Available online at www.sciencedirect.com
ScienceDirect
The Veterinary Journal xxx (2008) xxx–xxx
The Veterinary Journal
www.elsevier.com/locate/tvjl

Short Communication

Detection of *Ehrlichia canis* by polymerase chain reaction in dogs from Portugal

Nuno Alexandre [a,*], Ana Sofia Santos [b], Maria Sofia Núncio [b], Rita de Sousa [b], Fernando Boinas [c], Fátima Bacellar [b]

[a] *Departamento de Zootecnia, Universidade de Évora, Évora, Portugal*
[b] *Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Lisboa, Portugal*
[c] *Departamento de Sanidade Animal, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa, Portugal*

Accepted 9 March 2008

Abstract

Antibodies against *Ehrlichia canis*, the cause of canine monocytic ehrlichiosis, have been reported previously in clinically ill and stray dogs from Portugal. In this study, the *16S rRNA* gene of *E. canis* was detected by the polymerase chain reaction (PCR) in 12/55 (22%) of dogs with suspected tick-borne disease in the Algarve region in Portugal.

Keywords: *Ehrlichia canis*; Polymerase chain reaction; Canine; Portugal

Canine monocytic ehrlichiosis (CME) is a tick-borne disease caused by *Ehrlichia canis*, an intracellular bacterium transmitted by the brown dog tick *Rhipicephalus sanguineus* (Groves et al., 1975). Clinical manifestations of CME include fever, depression, anorexia, weight loss, epistaxis and ocular, gastrointestinal and respiratory signs (Harrus et al., 1997). Thrombocytopaenia, leucopaenia, anaemia and hypergammaglobulinaemia are the main laboratory findings (Harrus et al., 1997).

The indirect immunofluorescence assay (IFA) has been widely used to investigate canine ehrlichiosis in Portugal. Antibodies against *E. canis* were detected by IFA in 27/61 (44%) stray dogs from southern Portugal (Silveira, 1992). However, the IFA may cross-react with different species of *Ehrlichia* or microorganisms from other closely related genera. To confirm that *E. canis* is the agent causing canine ehrlichiosis in Portugal, the polymerase chain reaction (PCR) for the *16S rRNA* gene was performed on DNA extracted from the blood of 55 dogs (26 males, 29 females), aged 3 months to 10 years, exhibiting clinical signs of tick-borne disease that were presented to one hospital and seven veterinary clinics in the Algarve region from February to October 2004.

An automated cell counter (Hemavet 850, CDC Technologies) was used to perform a complete cell count on blood samples collected from affected dogs into ethylene diamine tetraacetic acid. After leucocyte concentration, buffy coat smears were stained with Diff-Quik (Medion Diagnostics GmbH) and examined under 1000× magnification (Davoust et al., 1999). DNA was extracted from the buffy coat (Flexigene DNA Kit, Qiagen GmbH) in parallel with negative controls.

Nested PCR was performed using the outer primers ECC-ECB and inner primers HE3-CANIS derived from the *E. canis 16S rRNA* gene (Dawson et al., 1996). In positive samples, two additional hemi-nested PCR reactions were performed using the universal outer primers EC12-EC9 and inner primers EC10 and EC11 (Anderson et al., 1991). PCR amplifications were carried out in a total volume of 50 μL containing 1 μM of each primer, 2.5 U of *Taq* polymerase, 20 mM of each dNTP, 10 mM

* Corresponding author. Tel.: +351 266760829; fax: +351 266760841.
E-mail address: nmla@uevora.pt (N. Alexandre).

1090-0233/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2008.03.025

Tris–HCL, 1.5 mM $MgCl_2$ and 50 mM KCl (Master Taq Kit, Eppendorf). Ten microlitres of buffy coat DNA extract were used for the initial amplification and 1 μL of PCR product in nested reactions. Amplification conditions have been described previously (Anderson et al., 1991; Dawson et al., 1996). Controls included both positive (*E. canis* Oklahoma strain DNA) and negative samples. DNA amplicons from positive samples were purified (Jetquick Purification Kit, Genomed GmbH) and sequenced using an ABI automated sequencer (Applied Biosystems). Sequence homology searches in GenBank were performed using BLASTN.

Antibodies against *E. canis* were detected by IFA (Davoust et al., 1999) using the Oklahoma strain of *E. canis* (kindly provided by J.E. Dawson, CDC) as the antigen source. Plasma diluted 1:160 in phosphate buffered saline was incubated for 30 min on antigen coated slides and bound antibodies were detected after a second incubation with fluorescein isothiocyanate-conjugated anti-dog immunoglobulin G (IgG) (Sigma Immunochemicals). A confirmed case of CME was defined by a positive PCR result with or without an IFA positive result. Probable CME cases were IFA positive but PCR negative.

