

Molecular diagnosis of diphyllbothriasis in Spain, most presumably acquired via imported fish, or sojourn abroad

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Abstract

Human diphyllbothriasis is sporadically detected in Spain. *Diphyllbothrium latum* and *Diplogonoporus balaenopterae* have been identified. In the study, four cases of presumably imported diphyllbothriasis in Spanish patients were appraised. Molecular diagnosis allowed us to identify 'exotic' fish tapeworms such as *Diplogonoporus balaenopterae* in one patient and *Diphyllbothrium pacificum* in the others.

Keywords: diphyllbothriasis, *Diphyllbothrium pacificum*, *Diplogonoporus balaenopterae*, Europe, molecular diagnosis, Spain

Original Submission: 2 September 2013; **Revised Submission:** 18 November 2013; **Accepted:** 22 November 2013

Article published online: 15 January 2014

New Microbe New Infect 2014; **2:** 1–6

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Introduction

Human diphyllbothriasis is caused by intestinal infection with adult stages of *Diphyllbothrium* spp. These so-called 'fish tapeworms' have a worldwide distribution [1], including relatively high prevalences in Arctic regions, and some parts of Europe, Asia, and North America. More recently, endemicity has been more deeply documented in South America, especially along the Pacific coast, and Africa. Although in some areas a decrease in incidence of human cases has been reported, new outbreaks and re-emergences were documented in other regions [2]. In this respect, Dupouy-Camet and Peduzzi [3] found that cases of diphyllbothriasis have been increasingly diagnosed in sub-alpine lakes of France, Italy and Switzerland, and sporadically in Austria, Spain, Greece, Romania, Poland and Norway. More recently, de Marval *et al.* [4] described an imported case of diphyllbothriasis in Switzerland and reviewed nine allochthonous *Diphyllbothrium* infections reported in the continent. While

older reports listed *Diphyllbothrium latum* as the predominant infecting organism, more recent reports, also elucidating imported cases, pointed at a more complex aetiological situation in that other species have become diagnosed, such as *Diphyllbothrium dendriticum* and *Diphyllbothrium nihonkaiense*. Such cases may be either linked to the globalization of fish trading or to travel and migrating behaviour of affected patients. Regarding Spain, as indicated above, few patients have been identified so far and most were infected with *D. latum* [5–8], but one case of *Diplogonoporus balaenopterae* was detected as well [9].

These pseudophyllidean cestodes show a relatively complex biology, with two intermediate hosts (crustaceans, fish), potential paratenic hosts (fish) and definitive hosts (fish-eating mammals and birds). Man becomes infected by the consumption of raw or inappropriately heated fish harbouring plerocercoid larvae that subsequently develop into adult tapeworms in the human intestine; unembryonated parasite eggs are shed by faeces and continue their development in water, such as to reach the intermediate hosts required to close the life-cycle [10]. In general, human infections are asymptomatic, although diarrhoea, abdominal pain, discomfort, weakness, constipation, headache and allergic reactions have also been described. During long-term chronic infections and/or high worm burdens, intestinal obstructions, proglotid ectopic locations and megaloblastic anemia with vitamin B12 deficiency can occur [2,5].

Diphyllobothrium latum, *D. dendriticum*, *D. nihonkaiense*, *Diphyllobothrium cordatum* and *Diphyllobothrium lanceolatum* are the species that most frequently infect humans, whereas *Diphyllobothrium ursi*, *Diphyllobothrium alascense*, *Diphyllobothrium dalliae*, *Diphyllobothrium cameroni*, *Diphyllobothrium hians*, *Diphyllobothrium orcini*, *Diphyllobothrium pacificum*, *Diphyllobothrium scoticum*, *Diphyllobothrium stemmacephalum* and *D. balaenopterae* have only rarely been detected [2, 11]. Each species shows a very similar morphology, but nevertheless peculiarities regarding host range and geographical distribution. Travelling, migration and international fish trading are the major parameters that have recently and are presently altering conventional geographical frontiers.

