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A PCR-RFLP assay for identification of *Anisakis simplex* from different geographical regions

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Anisakis simplex, a nematode from the family Anisakidae, is a parasite of fish and mammals. It is a casual agent of a human disease called anisakiosis. We found that the assay based on PCR amplification of the ITS-1–5·8 S–ITS-2 fragment of rDNA and subsequent restriction fragment length polymorphism, previously described on the basis of *A. simplex* isolated solely from one geographical region, can be used as a general test for identification of this worm species. The restriction patterns analysed for four restriction enzymes were found to be identical in the case of all *A. simplex* individuals isolated from as different geographical regions as Baltic Sea, Norwegian Sea, Bering Sea and Sea of Okchotsk. Moreover, our results support the previously proposed hypothesis, based on the studies of isoenzymes, that there is a remarkable genetic homogeneity within *A. simplex* from different geographical regions.

KEYWORDS: *Anisakis simplex*, PCR, restriction fragment length polymorphism (RFLP), ribosomal DNA (rDNA), species identification.

INTRODUCTION

Anisakis simplex is a parasitic nematode infecting fish and mammals.[1] The definitive hosts are marine mammals because they eat pelagic fish, which are the main hosts for *A. simplex* larvae. In a paratenic host, *A. simplex* larvae live in the intestinal mesentherium and in muscles. Larvae of *A. simplex* infect mammals exclusively by a trophic pathway. Therefore, this worm may also infect humans. In fact, *A. simplex* larvae have been found to be a casual agent of a human disease called anisakiosis.[2]

Identification of *A. simplex* is crucial for proper diagnosis of anisakiosis. However, it is often a problematic procedure since morphologically larvae of this worm are similar to larvae of other species from the family Anisakidae. Identification of *A. simplex* based on immunological reactions was proposed.[3] However, serological tests are also not precise due to cross-reactions with antigens from other worms.[4] Therefore, infections of humans caused by *A. simplex* are detected almost exclusively on the basis of endoscopic examination, though solid conclusions regarding a presence of the parasite in intestine is almost impossible.

It was reported that restriction fragment length polymorphism (RFLP) analysis of 25S rDNA could be helpful to identify *A. physeteris*.[5] Recently, it was proposed that RFLP analysis of the ITS-1–5·8 S–ITS-2 fragment of rDNA may be used to distinguish *A. simplex* from *Hysterothylacium aduncum* and *Contracaecum osculatum*.[6] This is a very promising assay,

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Table 1. Samples of *Anisakis simplex* from different geographical regions and their hosts

Geographical origin	Host species	Sample code	Number of samples
Baltic Sea	*Clupea harengus*	BS1, BS2, BS3	3
Norwegian Sea	*Clupea harengus*	NS1, NS2, NS3, NS4	4
Norwegian Sea	*Scomber scombrus*	NS5, NS6, NS7, NS8	4
Bering Sea	*Pleurogrammus monopterigus*	BeS1, BeS2, BeS3, BeS4	4
Sea of Okchotsk	*Micromesistus potassou*	SO1, SO2, SO3, SO4	4

however, in that report specimens of *A. simplex* which came solely from the Baltic Sea were analysed.[6] Therefore, we asked whether this test could be used for identification of *A. simplex* isolated from hosts living in different geographical regions. This question was also substantiated by the fact that in some regions (e.g. Japan) anisakiosis is a serious medical problem whereas in other regions (e.g. Eastern Europe) infections of humans by *A. simplex* are relatively rare.[7,8]

MATERIALS AND METHODS

Parasite materials

Larvae of *A. simplex* (i.e. *A. simplex* sensu stricto, formerly called *A. simplex* B) were isolated from different hosts taken from various geographical regions (Table 1). For control experiments, *H. aduncum* larvae, isolated from *Platichthys flesus* taken from the Baltic Sea, were used. The systematic classification of the worms was established on the basis of: (i) morphological structures of labiae and ventriculus; (ii) the shape of a tail;[9] and (iii) isoenzymatic studies performed as described by Nascetti *et al.* and Mattitucci *et al.*[10,11]

