

## Molecular Evidence of a New Strain of *Ehrlichia canis* from South America<sup>∇</sup>

Javier Vinasco,<sup>1</sup> Olga Li,<sup>3</sup> Arnaldo Alvarado,<sup>3</sup> Diego Diaz,<sup>3</sup> Luis Hoyos,<sup>3</sup> Luis Tabachi,<sup>3</sup>  
 Kamesh Sirigireddy,<sup>1</sup> Carolyn Ferguson,<sup>2</sup> and Manuel H. Moro<sup>1\*</sup>

Departments of Diagnostic Medicine/Pathobiology<sup>1</sup> and Division of Biology,<sup>2</sup> Kansas State University, Manhattan, Kansas 66506,  
 and Laboratory of Clinical Pathology, College of Veterinary Medicine, University of San Marcos, Lima, Peru<sup>3</sup>

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**Blood samples from dogs with clinical signs compatible with ehrlichiosis were examined for infection of *Ehrlichia canis* using PCR, multiplex real-time PCR, and DNA sequencing analysis. Eleven of 25 samples were positive for a new strain of *E. canis*. This is the first molecular identification of *E. canis* infection in dogs from Peru.**

*Ehrlichia canis* is a rickettsial gram-negative bacterium responsible for canine monocytic ehrlichiosis (CME) in dogs (6, 7, 19) and is mainly transmitted by ticks of the *Rhipicephalus* group (9, 15). *E. canis* has a wide distribution in the world but most frequently occurs in tropical and subtropical regions (2, 8, 11, 12, 21). Recently there have been reports of *E. canis* in South America (1, 6, 16, 17), including the first report of a human case of *E. canis* infection in Venezuela (24). The severity of disease depends on the host's immune status, age, breed, and any current coinfection. Clinical manifestations may vary geographically, but in general the main clinical signs are anemia, thrombocytopenia, and leukopenia (14, 27). Microscopic and serological evaluations have been used in conjunction with clinical signs for diagnosis of CME. Recently, molecular tools, like PCR, have become more sensitive and specific methods to aid in the diagnosis of CME. The specific PCR amplifies a segment of the 16S rRNA gene, and sequences of this gene have been used to identify different strains of *E. canis* (26). Here we report the first genetic characterization of *E. canis* in dogs from Peru.

Twenty-five dogs with clinical signs compatible with *Ehrlichia* sp. infection that were brought by their owners to the Veterinary Teaching Hospital (Universidad de San Marcos, Lima, Peru) from July 2002 to June 2003 were selected for the study. The ages of the animals ranged from 6 months to 11 years, and 80% were males. More than 90% had a history of tick exposure as well as either thrombocytopenia or nonregenerative anemia, fever, anorexia, weight loss, ecchymoses, petechiation, and/or epistaxis.

Serum samples were evaluated for *E. canis* (SNAP 3 DX; IDDEX Laboratories, Westbrook, ME) according to the manufacturer's protocol. All 25 samples were positive by *E. canis* enzyme-linked immunosorbent assay.

DNA was extracted from EDTA-treated blood samples using a QiaAmp kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's protocol. Specific *E. canis* amplification was accomplished using a previously described technique (26)

with some modifications. In this PCR, we used the forward primer CANIS (CAATTATTTATAGCCTCTGGCTATAGGA), reverse primer GAIUR (GAGTTTGCCGGGACTTCTTCT), and a HotStarTaq master mix kit (QIAGEN, Inc., Valencia, CA). Also, the multiplex real-time PCR (MRT-PCR) was used to detect *E. canis*, *E. chaffeensis*, *E. ewingii*, *Anaplasma platys*, and *A. phagocytophilum* (20), with DNA isolated as described above. All PCRs were performed using the Smart Cycler system (Cepheid, Sunnyvale, CA), as described previously. Eleven of the 25 blood samples tested positive for *E. canis* by specific PCR. The same 11 samples were also positive based on the MRT-PCR. None of the samples was positive for *E. chaffeensis*, *E. ewingii*, *A. platys*, or *A. phagocytophilum*.

