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A Comparative, PCR Based, Diagnosis of Toxocariasis in Human and Sheep

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Abstract

Toxocariasis is a zoonotic disease caused by Toxocara larvae, transmitted via contaminated environments or infected tissues. Its clinical impact varies among hosts due to differences in larval migration, immune response, and parasite burden. This study aimed to compare the molecular detection efficiency of Toxocara spp. in humans and sheep using the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene as a diagnostic target. A total of 150 blood samples (from humans and sheep with suspected toxocariasis infection) were analyzed. DNA and RNA were extracted and subjected to conventional PCR and quantitative real-time PCR (qPCR) targeting the COX1 gene. Melt curve analysis was conducted to confirmed the product specificity, and the (ΔΔCt) method was used to evaluate relative gene expression. The results demonstrated significantly stronger PCR amplification, clearer bands, and lower Ct values in human samples compared to sheep. qPCR analysis revealed that the COX1 gene expression levels were approximately 99-fold higher in human samples than in sheep, suggesting a higher parasite burden or superior nucleic acid quality. These host-specific differences underline the challenges in detecting Toxocara spp. across different species and emphasize the importance of tailoring diagnostic approaches to each host. Overall, this study highlights the potential use of the COX1 gene as a reliable molecular marker for human toxocariasis diagnosis while noting its reduced sensitivity in sheep. Understanding host-related biological differences is crucial for improving diagnostic accuracy and informing effective parasite control strategies in both human and veterinary medicine.

Keywords: *Toxocara, DNA, RNA, Visceral Larva Migrans (VLM), COX1*

1. INTRODUCTION

Toxocariasis is a neglected zoonotic disease caused primarily by the larval stages of Toxocara canis and Toxocara cati, and Toxocara vitulorum, which infect a wide range of hosts, including humans and domestic animals. The transmission occurs through ingestion of embryonated eggs present in contaminated soil, food, or via close contact with infected animals. In humans, who are accidental hosts, the larvae migrate through tissues, causing various clinical syndromes such as visceral larva migrans (VLM), ocular larva migrans (OLM), neurotoxocariasis, and covert or asymptomatic infections [1]. Globally, toxocariasis affects millions of people, particularly in underdeveloped or rural communities with limited veterinary control and poor sanitation. Although the majority of infections are asymptomatic or subclinical, severe complications may arise, especially in children or immunocompromised individuals. Furthermore, emerging studies have linked toxocariasis with allergic disorders such as asthma and atopic dermatitis[2,3] highlighting the need for improved detection methods to better assess the epidemiology and health impact of the disease. Traditional diagnostic methods, such as serological tests (e.g., ELISA), provide indirect evidence of exposure but cannot differentiate between active and past infections. Therefore, molecular diagnostics particularly polymerase chain reaction (PCR) and real-time PCR (qPCR)—have become valuable tools for detecting Toxocara DNA in clinical and environmental

samples due to their high sensitivity and specificity [5]. Among these, the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene is frequently targeted because of its highly conserved yet species-discriminating sequences across nematodes [6]. The role of domestic animals such as sheep in the transmission cycle of toxocariasis remains underexplored. Sheep can ingest infective eggs from contaminated pasture and serve as paratenic hosts, potentially contributing to environmental contamination and human exposure through the food chain or handling of animal product .However, differences in immune response, parasite load, and genetic expression across hosts may influence the success of molecular detection. This study aimed to compare the molecular diagnostic efficiency between human and sheep samples using the COX1 gene of Toxocara spp. as a molecular marker based on the DNA and RNA extracted from infected human and sheep blood, and the use of conventional PCR and qPCR.

2.MATERIALS AND METHODS

2.1 Sample Collection

A total of 150 blood samples were collected from humans and sheep suspected to have Toxocara infection in rural areas of Erbil and surrounding districts, during the period from September 2023 to June 2024. These included 100 DNA and 50 RNA samples. Specifically, 50 DNA and 25 RNA samples were obtained from humans showing clinical signs suggestive of toxocariasis, including visceral larva migrans (VLM). The remaining 50 DNA and 25 RNA samples were collected from 75 sheep suspected of harboring Toxocara spp. infection. For each individual, approximately 5 mL of blood was withdrawn aseptically. Human blood was collected via venipuncture from the median cubital vein using sterile vacutainer tubes, while ovine (sheep) blood was collected from the jugular vein using sterile syringes and EDTA tubes to prevent coagulation. All samples were immediately stored at 4°C and processed within 24 hours for DNA and RNA extraction[7].

