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Original Article

Optimization and Comparative Analysis of Phenol-Chloroform vs Salting-Out DNA Extraction Methods from Human Blood and Saliva Tissues: Assessing Yield, Purity, and Suitability for Downstream Molecular Applications

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Abstract

High quality DNA extraction is fundamental for molecular diagnostics, biomedical research, and forensic sciences. Among biological sources, blood provides high yields, whereas saliva offers a non-invasive alternative. Classical methods such as phenol-chloroform and salting-out remain widely used, but their comparative performance requires further evaluation. This comparative laboratory study analyzed 60 human samples (blood and saliva) using phenol-chloroform and salting-out extraction protocols. DNA yield and purity were assessed with Nanodrop spectrophotometry, and results were compared across sample types and methods. Phenol-chloroform yielded significantly higher DNA concentrations and superior purity than salting-out. From blood, the mean yield was ~308 ng/µL with A260/A280 ~1.89 using phenol-chloroform, compared with ~18 ng/µL (A260/A280 ~1.82) using salting-out. From saliva, phenol-chloroform produced ~64 ng/µL (A260/A280 ~1.87) versus ~38 ng/µL (A260/A280 ~1.75) with salting-out. Blood was the most reliable DNA source, but saliva provided adequate DNA quality for PCR-based and genotyping applications. Phenol-chloroform extraction remains the superior method for obtaining high-yield, high-purity DNA, particularly from blood. However, due to biosafety risks associated with organic solvents, the salting-out method offers a safer and low-cost alternative suitable for routine diagnostics and use in resource-limited settings.

Keywords: DNA Extraction, Phenol–Chloroform, Salting-Out, Blood, Saliva, Purity, Protocol Optimization.

Introduction

DNA serves as the essential hereditary molecule, carrying instructions that regulate growth, cellular function, and transmission of traits across all forms of life. Isolating DNA of sufficient purity and integrity is crucial for most procedures in molecular medicine, biomedical research, and forensic investigations. In health sciences, reliable DNA extraction underpins a wide spectrum of technologies, including polymerase chain reaction (PCR), genotyping, sequencing, mutation detection, genetic screening, biomarker discovery, and personalized medicine (He & Liu, 2017; Ahmed *et al*., 2019).

In clinical diagnostics, purified genomic DNA is required for the detection of hereditary diseases, infectious pathogens, and cancer-associated mutations (Ambardar, Gupta, Trakroo, Lal, & Vakhlu, 2016). For example, PCR-based detection of Mycobacterium tuberculosis, HIV, and HPV relies on DNA of adequate integrity and purity. Similarly, high-throughput sequencing platforms demand highly pure DNA to prevent interference in downstream bioinformatics analyses (Schwarzenbach, Hoon, & Pantel, 2011). In oncology, circulating tumor DNA is emerging as a biomarker for disease monitoring, and the reliability of such testing depends critically on robust DNA extraction protocols (Butler, 2015). Forensic sciences also rely heavily on DNA isolation methods. DNA extracted from blood, saliva, semen, or even degraded biological material forms the basis of human identification, paternity testing, and criminal investigations (Lahiri & Schnabel, 1993). Beyond diagnostics and forensics, DNA extraction supports agricultural biotechnology, vaccine development, and epidemiological research. Thus, developing and optimizing extraction methods that balance yield, purity, safety, cost, and feasibility is of central importance in health sciences. Among biological samples, blood and saliva represent the most common sources of DNA for research and diagnostics.

Blood is traditionally considered the reference source for DNA isolation since it contains plentiful nucleated cells and reliably provides large amounts of intact, high-molecular-weight DNA. (McGuigan & McNally, 2014). DNA derived from blood is widely used in clinical genetics, pharmacogenomics, and cancer research. However, venipuncture requires trained personnel, raises ethical concerns in vulnerable populations, and is less feasible for large-scale epidemiological studies (Quinque, Kittler, Kayser, Stoneking, & Nasidze, 2006). In comparison, saliva has gained attention as a convenient and non-invasive sample source. Collection is simple, painless, and does not require clinical expertise, making it particularly advantageous for paediatric patients, remote populations, or large cohort studies (Abraham, Maranian, Spiteri, Russell, Ingle, Luccarini *et al.*, 2012).

