne zoonosis, is the causative agent of canine monocytic ehrlichiosis (CME). It is a gram-negative obligate intracellular bacterium with a tropism of canine monocytes and macrophages. After the first description in Algeria in 1935 (Donatein and Lestoquard, 1935), CME was later recorded from numerous areas worldwide but at higher frequencies in tropical and subtropical regions (Keef et al., 1982; Ristic and Holland, 1993; Hua et al., 2000; Sukawat et al., 2001; Unver et al., 2001a; Unver et al., 2003).

After the incubation period of 8–20 days, the acute phase of CME may be characterized in dogs by fever, depression, dyspnea, anorexia, and weight loss, with laboratory findings of thrombocytopenia, leukopenia, mild anemia and hypergammaglobulinemia. Following the acute phase, a clinical apparent phase may last 40–120 days or years with persistent ehrlichial infection and mild thrombocytopenia. In the chronic phase, hemorhorages, epistaxis, hypotensive shock and edema may occur in addition to the clinical signs and laboratory findings of the acute phase, which are often exacerbated by coinfection with other microorganisms (Buhles et al., 1974; Codner and Farris-Smith, 1986; Rikihisa, 1991; Ristic and Holland, 1993). The dogs may remain infected with _E. canis_ without or sometimes even with a proper treatment (Jqbal and Rikihisa, 1994; Wen et al., 1997).

_E. canis_ infection among dogs in Turkey was previously detected by serological methods (Batmaz et al., 2001). However, the ehrlichial organisms in Turkey have so far not been characterized. _E. chaffeensis_ and _E. ewingii_, closely related to _E. canis_, may also be responsible for canine ehrlichiosis (CE) and the infections by these three organisms can not be distinguished from each other by fluorescent antibody test due to the antigenic cross reactions among them (Rikihisa et al., 1994; Unver et al., 1999). The characterization of an agent in dogs with CE is important for epidemiological studies and for designing diagnostic and prophylactic measures. Therefore, the current study was conducted to characterize the ehrlichial agent from dogs with CE in Turkey by 16S rRNA gene sequencing and to analyze the plasma of CE patients by Western blotting. The first report of molecular detection and characterization of an _Ehrlichia_ sp. in dogs from Turkey.

Materials and methods

Dog blood samples and DNA isolation

Blood specimens were collected in Ankara, Turkey, from 12 dogs of different ages and various breeds showing clinical signs compatible with those of CME. All the dogs had a history of tick bite. Heparinized 5 to 10-ml blood specimens were collected from each dog. After centrifugation of blood samples, plasma was collected and saved for serology. The Buffy coat layer was removed and the peripheral blood cells were washed two times with phosphate-buffered saline (137 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl, and 1.8 mM KH2PO4 [pH 7.2]).

DNA was isolated from these washed cells by using QIAamp blood kit (Qiagen Inc, Valencia, CA, USA), according to the manufacturer’s instructions. Before elution of DNA, QIAamp columns were washed three times with the buffer provided with the kit. DNA concentrations were determined by measuring the absorbance at 260 nm (A260) with a GeneQuant II RNA and DNA calculator (Pharmacia Biotech Inc., Cambridge, England).

PCR

The nested PCR was carried out to detect _E. canis_ DNA as described previously with primers ECC-ECB (outside pairs) and HE3-ECA (nested pairs) specific to the 16S rRNA gene of _E. canis_ (Wen et al., 1997). Briefly, the amplification was carried out in a 50-μl reaction mixture including PCR buffer, 1.5 mM MgCl2, lmol of primer pairs, 0.2 mM each of dNTP mixture, 1.5 μl of Taq DNA polymerase and 0.5 μg of DNA with 4 min of denaturation at 94°C followed by 40 cycles each consisting of 1 min of denaturation at 94°C, 1 min of annealing at 60°C and 1 min of extension at 72°C. The final extension was allowed to continue for 7 min. PCR products were electrophoresed in 1.5% agarose gels and visualized with ethidium bromide staining.