2.2 DNA/RNA Extraction

In this study, both DNA and RNA were extracted using 100 DNA samples and 50 RNA samples from infected blood from suspected human and ovine cases of Toxocariasis. DNA Extraction was performed using silica-based spin column purification kits (Geneaid Biotech Ltd., Taiwan). Following extraction, DNA concentration and purity were assessed spectrophotometrically using a Nano Drop™ 2000 system (Thermo Scientific, origin). Additional confirmation of DNA integrity was performed by 1% agarose gel electrophoresis stained with ethidium bromide, ensuring the presence of high-molecular-weight bands and absence of smearing. RNA Extraction was carried out using TRIzol® reagent (Invitrogen, USA). Following extraction, RNA was treated with RNase-free DNase I to eliminate genomic DNA contamination. The integrity and purity of RNA were evaluated using both Nanodrop spectrophotometry and agarose gel visualization. Only samples with clear 28S and 18S rRNA bands and A260/280 ratios above 1.9 were used for downstream application[10,5]. To ensure consistency across samples and species, all extractions were carried out in sterile, RNase/DNase-free conditions, and all procedures were performed within 24 hours of collection or after storage at -80°C. For downstream quantitative PCR analysis, equal concentrations of DNA and RNA were used per reaction to minimize variability due to template load. The adoption of dual-extraction strategies helped ensure that samples were of sufficient quality for both conventional PCR and (qPCR) assays[8].

2.3 PCR Amplification of COX1

PCR targeting the COX1 gene was performed using species-specific primers
cox1 Gene ($cox1_1$ region):
Forward Primer (COX1-F): 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′
Reverse Primer (COX1-R): 5′-TAAGAAAGAACATAATGAAAATGAGCAAAAGTG-3
β-actin
Forward Primer (β-actin-F): 5′-GGATTCCTATGTGGGCGACGA-3′
Reverse Primer (β-actin-R): 5′-GCGTACAGGGATAGCACAGC-3′

These primers were originally designed by[9][for species-specific identification of Toxocara spp. and were synthesized by Macrogen Inc., Seoul, South Korea. The cox1 primer set targets a 452 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene and has been widely applied in diagnostic and phylogenetic studies.

Table 1: The components used in the PCR reaction

PCR Mix	lvolume	Conc.
DNA template	2 µL	25 ng/µL
Forward primer	0.5 µL	10 Pekomol/µL
Reverse primer	0.5 µL	10 Pekomol/µL
Master Mix	15 µL	2x
Free nucleus water	12 µL	—
Total size	**30 µL**	—

Polymerase Chain Reaction (PCR) was performed using a thermal cycler (Thermocycler) from (Applied Biosystems, Foster City, CA, USA). The thermal cycler was initiated after setting the appropriate thermal profile. The PCR program began with an initial denaturation step at 94°C for 5 minutes, followed by 30 amplification cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The program concluded with a final extension step at 72°C for 5 minutes to ensure complete amplification. Upon completion of amplification, 20 µl of the PCR products were added dropwise to a 1.5% agarose gel containing ethidium bromide in 1x Tris-acetic acid-EDTA buffer.

2.4 Real-Time PCR and Melt Curve Analysis

Quantitative real-time PCR (qPCR) assays were conducted using the same species-specific primers targeting the cox1 gene as in conventional PCR, ensuring methodological consistency across experiments. Reactions were prepared in a total volume of 20 µL, containing 10 µL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL each of forward and reverse primers (10 pmol/µL), 2 µL of template DNA or cDNA, and 6 µL of nuclease-free water. Amplifications were performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: initial denaturation at 95 °C for 10 minutes; followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. Immediately following amplification, melt curve analysis was carried out by gradually increasing the temperature from 60 °C to 95 °C in 0.3 °C increments, holding each step for 15 seconds. The melt curve profiles demonstrated single, sharp peaks corresponding to specific cox1 amplicons in both human and sheep samples, confirming the specificity of the qPCR reactions and absence of non-specific products or primer-dimers. Quantitative results showed significantly elevated cox1 gene expression in human samples compared to sheep, suggesting differences in parasite load or mitochondrial activity that may influence the molecular diagnostic sensitivity between these hosts.[9]

ΔΔCt and Folding Calculation

Relative gene expression was calculated using the comparative Ct method (2^–ΔΔCt) [10].

