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RESEARCH ARTICLE

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Non-lethal detection of *Eubothrium crassum* (Cestoda) in farmed Atlantic salmon, *Salmo salar*, using anal swabs and real-time PCR

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Abstract

Detection of intestinal parasites in fish typically requires autopsy, resulting in the sacrifice of the fish. Here, we describe a non-lethal method for detecting the tapeworm *Eubothrium crassum* in fish using anal swabs and real-time PCR detection. Two assays were developed to detect cytochrome oxidase I (COI) mitochondrial DNA and 18S ribosomal DNA sequences of *E. crassum*, respectively. The assays were tested on swab samples from confirmed pathogen free Atlantic salmon (*Salmo salar* L.) and on samples from farmed Atlantic salmon, where the presence and intensity of parasites had been established through autopsy. The COI assay was shown to be specific to *E. crassum*, while the 18S assay also amplified the closely related *E. salvelini*, a species infecting Arctic charr (*Salvelinus alpinus* L.) in freshwater. The COI assay detected *E. crassum* in all field samples regardless of parasite load while the 18S assay failed to detect the parasite in two samples. The results thus demonstrates that this non-lethal approach can effectively detect *E. crassum* and can be a valuable tool in assessing the prevalence of infection in farmed salmon, aiding in treatment decisions and evaluating treatment effectiveness.

KEYWORDS
aquaculture, fish parasite, non-invasive detection, non-lethal detection, real-time PCR, tapeworm

1 | INTRODUCTION

Non-lethal (non-invasive) methods for pathogen detection are favoured over destructive methods, as the latter require autopsies and hence sacrificing the fish. Non-lethal methods, particularly when involving molecular techniques, might also be more cost-effective than methods relying on autopsies. At present, the detection of tapeworms and other intestinal parasites in fish relies on autopsy-based methods, while non-lethal methods are less widely applied. However, the use of non-lethal methods holds promise as a viable alternative to autopsies, also for intestinal parasites. These methods can be valuable for fish farmers in deciding when to treat against tapeworms and for evaluating treatment effectiveness. Furthermore, they offer potential applications in studying pathogen distribution in both wild fish and in aquaculture settings.

Non-lethal detection methods have been developed for several pathogens. For instance, gill swabs combined with real-time PCR have been used to detect the ectoparasitic amoeba *Paramoeba perurans*, the causative agent for amoebic gill disease (AGD) (Downes et al., 2017). Similarly, a method has been established for detecting salmon lice, *Lepeophtheirus salmonis*, in stomach samples from lumpfish (*Cyclopterus lumpus*) (Eysturskarð et al., 2017). Analysing faecal samples has also proved useful in assessing organisms in the stomach and intestines. This has been applied in several studies, including tapeworm detection in terrestrial animals (Øines et al., 2014), food content determination in birds (Oehm et al., 2011) and prey identification in scats from seals (Matejusová et al., 2008). More recently, a method detecting the Asian fish tapeworm, *Schyzocotyle acheilognathi*, in the endangered humpback chub, *Gila cypha*, using rectum swabs and PCR was developed (Campbell et al., 2019).

Eubothrium crassum is a tapeworm species known to infect Atlantic salmon, *Salmo salar*, trout, *Salmo trutta* and rainbow trout, *Oncorhynchus mykiss*, both in freshwater and sea water. A related species, *E. salvelini*, is found in Arctic charr, *Salvelinus alpinus* and brook trout, *Salvelinus fontinalis* (Scholz et al., 2003). While *E. crassum* has not been found in Arctic char and *E. salvelini* has not been found in salmon, rainbow trout can carry infections with both species (Scholz et al., 2003). *E. crassum* has been a recurring issue for Atlantic salmon farmers in Norway (Bristow & Berland, 1991; Saksvik et al., 2001), especially in the Western parts of the country (counties Rogaland and Vestland) (Hansen et al., 2022), often necessitating anthelmintic treatment the fish. The most common anthelminthic drug is praziquantel, and Norwegian fish farmers have in several years reported of an increased number of treatments against tapeworm infections. In addition, the treatments are also often reported not to give the desired effect (Sommerset et al., 2021). However, currently there is no straightforward method to determine the effect of such treatments except by counting the number of parasites in necropsied fish after treatment.