Sequence analysis of the 16S rRNA gene in dog blood samples

_E. canis_ Kutahya, in one of three PCR-positive samples was chosen as representative for 16S rRNA gene sequencing. Nearly the entire 16S rRNA gene was amplified as two fragments with primer pairs ECC (5’ AGAACAGACCGTCGGCGCAAGCC 3’)-16UnIR1 (5’ ATGCTTCGCGACCGTGATTCC 3’) and Africa1F (5’ ACGATGA GTGCTTAATGTGAG 3’)-1400R (5’ CAGTCACCTTGTCACGAC 3’), respectively. PCR was performed as described above except that the annealing temperature was 55°C. Two microliters of amplicons observed at the expected sizes of 843 and 695 bp on agarose gel electrophoresis were cloned into the PCRII vector of a TA cloning kit (Invitrogen Co., San Diego, CA, USA) as described by the manufacturer. Recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and the inserts were sequenced by a dyeode chain termination method with the universal synthetic primers M13 and T7 in the Plant-Microbe Genomics Facility at the Ohio State University, Columbus, OH, USA. The sequences of two clones from the sample were determined on both DNA strands. The alignment of DNA sequences was performed by using the DNAStar program (DNASTAR Inc., Madison, WI, USA).

Culturing of _E. canis_

The Oklahoma isolate of _E. canis_, used as antigen in indirect fluorescent antibody (IFA) assay and Western immunoblotting (WB), was propagated in DH82, a canine macrophage cell line. The cultured cells were maintained in Dulbecco’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 10 mM N-(2-hydroxyethyl)-piperezine-N’-(4-butanesulfonic acid) buffer in a humidified 37°C incubator with 5% CO2, 95% air as previously described (Rikihisa et al., 1994). The cells were examined for infectivity by microscopic examination of a Diff-Quik (American Scientific Product, Obetz, Ohio, USA)-stained cytospin preparation and harvested when they reached to 90–100% infectivity.

Indirect fluorescent antibody assay (IFA)

An IFA was performed as previously described (Rikihisa et al., 1994). DH82 cells infected with strain Oklahoma isolate of _E. canis_ were used for the preparation of antigen.
TABLE 1: Nucleotide sequence similarities of 16S rRNA gene of E. canis Kutahya compared to other selected Ehrlichia spp.

<table>
<thead>
<tr>
<th>Ehrlichia spp.</th>
<th>GenBank Accession</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. canis Kutahya</td>
<td>AF621071</td>
<td>100</td>
</tr>
<tr>
<td>E. ovina Turkey</td>
<td>AF318946</td>
<td>100</td>
</tr>
<tr>
<td>E. canis VDE</td>
<td>AF373613</td>
<td>100</td>
</tr>
<tr>
<td>E. canis Oklahoma</td>
<td>M73221</td>
<td>99.9</td>
</tr>
<tr>
<td>E. canis Kagoshima</td>
<td>AF536827</td>
<td>99.9</td>
</tr>
<tr>
<td>E. canis 611</td>
<td>U62470</td>
<td>99.8</td>
</tr>
<tr>
<td>E. canis Germanys</td>
<td>U58405</td>
<td>99.6</td>
</tr>
<tr>
<td>E. canis Gzh982</td>
<td>AF162860</td>
<td>99.6</td>
</tr>
<tr>
<td>E. canis Madrid</td>
<td>AY394465</td>
<td>98.6</td>
</tr>
</tbody>
</table>

* The values are percent 16S rRNA gene sequences identities for 1,388 bases which could be unambiguously aligned (bases 1-1,388 in the E. canis Kuta-

haya).

1 E. canis Kutahya in the present study.
2 E. ovina from sheep in Turkey (Jongejan et al., 1993).
3 E. canis culture isolate from a dog in Venezuela (VDE, Unver et al., 2001a).
4 E. canis type strain culture isolated from a dog in Oklahoma.
5 E. canis DNA from Kagoshima, Japan (Unver et al., 2003).
6 E. canis culture isolate isolated from a dog in Israel.
7 E. canis DNA from a sheep in South Africa (Allopp et al., 1997).
8 E. canis DNA from a dog in The People's Republic of China (Hua et al., 2000).
9 E. canis DNA from a dog in Madrid, Spain.

*Similarity was calculated based on the updated sequence reported by
Unver et al. (Unver et al., 2001a).

Nucleotide sequence accession numbers
The 16S rRNA gene sequence of E. canis Kutahya reported here has been assigned to the GenBank accession number AY621071.