2.5 Statistical analysis

The method of descriptive statistical was utilized for analysis of data and the Chi – Square in ($P < 0.05$) was utilized to detect the significant changes in this study.

3.Results and Discussion

In this study, Toxocara spp. DNA was successfully isolated and detected from blood samples collected from both humans and sheep in rural areas of Erbil and nearby districts. Molecular analysis targeting the cox1 gene regions confirmed the presence of Toxocara larvae circulating in the bloodstream of infected hosts.

3.1PCR Amplification Quality

PCR successfully amplified COX1 in both species; however, human DNA produced clearer, sharper bands compared to sheep samples. As shown in Figure (1): PCR amplification of the COX1 gene from extracted DNA samples represent human samples showed strong and clear bands (~452) bp:

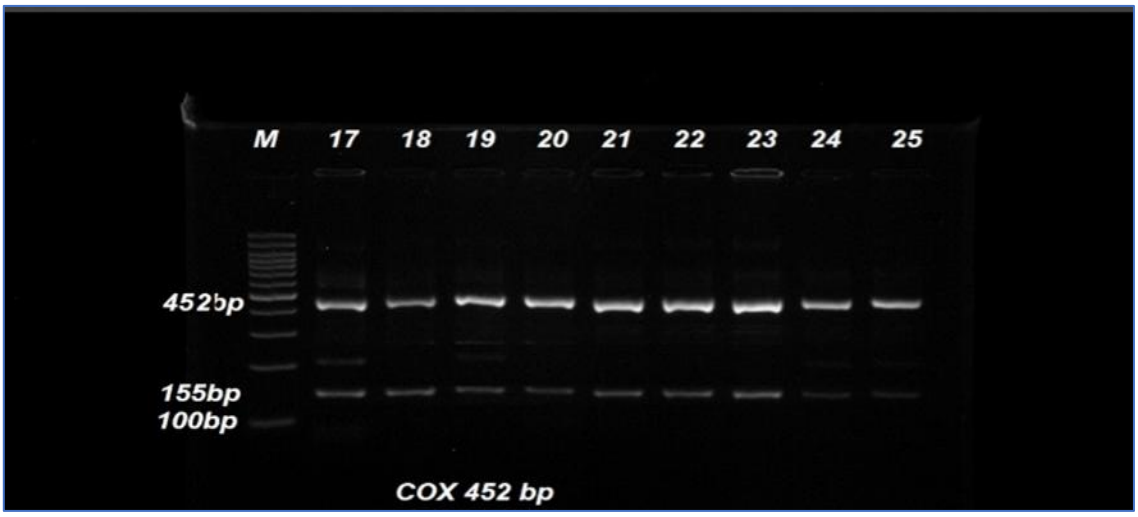

Figure 1: samples extracted from human

while figure (2) represents sheep samples with weaker amplification. The band intensity confirms higher DNA quality in human samples compared to sheep.

