Hammondia heydorni: evidence of genetic diversity among isolates from dogs

C. Sreekumar, a,* D.E. Hill, a K.B. Miska, a B.M. Rosenthal, a M.C.B. Vianna, a L. Venturini, b W. Basso, b S.M. Gennari, c D.S. Lindsay, d and J.P. Dubey a

a Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705, USA
b Catedra de Parasitologia, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118, 1900 La Plata, Argentina
c Departamento de Medicina Veterinaria Preventiva e Saúde Animal, Faculdade de Medicina Veterinaria Zootecnia, Universidade de Sao Paulo (USP), Avenida Prof. Orlando Marques de Paiva, 87, Cidade Universitaria, Sao Paulo, SP CEP 05508-000, Brazil
d Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061, USA

Received 6 November 2003; received in revised form 15 April 2004; accepted 21 April 2004
Available online 1 June 2004

Abstract

Canine isolates of Hammondia heydorni from Argentina, Brazil, and the United States were analysed for genetic diversity. A total of 14 isolates were tested for their ability to produce amplification using three PCR assays, one targeting the common toxoplasmatiid ITS-1 region and 2 amplifying novel, H. heydorni-specific loci, HhAP7 and HhAP10. While the ITS-1 fragments could be amplified from all isolates, only six isolates were capable of amplifying the fragments from the novel loci. The PCR products were further investigated for genetic diversity using restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) techniques. Polymorphism in the digestion pattern was evident only at the HhAP10 locus, differentiating two of the Argentinean isolates from the remainder. Mobility shifts on SSCP gels revealed that the two Argentinean isolates were not only different from the other four isolates, but also differed from each other, both at the HhAP7 and HhAP10 loci. The ITS-1 fragments of all isolates were identical by RFLP. However, two distinct mobility patterns resulted when the products were electrophoresed on SSCP gels. Based on the sequence data from the ITS-1 and the two random loci, the isolates could be broadly classified into two distinct groups, within which minor polymorphisms were evident. In contrast, very little heterogeneity occurred in the sequences of corresponding ITS-1 regions of Neospora caninum and Toxoplasma gondii isolates. Thus, it is concluded that there is a considerable degree of microheterogeneity among isolates of H. heydorni. This diversity should be taken into consideration while attempting to elucidate the systematics, diagnostics, and biology of H. heydorni in relation to N. caninum.

Published by Elsevier Inc.

Index Descriptors and Abbreviations: Apicomplexa, Hammondia heydorni; Neospora caninum; Dog; Toxoplasma gondii; Genetic diversity; RFLP; SSCP; Sequence comparison; APS, ammonium persulfate; DNA, deoxyribonucleic acid; ITS, internal transcribed spacer; LSU rDNA, large subunit ribosomal DNA; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSCP, single strand conformation polymorphism; TBE, tris borate ethylenediaminetetraacetic acid; TEMED, N,N,N',N'-tetramethylethylenediamine

1. Introduction

Hammondia heydorni (Protozoa: Apicomplexa) was among the first coccidians to be discovered from the dog. However, little is known about the biology, life cycle or genetics of this parasite. It is also not known whether additional species of Hammondia utilize dogs as their definitive host (Dubey et al., 2002). Interest in H. heydorni has been renewed by the discovery of Neospora caninum, a parasite of livestock and companion animals. Whereas H. heydorni has not been associated with any disease condition, N. caninum has been identified as an important cause of abortion in dairy cattle worldwide (Dubey, 2003). The domestic dog (see Lindsay et al., 1999, 2001; McAllister et al., 1998) and

* Corresponding author. Fax: 1-301-504-9222.
E-mail address: kumar@anri.barc.usda.gov (C. Sreekumar).

