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

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Abstract

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

Keywords: diphyllobothriasis, *Diphyllobothrium pacificum*, *Diplogonoporus balaenopterae*, Europe, molecular diagnosis, Spain
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Introduction

Human diphyllobothriasis is caused by intestinal infection with adult stages of *Diphyllobothrium* 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 diphyllobothriasis 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 dyphillobothriasis in Switzerland and reviewed nine allochthonous *Diphyllobothrium* infections reported in the continent. While older reports listed *Diphyllobothrium 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 *Diphyllobothrium dendriticum* and *Diphyllobothrium 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 diphyllobothriasis in Spanish patients are appraised. They were detected between 2008 and 2010, and molecular diagnosis was used to yield the correct identification of the diphyllobothrid 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 Dl/Dn-1805F (5′-CAGTGGGAATGGTGCTTGTAATGT-3′) and reverse species-specific primers Dl-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].