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Leishmania mini-exon Gene for Molecular Diagnosis and Genotypic of Cutaneous Leishmaniasis

Evan L. Khlef[1], Hawri M. Bakr[2], Bejan A. Dizayee[3], Hadi M. Alsakee[4]

[1,2] *College of Medicine/Hawler Medical University, Erbil, Iraq.*

[3]*Central Veterinary Laboratory in Kurdistan Region, Erbil, Iraq.*

[4] *College of Medicine, Hawler Medical University, International University of Erbil, Erbil, Iraq.*

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ABSTRACT

Background and objective: In Middle Eastern countries, cutaneous leishmaniasis is still a community health concern. This study aimed to identify the Leishmania species in the local area by an accrued method using Polymerase Chain Reaction (PCR) of the mini-exon gene.

Material and methods: Fourteen Gimsa-stained slides were collected for leishmaniasis from the private laboratory. These slides were prepared for patients with clinical manifestations of leishmaniasis. Genomic DNA was extracted using a modified Motazedian protocol. PCR technique was used to amplify all samples using specific primers for the mini-exon gene.

Results: All fourteen samples were positive for leishmaniasis by PCR amplification. Sanger sequencing has been achieved for the positive samples to identify the species. Seven samples out of 14 were identified as *L. infantum*, while the remaining seven samples were identified as L. major. The miniexon gene DNA data for Leishmania species (*L. major* and *L. infantum*) were submitted to the National Center for Biotechnology Information (NCBI). The sequences are given in GenBank accession numbers OP611207 (*L. major*) and OP611208 (*L. infantum*).

Conclusions: Molecular techniques such as PCR and sequencing enhance the accurate diagnosis and management of leishmaniasis.

Keywords: **Cutaneous leishmaniasis; Mini-exon gene; PCR.**

Name: **Evan L. Khlef** **E-mail:** **evan.latef@hmu.edu.krd**

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الجين المصغر في الليشمانيا للتشخيص الجزيئي والنمط الوراثي في الليشمانيات الجلدية

ايفان لطيف خليف[1] ، هاوري مصطفي بكر[2]، بيجان احمد دزيي[3] ، هادي مهدي الساقي[4]

[1،2]كلية الطب، جامعة هولير الطبية، أربيل، العراق

[3]المختبر البيطري المركزي في الإقليم، أربيل، العراق

[4]كلية الطب، جامعة هولير الطبية، جامعة أربيل الدولية، أربيل، العراق

الملخص

الخلفية والهدف: لا يزال داء الليشمانيات الجلدي (CL) مصدر قلق كبير للصحة العامة في دول الشرق الأوسط. كان الهدف من هذه الدراسة هو تحديد أنواع الليشمانيا باستخدام جين.mini-exon

المواد والطرق: تم فحص أربعة عشر شرائح مصبوغة ب Giemsa من المرضى الذين يعانون من عرض سريري من CL لتحديد أنواع الليشمانيا المسببة. تم استخراج الحمض النووي الجيني باستخدام بروتوكول Motazedian المعدل. تم تضخيم علامة تُعرف باسم جين-mini exonبواسطة تفاعل سلسلة البلمرة (PCR) باستخدام الاشعال المحدد LB-3C و L LC-3 لتأكيد الأنواع.

النتائج: اعطى تضخيم PCR نتائج إيجابية لجميع عينات الحمض النووي الـ 14. كشف تسلسل الحمض النووي لجين Mini-exon عن نوعين مختلفين من ليشمانيا، *L. Major* و *L. Infantum*. من بين 14 عينة، تم التعرف على سبعة على أنها *L. infantum* وسبعة تم تحديدها على أنها L. Major . تم تقديم تسلسل الحمض النووي لجين mini-exon من أنواع L. Major و *L. Infantum* إلى المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI) وتم إعطاء التسلسلات أرقام انضمام (*L. Major*) OP611207 و (*L. Infantum*) OP611208 من المركز الوطني لمعلومات التكنولوجيا الحيوية NCBI.

الاستنتاجات: تكشف نتائجنا عن مجموعة متنوعة وراثيا من أنواع الليشمانيا التي تسبب CL في هذا المجال.

