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Multiplex PCR for Differential Identification of Broad Tapeworms (*Cestoda*: *Diphyllobothrium*) Infecting Humans[▽]

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The specific identification of broad tapeworms (genus *Diphyllobothrium*) infecting humans is very difficult to perform by morphological observation. Molecular analysis by PCR and sequencing represents the only reliable tool to date to identify these parasites to the species level. Due to the recent spread of human diphyllobothriosis in several countries, a correct diagnosis has become crucial to better understand the distribution and the life cycle of human-infecting species as well as to prevent the introduction of parasites to disease-free water systems. Nevertheless, PCR and sequencing, although highly precise, are too complicated, long, and expensive to be employed in medical laboratories for routine diagnostics. In the present study we optimized a cheap and rapid molecular test for the differential identification of the most common *Diphyllobothrium* species infecting humans (*D. latum*, *D. dendriticum*, *D. nihonkaiense*, and *D. pacificum*), based on a multiplex PCR with the cytochrome *c* oxidase subunit 1 gene of mitochondrial DNA.

Human diphyllobothriosis is a widespread fish-borne zoonosis caused by tapeworms of the genus *Diphyllobothrium* Cobbold, 1858 (*Cestoda*: *Diphyllobothriidea*). Their life cycles are complex and involve two intermediate hosts (a copepod and a fish) and a definitive host (humans or other piscivorous mammals and aquatic birds). Infection takes place through the consumption of raw or undercooked fish harboring plerocercoid larvae and often remains unnoticed until the excretion of segments of the adult parasite (proglottids) in stools. Symptoms include various minor digestive problems occurring a few weeks after infection (mostly nausea, diarrhea, and abdominal pain); less commonly, prolonged or heavy infections can result in a pernicious anemia (7). About 14 species of *Diphyllobothrium* have been reported to infect humans. From a medical perspective, *Diphyllobothrium latum*, *Diphyllobothrium nihonkaiense*, *Diphyllobothrium dendriticum*, and *Diphyllobothrium pacificum* are the most important, because humans represent their preferred definitive hosts (17).

The identification of *Diphyllobothrium* tapeworms by physicians and medical laboratories is generally based on the morphological observation of operculated eggs and segments of adult worms (7). Morphoanatomical criteria allow identification to the genus level but are not reliable to assess species identity, because the different taxa are extremely similar one another, and species differentiation relies on characteristics of the scolex or the genital apparatus observed on mature proglottids, which are often unavailable during human infections (20). To this end, molecular methods have been recommended (17). In the past, biochemical techniques (isoenzymatic assay or immunoelectrophoresis) have been used as alternatives to traditional tools for species identification (5, 6). Matsuura et al. (13) previously discriminated between *D. latum* and *D. nihonkaiense* by using restriction fragment length polymorphism (RFLP) of ribosomal DNA (rDNA), as the profiles generated with three different restriction enzymes provided valuable species-specific markers. Since the late 1990s, the development of molecular biology based on the sequencing of nuclear and mitochondrial DNA (mtDNA) targets resulted in a better knowledge of the genus *Diphyllobothrium*. Notable results for the phylogenetic relationships among some taxa have been obtained by the sequencing of the 18S rRNA, cytochrome *c* oxidase subunit 1 (*cox1* or COI), and NADH dehydrogenase subunit 3 (NADH3) genes and internal transcribed spacer 1 (ITS1) and ITS2 (19, 27, 29, 31).

However, molecular methods are still rarely employed in routine laboratories due to economical (need of reagents and equipment such as a thermal cycler and sequencer) and technical (procedures are complicated and time-consuming and require trained personnel) reasons. Moreover, all human-infecting *Diphyllobothrium* species cause similar symptoms, and infections are successfully treated with praziquantel, which can lead one to think that a further, specific identification of the causative agent is useless for the practice.

