Parasitol Res (1998) 84: 505–508

ORIGINAL PAPER

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A DNA probe for the detection of *Dicrocoelium dendriticum* in ants of *Formica* spp. and *Lasius* spp.

Received: 30 August 1997 / Accepted: 12 November 1997

Abstract Repetitive DNA sequences present in the genome of *Dicrocoelium dendriticum* were identified by hybridization of genomic DNA that had been digested with different restriction enzymes with ^{32}P-labeled genomic *D. dendriticum* DNA. DNA fragments containing repetitive sequences were isolated from *Pst*I-digested *D. dendriticum* DNA and were subcloned into a plasmid vector. Plasmids containing repetitive sequences were identified by colony hybridization. One of these plasmids, designated Ddr-IV, was isolated and used as a probe in further studies. Ddr-IV is specific for *D. dendriticum* since it does not hybridize to DNA isolated from other trematodes. In addition, Ddr-IV was capable of detecting *D. dendriticum* metacercariae in ants (*Formica cunicularia*, *F. rufibarbis*, and *Lasius* sp.), which act as second intermediate hosts in the parasite's life cycle. Since metacercariae constitute the infectious stage of the parasite for grazing animals, Ddr-IV will provide a useful tool for epidemiology studies of dicrocoeliosis.

Introduction

Dicrocoelium dendriticum, also called the small liver fluke, is capable of infecting a wide range of different mammals, including humans (Soulsby 1982). The occurrence of dicrocoeliosis is worldwide and economic losses can be considerable. The life cycle includes two intermediate hosts: land snails (*Zebrina* spp. and *Cionella* spp.), in which sporocysts and cercariae are produced, and ants (*Formica* spp. and *Lasius* spp., among others), in which metacercariae are found. Some metacercariae enter the brain of the ant, causing tetanic spasm of the mouthparts when environmental temperatures are lowered (Soulsby 1982). Infected ants remain attached to herbage overnight and are thus available to grazing animals (Dawes and Hughes 1964; Wright 1971). Mammals acquire infections when they ingest leaves on which infected ants are attached. Cercariae enter the liver via the bile duct, and young flukes are found at 47–54 days after ingestion of the metacercariae. The small flukes penetrate into the fine branches of the bile ducts, in which they are attached by means of their suckers. In advanced infection there is extensive cirrhosis and scarring of the liver surface. Portal cirrhosis and marked proliferation of the bile-duct glandular epithelium also occur. The clinical picture in severe cases consists of anemia, edema, and marked emaciation (Soulsby 1982).

The percentage of metacercariae-containing ants represents the infection risk in a particular area and is thus an important parameter in the epidemiology of dicrocoeliosis. Ants very frequently also harbor larvae of other trematode species, which may have nonmammalian animals as definitive hosts. Their morphological identification, especially when many samples need to be examined, can be difficult and time-consuming.

In the present work a DNA probe was developed that can be used in squash-blot analysis and allows *D. dendriticum* larvae to be discriminated from larvae of other trematodes infecting ants.

Materials and methods

Parasite collection

Adult *Dicrocoelium dendriticum* parasites were isolated from the gallbladders of infected cattle at the slaughterhouse in Bern,

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Switzerland. Adult *Fasciola hepatica* parasites were isolated from the livers of experimentally infected rats. *Xiphidiocercaria* spp. and *Diplostoma* spp. were harvested from snails collected at the shore of Lake Neuchatel in Switzerland. These infected snails were kept in centrifuge tubes containing lake water and were allowed to shed their parasites for several days. Parasites were centrifuged and the pellets were stored at −20 °C until DNA preparation. Further trematode larvae of the genera *Trichobilharzia* and *Dendritobilharzia* were harvested from *Vivipara vivipara* and *Lymnea auricularia* snails collected at Lake Bienne. Uninfected and infected ants were collected from pastures in areas well known to be endemic for *D. dendriticum*.

