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EFFECTS OF PRESERVATION CONDITIONS ON DNA QUALITY TO BE USED IN PARASITIC STUDIES IN FISHERIES

ABSTRACT

Among the parasitic disease agents, Diplostomums are among the most common and highly pathogenic agents. In this study, DNA isolation and subsequent purity and concentration differences were investigated from parasite samples taken under different stock conditions. In the concentration differences, it was observed that the concentrations of the parasite samples kept at -20°C without buffer and the samples kept in 0.9% saline at -20°C were insignificantly higher ($p<0.05$). According to the results of the study, if the starting material is DNA, it has been observed that there is no problem in terms of DNA concentration if the tissue samples are kept at -20°C in a short time (3 days) without the need for a buffer solution.

Keywords: Fish, Fish Parasites, *Diplostomum* sp., DNA Isolation, Preservation Conditions

1. INTRODUCTION

Parasites affect human health both directly by causing diseases and indirectly by causing yield losses in animals, which are an important food source for humans [1]. The trematode genus, Diplostomum von Nordmann, 1832, represents a large group of widely distributed parasites with complex life-cycles involving freshwater Lymnaeid snails and fish as intermediate hosts and fish-eating birds as definitive hosts. Metacercarias in the eyes of freshwater fish are considered major pathogens since heavy infections may be a source of substantial losses of wild and farmed fish [2]. Advances in molecular parasitology, especially nucleic acid-based techniques, have provided powerful alternative diagnostic tools that increase specificity and sensitivity [3]. These techniques have found applications in parasitology, such as diagnosis, treatment, genetic typing, systematic (taxonomy and phylogeny), population genetics, ecology, epidemiology, antiparasitic drug and vaccine development, understanding drug resistance, and parasite genome studies [1, 4, 5, 6 and 7]. The use of appropriate genetic markers is of great importance for the control and protection of genetic resources in aquaculture [8 and 9]. Developing molecular techniques provide important advantages in the early diagnosis of bacterial, viral and parasitic fish diseases. Generally, the process is initiated by extracting the genetic material (DNA or RNA) from the relevant sample. It is then amplified with the aid of PCR, mostly using DNA-specific primers [10]. The basic step of these studies is DNA isolation and DNA amounts are also important in qPCR studies [11]. In order to apply molecular biology techniques in various studies with genetic material, first of all, high molecular weight DNA molecule must be obtained in a pure form [12]. There are 3

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successive basic steps in DNA isolation. The first of these is the process of breaking down cells and revealing high molecular weight DNA. This is followed by denaturation and proteolysis, separation of the DNA-protein complex and solubilization of the DNA. Finally, there is the separation of DNA from proteins, RNA and other macromolecules by simple enzymatic and chemical methods [13]. In order to obtain pure DNA, the cells from which the DNA will be isolated are lysed by various methods. The obtained extract is rotated very quickly in centrifuge to separate the part containing DNA. This part is kept at 37°C with a detergent and protein-degrading enzyme. During this process, proteins bound to DNA are broken down. In this way, DNA is separated from protein and other molecules and is obtained in pure form. Finally, DNA is precipitated in ethanol. It is stored at 4°C in a buffer solution that will preserve the DNA structure [13]. After these stages, DNA molecules of different origin or different properties must be separated. As with separating genomic DNA from cell lysate, chromatographic techniques can be used to separate single and double-stranded DNA molecules and plasmid DNA from genomic DNA. However, the gel electrophoresis method is the most widely used method for separating nucleic acids due to its higher separation power [14]. Among the most important steps in these studies are the collection, transfer, storage and preparation of the sample under appropriate conditions for sequencing [15].

2. RESEARCH SIGNIFICANCE

Diplostomum spathaceum is one of the trematode species commonly found in fish and causes the death of fish in cases where it is excessive. It is especially undesirable in aquaculture, as it is excessive in fish and causes death. In recent years, the rapid increase in molecular studies facilitates the better identification of this parasite, and this paves the way for us to find different methods to combat them. It is very important to take the sample used in molecular studies and to keep it until the study is done. With this study, it is aimed to find out which of the methods used gives a clearer DNA concentration.