The diagnosis of infection is generally carried out by coprological detection of parasite stages (proglottids or eggs) isolated from patients' faeces, but this approach is not always appropriate considering the close morphological similarity among the different fish tapeworm species. A species-specific diagnosis is, however, essential in order to define a clinical case, carry out an epidemiological analysis, to detect exotic species and to putatively control potential epidemic outbreaks. One option to circumvent the morphological diagnostic problems is to complement diagnosis with molecular biological techniques [12].

In the present work, four cases of presumably imported diphylobothriasis in Spanish patients are appraised. They were detected between 2008 and 2010, and molecular diagnosis was used to yield the correct identification of the diphylobothrid species involved in each case. Our results confirmed that, besides *D. latum*, 'exotic' fish tapeworms can be found in the Iberian country. Epidemiological consequences and public health impact are discussed.

Case Descriptions

Case #1

A 54-year-old man, resident in Cáceres (Spain), visited the doctor as he had been expelling tapeworm proglottids for a few years ago. The patient reported that he regularly ate smoked salmon and farmed gilthead bream. The clinical history did not reveal relevant data. Diagnostically, the proglottids were macro- and microscopically identified as *Diphyllobothrium* sp. and kept in formalin. Specific anti-cestode drug treatment was offered to the patient.

Case #2

A 50-year-old man visited the doctor as he had been expelling tapeworm proglottids for 1 year. No symptoms were recorded. Anamnestically relevant is a frequent travel record

(Egypt, Turkey, Scandinavia, all during the past year), and the regular consumption of fresh, smoked and/or cooked fish, acquired in markets and supermarkets. The proglottids were macro- and microscopically identified as *Diphyllobothrium* sp. and kept in formalin. Specific drug treatment was offered to the patient.

Case #3

The patient was a 52-year-old woman with no history of travel abroad. She regularly ate raw fresh fish, acquired in markets, with fish originating predominantly from the Pacific Ocean. No symptoms were recorded. Treatment was introduced, with a subsequent expulsion of a tapeworm. Proglottids were collected, identified as *Diphyllobothrium* sp. and kept in formalin.

Case #4

No anamnestic and epidemiological data about this patient are available. Proglottids were collected, identified as *Diphyllobothrium* sp. and kept in formalin.

Materials and Methods

Genomic DNA isolation from tapeworms proglottids

The parasitic material kept in formalin was washed with, and subsequently re-hydrated in phosphate-buffered saline (PBS) during several days. Genomic DNA (gDNA) of each sample was purified by DNeasy tissue kit (Qiagen, Hilden, Germany). First, samples were treated with proteinase K, incubated at 90°C for 45–60 min, and subsequently processed according to the manufacturer's recommendations. The gDNA was eluted from the column with nuclease-free water (Promega Corporation, Madison, WI, USA), and its concentration was determined spectrophotometrically (Nanodrop Technologies, Thermo Scientific, Waltham, MA, USA).

Molecular diagnosis: markers and PCRs

Both mitochondrial and nuclear markers were used. The following protocols were employed:

1. Mitochondrial *cob/nad4* genes, forward primer D1/Dn-1805F (5'-CAGTGGGAATGGTGCTTGTAATGT-3') and reverse species-specific primers D1-2211R (5'-TAACCTTTACTTATAACTACT-3', *D. latum*) and Dn-2380R (5'-AAACAGAAACACAGTATAGTG-3', *D. nihonkaiense*) [13].
2. Mitochondrial *cox1* gene, forward JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and reverse JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') primers [14, 15].

3. Mitochondrial *cox1* gene, with a generic reverse primer for *Diphyllbothrium* species MulRevCom (5'-ATGATAAGG GAYAGGRGCYCA-3' [1492–1512 nt]) and four forward species-specific primers, MulLat3 for *D. latum* (5'-GGGTG TTACGGGTATTAT ACTC-3' [1055–1077 nt]), MulDen4 for *D. dendriticum* (5'-GTGTTTTTCATTTGATGATGAC-CAGTC-3' [1174–1200 nt]), MulPac2 for *D. pacificum* (5'-ACATGTGTGTAGTAACCTTGGC-3' [765–786 nt]), and MulNih5 for *D. nihonkaiense* (5'-CTTTGTTGTCT GGCCTTCCT-3' [260–279 nt]) [12].
4. Ribosomal *ITS1* marker, using BD1 (5'-GTCGTAA-CAAGTTTCCGTA-3') and 4S (5'-TCTAGATG CGTTCCG AA(G/A)TGTCGATG-3') primers [15].
5. Ribosomal *18S* marker, with 18S81 (5'-TTCACCTA CGGAAACCTTGTTACG-3') and 18S83 (5'-GATACCG TCCTAGTTCTGACCA-3') primers [16].