DNA isolation

DNA was prepared from *D. dendriticum*, *F. hepatica*, and *Ostertagia ostertagii* as follows: adult parasites were rinsed thoroughly in phosphate-buffered saline, snap-frozen in liquid nitrogen, pulverized using a mortar and pestle, and incubated for 30 min at 37 °C in 25 ml of TNE [10 m*M* TRIS, 0.1 *M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.5] containing 0.5% sodium dodecyl sulfate (SDS). RNAse A (100 μg ml^{-1}) was added and the lysate was incubated for a further 80 min, after which it was incubated for 60 min in the presence of pronase E (1 mg ml^{-1}). The lysate was then consecutively extracted with an equal volume of phenol, chloroform, and ether. DNA was precipitated by the addition of 2.5 vol. of ethanol, washed twice in 70% ethanol, and dissolved in 10 m*M* TRIS (pH 7.5) and 0.1 m*M* EDTA. The same procedure, using reduced volumes, was followed for the isolation of DNA from trematode larvae harvested from various snails.

Isolation and cloning of repetitive DNA fragments

D. dendriticum DNA was digested with the restriction enzymes *Sal*I, *Hin*dIII, *Sma*I, *Pst*I, *Xba*I, *Eco*RI, *Bam*HI, *Ava*I and *Kpn*I (Boehringer Mannheim, Rotkreuz, Switzerland) and fragments were separated by electrophoresis in TAE buffer in preparative 0.8% agarose gel (Maniatis et al. 1982). Filters were hybridized with genomic *D. dendriticum DNA* that had been labeled with ^{32}P by nick translation to identify repetitive DNA sequences. DNA with a fragment size corresponding to that of the repetitive DNA was isolated using Whatman 3MM paper as previously described (Errington 1990). *Pst*I fragments isolated in this way were ligated into the plasmid vector pUC19 (Vieira and Messing 1982), which was linearized by digestion with *Pst*I (Maniatis et al. 1982). *Escherichia coli* bacteria were transformed with the recombinant plasmid and positive colonies were identified by transfer of the bacterial colonies to nitrocellulose filters and hybridization of the filters with genomic *D. dendriticum* DNA labeled with ^{32}P by nick translation. Plasmid DNA was isolated from positive colonies and digested with *Pst*I (Maniatis et al. 1982). For confirmation that the inserts contained repetitive sequences, fragments were separated in a 0.8% agarose gel, the DNA was blotted onto Hybond-N membranes (Amersham, Zürich, Switzerland), and the filters were hybridized with nick-translated genomic *D. dendriticum* DNA (see below).

The ends of the plasmid clone Ddr-IV containing the *D. dendriticum* repetitive DNA were sequenced by the dideoxy chain-termination method (Sanger et al. 1977; Hattori and Sakaki 1986) using a T7 sequencing kit (Pharmacia, Dübendorf, Switzerland).

Dot-blot analysis

In all, 10 ng of genomic DNA from *D. dendriticum*, other trematodes, and *O. ostertagii* was spotted onto a Hybond-N membrane. The filter was placed on two layers of 3MM paper soaked in 0.5 *M* NaOH and 1 *M* NaCl for 3 min for denaturation. The filters were then placed on two layers of 3MM paper soaked in 1 *M* TRIS-HCl (pH 7.5)/3 *M* NaCl for 3 min (neutralization). DNA was fixed to the membranes by UV irradiation for 2 min.

Filters were prehybridized for a minimum of 2 h at 65 °C in hybridization solution consisting of 5 × Denhardt's solution, 5 × SSC, 0.1% SDS, and 0.1% sodium pyrophosphate in the presence of 100 μg ml^{-1} of herring-sperm DNA. DNA was labeled with [^{32}P]-deoxycytidine triphosphate (^{32}P-[dCTP]) using a nick translation kit from Amersham (Zürich, Switzerland). DNA probes were added to the hybridization solution and filters were hybridized overnight at 65 °C. Filters were washed three times for 20 min at 65 °C in 0.1 × SSC, 0.1% SDS, and 0.1% sodium pyrophosphate.

Squash blots of ants

For preparation of squash blots, ants were crushed onto a Hybond-N filter. Alternatively, ants were crushed on a glass slide in a drop of phosphate-buffered saline and parasites were collected with a Pasteur pipette and transferred to a Hybond-N filter. Filters were placed (sample facing upward) for 2 min on Whatman 3MM paper that has been soaked in 80 m*M* TRIS (pH 6.9) and 1% SDS. The filters were then transferred to Whatman 3MM paper soaked in 0.5 *M* NaOH/1.5 *M* NaCl for denaturation of the DNA (5 min) and, subsequently, to 3MM paper soaked in 1 *M* TRIS (pH 7.5) and 1.5 *M* NaCl. After air-drying of the filters the DNA was fixed to the membrane by UV irradiation and filters were then stored at room temperature until used.