In such and some other circumstances, it can be preferable to detect *E. crassum* without sacrificing fish, both for assessing the presence of the parasite in fish farms and for the evaluation of treatments. Consequently, this study aimed to develop a non-lethal method for detection of *E. crassum* in fish using anal swabs and real-time PCR. Two assays were developed and tested, targeting the mitochondrial cytochrome oxidase I (COI) DNA and 18S ribosomal DNA sequences of *E. crassum*.

2 | MATERIALS AND METHODS

An overview of the full procedure for non-lethal detection of tapeworms using anal swabs and real-time PCR analyses is given in Figure 1 and consists principally of (1) and (2) sampling of tapeworm DNA with anal swabs, (3) DNA extraction, (4) real-time PCR analysis, and (5) and (6) interpretation and use of results.

2.1 | Samples

Table 1 gives an overview of all the samples used in the current study. Tissue samples from various tapeworm species and their respective fish hosts were included for specificity testing and optimization. These samples were either identified to species level in earlier studies or were identified in the current study. For the parasites, small pieces of tissue were used.

All swab samples were taken with COPAN FLOQSwabs, 520CSO1 (COPAN). The swab samples taken from the confirmed pathogen free fish (see Table 1) were obtained from the anal opening of a whole fish. The fish was placed on a clean paper and the outside of the fish was wiped off with a paper towel before the sample was taken.

To evaluate the performance of the developed assays on swab samples taken from the field, we included 13 samples from a separate experiment where the number of parasites in each fish had been counted (Sakariassen, 2019).

Briefly, the assessment of tapeworm presence in field samples involved a series of steps. The frozen intestines, received from a fish farm, were thawed in the laboratory. Subsequently, the pyloric caeca were carefully opened with a pair of scissors, and their contents were scraped onto a large Petri dish. A thorough examination of the intestinal content was then conducted to detect the presence of parasites. Additionally, to ensure that all the parasites had been scraped out, the pyloric caeca were pressed between two glass plates and examined.

The actual field samples tested in the present study were then selected from a larger number of samples to ensure a representation of a wide range of parasite numbers (see Table 4). The parasites in each fish were classified into different developmental stages (see Sakariassen, 2019). In infected fish there is often a bimodality in worm size (see Kennedy, 1996). In the current study, we differentiate between larger worms (>5 cm), which can include oviferous individuals releasing eggs, and smaller worms (<5 cm) which always are immature. The rationale behind this differentiation is that a larger proportion of the DNA obtained via the swabs originates from eggs or shed proglottids with eggs rather than free DNA or other parts of parasite tissue, and the detection probability may thus be expected to be higher in the presence of oviferous individuals.

Because only samples of the intestines were available from these field samples, the swabs were taken from the hindgut and not from the anal opening of a whole fish, as was done for the specific pathogen free fish.

All swabs were fixed in sample tubes containing 70% EtOH until DNA extraction. It would probably also be possible to gently