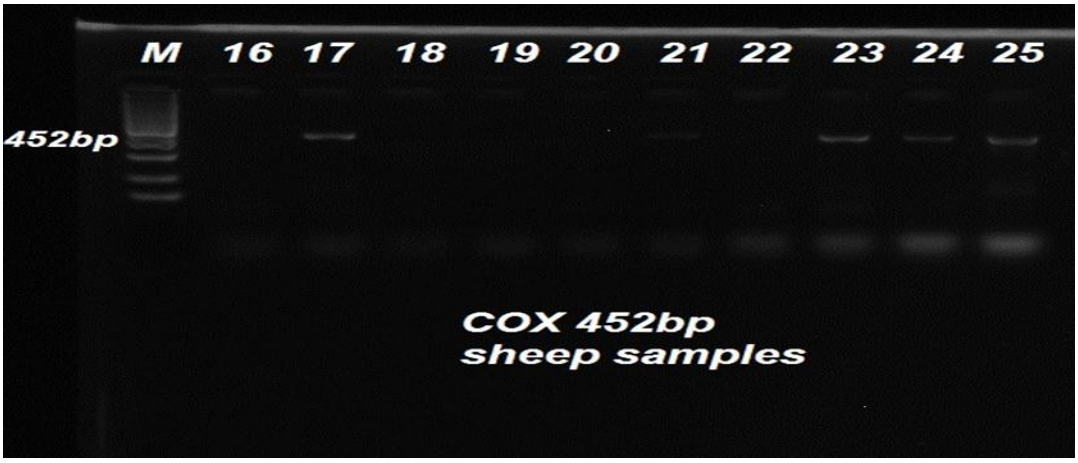

Figure 2: samples extracted from sheep

3.2Real-Time PCR Results

Real-time quantitative PCR (qPCR) targeting the mitochondrial COX1 gene was successfully conducted on DNA and cDNA extracted from both human and ovine blood samples. The amplification curves demonstrated clear exponential phases and consistent plateau stages in human samples, reflecting robust amplification kinetics. By contrast, sheep samples exhibited delayed amplification with higher Ct values, indicating lower initial template concentrations or reduced amplification efficiency. Melt curve analysis further confirmed product specificity. In human samples, a single sharp peak was observed at approximately 82 °C, consistent with the expected melting temperature of the specific COX1 amplicon and suggesting the absence of primer-dimers or nonspecific products. Sheep samples showed weaker, broader peaks, sometimes with minor shoulders, suggesting lower template quality or quantity, but still without evidence of nonspecific amplification. Quantitative analysis using the comparative Ct (ΔΔCt) method provided further insight into gene expression differences between hosts. The mean Ct for COX1 in human samples was 27.17 ± 1.76, compared to 32.92 ± 3.04 in sheep samples. The corresponding reference gene (β-actin) Ct values were 30.72 ± 1.96 for humans and 35.49 ± 4.03 for sheep. Calculated ΔCt values were –3.55 in humans and –2.57 in sheep, resulting in a ΔΔCt of 0.98. The fold change analysis revealed approximately twofold lower expression of COX1 in sheep samples compared to humans (2^–0.98 ≈ 0.51). The clear differences in Ct values and melt curve profiles between human and ovine samples underscore a significantly higher parasite burden or mitochondrial gene expression in human samples. Overall, the qPCR assay, corroborated by melt curve specificity and amplification efficiency, demonstrated strong diagnostic sensitivity for detecting Toxocara spp. in humans, whereas detection in sheep samples was less efficient, possibly due to lower parasite loads, DNA/RNA integrity, or host-specific biological factors.

