SHORT COMMUNICATION

COMPARISON OF LARVAE MORPHOLOGY AND DNA PRESERVATION IN PLASTINATION USING DIFFERENT FIXATIVES

Dilan Biroğul[1*], Betül Baygeldi[2], Duygu Neval Sayin Ipek[3]

[1]Department of Internal Medicine, Faculty of Dicle University, Dicle University, Diyarbakır, Türkiye
[2]Department of Anatomy, Faculty of Veterinary Medicine, Fırat University, Elazığ,Türkiye
[3]Department of Parasitology, Faculty of Veterinary Medicine, Dicle University, Diyarbakır, Türkiye

***Corresponding author:**
Dilan Biroğul
Address: Department of Internal Medicine, Faculty of Dicle University, Dicle University, Diyarbakır, Türkiye
ORCID: 0009-0000-2390-6321
E-mail: dilanbirogul@gmail.com

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ABSTRACT

This study was conducted to determine the extent to which the structures of parasite larvae treated with different fixatives were preserved through the silicone plastination procedure and to investigate the degree of DNA preservation in plastinated larvae. For morphometric evaluation, the length (anterior-posterior) and maximum width (widest trunk width) of the larvae were measured to record changes before and after the plastination process. The silicone plastination protocol was applied at room temperature using three different fixatives. Subsequently, the larvae were deplastinated. Total genomic DNA (gDNA) from third-instar plastinated and deplastinated larval samples was extracted using the GeneJET Genomic DNA Purification Kit. DNA concentrations were measured at 260 nm using a UV-Vis spectrophotometer. The PCR protocol was applied, and photographs were taken using a UV transilluminator. Following plastination, the larvae were completely dry, and the process rendered them harmless. They could be stored for an extended period and, most importantly, their morphological characteristics remained suitable for morphological analysis by graduate students. No visible morphological issues were observed during the dehydration and impregnation stages of plastination; however, after curing, some specimens exhibited significant structural collapse, resulting in alterations of their normal anatomical structure. Additionally, DNA concentrations in the deplastinated groups were found to be higher than in the plastinated groups. In the second step of PCR, a ~300 bp band was obtained in all plastinated and deplastinated groups. In conclusion, the silicone plastination method enables the long-term preservation of larval morphological structures with minimal alterations, while causing a partial reduction in DNA integrity.

Keywords: Anatomy, DNA, larvae, morphometry, PCR, preservation, plastination

INTRODUCTION

From the past to the present, the protection of parasite samples using various preservatives has been necessary for teaching or research. Most parasites used as teaching and research tools in schools, universities, research centers, and other institutions are usually protected with a 4-10% formaldehyde solution (formalin) and/or 70% alcohol (Rosilawati et al., 2014). However, besides causing shrinkage and color change in the samples, these chemicals have significant disadvantages such as toxicity, carcinogenicity, odor, the wetness of the samples, maintenance of liquid levels, storage capacity, limited time, and storage space (McLaughlin, 1994; Swemberg et al., 2013; Gonzálvez et al., 2017). The plastination technique (von Hagens et al., 1987) is a well-established and innovative technique that has been an alternative option in conserving biological organisms in recent years. The procedure occurs as a gradual exchange of fluids and adipose tissue in the tissues with polymers under certain conditions (Lattore et al., 2007; Sundar et al., 2019). This technique allows for the production of clean, dry, durable models that can be examined without gloves or other protective equipment, and does not require any special storage conditions. In addition, since they do not contain toxic substances, students and instructors are not exposed to formaldehyde, phenol, alcohol, etc., thereby preventing exposure to harmful substances. Plastination models are durable and resistant to microbial decomposition and degradation. They can be easily stored, even in humid environments, as the plastination solution prevents water absorption (Chaturvedi et al., 2014). At the same time, this method allows samples to maintain their original form, size, and enhanced cellular-level appearance (Kocevski et al., 2010).