Results

Isolation of a recombinant clone containing a highly repetitive DNA fragment

In a search for repetitive DNA fragments in the genome of *Dicrocoelium dendriticum*, genomic DNA isolated from adult parasites was digested with different restriction enzymes and subsequently blotted onto filters and hybridized with genomic *D. dendriticum* DNA labeled with ^{32}P by nick translation. A distinct banding pattern of repetitive DNA sequences could be detected in genomic DNA digested with *Pst*I (data not shown). DNA fragments corresponding to these regions were isolated and ligated into the plasmid vector pUC19, which was then used to transform *Escherichia coli*. Clones containing repetitive DNA sequences corresponding to each of these regions were obtained by screening of colonies of bacteria with genomic DNA labeled by nick translation. One clone, designated by the letters Ddr-IV (Dicrocoelium dendriticum repeat), containing a 2.1-kb fragment, was selected for further studies.

Southern-blot analysis of genomic *D. dendriticum* DNA digested with different restriction enzymes, using Ddr-IV as a probe, revealed a well-defined banding pattern, with strong signals appearing after short periods of exposure (Fig. 1). This confirms the repeated nature of the DNA fragments to which Ddr-IV hybridizes. The ends of the Ddr-IV fragment were sequenced and are shown in Fig. 2. Sequence comparison revealed no homology with sequences present in Genbank (release 38; Devereux et al. 1984).

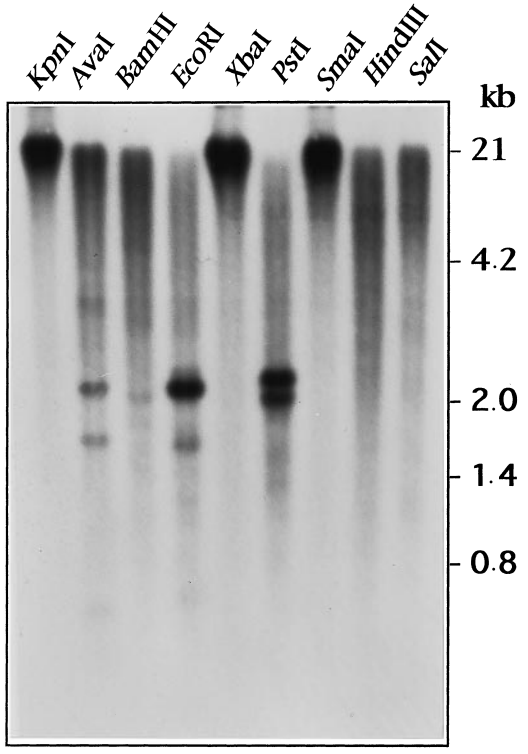

Fig. 1 Autoradiogram showing the presence of repetitive DNA fragments in the genome of *Dicrocoelium dendriticum*. Genomic DNA of *D. dendriticum* (2 µg per lane) was digested with different restriction enzymes and separated by agarose-gel electrophoresis, and Southern-blot analysis was performed using radiolabeled Ddr-IV as a probe. The filter was exposed overnight at −80 °C using an intensifying screen. A distinct banding pattern was found in the *Pst*I-, *Eco*RI-, and *Ava*I-digested genomic *D. dendriticum* DNA

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        10         20         30         40         50         60
CTGCAGCACCCGTCGTCGCTTCTGTAGTGGCCCCAAATGGCACAGCGCATGGTCCACTAG
        70         80         90        100        110        120
ACATTTGTATGAGAAAGAAAGGCATTAGTAGAGAACCGAAAACCCACCACCTGAAATAAA
       130        140        150        160        170        180
GAGACTTAGCATGCAACCATGCGGTTAGTAAATCGAATTCAGCGTGCCTACAAACGCTGC
       190        200        210        220        230        240
GCCGGTGCTGCCTACGTTCACTGCGGGACCCGTCGGCGCCTTCGACAGTGGTCCGAGTGA
       250        260
CCTCTCTCATGGCTCACTAGGCC <---------/≈ 1.6 kb/---------->
        10         20         30         40         50         60
GGATCCCGTCACCTCGGCACATTTGCCGTTTCGCAGCTGAGCAAGTGCTCTCTGCGTCTC
        70         80         90        100        110        120
ATCCAGTCTCGGCGTATCCGCCAGATCCGCCTTCACATCGTTCTGCTTCAGGCCATTCAA
       130        140        150        160        170        180
TGTAGGCCAGTCGATCTCTGAACGGTTGTTCAAGAGACAGTCAAAGTGTTTCCGCCAGAC
       190
CTGCATTATGTCTGCAG
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Fig. 2 Nucleotide sequence of the ends of the Ddr-IV fragments. The length of the unsequenced area is indicated as *<—/≈ 1.6 kb /—>*. The *Pst*I restriction sites that flank the Ddr-IV fragment are *underlined*

