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Original Article

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Genotypes Identification of *Echinococcus granulosus* Isolated from Iranian Dogs and Camels using Three Polymerase Chain Reaction-Based Methods of cox1 Gene

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Abstract

Aims: Hydatidosis is an important zoonotic disease that is caused by a tiny tapeworm, namely *Echinococcus granulosus.* In this study, three polymerase chain reaction (PCR)-based methods, including, high resolution melting (HRM) analysis, DNA sequencing, and PCR-restriction fragment length polymorphism (RFLP) have been used for genotype the identification of *E. granulosus* isolates from dogs and camels in Zarinshahr and Najafabad, Isfahan province, Iran. **Materials and Methods:** A total of 200 adult worms of 40 dogs and 51 samples of camel hydatid cysts were examined. Molecular characterization of isolates was performed using HRM assay, sequencing of DNA, and digestion Rsa1 pattern coding for the mitochondrial cox1 gene. For analysis of the HRM melting curve, we used the T_m within the range of 77.50°C–79.23°C. **Results:** HRM analysis revealed that 72.5%, 15%, and 12.5% dog's genotypes and 41.17%, 21.56%, and 35.29% camel genotypes were G1, G3, and G6, respectively. PCR-RFLP analysis, spare parts 310 bp and 138 bp of cox1 that shows the G1 genotype in all of the isolates. Sequence analysis as well as HRM assay was confirmed genotypes of G1, G3, and G6 in camels and dogs. Based on three methods of the cox1 gene the dominant genotype was G1. **Conclusion:** The PCR-RFLP only identified the G1 genotype, whereas the HRM analysis, as well as DNA sequencing, were detected three genotypes G1, G3, G6, therefore, these two methods have enough accuracy for the determination of genotypes of *E. granulosus*. This information leads to a better understanding of the biological characteristics of *E. granulosus* genotypes in Iran and shows the camel as a source of human hydatidosis.

Keywords: Camel, Cox1 gene, dog, *Echinococcus granulosus,* high resolution melting, polymerase chain reaction-restriction fragment length polymorphism, sequencing

Introduction

Hydatid Cyst disease (HCD) is an important zoonotic helminths disease that causes significant public health and the veterinary problem with a large socioeconomic burden in developing countries.[1] The causative agent of the disease is that the larval stage of a small tapeworm named *Echinococcus granulosus* with a complex of genotypes in numerous geographical areas worldwide.[2,3] It causes the average annual direct economic losses, at least US $1 million in humans and an annual production loss of at least 2 billion US$ in livestock.[4] The 2015 WHO FERG estimated HCD to be the cause of 19300 deaths and 871 000 disability-adjusted life-years (DALYs) globally each year.[5] HCD is endemic in Iran as other Mediterranean basin countries, where various domestic livestock is commonly infected and it makes up 1% of all human surgeries.[6] The prevalence rate of *E. granulosus* infection in stray dogs in several parts of Iran was determined as 23.6% (17.6%–30.1%).[6] Furthermore, the

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prevalence rate of HCD in animal and human cases has been reported as 15.6% (14.2%–17.1%) and 4.2% (3.0%–5.5%), respectively.[7] The most affected regions of Iran are the north-eastern, western, additionally central regions of the country. The average costs due to HCD losses in livestock species in Iran were estimated at US$232.3 million.[8] This initial valuation reveals the requirement for increased monitoring and the world management of HCD. There are wide biological, morphological, and molecular variations within *E. granulosus* isolate.[9] Molecular methods have proven to be valuable techniques in the detection, species, and genotypes of *Echinococcus* in endemic areas.[10,11] Molecular epidemiological studies and phylogenetic assay using the analysis sequences of the mitochondrial genes (cox1, nd1) and nuclear rRNA genes (ITS1) revealed that *E. granulosus* is a set of genotypes including G1 to G10 in different parts of the world.[11-14]

