COMPARISON OF DIAGNOSTIC METHODS FOR THE DETECTION OF PARASITES IN FISH

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ABSTRACT

In recent years, Aquaculture has developed very rapidly. However, fish parasitic diseases in fry and fingerling occur often. There are many methods which were used to diagnose fish parasites. In this paper, fifty wild fish belonging to three fish species: roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and bream (*Blicca bjoerkna*) were collected from Arreso Lake in Copenhagen, Denmark in 2005 to diagnose parasites. Parasitological investigation was implemented by normal observation, compression, digestion and PCR methods at the Fish Disease Laboratory, Pathology Department, Life Science University, Copenhagen, Denmark. The results show a high prevalence of eye fluke metacercaria in wild fish (100% of *Blicca bjoerkna* infected by *Diplostomum* sp.). Each method has advantages and disadvantages. The classical methods are simple, cheap and easy to apply in every fish laboratory. PCR methods produced results rapidly, sensitively and exactly. But this method costs much for equipment, and chemicals and needs exacting technique.

Keywords: Parasites, fish, diagnostic.

1. INTRODUCTION

World aquaculture production now accounts for 32% of total fisheries production, according to the FAO (2005). Globally, fish provide about 15% of all the animal proteins consumed, with variations from an average of 23% in Asia to approximately 18% in Africa and around 7% in Latin America. Total world fisheries production in 2003 was 132.5 million tonnes, of which 42.3 million tonnes were from aquaculture and 90.2 million tonnes were from capture fisheries. Total fish production has increased in recent years, mainly due to improvements in the aquaculture industries. However, intensive aquaculture systems with high stocking densities are vulnerable to infectious diseases.

Parasitic diseases in fish have become increasingly prevalent during the past few decades, in parallel with the growth and development of aquaculture industries throughout the world. Disease problems, including hazards caused by parasitic organisms, are the biggest threat to the continuing development of the industry (Buchmann, 2001). Fish parasitology is a rapidly expanding area, as *Gyrodactylus salaris* was introduced to Norway in the 1970s. Since its introduction in Norway the parasite has spread to a total of 45 salmon rivers. The affected salmon populations have experienced a significant decrease as a result (Buchmann, 2004).

The increasing importance of aquaculture products, including farmed fish, has emphasized the need for health control and proper fish disease diagnosis. Parasitological methods are vitally important for the parasitological study of fish. There are a wide variety of parasitological methods, and each method has its advantages

and disadvantages, depending on the purpose and target of study. For parasitological investigation, the classical methods (the normal observation, compression, and digestion methods) have been applied. To find blood parasites, the blood smear preparation or wet blood method has been used. PCR (Polymerase Chain Reaction) is a new method for parasite diagnosis. The use of the PCR method has allowed links to be elucidated between the various developmental stages such as cercariae, metacercariae and adults of specific trematodes (Cribb et al., 1998; Jousson et al., 1998; Anderson, 1999; Bartoli et al., 2000).

The objectives of the present study were to investigate the use of different methodologies in fish parasite studies. Thus, the aim is to compare classical and molecular methods for the diagnosis of fish parasites.

2. MATERIALS AND METHODS

2.1 Fish samples

Fish samples were collected during November, 2005 from Arreso lake, Copenhagen, Denmark by local fishermen.

Three species were used including roach (*Rutilus rutilus*), perch (*Perca fluviatilis*) and bream (*Blicca bjoerkna*). A total of 50 fish were examined (Figure 1).

2.2 Dissection of the fish

Fish species were identified, anaesthetized by MS 222 (100 ppm) and killed by cervical dislocation. Each fish was weighed (gram), measured (cm) and recorded. Gills, fins, the nostril, and scales were taken off; the eyes were removed from the fish and opened; then the lens and vitreous humour were exposed. All these organs were placed separately in petri dishes with PBS (phosphate buffered saline pH 7.0). The internal organs were exposed after a vertical incision was made from the anal opening to the lateral line and to the operculum (Buchmann & Bresciani, 2001). Liver, gall bladder, spleen, oesophagus, stomach, pyloric caeca, intestine, gonads, swim bladder, and urine bladder were cut and placed separately in petri dishes containing PBS.

