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RESEARCH

Identifying zoonotic risks: molecular insights into *Cryptosporidium* and *Enterocytozoon bieneusi* in pediatric cancer patients in Ahvaz, 2024

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Abstract

Opportunistic infections remain a major cause of morbidity and mortality among patients with immunodeficiency and malignancy. *Cryptosporidium* spp. and microsporidia, particularly *Enterocytozoon bieneusi*, are obligate intracellular parasites that can lead to severe complications and even death in immunocompromised individuals.In this cross-sectional study, 60 stool samples were collected from pediatric oncology patients aged 5–18 years who were hospitalized at Baghaei 2 Hospital in Ahvaz, southwestern Iran, in 2024. Following DNA extraction, molecular analysis was performed to detect the presence of *Cryptosporidium* spp. and *E. bieneusi*. For genotyping, amplification and sequencing of the 60-kDa glycoprotein (gp60) gene for *Cryptosporidium* spp. and the internal transcribed spacer (ITS) region for *E. bieneusi* were conducted. A total of 16 samples (26.6%) tested positive for either *C. hominis* or *E. bieneusi*. Co-infection with both pathogens was identified in two patients (3%). Statistical analysis revealed no significant association between gender and parasitic infection ($P=0.166$), nor between the type of malignancy and presence of protozoa ($P=0.717$). Genotyping of *E. bieneusi* showed the presence of BEB6, CHG3, D, and I genotypes, with BEB6 being the most prevalent. Subtyping of *C. hominis* revealed the presence of the IeA11G3T3 subtype. Overall, the molecular findings demonstrated a prevalence rate 26.6 % of parasitic infection among the studied population, with *E. bieneusi* being predominant. The detection of potentially zoonotic genotypes, particularly BEB6, underscores the likelihood of zoonotic transmission routes in these patients.

Keywords *Cryptosporidium* · *Enterocytozoon bieneusi* · Pediatrics · Cancer · Iran

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Introduction

Immunosuppression resulting from chemotherapy and corticosteroid use predisposes pediatric patients with malignancies to opportunistic infections, which can lead to severe complications and even death. Opportunistic infections are a leading cause of mortality among individuals with immune failure syndromes and malignancies (Abubakar et al. 2007; Thellier and Breton 2008). Cryptosporidiosis and microsporidiosis are two well-documented opportunistic infections frequently reported in immunocompromised patients (Laksemi et al. 2019). *Cryptosporidium* spp. and microsporidia disrupt the function of intestinal epithelial microvilli, resulting in acute, watery diarrhea. While this condition is typically self-limiting in immunocompetent individuals, it can progress to chronic gastroenteritis, persistent diarrhea, and even death in immunodeficient patients (Ye et al. 2014).

Reports indicate that chronic diarrhea associated with *Cryptosporidium* spp. and microsporidia may affect up to 22% of individuals in developed countries and 32% in developing regions (Becnel and Weiss 2014; Certad 2022; Kotler and Orenstein 1998; Ryan et al. 2021; Weber et al. 1994). Molecular epidemiological studies suggest both zoonotic and anthroponotic transmission pathways for *Cryptosporidium spp*. and *Enterocytozoon bieneusi*, with livestock and human-to-human routes playing significant roles (Colito et al. 2024; Mohammad Rahimi et al. 2021; Ryan et al. 2021).

Microsporidia comprise a diverse group of obligate intracellular organisms, among which *E. bieneusi* is the most commonly reported species in humans. It has been frequently isolated from various environmental sources, including water, vegetables, and animal reservoirs (Javanmard et al. 2018; Karimi et al. 2020; Mohammad Rahimi et al. 2021). *Enterocytozoon bieneusi* is recognized as a zoonotic pathogen, and many of its genotypes have been documented as being transmissible from animals to humans (Kotler and Orenstein 1998; Shen et al. 2020; Weber et al. 1994). Sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene has identified over 500 genotypes of *E. bieneusi*, with several classified as highly zoonotic (Nourrisson et al. 2024; Li et al. 2019a; Li and Xiao 2021).

Cryptosporidium spp. are globally distributed protozoan parasites infecting both humans and animals (Mahdavi et al. 2024; Wang et al. 2021). To date, more than 40 species and over 120 genotypes have been characterized, with *C. hominis* and *C. parvum* being the most prevalent species in humans (Ryan et al. 2021). *Cryptosporidium* is recognized as the second most common cause of gastrointestinal symptoms in children—after rotavirus—particularly in developing countries where sanitation infrastructure is lacking (Shrivastava et al. 2017; Wang et al. 2018).

Although numerous studies have examined the prevalence of cryptosporidiosis and microsporidiosis in various immunocompromised populations, data on these infections in pediatric oncology patients remain limited. Children undergoing chemotherapy or hematopoietic stem cell transplantation are particularly vulnerable due to impaired immune responses. This study aims to investigate the prevalence and molecular characterization of *Cryptosporidium spp.* and *E. bieneusi* among immunocompromised pediatric patients in southwestern Iran, a population at high risk for these opportunistic infections.