Among these genotypes, *E. granulosus* (G1-3) and *E. canadensis* (G6/7) are the major pathogens for human hydatidosis worldwide. In addition, the G1 genotype (sheep strain) is the predominant strain throughout the world, which formed fertile hydatid cyst in sheep as well as frequent isolate from human cases.[2,14] Based on molecular approaches, the presence of *E. granulosus* genotypes consists of (G1, G2, G3, G5, G6, and G7) are reported from different endemic regions of Iran.[15-24] It is critical to detect the genetic variation of adult *E. granulosus* genotypes in different parts of the world to give useful information about existing cycles in endemic areas, and additionally epidemiology, prevention, future vaccine studies, and sources of human infection. There are several molecular strategies developed to determine the genotype of *E. granulosus* such as those DNA-based methods which offer valuable information on molecular diversity. However, some of those methods are difficult, and not economical or careful adequate. The sequencing of the rDNA and mDNA genes for the determination *E. granulosus* genotypes is the golden standard; however, it's not workable to use extensive molecular epidemiological studies.[25]

The high resolution melting (HRM) analysis is a new powerful diagnostic tool, post-polymerase chain reaction (PCR) analysis method used for identifying genetic variation in nucleic acid sequences. The HRM method is based on PCR melting curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)–binding dyes along with next-generation real-time PCR instrumentation and analysis software.[26] Recently, this approach was applied as a new tool that generated by homologous PCR products of small sequence differences, for fast genotyping detection of parasitic protozoa and helminths as well as investigation the epidemiology of them in the endemic area.[27-32] This cost-effective, easy, and sensitive technique can provide valuable information for mutation screening and other investigative applications such as fast screening a large number of isolates to identify differences in DNA sequences using a single step and closed tube. A T_m-based HRM assay has been developed to discriminate in favor of genotypes within *E. granulosus* G1, G2, and G3.[33,34] In the present study, we aimed to determine *E. granulosus* genotypes in intermediate and definitive hosts from one of the endemic regions of Iran, where scant molecular information is out there. In the present study, we used the implementation of qualitative real-time PCR-HRM method, sequencing analyses, and PCR-RFLP techniques analysis of the mitochondrial cox1 gene for detection of the genotype of *E. granulosus* hydatid isolates collected from camel and dog in center Iran.

Methods

Ethical approval

The Ethics Committee of Kashan University of Medical Sciences approved the study (Paragraph 12 of enactment 97 of the Ethics Committee in Medical Sciences Research-October 9, 2012).

Study animals (Specimen collection)

Forty dead stray dogs that naturally infected in various geographical areas of Isfahan province, Iran were collected and examined for genotyping. Overall 200 adult parasites of *E. granulosus* were isolated from the dogs and fixed in 80% ethanol. Furthermore, a total of 51 Iranian camel (*Camelus dromedarius*) hydatid cysts (8 livers, and 43 lungs), were collected from Zarinshahr and Najafabad industrial abattoirs. Under sterile conditions, separation of protoscoleces (from fertile hydatid cysts) and/or germinal was done from each sample. The individual samples (protoscoleces and adult worm) were extensively washed three times in physiological saline and transferred into separate sterile test tubes, covered with 80% (v/v) ethanol and then kept at − 20°C until molecular examination.[35,36]

DNA extraction

Before DNA extraction, the residual ethanol was eliminated by washing all individual samples three times with sterile phosphate-buffered saline. Genomic DNA extraction from the protoscoleces and adult worm were performed using DNA extraction Tissue kit (Bioneer, Korea) according to the manufacturer's instructions with some modifications. The concentration of DNA obtained was measured by NanoDrop (BioTek™ Epoch™ Microplate Spectrophotometer, USA) and then, the DNA samples were stored at-20ºC until molecular analysis.

Polymerase chain reaction reaction

The specifically designed primer sets were checked by the Basic Local Alignment Search Tool (BLAST) on the NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for regions of similarity to other known sequences. Amplification of cox1 (448-bp) gene fragment was conducted by a pair of specific primers (T_m 58°C): cox1, F: (5'TTTTTTGGGCATCCTGAGGTTTAT 3', R: 5'TAAAGA AAGAACATAATGAAAA TG-3') in a final volume of 20 µl reaction mixtures containing 10 Pmol of each primer. The PCR conditions were as follows: One cycle of primary denaturation (10 min at 95°C), followed by

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40 cycles of denaturation (10 s at 95°C), annealing (26 s at 60°C), extension (25 s at 72°C). All PCR-amplified products were visualized under ultraviolet light on 1.5% agarose gel containing ethidium bromide.