Results
The dogs from which blood samples were collected had a recent history of tick infestation. The clinical findings varied from acute to chronic stage of the disease. Fever was the common symptom of dogs associated with leucopenia. The other signs were depression, anorexia, weight loss, dyspnea, ocular discharge, epistaxis, lymphadenopathy with mild enlarged lymph nodes. Three of 12 dog blood samples that were analyzed by PCR using E. canis-specific primers were positive by observing 389-bp PCR products (data not shown). E. canis detected in one of the PCR-positive samples was chosen as representative for further molecular characterization. For this purpose, nearly complete (1,410-bp) 16S rRNA gene sequence of this representative strain was obtained and the organism was designated as E. canis Kutahya'. This sequence was aligned and compared with GenBank-accessible selected sequences of E. canis from diverse geographical regions and Dhrlichia ovina from a sheep in Turkey (Jongejan et al., 1993). The 16S rRNA gene sequence of E. canis Kutahya in the current study was 100% identical to that of E. ovina from Turkey and Venezuelan Dog Ehrlichia (VDE, Unver et al., 2001a). The nucleotide sequence similarity data of the 16S rRNA gene of E. canis Kutahya to other selected Ehrlichia spp. are shown in Table 1.

Figure 1 shows the results of Western blot analysis of plasma samples from the representative dog in which E. canis Kutahya was detected and a dog experimentally infected with E. canis Oklahoma. In order to determine the reactive antibodies to ehrlichial antigens in plasma samples, two negative controls (DH82 and pET29-transformed E. coli lysate) and purified E. canis Oklahoma and affinity-purified rP30 of E. canis were used as antigens for each Western blotting. The plasma of the dog in which E. canis Kutahya was detected reacted with major antigens with approximate molecular sizes of 110-, 95-, 70-, 65-, 58-, 51-, 48-, 43-, 32- and 24-kDa of E. canis Oklahoma (Fig. 1A). This reaction pattern was similar to the pattern with the plasma from the experimentally infected dog (Fig. 1B). In addition to these antigens, plasma from the experimentally infected dog reacted with 30- to 28-kDa E. canis antigens with which the plasma from the E. canis Kutahya infected-dog did not react (Fig. 1A and B). The rP30 antigen was also used in WB since we have previously demonstrated that rP30 is a sensitive antigen in immunoblot analysis for serodiagnosis of human E. chaffeensis and canine E. canis (Ohashi et al., 1998; Unver et al., 1999) and, therefore, the reactivity of the test plasma or serum with rP30 is an important indicator in serodiagnosis of monocytic ehrlichiosis. Both two dog plasma samples strongly reacted with rP30 (Fig. 1). The bands reactive with both plasma in purified E. canis and rP30 antigens in WB were not found in the negative controls, DH82 and pET29-transformed E. coli lysate.

Discussion
Ehrlichial infection among dogs in Turkey was previously reported based on the result of IFA test using the
Israeli isolate of *E. canis* as antigen (Batmaz et al., 2001). Due to serological cross-reactivity of *E. canis* with *E. chaffeensis*, *E. ewingii* and *E. ruminantium* (Rikihisa et al., 1994; Unver et al., 1999), it has been inconclusive which *Ehrlichia* sp. and strain is responsible for canine ehrlichiosis in Turkey. Therefore, detection of *E. canis* in dogs with canine ehrlichiosis (CE) and comparative 16S rRNA gene sequence analysis of the agent in this study seemed to be a useful approach for better understanding the epidemiology of CE and for implementing diagnostic, control and preventive measures in Turkey.

Based on the 16S rRNA gene sequence comparison, *E. canis* Kutahya was found to be identical to that of *E. ovin* originated from a sheep in Turkey (Jongejan et al., 1993) and to that of *E. canis* strain from a dog in Venezuela (VDE, Unver et al., 2001a). Second most genetically related organisms to *E. canis* Kutahya based on 16S rRNA gene sequence analyses were Oklahoma and Kagoshima 1 strains of *E. canis*. High prevalence of *E. canis* infection among dogs was frequently reported from diverse geographical regions such as USA, Europe, Africa, Middle East and Far East Asia analyzed by serological methods or PCR and 16S rRNA sequence of *E. canis* strains were reported from diverse regions of the world (Murphy et al., 1998; Hua et al., 2000; Sukswat et al., 2001; Unver et al., 2001a; Unver et al., 2003). Interestingly, *E. canis* Kutahya in the current study is genetically more similar to the strains geographically far from Turkey (Venezuela and Japan) than those of geographically closer regions (Israel and Spain) (Table 1). These findings support the previous theory of a lack of geographic segregation of *E. canis* strains (Felek et al., 2003a; Felek et al., 2003b; Unver et al., 2001a). Analyses of more strains in this region and sequencing of more genes in addition to 16S rRNA gene or further molecular epidemiological typings such as groEL of outer membrane protein genes are expected to help validating this hypothesis in future studies. Moreover, culture isolation and propagation of the organism will help to characterize the agent in more detail and to develop an antigen in serologic tests to sensitively and specifically diagnose ehrlichiosis in Turkey.