The 16S rRNA gene of *E. canis* was amplified as two fragments for sequencing. Therefore, two sets of oligonucleotide primers targeting a highly conserved 16S rRNA gene in *E. canis* were used for PCR to obtain the near-full-length 16S rRNA gene. The nucleotide sequences of the forward and reverse primer pairs used for amplification of 16S rRNA were as follows: 15F and 842R, ATCATGGCTCAGAACGAACG and CTCATCGTTTACTCGGTGGACT, respectively; and 537F and 1442R, CAGCAGCCGGTAATACG and GTGACGGGCAGTGTGTACAAG, respectively (26). The PCR products were purified from the gel (Millipore Corp., Bedford, MA) and cloned with the pGEM-T cloning kit (Promega, Madison, WI). Plasmids were isolated from the clones with the QIAprep Spin miniprep kit (QIAGEN, Inc., Valencia, CA). Plasmids with inserts were tested by specific *E. canis* PCR. Inserted sequences were sequenced using vector primers T7 and SPS6, and a CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA) was used for sequencing. Both the sense and antisense strands of each amplicon were sequenced, and sequences were aligned and edited manually. The 16S rRNA gene sequences were subjected to BLAST analysis of GenBank.

Sequences were aligned manually using Se-Al (18); the matrix was trimmed slightly on each end and was complete for the included sequences. Two sequences of *Anaplasma* were used as an outgroup. All phylogenetic analyses were conducted using PAUP\* v4.0b10 (23), with gaps treated as missing data. Unweighted parsimony analysis was conducted by heuristic

\* Corresponding author. Mailing address: Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, Kansas 66506. Phone: (785) 532-4602. Fax: (785) 532-4851. E-mail: MMORO@vet.ksu.edu.

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TABLE 1. Comparison of nucleotide differences among 16S rRNA genes of *E. canis* from different sources

<i>E. canis</i> strain	Nucleotide at position <sup>a</sup> :															
	188	215	344	648	688	739	837	870	906	941	968	1001	1069	1227	1253	1296
PDE <sup>b</sup>	G	G	C	G	C	A	A	G	A	T	A	C	A	C	C	G
VDE <sup>c</sup>	●	●	●	A	●	●	●	●	●	C	●	T	●	●	●	●
VTE <sup>d</sup>	●	●	●	A	/	●	●	●	●	C	●	T	●	●	●	●
VHE <sup>e</sup>	●	●	●	A	●	●	●	● <sup>r</sup>	●	C	●	T	●	●	●	●
Oklahoma <sup>f</sup>	A	●	●	A	●	●	●	● <sup>r</sup>	●	C	●	T	●	●	●	●
Florida <sup>g</sup>	A	●	●	A	●	●	●	-	●	C	●	T	●	●	T	●
611 <sup>h</sup>	●	●	●	A	●	●	●	●	●	C	C	T	●	●	●	A
Gzh982 <sup>i</sup>	●	●	●	A	●	●	-	●	●	C	●	T	●	T	●	●
Germishuys <sup>j</sup>	●	●	-	A	●	●	●	●	●	C	●	T	●	●	●	●
Gxht67 <sup>k</sup>	A	A	●	A	T	●	●	●	G	C	●	T	G	/	/	/
Gdt3 <sup>l</sup>	A	●	●	A	●	●	●	●	●	C	●	T	●	/	/	/
95E10 <sup>k</sup>	/	/	/	/	/	●	●	●	●	T	●	T	●	●	●	●
Ovina <sup>l</sup>	●	●	●	A	●	●	●	●	●	C	●	T	●	●	●	●
Kagoshima <sup>m</sup>	●	●	●	A	●	●	●	●	●	C	●	T	●	●	●	●
Madrid <sup>n</sup>	●	●	●	A	●	C	●	●	●	C	●	T	●	●	●	●
Greece1 <sup>o</sup>	●	●	●	A	●	●	●	●	●	C	●	T	●	●	●	●
Greece0 <sup>o</sup>	●	●	●	A	●	●	●	●	●	C	●	T	●	●	●	●
Thai <sup>p</sup>	●	●	●	A	●	●	●	●	●	C	●	T	●	●	●	●
Okinawa <sup>q</sup>	●	●	●	/	/	/	/	/	/	/	/	/	/	/	/	/

<sup>a</sup> Bullets represent positions conserved relative to the sequence based on the PDE sequence. Slashes (“/”) indicate the sequences were unavailable. Data are presented as modified from those in the study by Unver et al. (24).

<sup>b</sup> *E. canis* DNA from a dog in Peru reported in this study.

<sup>c</sup> *E. canis* DNA from a dog in Venezuela (24).

<sup>d</sup> *E. canis* cDNA from *R. sanguineus* ticks from Venezuela (24).