Despite significant progress made in molecular biology, there are few studies in the literature that examine nucleic acids in post-plastination tissues and investigate the preservation, extraction, and detection of DNA in plastinated samples (DeJong and Henry, 2007; Sagoo and Adds, 2013; Nagaraj et al, 2018). Although the plastination technique is an excellent alternative for educational materials, further studies on its suitability for various examination techniques are needed to demonstrate its potential as a museum or research material.

This study was conducted to determine the extent to which the structures of parasite larvae treated with different fixatives were preserved through the silicone plastination procedure and to investigate the preservation of DNA in plastinated larvae.

MATERIAL AND METHODS

The larvae used in this study were collected from the wounds of an injured dog that was brought to the Animal Hospital of the Faculty of Veterinary Medicine.

Plastination protocol

Firstly, for morphometric evaluation, length (from front to back) and maximum width (widest trunk width) were measured to record changes before and after the plastination process. The standard silicone plastination method (Biodur® S10) was used (von Hagens et al., 1987). The collected larvae were divided into three groups according to the fixatives used before plastination: Group A was fixed in 70% alcohol, Group B in pure acetone, and Group C in 10% formaldehyde. After fixation, the first step in the plastination process was the dehydration stage. Larvae were placed in cold (-25 ºC) acetone baths. The second step involved impregnating the samples with the silicone mixture (S10 + S3) at room temperature by reducing the pressure (increasing the vacuum). During impregnation, the acetone in the tissue evaporates from the cells and is replaced with the silicon mixture. Finally, the silicone in the tissue was cured (polymerized) using the hardener S6.A plastinated image of larvae fixed with different fixatives is shown in Figure 1.

Deplastination procedure

The stages of the deplastination procedure have been previously described (Ripani et al., 1996). For deplastination, larvae samples were sequentially incubated in 99% alcohol (Merck, #107017) for 24

hours, followed by incubation in methylbenzene (Merck, #108327) for 24 hours. To monitor the effectiveness of methylbenzene deplastination tissue, softening was checked regularly until the pin could easily be inserted into the tissue. After the methylbenzene application, the larvae were placed in 70% alcohol.

gDNA Extraction protocol

Total genomic DNA (gDNA) from third-instar plastinated and deplastinated larval samples was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA). For gDNA isolation, all larval samples were washed five times with PBS and cut into small pieces. 50 mg of each sample was placed in 1.5 ml falcon tubes and 20 µl of Proteinase K (20 mg/ml) and 180 µl of lysis buffer and incubated at 56°C for 24 hours to 72 hours. At the end of the incubation period, gDNA was extracted by following the kit's protocol, and the samples were stored at -20 °C until use.

Quantitative analysis of extracted DNA

The samples DNA concentrations were measured at 260 nm using a UV-Vis Spectrophotometer (NanoDrop™ 2000, Thermo Scientific).

Semi Nested PCR

In the first step, two primers designed to amplify DNA from a wide range of insects, including those belonging to the Oestridae family (Otranto et al., 2003), were used: UEA7 and UEA10. PCRs were carried out in a total volume of 50µl containing 5µl 10× PCR buffer, 5µl 25mM $MgCl_2$, 250µM each of dNTP, 20 pmol of each primer, 10 µl of template DNA, and 1.25U of Taq DNA polymerase (Thermo Scientific). The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

The second step was performed using the primers UEA9 and UEA10. In this step, the first-round PCR products were diluted at a 1:10 ratio. The annealing temperature for the second step was set to 58 °C, while all other conditions remained the same.

Positive and negative samples were included in all PCR reactions. All PCR amplicons were electrophoresed in a 2% agarose gel stained with ethidium bromide. The gel was then visualized under a UV transilluminator, and photographs were taken.

RESULTS

The final state of the larvae after plastination: all the samples in the three groups (A, B, C) were dry, and plastination rendered them harmless. They could be stored for a long time and, most importantly, their morphological characteristics could be studied by postgraduate students. They were able to make precise measurements for both length and width. A significant darkening of the color of the larvae in group A and group B was observed, while the larvae in group C retained their original color (Figure 1). No visible morphological problems were observed in all three groups, but, after curing, significant collapse occurred in some samples in groups A and B, which led to changes in their normal anatomy.