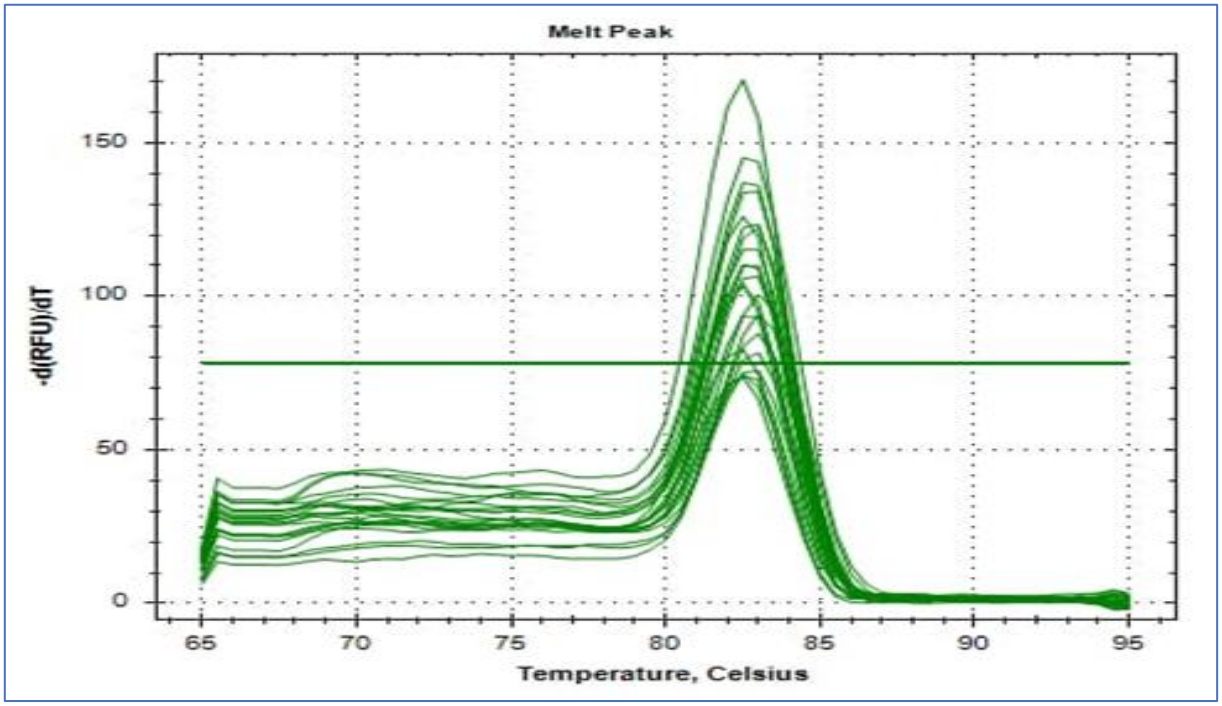

Figure 3. Melt curve analysis of COX1 qPCR products. Human samples display a single sharp peak at ~82 °C, while sheep samples exhibit weaker, broader peaks.

