Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 141 (2006) 368–372
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

A simple molecular method for discriminating common filarial nematodes of dogs (*Canis familiaris*)

Maurizio Casiraghi [a], Chiara Bazzocchi [b], Michele Mortarino [b], Eleonora Ottina [b], Claudio Genchi [b,*]

[a] *Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano Bicocca, Milan, Italy*
[b] *Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Patologia Generale e Parassitologia, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy*

Received 7 February 2006; received in revised form 29 May 2006; accepted 16 June 2006

Abstract

Accurate diagnosis of canine filariosis is essential for choosing correct therapeutic approach. Therefore, reliable methods for discriminating among the different filarial infections in dogs are needed. The authors report simple and highly specific molecular methods that identify the three most common filarial nematodes of European dogs: *Dirofilaria immitis*, *D. repens* and *Acanthocheilonema* (syn. *Dipetalonema*) *reconditum*, based on (1) PCR amplifications of mitochondrial DNA (12S rDNA and *coxI*) with general filarial primers followed by digestion with restriction enzymes that generates band polymorphisms clearly discriminating the three species and (2) PCR amplifications with species-specific primers to support the restriction analysis, in particular in the case of multiple infections.

Keywords: *Dirofilaria immitis*; *Dirifilaria repens*; *Acanthocheilonema reconditum*; PCR; Restriction enzyme; mtDNA

Filarial nematodes are common parasites of many vertebrates (Anderson and Bain, 1976; Anderson, 2000). In European dogs, the most common species of filariae are *Dirofilaria immitis*, *D. repens* and *Acanthocheilonema* (syn. *Dipetalonema*) *reconditum*.

Filarial infection in dogs can be diagnosed through morphological observation of circulating microfilariae (mf), detection of circulating antigens (currently available only for *D. immitis*), histochemical or immuno-histochemical staining of circulating mf, or through molecular approaches. Morphological identification of circulating mf, however, is not always easy and potentially misleading: (1) it requires experienced personnel, not always available in non-endemic areas, (2) in dogs treated incorrectly with preventive drugs, is not infrequent to observe mf with morphological alterations (Venco, L., personal communication) and (3) it may be difficult to detect multiple infections with more than one species of filarial worms. Indeed, in the case of a mixed infection, it is possible to underestimate the presence of more than one filarial species.

The discrimination of *D. immitis* mf from those of *Dirofilaria* spp. or *Acanthocheilonema* spp. is also possible by acid phosphatase histochemical staining (Chalifoux and Hunt, 1971). More recently, an immunohistochemical staining method has been proposed for the microfilarial differentiation of *D. immitis*,

* Corresponding author. Tel.: +39 02 50318101; fax: +39 02 50318095.
E-mail address: claudio.genchi@unimi.it (C. Genchi).

0304-4017/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2006.06.006

D. repens and *Acanthocheilonema dracunculoides* (Peribáñez et al., 2001). Molecular methods for the discrimination of canine filarial nematodes based on species-specific PCRs have been reported (Favia et al., 1996; Mar et al., 2002; Rishniw et al., 2006). In the present work, the authors report a simple and highly specific molecular approach for the discrimination of the three principal filarial nematodes of European dogs. This method is based on PCR amplification of mitochondrial DNA (mtDNA; 12S rDNA and *coxI* genes) with general filarial primers (Casiraghi et al., 2001, 2004), followed by digestion with restriction enzymes that generates polymorphisms discriminating the three species. In addition, species-specific primers have been designed to support the restriction analysis, particularly useful for multiple infections.

Crude DNA preparations were obtained through proteinase-K treatment, according to Bandi et al. (1994) starting from pooled samples of mf obtained through blood filtration and from adult specimens. In addition, agarose gel quantification of the extracted DNAs was carried out and a ''mixed infection'' was artificially created by combining DNA preparations derived from all filarial species.

In order to validate the efficiency of our methods, blood samples collected from 22 microfilaremic dogs were blind tested. The blood donors came from Italy (12 samples), France (5 samples) and Spain (5 samples).

Restriction maps of the 12S rDNA and *coxI* gene sequences from *D. immitis*, *D. repens* and *A. reconditum* available in databases (*D. immitis* accession number: 12S rDNA AJ544831 and *coxI* AJ271613; *D. repens*: 12S rDNA AJ544832 and *coxI* AJ271614; *A. reconditum*: 12S rDNA AJ544853 and *coxI* AJ544876) were generated using REBsites (http://tools.neb.com/REBsites/index.php3). Based on these maps, ApoI and Hyp8I restriction enzymes (MBI Fermentas) were selected for the 12S rDNA and *coxI*, respectively (isoschizomer XapI instead of ApoI was used).