Materials and methods

Ethics approval and consent to participate

All experimental protocols were conducted in accordance with the ethical principles and national standards for medical research in Iran. The study adhered to relevant ethical guidelines, including the Declaration of Helsinki. Ethical approval for the study was obtained from the Thalassemia and Hemoglobinopathy Research Center, Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1402.707). Verbal informed consent was obtained from all participants or their legal guardians prior to enrollment in the study.

Consent to participate: Not applicable.
Clinical trial number: Not applicable.

Geographical area

Khuzestan province is located in the southwest of Iran, spanning latitudes 29°58′ to 33°04′ N and longitudes 47°41′ to 50°39′ E. The province shares its western border with Iraq and its southern border with the Persian Gulf, covering an area of approximately 63,238 square kilometers. As of the most recent data, Khuzestan has a population of approximately 4.71 million people. The provincial capital, Ahvaz, is the largest city in the region, with an estimated population of 1.2 million residents (Wikipedia, Khuzestan Province).

Sample collection

Stool samples were collected over a two-week period in 2024 from 60 pediatric patients (aged 5–18 years) diagnosed with various types of malignancies and admitted to Baghaei 2 Hospital in Ahvaz, southwestern Iran. Demographic and clinical data were recorded using a researcher-designed questionnaire, including age, sex, type of malignancy, date

of diagnosis, number of chemotherapy sessions, and clinical symptoms. Sample collection was carried out randomly in consultation with an oncologist and following informed consent from the patients or their legal guardians.

All stool samples were immediately preserved in 70% ethanol and stored at − 20 °C until further molecular analysis. The preserved specimens were transported to the Foodborne and Waterborne Diseases Research Center at the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, in Tehran. For transportation, samples were shipped via air in a secure biosafety container with dry ice to maintain the cold chain.

DNA extraction and PCR amplification

Total genomic DNA was extracted from stool samples following a standardized protocol. Briefly, the samples were first washed with sterile phosphate-buffered saline (PBS; pH 7.5), and the supernatant was discarded. DNA extraction was then performed using a commercial kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. The purified DNA was stored at − 20 °C until further use.

The presence of *E. bieneusi* was assessed by conventional PCR targeting a ~ 103-bp fragment of the internal transcribed spacer (ITS) region of the rRNA gene, using primers previously described by Verweij et al. (2007). The primer sequences were: EbITS-89 F (5′-TGTGTAGGCGTG AGAGTGTATCTG-3′) and EbITS-191R (5′-CATCCAACC ATCACGTACCAATC-3′). PCR was performed in a final volume of 15 µL, containing 7.5 µL of 2X red master mix (Ampliqon, Denmark), 3 µL of template DNA (minimum 10 ng), and 5 pmol of each primer. The thermal cycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s, with a final extension step at 72 °C for 10 min.

Cryptosporidium spp. were detected using a nested PCR targeting the 18S rRNA gene, based on the protocol and primers described by Xiao et al. (1999).

For all PCR reactions, distilled water was used as a negative control, and reference DNA served as a positive control. PCR products were resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized using a UV transilluminator.

Genotyping and phylogenetic analysis

To characterize the genotypes of *E. bieneusi*-positive samples, a nested PCR targeting the internal transcribed spacer (ITS) region of the rRNA gene was performed, following the protocol described by Mirjalali et al. (2015). In the primary PCR, primers EbGeno-Fe (5′-TTCAGATGGTCATAG GGATG-3′) and EbGeno-Re (5′-ATTAGAGCATTCCGT GAGG-3′) were used to amplify a 465-bp fragment. In the secondary PCR, primers EbGeno-Fi (5′-TCGGCTCTGAAT ATCTATGG-3′) and EbGeno-Ri (5′-ATTCTTTCGCGC TCGTC-3′) were employed to amplify a 410-bp fragment.

Genotyping of *Cryptosporidium* spp. was conducted by amplifying the 60-kDa glycoprotein (GP60) gene using primers and PCR conditions described by Plutzer and Karanis (2007).

All PCR-positive samples were subjected to sequencing using an ABI 3130 XL Genetic Analyzer (Applied Biosystems). The resulting sequences were submitted to the GenBank database under the accession numbers PP920015–PP920030 for *E. bieneusi*, and PP921519 for *C. hominis*.

Phylogenetic analysis

The obtained sequences were aligned with each other and with reference sequences retrieved from GenBank. Ambiguous regions were manually edited, and sequences were trimmed to uniform lengths. Sequence editing was performed using BioEdit software version 7.2.6. Phylogenetic analysis was conducted to determine the evolutionary relationships of the identified *E. bieneusi* genotypes. A Maximum Likelihood (ML) phylogenetic tree was constructed using the Tamura 3-parameter substitution model, as implemented in MEGA X software (Kumar et al. 2018).

Statistical analysis

Statistical analyses were performed using SPSS software version 23 (SPSS Inc., IBM, Chicago, IL, USA). Pearson's chi-squared (χ^2) test for independence and Fisher's exact two-sided test were applied to assess associations between categorical variables. A P-value of < 0.05 was considered statistically significant.

