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Occurrence, molecular identification, and *in vitro* features of emerging zoonotic parasites in Mediterranean marine fish

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Los abajo firmantes, como directores de esta Tesis Doctoral realizada por la doctoranda Dña. Samantha Moratal Martínez y titulada **“Occurrence, molecular identification, and *in vitro* features of emerging zoonotic parasites in Mediterranean marine fish”**, hacen constar que reúne los requisitos necesarios para su defensa y aprobación y, por tanto, para optar al grado de doctor con mención internacional.

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ABSTRACT

Fish has core relevance in humans' diets all over the world, both for its importance in food security and for its enormous nutritional benefits. However, fish consumption is not exempt from risks, such as the presence of chemical pollutants or disease-causing biological agents, such as parasites. In particular, marine fish serve as hosts to a wide variety of parasites, including foodborne emerging zoonotic parasites, as nematodes of the genus *Anisakis*, of significant public health concern. Furthermore, they can also harbor other emerging zoonotic parasites originating from terrestrial environments, capable of reaching and contaminating marine waters. In both cases, the presence of these parasites in marine fish intended for human consumption represents a potential risk of transmission to humans. Given the distinct nature of these two groups of parasites and their varying research requirements, this doctoral thesis is structured into two parts.

The **first part**, consisting of two chapters, focused on the molecular detection and characterization of emerging unicellular zoonotic parasites of terrestrial origin in marine fish from the Spanish coast of the western Mediterranean. In the **first chapter**, different PCR techniques were employed to determine the prevalence of *Cryptosporidium* spp., *Blastocystis* sp., and zoonotic microsporidia species in the gastrointestinal tract of three groups of marine fish from the coast of the Comunidad Valenciana (total N = 408): aquaculture fish, wild fish from the surroundings of the aquaculture facilities, and wild fish from extractive fisheries. Zoonotic species and subtypes were detected for all three parasites/parasite groups under study, namely *Cryptosporidium ubiquitum*, *Blastocystis* sp. subtypes (STs) ST5, ST6, and ST7, and *Encephalitozoon hellem/Encephalitozoon intestinalis*. However, the prevalences were very low, suggesting a limited risk of transmission through fish consumption in the studied fish populations.

In the **second chapter**, a metabarcoding dietary approach based on the V8-V9 region of the 18S rRNA gene was used to investigate the parasite community present in digestive samples from two ecologically and economically important pelagic fish species in the western Mediterranean, the European pilchard (*Sardina pilchardus*)

and the round sardinella (*Sardinella aurita*) (total N = 47). Organisms belonging to four parasitic classes were identified, with higher significant prevalence in European pilchards for Cestoda and Myxozoa. Unicellular zoonotic parasites (*Cryptosporidium* sp., *Blastocystis* sp., and *Kudoa thyrsites*) were found in very low presence and number of reads. However, false negatives were detected for *Cryptosporidium* and *Blastocystis*. Therefore, this technique proved useful for exploratory studies of parasite communities in both species but presented limitations in terms of coverage, taxonomic depth, and detection of rare or low abundant organisms. The use of alternative primers and hierarchical metabarcoding is suggested as an improvement for future studies.

The **second part**, corresponding to the **third chapter**, focused on *Anisakis pegreffii*, the most relevant zoonotic parasite transmitted through fish consumption in the Mediterranean basin. The primary objective was to contribute to the development of the *in vitro* life cycle of *A. pegreffii*, crucial for understanding key aspects of the host-parasite interaction and for providing biological material for subsequent research. Therefore, the fecundity and fertility window of two populations of *in vitro* cultured adult *Anisakis* (*Anisakis simplex* sensu lato (s.l.) and *A. pegreffii*) were evaluated; a protocol to obtain third-stage larvae (L3, the infective stage) from eggs laid by *A. pegreffii* adults in culture was developed; ultrastructural characteristics of recently hatched second-stage larvae (L2) of *A. pegreffii* were studied; and chromosomal extensions were obtained for karyotyping from gonadal tissue of adult *A. pegreffii*. Results showed high average fecundity, yielding sufficient biological material (eggs and larvae) for additional experiments. The *in vitro* protocol successfully produced L3 larvae that were maintained in culture for up to 17 weeks. Ultrastructural examination revealed relevant features, including the presence of the excretory gland cell (of utmost relevance in *Anisakis* pathogenicity) in larvae as young as 48 hours old, as well as the release of extracellular vesicles and cell-free mitochondria. Finally, a diploid karyotype, potentially $2n = 18$, was obtained.