Each protocol was based on the original amplification conditions already described in the respective publications as mentioned above. gDNA from each sample was amplified by the different protocols. Also, two PCR controls were used; one with *Taenia saginata* gDNA as a non-related cestode genomic template and other with water, no DNA was included. PCRs were carried out in a GeneAmp™ PCR thermocycler, System 2700, and the amplification products were electrophoretically resolved in 1.5% (w/v) agarose gels. Amplicons were purified by QIAquick Gel extraction Kit (Qiagen), following the manufacturer's instructions.

DNA sequencing and DNA sequence analyses

All DNA fragments were automatically sequenced by standard Sanger chemistry using a Model 377 ABI PRISM system (Applied Biosystems, Foster City, CA, USA). The sequences obtained for each sample were assembled and edited using the program LaserGene 7 (DNASar, Madison, WI, USA) for visual inspection of data, for mismatches of aligned positions to confirm, or manually correct, automatic readings. All sequences generated in this study were deposited in GenBank (*18S*: HG315734–HG315737; *ITS1*: HG315730–HG315733; *COI*: HF969328, HF969325–HF969327). These sequences were compared among them and with similar sequences from GenBank using the BLASTn algorithm [17].

Sequences obtained in this study were aligned using the Clustal X program [18], together with other diphyllbothrid sequences available in GenBank. Subsequent genetic analyses of the different parasite-specific molecular markers were performed by the program PAUP* 4.0b10 [19]. Genetic relationships among the samples were assessed using a distance method. Phylogenetic trees were inferred from the alignments by the neighbour-joining method using the Kimura

2-parameters evolutionary model [20]. Finally, we used a bootstrap test (10 000 pseudoreplicates) to assess node support in resulting topologies [21].

Results and Discussion

In the present investigation, four cases of presumably imported human cases of diphyllbothriasis were species-specifically diagnosed upon molecular biological tools. A species-specific morphological identification of the proglottids was not feasible due to the poor preservation mode used for these parasite samples. As a finding, *D. balaenopterae* was identified in one patient and *D. pacificum* in the others, thus being the first time that *D. pacificum* could be detected in Spain. In the past, few patients were found to be infected by *D. latum* [5–8], and one case of *D. balaenopterae* was found [9], most of these diagnoses having used conventional morphological criteria for the identification [5–7].

As an initial working hypothesis, we had suspected *D. latum* to be the origin of the four infections; this was based on the previous reports [5–8]. However, the use of species-specific primers for *D. latum* mitochondrial *cob/nad4* gene amplification [13] already provided inconclusive results, with 400 bp amplicons for samples #2 and #3, matching rather the size revealed by *T. saginata* DNA employed as a non-related negative DNA control (data not shown). Subsequent sequencing of the respective DNA fragments yielded 81% identity with *D. latum* mitochondrial genome (accession AB269325.1). Also, the *cox1*-multiplex PCR [12] did not yield any amplification band.

These data prompted us to subsequently apply other DNA markers to elucidate the correct nature of these tapeworms [12]. The *18S* (918 bp) and *ITS1* (673 bp) ribosomal partial sequences [15, 16] were analysed. Similarities between samples #2, #3, #4 and *D. pacificum* were 100% with respect to these ribosomal sequences previously described (FM204788, DQ925310). Conversely, ribosomal markers of sample #1 showed ambiguous results, with an equally matching homology rate (99–100%) to *D. balaenopterae* (02NPSE001 AB4745569; 97NP0282 AB449351) and to *D. grandis* (Dgk1 AB298510; Dgk2 AB298511) for *ITS1* (673 bp), and an undeterminable homology in the case of *18S* (682 bp) sequence, as this marker had only been sequenced for *D. grandis* (AB353272) (Fig. 1a and b). In both subsequently elaborated cladograms, the clades formed by sample #1 with *D. balaenopterae* and/or *D. grandis*, and the samples #2, #3, and #4 with *D. pacificum* were supported by bootstrap values of 100%.