Figure 1. Fish samples used for parasitological methods

2.3 Parasitological investigation

The normal observation

Before any dissection, the exterior of the fish was observed under the dissecting microscope at 7x-40x magnification. Scrapings of the body surface were done with a cover slip to remove epithelial cells and mucus with parasites for examination in the compound microscope (40x-1000x). The fins, gills, eye lenses and vitreous humour were examined in the dissecting microscope (7x-40x). All parasites were recovered and placed in separate vials with PBS.

The content of selected separate organs (oesophagus, stomach, pyloric caeca and intestine) was scraped from the lumen and epithelial lining and inspected under the microscope. Parasites were found and transferred by pipettes, pincers or forceps to separate glass vials with PBS. In addition,

parasites were kept in Eppendorf tubes with ethanol (70%) or neutral formalin (4%).

The compression method

Different parts of the fish (muscles, fins, gonad, liver, spleen, etc.) were taken. Each part was compressed between 2 glass slides. Thus, by applying a little pressure to the tissue it is flattened until the presence of parasites is revealed (Buchmann, 2005). The two glass slides were placed under the dissecting microscope (7x-40x magnification). Parasites were observed, recovered and placed in separate vials with PBS. For later study, all of the parasites were kept in Eppendorf tubes with ethanol (70%) or neutral formalin (4%).

The digestion method

For larger fish, different parts (e.g. fins, muscles, and bone structures) were taken. For small fish, the whole fish body or whole head (except eyes) were used. Then, each different part of each fish was weighed, ground in a mortar with pestle and transferred into a beaker (1:5 to 1:10 w/v) with pepsin solution (2% pepsin, pH 2) at acid conditions. They were mixed well and placed in a 37^{o}C incubator for 2-3 hours (longer for hard tissues) with occasional stirring. Samples were added to saline water (0.85%), shaken, and allowed to settle. Digest was poured through a 1x1 mm mesh brass sieve, washed with saline and settled until sediment was easily observed. The supernatant part was discarded very carefully and the sediment kept. This procedure was repeated several times (typically between seven and eight) or until the supernatant became clear. The encysted metacercariae were found and isolated. Then, these encysted metacercariae were excysted using a trypsin solution at slightly basic conditions (0.5% Bile: 0.25% Trypsin: 0.5% Chymotrypsin; pH: 8.4), and placed in a 37^{o}C incubator for 5-10 minutes (Buchmann, 2005). The metacercariae out of the cyst were collected and placed in separate vials with physiological saline. They were observed and identified using a compound microscope. Stretching of these metacercariae was done by hot formalin for two minutes. Then, they were kept in Eppendorf tubes with neutral formalin (4%).

Diagnosis of parasitic infections

* *Diagnosis based on morphological criteria*

Morphological characteristics of parasites are important values. Features observed were shape, total length and width, external structures of parasites (spines, lobes, etc.), different appendices, sclerotinized structures (hamuli, attachment hook, etc.), sex organs (testes or ovaries). Parasite morphological diagnosis followed the key of Bykhovskaya-Pavlovskaya *et al.* (1964). Infection was described by prevalence (the percentage of the hosts which are infected with a certain parasite) and mean intensity (the mean number of parasites in the infected fish only) (Buchmann & Bresciani, 2001).

* *Diagnosis based on PCR techniques*

Metacercariae of eye flukes were collected from fish eyes, other metacercariae were collected by digestion method in the fish parasitological laboratory of KVL and preserved in 70% ethanol.

- Extraction of total genomic DNA

Total genomic DNA was extracted using commercial DNA extraction kits (QIAamp DNA kit, Qiagen Inc., USA). The extracted genomic DNA used as the template in PCR reactions was diluted to a final concentration of 100-150 ng/µl, and the template for this concentration was used in a normal PCR reaction of 50 µl volume (25µl of master mix,

2µl of each primer, 1 or 3 µl of template and 18 or 20 µl of water).

- Polymerase Chain Reaction (PCR)

A master mix was prepared for PCR in a 1.5 ml Eppendorf tube which included H_20, PCR buffer (10X), dNTPs, Primer 1 (forward) NC2: 5'-TTAGTT TCT TTT CCT CCG CT-3' and Primer 2 (reverse) NC5: 5'-GTA GGT GAA CCT GCG GAA GGA TCATT-3'(Maniatis et al., 1989). All materials were kept on ice all the time. The master mix was divided with 50 µl going into each of the PCR tubes. One or 3 µl of DNA was added. PCR was performed in the PCR machine (Gene Amp PCR system 9700) with an initial 95^{o}C step for 5 minutes and 30 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds; followed by a final extension at 72^{o}C for 7 minutes.