Specificity of Ddr-IV

In a test of the specificity of Ddr-IV, DNA was isolated from different trematodes, dotted onto a Hybond-N filter, and hybridized under stringent conditions with radiolabeled Ddr-IV. Figure 3 shows clearly that the probe Ddr-IV is specific for *D. dendriticum* and does not hybridize to DNA isolated from other trematodes such as *Fasciola hepatica*, *Xiphidiocercaria* sp., or *Diplostoma* sp. or from nematodes such as *Ostertagia ostertagii*. In addition, *Ddr*-IV did not hybridize to DNA isolated from the cercarial stage of *Trichobilharzia* sp. or *Dendritobilharzia* sp. harvested from the water snails *Viviparа vivipara* and *Lymnea auricularia* (data not shown).

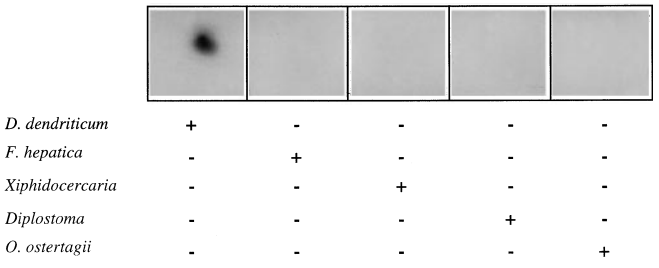

Fig. 3 Autoradiogram of dot blots showing the specificity of Ddr -IV. Genomic DNA was isolated from adult *D. dendriticum* and *Fasciola hepatica* and from trematode larvae belonging to the genera *Xiphidocercaria* and *Diplostoma*. DNA was also isolated from *Ostertagia ostertagii* as a member of the Nematoda. Genomic DNA was dotted onto a Hybond-N filter and hybridized with Ddr-IV labeled with ^{32}P by nick translation. Filters were washed under stringent conditions and exposed overnight at −80 °C using an intensifying screen. No additional signal appeared upon longer exposure

Detection of trematodes in ants

To test whether Ddr-IV could be used to detect *D. dendriticum* larvae present in ants we prepared squash blots from uninfected control ants and ants carrying *D. dendriticum* larvae (Fig. 4). All the ants were dissected under a microscope prior to the squash-blot analysis for determination as to whether they were infected. Signals were obtained only where squashed, infected ants were applied, and no hybridization with ant DNA could be detected. The probe proved to be highly sensitive, since isolated metacercariae collected in droplets of phosphate-buffered saline from infected ants could also be detected using Ddr-IV (data not shown).

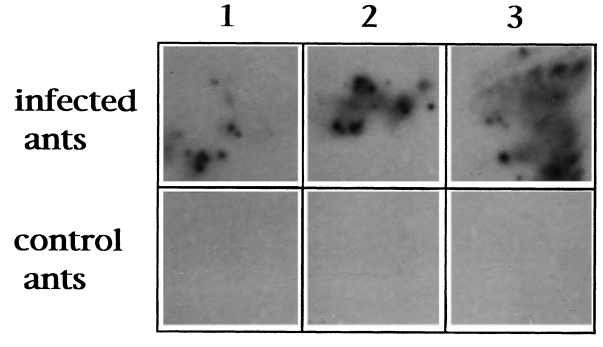

Fig. 4 Detection of *D. dendriticum* DNA in ant squash blots. The probe used was Ddr-IV labeled with ^{32}P by nick translation. The filter was exposed overnight at −80 °C using an intensifying screen