Highlights

- *Diplostomum* sp is one of the parasitic groups found in fish. Although these parasites are micron in size, they are collected in a size of approximately 1 g, and the net result in the DNA concentration study results in a more comfortable study in larger parasite groups.
- Although it is explained in the literature that the samples taken should be left to 75-95% ethyl alcohol to obtain genomic DNA, it was observed that clearer gDNA was obtained in the frozen storage of the samples in this study.
- With this study, it sheds light on other studies that it is possible to obtain clearer gDNA by using different samples other than parasites, without using ethyl alcohol, only by freezing the sample.

3. MATERIAL

Dead *Chondrostoma regium* fish caught by fishermen from the Fırat River were taken and brought to the Fish Diseases Laboratory of the Fırat University Fisheries Faculty.

4. METHODS

The lengths of the fish were measured on the length measuring board and their weights were weighed on the balance. Petri dishes and

Eppendorf tubes were prepared. The eyes of the fish were removed with the help of forceps, scalpel and scissors and left in petri dishes prepared with 0.9% saline in the eyepiece. Some of the Eppendorf tubes were left empty and the others were prepared separately by leaving 75% ethyl alcohol and 0.9% saline. Micron-sized Diplostomas were collected one by one from petri dishes containing saline under a stereo microscope. The collected parasites were left separately in eppendorfs containing ethyl alcohol and saline, and some of them were left in eppendorfs that did not contain any substance and stored in a deep freezer at -20°C. Samples were placed in tubes in 3 replications. Samples were analyzed under cold chain at Van Yüzüncü Yıl University Faculty of Fisheries Disease Laboratory and with the academic support of Biotechnology Application and Research Center. Moreover; According to [16], prevalence, mean intensity, and mean abundance values were calculated.

Mean Abundance=The total number of parasites/The number of fishes examined

Mean Intensity=The total number of parasites/The number of hosts infected

Prevalence=The number of host infected/The number of total fishes examinedx100

4.1. DNA Isolation

Total DNA isolations were isolated in an automated isolation robot (Qiacube Lt) using the Qiamp DNA mini kit (Qiagen) in accordance with the manufacturer's instructions. The isolation procedure was started by adding Buffer AL (and in most protocols, ethanol) to the lysate to allow optimal binding of the DNA to the membrane. Lysates were transferred to a QIAamp MinElute column, where the DNA was adsorbed on the silica gel membrane (by Centrifugation) while the lysate was drawn. Next; The salt was transferred to the QIAamp MinElute column membrane, which can also inhibit protein and other contaminants. Washes were performed by adding Buffers AW1 and AW2 under the conditions of binding to the silica gel membrane for cleaning of genomic DNA. With the elution step, total DNAs were transferred to centrifuge tubes as 50ul.

4.2. Nanospectrophotometric Measurement

After DNA isolation, measurements of DNA purity and concentration were performed using QIAxpert (Qiagen). The device can measure DNA, RNA and protein concentration and quality by scanning the entire UV/VIS spectrum in the wavelength range of 230-750nm. Since the sample volume to be used in the device can measure between 1.5ng and 10,000ng/ul, the maximum concentration is 2ul. Samples were measured as 2ul. Since the nanospectrophotometer can separate DNA, RNA and impurity fractions with a single measurement thanks to its microfluidic structure, results in ng/µl were obtained for each fraction. Since the measurement result can separate dsDNA from other nucleic acids, the probability of incorrect nucleotide is reduced to zero. Since the device has a UV/VIS Polychromatic system for detection and a reference channel, the results were taken simultaneously by measuring with 16-well slides.