DNA isolation and amplification

Genomic DNA from worms (a whole larva was used for one sample) was isolated by a SDS/proteinase K procedure as described by Sambrook *et al.*[12] For isolation of DNA from fish (*Scomber scombrus* from the Norwegian Sea) tissues and from mixtures of worm and fish tissues, the same method was used. DNA samples were stored in the TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8·0) at −60°C. For amplification of the rDNA region containing ITS-1, 5·8 S rRNA gene, ITS-2 and ~70 bp of 28 S rRNA gene, the previously described[6] primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) were used. Each reaction mixture (total volume of 50 µl) contained 1 µl of genomic DNA isolated as described above, 1 unit of DyNAzyme™ II DNA Polymerase (Finnzymes), dNTPs (250 µM each) and 100 pmol of each primer. The following program was employed: one cycle of denaturation at 94°C for 5 min, annealing at 60°C for 30 s, and extension at 72°C for 90 s, then 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s, and then the final cycle of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 5 min. PCR products were separated electrophoretically on 1% (w/v) agarose gels and visualized by staining with ethidium bromide as described by Sambrook *et al.*[12]

Restriction fragment length polymorphism (RFLP) analysis

The amplified DNA samples were digested with *Bsu*RI, *Rsa*I, *Taq*I and *Alu*I restriction enzymes and

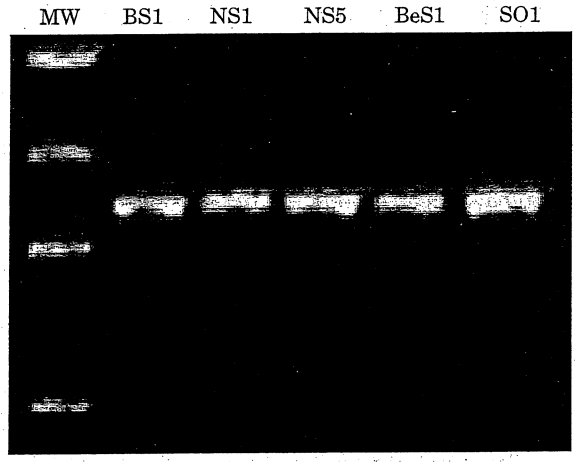

Fig. 1. Products of PCR amplification of the rDNA fragment encompassing the ITS-1–5·8 S–ITS-2 region from *Anisakis simplex* worms originating from different geographical regions. Agarose gel electrophoresis was performed and the gel was stained with ethidium bromide (0·5 µg/ml) and photographed over a transilluminator. Abbreviations of samples correspond to those presented in Table 1. DNAs isolated from other worms listed in Table 1 gave the same results. MW, molecular weight standards.