RESUMEN

El pescado tiene una relevancia fundamental en la dieta de los seres humanos en todo el mundo, tanto por su importancia en la seguridad alimentaria como por sus enormes beneficios nutricionales. Sin embargo, el consumo de pescado no está exento de riesgos, como la presencia de contaminantes químicos o agentes biológicos causantes de enfermedades, como los parásitos. En particular, los peces marinos son hospedadores de una amplia variedad de parásitos, incluyendo parásitos zoonóticos emergentes de transmisión alimentaria, como es el caso de los nematodos del género *Anisakis*, que representan una gran preocupación para la salud pública. Además, también pueden albergar otros parásitos zoonóticos emergentes que tienen su origen en el medio terrestre y que son capaces de alcanzar y contaminar las aguas marinas. En ambos casos, la presencia de estos parásitos en peces marinos destinados al consumo constituye un potencial riesgo de transmisión a los seres humanos. Dada la diferente naturaleza de estos dos grupos de parásitos y sus diferentes necesidades de investigación, la presente tesis doctoral se estructura en dos partes.

La **primera parte**, compuesta por dos capítulos, se enfocó en la detección y caracterización molecular de parásitos unicelulares zoonóticos emergentes de origen terrestre en peces marinos de la costa española del Mediterráneo occidental. En el **primer capítulo**, se utilizaron diferentes técnicas de PCR para determinar la prevalencia de *Cryptosporidium* spp. *Blastocystis* sp. y especies zoonóticas de microsporidios en el tracto gastrointestinal de tres grupos de peces marinos de la costa de la Comunidad Valenciana (N total = 408): peces de acuicultura, peces silvestres de las inmediaciones de las explotaciones de acuicultura y peces silvestres procedentes de pesca extractiva. Se detectaron especies y subtipos zoonóticos para los tres parásitos/grupos parasitarios objeto de estudio, a saber, *Cryptosporidium ubiquitum*, *Blastocystis* sp. subtipos (STs) ST5, ST6 y ST7, y *Encephalitozoon hellem/Encephalitozoon intestinalis*. Sin embargo, las prevalencias fueron muy bajas, lo que sugiere un riesgo limitado de transmisión por consumo de pescado en las poblaciones de peces estudiadas.

En el **segundo capítulo** se empleó un enfoque de metabarcodificación para dieta basado en la región V8-V9 del gen 18S rRNA para estudiar la comunidad de parásitos presente en muestras digestivas de dos especies de peces pelágicos de gran importancia ecológica y económica en el Mediterráneo occidental, la sardina Europea (*Sardina pilchardus*) y la alacha (*Sardinella aurita*) (N total = 47). Se identificaron organismos pertenecientes a cuatro clases parasitarias, con una prevalencia significativamente mayor en las sardinas para Cestoda y Myxozoa. Se detectaron parásitos zoonóticos unicelulares (*Cryptosporidium* sp., *Blastocystis* sp., y *Kudoa thyrsites*) en baja presencia y cantidad de lecturas. Sin embargo, se detectaron falsos negativos para *Cryptosporidium* y *Blastocystis*. Por lo tanto, esta técnica resultó útil para el estudio exploratorio de las comunidades parasitarias de ambas especies, pero presentó limitaciones en términos de cobertura, profundidad taxonómica y detección de organismos raros o poco abundantes. Se sugiere el empleo de otros primers y la metabarcodificación jerarquizada como mejora para futuros estudios.