Several studies have shown that saliva provides DNA of sufficient yield and purity for PCR amplification, SNP genotyping, and next-generation sequencing (Prinz, Boll, Baum, & Shaler, 1993). Saliva also has practical value in forensic sciences, since it can be recovered from bite marks, cigarette butts, or crime scenes (Pandey *et al*., 2018). However, saliva samples often contain bacterial contamination, variable cell counts, and inhibitors, which may compromise DNA purity if extraction methods are not optimized Marmur, 1961). Given these complementary advantages, comparative studies of blood- and saliva-derived DNA are critical to guide the selection of sample sources in health sciences research. DNA extraction methods are broadly categorized into organic solvent-based techniques, inorganic precipitation methods, and commercial kits. The phenol-chloroform method, first introduced in the 1950s, is one of the oldest and most widely used organic solvent–based protocols (Aljanabi & Martinez, 1997). It relies on the differential solubility of nucleic acids and proteins in aqueous and organic phases. The method is praised for producing high yields of pure, high-molecular-weight DNA, suitable for demanding downstream applications such as sequencing and cloning (Boom, Sol, Salimans, Jansen, Wertheim-van Dillen, & van der Noordaa, 1990). However, the use of phenol (corrosive) and chloroform (carcinogenic, hepatotoxic) poses serious biosafety hazards.

The method is also labor-intensive, involving multiple centrifugation steps that increase the risk of contamination or sample loss (Miller, Dykes, & Polesky, 1988). Despite these drawbacks, phenol-chloroform extraction remains the benchmark for DNA quality in many molecular biology laboratories. The salting-out method, developed in the late 1980s, provides a safer and more economical alternative (Suguna *et al*., 2014). In this approach, an elevated quantity of salts, i.e. sodium chloride and ammonium acetate are used to aggregate and remove proteins, after which DNA remains soluble and is recovered through alcohol precipitation. Salting-out avoids hazardous solvents and requires

inexpensive reagents, making it particularly attractive for laboratories in resource-limited settings (Ivanova, Dewaard, & Hebert, 2006). However, it often yields lower quantities of DNA, and purity may be compromised if protein precipitation is incomplete. Commercial silica column-based kits and magnetic bead methods are increasingly popular due to their speed, reproducibility, and automation compatibility (Kaneko, Kawana, Fukushima, & Suzutani, 2007). Nevertheless, their high cost and dependence on proprietary reagents limit widespread adoption in low-resource environments. Thus, while newer technologies exist, classical methods such as phenol-chloroform and salting-out remain highly relevant in health sciences due to their cost-effectiveness and adaptability.

Recent Advances and Challenges

Recent years have witnessed significant innovations in DNA extraction techniques. Automated systems, microfluidic devices, and paper-based extraction platforms have been developed to improve throughput, minimize reagent use, and enable point-of-care molecular diagnostics (Kaneko, Kawana, Fukushima, & Suzutani, 2007; Lee *et al*., 2010). For example, paper-based nucleic acid extraction devices have enabled rapid DNA isolation from dried blood spots in under 10 minutes without electricity, demonstrating potential for field diagnostics (Zou, Mason, Wang, Wee, Turni, Blackall *et al*., 2017). Similarly, saliva-based DNA testing has gained attention as a non-invasive alternative for cancer screening, such as early detection of aggressive prostate cancers (Wu *et al*., 2015). Despite these advances, classical methods remain widely used, particularly in low- and middle-income countries where commercial kits are prohibitively expensive. Comparative evaluations of simple, robust protocols for different sample types remain essential to inform laboratory practice in such contexts. The present study aims to perform a comparative evaluation of the salting-out and phenol-chloroform DNA extraction methods using human blood and saliva samples. The focus is on assessing DNA yield and purity using Nanodrop spectrophotometry, with protocol modifications explored to optimize performance. By analyzing both sample types, this study seeks to provide evidence-based recommendations for the selection of DNA extraction protocols in clinical diagnostics, biomedical research, and forensic applications.

Materials and Methods

Study Design and Ethical Approval

This comparative laboratory study was conducted in the Department of Zoology, Government College No. 1, Dera Ismail Khan, during the academic session 2021–2025. The aim was to compare the efficiency of two classical DNA extraction methods—phenol-chloroform and salting-out—using human blood and saliva as sample sources. Ethical approval for the collection of human samples was obtained from the institutional review board, and informed consent was obtained from all participants.