0014-4894/$ - see front matter. Published by Elsevier Inc.
the coyote (Gondim et al., 2004) are the known definitive hosts for *N. caninum*. The parasite can be transmitted transplacentally, by the ingestion of infected tissues, and by the ingestion of food and water contaminated with oocysts excreted in the feces of dogs. However, the role of the dog in the epidemiology of *N. caninum* is currently unclear, as experimentally infected dogs have been shown to excrete only few oocysts and because the parasite has been isolated only a few times from naturally infected dogs (Basso et al., 2001; McGarry et al., 2003; Slapeta et al., 2002b). The oocysts of *N. caninum* resemble those of *H. heydorni* and there is no simple method to distinguish them. This morphological similarity, coupled with the lack of knowledge on the biology of *H. heydorni*, has led to an uncertainty regarding the systematic positions and distinctness of both *N. caninum* and *H. heydorni* (Mehlhorn and Heydorn, 2000; Heydorn and Mehlhorn, 2002). Thus, more information is needed about the biology and genetic make-up of *H. heydorni* to clearly differentiate it from *N. caninum*.

Recently, molecular diagnostic tools have been described for the specific detection of *H. heydorni*, based on the PCR amplification of ITS-1 (Slapeta et al., 2002a) and two random genetic loci (Sreekumar et al., 2003). It has been observed that these tools do not provide means for diagnosing all isolates currently considered to represent *H. heydorni* (Sreekumar et al., 2003). Recent literature also suggests that there are both phenotypic and genetic differences among isolates of *H. heydorni* obtained from foxes and dogs (Mohammed et al., 2003; Schares et al., 2003). However, no studies have yet explicitly explored this variation. Restriction fragment length polymorphism (RFLP) analysis of PCR-derived products can detect polymorphism between closely related genomes. Single strand conformation polymorphism (SSCP) (Orita et al., 1989) can be used to detect point mutations in PCR products. In this technique, PCR is used to amplify the region of interest and the resultant DNA is separated as single-stranded molecules and electrophoresed in a non-denaturing polyacrylamide gel. Single strands of DNA differing by as few as a single base may fold differently and such changes in the tertiary structure result in different mobilities for the two strands (Orita et al., 1989). Here, we investigate the genetic diversity among isolates of *H. heydorni* obtained from dogs, using RFLP, SSCP, and direct sequencing of three different *H. heydorni*-specific PCR products.

**2. Materials and methods**

**2.1. Parasites**

The *H. heydorni* isolates used in this study were obtained as oocysts from dogs from the USA, Argentina, and Brazil (Table 1). They were presumed to be *H. heydorni* based on the morphology (measuring ~10 μm × 13 μm) and the failure to amplify the target fragment from their DNA using previously published *N. caninum*-specific primers (Yamage et al., 1996). For comparison, *N. caninum* (Spanish isolate) and *Toxoplasma gondii* (B1 isolate) DNA were included.

**2.2. Genetic analysis**

DNA extraction and PCR conditions have been described previously (Sreekumar et al., 2003). Amplification products obtained with the common toxoplasmatiid ITS-1 primers and the two sets of RAPD-derived primers, HhAP7 and HhAP10 (Sreekumar et al., 2003), were used for further study. The PCR products were initially electrophoresed in a 2% agarose gel. The specific amplicons were enriched by purifying the bands from the gel using MinElute gel extraction kit (Qiagen, Valencia, CA) followed by re-amplification using the respective primers and used for subsequent studies.

**2.2.1. PCR-RFLP**

Restriction enzymes Dde1 (ITS-1 and HhAP7) and Sau3A1 (HhAP10), with a single recognition site on the respective amplicons, were selected using the GeneTool Software (Bio Tools, Alberta, Canada). Each digestion was performed in 20 μl aliquots using 10 μl of PCR product and 10 U of enzyme. After incubation at 37°C for 4 h, the products were mixed with loading dye, electrophoresed in a 1.5% agarose gel, and documented.