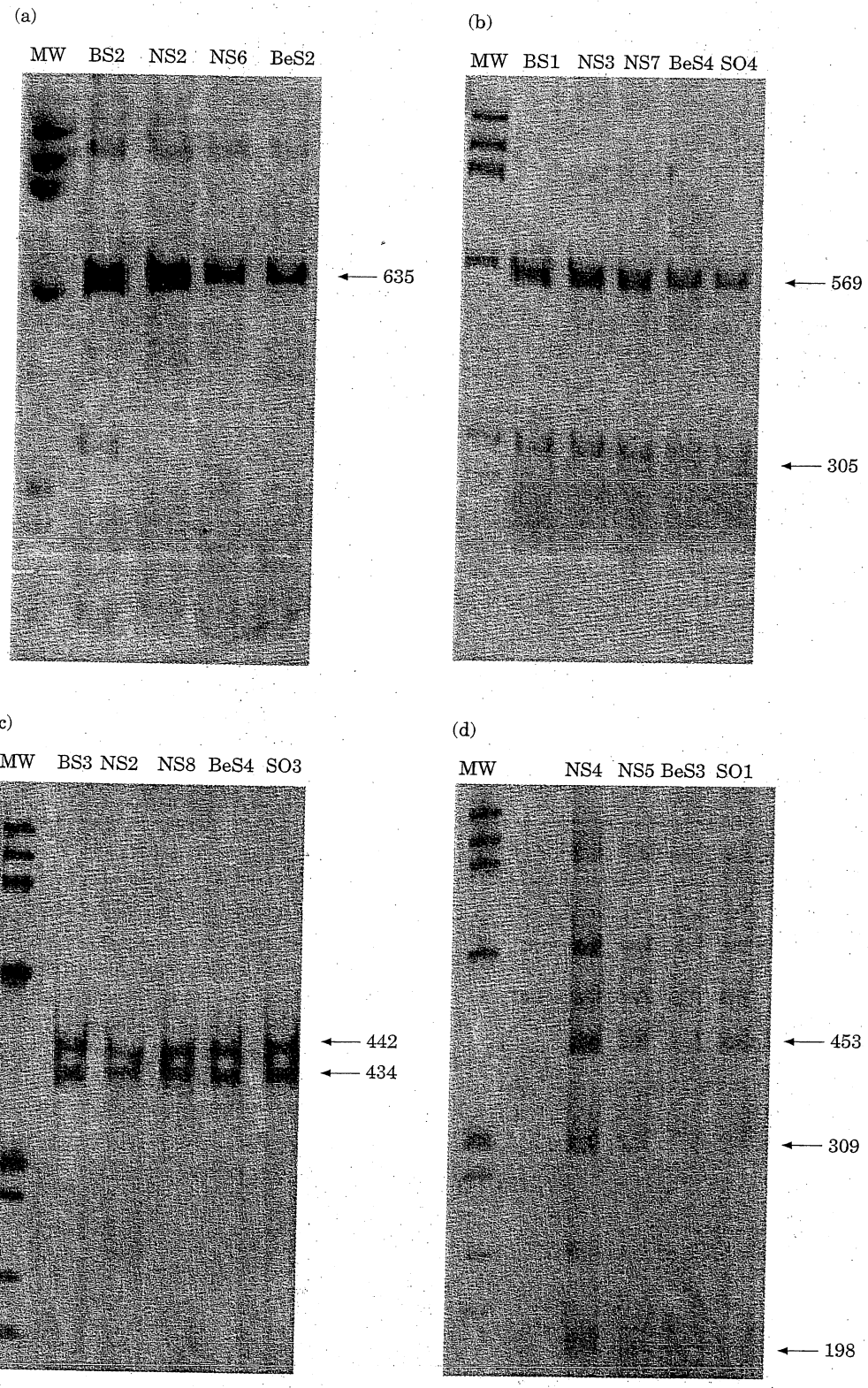

Fig. 2. Restriction fragment length polymorphism of the *Anisakis simplex* DNA fragment encompassing the ITS-1–5·8 S–ITS-2 region from worms originating from different geographical regions. The PCR products (see Fig. 1) were digested with (a) *Bsu*RI, (b) *Rsa*I, (c) *Taq*I and (d) *Alu*I, and separated by polyacrylamide gel electrophoresis. The gels were stained with silver nitrate. Abbreviations of samples correspond to those presented in Table 1. DNAs isolated from other worms listed in Table 1 gave the same results (data not shown). Numbers near arrows correspond to lengths of particular DNA fragments (in bp). MW, molecular weight standards.