<sup>e</sup> Venezuelan human culture isolate (17).

<sup>f</sup> *E. canis* type strain culture isolated from a dog in Oklahoma (4).

<sup>g</sup> *E. canis* culture isolated from a dog in Florida (4).

<sup>h</sup> *E. canis* culture isolated from a dog in Israel (13).

<sup>i</sup> *E. canis* DNA from a dog in The People’s Republic of China (10).

<sup>j</sup> *E. canis* DNA from a sheep in South Africa (3).

<sup>k</sup> *E. canis* DNA from a dog in Virginia.

<sup>l</sup> *E. ovina* DNA from sheep in Turkey (5).

<sup>m</sup> *E. canis* DNA from a dog in Kagoshima, Japan (25).

<sup>n</sup> *E. canis* DNA from a dog in Spain (2).

<sup>o</sup> *E. canis* DNA from a dog in Greece.

<sup>p</sup> *E. canis* DNA from a dog in Thailand.

<sup>q</sup> *E. canis* DNA from a dog in Okinawa, Japan (22).

<sup>r</sup> Corrected bases based on the sequence data that were obtained by resequencing (24).

search with 100 random additions (with tree bisection-reconnection branch swapping and the Multrees option on), followed by full heuristic bootstrapping to evaluate support for branches (8a; with 1,000 replicates, each with 10 random additions and tree bisection-reconnection branch swapping). A neighbor-joining analysis was also conducted, with an uncorrected *p* (uncorrected number of changes between two sequences) distance measure. The aligned sequence data matrix including partial sequences of the 16S rRNA gene (PDE and other sequences from GenBank) was 1,300 bp long. All sequences from Peru were identical (100%) in all samples analyzed and named “PDE,” for Peruvian dog *Ehrlichia* (GenBank accession no. DQ915970). The PDE sequence was nearly identical (99.9%) to the *E. canis* VHE, VDE, VTE, Ovina, and Kagoshima sequences (Table 1). Specific differences between PDE and almost all *E. canis* reported strains in this article were found at nucleotide positions 648, 941, and 1001 (Table 1).

There were 134 variable characters, 100 of which were potentially parsimony informative. Parsimony analysis yielded two most parsimonious trees of 141 steps each, with a consistency index of 0.99. The new strain, PDE, grouped within a well-supported *E. canis* clade (Fig. 1). However, relationships within *E. canis* were not well resolved and did not enable any

robust inferences regarding patterns of diversification. Indeed, all *E. canis* sequences and the included sequence of *E. ovina* were nearly identical (Table 1 and Fig. 1).

Canine ehrlichiosis is a potentially fatal disease, and diagnosis cannot be made based on clinical signs or serological results alone. Clinical signs may vary between different geographical regions, and serology does not differentiate between current infection and previous exposure to *Ehrlichia*, whereas PCR might indicate an active infection. Therefore, the assessment of clinical manifestations, serologic evaluation, and molecular evidence are all important for accurate diagnosis of ehrlichiosis.

The presence of dogs with *E. canis* in Peru raises concern that dogs may act as a reservoir of agents of human ehrlichiosis in this region, as purported for Venezuela (24). The possibility that dogs may facilitate transmission of these bacteria to humans increases their zoonotic importance.

The present work is the first to document infection of dogs with *E. canis* from Peru using molecular methods. It is clear that the new strain reported here, PDE, represents *E. canis*. While sequencing of the 16S gene enables positive identification of the bacterium, there is too little sequence variation within this evolutionarily conserved coding region to enable

