Original article

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COMPARATIVE ANALYSIS OF DNA EXTRACTION METHODS FROM FORMALIN-FIXED AND PARAFFIN-EMBEDDED ECHINOCOCCUS CYST TISSUE FOR MOLECULAR IDENTIFICATION

Aidana Tautanova[1]*, Karina Jazina[2], Rabiga Uakhit[2], Ainura Smagulova[2], Marat Dusmagambetov[1]

[1] Department of Microbiology and Virology, Astana Medical University, Astana, Kazakhstan
[2] Laboratory of Biodiversity and Genetic Resources, National Center for Biotechnology, Astana, Kazakhstan

*Corresponding Author: Tautanova A., tautanovaidana@gmail.com.

ABSTRACT

Archived formalin-fixed and paraffin-embedded (FFPE) samples of echinococcal cysts represent a valuable biological resource for molecular epidemiological studies of infections caused by parasites of the genus *Echinococcus*. However, formalin fixation and prolonged storage significantly affect DNA quality, leading to DNA fragmentation and the formation of cross-links between nucleic acids and proteins. The aim of this study was to comparatively evaluate the efficiency of DNA extraction methods from FFPE material of echinococcal cysts and to assess their suitability for molecular identification of the pathogen. The chitinous laminated layers of echinococcal cysts isolated from FFPE samples were selected as the study material. The results demonstrated that the DNA extraction method incorporating xylene-based deparaffinization yielded higher DNA concentrations ranging from 13.1 to 1161.8 ng/µL and superior nucleic acid purity. In contrast, DNA extracted using the Raissol Bio kit showed low concentrations ranging from 0.6 to 10.2 ng/µL and evidence of protein and organic contamination, which potentially inhibited PCR amplification. The application of xylene deparaffinization ensured complete paraffin removal and preservation of DNA integrity, enabling successful molecular identification. For molecular-genetic identification, a marker fragment of the mitochondrial *cox1* gene was used. The obtained results highlight the potential of optimized DNA extraction protocols for FFPE material and expand the possibilities for laboratory diagnostics and retrospective molecular epidemiological investigations of echinococcosis.

Keywords: echinococcosis, DNA extraction, *Echinococcus* cysts, FFPE, paraffin blocks, deparaffinization, molecular identification.

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1. INTRODUCTION

Echinococcosis is classified as a socially significant zoonotic parasitic disease widely distributed in regions with developed livestock farming and persistent natural and synanthropic transmission foci [1, 2]. The disease is characterized by a chronic course, a high proportion of surgical interventions, and substantial economic burden, which sustains ongoing interest in its epidemiology and the molecular characterization of the causative agent [3].

Modern molecular genetics methods represent a key tool for the identification of species and genotypes of *Echinococcus* spp., investigation of transmission pathways, and assessment of epidemiological risks [4-6]. In most studies, mitochondrial genes such as cox1 and nad1, which possess high copy numbers and strong discriminatory power for the analysis of parasitic material, are used as molecular markers [7-9].

At the same time, the availability of fresh or freshly frozen parasitic material remains a significant limitation for conducting comprehensive molecular epidemiological studies, particularly when analyzing retrospective clinical cases [10]. In routine clinical practice, *Echinococcus* cysts, following surgical removal, are typically subjected to standard histopathological processing involving formalin fixation and paraffin embedding, resulting in the formation of formalin-fixed, paraffin-embedded (FFPE) tissue material [11].

Formalin fixation is associated with DNA fragmentation, the formation of cross-links between nucleic acids and proteins, and the accumulation of PCR inhibitors, which significantly complicates subsequent molecular analysis [12-14]. Nevertheless, a number of studies have demonstrated the feasibility of obtaining amplifiable DNA from FFPE material provided that protocols for deparaffinization, lysis, and nucleic acid purification are optimized [15-18].

In recent years, FFPE material has been considered a promising source of genetic information for molecular investigations of parasitic diseases, including echinococcosis, as it allows the inclusion of archival samples accumulated in pathology laboratories into analysis [19-21]. However, comparative studies evaluating the efficiency of different DNA extraction methods specifically from FFPE Echinococcus cyst material remain limited, particularly for Central Asia and the Republic of Kazakhstan [22, 23].

In this context, conducting a comparative analysis of DNA extraction methods from formalin-fixed, paraffin-embedded *Echinococcus* cyst tissue, with assessment of their suitability for subsequent molecular genetics identification of the pathogen and their application in molecular epidemiological studies, represents a relevant research objective.