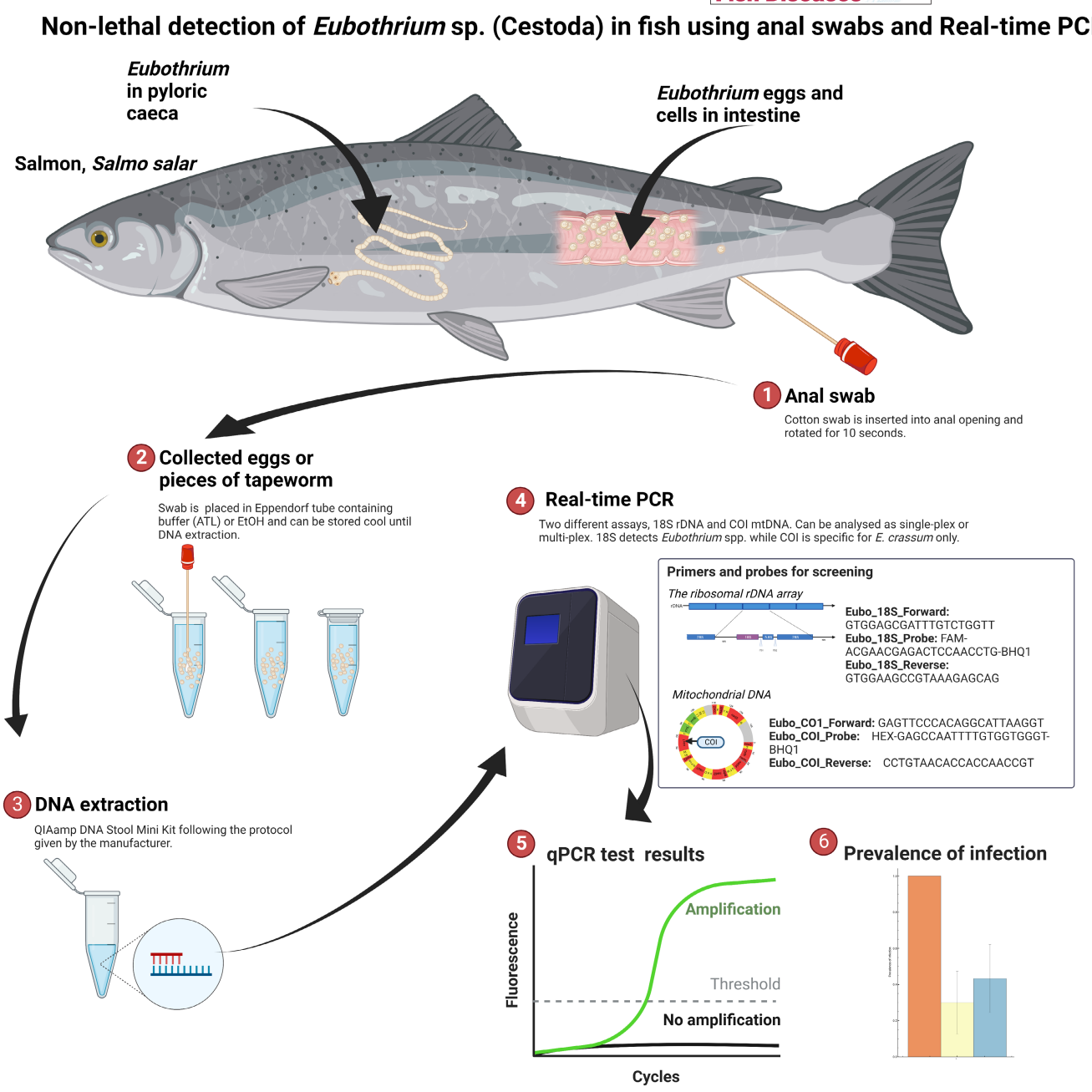

FIGURE 1 A protocol for the non-lethal detection of *Eubothrium crassum* in fish using anal swabs and real-time PCR. Created with BioRender.com

squeeze out faeces and sample directly without a swab, but this procedure was not tested in the current study.

2.2 | DNA extraction

DNA extraction from tissue samples were performed with a DNeasy kit on a QiaCube extraction machine (Qiagen®) in accordance with the manufacturer's instructions. For extraction of swabs, the sample tubes containing the swabs were first shaken to release the content on the swabs and subsequently centrifuged briefly. Next, the swabs were removed from the tubes and most of the EtOH was removed with the use of a pipette. Any remaining EtOH was then evaporated on a heating block at 56°C. Finally, DNA was extracted from the pellet using the QIAamp Fast DNA Stool Mini Kit (Qiagen®) in accordance with the manufacturer's instructions.