INTRODUCTION

Leishmaniasis is a worldwide disease that occurs in eighty-nine countries. Its World Health Organization (WHO) spotlighted tropical disease, with more than six hundred people at risk [1, 2]. Its rate is increasing globally. The reason for increased travel and population migration of infected people into nonendemic regions [3-6]. Leishmaniasis is endemic, particularly in the Mediterranean, Aegean, and Southeast Anatolian regions [7].

The culture method for diagnosing leishmaniasis is considered a standard method but has low sensitivity. The microscopic method requires skilled staff and has no ability to diagnose species. Molecular techniques are preferred because of their high specificity and sensitivity. The Polymerase Chain Reaction (PCR) method gives 100% specificity and 92-98% sensitivity. In addition, *Leishmania* spp. could be detected within 24 hours, and the species and subspecies could identified [8-10]. An entrenched PCR method targeting the mini-exon gene is helpful for directly diagnosing different *Leishmania* species. The mini-exon gene of Kineto plastid protists exists in 100-200 tandemly repeated copies per nuclear genome [11, 12]. Each repeat consists of three major parts: i) a transcribed 39 bp long mini-exon; ii) a moderately conserved intron; and iii) a non-transcribed highly variable spacer [13]. Its variant in size allows it to differentiate between the major Old and New World *Leishmania* complexes [14-16].

In South America, the Mediterranean Basin, and West and Central Asia, leishmania infantum is the causative agent of visceral leishmaniasis. It can also cause cutaneous leishmaniasis, particularly in Middle Eastern countries [17-19].

This study aimed to use PCR of the mini-exon gene to identify the Leishmania species in the local area.

MATERIALS AND METHODS

Sample collection

Fourteen [14] Giemsa-stained slides were collected. The slides prepared for patients show clinical manifestations of cutaneous leishmaniasis. The Patients were referred to a private diagnostic laboratory for further evaluation. The study was conducted between March 2014 and May 2015.

DNA extraction from the samples

DNA was performed from Giemsa-stained slides according to the procedure described by [20], with some modifications; the modification includes neglecting the boiling step, using Qiagen buffers and QIAamp spin column. The smears were scraped off the glass slides. The scraped material was transferred to clean Eppendorf tubes and processed according to Motazedian's protocol. The protocol was modified by neglecting the boiling step and combining it with the QIAamp procedure (QIAGEN, Germany), initially developed to isolate DNA from tissue specimens. Briefly, the scraped material was resuspended in 180 µl of ATL buffer containing 30 µl of proteinase K and incubated at 55°C for one hour. Next, 200 µl of ice-cold ethanol was added. The DNA was prepared using QIAamp spin columns and then well preserved at -80°C until the day of investigation. The concentration and purity of each extracted DNA sample were measured using a Nanodrop spectrophotometer [20]. The mean value of the DNA concentration was one (1) at A_{260}, and the A_{260}/A_{280} ratio was 1.85.

Primer design

DNA sequences of the spliced leader RNA (SL RNA) mini-exon gene of L. major OU755536.1 and L. infantum LR812935.1 were obtained from NCBI. The two sequences were used as a control to design the primers. The location of the SL mini-exon sequence was detected, and the primers were designed for the sequence. Figure (1) shows the loci detection for the primers on both control sequences. The reverse primer is designed to hold the restriction enzyme site Acil.

Polymerase Chain Reaction

Amplification of the mini-exon gene was accomplished using forward (LB-3C 5'-TTT ATT GGT ATG CGA AAC TTC-3') and reverse (LC-3 L 5'-GCC CGC G(C/T) G TCA CCA CCA-3') primers in a thermal cycler (Master Cycler, Eppendorf, Germany). PCR was performed in a 25 µl mixture of 10 µl reaction buffer, 0.2 mM deoxynucleotide triphosphate, 2.5 mM MgCl2, 0.4 mM primers, and 0.3 U of Taq polymerase (Qiagen, Australia). Before adding 200 ng of the DNA template, the samples were preheated at 94°C for 10 min. The PCR consisted of 32 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C. The final extension step was performed at 72°C for 5 min. Ten microliters (10 µL) of PCR products were electrophoresed on a 1% agarose gel containing 4 µL of 10 mg/ml ethidium bromide at 80 volts for 45 min [21-23]. After the gel electrophoresis was finished, the result was documented, and the positive DNA band was sliced out of the gel and preserved in a clean Eppendorf tube for the gel purification step; for that reason, ten microliters (10 µL) of the sample were run.