In contrast, the specific diagnostic identification of *Diphyllobothrium* parasites isolated from patients is of great importance from an epidemiological point of view. Human diphyllobothriosis has been estimated to affect 20 million people worldwide (4, 14), and recent studies indicate a recrudescence in some well-developed countries (26). Thanks to the use of molecular tools, locally acquired infections with allochtonous species are being reported more and more frequently. For instance, *D. nihonkaiense*, usually present in Japan and South Korea (8), has recently been detected in Switzerland (18, 23), France (15, 30), Finland (26), and North America (25). *D. dendriticum*, common in northern Europe, was found in a Swiss

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TABLE 1. *Diphyllobothrium* samples sequenced for the complete *cox1* gene

Species	Stage	Host	Origin[b]
D. latum	Adult	Human	St. Gallen, SWI
D. latum	Plerocercoid	*Perca fluviatilis*	POL
D. dendriticum	Plerocercoid	Freshwater fish	EST
D. dendriticum	Plerocercoid	Freshwater fish	EST
D. dendriticum	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR
D. dendriticum	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR
D. dendriticum	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR
D. dendriticum	Plerocercoid	*Coregonus autumnalis*	Olkhon Island, RUS
D. pacificum	Adult	Human	Lima, PER
D. pacificum	Adult	Human	Lima, PER
D. pacificum	Adult	Human	PER
D. pacificum	Adult	Human	Lima, PER
D. pacificum[a]	Plerocercoid	*Sciaena deliciosa*	Lima, PER
D. pacificum[a]	Plerocercoid	*Sciaena deliciosa*	Lima, PER

[a] Identified by morphological observation.
[b] SWI, Switzerland; POL, Poland; EST, Estonia; GBR, Great Britain; RUS, Russia; PER, Peru.

patient (24). A human case due to *D. latum* was reported in Taiwan (11). In Sao Paulo, Brazil, an outbreak with a significant economic and public health impact linked to the ingestion of raw salmon was proven to be caused by *D. latum* (16). In most of these cases the infective source consisted of imported fish; on the other hand, the origin of the intermediate host even brought into question the known geographical repartition of some species, i.e., *D. nihonkaiense*, harbored by Pacific salmons in North America (a hypothesis which is currently under verification). The molecular identification of broad tapeworms infecting humans can therefore contribute to map the present species distribution, to understand their life cycles (for example, the intermediate hosts of *D. pacificum* are still unknown), and to clarify which fish represent the most important sources of human infections.

To date, the sequencing of some genomic DNA targets allowed the identification of many *Diphyllobothrium* species (17), and the use of RFLP enabled the differentiation between *D. latum* and *D. nihonkaiense* (13). Nevertheless, only a PCR-based method for routine discrimination between *D. latum* and *D. nihonkaiense* has been developed for use in diagnostic laboratories (9). In this study, we adapted an easy and rapid molecular technique (one that does not require the sequencing of PCR products) to be routinely used for the differential diagnosis of all principal *Diphyllobothrium* species infecting humans. The method is based on a multiplex PCR, which was successfully employed for cestode identification in previous studies (9, 28).

MATERIALS AND METHODS

Target gene. The cytochrome *c* oxidase subunit 1 (*cox1*) gene of mitochondrial DNA (mtDNA) represents a suitable gene for the inter- and intraspecific identification of various *Diphyllobothrium* species (17, 27, 29, 31). It was therefore used as a target for multiplex PCR. Due to its variability, the primers for the multiplex PCR were designed on the basis of several representative sequences for each species, i.e., samples from different geographical origins and developmental stages. The alignment was built by using (i) sequences deposited in public databases (DDJB/EMBL/GenBank accession numbers AM747494 [*D. pacificum*]; AB268585, NC_009463, AM412559, AM412560, EF420138, and AB015755 [*D. nihonkaiense*]; NC_008945, AB269325, DQ985706, FM209180, and FM209181 [*D. latum*]; and AM412738 and AB374223 [*D. dendriticum*]) and (ii) sequences obtained from other samples (Table 1); among the latter, two plerocercoid larvae isolated from Lorna drum (*Sciaena deliciosa*; *Perciformes*: *Sciaenidae*) in Peru had been identified as *D. pacificum* according to morphological characteristics only (2). The other specimens had been previously identified by genetic analysis (18S rRNA gene and ITS1-ITS2 regions) (26).

Cox1 sequencing and analysis. DNA from parasite samples was extracted from 25 mg tissue (about two proglottids) or the whole plerocercoid larva with the DNeasy blood and tissue kit according to the manufacturer's protocol for the purification of total DNA from animal tissues (Qiagen, Hilden, Germany). The complete *cox1* gene was amplified by PCR in a T3000 thermocycler (Biometra, Goettingen, Germany) by using primers Cox1Forward (5′-TATCAAATTAAG TTAAGTAGACTA-3′) and Cox1Reverse (5′-CCAAATAGCATGATGCAAA AG-3′) in 50-μl reaction mixtures containing 2.5 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP) (Top Bio, Prague, Czech Republic), and 0.3 μM each primer. The thermal cycle consisted of 1 cycle at 95°C for 5 min; 40 cycles of 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min; and a final cycle at 72°C for 10 min. The PCR products were visualized on a 0.8% agarose gel and purified by using the QIAquick PCR purification kit according to the manufacturer's instructions for the direct purification of DNA fragments with a microcentrifuge (Qiagen, Hilden, Germany). Sequencing was performed with an ABI Prism 3130xl automatic sequencer by using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The products of the sequencing reaction were purified by ethanol precipitation with sodium acetate. The sequences obtained were analyzed and corrected with the software MEGA v. 4.0 (21).