Polymerase chain reaction-restriction fragment length polymorphism assay

For determination of the enzymatic digestive pattern, the PCR products were digested with base cutting restriction enzymes of *Rasa1* for cox1, as suggested by the manufacturer. The digestion restriction enzymes were performed by incubating RFLP products at 37°C during 3 h. The digestion products were separated by electrophoresis through 3.5% (w/v) TBE agarose gel (50–72 mV constant voltage) and stained with ethidium bromide and visualized under UV trans-illumination.

High resolution melting assay

The HRM assay of the cox1 gene was carried out on the Rotor-Gene 6000 real-time PCR detection system (Qiagen-Germany) using the two pairs of primers were applied for analysis.

PCR amplification was performed in a final volume of 20 µl reaction, containing 8 µl master mixes, 6 µl distilled nuclease-free water, 1 µl each from primer, and 5 µl of DNA extracted. The protocol cycling reactions were performed using the following the manufacturer's recommendations the reaction mixture was heated at 95°C for 10 min for denaturation of DNA. Then, 40 cycles of amplification were performed, everyone enclosed 95°C (10s), 60°C (26s), and 72°C (25s), followed by a final extension at 72°C for 5 min. The raising of the melting temperature was carried out from 70°C to 85°C at intervals of 0.2°C per sec with continuous fluorescence monitoring through the ramp. The fluorescence signals were measured after each amplification cycle. Two replications of each sample were analyzed. Each extract was run in duplicate, within the presence of reference materials used for HRM profile analysis as well as the positive and negative controls. All tests were performed in triplicate to confirm the repeatability of the T_m result by estimating the T_m varies.

Sequencing

PCR products of the cox1 gene were extracted from the gel, purified employing a commercial purification kit (Bioneer, South Korea) and were directly sequenced by ABIPRISMTM 3130 Genetic Analyzer automated sequencer (Applied Biosystem, USA). To confirm the detected genotype and HRM finding, 25 products of PCR obtained from camel and dog samples were sequenced for cox1 mitochondrial DNA. The obtained sequences were aligned with existing sequences of known genotypes, justified and edited in consensus positions compared with previously published sequences on the mitochondrial cox1 in NCBI (www.ncbi.nlm.nih.gov) using BLAST genotype demonstration was operated by the Maximum Composite Likelihood method using MEGA5 software.

Results

From 51 hydatid cysts in which, 84.3% (43/51) belonged to the lung and 15.7% (8/51) to the liver as well as 200 specimens of adult worms were tested.

Restriction fragment length polymorphism-polymerase chain reaction assay

A DNA fragment of 448-bp from the cox1 mitochondrial gene was amplified thoroughly isolates and no amplification was seen in the control groups. The digestion PCR product of cox1with *Rasa1* produced two fragments of 138 bp and 310 bp were detected. This enzyme digestion patterns indicating the presence of G1 genotype within the isolates [Figure 1].

Sequence analysis

Sequence analysis showed that the length of the cox1 region was 448 bp. The Blast of sequences indicated the presence of G1, G3, likewise G6 genotypes of *E. granulosus* between the two hosts of the present study.

High-resolution melting analysis

The results represented G1, G3, G6 genotypes in camel and dog isolates. HRM analyses indicated very low T_m variation and this way, the characteristics of a specific species can be determined in this way, the characteristics of a specific species can be determined. The results of PCR-HRM for all of the isolate were clearly confirmed by sequence the amplicons. Blast analysis showed identity values greater than 99% of all samples. We reported, for the first time, the mixed G1/G6 genotype in camel isolates. The findings of the present study exhibited that the PCR-RFLP molecular method only identified the G1 genotype, whereas the HRM molecular method was able to identify three genotypes G1, G3, G6, and in this regard the latter method has more power and accuracy for determination of genotypes of *E. granulosus* within the host and host interfaces [Figures 2 and 3].