The aim of the present study is to perform a comparative analysis of different approaches to DNA extraction from FFPE Echinococcus cyst material and to evaluate their methodological significance for the molecular epidemiology of echinococcosis.

2. MATERIAL AND METHODS

2.1. Sample collection and preparation

A total of 10 paraffin section samples provided by the "Olymp" clinical diagnostic laboratory were obtained for the study. The objects of investigation were the chitinous laminated layers of *Echinococcus* cysts isolated from FFPE samples. Paraffin blocks were sectioned into slices with a thickness of 2-5 mm and used for subsequent DNA extraction using different methods.

2.2. DNA extraction with xylene deparaffinization

Deparaffinization of FFPE sections was performed by treatment with xylene (1 mL), followed by incubation at 90°C for 10 minutes and centrifugation at 16,000 rcf for 5 minutes; the procedure was repeated at least twice. To remove residual xylene, sequential rehydration of the pellet was carried out using 96% ethanol in 2-3 cycles followed by drying at 37-40°C until complete evaporation of the alcohol. Tissue lysis was performed in lysis buffer with the addition of 10-15 µL Proteinase K at 56°C for 16-18 hours, with possible extension of incubation time up to 20 hours. To inactivate Proteinase K and partially reverse formalin-induced cross-links, samples were additionally incubated at 95°C for 10 minutes. DNA purification was carried out using phenol-chloroform extraction followed by isopropanol precipitation at -20°C. The DNA pellet was washed with 70% ethanol, dissolved in TE buffer, and used for PCR amplification of short fragments of the mitochondrial genes of *Echinococcus* (*cox1*).

2.3. DNA extraction using the Raissol Bio kit

Pre-homogenized *Echinococcus* cyst samples were supplemented with 150 µL of starting buffer and 100 µL of lysis buffer, followed by incubation at 60°C for 15 minutes with periodic mixing until complete dissolution of paraffin. Samples were then centrifuged at 12,000 rpm for 30 seconds, the upper organic phase was removed, and the samples were further incubated at 60°C for at least 3 hours.

After repeated centrifugation at maximum speed for 3 minutes, the supernatant was transferred to a new tube and treated with RNase (5 µL) at room temperature for 15 minutes. For DNA binding, 600 µL of binding buffer and 60 µL of magnetic particles were added, followed by incubation for 5 minutes at room temperature. Samples were placed on a magnetic rack for 2 minutes, after which the supernatant was removed. The pellet was sequentially washed with wash buffers 1-3 according to the manufacturer's protocol using a magnetic rack. After complete drying of the magnetic particles, DNA was eluted in 50-100 µL of elution buffer.

2.4. DNA quality assessment, PCR analysis and sequencing

DNA concentration and purity were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific) at wavelengths of 260 and 280 nm. An A260/A280 ratio within the range of 1.6-2.2 was considered indicative of acceptable DNA purity.

PCR amplification was performed using the HS-Taq PCR Biomasters (2×) kit (Biolabmix, cat. no. MH010-1020). The primers EgCOI1 (TTTTTTGGCCATCCTGAGGTTTAT) and EgCOI2 (TAACGACATAACATAATGAAAATG), targeting the mitochondrial marker gene cox1, were used for PCR analysis [24]. The PCR program included an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles consisting of denaturation for 30 seconds at 94°C, primer annealing for

Figure 1 – Paraffin blocks containing *Echinococcus* cysts.

30 seconds at 55°C, and extension for 30 seconds at 72°C, with a final extension step of 7 minutes at 72°C. PCR amplification results were used as a functional criterion for evaluating the efficiency of different DNA extraction methods from FFPE Echinococcus cyst material. Successful amplification was considered indicative of the suitability of the extracted DNA for subsequent molecular studies.

Amplification products were visualized after electrophoresis in a 1% agarose gel using a transilluminator. Successfully obtained amplification products were purified using Exonuclease I (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Sequencing was performed using a 96-capillary DNA analyzer 3730xl (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were visually inspected using BioEdit software (version 7.0) and subsequently analyzed using BLAST search against the GenBank database (https://www.ncbi.nlm.nih.gov/).

3. RESULTS

As a result of the conducted work, the DNA extraction method with xylene deparaffinization was optimized. Table 1 presents the data on DNA concentration and purity obtained using two different extraction methods.

