

Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



Volume 151, issue 1, 25 January 2008

ISSN 0304-4017

veterinary parasitology

An International Scientific Journal



Official Organ of the American Association of Veterinary Parasitologists (AA.V.P.),
the European Veterinary Parasitology College (E.V.P.C.) and the World
Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)

This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



ELSEVIER

Available online at www.sciencedirect.com



Veterinary Parasitology 151 (2008) 61–67

**veterinary
parasitology**

www.elsevier.com/locate/vetpar

Mitochondrial DNA variation of the dog hookworm *Ancylostoma caninum* in Brazilian populations

Rodrigo R. Miranda^a, Jacob A. Tennessen^b, Michael S. Blouin^b, Élide M. Rabelo^{a,*}

^a Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos 6627, Campus Pampulha, CEP 31270-901 Belo Horizonte, Minas Gerais, Brazil

^b Department of Zoology, 3029 Cordley Hall, Oregon State University, Corvallis, OR 97331, USA

Received 2 September 2007; received in revised form 22 September 2007; accepted 24 September 2007

Abstract

The mitochondrial cytochrome oxidase I gene was partially sequenced for 164 *Ancylostoma caninum* individuals, originating from five different localities in Brazil, with the aim of describing the genetic diversity and genetic structure of Brazilian hookworm populations. Allelic and nucleotide diversity were moderate (overall $h = 0.88$ and $\pi = 0.016$) and were similar among cities. There was moderate genetic differentiation among the populations sampled ($\approx \Phi_{ST} = 0.12$) and a weak but nonsignificant correlation between geographical and genetic distance. This genetic structure was similar to that observed among populations of the human hookworm, *Necator americanus*, but distinct from that typically found in trichostrongylid nematode parasites of livestock. Thus, a pattern of different genetic structures among different groups of nematodes is emerging. We also observed a few individuals that had a highly divergent mtDNA sequence (almost 7% sequence divergence from the other sequences). These results in combination with data from other studies suggest that *A. caninum* populations worldwide consist of a mix of previously differentiated populations, or perhaps even cryptic species. This study contributes to the knowledge of genetic structure and diversity of hookworms, which in turn will be useful in developing methods for their control.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Hookworm; Gene flow; Genetic structure; Mitochondrial DNA; Nematode; Population genetic analysis

1. Introduction

Bloodfeeding hookworms, parasitic nematodes of the gastrointestinal tract, remain a leading cause of anemia throughout much of the developing world (Gilles, 1985; Keymer and Bundy, 1989; Hotez and Pritchard, 1995). *Ancylostoma* is a cosmopolitan genus that infects humans and other animals. Some species are specific to man but others are zoonotics. The dog worm *Ancylostoma caninum* can cause clinical disease

resulting from hookworm infection characterized by iron deficiency anemia due to chronic gastrointestinal blood loss caused by the adult worms. It is also a zoonotic problem causing eosinophilic enteritis (Prociv and Croese, 1996) and a dermatological infection (cutaneous larva migrans or ground itch) in humans (together with *Ancylostoma braziliense* in the latter disease). Anthelmintic drugs are effective, but rapid reinfection and the high cost of treatment hamper control efforts (Albonico et al., 1995). An effective vaccine against hookworms would obviate the need for the widespread use of anthelmintics, however vaccine development efforts are still underway (Hotez et al., 1999, 2003; Bungiro and Cappello, 2004). In a vaccine development program it is important to know whether

* Corresponding author. Tel.: +55 31 34992871; fax: +55 31 34992970.

E-mail address: rabelo@icb.ufmg.br (& M. Rabelo).

populations are likely to differ at the loci targeted by highly specific recombinant vaccines, and thus to predict the geographical scale over which new vaccines need to be tested (Gupta et al., 1994; Anderson et al., 1998). Genetic structure informs us about patterns of gene flow and effective size, both key parameters that influence the development and spread of anthelmintic resistance (Anderson et al., 1998; Blouin, 1998; Viney, 1998). Thus, understanding the genetic structure of parasitic nematodes will aid in designing methods for their control.