Finally, the amplification of a 416 nt fragment of the mitochondrial *cox1* gene [14, 15], with distinct primers from

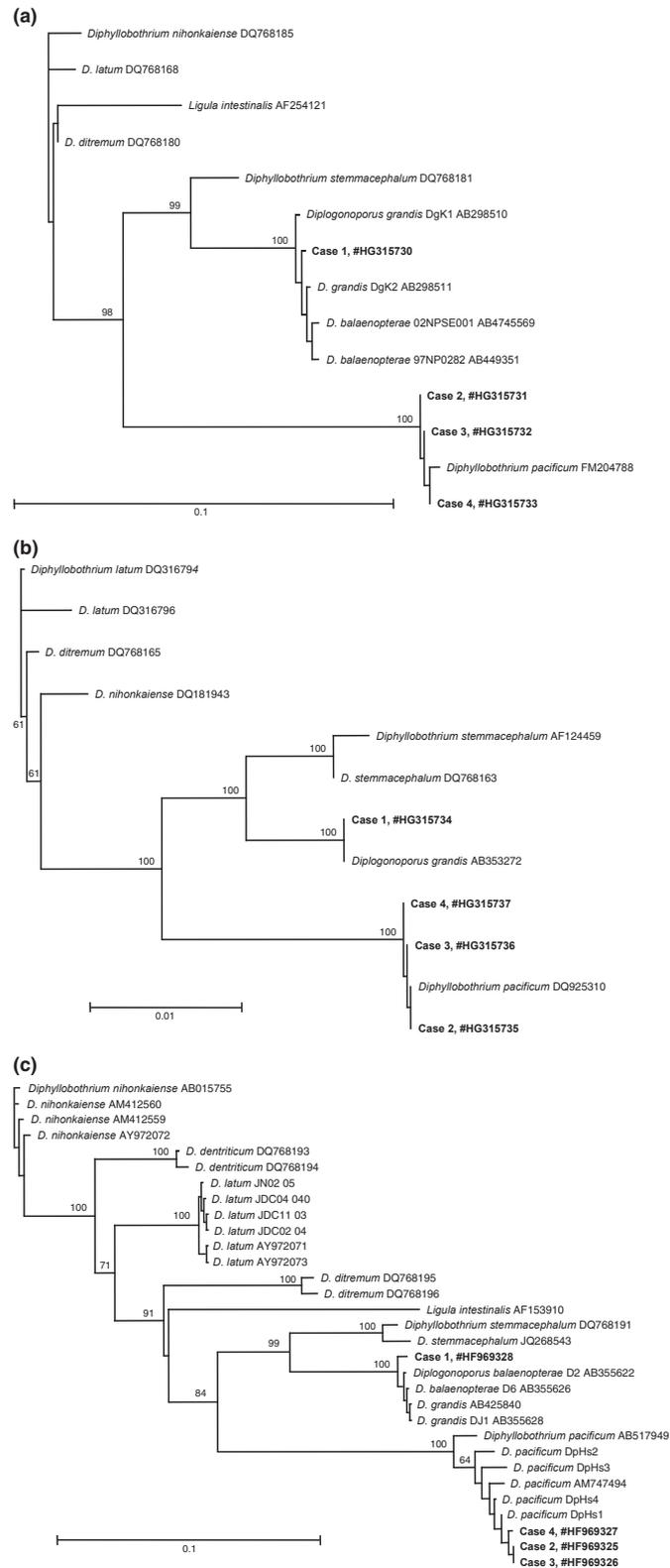


FIG 1. Molecular diagnosis by phylogenetic trees inferred by neighbour-joining method, based on *ITS1* (a), *18S* (b) and *cox1* (c) partial sequences obtained from diphyllobothrid samples isolated from infected Spanish human patients (Cases #1, #2, #3, and #4). GenBank accession numbers of all the sequences used to construct the trees are indicated. The numbers at the branches indicate the bootstrap values for 10 000 replicates (only bootstrap scores higher than 50% are shown).