DNA Sequencing and Bioinformatics analyses of the Mini-exon gene

The PCR products of our mini-exon genes were sequenced by GENEWIZ company (NJ, USA). The sequencing methodology involved the Sanger dideoxy sequencing method. The sequencing process used PCR amplicons, which were purified using the Qiagen GEL purification kit, following the manufacturer's instructions provided with the kit. The purified samples were dehydrated by concentrated centrifuge (Eppendorf). The ready-to-send samples were sent to GENEWIZ company (NJ,

USA). The result of the sequencing is sent back to us via email. By using the MUSCLE program, the result was aligned to the control (MHOM/Israel/83/LT252, accession number X69449) sequence of the mini-exon gene to confirm the results and to detect the transcribed 39 bp sequence and the non-transcript sequence of the mini-exon figure (2). Each confirmed mini-exon sequence was aligned to entire NCBI sequences using BLAST (blastn)/National Center for Biotechnology Information (NCBI) to confirm the *Leishmania* species figure (3).

NCBI submission

The confirmed DNA sequences of the mini-exon genes of *Leishmania* species (*L. major* and *L. infantum*) were submitted to the National Center for Biotechnology Information (NCBI). Further confirmation was achieved by assigning accession numbers OP611207 and OP611208 for *L. major* and *L. infantum*, respectively.

Ethics statement

The Hawler Medical University/College of Medicine Ethics Committee approved the Ethical statement of this study.

RESULTS AND DISCUSSION

Fourteen Giemsa-stained slides, each confirmed positive for cutaneous leishmaniasis, were obtained from patients with clinical symptoms revealing the disease. DNA extraction was performed using a modified Motazedian protocol. Amplification of the mini-exon gene was achieved through PCR using specific LB-3C and LC-3 L DNA primers (Figures (4), (5) and (6)). All the 14 DNA samples yielded positive results. DNA sequencing of the mini-exon gene revealed the presence of two distinct *Leishmania* species, *L. major* and *L. infantum*. Among the 14 samples examined, *L. infantum* was identified in seven (7), while the remaining seven (7) were determined to be *L. major*, as shown in Table (1). The recognized DNA sequences of the mini-exon gene from each *Leishmania* species (*L. major* and *L. infantum*) were submitted to the National Center for Biotechnology Information (NCBI) under accession numbers OP611207 and OP611208 for *L. major* and *L. infantum*, respectively.

Table 1: The number of positive cases of *L. major* and *L. infantum*

Type of sample	Total number of collected samples	Total number of positive samples	*L. major*	*L. infantum*
Giemsa-stained slides	14	14	7	7

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Fig. 1: The primer design for both *L. major* and *L. infantum*.