This study showed that based on the HRM technique, 41/17% (21/51), 21.56% (11/51) and 35.29% (18/51) of

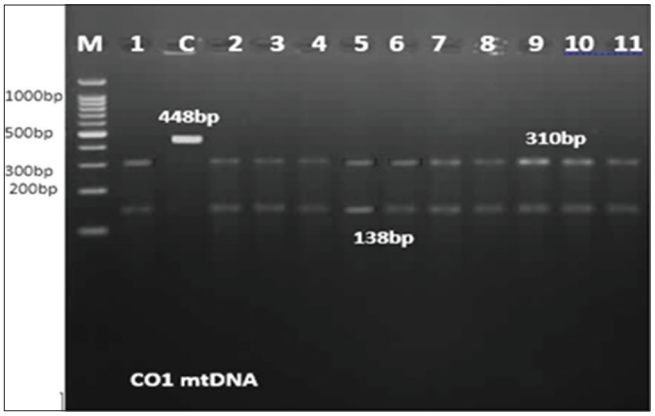

Figure 1: 3.5% agarose gel showing the polymerase chain reaction-restriction fragment length polymorphism patterns of *Echinococcus granulosus*, cox1 gene. M: DNA marker; C: Positive control, and 1–11 digestion samples

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the genotypes of camel isolates were G1, G3, and G6 type, respectively. Whereas, in dogs isolates the frequency of these genotypes was 72.5% (29/40), 15% (6/40), and 12.5% (5/40), respectively. Table 1 shows the results of genotypes by DNA sequencing and HRM analysis.

Discussion

HCD is a crucial parasitic zoonotic disease with important economic and health effects on the communities in endemic countries similar to Iran. Genotype identification of *E. granulosus* in endemic foci is enormously essential for successful control and effective management programs.[2,34,35] The presence of varied genotypes within the endemic regions reinforces the requirement of quick and specific techniques for precise assurance of *Echinococcus* species comparable to those proposed in this work. HRM analysis has been successfully adjusted to differentiating *E. granulosus* species and its genotypes.[37-39] In this regard, some researches on *E. granulosus* genotypes within the intermediate hosts and definitive hosts are performed in some parts of Iran by the HRM assay. These studies suggested the HRM method as a useful tool for routine detection and identification of echinococcosis in endemic regions.[32,37,39]

The HRM analysis has been used as a cost-effective and sensitive screening tool for detection, diagnosis,

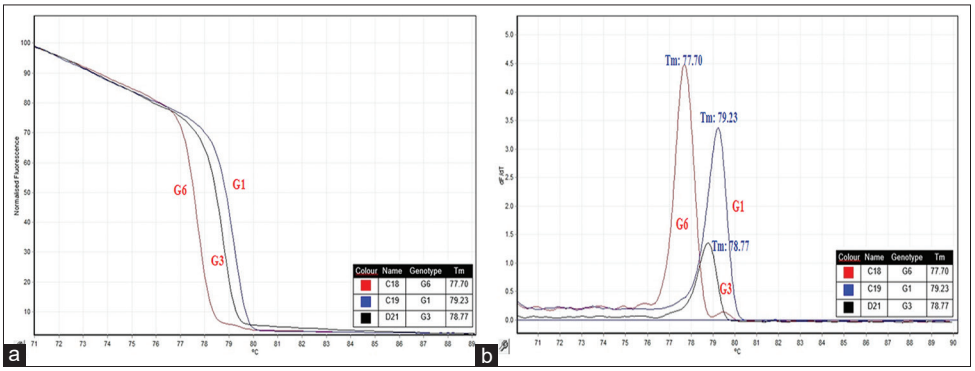

Figure 2: (a) High resolution melting based on (Eva Green) Aligned Melt curve identified *Echinococcus granulosus* genotypes using cox1 gene, and (b) Melting high resolution melting curve of cox1 gen for identification *Echinococcus granulosus*