Sample Collection

Sum of 60 fresh samples were obtained from healthy adult volunteers (aged 18–35 years) with no history of chronic illness. To ensure diversity, samples were collected from both male and female participants.

Blood Samples: Approximately 1 mL of circulating blood was obtained via venipuncture in the sterile anticoagulating tubes, i.e. EDTA EDTA-coated tubes to prevent coagulation. Tubes were gently inverted several times to ensure mixing of the anticoagulant. Samples were stored on ice and processed within 4 hours of collection.

Saliva Samples: Unstimulated saliva (1–2 mL) was collected into sterile polypropylene tubes. Participants were asked to refrain from eating, drinking, or performing oral hygiene procedures for at least 30 minutes before collection. Samples were immediately centrifuged to pellet buccal epithelial cells and stored at –20 °C until DNA extraction.

Reagents and Chemicals

All chemicals used were of analytical grade.

Buffers and Reagents for Phenol-Chloroform Method:

Tris(hydroxymethyl)aminomethane hydrochloride (10 millimolar, pH should be 8.0), Ethylenediaminetetraacetic acid (1–2 millimolar), Sodium Chloride (100–150 millimolar), SDS (10%), Proteinase K (20–50 µL, 20 mg/mL), Phenol (equilibrated, pH adjusted at 8.0), Chloroform: Isoamyl alcohol (24:1 v/v), Sodium acetate (3 M), Absolute ethanol and 70% ethanol, Tris-EDTA solution (10 millimolar of Tris-HCl, 1 millimolar of EDTA, pH should be 8.0)

Buffers and Reagents for Salting-Out Method:

Tris(hydroxymethyl)aminomethane hydrochloride (10 millimolar, pH should be 8.0), KCl (50 millimolar), Ethylenediaminetetraacetic acid (1–5 millimolar), Sodium Chloride (6 Molar stock solution), SDS (10%), Proteinase K (10–20 µL, 20 mg/mL), Isopropanol, 70% ethanol, Tris-EDTA solution (10 millimolar of Tris-HCl, 1 millimolar of EDTA, pH should be 8.0)
All solutions were freshly prepared, autoclaved, and stored at 4 °C.

DNA Extraction Protocols

1. Phenol-Chloroform Protocol

From Blood

Genomic DNA was extracted following the protocol described by Sambrook *et al.* (2001) with slight modifications. All buffers and solutions were prepared fresh according to the referenced protocol. 1 mL of whole blood was mixed with 1 mL of sterile distilled water in a 2 mL microcentrifuge tube. Centrifugation of blood samples took place at 4000 revolutions per minute for 10 minutes to lyse erythrocytes; supernatant discarded, and the step was repeated 2–3 times. Pellet was mixed back in 800 microlitre Lysis-Buffer II (Tris(hydroxymethyl)aminomethane hydrochloride, EDTA, Sodium Chloride salt) and supplemented with 100 µL 10% SDS and 25 µL Proteolytic enzyme. Incubated at 56 degrees Celsius for 2 hours to ensure protein digestion. 400 µL phenol was added, vortexed, then spun at 4000 revolutions per minute for 5 minutes. Aqueous layer carefully transferred to a new tube, mixed with 400 microlitres of chloroform: isoamyl alcohol (24:1 v/v) and 50 microlitres of sodium acetate, and spun at 4000 revolutions per minute for 5 minutes; aqueous phase transferred to a fresh tube. DNA precipitated with 3 volumes of cold absolute ethanol, gently inverted, and centrifuged at 3000 rpm for 3 min. Pellet washed with 1 mL 70% ethanol, centrifuged, and air-dried. DNA dissolved in 100 µL sterile TE buffer and stored at –20 °C.

From Saliva

This is the classic organic extraction method, known for high purity and integrity. 1–2 mL saliva centrifuged at 3000 revolutions per minute for 10 minutes, and the upper layer is discarded carefully. Pellet resuspended in 500 µL lysis buffer; 20 µL Proteinase K enzyme added, maintained at 55–60 °C for 1 to 2 hours. Proportionally the same volume of phenol, chloroform, and isoamyl alcohol was added, vortexed for 10 s, and spun at 12000 revolutions per minute for 10 minutes. Above aqueous phase collected, extraction repeated if cloudy. 1/10th capacity of 3 Molar sodium acetate and two volumes of 100% ethyl alcohol were added; incubated –20 degrees Celsius for 30 to 60 minutes. Centrifuged at a speed of 12000 revolutions per minute for 15 minutes; the pellet was washed with 70% of ethyl alcohol and dried in open air. DNA mixed again in 50-100 microlitre of TE buffer solution then stored at –20 °C.