those used in the *cox1*-multiplex approach [12], yielded the best results. The DNA sequence of the *cox1* fragment from the case #1 worm (HF969328) showed an 85.3% identity with the sequences obtained from cases #2 (HF969325), #3 (HF969326), and #4 (HF969327). Importantly, sample #1 had 99% identity with *D. balaenopterae* and *D. grandis*, respectively. These results, together with the phylogenetic analysis (Fig. 1a–c), allowed us to identify sample #1 as *D. grandis* or *D. balaenoptera*. It should be noted that there is controversy on the taxonomic status of *D. balaenopterae* versus *D. grandis*, and this still unresolved problem also became apparent in our results (Fig. 1a and c). Based on the conclusions by Yamasaki et al. [22] following the mitochondrial genome sequencing of both *D. balaenopterae* and *D. grandis*, we concluded that at present, the best interpretation is that *D. grandis* is a junior synonym of *D. balaenopterae*, and consequently we agreed for a *D. balaenopterae* species-specific diagnosis. *Diplogonoporus balaenopterae* is more frequently diagnosed in the coast of Japan [23]; it has already been described in Spain and probably associated with the importation of fish into the country [9]. Regarding the other three samples, using DNA similarity searches and DNA phylogenetic trees (Fig. 1a–c), they were all confirmed as *D. pacificum*. *Diphyllbothrium pacificum* is commonly detected in fish from the Pacific coast of South America, where sporadic human cases have mainly been detected in Peru and Chile [2, 24, 25]. As in Case #1, importation of infected fish could be the origin of infection.

Our findings strongly indicate that *Diphyllbothrium* spp. becomes imported into Spain via fresh fish, including *D. pacificum* and *D. balaenopterae*. Based on the data provided by some patients, imported fresh infected salmon and other infected fish species, distributed and sold in Spanish markets and supermarkets, could be the origin of human diphyllbothriasis. The rather rare species detected contrast with the profile of allochthonous *Diphyllbothrium* identified in the European continent, such as *D. nihonkaiense* and *D. dendriticum* [4, 8]. This new observation may be a consequence of the distinct commercial networks maintained by Spain that import South American fish, especially from Chile, where infection with *D. pacificum* is frequent [24]. The probability that infection was acquired via imported fresh fish is very likely for at least three of the four Spanish patients, as they had never travelled abroad. Taking into account the increasing globalization of the fish industry and personal migration, the probability of introducing exotic *Diphyllbothrium* species could be steadily increasing in Spain, and considering that adequate environmental conditions exist to maintain the full life cycle of some of the species, they may promote the eruption of epidemic outbreaks of the infection [25].

The best control measure to avoid human diphyllbothriasis is to abstain from the consumption of raw, smoked or pickled

fish. Fish should be well cooked, or adequately frozen prior to consumption, in order to prevent the infection. The same preventive control measures can be applied for anisakiasis. Therefore, it is necessary to inform consumers about the risks related to some culinary habits, with respect to diphyllbothriasis and anisakiasis [2].

Conclusion

Human diphyllbothriasis cases with exotic species have been identified in Spain using molecular tools. Previous reports also described allochthonous diphyllbothriasis, but the diagnostic application of DNA sequencing of *cox1* fragments highlighted the relevance and new need to assess species-specifically imported infections. It also became obvious that warning of the danger of eating uncooked or raw uninspected fish is vital for proper control of the problem.

Acknowledgements

We thank the patients for providing the parasitic material and the epidemiological data described. Also, we gratefully acknowledge the financial support by the Instituto de Salud Carlos III (ISCIII), Fondo de Investigaciones Sanitarias (FIS), through VI NP of I+D+I (2008–2011), ISCIII—General Sub-Direction of Networks and Centers for Collaborative Research (RETIC-RICET, RD06/0021/0019, RD12/0018/011) and FIS projects PI 10/01627. Jacinta Pastor-Valle was supported by a grant from Sociedad Española de Medicina Tropical y Salud Internacional (SEMTSI). GenBank accession numbers: HG315730–HG315737; HF969325–HF969328.

Transparency Declaration

The lead author (the manuscript's guarantor) affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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