5. FINDINGS

It was determined that the fish samples collected from the Fırat River had a total length of 39.8-44.0cm, a fork length of 38.2-39.0cm, and a weight of 580-837.0g. No pathological changes or disease symptoms were found in the necropsy of the fish, and only the evaluation was made in terms of parasitic infestation (Figure 1). A

total of 246 parasites were collected from the eyes of the fish. According to [16], prevalence, mean intensity, and mean abundance values were calculated and the prevalence was found to be 100, the mean intensity was 24.6, and the mean abundance was 24.6.

Microscopic identification of *Diplostomum* sp species was carried out as a result of the parasite examination. Morphologically, it was identified as *Diplostomum spathaceum*. Morphological features were observed as protruding posterior part and rounded anterior part. The ventral sucker was determined to be approximately twice as large as the oral sucker. In addition, it was observed that the esophageal branches reached up to the inside of the two intestinal hoses and merged and took a V shape (Figure 2).

Figure 1. Fish species sampled in the study and eye tissues sampled for parasites

The parasite images displayed microscopically after macroscopic examination are given below (Figure 2).

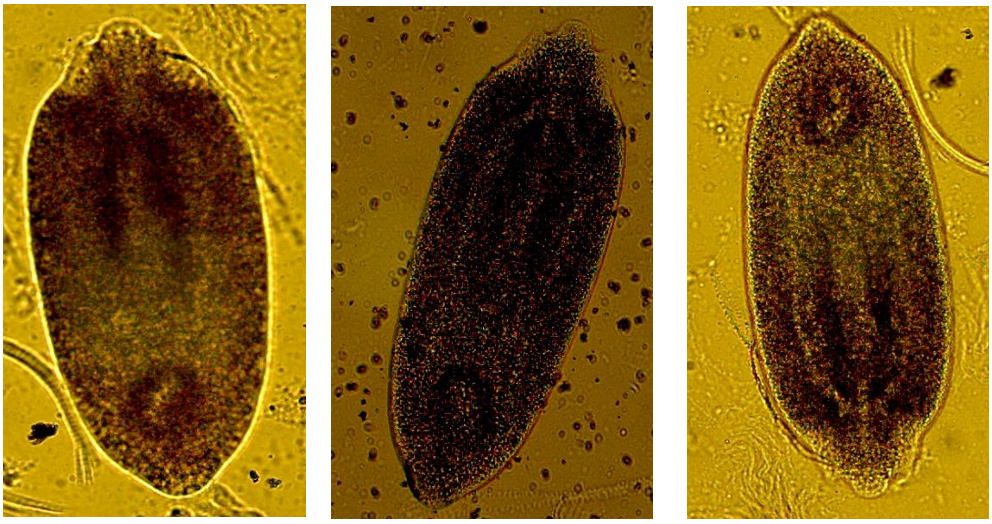

Figure 2. Images of *Diplostomum* sp parasites isolated in the study

5.1. DNA Isolation and Concentration Differences

The absence of parasites isolated in the study, that is, isolation from the parasite itself, reduced the possibility of contaminants or non-specific isolation products during isolation. It has been observed that parasite specimens maintain their vitality for at least 24 hours at -20°C. The DNA purity and concentration results after isolation with the QIACube Lt are given below (Figure 3).