Discussion

DNA probes for the identification and detection of parasites either in their definitive hosts or in different intermediate hosts have proved to be useful diagnostic and epidemiology tools (Kukla et al. 1987; Schofield 1987; Delves et al. 1989; Walker et al. 1989; Webster et al. 1989). To date, the detection of *Dicrocoelium dendriticum* larvae in the snail and ant intermediate host has been dependent on the microscopic examination of individual snails and ants. This can be very time-consuming, especially if large numbers of intermediate hosts – possibly originating from different geographic areas – need to be examined. In addition, larvae of different trematode species can be very similar and it may be hard for the untrained microscopist to distinguish them. The use of diagnostic probes circumvents this problem. In the present work a DNA probe was generated that is specific for *D. dendriticum*. This probe could be used to detect *D. dendriticum* larvae in infected ants. The probe hybridizes to abundant repetitive sequences and is therefore very sensitive and capable of detecting single metacercariae. The *Dicrocoelium*-specific probe described herein will thus provide a useful reagent for epidemiology studies. Recently, a specific DNA probe for *Fasciola hepatica* was described that can be used to detect *F. hepatica* larvae in *Lymnea truncatula* by the squash-blot method (Heussler et al. 1993).

Another method that has recently been described is a nucleic acid-based test for the detection of *F. hepatica*, in which an oligonucleotide probe with specificity for the small-subunit ribosomal RNA of *F. hepatica* is used (Shubkin et al. 1992). This test involves the isolation of RNA from infected snails followed by slot-blot analysis.

By using the squash-blot technique described herein, one can avoid the cumbersome extraction of RNA from each of the ants to be tested. Squash blots of ants are easily made in the field and, thus, ants (or snails) do not need to be collected, transported, and analyzed individually. This is of particular relevance for epidemiology studies that are carried out in isolated areas or in developing countries, where immediate analysis of large numbers of samples is frequently impossible and diagnostic experience is not always at hand. Filters prepared according to the method described herein could be stored for a long time in that Ddr-IV could detect *D. dendriticum* DNA on filters that had been kept at room temperature for several months.

The data presented herein were obtained using radioactively labeled probes, which have a limited life span and depend for their production and processing on the availability of an adequately equipped laboratory. Recent data from our laboratory, however, suggest that because of the repetitive nature of the target sequences the generation of nonradioactive probes of sufficient sensitivity should also be possible.

Acknowledgements This work was supported in part by a grant from the Swiss National Science Foundation (NF grant 31-28712.90). Bruno Gottstein (Institute for Parasitology, University of Bern) is kindly thanked for reading this manuscript. B. Hörning and K. Pfister (Institute of Parasitology, University of Bern) are thanked for their support.

References

Dawes B, Hughes DL (1964) Fascioliasis: the invasive stage of *Fasciola hepatica* in mammalian hosts. Adv Parasitol 2: 97–168

Delves CJ, Goman M, Ridley RG, Matile H, Lensen THW, Ponnudurai T, Scaife JG (1989) Identification of *Plasmodium falciparum*-infected mosquitoes using a probe containing repetitive DNA. Mol Biochem Parasitol 32: 105–112

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the Vax. Nucleic Acids Res 12: 387–395

Errington J (1990) A rapid and reliable one-step method for isolating DNA fragments from agarose gels. Nucleic Acids Res 18: 5324

Hattori M, Sakaki Y (1986) Dideoxy sequencing method using denatured plasmid templates. Anal Biochem 152: 232–238

Heussler V, Kaufmann H, Strahm D, Liz D, Dobbelaere D (1993) DNA probes for the detection of *Fasciola hepatica* in snails. Mol Cell Prob 7: 261–267

Kukla BA, Majiwa PAO, Young JR, Moloo SK (1987) Use of species-specific DNA probes for the detection and identification of trypanosome infection in tsetse flies. Parasitology 95: 1–16

Maniatis T, Fritch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson T (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Schofield CJ (1987) DNA probes in the field. Parasitol Today 3: 371–383

Shubkin CD, White MW, Abrahamsen MS, Rognlie MC, Knapp SE (1992) A nucleic acid-based test for detection of *Fasciola hepatica*. J Parasitol 78: 817–821

Soulsby EJL (1982) Helminths, arthropods and protozoa of domesticated animals, 7th edn. Baillière Tindall, London, pp 24–26

Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19: 259–268

Walker TK, Rollinson D, Simpson AJG (1989) A DNA probe from *Schistosoma mansoni* allows rapid determination of the sex of larval parasites. Mol Biochem Parasitol 33: 93–100

Webster P, Mansour TE, Bieber D (1989) Isolation of a female specific, highly repeated *Schistosoma mansoni* DNA probe and its use in an assay of cercarial sex. Mol Biochem Parasitol 36: 217–222

Wright CA (1971) Flukes and snails. Allen and Unwin, London