Quantitative analysis of extracted DNA

Table 1 shows the results of the DNA quantification obtained using the UV-vis spectrophotometer. The DNA concentrations in the deplastinated groups were higher than in the plastinated groups.

Semi-nested PCR

In the first step of semi-nested PCR, the gel results of PCR products obtained from all groups are shown in Figure 2. As a result of the first step PCR, □680 bp bands could not be obtained from the groups fixed with 10% formaldehyde and the freshly plastinated and deplastinated groups. However, after alcohol fixation, plastinated and deplastinated bands were obtained from larvae. In the second step of PCR, □a 300bp band was obtained in all plastinated and deplastinated groups.

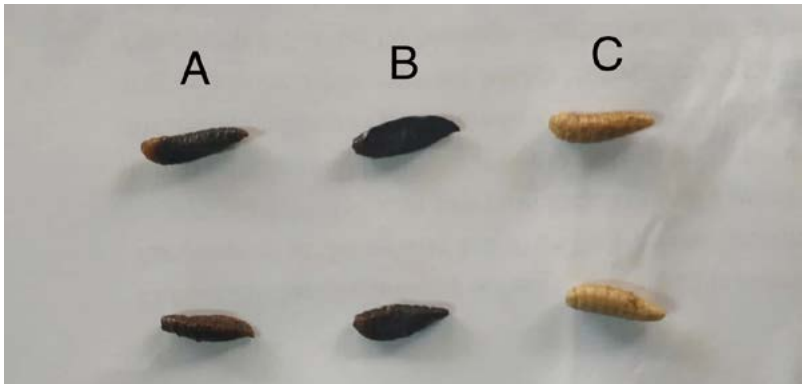

Figure 1 Classification of plastinated larvae (Groups A,B,C)

Table 1 Genetic material obtained from three groups' plastined and deplastined samples (Group A: %70 Alcohol; Group B: Pure Acetone; Group C: %10 formaldehyde)

Sample name	Amount of DNA
Group A	19.2 ug/ml
Deplastinated Group A	20.2 ug/ml
Group B	12.8 ug/ml
Deplastinated Group B	39 ug/ml
Group C	7 ug/ml
Deplastinated Group C	17.7 ug/ml

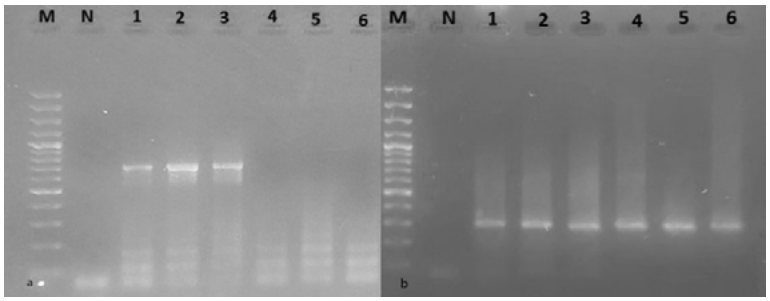

Figure 2 a, b. Agarose gel electrophoresis of amplification products of first and second PCR. Lane M, 100 bp marker; N, negative control; lane 1–6, samples (1, A; 2, Deplastine Group A; 3, B; 4, Deplastine Group B; 5, C; 6, Deplastine Group C).

DISCUSSION AND CONCLUSION

The plastination technique, which quickly gained popularity in the technical and medical fields, especially among anatomists, was developed by Gunther von Hagens in 1977 to preserve biological specimens (Lattore et al., 2007). Studies focusing mainly on anatomy support using biological samples obtained by using the plastination technique as educational material for students (Kumar et al., 2017). Studies investigating the use of the plastination technique as a suitable tool for protecting and preserving various endo- and ectoparasites have increased (Ramakrishna et al., 2010a; 2010b; Menaka et al., 2010). For the first time, Asadi and Mahmodzaeh (Ramakrishna et al., 2010a) used the S10 plastination technique to plastinate *Ascaris lumbricoides*. Since then, scientists have made necessary changes to the plastination protocol to develop ideally plastinated parasites based on size and morphology (Kocevski et al., 2010; Menaka et al., 2015; Gonzálvez et