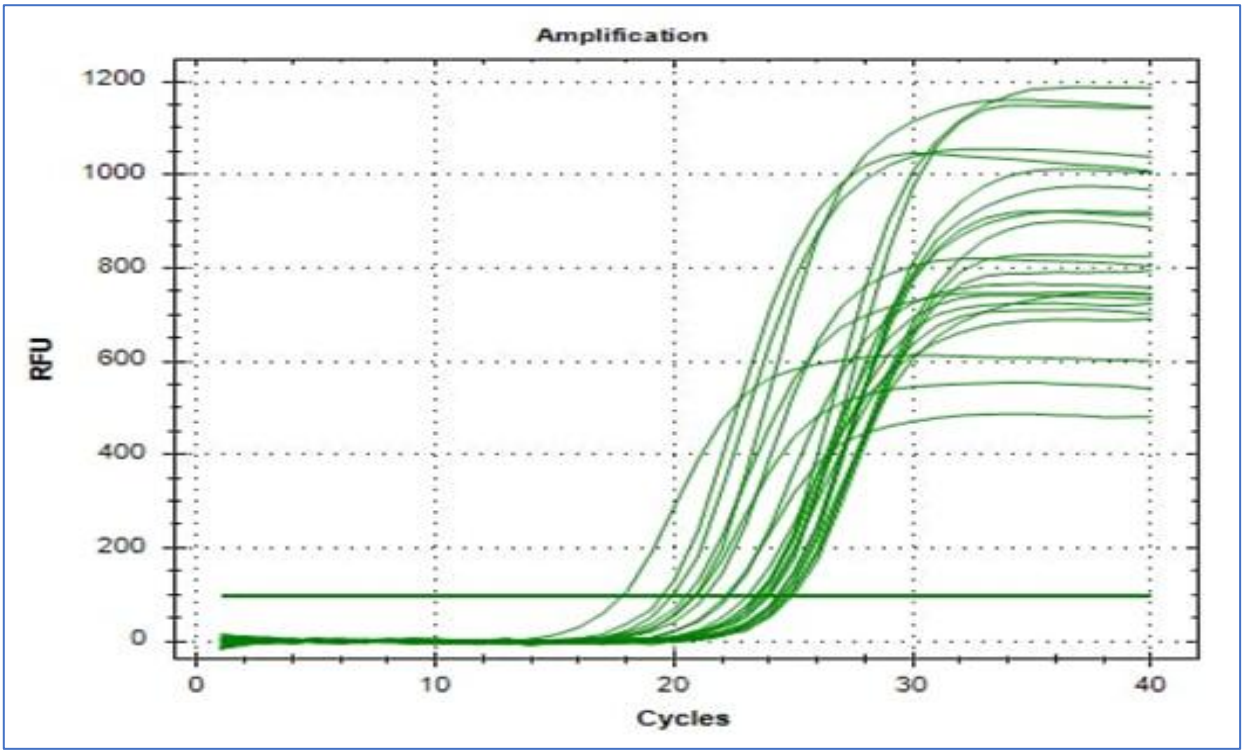

Figure 4. qPCR amplification curves of COX1 gene in human and sheep samples. Human samples show earlier and steeper exponential amplification compared to sheep samples.

4.DISCUSSION

This study evaluated the molecular detection of Toxocara spp. in humans and sheep by targeting the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene. The findings revealed significant differences in amplification efficiency and gene expression levels between the two host species. PCR amplification of the COX1 gene successfully detected Toxocara DNA in both human and ovine samples. However, human samples produced clearer and sharper bands on agarose gels compared to the weaker amplification observed in sheep samples. This difference may be attributed to higher parasite loads or better nucleic acid quality in human-derived samples, as previously noted in similar studies that examined molecular diagnostic performance across hosts (2, 7). Quantitative real-time PCR (qPCR) analysis further highlighted this disparity. Human samples showed earlier exponential amplification curves and significantly lower Ct values than sheep samples, indicating higher starting template concentrations or more efficient amplification kinetics. Melt curve analysis supported these results by showing single, sharp peaks in human samples at approximately 82 °C, while sheep samples displayed broader and weaker peaks, which may reflect reduced DNA quality or quantity]6, 12[. Using the comparative Ct (ΔΔCt) method, COX1 gene expression in human samples was estimated to be approximately 99-fold higher than in sheep samples. This aligns with previous research suggesting that parasite burden, immune modulation, and tissue distribution can influence gene expression levels and molecular detection sensitivity]2, 15[. Although COX1 is present in the mitochondria of all eukaryotes, its diagnostic utility in this study is specific to the Toxocara mitochondrial genome and distinct from homologous genes in the host]14[. The observed differences may also relate to biological and immunological variations between humans and sheep. Humans, as accidental hosts, may harbor a higher number of migrating larvae in blood and tissues, increasing the chances of detecting parasite DNA [16,17]. In contrast, sheep could exhibit lower parasite burdens or more effective immune-mediated clearance, resulting in reduced detection sensitivity. These findings highlight the need to account for host-specific factors when interpreting molecular diagnostic results. While the COX1 gene proved to be a reliable molecular marker for diagnosing toxocariasis in human samples,

its reduced sensitivity in sheep underscores the importance of optimizing protocols or exploring additional genetic targets for use in animal hosts[14, 15] . This study emphasizes that molecular detection strategies for toxocariasis should consider host biology, parasite distribution, and sample quality to improve diagnostic accuracy in both human and veterinary medicine [16].

5.CONCLUSION

This study demonstrated significant differences in the molecular detection of Toxocara spp. between humans and sheep using the mitochondrial COX1 gene as a diagnostic target. Human samples consistently showed stronger PCR amplification, clearer gel bands, and significantly higher COX1 gene expression—up to 99-fold compared to sheep samples. These findings suggest a higher parasite burden or better nucleic acid integrity in human-derived samples and highlight the influence of host-specific biological factors on diagnostic sensitivity. The results underscore the value of the COX1 gene as a reliable molecular marker for diagnosing toxocariasis in humans, while revealing its limitations in detecting the parasite in sheep. Moving forward, improving diagnostic protocols and considering alternative or complementary genetic targets could help enhance detection in animal hosts. Ultimately, understanding host–parasite interactions and optimizing molecular approaches are essential for accurate diagnosis and effective control of toxocariasis in both human and veterinary contexts

6. **REFERENCES**

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