PCR reactions for the amplification of 12S rDNA and *coxI* genes were performed in a final volume of 25 μl using general primers and thermal profiles described in Casiraghi et al. (2001, 2004). Five microlitres of the PCR reactions were loaded on a 2% agarose gel to verify the presence of the amplification fragments and the sensibility of the reactions and 10 μl were purified (QIAquick gel extraction kit, QIAGEN) and directly sequenced, using ABI technology, to confirm the specificity of the PCR reactions. The remaining 10 μl of the PCR reactions were digested. Both digestions were performed in a final volume of 20 μl, with 5 U of XapI or Hyp8I incubated at 37 °C for 90 min. The digestion products were loaded on 3% agarose gels.

Sequences of the gene coding for 12S rDNA and *coxI* of *D. immitis*, *D. repens* and *A. reconditum* available in databases were aligned using ClustalX (Thompson et al., 1997). In the case of 12S rDNA, species-specific forward primers were designed in non-conserved regions and these primers were used in PCR combined with the filarial nematode reverse general primer (12S R, Casiraghi et al., 2004). The primer sequences are: D.imm-12S F (5′-ATTTGTTGTAATATTACGA-3′) for *D. immitis*; D.rep-12S F (5′-ATGTTTTGATTTTTTTGTAT-3′) for *D. repens* and A.rec-12S F (5'-TTGTAATATTTATTTTTGTG-3′) for *A. reconditum*. In the case of *coxI*, species-specific reverse primers were designed in non-conserved regions and these primers were used in PCR combined with the filarial nematode forward general primer (COIintF, Casiraghi et al., 2001). The primer sequences are: D.imm-coxI R (5′-GCACTGACAATACCAAT-3′) for *D. immitis*; D.rep-coxI R (5′-TCAAACAGAAGTACCTAAA-3′) for *D. repens* and A.rec-coxI R (5'-CTGTGATGATTGGTTCT-3′) for *A. reconditum*.

PCRs were performed in a 20 μl volume under the following final conditions: 1× buffer containing 1.5 mM $MgCl_2$ (Eppendorf), 0.2 mM dNTP, 1 μM each primer, and 1 U Taq DNA Polymerase (MasterTaq® Eppendorf). The thermal profile used for the amplification of *D. immitis* 12S rDNA was: 94 °C 45 s, 48 °C 45 s, 72 °C 90 s for 38 cycles. For the amplification of the *D. repens* gene, the thermal profile was analogous to that for *D. immitis*, but the annealing temperature was set at 50 °C. For *A. reconditum*, a touch-down thermal profile was used: 94 °C 45 s, 55 °C 45 s, 72 °C 90 s for five cycles and 94 °C 45 s, 48 °C 45 s, 72 °C 90 s for 37 cycles. The thermal profile used for the amplification of *D. immitis coxI* gene was: 94 °C 45 s, 50 °C 45 s, 72 °C 90 s for 40 cycles. For the amplification of *D. repens* gene the thermal profile was analogous to that of *D. immitis* gene, but the annealing temperature was set at 48 °C. For *A. reconditum* gene was used a touch-down thermal profile: 94 °C 45 s, 58 °C 45 s, 72 °C 90 s for 10 cycles and 94 °C 45 s, 53 °C 45 s, 72 °C 90 s for 30 cycles.

PCR products were loaded on 2% agarose gels. The sizes of the 12S rDNA and *coxI* amplification fragments were approximately 450 and 650 bp, respectively, for the three species. The amplification products were gel purified and directly sequenced using ABI technology to verify the specificity of the PCR reactions.