- Gel loading: 1.5% agarose in 10% TAE buffer was placed in an erlenmeyer flask. Then it was placed in the microwave on full power until boiling (2 minutes). It was mixed again and placed once again in the microwave on full power until boiling. It was cooled to 45-50^{o}C (not hotter to avoid plastic deformation) on the table and poured into the gel frame which had been sealed at the ends with autoclave tape. The gel comb making the wells was added. The gel then polymerized. The combs were removed and the gel was placed in the electrophoresis chamber. One x TAE buffer was poured into the chamber until the gel was covered. Each well received 3 µl of loading buffer and 5 µl of the digested product or 5 µl undigested PCR product. The first and last lanes on the gel were loaded with 6 µl size markers (100bp). The samples were run for about 45 minutes at 100 V. The gel was stained 20 minutes in TAE buffer, which contained ethidium bromide (0.01%). The DNA bands were visualized under UV illumination and a photo of the gel was taken by the machine. The gel was discarded in special containers for toxic material. After that, the banding pattern was analyzed.

3. RESULTS

Fish parasite prevalence

By the normal observation method, 32 fish of the three species were examined. The parasite prevalence was 59.4%. All bream samples were infected with parasites, while parasite prevalence of perch and roach samples ranged from 53.3 to 57.1% (Table 1).

Table 1. The results of fish parasitological examination by normal observation (for all parasites).

Name of fish species	No. of fish examined	Total body length (cm)(SD)	Total body weight (g) (SD)	No. of fish infected with parasites	Prevalence parasites
Perch *Perca fluviatilis*	15	10.24 ± 4.59	14.61± 9.94	8	53.3 %
Bream *Blicca bjoerkna*	3	14.33 ± 6.66	18.67± 6.11	3	3/3
Roach *Rutilus rutilus*	14	10.66 ± 2.81	14.64 ±7.65	8	57.1 %
Total	32	11.77±4.69	15.97±7.9	19	59.4 %

Eighteen samples of the three fish species were examined by the digestion method; the prevalence of metacercaria infection was 27.8%. The prevalence between fish species ranged from 2/8 to 1/3 (Table 2).

Table 2. The result of fish metacercariae testing by the digestion method (for all metacercaria).

Name of fish species	N° of fish examined	Total body length (cm)(SD)	Total body weight (g) (SD)	N° of fish infected with metacercariae	Prevalence metacercaria infection
Perch *Perca fluviatilis*	7	12.27± 1.68	21.71± 7.66	2	2/7
Bream *Blicca bjoerkna*	3	11.83± 0.76	15.23± 2.36	1	1/3
Roach *Rutilus rutilus*	8	15.30± 3.89	49.11± 4.12	2	2/8
Total	18	13.13±2.11	28.68±4.71	5	27.8%

Three fish species with a total of 6 samples were tested by the compression method, no parasites were found.

3.2 Results of parasitological examinations

Results from the normal observation method are shown in Table 3. In perch and roach, the prevalence was 13.3%, 28.6% respectively (eye lens), and 26.7% and 42.9% respectively (vitreous humour). In bream samples, this value was in all samples (eye lens) and 1/3 (vitreous humour). *Diplostomum* sp. was identified from eye lenses, and *Tylodelphys* sp. was identified from vitreous humours of all three fish species. In addition, other parasites were found including tapeworm, crustaceans, roundworm, metacercariae of trematodes. The crustacean *Argulus* sp. was found on the skin of bream and roach with a prevalence of 1/3 (bream) and 7.1% (roach). Tapeworm was found in the intestine of perch with a prevalence of 15%. A roundworm *Philometra* sp. was found on roach fins with a 7.1% prevalence. There was a 20% prevalence of metacercariae1 (trematodes) present in the abdominal cavity of perch. The names of cestodes1 and metacercariae1 were not determined. The mean intensity of eye flukes of bream ranged from 13.3 to 16.0, perch from 5.0 to 8.5 and in roach from 3.3 to 6.7. Intensities of other parasites had low values, ranging from 1 to 4.