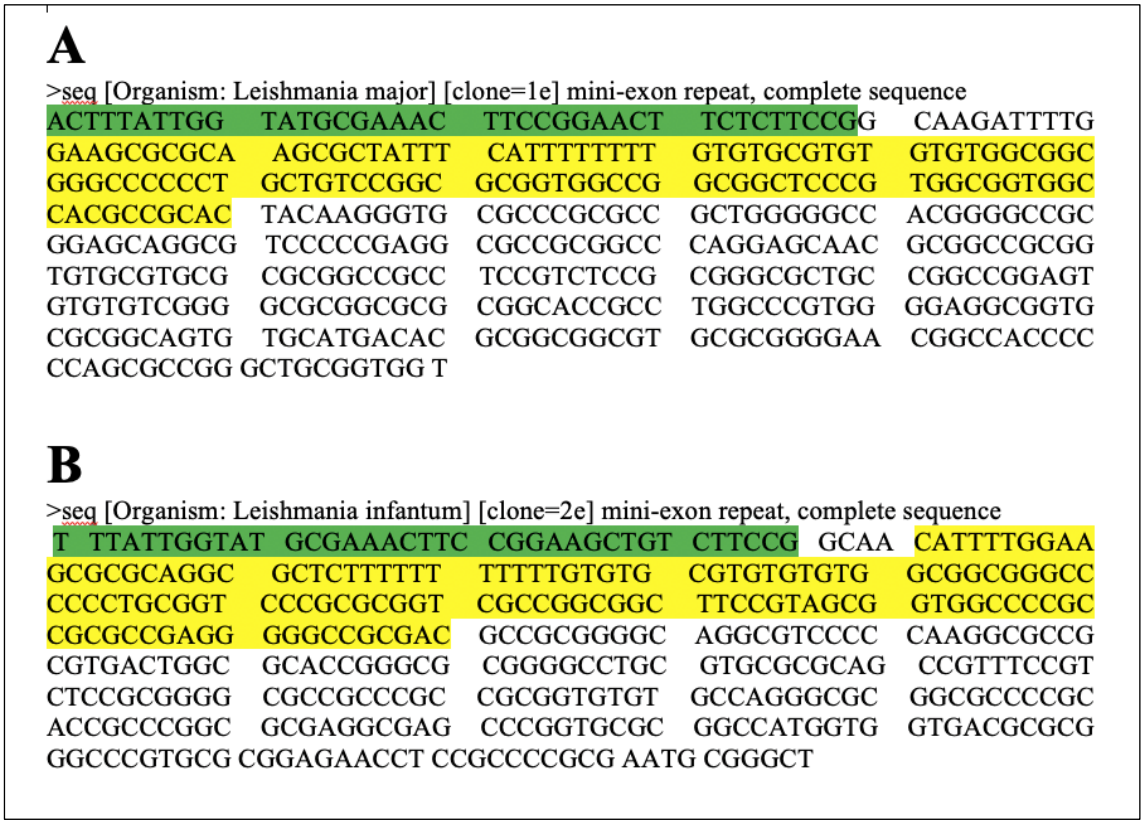

Fig. 2: Result of the sequencing and detecting the transcribed 39 bp sequence (Green) and the non-transcript sequence (Yellow) of the mini-exon in both Leishmania species (*L. major* (A) and *L. infantum* (B)).