Primer design. Five primers for the multiplex PCR were designed on the basis of the alignment of *cox1* sequences by using the software MEGA v. 4.0 (21). One reverse primer was common for all *Diphyllobothrium* species (MulRevCom, located at positions 1492 to 1512). Four forward primers were specific: MulLat3 (positions 1055 to 1077) for *D. latum*, MulDen4 (positions 1174 to 1200) for *D. dendriticum*, MulPac2 (positions 765 to 786) for *D. pacificum*, and MulNih5 (positions 260 to 279) for *D. nihonkaiense* (Table 2). The expected product sizes were 437 bp for *D. latum*, 318 bp for *D. dendriticum*, 727 bp for *D. pacificum*, and 1,232 bp for *D. nihonkaiense*.

Multiplex PCR differential identification. The multiplex PCR was tested on 24 samples of *D. latum*, 17 samples of *D. dendriticum*, 17 samples of *D. pacificum*, and 21 samples of *D. nihonkaiense* (Table 3). To estimate the specificity of the primers, three other *Diphyllobothrium* species were also tested: *Diphyllobothrium ursi* (a species infecting bears in the Pacific coast of North America, which occasionally infects humans), *Diphyllobothrium ditremum* (present in northern Europe and North America, infecting mainly fish-eating birds), and *Diphyllobothrium hottai* (present along the northern coast of Japan; the definitive hosts are unknown).

DNA extraction and the multiplex PCR procedure were performed as de-

TABLE 2. Primers designed for multiplex PCR

Primer	Specificity	Strand	Sequence (5′→3′)
MulRevCom	Common	Reverse	ATGATAAGGGAYAGGRGCYCA
MulLat3	*D. latum*	Forward	GGGGTGTTACGGGTATTATACTC
MulDen4	*D. dendriticum*	Forward	GTGTTTTTCATTTGATGATGACCAGTC
MulPac2	*D. pacificum*	Forward	ACATGTGTGTAGTAACCTTGGC
MulNih5	*D. nihonkaiense*	Forward	CTTTGTTGTCTGGCCTTCCT