al., 2017). To develop a more helpful plastination technique for *Toxocara* (*Neoascaris*) *vitulorum*, the impregnation of the melamine polymer was carried out at normal ambient pressure, in contrast to the low-pressure requirement (below five mmHg) of the traditional silicone plastination technique (Kocevski et al., 2010; Menaka et al., 2015). The temperature during plastination was kept at -20°C. This ultra-low temperature causes fixation and expansion in the samples and prevents their separation (Sagoo and Adds, 2013). Plastinated parasites were generally dry, non-sticky, shiny, odorless, chemical-free, and harmless, with some degree of flexibility and detectable morphological structure, and retained their natural form but lost their natural color. The results indicate that plastination is an effective method for preserving parasites and is widely used as an educational tool in parasitology. It is evident that the required times for each step of the plastination protocol vary depending on size and morphology of the different parasite species tested. This is consistent with reports of other specimens requiring modifications to the protocol to achieve optimal morphological and morphometric results (Gonzálvez et al., 2017). These plastination protocol modifications have also been described in other fields, such as embryology, herpetology, and zoology. For example, a sample's cuticle, tissue, or skin acts as a barrier that can interfere with acetone and polymer exchange during the dehydration and impregnation phases. (Asadi, 1998; Schaap, 1998; Wendel et al., 2008; Tiwari et al., 2012; October et al., 2017). Nematodes, acanthocephalans, and small trematodes often collapse and change their morphology and morphometry at a macroscopic level. No morphological or morphometric differences were observed between the cut and uncut specimens, and no correlation was found between the level of slump and the present cuticle cuts (Gonzálvez et al., 2017). Samples with a strong, thick surface layer (such as cuticle or membrane) should be cut to prevent the collapse of the entire sample. However, this protocol modification was found to be unnecessary for parasites without a body cavity (e.g., *Fasciola hepatica*) or with a very thin cuticle (e.g., *Dirofilaria immitis*) (Cheng, 1978). Ramakrishna et al. also used the plastination technique to preserve ecto- and endo-parasites (Ramakrishna et al., 2010b; Menaka et al., 2015). In this study, all samples fixed with formaldehyde and plastinated with silicone preserved their color.

Menaka et al. (2015) reported that plastinated larvae both retained their color and did not cause any respiratory irritation or allergies, as observed. Based on this study, silicone plastination can be effectively used to preserve fly larvae.

Shrinkage and flexibility are the two main limitations of this technique, especially for biological samples composed of soft tissues. These limitations were also noted in macroparasites plastinated using the Biodur TM S10/S3 polymer by Sagoo and Adds (2013) and the melamine polymer by Kumar et al. (2017). According to Latorre et al. (2007), shrinkage and color changes are the main causes of failures in the plastination technique. However, plastinated products remain intact in environmental conditions, and we share this opinion.

Although the number of studies on preserving DNA in plastinated and deplastinated tissues is limited, no study has yet been found focusing on arthropods with chitinous tissue. Our study observed that the amount of DNA extracted from the deplastinated groups was higher than from the plastinated groups. We believe this is due to the fact that DNA extraction was more successful in the deplastinated group, as the methylbenzene used in the deplastination process disrupts the cell membrane structure. Although the amount of DNA extracted from deplastinated larvae was similar to the amount of DNA extracted from deplastinated dog liver tissue in Ottone et al. (2020), it was higher than the amount of DNA extracted from deplastinated rat muscle tissue in the same study.

Nagaraj et al. (2018) reported that the amount extracted from plastinated tissue samples was 878 ng/µl, which is considerably higher than the amount obtained in our study. This difference is likely due to variations in the extraction methods used and differences in the plastinated tissues themselves.