---

### Table 1

*Isolates of *H. heydorni* used and their PCR profiles*

<table>
<thead>
<tr>
<th>Isolates</th>
<th>NP6/NP21&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR with HhAP&lt;sup&gt;b&lt;/sup&gt; and HhAP10&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CT1/CT2&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N. caninum</strong></td>
<td><strong>H. heydorni</strong></td>
<td><strong>Toxoplasmatiid</strong></td>
<td><strong>ITS-1</strong></td>
</tr>
<tr>
<td>1. Manhattan (Kansas)-1</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>2. Virginia -1</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>3. Mississippi-1</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>4. Mississippi-2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>5. Brazil-1</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>6. ARG-1</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>7. ARG-28</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>8. ARG-32</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>9. ARG-33</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>10. ARG-36</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>11. ARG-45</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>12. ARG-50</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>13. ARG-51</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>14. ARG-52</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

<sup>a</sup> Yamage et al. (1996).
<sup>b</sup> Sreekumar et al. (2003).
<sup>c</sup> Dubey et al. (2004).
using ProExpress Gel Documentation system (Perkin-Elmer, Wellesley, MA).

2.2.2. PCR-SSCP

The SSCP mobility shifts in PCR products were detected as described by Strippoli et al. (2001). Briefly, 8 μl of enriched PCR products were mixed with 1 μl of denaturation buffer (500 mM Tris-HCl and 100 mM NaOH) and heated to 95 °C for 5 min. The tubes were then snap chilled in ice and mixed with 1 μl SSCP loading dye (0.25% bromophenol blue and 0.25% xylene cyanol in formamide). The products were electrophoresed in 20 cm × 20 cm × 1 mm polyacrylamide gels (5.5% acrylamide (39):bisacrylamide (1) mix, 5% glycerol, in 0.5% TBE, polymerized with 0.08% each of TEMED and APS) with 1% TBE run buffer at 400 V and 20 °C until the xylene cyanol dye front passed out from the gel. The ITS fragments were electrophoresed at 125 V and 10 °C overnight. Gels were subsequently stained with ethidium bromide (1 μg/ml) and documented as described above.

2.2.3. Sequence analysis

The PCR products were directly sequenced in both directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, CA) using an ABI 377 sequencer. The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes, Ann Arbor, MI).

The sequences were aligned using Clustal X software (Thompson et al., 1997). Neighbor joining trees were reconstructed from pairwise Kimura 2-parameter distances of full-length H. heydorni ITS-1 sequences to infer the structure of variation among them. Additionally, the ITS-1 variation was placed within a larger comparative context by constructing a 277 bp alignment comprising these and other toxoplasmatiid homologues.

Average pairwise nucleotide polymorphism (π) and their standard errors were estimated from a homologous 277 nucleotide portion of ITS-1 as a means of estimating the comparative genetic diversity among parasites corresponding to T. gondii, N. caninum, H. hammondi, and H. heydorni using the bootstrap method of MEGA 2.1 (Kumar et al., 2001).

3. Results

The ITS-1 fragments were successfully amplified from all 14 isolates (Fig. 1). No polymorphisms were evident in the RFLP patterns of the ITS-1 PCR products. However, two distinct mobility patterns resulted among the 14 isolates when the ITS-1 fragments were electrophoresed on an SSCP gel (Fig. 1). Whereas isolates Manhattan-1, Virginia-1, ARG-50, ARG-1, and ARG-33 shared one electrophoretic pattern, the bands from isolates ARG-45, ARG-51, Mississippi-1, Brazil-1, Mississippi-2, ARG-28, ARG-32, ARG-36, and ARG-52 co-migrated differently. Sequence analyses subsequently confirmed the presence of polymorphism indicated by PCR-SSCP (below).

The same 6 of 14 isolates supported amplification of the HhAP7 and HhAP10 loci (Fig. 2); 4 of these were from Argentina and the other two from the USA. No polymorphisms were evident in the RFLP patterns of the HhAP7 (Fig. 2) PCR products. PCR-RFLP of the HhAP10 amplicon revealed differences between the isolates (Fig. 2). Although the anticipated digestion pattern occurred in four isolates, no digestion occurred in isolates ARG-45 or ARG-51.

The SSCP patterns of HhAP7 and HhAP10 amplicons showed reproducible polymorphisms between the isolates (Fig. 2). While the mobility patterns for four of the isolates, Manhattan-1, Virginia-1, ARG-50, and Arg-51, were similar, the band mobilities for the other 2 isolates, ARG-45 and ARG-51, were distinct. The mobility patterns for these two isolates differed from one another and from those of the other four isolates.