TABLE 3. *Diphyllobothrium* samples tested by multiplex PCR

Species	Stage	Host[b]	Origin[c]	Yr of isolation	Lane[a]
D. latum	Plerocercoid	*Esox lucius*	RUS	2005	1
	Plerocercoid	*Perca fluviatilis*	POL	2007	2
	Adult	Human	Kotka, FIN	2004	3
	Adult	Human	Grenoble, FRA	2005	4
	Plerocercoid	Fish (NI)	Lake Peipsi, EST	2004	5
	Plerocercoid	Fish (NI)	Lake Peipsi, EST	2006	6
	Plerocercoid	*Perca fluviatilis*	Como, ITA	2006	7
	Plerocercoid	*Perca fluviatilis*	Lake Maggiore, SWI	2006	8
	Plerocercoid	*Perca fluviatilis*	Lake Geneva, SWI	2007	9
	Plerocercoid	*Perca fluviatilis*	Lake Geneva, SWI	2007	10
	Plerocercoid	*Perca fluviatilis*	Lake Maggiore, SWI	2007	11
	Eggs	Human	Geneva, SWI	2005	12
	Eggs	Human	Geneva, SWI	2005	13
	Adult	Human	Le Lignon, SWI	2005	14
	Adult	Human	Vaud, SWI	2005	15
	Adult	Human	Geneva, SWI	2006	16
	Adult	Human	St Gallen, SWI	2006	17
	Adult	Human	Vésenaz, SWI	2006	18
	Adult	Human	Baveno, ITA	2006	19
	Eggs	Human	Bubendorf, SWI	2006	20
	Eggs	Human	Montreux, SWI	2006	21
	Eggs	Human	Chiasso, SWI	2006	22
	Adult	Dog	Bern, SWI	2002	23
	Adult	*Vulpes vulpes*	Grisons, SWI	2002	24
D. dendriticum	Plerocercoid	*Coregonus lavaretus*	Lake Peipsi, EST	2004	25
	Plerocercoid	*Coregonus lavaretus*	Lake Peipsi, EST	2004	26
	Plerocercoid	*Coregonus lavaretus*	Lake Peipsi, EST	2004	27
	Plerocercoid	*Coregonus lavaretus*	Lake Peipsi, EST	2004	28
	Plerocercoid	*Coregonus lavaretus*	Lake Peipsi, EST	2004	29
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2001	30
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2002	31
	Plerocercoid	*Salmo trutta*	Loch Doyne, GBR	2002	32
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	33
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	34
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	35
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	36
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	37
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	38
	Plerocercoid	*Coregonus lavaretus*	Loch Lomond, GBR	2004	39
	Plerocercoid	*Coregonus autumnalis*	Olkhon Island, RUS	2005	40
	Adult	Human	Bern, SWI	2006	41
D. pacificum	Adult	Human	Piura, PER	2004	42
	Adult	Human	Lima, PER	2005	43
	Adult	Human	Tumbes, PER	1994	44
	Adult	Human	Tumbes, PER	2003	45
	Adult	Human	Lima, PER	2006	46
	Adult	Human	Lima, PER	2006	47
	Adult	Human	Tumbes, PER	2006	48
	Adult	Human	Tumbes, PER	2006	49
	Adult	Human	La Libertad, PER	2006	50
	Adult	Human	Tumbes, PER	2007	51
	Adult	Human	Tumbes, PER	2007	52
	Adult	Human	Tumbes, PER	2007	53
	Adult	Human	Lima, PER	2007	54
	Adult	Human	La Libertad, PER	2007	55
	Adult	Human	La Libertad, PER	2007	56
	Adult	Human	La Libertad, PER	2007	57
	Adult	Human	La Libertad, PER	2008	58
D. nihonkaiense	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	59
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	60
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	61
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	62
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	63
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	64

Continued on following page

TABLE 3—*Continued*

Species	Stage	Host[b]	Origin[c]	Yr of isolation	Lane in Fig. 1[a]
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	65
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	66
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	67
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	68
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	69
	Plerocercoid	*Oncorhynchus keta*	Hokkaido, JPN	2006	70
	Plerocercoid	*Oncorhynchus masou*	Hokkaido, JPN	2006	71
	Adult	Human	JPN	2006	72
	Adult	Human	JPN	2007	73
	Adult	Human	JPN	2007	74
	Adult	Human	JPN	2006	75
	Adult	Human	JPN	2007	76
	Adult	Human	JPN	2007	77
	Adult	Human	JPN	2006	78
	Adult	Human	JPN	2007	79

[a] The lane numbers correspond to those reported in Fig. 1.
[b] NI, not identified.
[c] RUS, Russia; POL, Poland; ITA, Italy; SWI, Switzerland; EST, Estonia; FRA, France; GBR, Great Britain; PER, Peru; JPN, Japan.

scribed above for the *cox1* gene analysis. Eggs were isolated from fecal samples by formalin-ether sediment concentration. One thousand to 4,000 eggs per sample were resuspended in 30 μl of Tris-EDTA (TE) solution and subsequently used for DNA extraction. DNA was amplified by multiplex PCR using primers MulRevCom, MulLat3, MulDen4, MulPac2, and MulNih5 in a single reaction at the same concentration (0.3 μM each). Different annealing temperatures (ranging from 50°C to 60°C) were tested to avoid unspecific amplification. The thermal cycle was optimized as follows: 94°C for 15 min; 25 cycles of 94°C for 30 s, 60°C for 1 min 30 s, 72°C for 1 min 30 s; 72°C for 10 min; storage at 4°C. A negative control (5 μl sterile water) was included for each species. The presence of the amplicon was verified by loading 5 μl of PCR product into a 1.5% agarose gel, which was electrophoresed for 50 min at 100 V in 1% Tris-acetate-EDTA (TAE) buffer and stained in ethidium bromide. For each species, four of the PCR products obtained by multiplex PCR were purified and directly sequenced as described above.