The restriction patterns of *D. immitis*, *D. repens* and *A. reconditum* 12S rDNA and *coxI* genes are shown in Fig. 1. The theoretical digestion pattern for the 12S

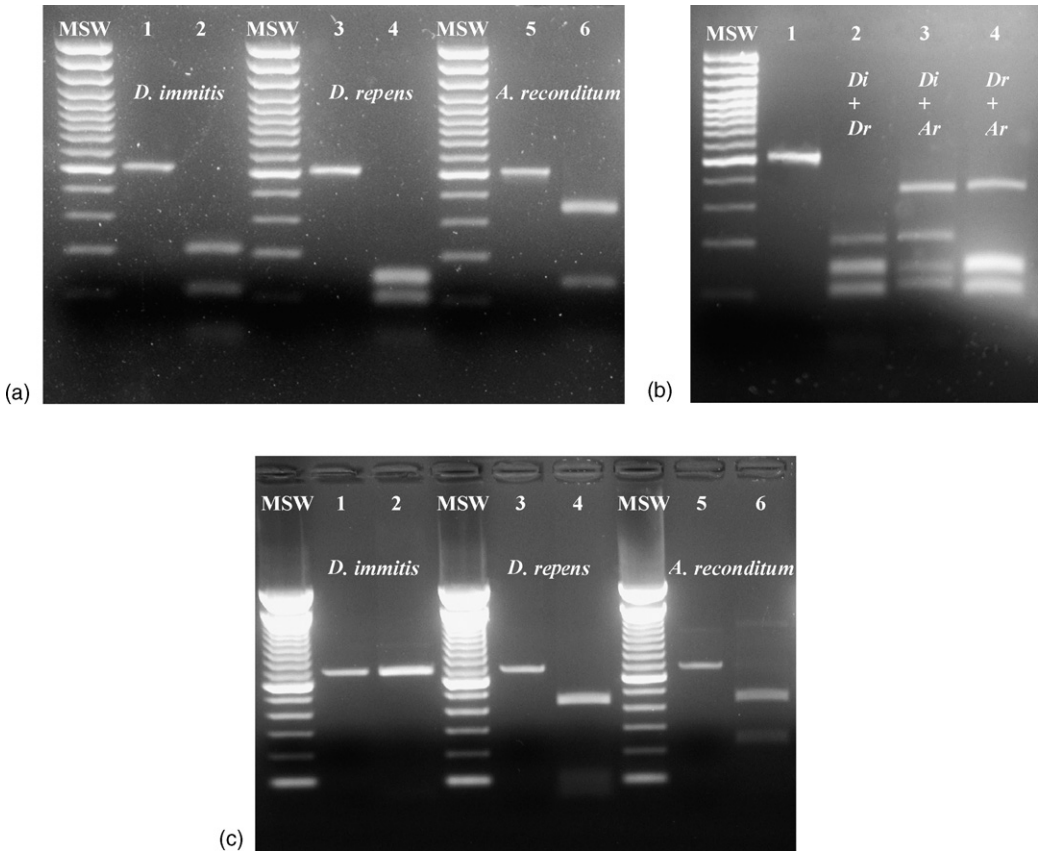

Fig. 1. PCR amplicons and digestion patterns using XapI (12S rDNA) and Hyp8I (*coxI*) restriction enzymes. (a) XapI digestion patterns of 12S rDNA gene of *D. immitis*, *D. repens* and *A. reconditum*; lanes 1, 3 and 5: undigested PCR products; lanes 2, 4 and 6: PCR products digested using XapI. (b) XapI digestion patterns of 12S rDNA gene of mixed PCR products of *D. immitis* + *D. repens* (lane 2), *D. immitis* + *A. reconditum* (lane 3) and *D. repens* + *A. reconditum* (lane 4); lanes 1 contains the undigested product of *D. immitis* as a size control. (c) Hyp8I digestion patterns of *coxI* gene of *D. immitis*, *D. repens* and *A. reconditum*; lanes 1, 3 and 5: undigested PCR products; lanes 2, 4 and 6: PCR products digested using Hyp8I. MSW: molecular standard weight (100 bp ladder, GeneRuler DNA Ladder Plus, MBI Fermentas).