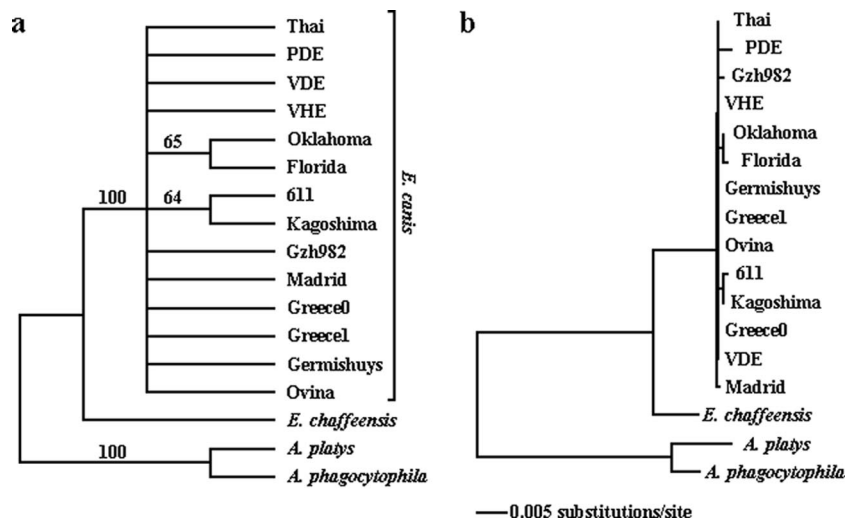


FIG. 1. Phylogenetic trees derived from the nucleotide sequences of 16S rRNA gene sequences from *E. canis*, *E. chaffeensis*, *A. platys*, and *A. phagocytophila*. (a) Strict consensus of two most parsimonious trees resulting from analysis of 16S rRNA data, with bootstrap numbers ( $\geq 50$ ) above branches. (b) Neighbor-joining tree. The sequence of interest in this study, PDE, represents *E. canis* (falling within a well-supported clade and cluster of *E. canis* samples). There are very poor resolution and weak support for any relationships within *E. canis* based on the 16S rRNA gene.

inference of the phylogenetic history of *E. canis* strains. An interesting future investigation would be the exploration of phylogeographic patterns of these strains: sequencing of a non-coding region of DNA for a diversity of *E. canis* samples might yield valuable information on the epidemiology of the disease.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the 16S rRNA nucleotide sequences of organisms used for comparison in this study are as follows: *E. canis* VDE, AF373613; *E. canis* VTE, AF373614 and AF373615; *E. canis* VHE, AF373612; *E. canis* Venezuela, AF287154; *E. canis* Oklahoma, M73221; *E. canis* Florida, M73226; *E. canis* 611 (Israel), U26740; *E. canis* Gzh982, AF162860; *E. canis* Germishuys, U54805; *E. canis* Gxht67, AF156786; *E. canis* Gdt3, AF156785; *E. canis* 95E10, U96437; *E. canis* Okinawa, AF308455; *E. canis* Madrid, AY394465; *E. canis* Kagoshima, AF536827; *Ehrlichia ovina* Turkey, AF318946; *E. canis* Turkey, AY621071; *E. canis* Greece, EF011110 and EF011111; *E. chaffeensis*, M773222; *E. ewingii*, M73227; *A. platys*, AF156784; *A. phagocytophila*, DQ449948; *Neorickettsia helminthoeca*, U12457; and the new *E. canis* PDE reported here, DQ915970.