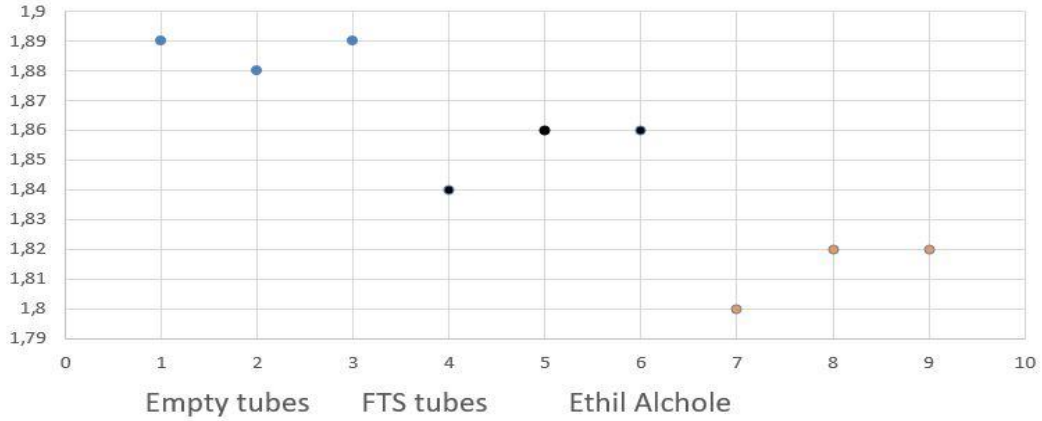

Figure 3. Purity ratios of samples after DNA isolation (1-3: Unbuffered samples, 4-6: Physiological saline samples, 7-9: Ethyl alcohol samples)

Concentration values were measured after the purity ratios of the isolated DNAs were within normal values between 1.8<x<2.0. The results obtained in nanogram/microliter value are given below (Figure 4).

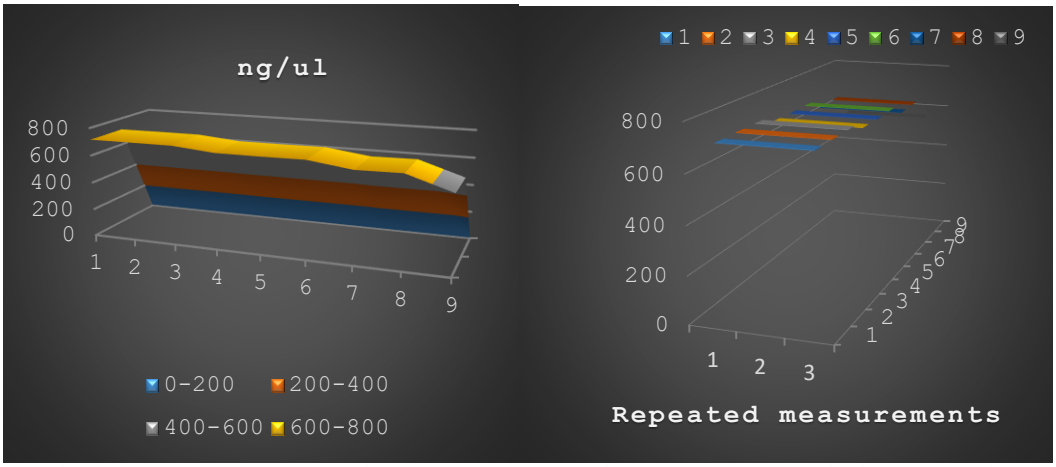

Figure 4. Concentration results of isolated parasitic DNAs in different groups
(1-3: Unbuffered samples, 4-6: Physiological saline samples, 7-9: Ethyl alcohol samples)

As a result of the study, the starting amount equaled at 25mg weight and after the isolation performed with the automatic isolation device without manipulation, the DNAs were again automatically and simultaneously measured with the QIAxpert device, and as a result of the reading of the samples with 3 replications, the purity rates were found to be between 1.8<x<2.0 after isolation in 3 different storage conditions. he has met. In the concentration differences, it was observed that the concentrations of the parasite samples kept at -20°C without buffer and the samples kept in 0.9% saline at -20°C were insignificantly higher ($p<0.05$).

6. DISCUSSIONS

Since fish are at the top of the feeding pyramid in the waters, they are constantly faced with parasite infestation [17]. The larvae of the *Diplostomum* species cause degenerations in the eyes of the fish

and form the "Larval Eye Diplostomidosis" known as eye worm disease. These metacercariae found in the eyes of fish can kill the fish during entry through the skin and cause eye staining, opacity in the eye lens, swelling of the cornea (keratoglobus) due to the collection of aqueous exudates in the anterior eye chamber, clouding of the eye lens (parasitic cataract), increased intraocular pressure, exophthalmus, cornea. It can cause tearing and ultimately blindness of the fish [18, 19 and 20]. *Diplostomum* has been used in our study because it is a dangerous parasite species in fish, especially in aquaculture, and in molecular studies to be carried out especially for *Diplostomum* and other parasite groups, it is aimed to find the best DNA concentration by using which concentration until genomic DNA is obtained after the sample is taken. As a result of the analysis, we observed that freezing the sample gives clearer results than other methods.