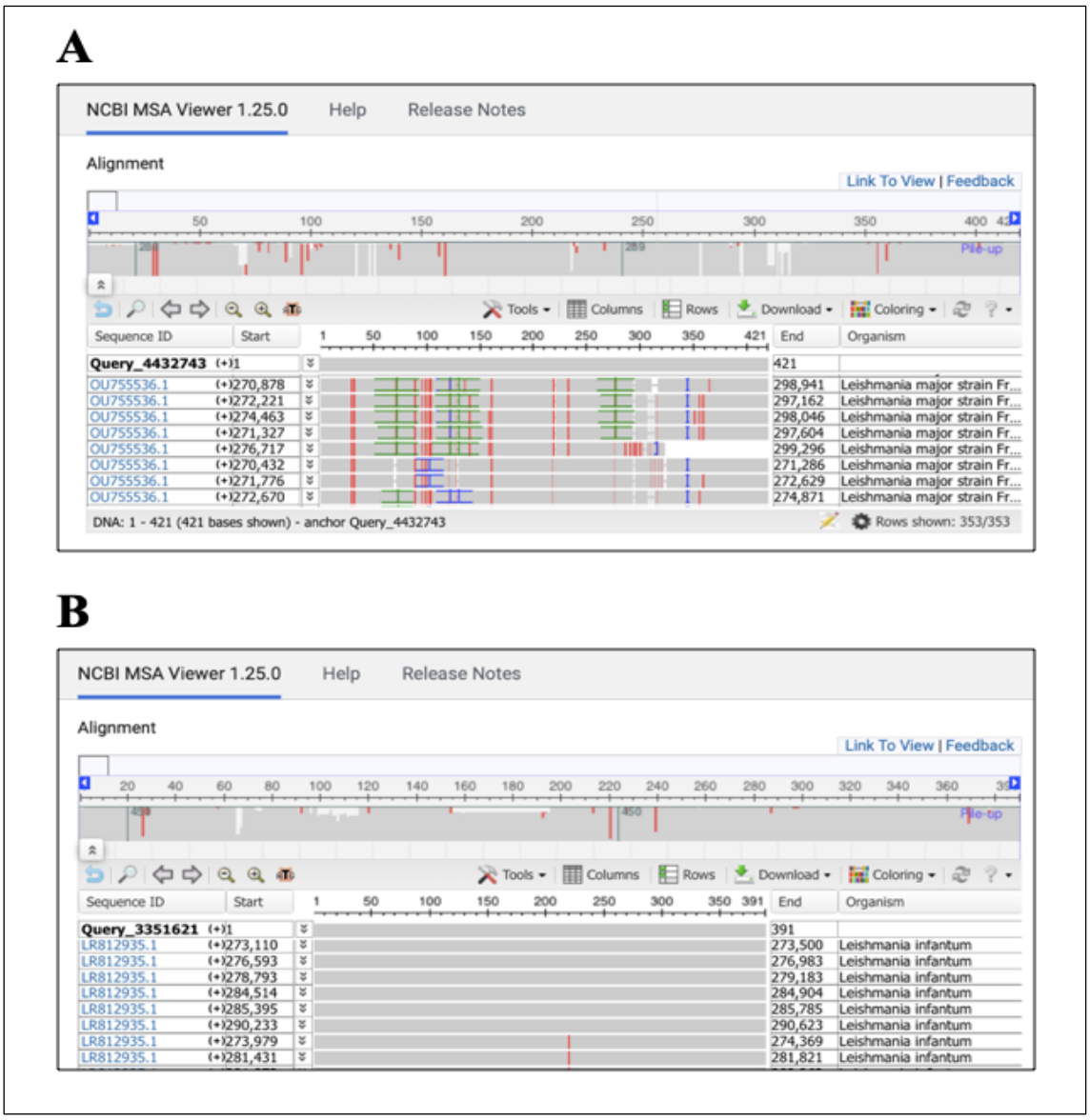

Fig. 3: Alignment to entire NCBI sequences using BLAST (blastn/NCBI) to confirm the *Leishmania* species. (*L. major* (A) and *L. infantum* (B))

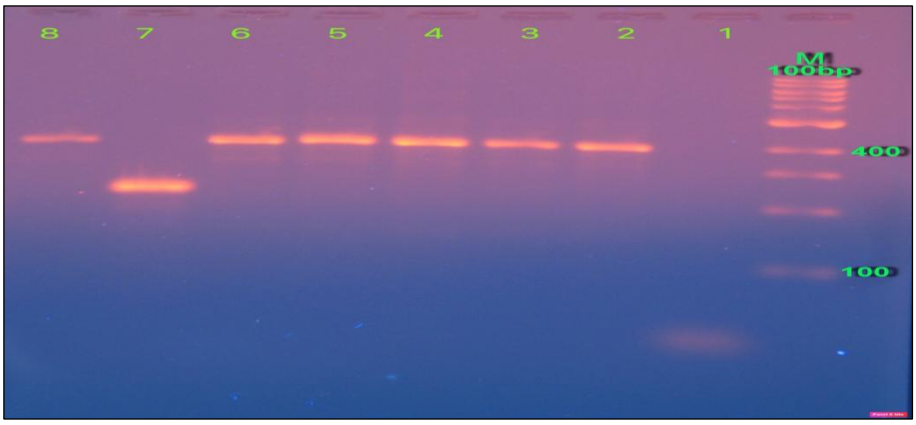

Fig. 4: PCR results showed a positive reaction with 400bp. Line 1 is negative control. Lines 2-8 are positive results for *L. major*. Line 7 is a positive result with a partially amplified band, confirmed by sequencing.