2.3 | Assay design and optimalization

Two PCR assays were designed to detect *E. crassum*, one that target the mitochondrial cytochrome c oxidase subunit I (COI) gene and one that target the 18S ribosomal RNA gene.

TABLE 1 Samples used in the development and testing of the non-lethal method for detection of *Eubothrium crassum* in fish.

Sample type	Purpose	Species	*n* samples	Locality	Host
Tissue	Sensitivity and optimalization	*E. crassum*	2	Salmon farm, County Rogaland	*Salmo salar*
Tissue	Specificity	*E. salvelini*	1	Lake Kjemsjøen, County Innlandet	*Salvelinus alpinus*
Tissue	Specificity	*Schistocephalus solidus*	1	Lake Luktvatnet, County Nordland	*Gasterosteus aculeatus*
Tissue	Specificity	*Dibothriocephalus ditremus*	1	Lake Fustvatnet, County Nordland	*Salvelinus alpinus*
Tissue	Specificity	*Dibothriocephalus dendriticus*	1	Lake Luktvatnet, County Nordland	*Salvelinus alpinus*
Tissue	Specificity	*Proteocephalus longicollis*	1	County Agder	*Coregonus lavaretus*
Tissue	Specificity	*Salmo salar*	1	NIVA, Solbergstrand, County Akershus	—
Tissue	Specificity	*Salmo trutta*	1	Lake Luktvatnet, County Nordland	—
Tissue	Specificity	*Salvelinus alpinus*	1	Lake Luktvatnet, County Nordland	—
Tissue	Specificity	*Oncorhynchus mykiss*	1	Aquarium, NVI[a]	—
Swab	Field sample	*Salmo salar*	10	Vikan, County Trøndelag[b]	—
Swab	Field sample	*Salmo salar*	13	Salmon farm, County Rogaland[c]	—

Abbreviation: NVI, Norwegian Veterinary Institute.

[a]Samples taken from fish that were euthanized for use in another trial.

[b]Certified pathogen free fish. Samples taken from fish that were euthanized for use in another trial. Average fish length: 280 mm (range 260–300 mm) and weight: 241 g (range 192–186 g).

[c]Samples taken from fish that were euthanized for use in another trial. Average fish length: 413 mm (range 370–450 mm) and weight: 873 g (range 514–1234 g).

TABLE 2 Primers and probes for real-time PCR detection of *Eubothrium crassum*.

Target gene	Primer name	Primer/probe	Length (bp)	Sequence (5′–3′)
E. crassum 18S	Eub-F	Forward	20	GTGGAGCGATTTGTCTGGTT
	Eub-R	Reverse	20	GTGGAAGCCGTAAAGAGCAG
	Eub-P	Probe	20	FAM-ACGAACGAGACTCCAACCTG(BHQ1)
E. crassum COI	Eub-4F	Forward	22	GAGTTCCCACAGGCATTAAGGT
	Eub-3R	Reverse	20	CCTGTAACACCACCAACCGT
	Eub-3pr	Probe	20	HEX-GAGCCAATTTTGTGGTGGGT(BHQ1)

TABLE 3 PCR results from analysis of *Eubothrium salvelini* DNA.

Sample	Experiment	18S C_q values	COI C_q values
E. salvelini (undiluted)	1	18.7/17.5/18.2	No C_q/No C_q/No C_q
E. salvelini (10× diluted)	1	21.5/20.8/21.3	No C_q/No C_q/No C_q
E. salvelini (undiluted)	2	17.5/17.9/17.2	No C_q/No C_q/No C_q
E. salvelini (10× diluted)	2	20.2/20.2/20.0	No C_q/No C_q/No C_q

TABLE 4 Qualitative 18S and COI PCR results for a set of anal swab samples taken during a field study and the parasite counts for each sample.