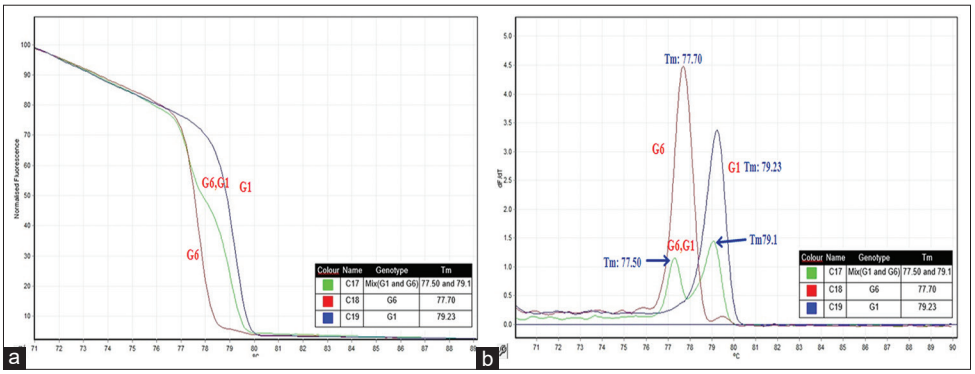

Figure 3: (a) High resolution melting based on (Eva Green) Aligned Melt curve analyses and identified *Echinococcus granulosus* genotypes using cox1 gene and (b) Melting high resolution melting curve of cox1 amplicons for *Echinococcus granulosus*, showing a G1 and G6 genotypes mix in camel isolates of Isfahan region

Table 1: Comparison high resolution melting analysis and sequencing in the identification of *Echinococcus granulosus* genotypes using genetic marker cox 1 in camel and dog isolates

Genotype	Camel isolates				Dog isolates			
	HRM		Sequencing		HRM		Sequencing	
	n (%)	CI	*n* (%)	CI	*n* (%)	CI	*n* (%)	CI
G1	21 (41.17)	41.17±6.89	10 (40)	40±9.8	29 (72.5)	72.5±7.1	18 (72)	72±8.98
G3	11 (21.56)	21.57±5.75	5 (20)	20±8	6 (15)	15±5.64	5 (20)	20±8
G6	18 (35.29)	35.29±6.69	10 (40)	40±9.8	5 (12.5)	12.5±5.22	2 (8)	8±5.42
G1, G6	1 (1.96)	1.96±1.9	-	-	-	-	-	-
Total	51 (100)		25 (100)		40 (100)		25 (100)	

CI: Confidence interval, HRM: High resolution melting

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characterization, and genotyping of a spread of human parasitic infections, chiefly in molecular investigations on protozoa and helminth pathogens including *E. granulosus*.[31,32,37-47] In this study, the three molecular techniques consist of HRM assay, PCR-RFLP, moreover as sequence tools were evaluated. The results exhibited that HRM was an effective and fast procedure that conceded the recognition of *E. granulosus* genotypes in the great number of samples collected from different hosts of numerous regions.

In a similar study, Moghaddas *et al*. studied the molecular characteristics of *E. granulosus* isolates from the camel in eastern Iran, using PCR-RFLP analysis of cox1 and showed more than half of *E. granulosus* isolates belonged to G1 genotype.[49] They show a relatively high prevalence of G6 genotype compared to the present findings as well as Eskandari *et al*. that took place in some central regions of Iran.[39] The PCR-RFLP pattern used Rsa1 digestion enzyme admits speedy detection of the species/genotype *E. granulosus* with low DNA concentration permitting analyzing the specimen with few parasite components accessible. It may be utilized as a rapid screening, followed by a sequence or HRM curve analysis just in negative cases. We recommended that coincident use of alternative and HRM assay is beneficial in the protocol molecular detection of *E. granulosus* genotypes. A comparative outcome has been confirmed by different investigations. The results of many studies obtained by PCR-HRM were affirmed by sequence the amplicons. BLAST analysis demonstrated character values more than 90% of all isolates.[38]