2. Salting-Out Protocol

Procedure For Blood

The extraction of deoxyribonucleic acid from blood samples was performed following the protocol described by Suguna et al. (2014).

300 µL blood was mixed with 900 µL TKM1 buffer (Tris-HCl, KCl, $MgCl_2$) + 50 µL 1× Triton-X and incubated at 37 degrees Celsius for 5 minutes. The whole mixture spun at speed of 8000 revolution per minute for 3 minutes; supernatant discarded (step repeated 2–3 times), pellet resuspended in 300 µL TKM2 buffer + 40 µL 10% SDS and incubated at 37 degrees Celsius for 5 minutes. 100 µL of 6M NaCl was added, vortexed vigorously, and spun at a speed of 8000 revolution per minute for 5 minutes; the upper layer was collected into a new tube carefully. 300 µL cold isopropanol was added to precipitate DNA and centrifuged at 8000 rpm for 10 min. Pellet was treated with a solution of 70% ethanol, centrifuged again then dried. DNA mixed in 50 microlitre of TE buffer solution and stored at –20 °C.

Procedure For Saliva

This is a non-toxic, cost-effective method for extracting DNA from saliva, adapted from Miller et al., 1988.
1–2 mL of saliva centrifuged at 3000 rpm for 10 min; pellet collected carefully. This pellet was resuspended in 500 µL lysis buffer; 30 µL SDS (10%) was added to it. 10 µL Proteinase K; incubated at 56 °C for 1–2 h, mixed in it. 200 µL saturated NaCl added, vortexed 30 s, centrifuged at 12,000 rpm for 10 min. So, supernatant transferred; equal volume of isopropanol added, inverted 5–10 times and centrifuged at 12,000 rpm for 10 min; pellet washed with 500 µL 70% ethanol. DNA air-dried, resuspended in 50–100 µL TE buffer.

DNA Quantification and Purity Assessment

DNA concentration and quality were evaluated with a Nanodrop spectrophotometer (Thermo Scientific) by measuring absorbance at 260 and 280 nm. The following ratios were calculated: A260/A280: Indicator of protein contamination; a ratio close to 1.8 generally indicates pure DNA. A260/A230: Indicator of salt or organic contamination; ratios between 2.0 and 2.2 are typically regarded as optimal. DNA yield was reported in ng/µL. Each sample was measured in triplicate to ensure reproducibility.

Statistical Analysis

Data was analysed using SPSS v25 (IBM Corp.). Mean DNA yield and purity ratios were calculated for each protocol. Comparisons between methods and sample types were performed using one-way ANOVA, with $p < 0.05$ considered statistically significant. Graphs were generated using Microsoft Excel.

Results and Findings

DNA was successfully extracted from all collected blood and saliva samples using both the phenol-chloroform and salting-out protocols. Yield and purity were assessed using Nanodrop spectrophotometry, and values were recorded for concentration (ng/µL), A260/A280 ratio, and A260/A230 ratio. The results are presented in Tables 1–4.

Table 1
DNA Yield and Purity from Blood Using Salting-Out Method & Phenol-Chloroform Method

	Salting-out Method			Phenol-Chloroform Method		
Sample No.	***Concentration (ng/µL)***	***A260/A280***	***A260/A230***	***Concentration (ng/µL)***	***A260/A280***	***A260/A230***
1	19.2	1.84	1.43	310.2	1.89	1.97
2	16.8	1.80	1.39	298.7	1.92	1.95
3	21.4	1.78	1.41	320.5	1.88	1.96
4	17.9	1.76	1.35	305.4	1.91	1.98
5	15.3	1.88	1.44	335.6	1.90	2.00
6	18.7	1.85	1.42	289.7	1.95	1.94

7	14.6	1.92	1.47	275.4	1.78	1.92
8	20.5	1.82	1.40	318.9	1.83	1.96
9	19.9	1.79	1.38	299.5	1.85	1.93
10	16.2	1.86	1.45	322.0	1.99	2.01