Table 1 presents comparative indicators of the concentration and purity of DNA extracted from FFPE Echinococcus cyst samples using two different methods. DNA concentration obtained using the xylene deparaffinization method was relatively high, ranging from 13.1 to 1161.8 ng/µL, with an average A260/A280 purity ratio of 1.66. In contrast, DNA extraction using the Raissol Bio kit demonstrated low concentrations, ranging from 0.6 to 10.2 ng/µL, while the A260/A280 ratio did not exceed 1.26.

Clear amplicon bands of approximately 430 bp, corresponding to the positive control, were observed in samples No. 1, 2, 4, 6, 7, 8 and 10, indicating successful amplification of DNA extracted using the xylene deparaffinization method. Samples No. 3, 5 and 9 did not amplify. These samples were characterized by low DNA concentration and low A260/A280 values, indicating reduced DNA quality and the possible presence of contaminants.

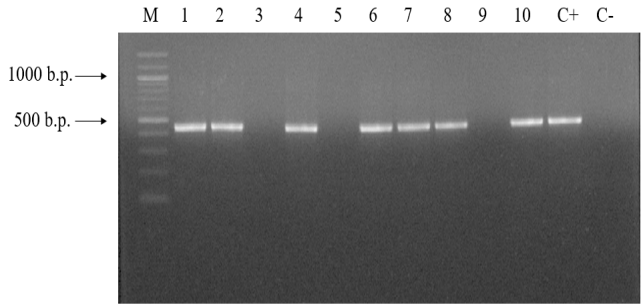

Figure 2 – PCR results of DNA from *Echinococcus* cysts extracted using the xylene deparaffinization method: M - molecular marker; 1-10 - studied samples; C+ - positive control; C- - negative control.

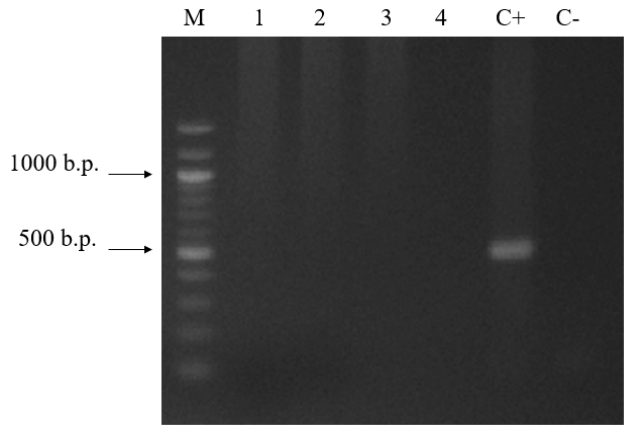

Figure 3 – PCR results of DNA from *Echinococcus* cysts extracted using the Raissol Bio kit: M - molecular marker; 1-4 - studied samples; C+ - positive control; C- - negative control.

On the electrophoregram of PCR products obtained using DNA extracted with the Raissol Bio kit, no amplicon bands were detected in the studied samples (Figure 3). The absence of bands indicates inefficient amplification, likely associated with insufficient DNA quantity and reduced purity of the extracted nucleic acids.

Table 1 – Qualitative and quantitative characteristics of extracted DNA.

No.	DNA Extraction with Xylene Deparaffinization		DNA Extraction Using the Raissol Bio Kit	
	Concentration, ng/µL	A260/A280	Concentration, ng/µL	A260/A280
1	214.1	1.53	0.8	1.21
2	1161.8	1.66	6.5	0.96
3	13.1	1.17	0.6	1.18
4	204.5	1.84	3.4	0.83
5	18.7	1.12	10.2	1.26
6	249.3	1.79	1.3	1.00
7	176.4	1.68	3.8	0.94
8	221.8	1.88	0.9	1.18
9	9.4	0.90	6.4	0.67
10	192.3	1.56	2.7	0.98

4. DISCUSSION

One of the major limitations of molecular epidemiological studies of echinococcosis remains the restricted availability of fresh parasitic material, particularly when analyzing retrospective clinical cases and archival data [1, 10]. The present study was conducted to compare the efficiency of two DNA extraction methods applied to FFPE samples of *Echinococcus* cysts.