La **segunda parte**, correspondiente con el **capítulo 3**, se centró en *Anisakis pegreffii*, el parásito zoonótico transmitido por consumo de pescado de mayor relevancia en salud pública en la cuenca Mediterránea. El objetivo principal fue contribuir al desarrollo del ciclo biológico *in vitro* de *A. pegreffii*, crucial para la comprensión de aspectos clave de la interacción hospedador-parásito y para proporcionar material biológico para investigaciones posteriores. Así pues, se evaluó la fecundidad y ventana de fertilidad de dos poblaciones de *Anisakis* adultos cultivados *in vitro* (*Anisakis simplex* sensu lato (s.l.) y *A. pegreffii*); se desarrolló un protocolo para obtener larvas de tercer estadio (L3, la fase infectante) a partir de huevos depositados por adultos en cultivo de *A. pegreffii*; se estudiaron las características ultraestructurales de las larvas de segundo estadio (L2) recién eclosionadas de *A. pegreffii*; y se obtuvieron extensiones cromosómicas para cariotipado a partir del tejido gonadal de adultos de *A. pegreffii*. Los resultados mostraron una elevada fecundidad media, dando lugar a suficiente material biológico (huevos y larvas) para experimentos adicionales. El protocolo *in vitro* logró producir larvas L3 que se

mantuvieron en cultivo hasta 17 semanas. El estudio ultraestructural reveló características relevantes, como la presencia de la célula de la glándula excretora (sitio de mayor relevancia en la patogenicidad de *Anisakis*) en larvas de tan solo 48 horas, o la descarga de vesículas extracelulares y mitocondrias libres. Por último, se obtuvo un cariotipo diploide, potencialmente $2n = 18$.

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°C	degree Celsius
µg	microgram
µL	microlitre
µM	micromolar
16S/18S rRNA	16S/18S ribosomal ribonucleic acid
ADS	Asociación de Defensa Sanitaria
ASVs	Amplicon sequence variants
BLAST	Basic Local Alignment Search Tool
bp	base pairs
COI	cytochrome c oxidase subunit 1 gene
cox2	cytochrome c oxidase subunit 2 gene
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS	chicken serum
Ct	cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EGC	excretory gland cell
EIDs	(re)emerging infectious diseases
ESPs	excretory/secretory products
EVs	extracellular vesicles
EWUP	emerging waterborne unicellular parasites
FAO	Food and Agriculture Organization
FISH	fluorescence <i>in situ</i> hybridization
g	gram
GFCM	General Fisheries Commission for the Mediterranean
GP60	60-kDa glycoprotein gene
h	hour
ID	identity
ITS	internal transcribed spacer region gene
L	litre
L2	second-stage larva/e
L3	third-stage larva/e

L3P	large third-stage larva phenotype
L4	fourth-stage larva/e
M	molar
m	metre
mg	miligram
min	minute
mL	mililitre
ML	Maximum-Likelihood
mm	milimetre
mM	milimolar
mOsm	milliosmoles
NCBI	National Center for Biotechnology Information
ng	nanogram
NGS	next generation sequencing
NIAID	National Institute of Allergy and Infectious Diseases
OTUs	operational taxonomic units
<i>p</i>	<i>p</i> -value
PCR	polymerase chain reaction
pM	picomolar
pmol	picomol
P/S	penicillin/streptomycin
QC	query cover
RFLP	restriction fragment length polymorphism
s	second
S3P	small third-stage larva phenotype
SAR	Stramenopiles, Alveolata, and Rhizaria
SDGs	Sustainable Development Goals
SDM	Schneider's <i>Drosophila</i> medium
SE	standard error
s.l.	sensu lato
SNV	single nucleotide variant
s.s.	sensu stricto

SSC	sodium citrate buffer
SSS	sea salt solution
ST	subtype
STD	standard deviation
TEM	transmission electron microscopy
T_m	melting temperature
WHO	World Health Organization