Mean ± SD: 18.05 ± 2.12 ng/µL; A260/A280 = 1.82 ± 0.05 (Salting-out Method)
Mean ± SD: 307.6 ± 16.4 ng/µL; A260/A280 = 1.89 ± 0.06 (Phenol-Chloroform Method)

Table 2

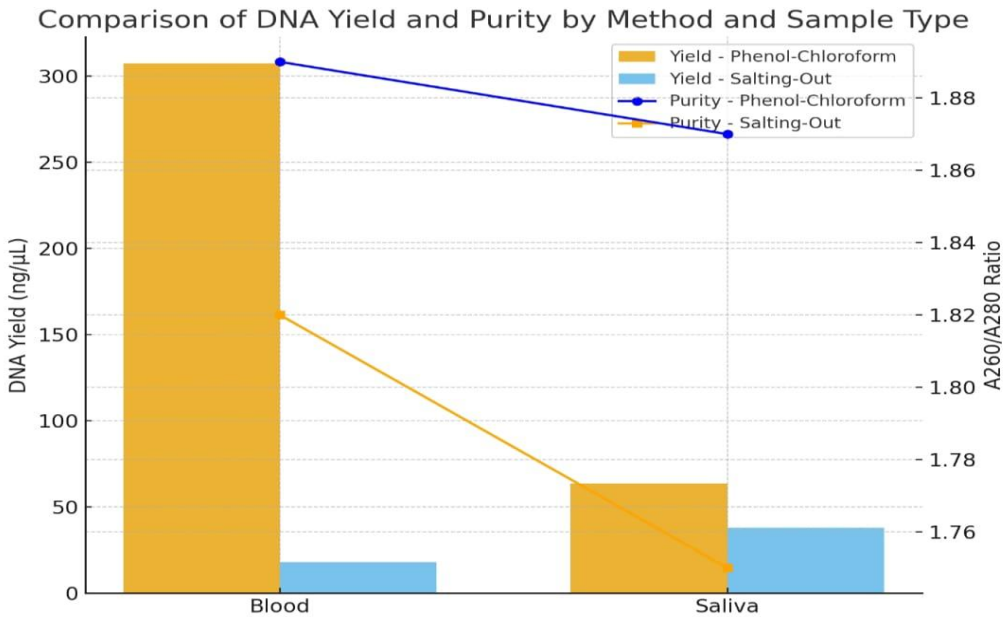
DNA Yield and Purity from Saliva Using Salting-Out Method & Phenol-Chloroform Method

	Salting-Out Method			Phenol-Chloroform Method		
Sample No.	***Concentration (ng/µL)***	***A260/A280***	***A260/A230***	***Concentration (ng/µL)***	***A260/A280***	***A260/A230***
1	34.2	1.71	1.31	66.5	1.91	1.98
2	39.5	1.69	1.28	59.7	1.83	1.96
3	41.7	1.78	1.33	55.2	1.86	1.97
4	30.8	1.76	1.35	68.4	1.90	1.95
5	37.2	1.79	1.36	71.9	1.88	1.96
6	44.2	1.80	1.39	62.8	1.84	1.94
7	33.6	1.74	1.34	57.4	1.89	1.93
8	42.8	1.77	1.32	64.3	1.87	1.95
9	38.4	1.75	1.30	60.5	1.85	1.92
10	36.1	1.72	1.29	69.1	1.92	1.97

Mean ± SD: 37.9 ± 3.8 ng/µL; A260/A280 = 1.75 ± 0.04 (Salting-Out Method)
Mean ± SD: 63.6 ± 5.2 ng/µL; A260/A280 = 1.87 ± 0.03 (Phenol-Chloroform Method)

Graph 1

Comparison of DNA yield and purity by two different protocols from human blood and saliva



