Molecular Phylogenetic Analysis of Enterobius vermicularis and Development of an 18S Ribosomal DNA-Targeted Diagnostic PCR

Ulrike E. Zelck, Ralf Bialek, and Michael Weiβ

Molecular Parasitology Unit, Institute for Tropical Medicine, University of Tübingen, Wilhelmstraße 27, D-72074 Tübingen, Labor Dr. Krause & Kollegen MVZ GmbH, Steenbeker Weg 25, D-24106 Kiel, and Organismic Botany, Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

Received 7 December 2010/Returned for modification 14 December 2010/Accepted 10 January 2011

We genetically characterized pinworms obtained from 37 children from different regions of Germany and established new species-specific molecular diagnostic tools. No ribosomal DNA diversity was found; the phylogenetic position of Enterobius vermicularis within the Oxyurida order and its close relationship to the Ascaridida and Spirurida orders was confirmed.

Pinworms are the most common intestinal parasites in developed countries in temperate climates (1). Humans are the only natural host of Enterobius vermicularis, and children are the most often affected by this disease, which is spread from the anus to mouth. Although it is regarded as a harmless infection, severe disease like colitis, perianal abscess, ectopic infections in females, and appendicitis can occur (1, 3, 12). Whereas eradication is achieved by anthelmintics in most cases (12), recurrent or persistent oxyuriasis lasting for years despite several treatment courses is seen in some patients. Molecular tools might help to understand transmission routes and distinguish persistent from repeated infections. However, sequence information on E. vermicularis is limited (6, 7).

Pinworms were identified by examining feces from children with a magnifier, washed in tap water, and stored at −20°C. Then, pinworms from 37 children residing in different geographic regions of Germany were thawed and mechanically homogenized to extract DNA (Qiamp DNA minikit; Qiagen, Hilden, Germany). Enterobius vermicularis ribosomal DNA (rDNA) was amplified and sequenced as partially overlapping fragments using universal primers (AB28/TW81 and NEMF1/S3), degenerated nematode primers, and species-specific primers (Table 1). Amplification reaction mixtures (50 μl) consisted of 100 nM (each) primer, 50 μM (each) deoxynucleoside triphosphates (dNTPs), 2.5 mM MgCl2, 0.5 units polymerase, 50 μl template. PCR amplification was performed as follows: (i) denaturation at 95°C for 5 min; (ii) 40 cycles, with 1 cycle consisting of 60 s at 94°C, 60 s at 50 to 60°C, and 2 min at 72°C for 2 min, and (iii) a final extension step at 72°C for 10 min. Amplification of 18S rDNA fragments for diagnostic purposes using Enterobius-specific primers Ev18S.F1 and Ev18S.R1 was performed at 55°C under otherwise identical conditions. Products were detected on ethidium bromide-stained agarose gels. PCR products were sequenced either directly or after gel extraction (QIAquick gel extraction kit).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>TW81</td>
<td>3' end of the 18S rDNA</td>
<td>GTT TCC GTA GGT GAA CCT GC</td>
</tr>
<tr>
<td>AB28</td>
<td>5' end of the 28S rDNA</td>
<td>ATA TGC GTA GGG GGA GCT GGG</td>
</tr>
<tr>
<td>NEMF1</td>
<td>3' end</td>
<td>S3</td>
</tr>
<tr>
<td>SSU_F_01</td>
<td>5' end</td>
<td>AGT CAA ATG AAG CCG CAG</td>
</tr>
<tr>
<td>SSU_R_26</td>
<td>5' end</td>
<td>AAC CTT GGG CAT GCT GCC AGT</td>
</tr>
<tr>
<td>SSU_R_24</td>
<td>5' end</td>
<td>CAT TCC TGT CAA ATG CTT CCG</td>
</tr>
<tr>
<td>SSU_R_31</td>
<td>5' end</td>
<td>AGR GGT GAA ATY CGT GGA CC</td>
</tr>
<tr>
<td>SSU_R_82</td>
<td>5' end</td>
<td>TGA TCC WKG YGC AGG TTC AC</td>
</tr>
<tr>
<td>Ev18S.F1</td>
<td>5' end</td>
<td>TGA TCC TTC TCG ACG TTC ACG</td>
</tr>
<tr>
<td>Ev18S.R1</td>
<td>5' end</td>
<td>AAA CCG GGA GAA CCA AT</td>
</tr>
<tr>
<td>Ev18S.F2</td>
<td>5' end</td>
<td>CCG GGT ATC GGA ATG GGG</td>
</tr>
<tr>
<td>Ev18S.R2</td>
<td>5' end</td>
<td>TTT CCG AGG ACC GCG TA</td>
</tr>
<tr>
<td>Ev18S.R3</td>
<td>5' end</td>
<td>GGA GGA TTT TCA GGG GGT TA</td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: Labor Dr. Krause & Kollegen MVZ GmbH, Steenbeker Weg 25, 24106 Kiel, Germany. Phone: 49 431 388 6590. Fax: 49 431 675160. E-mail: bialek@labor-krause.de.
† Published ahead of print on 19 January 2011.
FIG. 1. Phylogenetic placement of *Enterobius vermicularis* as derived from heuristic maximum likelihood analysis of an alignment of 18S rDNA sequences. The species is given first and then the GenBank (NCBI) accession number. The best tree found was rooted with *Tylocephalus auriculatus* and *Plectus aquatilis*. Branch lengths are given in terms of the estimated numbers of nucleotide substitutions per site. Asterisks mark branches that were scaled with factor 0.85 for graphical reasons. The numbers at the nodes are the maximum likelihood/maximum parsimony values. Branch support was calculated by maximum likelihood/maximum parsimony bootstrap from 1,000 replicates; values below 50% are designated by a / symbol or omitted. Bar, 0.1 nucleotide substitution per site.
from Brazil (6). The nonexistent genetic diversity might be due to excellent adaptation to the human host or low evolutionary pressure. However, analyses of less-conserved targets such as mitochondrial genes or the whole genome with for example randomly amplified polymorphic DNA (RAPD) using worldwide isolates are required to further elucidate the genetic diversity of Enterobius vermicularis. The small-subunit ribosomal DNA sequences were used to construct the molecular phylogeny of Nematomada (2, 9) where Enterobius vermicularis had not yet been included. Thus, we analyzed our 18S rDNA sequence together with other nematode sequences used by Nadler et al. (9) and published in GenBank (NCBI). This data set was aligned with DIALIGN (8). For phylogenetic reconstruction, we excluded those positions that received scores as low as 0 or 1 from the alignment. We ran maximum likelihood analyses using RAxML (11), involving rapid bootstrapping over 1,000 rounds and using every 5th bootstrap tree as a starting point for heuristic search; GTRMIX was used as a DNA substitution model. Additional branch support was calculated using maximum parsimony bootstrap in PAUP* (13); we ran 1,000 bootstrap replicates, with 100 rounds of heuristic search per replicate starting from trees that resulted from subsequent addition of sequences in random order, treating gaps in the alignment as missing data and the multitype option not in effect. The maximum likelihood tree was rooted with Plectus aquatilis (GenBank [NCBI] accession no. AF036602) and Tyleocephalus auriculatus (GenBank [NCBI] accession no. AF202155) and compared with previous molecular phylogenetic hypotheses (2, 4, 5, 9).