A. caninum occurs in many parts of the world and represents a good biological model for studies with hookworms. It is also itself a public health concern. Yet little is known about population genetic structure in this species. In this paper we describe the genetic diversity and population structure of *A. caninum* from five localities in Brazil and compare our results with those from previous studies on hookworms and other nematode species.

2. Material and methods

At each of five sites, adult worms were collected at necropsy from the small intestines of five dogs aged 6–24 months of undefined breeds, which are routinely submitted to euthanasia following the approved procedures of the Municipality Health Center from Brazilian cities by the Zoonoses Control Center (CCZ). Distances ranging from 426 to 2599 km separated the sites (Fig. 1). Sample sizes ranged from 11 to 44 per locality (Table 2).

Adult hookworms of *A. caninum* were washed in physiological saline, identified morphologically to species and frozen (-86°C) until used. DNA was extracted using the technique described by Waldschmidt et al. (1997). Individual adult worms were ground in a microcentrifuge tube with a glass pestle and liquid N_2 and then homogenized with 400 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 2% sodium dodecyl sulfate (SDS), 0.75 M NaCl, 10 mM EDTA) and 100 $\mu\text{g}/\text{ml}$ proteinase K. Samples were then incubated at 65°C for 30 min, deproteinized with chloroform:isoamyl alcohol (24:1) and centrifuged to $12,000 \times g/5 \text{ min}/25^{\circ}\text{C}$. The organic phase was transferred to another microcentrifuge tube and incubated with RNase A (100 $\mu\text{g}/\text{ml}$) for 30 min at 37°C . Samples were submitted to a second deproteinization with chloroform:isoamyl alcohol (24:1) and precipitated with 400 μl of isopropanol after centrifugation to $12,000 \times g/30 \text{ min}/4^{\circ}\text{C}$. The resulting pellet was resuspended in 30 μl of DNase-free bidistilled water. The integrity and purity of DNA



Fig. 1. Brazilian map showing the arrangement of the study sites (squares) and geographical distances in km between localities (dashed lines).

samples were checked on 0.8% (w/v) agarose gels. One microlitre of template was used as a polymerase chain reaction (PCR) in 50 μl containing 2 mM MgCl_2 , 1 U *Taq* DNA polymerase (Phonetrria, MG, Brazil), 100 ng of each conserved cytochrome oxidase I primers HCO and LCO (Folmer et al., 1994) and subjected to 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR products were precipitated with 20% polyethylene glycol (PEG 8000) and sequenced twice in both directions with the same primers for PCR amplification to improve the credibility of the sequences. The consensus sequences were obtained using the programs Phred v.0.20425 (Ewing and Green, 1998; Ewing et al., 1998), Phrap v.0.990319 (<http://www.phrap.org>) and Consed 12.0 (Gordon et al., 1998).

Four hundred and sixty-seven bases of the cytochrome *c* oxidase I gene (COI) were analyzed, corresponding to positions 96–562 of the *A. caninum* COI gene sequence (accession number U57030).

Haplotype diversity (h), nucleotide diversity (π) and mean number of pairwise differences (K) were estimated using DNASP version 4.0 program (Rozas et al., 2003), while genetic distances between the populations were obtained using a distance analysis (Kimura's two-parameter distance estimator, K_2P). Population genetic structure and differentiation were described using analysis of molecular variance (AMOVA available in ARLEQUIN 3.11 program, Excoffier et al., 2005). This same program was also

used to estimate F -statistics (Φ_{ST}) from the uncorrected number of nucleotide differences between sequences, to obtain traditional F -statistics and to conduct Mantel test and neutrality tests using Fu's F (Fu, 1997) and Tajima's D (Tajima, 1989) statistics. A haplotype network was constructed using the median-joining network (Bandelt et al., 1999; Network 4.0.00 available at: <http://www.fluxus-engineering.com>). In the haplotype network, we also included three non-Brazilian *A. caninum* sequences (two from US laboratory strains and one from China) and two other hookworm sequences (*Ancylostoma duodenale* and *Necator americanus* sequences to be used as out-group) (J. Hawdon, unpublished and GenBank accession number EU007444–EU007447 and AJ417719).