Table 2. Restriction patterns of the rDNA fragment encompassing the ITS-1–5·8 S–ITS-2 region (960 bp) amplified by PCR from genomic DNA of *Anisakis simplex*

Restriction enzyme	Lengths of DNA fragments (bp)[a]
*Bsu*RI	635*, 179, 146
*Rsa*I	569*, 305*, 86
*Taq*I	442*, 434*, 84
*Alu*I	453*, 309*, 198*

[a] Agarose gel electrophoresis allowed for estimation of approximate lengths of the major restriction fragments (marked in the table by asterisks). The exact lengths of all fragments are based on the analysis of previously published[6] DNA sequence of the ITS-1–5·8 S–ITS-2 region of *A. simplex* originated from Baltic Sea.

the products were separated electrophoretically on 29% (w/v) polyacrylamide gels according to a previously published procedure.[12] DNA bands were visualized by staining with silver nitrate.[12] The ϕX174 DNA digested with *Bsu*RI (*Hae*III) was used as a DNA marker (Marker 9, Fermentas).

RESULTS

Total DNA was isolated from *A. simplex* larvae, which were isolated from fish taken from different geographical regions (see Table 1). A chromosomal DNA region encompassing ITS-1, 5·8 S rDNA, and ITS-2 was amplified using PCR with previously described primers.[6] In some cases a second amplification (using the material obtained after the first PCR amplification as a template) was necessary to obtain sufficient amount of DNA (data not shown). The length of the PCR products in all samples was the same (960 bp) and identical to that reported previously,[6] irrespective of the geographical region from which the worms originated (Fig. 1). In the control experiments, amplification of the corresponding DNA region from larvae of *H. aduncum*, a species closely related to *A. simplex*, using the same primers, gave a longer PCR product (about 1050 bp) (data not shown), according to previously published results.[6]

Amplified DNA fragments were digested with *Bsu*RI (Fig. 2a), *Rsa*I (Fig. 2b), *Taq*I (Fig. 2c) and *Alu*I (Fig. 2d) and separated by polyacrylamide gel electrophoresis. The results show identical restriction patterns of DNA samples irrespective of the geographical region from which the worms came. Therefore, the specific restriction pattern for each enzyme could be established for *A. simplex* DNA region encompassing ITS-1, 5·8 S rDNA, and ITS-2. These patterns are presented in Table 2. All the patterns are compatible with the DNA sequence of this region reported previously.[6] In the control experiments the PCR-amplified DNA fragment from *H. aduncum* (~1050 pb) was digested with *Bsu*RI and *Rsa*I, and restriction patterns identical to those described by Zhu *et al.*,[6] but significantly different from the *A. simplex* patterns were observed (data not shown). These results indicate that the assessed PCR-RFLP test can be used for detection of *A. simplex* coming from different geographical regions and to distinguish this species from other closely related species irrespective of the source of the biological material.

To test the sensitivity of the assay, we mixed different amounts of worm and fish tissues, isolated DNA and performed the PCR amplification. We were not able to detect any PCR product when only fish tissues (either muscle or intestine and liver) were analysed (Fig. 3), even when 1 g of the tissue was used for template DNA isolation (for comparison, a weight of one *A. simplex* larva is approximately 4 mg). However, the specific PCR products of expected size were easily detected in such experiments even at the worm tissue to fish tissue ratio as low as 1:200 (Fig. 3).