Table 3. Parasites recovered by normal observation method.

Fish Species	Parasite species	Infected organs	Number of fish examined	Number of infected fish	Prevalence	Mean intensity
Perch *Perca fluviatilis*	*Diplostomum* sp.	Eye lens	15	2	13.3%	5.0
	Tylodelphys sp.	V. humour	15	4	26.7%	8.5
	Metacercariae[1]	A. cavity	15	3	20.0%	3.3
	Cestodes[1]	Intestine	15	1	15.0%	1.0
Bream *Blicca bjoerkna*	*Diplostomum* sp.	Eye lens	3	3	3/3	13.3
	Tylodelphys sp.	V. humour	3	1	1/3	16.0
	Argulus sp.	Skin	3	1	1/3	4.0
Roach *Rutilus rutilus*	*Diplostomum* sp.	Eye lens	14	4	28.6%	3.3
	Tylodelphys sp.	V. humour	14	6	42.9%	6.7
	Philometra sp.	Fins	14	1	7.1%	1.0
	Argulus sp.	Skin	14	1	7.1%	1.0

V. humour: Vitreous humour; A. cavity: Abdominal cavity; Metacercariae[1], Cestodes[1].

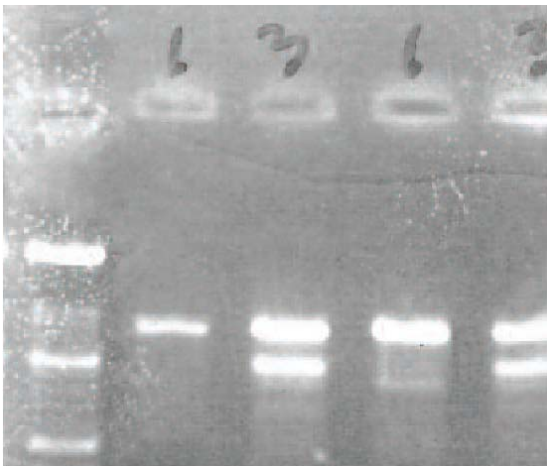
With the use of the digestion method, metacercaria 2 was found from fins. When bream (whole fish, except fin) was digested, a cyst1 was found; metacercariae were found in roach at a 33.3% prevalence. No parasites were found in perch. In roach, the metacercaria 3, 4 could be *Opisthorchis* sp.. The infection prevalence with metacercariae 3 was 12.5% (whole head), and 12.5% for metacercariae 4 (whole head except eyes) (Table 4). Mean intensity of metacercariae ranged from 1 to 5 (Table 4).

Table 4. Parasites recovered by the normal observation method.

Fish Species	Parasite species	Infected organs	Number of fish examined	Number of infected fish	Prevalence (%)	Mean intensity
Bream *Blicca bjoerkna*	Cyst	Whole fish (except fins)	3	1	33.3	3.0
	Metacercariae	Fins	8	2	25.0	2.0
Roach *Rutilus rutilus*	Metacercariae	Whole head (except eyes)	8	1	12.5	1.0
	Metacercariae	Whole head	8	1	12.5	5.0

3.3 The use of the PCR method for identification of trematodes with the NC2-NC5 primer pair

The results from testing eye flukes by the PCR method are shown in Figure 2. The NC2-NC5 primer pair shows the difference between metacercariae in vitreous humour of eyes (one band) and metacercariae in lens of eyes (two bands). No difference between 1 and 3 µl of DNA was observed.



Figue 2. Testing of NC2-NC5 primer pair from eye flukes lanes 1 & 3: metacercariae of *Tylodelphys* sp.; lans 2 & 4: metacercaria of *Diplostomum* sp.