3. Results

A total of 30 haplotypes were identified among the 164 Brazilian *A. caninum* COI sequences (accession number EF566762–EF566791). The three common

haplotypes h1, h3 and h4 were found in 42 (25.6%), 25 (15.2%) and 25 (15.2%), of the 164 *A. caninum* individuals examined, respectively (Table 1), while the other 27 haplotypes were each found in ≤ 13 individuals (0.6–7.9%). Haplotype diversity (h), nucleotide diversity (π) and the mean number of pairwise differences (K) were similar among the populations (Table 1). Nucleotide variation was detected at 66 (14.1%) of the 467 nucleotide sequenced. Most of the nucleotide variation in the COI gene was restricted to the third-base codon position (data not shown). The mean A + T content of all 30 COI haplotypes was 70.78%. The percentage of transitions was significantly greater than that of transversions (Table 1). Fu's and Tajimas's tests indicated that the populations are under mutation-drift equilibrium and there is no evidence for substantial positive natural selection occurring at this locus (Table 1). The molecular variance (AMOVA) analysis indicated that 11.75% of the total variation in haplotypic identity was distributed among sites ($P < 0.05$) indicating a moderate differentiation among

Table 1
Molecular diversity indices from Brazilian populations of *Ancylostoma caninum*

mtDNA COI (467 nts)						
Population	BH	CG	CT	RP	SL	Entire dataset
Molecular diversity						
Sample sizes	37	44	30	11	42	164
Number of haplotypes	14	7	10	4	13	30
Number of polymorphic sites	32	17	45	16	28	66
Haplotype diversity (h)	0.90 \pm 0.02	0.77 \pm 0.03	0.74 \pm 0.08	0.74 \pm 0.10	0.84 \pm 0.03	0.88 \pm 0.001
Nucleotide diversity (π)	0.018 \pm 0.009	0.014 \pm 0.007	0.019 \pm 0.010	0.016 \pm 0.009	0.011 \pm 0.006	0.016 \pm 0.014
Mean number of pairwise differences (K)	8.33 \pm 3.95	6.49 \pm 3.13	8.77 \pm 4.16	7.33 \pm 3.72	5.42 \pm 2.67	7.522
Number of transitions	29	15	32	15	22	–
Number of transversions	4	2	13	1	7	–
Transitions/transversions	7.25	7.5	2.46	15	3.14	–
Number of indels	0	0	0	0	0	0
Frequencies of most common haplotypes						
h1	0.22	0.34	0.50	0.27	0.02	45
h3	0.19	0.18	0.10	0.18	0.12	25
h4	0.13	0.14	0.07	0	0.29	25
h12	0.03	0.27	0	0	0	13
Nucleotide composition						
Nucleotide composition						
%C	18.96	18.85	19.03	19.14	18.77	18.95
%T	25.24	25.38	25.30	25.05	25.53	25.30
%A	45.59	45.53	45.36	45.55	45.37	45.48
%G	10.21	10.24	10.31	10.26	10.32	10.27
Test of selective neutrality						
Tajima's D (P -values)	0.15 (0.65)	1.93 (0.97)	–1.00 (0.15)	1.30 (0.93)	–0.65 (0.28)	–1.09 (0.12)
Fu's F_s (P -values)	0.41 (0.60)	5.78 (0.96)	2.58 (0.85)	4.63 (0.97)	–0.35 (0.49)	–2.32 (0.35)

Geographic localizations: BH, latitude 19°55'S and longitude 43°56'W; CG, latitude 20°26'S and longitude 54°38'W; CT, latitude 25°25'S and longitude 49°14'W; RP, latitude 21°12'S and longitude 47°48'W; SL, latitude 2°31'S and longitude 44°16'W.

what was found in human hookworms, *N. americanus*, sampled across a similar geographic area in China (Hawdon et al., 2001), and is also similar to the genetic structure observed in *Ascaris suum/lumbricoides* populations from humans and pigs (Anderson, 1995; Anderson and Jaenike, 1997).