Detection of *E. canis* among febrile dogs of systemic illness in this study suggests that veterinarians need to consider ehrlichiosis as differential diagnosis febrile patients of systemic illness with a history of tick-bite or travel to tick-infested regions. We previously showed that the same organism is found in both humans, dogs and ticks in Venezuela (Perez et al., 1996; Unver et al., 2001a) showing the zoonotic potential of VDE. Therefore, an attention needs also be paid by physicians in this area. Since veterinarians and public health professionals have insufficient awareness of ehrlichiosis and very limited laboratories can perform diagnosis of ehrlichiosis in Turkey, the disease may be unreported or undiagnosed in this region. Since CE progresses without showing severe clinical symptoms in most of PCR-positive dogs (Wen et al., 1997), the recognition of this disease based on the manifestation of significant clinical signs may also cause CE cases to remain underrecognized in Turkey. In addition to *E. canis*, *E. chaffeensis* and *E. ewingii* were reported from naturally infected dogs (Murphy et al., 1998; Sukswat et al., 2001), therefore, these two organisms should also be considered during diagnosis of CE.

*E. canis* infection in a sheep showing the clinical signs indistinguishable from heartwater has been reported from South Africa (Allsopp et al, 1997). The 16S rRNA gene sequence of *E. canis* Kutahya from a dog in this study was identical to *E. ovin* from a sheep in Turkey. This may support our previous hypothesis that *E. canis* infection may not be limited to *Canidae* as previously thought (Ristic and Husxoll, 1984; Unver et al., 2001a). Further antigenic and genetic characterizations are need to conclude whether *E. canis* Kutahya from a dog and *E. ovin* from a sheep in Turkey are the same organisms.

Designation of the name of *E. canis* strain described here as Kutahya is based on the particular location in which the infected dog has been raised. Since the results of this study still present inadequate evidence to conclude whether *E. canis* Kutahya, VDE and *E. ovin* from Turkey (Jongejan et al., 1993) are the same organisms prior to the further characterizations, a surrogate name as *E. canis* Kutahya was used in this study.

Western blot analysis of plasma samples from dogs clinically infected with *E. canis* Kutahya and experimentally infected with *E. canis* -Oklahoma shows that these two plasmas have similar reactive antibody profiles indicating similar antigenic characteristics between the two organisms. However, different reactive antibody patterns between the two plasma samples were observed regarding their recognition of 30-kDa-range antigens of *E. canis* encoded by a multigene family differentially transcribed in dogs and ticks (Ohashi et al., 1998; Unver et al., 2001b). The plasma of the *E. canis* Oklahoma-infected dog reacted with 28- to 30- and 32-kDa of purified *E. canis* antigens, while the plasma from *E. canis* Kutahya-infected dog reacted with the 32-kDa antigen.

![FIGURE 1: Western immunoblot analysis of the plasma from the dog naturally infected with *E. canis* Kutahya (1:640 *E. canis* IFA titer) (A), the dog (10184) experimentally infected with *E. canis* Oklahoma (1:5,120 *E. canis* IFA titer) (B). Lanes: DH82, dog macrophage cell line DH82 (negative control); *E. canis*, purified *E. canis*; pET, pET29 transformed E. coli (negative control); rP30, affinity-purified recombinant fusion protein of *E. canis* (27 kDa). The numbers on the left of each panel indicate molecular masses in kilodaltons based on the Precision Plus Protein Standards (Bio-Rad). The arrowhead indicates the position of rP30.](image-url)
only. This may be due to differences between $p30$s of Kutahya and Oklahoma strains at DNA, mRNA and/or protein levels.

The brown dog tick, *Rhipicephalus sanguineus*, has been reported to be the primary vector responsible for transmission of *E. canis* worldwide (Groves et al., 1975; Ristic and Holland, 1993) and an experimental study showed that *Dermacentor variabilis* can also transmit *E. canis* among dogs (Johnson et al., 1998). Turkey, especially Southern Turkey, is highly endemic for numerous tick species such as *R. sanguineus, Haemaphysalis concinna, Hyalomma anatolicum, Hyalomma marginatum, D. marginatus* and *Ixodes ricinus* (Leeson, 1951; Goddard, 1993). Analyses of different tick spp. for presence of *E. canis* and other ehrlichial organisms are needed to assess potential tick spp. responsible for transmission of *Ehrlichia* spp. and to better understand epidemiology of ehrlichiosis in Turkey and surrounding regions.

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References


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