rDNA gene using XapI for *D. immitis* predicted five fragments sizing 183, 107, 84, 39 and 30 bp, for *D. repens* five fragments sizing 144, 110, 96, 77 and 16 bp and for *A. reconditum* two fragments sizing 331 and 112 bp. As shown in Fig. 1a, electrophoresis on 3% agarose gel did not allow visualization of bands with a lower size than those of the digested products (i.e. the 30 and 39 bp bands for *D. immitis* and the 16 bp band for *D. repens*) nor to discriminate the bands with sizes similar to the digested products (i.e. the 77 and 96 bp bands for *D. repens*). Thus, we were able to identify only three out of the five bands of the theoretic digestion patterns for *D. immitis* and *D. repens*. Nevertheless, the differential patterns visualized on the gel (Fig. 1a) allowed easy and unambiguous discrimination of the three filarial species. Fig. 1b reports the digestion patterns of XapI on the artificially created ‘‘mixed infection’’. Even if PCR products from more than one species were digested, it was still possible to recognize patterns that allowed us to distinguish which two filarial nematodes were present. The theoretical digestion pattern for the *coxI* gene using Hyp8I for *D. immitis* predicted two fragments sizing 635 and 13 bp, for *D. repens* six fragments sizing 430, 71, 50, 49, 36 and 13 bp and for *A. reconditum* three fragments sizing 430, 205 and 13 bp. As shown in Fig. 1c, electrophoresis on 3% agarose gel did not allow us to visualize the bands with a lower size than that of the digested products (i.e. the 13 bp band for *D. immitis*; 13, 36, 49, 50 and 71 bp bands for *D. repens*; 13 bp band for *A. reconditum*). Thus, we were able to identify only one out of the two bands of the theoretic digestion patterns for *D. immitis*; one out of the six bands for *D. repens* and two out of three bands for *A. reconditum*. Nevertheless, the differential patterns visualized on the gel (Fig. 1c) allowed a discrimination of the three filarial species.

The discriminating efficiency of the digestion method was then evaluated on an epidemiological

study conducted on blood samples collected from 22 microfilaremic European dogs. By analyzing the restriction patterns, 12 samples were identified as *D. immitis* (4 samples from Italy, 5 from France and 3 from Spain), 7 samples as *D. repens* (5 samples from Italy and 2 from Spain) and 3 samples as *A. reconditum* (from Italy). These results have been confirmed by direct sequencing of the PCR products (data not shown).

Fig. 2 shows three of the species-specific amplification patterns for the filarial nematode species analyzed including the artificial ''mixed infection''. In particular, species-specific primer pairs designed on 12S rDNA gene gave the best results for the identification of *D. immitis* and *D. repens* (Fig. 2a and b, respectively), while the species-specific primer pair designed on the *coxI* gene gave the best results for the identification of *A. reconditum* (Fig. 2c). Species-specific primer sequences designed on *D. immitis* and *D. repens coxI* gene also generated specific patterns (data not shown). Species-specific primers designed on *A. reconditum* 12S rDNA gene did not generate amplifications on *D. immitis* or *D. repens* DNA, while they generated amplicons of the expected size, along with non-specific bands, on *A. reconditum* DNA, allowing discrimination (data not shown).

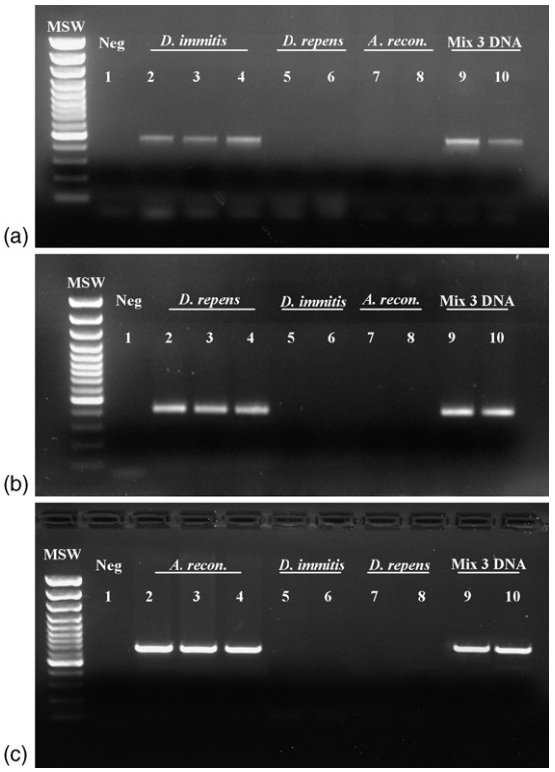

Fig. 2. Species-specific PCR amplifications of *D. immitis* and *D. repens* 12S rDNA and of *A. reconditum coxI*. (a) PCR amplification using *D. immitis* 12S rDNA specific primers on *D. immitis* DNA (lanes 2–4), *D. repens* (lanes 5 and 6), *A. reconditum* (lanes 7 and 8) and mixed DNAs (lanes 9 and 10) and lane 1 negative control. (b) PCR amplification using *D. repens* 12S rDNA specific primers on *D. repens* DNA (lanes 2–4), *D. immitis* (lanes 5 and 6), *A. reconditum* (lanes 7 and 8) and mixed DNAs (lanes 9 and 10) and lane 1 negative control. (c) PCR amplification using *A. reconditum coxI* specific primers on *A. reconditum* DNA (lanes 2–4), *D. immitis* (lanes 5 and 6), *D. repens* (lanes 7 and 8) and mixed DNAs (lanes 9 and 10) and lane 1 negative control. MSW molecular standard weight (100 bp ladder, GeneRuler DNA Ladder Plus, MBI Fermentas).