As a result of the optimization study performed with automatic isolation robots in molecular-based studies, it has been reported that the studies performed with the automatic isolation robot are completed in 80% shorter time than the manual studies, as stated on the Qiagen website. In previous studies carried out by some researchers, the importance of both the fish species and the parasite species used in the study was mentioned. Again, there are other studies that show the importance of isolation and concentration in different studies performed with the same method as the method used in this study. The type-genus *Diplostomum* von Nordmann, 1932 (subfamily Diplostominae Poirier, 1886) is highly speciose and globally distributed [2, 21, 22, 23, 24 and 25]. Metacercariae of various *Diplostomum* spp. are major helminth pathogens of freshwater fish, both wild and farmed. They decrease the general fitness of their hosts and increase their sensitivity to secondary infections and vulnerability to predation [22, 26, 27 and 28]. Therefore, the study of biology, ecology and diversity of *Diplostomum* spp. is important from both theoretical and practical viewpoint [28].

Eye flukes, metacercariae of digenean trematodes within the family Diplostomidae, are widespread in freshwater fish populations. The last host, carrying the adult trematode in the gut, is a piscivorous bird; freshwater snails serve as first intermediate hosts, releasing infective cercariae; and fish act as second intermediate hosts. Species within the genus *Diplostomum* choose various sites in the fish eye, such as the lens, and may reduce vision and elicit cataract in the host. Heavy infestations are associated with weight loss as the infections decrease the visual abilities of the host and thereby its food search capacity [29, 30, 31 and 32]. Researchers [33] stated that when comparing the methods used in their study in terms of economy, time and quality, the results obtained with the isolation robot are more reliable and efficient in terms of working quality and efficiency. In this respect, it is compatible with our study. Some researchers [34] stated that in their studies on the molecular identification of *Diplostomum spathaceum*, they put the fish's eyes in 0.9% physiological salt water and take the parasites one by one, leave them in 96% ethanol and keep them at -20°C. A few researchers [28] left the Diplostomums they obtained in 96% ethyl alcohol and kept the DNA concentration at +4°C until working. It is partially compatible with our study. Some researchers [35] explained in their study on the diagnosis of fish parasites that *Diplostomum* sp parasite is an intense parasite species. In this study, we can say that the presence of this parasite in all autopsied fish is compatible with both studies. They study molecular identification of *Diplostomum* sp [2, 24, 28, 36, 37, 38 and 39]. Stating that it is a common parasite species in 2022, they emphasized that it is important to

diagnose the species. Morever some researchers, [34] in their molecular characterization study of Diplostomum, they found that this parasite is *Diplostomum spathaceum*. In our study, it was understood that it was the same *D. spathaceum* morphologically. This study is similar to our study.

7. CONCLUSION AND RECOMMENDATIONS

In this study, it was determined that the DNA concentrations obtained by subjecting different types of concentrations by using *Diplostomum* sp parasite, one of the most intense parasite species of trematodes, gave the best results in a short time, and it was determined that the method subjected to analysis without using any substance. According to the results of the study, if the starting material is DNA, it has been observed that there is no problem in terms of DNA concentration if the tissue samples are kept at -20°C in a short time (3 days) without the need for a buffer solution.

CONFLICT OF INTEREST

The author(s) has no conflict of interest in the study.

FINANCIAL DISCLOSURE

The authors declare that this study has received no financial support.

DECLARATION OF ETHICAL STANDARDS

Ethics committee certificate is not required in this study as dead fish taken from fishermen were used.

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