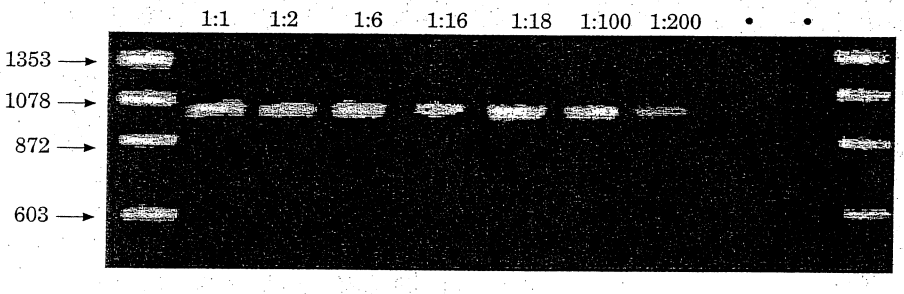

Fig. 3. Products of PCR amplification of the rDNA fragment encompassing the ITS-1–5·8 S–ITS-2 region of *Anisakis simplex*. Agarose gel electrophoresis was performed and the gel was stained with ethidium bromide (0·5 μg/ml) and photographed over a transilluminator. The mixed material of worm and fish tissues was used for isolation of template DNA. A worm tissue/fish tissue ratio is indicated above each particular lane, but the first and the last lanes represent molecular weight standards (numbers near arrows correspond to lengths of particular marker DNA fragments in bp), and lanes marked '●' indicate samples in which solely fish tissues were used for template DNA isolation.

DISCUSSION

Since *A. simplex* is a parasite transmissible to humans and can cause a dangerous disease, anisakiosis,[2] it is very important to perform proper identification of this parasite. This is not a trivial problem because larvae of *A. simplex* are morphologically very similar to larvae of other worms from the Anisakidae family, and even serological reactions may give cross-reactions with members of other species.[4] Moreover, often only small portions of worms are available for identification in a clinical practice.

Recently, an assay based on RFLP of the ITS-1–5·8 S–ITS-2 region was proposed as a specific test to distinguish *A. simplex* from *H. aduncum* and *C. osculatum*.[6] However, for that analysis worms coming solely from the Baltic Sea were considered. Therefore, it seemed important to test whether *A. simplex* worms from other geographical regions reveal the same properties of the RFLP.

We found that DNA of *A. simplex* isolated from hosts living in as diverse regions as Baltic Sea, Norwegian Sea, Bering Sea and Sea of Okchotsk, after PCR amplification of the ITS-1–5·8 S–ITS-2 region, revealed exactly the same restriction patterns for all restriction enzymes tested. Therefore, it seems that the assay proposed by Zhu *et al.*[6] and investigated in this report can be considered as a general test for identification of *A. simplex* irrespective of the geographical region from which a sample of material was obtained.

The results demonstrated in this report indicate that the assessed test is specific for *A. simplex* (when DNA isolated form a closely related species, *H. aduncum*, was used, the PCR product and its restriction patterns were considerably different from those of *A. simplex*). Moreover, this test is highly sensitive because we were able to obtain the specific PCR product when ratio of the amount of tissues taken from the worm and from a fish, used for template DNA isolation, was 1:200. Therefore, even a small part of a worm isolated from an animal together with a relatively large piece of the host tissue should be still sufficient for detection of the presence of the parasite and its identification. It is also worth noting that no PCR product was obtained when only fish tissues were used for DNA isolation and subsequent PCR reaction with primers specific for the worm's genome region encompassing ITS-1–5·8 S–ITS-2.

Our results may also have another significance. It is known that anisakiosis is a serious problem in some countries (especially in Japan, and to some extent in Korea, Netherlands, Germany, France and USA) but not in other geographical regions (e.g. Eastern Europe, Scandinavia, Southern America).[7,8] Isoenzymatic studies suggested that there is a remarkable genetic homogeneity within *A. simplex* from different geographical regions.[10,11] This may suggest that the problems with a frequently occurring disease caused by *A. simplex* in some countries but not in others may be due to a specific diet rather than to any potential differences between worms living in fish from different seas. In certain countries (e.g. Japan) a raw fish is a common dish, thus increasing significantly a probability of infection by *A. simplex*, whereas in other geographical regions (e.g. Eastern Europe) dishes based on a raw fish are prepared very rare. Specific preparation of fish (e.g. cooking, backing and others) kills the worms efficiently and prevents infection. Our data, demonstrating a lack of differences in the RFLP of the very variable region of DNA between *A. simplex* originated from different geographical regions (including both, countries in which anisakiosis is a serious medical problem, i.e. Japan, and countries in which anisakiosis is sporadic, i.e. Poland), may support this hypothesis.

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