The xylene-based deparaffinization method demonstrated higher DNA concentration and purity, which enabled successful PCR amplification and sequencing, whereas the Raissol Bio kit yielded low DNA concentrations and contaminated samples that hindered amplification. The observed differences in method performance are likely associated with variations in paraffin removal efficiency and preservation of DNA structural integrity. The low purity of DNA extracted using the Raissol Bio kit, reflected by A260/A280 ratios below 1.6, suggests the presence of protein or organic contaminants that may inhibit PCR. In contrast, the xylene-based deparaffinization method ensured effective paraffin removal and preservation of DNA quality, thereby explaining the successful generation of amplicons.

The results of the present study demonstrate that FFPE Echinococcus cyst material may be considered not merely an auxiliary but a reliable source of DNA for molecular genetics identification of *Echinococcus* spp., provided that an optimal extraction method is selected. These findings are consistent with previously published studies [18, 20, 21]. In this study, the mitochondrial marker gene cox1 was used for molecular identification of the causative agent of echinococcosis. This gene is widely employed for differentiation of Echinococcus species and genotypes due to its high variability and strong phylogenetic informativeness.

From a practical perspective, the ability to obtain amplifiable DNA from FFPE material opens new opportunities for reconstructing the historical circulation of *Echinococcus* genotypes in endemic regions, correlating molecular data with clinical and morphological characteristics of *Echinococcus* cysts, and conducting multicenter and retrospective studies without the need for repeated biological sampling [6, 9, 19].

This approach is of particular importance for countries of Central Asia, including the Republic of Kazakhstan, where echinococcosis remains a socially significant disease and access to fresh parasitic material is often limited by logistical and ethical constraints [22, 23]. The use of FFPE samples establishes a methodological foundation for integrating pathology archives into modern molecular epidemiological investigations of parasitic diseases, as emphasized in international methodological reviews [15-18].

The main limitation of the present study is the relatively small number of analyzed FFPE samples. In addition, the duration and conditions of fixation and storage were not standardized, which may influence DNA fragmentation, purity and PCR amplification efficiency. Further studies involving larger sample sets and controlled fixation parameters are needed to validate the proposed extraction approach.

Overall, the xylene-based deparaffinization method provided higher DNA concentration and purity from FFPE Echinococcus cyst samples compared with the Raissol Bio kit-based approach, making it preferable for subsequent PCR analysis and molecular identification. The findings of this study create a basis for broader utilization of archival FFPE material in population-genetic and epidemiological studies of *Echinococcus* spp.

AUTHORS' CONTRIBUTIONS

Conceptualization, A.T. and R.U.; methodology, A.T., K.J., R.U. and A.S.; validation, A.T., K.J. and R.U.; formal analysis, A.T. and A.S.; investigation, A.T. and K.J.; resources, A.T. and M.D.; data curation, A.T.; writing–original draft preparation, A.T.; writing–review and editing, R.U., A.S. and K.J.; visualization, A.T. and A.S.; project administration, A.T. and M.D. All authors have read and approved the submitted version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ МЕТОДОВ ВЫДЕЛЕНИЯ ДНК ИЗ ФОРМАЛИН-ФИКСИРОВАННОЙ И ПАРАФИН-ЗАЛИТОЙ ТКАНИ ЭХИНОКОККОВОЙ КИСТЫ ДЛЯ МОЛЕКУЛЯРНОЙ ИДЕНТИФИКАЦИИ

Айдана Таутанова[1]*, Карина Джазина[2], Рабига Уахит[2], Айнура Смагулова[2], Марат Дусмагамбетов[1]

[1] Кафедра микробиологии и вирусологии, Медицинский университет Астана, Астана, Казахстан
[2] Лаборатория биоразнообразия и генетических ресурсов, Национальный центр биотехнологии, Астана, Казахстан

*Корреспондент автор: Таутанова А., tautanovaidana@gmail.com.