#### REFERENCES

- Adrianzen, J., A. Chavez, E. Casas, and O. Li. 2003. Seroprevalencia de la dirofilariosis y ehrlichiosis canina en tres distritos de Lima. *Rev. Inv. Peru* **14**:43–48.
- Aguirre, E., A. Sainz, S. Dunner, I. Amusatgeui, L. López, F. Rodriguez-Franco, I. Luaces, O. Cortés, and M. A. Tesouro. 2004. First isolation and molecular characterization of *Ehrlichia canis* in Spain. *Vet. Parasitol.* **125**: 365–372.
- Allsopp, M., E. S. Visser, J. L. du Plessis, S. W. Vogel, and B. A. Allsopp. 1997. Different organisms associated with heartwater as shown by analysis of 16S ribosomal RNA gene sequences. *Vet. Parasitol.* **71**:283–300.
- Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson. 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. *J. Clin. Microbiol.* **29**:2838–2842.
- Bekker, C. P., S. de Vos, A. Taoufik, O. A. Sparagano, and F. Jongejan. 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Vet. Microbiol.* **89**:223–238.
- Dagnone, A. S., H. S. de Moraes, M. C. Vidotto, F. S. Jojima, and O. Vidotto. 2003. Ehrlichiosis in anemic, thrombocytopenic, or tick-infested dogs from a hospital population in South Brazil. *Vet. Parasitol.* **117**:285–290.
- de Castro, M. B., R. Z. Machado, L. P. de Aquino, A. C. Alessi, and M. T. Costa. 2004. Experimental acute canine monocytic ehrlichiosis: clinicopathological and immunopathological findings. *Vet. Parasitol.* **119**:73–86.
- Ewing, S. A. 1972. Geographic distribution and tick transmission of *Ehrlichia canis*. *J. Med. Entomol.* **9**:597–598.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783–791.
- Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll. 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). *Am. J. Vet. Res.* **36**:937–940.
- Hua, P., M. Yuhai, T. Shide, S. Yang, W. Bohai, and C. Xiangrui. 2000. Canine ehrlichiosis caused simultaneously by *Ehrlichia canis* and *Ehrlichia platys*. *Microbiol. Immunol.* **44**:737–739.
- Inokuma, H., T. Beppu, M. Okuda, Y. Shimada, and Y. Sakata. 2003. Epidemiological survey of *Anaplasma platys* and *Ehrlichia canis* using ticks collected from dogs in Japan. *Vet. Parasitol.* **115**:343–348.
- Keefe, T. J., C. J. Holland, P. E. Salyer, and M. Ristic. 1982. Distribution of *Ehrlichia canis* among military working dogs in the world and selected civilian dogs in the United States. *J. Am. Vet. Med. Assoc.* **181**:236–238.
- Keysary, A., T. Waner, M. Rosner, C. K. Warner, J. E. Dawson, R. Zass, K. L. Biggie, and S. Harrus. 1996. The first isolation, in vitro propagation, and genetic characterization of *Ehrlichia canis* in Israel. *Vet. Parasitol.* **62**: 331–340.
- Kuehn, N. F., and S. D. Gaunt. 1985. Clinical and hematologic findings in canine ehrlichiosis. *J. Am. Vet. Med. Assoc.* **186**:355–358.
- Lewis, G. E., Jr., M. Ristic, R. D. Smith, T. Lincoln, and E. H. Stephenson. 1977. The brown dog tick *Rhipicephalus sanguineus* and the dog as experimental hosts of *Ehrlichia canis*. *Am. J. Vet. Res.* **38**:1953–1955.
- López, J., M. Rivera, J. C. Concha, S. Gatica, M. Loeffholz, and O. Barriga. 2003. Ehrlichiosis humana en Chile, evidencia serológica. *Rev. Med. Chile* **131**:67–70.
- Perez, M., Y. Rikihisa, and B. Wen. 1996. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *J. Clin. Microbiol.* **34**:2133–2139.
- Rambaut, A. 1996. Se-Al: Sequence Alignment Editor, v2.0a11. University of Oxford, Oxford, United Kingdom.
- Rikihisa, Y., S. A. Ewing, J. C. Fox, A. G. Siregar, F. H. Pasaribu, and M. B. Malole. 1992. Analyses of *Ehrlichia canis* and a canine granulocytic *Ehrlichia* infection. *J. Clin. Microbiol.* **30**:143–148.
- Sirigireddy, K. R., and R. R. Ganta. 2005. Multiplex detection of Ehrlichia and Anaplasma species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J. Mol. Diagnostics* **7**:308–316.
- Stephenson, E. H., and M. Ristic. 1978. Retrospective study of an Ehrlichia canis epizootic around Phoenix, Arizona. *J. Am. Vet. Med. Assoc.* **172**:63–65.
- Suto, Y., A. Suto, H. Inokuma, H. Obayashi, and T. Hayashi. 2001. First confirmed canine case of *Ehrlichia canis* infection in Japan. *Vet. Rec.* **148**: 809–811.
- Swofford, D. L. 2002. PAUP\* v4.0b10: phylogenetic analysis using parsimony (\* and other methods). Sinauer, Sunderland, MA.

24. **Unver, A., M. Perez, N. Orellana, H. Huang, and Y. Rikihisa.** 2001. Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. *J. Clin. Microbiol.* **39**:2788–2793.
25. **Unver, A., Y. Rikihisa, M. Kawahara, and S. Yanamoto.** 2003. Analysis of 16S rRNA gene sequences of *Ehrlichia canis*, *Anaplasma platys*, and *Wolbachia* species from canine blood in Japan. *Ann. N. Y. Acad. Sci.* **990**:692–698.
26. **Warner, C. K., and J. E. Dawson.** 1996. Genus- and species-level identification of *Ehrlichia* species by PCR and sequencing, p. 100–105. *In* D. H. Persing (ed.), *PCR protocols for emerging infectious diseases*. ASM Press, Washington, DC.
27. **Woody, B. J., and J. D. Hoskins.** 1991. Ehrlichial diseases of dogs. *Vet. Clin. N. Am. Small Anim. Pract.* **21**:75–98.