Discussion

This study compared two classical DNA extraction methods—phenol-chloroform and salting-out—using human blood and saliva samples. The findings show that phenol-chloroform consistently produced higher DNA yields and superior purity compared with salting-out. From blood, phenol-chloroform yielded an average of ~308 ng/µL, whereas salting-out produced only ~18 ng/µL. Similarly, saliva extractions averaged ~64 ng/µL with phenol-chloroform versus ~38 ng/µL with salting-out. These results agree with earlier reports demonstrating the efficiency of organic solvent–based extraction in maximizing DNA recovery (Koh *et al*., 2019). Blood was confirmed as the most reliable DNA source, yielding significantly higher concentrations than saliva. This is expected since blood contains abundant nucleated leukocytes, whereas saliva provides fewer host cells and often includes bacterial DNA (Abraham, Maranian, Spiteri, Russell, Ingle, Luccarini *et al*., 2012). However, saliva still produced DNA of acceptable purity (A260/A280 ~1.75–1.87), supporting its role as a practical non-invasive sample type. Recent studies confirm saliva's expanding utility in genetic testing, cancer biomarker discovery, and large-scale epidemiological studies (Prinz, Boll, Baum, & Shaler, 1993; Bahlo, Stankovich, Danoy, Hickey, Taylor, Browning, & Rubio, 2010; Bruinsma, Joo, Dowty, Hopper, English, & Makalic, 2018). Its ease of collection also makes it valuable in forensic applications where biological material is scarce or degraded (Pandey *et al*., 2018).

Regarding purity, DNA from phenol–chloroform extractions exhibited A260/A280 ratios near the ideal range of 1.8–2.0, reflecting limited protein contamination. DNA isolated by salting-out showed somewhat reduced ratios, consistent with partial protein carryover. A260/A230 ratios were also higher in phenol-chloroform, reflecting fewer salt and organic contaminants. These differences highlight why phenol-chloroform is still favoured in protocols requiring high-integrity DNA for downstream applications such as sequencing, methylation studies, and whole-genome analysis (Chacon-Cortes & Griffiths, 2014). Nevertheless, the phenol-chloroform method is limited by its reliance on hazardous chemicals, making it less suitable for routine or resource-limited laboratories (Miller, Dykes, & Polesky, 1988). Here, the salting-out method remains important. While it provides lower yields, it is inexpensive, avoids toxic solvents, and produces DNA adequate for many PCR-based diagnostics, genotyping, and routine forensic analyses (Nasiri, Forouzandeh, Rasaee, & Rahbarizadeh, 2005). Studies in low- and middle-income countries support salting-out as a practical alternative where laboratory safety infrastructure is limited (Gudiseva, Danford, Veeraraghavan, & Chavali, (2016).

Conclusion

It was concluded from the study that tetanus toxoid (TT) coverage is low in district Barkhan Balochistan. Only This study demonstrates that the phenol-chloroform method yields significantly higher concentrations and purer DNA than the salting-out method, particularly from blood samples. While blood remains the gold standard source, saliva provided adequate DNA quality for most molecular and forensic applications, highlighting its value as a non-invasive alternative. Despite its efficiency, phenol-chloroform is limited by the hazards of organic solvents, making salting-out a safer, low-cost option for routine diagnostics in resource-limited settings. Ultimately, the choice of protocol should be guided by specific requirements of subsequent applications, available resources, and biosafety considerations.

Recommendations

Based on the findings, it is recommended that future research focus on optimizing DNA extraction protocols to enhance yield and purity while minimizing chemical hazards. The development and evaluation of non-toxic, cost-effective alternatives to phenol-chloroform are encouraged to ensure laboratory safety without compromising DNA quality. Further comparative studies using various biological sources and standardized extraction conditions are necessary to validate these methods for diagnostic and research applications. Additionally, integrating automation and conducting cost–benefit analyses will support the selection of efficient, reproducible, and safe DNA extraction techniques suitable for both high-precision and routine molecular laboratories.

Limitations

This study has a few limitations. DNA quality was assessed only by spectrophotometry; downstream validation with PCR or sequencing would strengthen the conclusions. In addition, commercial kits, though costly, were not included in the comparison. Despite these limitations, the findings provide clear guidance: phenol-chloroform is best for high-quality applications, while salting-out offers a safer and cheaper option for routine diagnostics.

Declarations

Ethical Approval and Consent to Participate: This study strictly adhered to the Declaration of Helsinki and relevant national and institutional ethical guidelines. Informed consent was not required, as secondary data available on websites was obtained for analysis. All procedures performed in this study were by the ethical standards of the Helsinki Declaration.

Consent for Publication: Here, we, the authors, give our consent for publication.

Availability of Data and Materials: Data will be provided upon written request from the corresponding author.

Competing Interest: There is no conflict of interest among the authors.

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Authors' Contribution: All authors have equal contributions. All authors have reviewed and approved the final version of the paper.

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