Sequence analyses confirmed the presence of polymorphism indicated by PCR-RFLP and PCR-SSCP. The sequences of the 517 bp HhAP7 amplicons (Fig. 3) from Manhattan-1, Virginia-1 (AY373531), ARG-1 (AY373530), and ARG-50 (AY373528) isolates were identical. The sequences for isolates ARG-45 (AY373527) and ARG-51 (AY373529) differed from the other 4 isolates by 17 and 14 nucleotides, respectively. The two fragments were in turn differentiated from each other by a 3-nucleotide insertion in the amplicon from...
isolate ARG-45, which was absent from the ARG-51 fragment.

Comparison of the sequences of the 369 bp HhAP10 region (Fig. 3) revealed that the fragments from isolates Manhattan-1, Virginia-1 (AY373532), ARG-1 (AY373533), and ARG-50 (AY373535) differed by 14 nucleotides from the fragments of ARG-45 (AY373534) and by 13 nucleotides from that of ARG-51 (AY373536). There was a single nucleotide difference in the HhAP10 amplicons between the isolates ARG-45 and ARG-51. The substitution of an A residue with a C at the 105th position in isolates ARG-45 and ARG-51 (GCTC instead of GATC) had resulted in the loss of the recognition sequence for the enzyme Sau3A1, leading to the lack of digestion.

The sequences of the ITS-1 fragments (Fig. 3) from isolates Manhattan-1 (AY579757), Virginia-1 (AY579758), ARG-50 (AY579760), and ARG-1 (AY579762) were identical. The sequence of isolate ARG-33 (AY579767) differed at a single nucleotide from the previous four. The sequences of isolates ARG-45 (AY579759), Mississippi-1 (AY579763), ARG-28 (AY579765), ARG-32 (AY579766), ARG-36 (AY579768), and ARG-52 (AY579769) were identical. These six sequences differed from the other three identical sequences, ARG-51 (AY579761), Brazil-1 (AY579764), and Mississippi-2 (AY530018) by a single nucleotide polymorphism.

Neighbor joining trees reconstructed from pairwise Kimura 2-parameter distances of full-length ITS-1 sequences grouped all the H. heydorni isolates within two distinct clades (Fig. 4), with minor polymorphism evident within the clades. Alignment of a 277 bp stretch of the ITS-1 region from isolates of H. heydorni and other toxoplasmatid homologues (Fig. 5) clearly grouped the different taxa into distinct clades.

Over the homologous portion of ITS-1, no polymorphism was evident among the available exemplars of either N. caninum (AY582109 (Spanish isolate), AY463245, AF249970, AF249969, AF249968,
4. Discussion

PCR-RFLP, PCR-SSCP, and sequence analyses revealed polymorphism among the 14 isolates of *H. heydorni*. At the HhAP7 and HhAP10 loci, whereas four of the geographically disparate isolates (ARG-1, ARG-50, Virginia-1, and Manhattan-1) were identical in their genetic makeup, the two other isolates (ARG-45 and ARG-51) were unique. Based on the ITS-1 sequences the 14 isolates could be broadly grouped into 2 clades (Fig. 4), with only minor polymorphisms evident between the isolates within each clade. Thus the pattern of polymorphism was consistent across three independent loci (ITS-1, HhAP7, and HhAP10).

Earlier studies have pointed to the presence of phenotypic differences among isolates of *H. heydorni*. Some of the isolates have been known to be infective to gerbils (Schara et al., 2003) while others were not (J.P. Dubey, unpublished). Schara et al. (2003) speculated that the ability to induce tissue cyst formation in vitro may be unique to the fox-derived isolates and thus could be a trait lacking in the dog-derived isolates. Recent evidence has also pointed to the presence of molecular differences among isolates of *H. heydorni* from dogs and foxes. Mohammed et al. (2003) concluded that the isolates of *H. heydorni* from foxes and dogs constitute genetically different populations based on the rDNA sequences.