АБСТРАКТ

Архивные формалин-фиксированные и парафин-залитые (FFPE) образцы эхинококковых кист представляют ценный биологический ресурс для молекулярно-эпидемиологических исследований инфекций, вызываемых паразитами рода *Echinococcus*. Вместе с тем формалиновая фиксация и длительное хранение существенно влияют на качество ДНК, приводя к ее фрагментации и образованию сшивок между нуклеиновыми кислотами и белками. Целью настоящего исследования было сравнительно оценить эффективность методов выделения ДНК из FFPE-материала эхинококковых кист и определить их пригодность для молекулярной идентификации возбудителя. В качестве материала исследования использовали хитиновые ламинированные оболочки эхинококковых кист, выделенные из FFPE-образцов. Полученные результаты показали, что метод выделения ДНК с депарафинизацией ксилолом обеспечивает более высокую концентрацию ДНК в диапазоне от 13,1 до 1161,8 нг/мкл и лучшую чистоту нуклеиновых кислот. В отличие от этого, ДНК, выделенная с использованием набора Raissol Bio, характеризовалась низкой концентрацией от 0,6 до 10,2 нг/мкл и признаками белкового либо органического загрязнения, что могло ингибировать ПЦР-амплификацию. Использование ксилоловой депарафинизации обеспечило удаление парафина, сохранение качества ДНК и возможность успешной молекулярной идентификации. Для молекулярно-генетической идентификации использовали маркерный фрагмент митохондриального гена cox1. Результаты подтверждают перспективность оптимизированных протоколов выделения ДНК из FFPE-материала для лабораторной диагностики и ретроспективных молекулярно-эпидемиологических исследований эхинококкоза.

Ключевые слова: эхинококкоз, выделение ДНК, кисты *Echinococcus*, FFPE, парафиновые блоки, депарафинизация, молекулярная идентификация

МОЛЕКУЛАЛЫҚ СӘЙКЕСТЕНДІРУ ҮШІН ФОРМАЛИНМЕН БЕКІТІЛГЕН ЖӘНЕ ПАРАФИНГЕ ЕНГІЗІЛГЕН ЭХИНОКОКК КИСТАСЫ ТІНІНЕН ДНҚ БӨЛІП АЛУ ӘДІСТЕРІН САЛЫСТЫРМАЛЫ ТАЛДАУ

Айдана Таутанова[1]*, Карина Джазина[2], Рабига Уахит[2], Айнура Смагулова[2], Марат Дусмагамбетов[1]

[1] Микробиология және вирусология кафедрасы, Астана медицина университеті, Астана, Қазақстан
[2] Биоалуантүрлілік және генетикалық ресурстар зертханасы, Ұлттық биотехнология орталығы, Астана, Қазақстан

*Хат-хабарларға жауапты автор: аутанова А., tautanovaidana@gmail.com.

АБСТРАКТ

Архивтік формалинмен бекітілген және парафинге енгізілген (FFPE) эхинококк кисталарының үлгілері *Echinococcus* туысына жататын паразиттер қоздыратын инфекциялардың молекулалық-эпидемиологиялық зерттеулері үшін құнды биологиялық ресурс болып табылады. Алайда формалинмен бекіту және ұзақ сақтау ДНҚ сапасына айтарлықтай әсер

етіп, оның фрагментациясына және нуклеин қышқылдары мен ақуыздар арасында айқаспалы байланыстардың түзілуіне әкеледі. Бұл зерттеудің мақсаты эхинококк кисталарының FFPE-материалынан ДНҚ бөліп алу әдістерінің тиімділігін салыстырмалы бағалау және олардың қоздырғышты молекулалық сәйкестендіруге жарамдылығын анықтау болды. Зерттеу материалы ретінде FFPE-үлгілерден бөлініп алынған эхинококк кисталарының хитинді ламинацияланған қабаттары пайдаланылды. Нәтижелер ксилолмен депарафинизациялауды қамтитын ДНҚ бөліп алу әдісі ДНҚ-ның жоғары концентрациясын, яғни 13,1-ден 1161,8 нг/мкл-ге дейінгі аралықты, және нуклеин қышқылдарының жақсы тазалығын қамтамасыз ететінін көрсетті. Ал Raissol Bio жинағы арқылы алынған ДНҚ төмен концентрациямен, 0,6-дан 10,2 нг/мкл-ге дейін, және ПТР-амплификацияны тежеуі мүмкін ақуыздық немесе органикалық ластану белгілерімен сипатталды. Ксилолды депарафинизация парафинді тиімді жоюға, ДНҚ сапасын сақтауға және молекулалық сәйкестендіруді сәтті жүргізуге мүмкіндік берді. Молекулалық-генетикалық сәйкестендіру үшін митохондриялық cox1 генінің маркерлік фрагменті қолданылды. Алынған нәтижелер FFPE-материалынан ДНҚ бөліп алудың оңтайландырылған хаттамалары зертханалық диагностика және эхинококкоздың ретроспективті молекулалық-эпидемиологиялық зерттеулері үшін перспективалы екенін көрсетеді.

Түйін сөздер: эхинококкоз, ДНҚ бөліп алу, *Echinococcus* кисталары, FFPE, парафин блоктары, депарафинизация, молекулалық сәйкестендіру