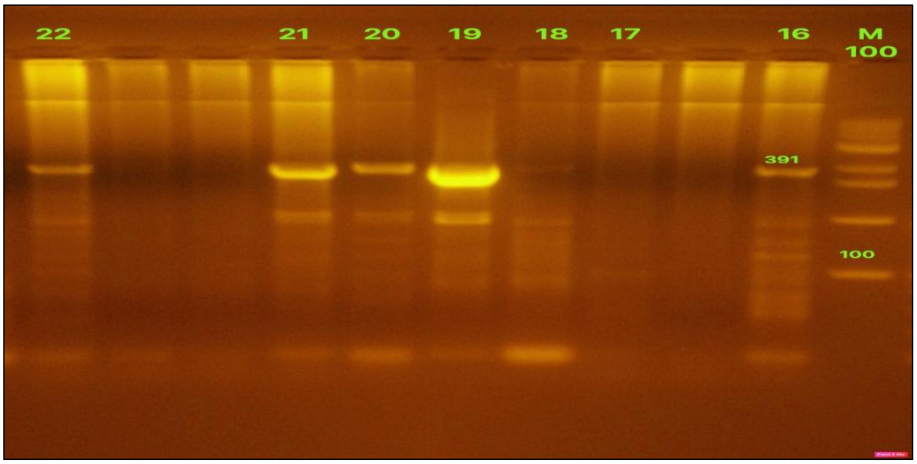

Fig. 5: PCR results showed a positive reaction with 391bp. Lines 16, 19, 20, 21, and 22 are positive for *L. infantum.*

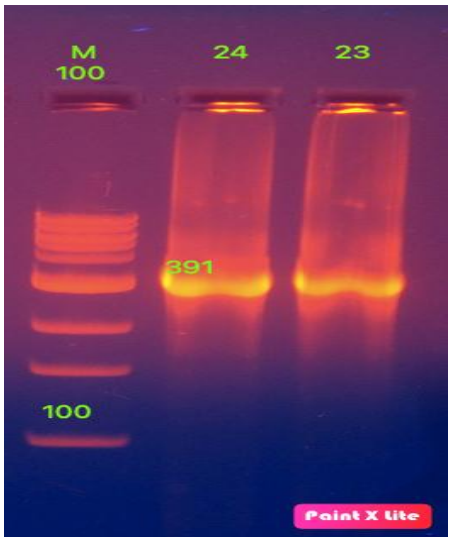

Fig. 6: PCR results showed a positive reaction with 391bp. Lines 23, and 24 are positive for *L. infantum.*

The diagnosis of leishmaniasis is based on the signs and symptoms of the disease, and its appearance is not controllable [12, 24]. Identifying Leishmania species in patients is required to develop good treatment strategies for this parasitic infection [25-27]. The molecular-based method is more accurate than culture methods and smears for disease diagnosis. The accuracy of culture methods ranges from 40% to 75%, while that of microscopic examination methods ranges from 74% to 90% (28, 29). In contrast, mini-exon PCR is the most accurate molecular technique for diagnosing leishmaniasis. The accuracy of mini-exon PCR methods ranges from 53.8-98% [30].

Another study reported that one of the most common genetic markers for detecting *Leishmania* species is the mini-exon gene, which has recently been used to identify *Leishmania* species [31, 32]. Marfurt et al. concluded that the mini-exon PCR test was more accurate than conventional diagnostic methods and could detect infections in many clinical samples, including paraffin-embedded tissue sections [21]. Another study showed that suitable oligonucleotide primers will increase the chance of accurately diagnosing clinical cases [33].

Our study identified two distinct Leishmania species, *L. major* and *L. infantum*, as causative agents of Cutaneous leishmaniasis. Other studies have shown that *L. major* and *L. tropica* cause the most cutaneous leishmaniasis (CL) cases in the Mediterranean Basin [34-36].

In this study, *L. infantum* was one of the causative agents of cutaneous leishmaniasis. During our study, we had an opportunity to meet Syrian refugees. Many studies have shown that after the civil war in Syria, the number of cutaneous leishmaniasis patients increased with the settlement of refugees [37-39].

CONCLUSION

As a result, mini-exon amplification is highly accurate and specific compared to other methods for diagnosing and differentiating *Leishmania* spp. However, the mini-exon RFLP technique is required

to discriminate *L. infantum*. *Leishmania infantum* is the causative agent of visceral and cutaneous leishmaniasis in the region.

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Authors' contributions: E.L.K. conceived the design, conducted the experiments, and wrote the manuscript. H.M.B., B.A.D, and H.M.A provided the experiment requirements (materials, machine, and lab space).

Availability of data and materials: The data are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate: Approved by the Ethics Committee of the Hawler Medical University/ College of Medicine.

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

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