Nucleotide sequence accession numbers. The nucleotide sequences determined in the present study have been deposited in the DDJB/EMBL/GenBank databases under the accession numbers GU997612, GU997613, GU997614, and GU997615 (*D. latum*); AB548647, AB548648, AB548649, and AB548650 (*D. nihonkaiense*); GU997616, GU997617, GU997618, and GU997619 (*D. dendriticum*); and AB548651, AB548652, AB548653, and AB548654 (*D. pacificum*).

RESULTS

A total of 23 samples of *D. latum*, 17 samples of *D. dendriticum*, 17 samples of *D. pacificum*, and 21 samples of *D. nihonkaiense* were successfully amplified by multiplex PCR, yielding specific products of the expected sizes (Fig. 1). One sample of *D. dendriticum* (no. 25) yielded a faint band, and one sample of *D. latum* (no. 4) did not yield any amplicons. There were no amplification products from three other *Diphyllobothrium* species (data not shown) as well as from the negative control. The sequenced PCR products were confirmed as the target site of each species.

The amplification of diagnostic products by multiplex PCR was successfully obtained with an equal ratio of each reverse and forward primer. The optimal annealing temperature was set at 60°C, because the amplification at lower temperatures was unspecific and in some cases yielded multiple bands.

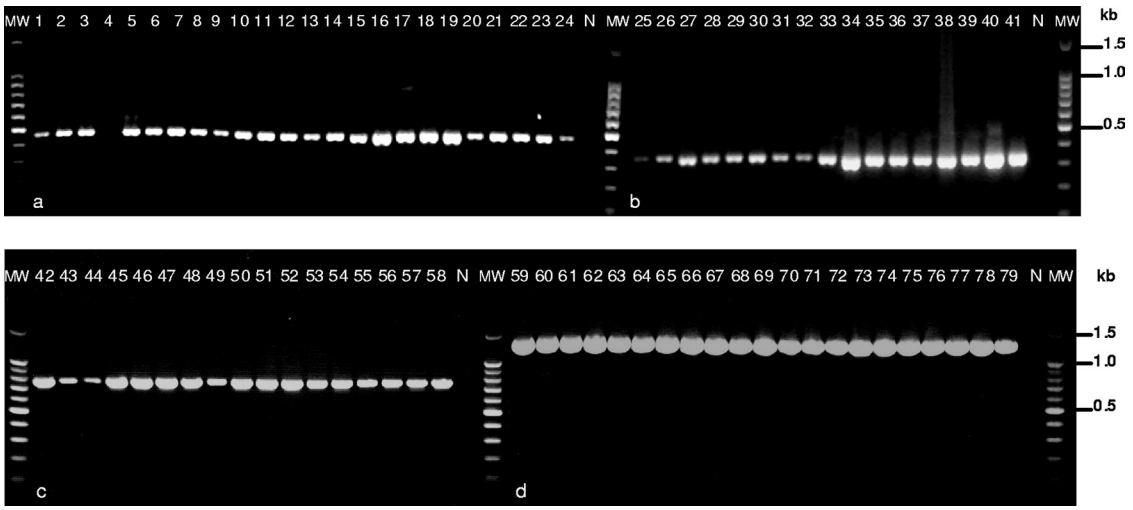

FIG. 1. Differential identification by multiplex PCR of human-infecting species of *Diphyllobothrium*. (a) *D. latum* (lanes 1 to 24). (b) *D. dendriticum* (lanes 25 to 41). (c) *D. pacificum* (lanes 42 to 58). (d) *D. nihonkaiense* (lanes 59 to 79). N, negative control; MW, molecular weight (100-bp ladder; Promega, Madison, WI).

The analysis of *cox1* gene sequences obtained from two plerocercoid larvae isolated from *S. deliciosa* allowed us to identify them as *D. pacificum*, the main species infecting humans in South America (17). As an additional tool for identification, their DNA was also tested by PCR with primers for the amplification of ITS1 and ITS2 according to a method described previously by Luton et al. (12); the analysis of the two obtained partial sequences (1,247-bp fragment) by a BLAST search (1) supported their identity (99.8% similarity with the reference sequence of *D. pacificum* under DDJB/EMBL/GenBank accession number FM204788).

DISCUSSION

In light of the recent appearance of both local and imported diphyllobothriosis in several regions, a reliable technique for the routine identification of broad tapeworms infecting humans is necessary for the surveillance, control, and better understanding of the epidemiology of this zoonosis. In particular, *D. latum*, *D. dendriticum*, *D. nihonkaiense*, and *D. pacificum* are of great medical importance and represent an emerging public health problem, especially in countries where the demand for fish is increasing and new culinary habits involving raw or uncooked preparations (sushi, sashimi, ceviche, carpaccio, etc.) are gaining ground. General practitioners and health workers usually identify *Diphyllobothrium* species on the basis of their morphological characteristics, often according to previously reported cases in the geographical area where their patients live. It has been widely proven that such methods may easily lead to misidentification, because entire worms with scolex and mature proglottids are almost never available, and that specific identification can be achieved only by using molecular data.