Our overall molecular variation ($\pi = 0.014$) was similar to that observed by Hu et al. (2002) in a sample of 38 *A. caninum* from one city in Australia, and to that of *N. americanus* from Chinese human populations (Hawdon et al., 2001; $\pi = 0.012$). Together, these results suggest a fairly similar population biology among hookworms world wide. In contrast, trichostrongylid nematodes of domestic ruminants are characterized by higher genetic diversities and ultralow structure, with worms from different locations effectively united into one large panmictic population (Blouin, 1998; summarized in Table 5 in Höglund et al., 2006). The low genetic structure of trichostrongylids of domestic ruminants is probably due to their extremely large effective population sizes and to the extensive livestock movements between farms (Blouin, 1998). Effective sizes in hookworms and *Ascaris* are probably much smaller as a result of their much smaller infection intensities, which result in lower genetic diversity in populations and in each species as a whole (e.g. Fig. 1 in Criscione and Blouin, 2005). Host movement is also probably more extensive in livestock animals of economic significance than in dogs. The dog hosts of *A. caninum* are domestic animals and their movement is associated with human mobility and also with the socioeconomic conditions of their human owners. For example, it can be assumed that people experiencing better economical conditions are more likely to take their pets along with them when they move, in comparison with less economically favored families. In Brazil, rural–urban migration has continued over the last 50 years. During 1950–1980, most of the rural–urban migration occurred in the Southeast and the South. In the last three decades, the rural areas of the Northeast and agricultural frontier areas in the Midwest and the North contributed with high numbers of migrants to other regions of Brazil (Camarano and Abromovay, 1998). We sampled worms from public zoonotic central controls in different regions of Brazil. Most of the dogs captured and submitted to necropsy in these centers came from the area surrounding the cities. It is plausible to assume that these areas include some of the many migrants that characterize recent Brazilian demographic history. Therefore, we could be sampling and sequencing haplotypes that represent a recent mix of different regions of Brazil. For example, the

haplotype h18 found in site CT is phylogenetically different from the other ones sequenced in this and other localities, and it could be a result of a demographic event including dogs that migrated to CT from Brazilian regions not sampled in this study or from other regions of the world. CT (Curitiba) is located in southern Brazil, a region that received many migrants, especially Europeans, in the last century. Considering the extensive migration that has occurred in Brazil in the last century, the absence of a strong positive correlation between genetic and geographical distances among populations in a human commensal species is, perhaps, not surprising (and again, mirrors the pattern seen in hookworms of humans themselves). Thus, our results are consistent with the hypothesis that recent migrations have played a major role in determining the patterns we have observed.

Hu et al. (2002) used SSCP and sequencing of the partial COI mtDNA gene to assess molecular diversity and genetic structure in hookworms. They found two genetically distinct subpopulations of *A. caninum* in dogs from Townsville, Australia. Because *A. caninum* from Townsville can infect other hosts such as cats and humans (Prociv and Croese, 1996), the authors have speculated about the possibility of genetically distinct subpopulations within *A. caninum* that selectively infect these non-canine hosts. They also suggest that recent contact between allopatrically evolved populations or subpopulations due to host movement from other geographical areas could explain the pattern of haplotypic variability within *A. caninum*. We observed high genetic differentiation among three mtDNA clades with a clear signal of a very old divergence between one group and the other two. One hypothesis to explain the genetic pattern found is that the clades represent previously differentiated allopatric populations that have recently been mixed by host movement, as suggested by Hu et al. (2002). An alternative hypothesis is suggested by the observation that, in nematodes, a divergence at the COI gene of greater than 5% is characteristic of distinct species (Blouin, 2002). Therefore, it is possible that the third clade represents a cryptic species. We have no direct evidence of host-specialized subpopulations or ecological specializations among our samples, but that hypothesis deserves further consideration.