Therefore, in the present work, we have successfully designed the appliance of the HRM assay as an advantageous technique to amplify of a conserved region of the cox1 gene. This mitochondrial gene is a good molecular marker for separation *E. granulosus* species in dogs and its metacestode in camel in the region of cystic echinococcosis. This is crucial to develop a method that will be able to differentiate *E. granulosus* strains and is an important factor of the successful control of the disease. Global, the cox1 gene give sharper phylogenetic information than other mitochondrial gene because of differences in its amino acid sequence appear more slowly than those in some other mitochondrial gene.[46] Conventional melting curve analysis has been used to distinguish between G1 and G2/G3 genotypes using 12S rRNA gene amplification.[48] HRM has been with success performed in amplicons of 38–1000 bp, however, in most investigations fragment sizes from 100 to 300 bp have been used.[50-52] In the present study, we successfully performed in amplicon of 448 bp by means of the HRM technique. The HRM assay is a trustworthy technique giving less contamination, more cost-effective for every sample, simple, and quick to obtain the results using totally different plotted melt curves for sequence variations.[52] However, this technique needs advanced equipment and facilities which may not available in many laboratories.

The application of fast and powerful tools for recognizing diverse genotypes of Echinococcus has been the subject of some researches.[39,53] In distinction, HRM to make possible the use of DNA extracted from any parasite material similarly we appeared in this work. In qualification, HRM investigations empower the work of DNA removed from any parasite material as we appeared on the approval test. Recently, the application of cox1 and nd1 HRM analysis on sheep, goats, cattle, and camels hydatid isolates was described in genotyping *E. granulosus* in Iran to discriminate G6 from the G1 and G3 genotypes, but it did not show sufficient results in characteristic G1 from G3.[37,39] HRM analysis has some benefits, such as ideal for large-scale genotyping projects and it's able to accurately genotype huge variety of samples of a short time, with a high level of accuracy. HRM assay can be used to identify any changes in the gene sequences and is a lot of more powerful than alternative methods that detect only a single allele.[53] This close-tube method permits the detection of any alteration in DNA with simple screening.[54] However, there are some limitations, such as the requirement for a small amplicon that makes the choice of primer less flexible as well as the nature mutation cannot be easily detected.[39] Hence, it is best to use the sequencing method. The length of the DNA amplicon may impact the sensitivity and specificity of later HRM analysis. Amplicon lengths of 100–300 bp are typically suggested for HRM analysis of various sequence variants, as well as single nucleotide polymorphisms, inversions, insertions, and deletions.[26] Recently, many molecular epidemiological studies reported different genotypes of *E. granulosus* such as G1/G2/G3/G4/G5 and G7 in numerous different regions, including Asia, Europe, Africa, and America.[55-58] The present survey targeting the cox1 gene showed that the predominant genotype in the camel and dog isolate was the G1 genotype. This result is similar to several previous studies that conducted in some parts of Iran and different regions of the world.[36,56,59-64] In addition to the G1 genotype, the G3 and G6 genotypes have also been detected in the present study. This outcome is obtained in some previous studies conducted in various geographical of Iran.[59,65,66]

HRM-PCR assay of the cox1 gene provides a potentially powerful screening, rapid, simple, accurate, low-cost, and faster molecular tool than conventional DNA-based assays for the identification genotypes and differentiate the genetic variation of *E. granolosus* in definitive and intermediate hosts. Camel strains (G6) in this area suggest that possibility intermediate host play a secondary role in the conservation of the camel-dog cycle. It is clear that distinguishing molecular variation in the genotype of *E. granulosus* will have an effect on the biology pattern and development. This is increasing our knowledge regarding the essential molecular knowledge required for the diagnosis, taxonomy, and genotypes of parasite, epidemiology, and implementation of strategies for monitoring, therapy and management of echinococcosis additional development of vaccines.

Conclusion

HRM-PCR assay of the cox1 gene provides a potentially powerful screening, rapid, simple, accurate, low-cost, and

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faster molecular tool for identification genotypes of *E. granolosus* in definitive and intermediate hosts. As this study has shown, G1 is the predominant genotype infecting camel and dog in center Iran. This evidence increasing our knowledge regarding the essential molecular knowledge required for the diagnosis, taxonomy, and genotypes of parasite, epidemiology, and implementation of strategies for monitoring, therapy, and management of echinococcosis additional development of vaccines. To fully understand *E. granulosus* transmission, further comparative studies of its genetic structure of different regions should be conducted.

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Conflicts of interest

There are no conflicts of interest.

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