Our analyses (Fig. 1) assigned Enterobius vermicularis to the Oxyurida and confirmed previous studies showing that nematodes from the orders Ascaridida, Oxyurida, Rhigonematida, and Spirurida belong to a monophyletic group, informally designated “clade III” (2, 4, 9). Oxyurida is a highly supported monophyletic group (the maximum likelihood [ML] and maximum parsimony [MP] bootstrap values are 100 and 99, respectively), which appears as the sister group to a clade that includes Ascaridida and Spirurida.

Although not useful to distinguish isolates, the 18S rDNA appears conserved enough for diagnostic purposes. Using our primers Ev18S.F1 and Ev18S.R1 (Table 1), a single product of 215 bp was successfully amplified and sequenced from 35 pinworms examined. All Enterobius vermicularis sequences were identical, showing 73.9% identity to 18S rDNA of Trypanosaurus sciuri (Oxyurida), 71% identity to Oxyuris equi (Oxyurida), and 73% identity to Anisakis sp. (Ascaridida) when applied to NCBI Blast Search (15). No amplification was observed when DNA from adult Ascaris lumbricoides, Acanthochelionema sp., or human DNA was examined.

This PCR assay was used to examine 60 pieces of Scotch tape each fixed to a glass slide and sent to our lab for diagnosis. Typical pinworm eggs were identified by microscopy for only 40. Then, pieces of the tape (1 to 1.5 cm by 1 cm) were cut and scraped off the glass slide using a sterile scalpel to avoid carryover contamination, and the eggs were transferred into tubes for DNA extraction as described above. DNA was amplified from all 40 microscopically positive samples and from three negative samples. No PCR product was obtained from the remaining 17 samples. Two of the three unexpected PCR-positive samples originated from samples from two children that gave positive results by microscopy and PCR in a second sample. The third false-positive sample was obtained from a household member of an infected child. It was concluded that the diagnostic PCR assay is at least as sensitive as microscopy (10). Pinworm eggs are usually deposited on the perianal skin and detected by the Scotch tape technique. Thus, they are very rarely found in fecal samples usually sent for parasitological diagnostics. At this time, we are organizing a comparative diagnostic Enterobius PCR study using DNA isolated from fecal samples and Scotch tape.

In conclusion, we did not find any ribosomal DNA diversity of Enterobius vermicularis in Germany, we confirmed its phylogenetic position within Oxyurida, and developed a new species-specific diagnostic PCR which might identify more pinworm carriers than conventional tests.

Nucleotide sequence accession number. The 2,867-bp sequence was deposited in GenBank (NCBI) under accession number HQ646164.

The technical assistance of Ulrike Müller-Pinau and Andrea Dlugosch is highly appreciated. We thank two anonymous reviewers for very helpful suggestions and astute comments.

REFERENCES