Anthelmintic resistance is a widely recognized issue in parasites of livestock animals, and drug resistance in hookworms and other parasites of companion animals has been described (Kopp et al., 2007). Drug resistance evolution is promoted by high effective sizes, and the spread of resistance alleles is promoted by high gene flow. Our data and those of Hu et al. (2002) and Hawdon

et al. (2001) suggest that for hookworms, neither effective sizes nor rates of gene flow are as high as those observed in trichostrongylids of livestock. Thus, the appearance and spread of resistance may not occur as rapidly in hookworms under similar selective pressures. On the other hand, the higher genetic differentiation among populations suggests that more care must be given to designing vaccines that will target antigenic variation species-wide (unlike the situation with many trichostrongylids, in which one population may be representative of most). Continued advances in our understanding of the population biology of parasitic nematodes will be important for continued advances in their control (Anderson et al., 1998; Viney, 1998; Gasser and Newton, 2000; Hawdon et al., 2001; Hu et al., 2004). This work adds to a growing literature on the population genetics of parasitic nematodes, from which we are now able to begin making generalizations about the similarities and differences among groups of nematodes.

5. Conclusion

In this study, the genetic structure of *A. caninum* originating from five different localities in Brazil was analyzed. We found similar genetic diversities, and moderate genetic differentiation, among the populations sampled. Overall levels of diversity and patterns of genetic structure mirror those observed in two other studies on hookworms. Thus, a consensus is emerging about what hookworm genetic structure is likely to be in different regions worldwide. This knowledge has important implications for understanding ecology, development of vaccine programs and evaluation of drug resistance.

Acknowledgements

The authors thank the people from Zoonoses control Centers (CCZs) of the Brazilian cities for their cooperation and help to obtain the worms. We also thank Dr. Rodrigo Redondo for his comments in this work and M.S. Hudson A. Santos for his help with the parasite identification. CNPq/Brazil funded Rodrigo Miranda with Ph.D. scholarship. This work was also supported by CNPq/Brazil grant (process number 476406/2004-8).