However, the polymorphisms observed in this study appeared to be not limited by geographical or biological constraints. Each of the two major clades encompassed isolates from the USA and Argentina, and a fox isolate was not differentiable from others originating in domestic dogs, suggesting that neither geography nor host species constitute biologically significant barriers to the distribution of these parasite genotypes.

The corresponding ITS-1 sequences of *T. gondii* and *N. caninum* revealed comparatively little inter-isolate polymorphism, corroborating previous findings (Homan et al., 1997). Recent studies have provided evidence of molecular distinction between *N. caninum* and *H. heydorni*. Slapeta et al. (2002b), based on ITS-1 and LSU rDNA sequences, emphasized that *H. heydorni* is distinct from *N. caninum*. Similar results have been obtained from the analyses of different loci like the α-tubulin genes (Siverajah et al., 2003). The evidence provided herein further assert the point that *H. heydorni* is indeed a distinct organism from *N. caninum*.

The pairwise nucleotide polymorphism among isolates presumed to represent *H. heydorni* (\(\pi = 0.005927\), \(\sigma = 0.00338\)) was considerably greater than that observed among isolates (AY582110 (B1 isolate), U16161, X75453, X75429, L49390, AF252408, AY488170, AY488169, AY488167, AY488166, and AY143138) of *T. gondii* (\(\pi = 0.00567\); \(\sigma = 0.00565\)). The comparatively greater variation in isolates presumed to represent *H. heydorni* accounts for their especially great horizontal depth depicted in Fig. 5. Despite their apparently higher genetic variability, the isolates of *H. heydorni* appear monophyletic with respect to other toxoplasmatiids (Fig. 5). Thus, whether or not epidemiologically significant phenotypic variation is eventually discovered among them, these provisionally named *H. heydorni* isolates nonetheless would appear to share an especially close evolutionary relationship. The same cannot be said for all parasites currently assigned to the genus *Hammondia*. Our data buttress previous findings that the common ancestor of *H. heydorni* and *H. hammondi* also gave rise to *T. gondii* and *N. caninum*, creating conflict between parasite phylogeny and their current taxonomic nomenclature (Jenkins et al., 1999; Slapeta et al., 2002b). Until we learn more about the structure of these parasite taxa, it will be difficult to determine the reasons for the comparatively scant variation among other toxoplasmatiid taxa.

Although our limited sample size is insufficient to address the population structure of *H. heydorni* in any detail, it identified at least two presumptive genotypes to be geographically widespread. Also, the apparent similarity of the isolates at each of three loci indicates that such taxa may be comprised of non-recombinant, clonal strains. Further evidence is therefore needed to corroborate, or refute, a clonal genetic structure for canine-excreted coccidia presently presumed to represent either *N. caninum* or *H. heydorni*.
The present study underscores our incomplete understanding of canine-excreted coccidia and indicates that the population structure of *H. heydorni* may be complex. The biological significance of genetic differentiation is seldom clear when applied to organisms, such as these, whose phenotypes are only poorly known. In the case of parasites known or suspected to pose risk to veterinary or human health, such diagnostic tools should help frame future investigation. Here, increasingly discriminatory tools have, perhaps inevitably, enabled recognition of more genetically distinct forms.

The application of SSCP technique for mutation analysis of parasite genome is well documented (Gasser, 1997). The technique has been commonly used for genetic analysis of apicomplexan parasites (Gasser et al., 2003; Sallenave-Sales et al., 2003). In the present study, using the SSCP technique, it was possible to unequivocally differentiate haplotypes demarcated by as few as 3 and 1 nucleotide polymorphisms in the 517 bp HhAP7 and the 369 bp HhAP10 PCR products, respectively. The results reiterate the simplicity of this technique and its efficacy to identify single nucleotide differences between PCR products without resorting to time consuming and expensive sequencing protocols.

Acknowledgments

The authors thank Valsin Fournet and Jenifer Herrmann, Animal Parasitic Diseases Laboratory, USDA, for their assistance with the figures and sequencing, respectively.

References


Lindsay, D.S., Dubey, J.P., Duncan, R.B., 1999. Confirmation that the dog is a definitive host for *Neospora caninum*. Veterinary Parasitology 82, 327–333.