CME was confirmed by PCR in 12/55 (22%) dogs (8 males, 4 females) with suspected tick-borne disease. Nucleotide sequences of the *16S rRNA* gene from all 12 positive dogs were identical. Nested PCR with species-specific primers produced a 334 base pair (bp) fragment from the 5′ end of the *16S rRNA* gene (GenBank EF051166). Hemi-nested reactions with universal primers generated a 684 bp fragment from the 3′ end of the *16S rRNA* gene (EU491504). These sequences had 100% identity with *E. canis* strains from dogs in Turkey (AY621071), Taiwan (EU143637), Thailand (EU263991), Brazil (EF195134), Venezuela (AF373613) and Greece (EF011111), 99.9% identity (334/334 and 666/667) with a Spanish strain (AY394465) and 99.7% identity (333/334 and 577/579) with an Israeli strain (U26740). Partial *16S rRNA* gene sequences of the Portuguese isolates had a nucleotide deletion at position 876 relative to the *E. canis* prototype strain Jake (CP000107).

IgG against *E. canis* was detected by IFA in the plasma of 5/12 (41.6%) cases confirmed as CME by PCR. Five other IFA positive dogs had negative PCR results. *E. canis* morulae were observed in buffy coat smears of 1/12 PCR positive dogs by direct microscopy and 0/43 PCR negative dogs. PCR positive dogs exhibited pyrexia (>39 °C; $n = 12$), anorexia ($n = 12$), lymphadenopathy ($n = 8$), epistaxis ($n = 2$), icterus ($n = 1$), petechial haemorrhages on the skin ($n = 1$) and vomiting ($n = 1$). Haematological findings included thrombocytopaenia ($<200 \times 10^3/\mu L$; $n = 12$), anaemia ($<5.5 \times 10^6/\mu L$; $n = 9$) and leucopaenia ($<6 \times 10^3/\mu L$; $n = 6$).

PCR has been widely used in the laboratory diagnosis of CME, especially during the acute phase of the disease before antibodies are detectable (Wen et al., 1997). *E. canis* DNA can be detected by nested PCR in the buffy coats of experimentally infected dogs from the fourth day of infection (Wen et al., 1997). In this study, *E. canis* infection was detected by nested PCR in 7/12 dogs that were seronegative by indirect IFA results, reinforcing the value of molecular techniques in the early diagnosis of CME. PCR should be used in conjunction with serology for diagnosis of CME (Neer et al., 2002).

Previously, evidence of *E. canis* infection in Portugal was based on serology (Silveira, 1992). In the present study, we have amplified and sequenced the *16S rRNA* gene of *E. canis*, providing the first molecular confirmation that this agent is involved in canine disease in Portugal.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

This research was partially supported by the Portuguese government through the Fundação para a Ciência e a Tecnologia Grant BD/8610/2002. We thank to all veterinarians directly involved in sample collection. Also, we would like to thank the staff of the Unidade Laboratorial de Utilização Comum, Instituto Nacional de Saude Dr. Ricardo Jorge for assistance with sequencing.

References

Anderson, B.E., Dawson, J.E., Jones, D.C., Wilson, K.H., 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. Journal of Clinical Microbiology 29, 2838–2842.

Davoust, B., Boni, M., Parzy, D., 1999. Apport du laboratoire au diagnostic de l'ehrlichiose monocytaire canine. Revue Française des Laboratoire 310, 25–32.

Dawson, J.E., Biggie, K.L., Warner, C.K., Cookson, K., Jenkins, S., Levine, J.F., Olson, J.G., 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from Southeast Virginia. American Journal of Veterinary Research 57, 1175–1179.

Groves, M.G., Dennis, G.L., Amyx, H.L., Huxsoll, D.L., 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). American Journal of Veterinary Research 36, 937–940.

Harrus, S., Bark, H., Waner, T., 1997. Canine monocytic ehrlichiosis: an update. Compendium on Continuing Education for the Practicing Veterinarian 19, 431–444.

Neer, T.M., Breitschwerdt, E.B., Greene, R.T., Lappin, M.R., 2002. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. Journal of Veterinary Internal Medicine 16, 309–315.

Silveira, C.A., 1992. Ehrlichiose canina. Estudo clínico de uma população animal, na região urbana e rural de Setúbal – Implicações em saúde pública e saúde pública veterinária. M.Sc., thesis, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa, Portugal.

Wen, B., Rikihisa, Y., Mott, J.M., Greene, R., Kim, H.Y., Zhi, N., Couto, C.G., Unver, A., Barstch, R., 1997. Comparison of nested-PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. Journal of Clinical Microbiology 35, 1852–1855.