In our study, following commercial mass DNA extraction, the first step of semi-nested PCR yielded an amplicon only in alcohol-fixed groups, while in the second step, the amplicons were obtained from all groups. Ottone et al. (2020) reported successfully amplifying the beta-actin gene from DNA extracted from deplastinated dog liver and rat muscle tissues using real-time PCR, though amplification occurred in the final cycles. Similarly, while not all samples produced amplicons in the first PCR step in our study, successful amplification in the second step supports these findings. This issue is likely caused by the presence of PCR inhibitors. However, the successful amplification of DNA in the first PCR step from alcohol-fixed tissues suggests that these inhibitors are either present in low amounts or that alcohol fixation better preserves the DNA structure.

Consequently, the results obtained in this study validate the use of plastinated parasites as an alternative tool for teaching, learning and scientific research in applied parasitology courses. The traditional method of fixing samples with formaldehyde poses significant health risks. However, the cost of plastination equipment and chemicals is considerably higher. Moreover, a thorough understanding of the plastination technique is required to produce high-quality samples. Plastination provides a dry, odorless, non-irritating, non-carcinogenic, and non-toxic material, ensuring safe procedures in higher education. Additionally, easy handling and storage of plastinated material offer an added advantage, as it is a durable teaching tool in educational institutions. When plastinating arthropods with chitinous tissue, the DNA in alcohol-fixed groups was better preserved, while no degradation was observed in any of the three methods.

These findings indicate that the larvae preserved using this plastination technique can be utilized for educational purposes as well as in future genetic studies, including forensic medicine, taxonomy, and evolutionary research. Additionally, the limitations of plastination can be overcome by using formaldehyde fixation prior to plastination, which better preserves morphological features. High-quality DNA can still be obtained from these larvae using alternative extraction methods.

AUTHOR CONTRIBUTIONS

Conceptualization, writing—original draft preparation, resources, DB and BB; technical assistance, DB and DNSI; data validation, review and editing, DB, BB, DNSI; supervision, writing—review and editing, DB.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this article..

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KOMPARACIJA MORFOLOGIJE LARVI I PREZERVACIJA DNA KOD PLASTINACIJE UPOTREBOM RAZLIČITIH FIKSATIVA

SAŽETAK

Istraživanje je provedeno sa ciljem određivanja opsega prezervacije strukture larvi parazita tretiranih sa različitim fiksativima u procesu silikonske plastinacije, kao i stepen prezervacije DNA u plastiniranim larvama. U morfometrijskoj evaluaciji su izmjereni dužina (anterior-posterior) i maksimalna širina (najveća širina trupa) larvi kako bi se zabilježile promjene prije i poslije plastinacije. Na sobnoj temperaturi je primijenjen protokol silikonske plastinacije korištenjem tri različita fiksativa. Larve su na kraju deplastinirane. Iz plastiniranih i neplastiniranih larvalnih uzoraka larvi trećeg stadija je ekstrahirana ukupna genomska DNA (gDNA) korištenjem GeneJET kita za purifikaciju genomske DNA. Na 260 nm suizmjerene koncentracije DNA korištenjem UV-Vis spektrofotometra. Primijenjen je PCR protokol, pri čemu su korištenjem UV transiluminatora napravljene fotografije. Larve su nakon plastinacije bile potpuno suhe i bezopasne. Mogle su biti uskladištene duži vremenski period i, što je najvažnije, njihove morfološke karakteristike su ostale pogodne za morfološku analizu od strane studenata. U dehidracijskoj i impregnacijskoj fazi plastinacije nisu uočeni morfološki problemi, međutim, nakon očvršćivanja neki uzorci su pokazali signifikantni strukturni kolaps, koji je imao za posljedicu promjene u nihovoj normalnoj anatomskoj strukturi. Osim toga, koncentracije DNA u deplastiniranim grupama su bile više od onih u plastiniranim. U drugom koraku PCR-a dobivena je traka od ~300 bp za sve plastinirane I neplastinirane grupe. U zaključku, metoda silikonske plastinacije omogućava dugotrajnu prezervaciju morfoloških struktura larvi uz minimalne promjene, uzrokujući djelomično smanjenje integriteta DNA.

Ključne riječi: Anatomija, larve, morfometrija, PCR, plastinacija, prezervacija DNA