References

- Albonico, M., Smith, P.G., Ercole, E., Hall, A., Chwaya, H.M., Alawi, K.S., Savioli, L., 1995. Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area. *Trans. R. Soc. Trop. Med. Hyg.* 89, 538–541.
- Anderson, T.J.C., 1995. *Ascaris* infections in humans from North America: molecular evidence for cross-infection. *Parasitology* 110, 215–219.
- Anderson, T.J.C., Jaenike, J., 1997. Host specificity, evolutionary relationships and macrogeographic differentiation among *Ascaris* populations from humans and pigs. *Parasitology* 115, 325–342.
- Anderson, T.J.C., Blouin, M.S., Beech, R.N., 1998. Population biology of parasitic nematodes: applications of genetic markers. *Adv. Parasitol.* 41, 219–283.
- Bandelt, H.-J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16, 37–48.
- Blouin, M.S., 1998. Mitochondrial DNA diversity in nematodes. *J. Helminthol.* 72, 285–289.
- Blouin, M.S., 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *Int. J. Parasitol.* 32, 527–531.
- Bungiro, R., Cappello, M., 2004. Hookworm infection: new developments and prospects for control. *Curr. Opin. Infect. Dis.* 17, 421–426.
- Camarano, A.A., Abromovay, R., 1998. Texto para discussão no. 621 Êxodo rural, envelhecimento e masculinização no Brasil: Panorama dos últimos 50 anos. IPEA, Brasília, 28 pp., http://www.econ.fea.usp.br/abramovay/artigos_cientificos/1999/Exodo_rural.pdf.
- Criscione, C., Blouin, M.S., 2005. Effective sizes of macroparasite populations: a conceptual model. *Trends Parasitol.* 21, 212–217.
- Ewing, B., Green, P., 1998. Basecalling of automated sequencer traces using Phred II: error probabilities. *Genome Res.* 8, 186–194.
- Ewing, B., Hillier, I., Wendi, M., Green, P., 1998. Basecalling of automated sequencer traces using Phred I: accuracy assessment. *Genome Res.* 8, 175–185.
- Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinform. (Online)* 1, 47–50.
- Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3, 294–299.
- Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 147, 915–925.
- Gasser, R.B., Newton, S.E., 2000. Genomic and genetic research on rursate nematodes: significance, implications and prospects. *Int. J. Parasitol.* 30, 509–534.
- Gilles, H.M., 1985. Selective primary health care: strategies for control of disease in the developing world hookworm infection and anemia. *Rev. Infect. Dis.* 7, 111–118.
- Gordon, D., Abajian, C., Green, P., 1998. Consed: a graphical tool for sequence finishing. *Genome Res.* 8, 195–202.
- Gupta, S., Trenholme, K., Anderson, R.M., Day, K.P., 1994. Antigenic diversity and the transmission dynamics of *Plasmodium falciparum*. *Science* 263, 961–963.
- Hawdon, J.M., Li, T., Zhan, B., Blouin, M.S., 2001. Genetic structure of populations of the human hookworm, *Necator americanus*, in China. *Mol. Ecol.* 10, 1433–1437.
- Höglund, J., Morrison, D.A., Mattsson, J.G., Engström, A., 2006. Population genetics of the bovine/cattle lungworm (*Dictyocaulus*

- viviparus*) based on mtDNA and AFLP marker techniques. *Parasitology* 113, 89–99.
- Hotez, P.J., Pritchard, D.I., 1995. Hookworm infection. *Sci. Am.* 272, 68–75.
- Hotez, P.J., Ghosh, K., Hawdon, J.M., et al., 1999. Experimental approaches to the development of a recombinant hookworm vaccine. *Immunol. Rev.* 171, 163–171.
- Hotez, P.J., Zhan, B., Bethony, J.M., et al., 2003. Progress in the development of a recombinant vaccine for human hookworm disease: the human hookworm vaccine initiative. *Int. J. Parasitol.* 33, 1245–1258.
- Hu, M., Chilton, N.B., Zhu, X.Q., Gasser, R.B., 2002. Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome *c* oxidase subunit 1 reveals significant substructuring in hookworm populations. *Electrophoresis* 23, 27–34.
- Hu, M., Chilton, N.B., Gasser, R.B., 2004. The mitochondrial genomics of parasitic nematodes of socio-economic importance: recent progress, and implications for population genetics and systematics. *Adv. Parasitol.* 56, 134–212.
- Keymer, A., Bundy, D., 1989. Parasitology. Seventy-five years of solicitude. *Nature* 337, 114.
- Kopp, S.R., Kotze, A.C., McCarthy, J.S., Coleman, G.T., 2007. High-level pyrantel resistance in the hookworm *Ancylostoma caninum*. *Vet. Parasitol.* 143, 299–304.
- Prociv, P., Croese, J., 1996. Human enteritic infection with *Ancylostoma caninum*: hookworms reappraised in the light of a “new” zoonosis. *Acta Trop.* 62, 23–44.
- Rozas, J., Sánchez-Delbarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19, 2496–2497.
- Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123, 585–596.
- Viney, M.E., 1998. Nematode population genetics. *J. Helminthol.* 72, 281–283.
- Waldschmidt, A.M., Salomão, T.M.F., Barros, E.G., Campos, L.A.O., 1997. Extraction of genomic DNA from *Melipona quadrifasciata* (Hymenoptera: Apidae Meliponinae). *Braz. J. Genet.* 20, 421–423.