Sample	18S assay	COI assay	Total parasite count ($n > 5$ cm[a])
1	4/4	4/4	72 (34)
2	3/4	3/4	30 (26)
3	4/4	4/4	8 (8)
4	4/4	4/4	3 (3)
5	4/4	4/4	67 (33)
6	4/4	3/4	6 (6)
7	2/4	4/4	12 (12)
8	4/4	3/4	5 (5)
9	0/4	3/4	25 (19)
10	4/4	4/4	5 (5)
11	4/4	4/4	74 (37)
12	0/4	4/4	23 (21)
13	4/4	4/4	4 (4)

Note: The numbers listed under each assay reflect the number of detected/number of analyses (two PCR parallels in two independent PCR experiments).

[a]Stages > 5 cm that can be oviferous.

The published COI sequence from *Eubothrium* sp. in GenBank (KR780781), in addition to several unpublished sequences from *E. crassum* obtained by the current authors (n total = 23, manuscript in preparation), were aligned and variable regions identified. KR780781 was initially used as template for PCR design with Primer3Plus (Untergasser et al., 2007). The details for the primers and probes can be found in Table 2. This assay was designed to produce a 130 base pair amplicon. A comparison to *E. salvelini* (KR780824) showed three SNPs in the forward primer, two in the probe and five in the reverse primer, rendering it unlikely that DNA from this species would amplify with this assay. A primer-BLAST (Ye et al., 2012) search limited to *Bothriocephalidea* gave no further hits that might produce a signal.

For the design of the 18S assay, all published 18S rDNA sequences from *Eubothrium* sp. in GenBank and from *E. crassum* obtained by the current authors, were aligned and variable regions identified. The 18S ribosomal RNA gene sequence (AJ287509) of *E. crassum* was initially used as template for PCR design with Primer3Plus (Untergasser et al., 2007). The details for the primers and probes can be found in Table 2. This assay was designed to produce a 120 base pair amplicon. Primer-BLAST (Ye et al., 2012) was subsequently used to check specificity in silico. Primer-BLAST identified another *E. crassum* sequence, KR780924, and two sequences of *E. salvelini* (KR780963 and AF267291) in GeneBank that gave a perfect match to the designed assay. Since *E. salvelini* is a freshwater species which has not been found to infect Atlantic salmon, we considered this design suitable for use in studies of farmed Atlantic salmon in salt water.

The closest non-perfect hit was to *E. fragile* (KR780946) with two SNPs in the reverse primer. This parasite might thus also be detected by our assay. However, the host Twaite shad, *Alosa fallax*, is a rare species in the Northern Atlantic, which is the intended area of use for the designed assay. *E. rugosum* (KR780961) and *E. tulipai* (KR780951) both showed 3 SNPs in the reverse primer and it is thus unlikely that DNA from these species would produce a signal.

The specificity of the assays was investigated in three experiments. A dedicated analysis on DNA from *E. salvelini*, an analysis of a collection of other tapeworms present in salmonids in Norway and their hosts, and finally an analysis of a set of anal swab samples taken from *S. salar* that were certified pathogen free (see Table 1).

The PCRs were run as duplex PCR and were performed with Brilliant III ultrafast mastermix (Agilent) with the following PCR protocol: initial enzyme activation for 3 min at 95°C followed by

45 cycles of 10 s at 95°C and 20 s at 60°C. The gradient experiments were run on a CFX96 instrument (Bio-rad) and all other experiments were run on an Aria instrument (Agilent). All samples giving C_q values above 38 were considered negative.

Gradient PCR was performed with annealing temperatures varying from 55 to 65°C. The 18S showed a similar C_q over the entire temperature span, while the C_q values for the COI assay were constant from 57 to 63°C. To estimate the amplification efficiency and linearity for the two assays, two samples identified as *E. crassum* were used to make dilution curves with five times 10× dilutions. The two standard curves were analysed twice with three PCR replicates each time.