4. DISCUSSION

The normal observation, compression and digestion methods were the simplest methods for examining parasite infections of fishes. In this study, with the normal observation method, each fish species attained over 50% parasite prevalence. The parasites discovered were found in different organs, such as eye lens, vitreous humours, intestine, fins, skins and the abdominal cavity of fish. The detected parasite types include eye flukes, tapeworm, roundworm, crustacean and metacercariae of trematodes. However, names of cyst1, metacecaria1,2 and cestodes1 were not determined because their morphological characteristics were damaged after the examination. The mean intensity of eye flukes was almost higher than the other parasite types. Some advantages of the normal observation method were recorded, it is easy to do and easy to apply at a fish farm, cheap, no need for any chemicals for examination. The compression and digestion methods were also applied. Thus, metacercariae of digeneans, third stage larvae of nematodes, plerocercoids of cestodes, cysts

of myxosporeans may be hidden in different types of tissues. The compression technique can be used to obtain a fast and preliminary visual impression (Buchmann, 2005). Some other advantages were also recorded from this technique. The exact location, or infection site, of metacercariae can be determined. It is economical, without the need to use expensive reagents. Features of the host tissue wall surrounding the metacercarial cyst can be useful in identification, but this can be lost in digestion.

The digestion method is also applied when parasitic stages of various species are difficult to discern, and a number of parasite forms are located in fish tissue such as fins, flesh, skin, etc. Cyst1, metacercaria2,3,4 were the parasitic stages of various species identified by this method. With this technique: a large number of samples can be processed; metacercariae can be isolated and collected; and exact numbers of metacercariae can be prepared for experimental infection. This method was previously used to estimate the number of *Cryptocotyle* spp. metacercariae in the skin of fish (Lysne, 1995). Some encysted metacercariae were found and were excysted by artificial digestion (trypsin solution) using this technique. Morphology was excellent and aided further identification.

It is often difficult to identify different stages of trematodes based on morphology (eggs, cercariae, metacercariae and adult worms). Eggs in the faeces of the definitive hosts have been difficult to identify due to the fact that the eggs are very small and can not be assigned to a specific species using light microscopy (Pauly et al., 2003). To find a relationship between metacercariae and adult worms, it is often necessary to conduct an infection experiment with sensitive final hosts. Such work takes a lot of time and money. The PCR method can help in this regard (Sirisinha et al., 1991). DNA technology has had a major impact in many areas of parasitology, including the identification and classification of parasites, the diagnosis of infections, the epidemiology of parasites, the analysis of population genetic structures, gene expression and organization, the study of drug resistance and vaccine development. In particular, the advent of the PCR has revolutionized parasitological research and has found broad applicability, mainly because its sensitivity permits the amplification of genes or gene fragments from minute amounts of parasite material. While specific determination of larval stages by morphological traits is often difficult and ambiguous, experimental demonstration of the life history is frequently unachievable due to the unidentified nature of the specific intermediate or definitive host. The use of molecular methodologies has allowed links to be elucidated between the various developmental stages as cercariae, metacercariae and adults of specific trematodes (Cribb et al., 1998; Jousson et al., 1998; Anderson, 1999; Bartoli et al., 2000). Currently the morphological characteristics of either the metacercariae recovered from fish or adult worms from humans are indistinguishable, and limited information on genetic studies is available. Up to now, the detection of eggs, cercariae, metacercariae and adult worms of certain species has been implemented by the PCR method. PCR assays have proven useful in demonstrating genetic links between metacercariae and adult worms of Heterophyidae species. These tools may be used for early diagnosis as they were shown to be sensitive in the identification of early infection in fish and useful for studying trematode life history. The ITS rDNAregion have been utilized for species-specific identification (Cribb et al., 1998; Jousson et

al., 1998; Anderson, 1999). Our primer sets were designed for identification of different flukes and they were useful for detection of eye flukes. Although the PCR method gives rapid, sensitive and exact results, it is still a new method in parasitic studies so many things are still limited, such as primer design or the PCR process, and this method requires a lot of money to be spent on expensive equipment and chemicals. Thus, it is difficult to develop in poor countries. For parasitic studies need to combine all the convenient methods well.

5. CONCLUSIONS

During the time devoted to the practical work of parasitological methods, three wild fish species, with a total of 50 fish, were collected and tested for parasites using the following classical methods: normal observation, compression, and digestion methods. These methods are simple, cheap and easy to apply in every fish laboratory. A new and model method, PCR, has been implemented for detection of metacercariae of *Tylodelphys* sp. and *Diplostomum* sp. by the NC2-NC5 primer pair.This method produced results rapidly, sensitively and exactly. But until now, this method has had some limitations due to the primer design or PCR process for parasite studying, which costs a lot of money.

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