The molecular approaches presented in our work allowed easy and highly specific discrimination of the main canine filarial nematodes found in Europe. These methods could be quite useful, since practicing veterinarians are encountering more often cases in which the morphological and/or antigenic identification of filariasis is complex or misleading. Furthermore, the risk of spreading of filarial infections in non-endemic areas (Genchi et al., 2005) suggests the need to provide reliable methods for people with little experience on morphological identification of mf or adult worms. Filarial nematodes are characterized by a very low intraspecific variation of mtDNA. Thus the usefulness of mtDNA as a target for the identification of samples coming from a vast geographic area is not surprising. Recently, *D. immitis* samples collected in Japan, Cuba, USA and different areas in Italy were characterized by an identical 12S rDNA and *coxI* gene sequence (Casiraghi et al., personal communication).

Acknowledgments

The authors wish to express their thanks to L. Venco, S. Giannetto and J.W. McCall for providing filarial nematode samples. The authors wish to particularly thank Laura Kramer for her helpful reading and comments on the manuscript.

References

Anderson, R.C., 2000. Nematode Parasites of Vertebrates—Their Development and transmission. CAB International, Wallingford, Oxon, UK, pp. 1–650.

Anderson, R.C., Bain, O., 1976. Keys to genera of the order Spirurida. Part 3: Diplotriaenoidea, Aproctoidea and Filarioidea. In: Anderson, R.C., Chabaud, A.G., Willmott, S. (Eds.), CIH Keys to the Nematode Parasites of Vertebrates, vol. 3. Commonwealth Agricultural Bureaux, Farnham Royal, pp. 59–116.

Bandi, C., Damiani, G., Magrassi, L., Gigolo, A., Fani, R., Sacchi, L., 1994. Flavobacteria as intracellular symbionts in cockroaches. Proc. R. Soc. Lond. B 257, 43–48.

Casiraghi, M., Anderson, T., Bandi, C., Bazzocchi, C., Genchi, C., 2001. A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. Parasitology 122, 93–103.

Casiraghi, M., Bain, O., Guerriero, R., Martin, C., Pocacqua, V., Gardner, S.L., Franceschi, A., Bandi, C., 2004. Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. Int. J. Parasitol. 34, 191–203.

Chalifoux, L., Hunt, R.D., 1971. Histochemical differentiation of *Dirofilaria immitis* and *Dipetalonema reconditum*. J. Am. Vet. Med. Assoc. 5, 601–605.

Favia, G., Lanfrancotti, A., Torre, A.D., Cancrini, G., Coluzzi, M., 1996. Polymerase chain reaction-identification of *Dirofilaria repens* and *Dirofilaria immitis*. Parasitology 113, 567–571.

Genchi, C., Rinaldi, L., Cascone, C., Mortarino, M., Cringoli, G., 2005. Is heartworm really spreading in Europe? Vet. Parasitol. 113, 137–148.

Mar, P.H., Yang, I.C., Chang, G.N., Fei, A.C., 2002. Specific polymerase chain reaction for differential diagnosis of *Dirofilaria immitis* and *Dipetalonema reconditum* using primers derived from internal transcribed spacer region 2 (ITS2). Vet. Parasitol. 106, 243–252.

Peribáñez, M.A., Lucientes, J., Arce, S., Morales, M., Castello, J.A., Grazia, M.J., 2001. Histochemical differentiation of *Dirofilaria immitis*, *Dirofilaria repens* and *Acanthocheilonema dracunculoides* microfilariae by staining with a commercial kit, Leucognost-SP®. Vet. Parasitol. 102, 173–175.

Rishniw, M., Barr, S.C., Simpson, K.W., Frongillo, M.F., Franz, M., Alpizar, J.L.D., 2006. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet. Parasitol. 135, 305–314.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4876–4882.