Correlations were examined using Spearman's Rank Order Correlation Coefficients in Statistica 13 (TIBCO).

3 | RESULTS AND DISCUSSION

Based on the gradient PCR experiment performed, an annealing temperature of 60°C was selected. The amplification efficiency was found to be 91.4% for the COI assay and 98.3% for the 18S assay. Linearity expressed as R^2 were determined to be above 0.995 for both assays.

As indicated by the in silico specificity analysis, DNA from *E. salvelini* was detected by the 18S assay but not by the COI assay (Table 3). None of samples from the other tapeworms, salmonid hosts or from the anal swabs taken from the confirmed pathogen free fish tested positive with the 18S or COI assays (results not shown).

For the 13 swab samples taken in the field, *E. crassum* was detected in all samples regardless of parasite load for the COI assay (Table 4). The 18S assay failed to detect the parasite in two of the 13 samples and was generally less sensitive than the COI assay.

The reason for the difference in sensitivity between the two assays is unknown, but as the COI assay was both specific and sensitive, this was not followed up further and we would recommend using the COI assay. The apparent higher sensitivity of the COI assay compared to the 18S assay is in contrast to several other eDNA studies targeting other organisms. There, the ribosomal assays have been shown to be more sensitive than mitochondrial assay (see e.g. Minamoto et al., 2017), something which might be attributed to differences in DNA copy numbers.

There were no correlations between C_q values (data not shown) and 'total' or 'large' worm counts ($-0.09 < Rs < 0.24$; $p > 0.44$). This is not surprising because the faecal content contains more eggs, or proglottids with eggs, than free DNA from the tapeworms present and the eggs are probably released from the adult worms at regular intervals. The concentration of DNA in a swab sample is therefore likely correlated more strongly with the number of eggs present rather than the number of adult worms in the intestine. The PCR method detects *E. crassum* irrespective of the origin of the DNA, and will as such, also detect non-established infections/infections in digested copepods with intermediate stages. However, this application was beyond the scope of the present study since for a study of prevalence in a fish farm, the important thing is that the parasite is detected, and as such, the method works well.

In conclusion, the non-lethal method presented herein is a valuable tool for assessing the prevalence of infection with *E. crassum* without sacrificing any fish. This can potentially be carried out at any time point in the grow out phase of Atlantic salmon in the sea, but this needs further testing. In addition, the DNA extracted from the swab could be analysed for the presence of other pathogens in the intestines of Atlantic salmon, such as the flagellate *Spironucleus* spp. The real-time PCR should also be suitable for use in detecting DNA of *E. crassum* in water samples (eDNA monitoring) and pro- and plerocercoids in the copepod intermediate hosts. Being more sensitive than conventional PCR and sequencing, the qPCR assays presented here can be used for species identification in formalin fixed samples, for example when tapeworms are detected in histological sections. As shown for humpback chub (Campbell et al., 2019), the current method can also be applied in ecological studies of wild fish, for example from endangered populations, where a non-lethal method is the only alternative.

AUTHOR CONTRIBUTIONS

Haakon Hansen: Conceptualization; funding acquisition; writing – original draft; methodology; writing – review and editing; formal analysis; project administration; data curation. **Bjørn Spilsberg:** Methodology; validation; writing – review and editing; formal analysis. **Sigmund Sevatdal:** Funding acquisition; writing – review and editing; investigation. **Trine Sakariassen:** Formal analysis; writing – review and editing; investigation. **Christoph Hahn:** Writing – review and editing; methodology; formal analysis. **Saima Nasrin Mohammad:** Writing – review and editing; methodology. **Egil Karlsbakk:** Writing – review and editing; formal analysis; conceptualization; investigation.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data generated or analysed during this study are included in this published article and its additional files.

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