Our results indicate that the multiplex PCR assay is a promising method for the routinely identification of the principal species of *Diphyllobothrium* infecting humans. It represents a suitable alternative for medical laboratories and physicians, replacing the more expensive and time-consuming PCR and sequencing, and can be employed by nonspecialized personnel. Compared to other techniques described in the literature (5, 6, 13), the multiplex PCR is easier, faster, cheaper, and highly reliable, due to the specificity of *cox1* gene sequences. On the contrary, immunoelectrophoretic methods require manual expertise, are rather work intensive, and require large amounts of polyclonal antibodies. Other molecular methods based on the random restriction of DNA, such as RFLP, can be used even when no previous data on DNA sequences are available for a particular organism. However, they are not necessarily species specific and are often difficult to interpret, as profiles generated from a whole DNA sample can include several fragments; such methods are better employed to discriminate relatively few DNA sequences.

The use of the multiplex PCR allows the differential identification of proglottids and eggs shed by patients, which in turn can help trace the origins of the infective plerocercoids present in fish (through the analysis of anamnestic data). Moreover, it has been demonstrated that allochtonous *Diphyllobothrium* species can be introduced into new habitats that satisfy their ecological requirements (i.e., the presence of adequate hosts and suitable biotic and abiotic factors), as was the case for *D. latum* and *D. dendriticum* in South America (22). Given that a single person harboring a broad tapeworm produces up to 1 million eggs per day and is able to infect a lake, a correct diagnosis may help prevent the introduction of exotic parasites in disease-free water systems. Specific identification by molecular methods is also of key importance to clarify the geographical range of the zoonosis and to prevent its spread. It should be applied especially in cases of atypical specimens and/or in cases of patients who have been abroad.

The differential PCR test can be applied to any developmental stage of *Diphyllobothrium* parasites provided that the DNA is extracted from frozen or ethanol-preserved samples (17). Other fixatives such as formalin or denatured alcohol irreversibly affect the quality of DNA and the chemical reactions for its amplification. Although some authors reported successful PCRs of short DNA fragments from formalin-fixed materials, the results were highly dependent on the formaldehyde concentration and time of sample fixation (3, 10). In our study, the lack of an amplicon for sample no. 4 is most probably due to the fact that it was stored in formalin at a high concentration (10%). The fact that sample no. 25 yielded a faint signal might be due to a handling problem, because a higher specific signal was produced in previous multiplex PCRs carried out to test different annealing temperatures.

Besides medical purposes, multiplex PCR can be useful in other fields, for example, in the study of *Diphyllobothrium* life cycles, through the identification of larval stages (sharing, like adults and eggs, similar morphologies among the different taxa). In the present study, we had the opportunity to identify plerocercoids of *D. pacificum* isolated from the fish *S. deliciosa*, providing the first evidence of this parasite in this intermediate (or paratenic) host.

A limitation of the present study concerned the evaluation of the cross-reaction with other *Diphyllobothrium* species (both human-infecting and non-human-infecting strains), an aspect that should be considered especially in the analysis of samples collected in the field. Also, the occurrence of broad tapeworms belonging to species other than *D. latum*, *D. dendriticum*, *D. nihonkaiense*, and *D. pacificum* in humans is quite rare but not impossible (17). Due to the lack of samples, we were able to evaluate the primers' specificity using only *D. ursi*, *D. ditremum*, and *D. hottai*. Moreover, molecular data are currently available for only a few species in the genus. Thus, even though *cox1* sequences of other *Diphyllobothrium* and other human-infecting cestodes (such as taeniids and *Diplogonoporus grandis*) are theoretically different enough at the primer binding site, further investigation is required to evaluate the possibilities of cross-reactions.

In conclusion, further research is needed to optimize a broader, cheap, and rapid molecular diagnostic test for the identification of all *Diphyllobothrium* parasites to be used, for example, for the identification of larvae isolated from copepods and fish. Such a tool would noticeably facilitate the comprehension of parasitic life cycles and be employed in ecological and epidemiological studies.

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