Results

Demographic data

This study included 60 pediatric patients diagnosed with malignancies, comprising 38 males (63%) and 22 females (37%). The mean age of participants was 9.72 ± 3.84 years (range: 5–18 years). All patients were undergoing chemotherapy at the time of sample collection, with an average of 10 chemotherapy cycles administered per patient.

Among the total, 16 patients (26.6%) tested positive for *E. bieneusi* and/or *C. hominis*. Of these, 4 patients presented with clinical symptoms including abdominal pain, diarrhea, and constipation. Acute lymphoblastic leukemia

(ALL) was the most common malignancy, diagnosed in 35 patients (58%), followed by acute myeloid leukemia (AML) in 9 patients (15%). The remaining 16 patients (26.6%) had other malignancies, including osteosarcoma, neuroblastoma, Ewing sarcoma, sarcoma, gastric cancer, rhabdomyosarcoma, Wilms tumor, brain tumor, abdominal tumor, non-Hodgkin lymphoma, and Hodgkin lymphoma.

Opportunistic protozoan infections

Molecular analysis confirmed the presence of *E. bieneusi* and *C. hominis* in 16 of the 60 patients (26.6%). All 16 positive cases were infected with *E. bieneusi*, while co-infection with *C. hominis* was detected in 2 of these patients (3%).

Statistical analysis revealed no significant association between gender and the presence of parasitic infection ($P = 0.166$). Additionally, there was no significant correlation between type of malignancy and overall parasitic infection ($P = 0.717$). When analyzed separately, *E. bieneusi* ($P = 0.847$) and *C. hominis* ($P = 0.684$) also showed no statistically significant association with specific malignancy types (Table 1).

Genotyping and molecular analysis

Sequence analysis of the ITS region of *E. bieneusi* revealed the presence of four genotypes: BEB6 ($n = 7$), CHG3 ($n = 5$), D ($n = 3$), and I ($n = 1$), with BEB6 being the most frequently detected.

Both *C. hominis*-positive samples underwent gp60 gene amplification for subtyping. Of these, only one sample was successfully amplified and sequenced, identifying the subtype IeA11G3 T3. The second sample failed to yield a gp60 amplicon, which may suggest infection with an alternate *Cryptosporidium* species, such as *C. felis* (Xiao 2010). A summary of genotypes and subtypes identified is presented in Table 2.

Phylogenetic analysis

Phylogenetic analysis was conducted using the obtained ITS sequences for *E. bieneusi* alongside reference sequences representing all 15 established genotype groups. The sequences from this study clustered within their corresponding reference genotype groups, confirming their classification and genetic relatedness (Fig. 1).

All positive cases were reported to the appropriate hospital department and consulting oncologist to ensure timely implementation of appropriate treatment and follow-up care for the affected patients.

Discussion

In the present study, *E. bieneusi* and *C. hominis* were detected in children with malignancies. Numerous studies worldwide have investigated the transmission dynamics of microsporidia and *Cryptosporidium* spp.; however, prevalence rates and dominant species, genotypes or subtypes have varied depending on the study population, sample types, and diagnostic methodologies used. Nevertheless, *E. bieneusi* and *Cryptosporidium* spp. remain the most frequently reported protozoa in immunocompromised individuals (Tavalla et al. 2017).

Table 1 Variables and the prevalence of identified protozoa in each group

Variables		Number (%)	*E. bieneusi*	*C. hominis*
Gender	Male	38 (63%)	9 (56.25%)	0
	Female	22 (37%)	7 (43.75%)	2 (100%)
Malignancy types	Acute lymphocytic leukemia	35 (58%)	9 (56.25%)	2 (100%)
	Acute myeloid leukemia	9 (15%)	2 (12.5%)	0
	Neuroblastoma	3 (5%)	0	0
	Ewing sarcoma	2 (3.3%)	1 (6.25%)	0
	Rhabdomyosarcoma	2 (3.3%)	0	0
	Osteosarcoma	2 (3.3%)	1 (6.25%)	0
	Hodgkin lymphoma	1 (1.6%)	0	0
	Wilms tumor	1 (1.6%)	0	0
	Abdominal cancer	1 (1.6%)	1 (6.26%)	0
	Gastric cancer	1 (1.6%)	0	0
	Sarcoma	1 (1.6%)	1 (6.25%)	0
	Non-Hodgkin lymphoma	1 (1.6%)	1 (6.25%)	0
	Brain tumors	1 (1.6%)	0	0
Symptoms	Yes	4 (6.7%)	4 (25%)	0
	No	56 (93.3%)	12 (75%)	0

Table 2 The presence of studied protozoa and their genotypes in patients

Isolate code	Identified protozoa		Malignancy types
	E. bieneusi	*C. hominis*	
h1	CHG3 (PP920015)	-	Osteosarcoma
h2	BEB6 (PP920016)	-	Acute lymphocytic leukemia
h3	BEB6 (PP920017)	*	Acute lymphocytic leukemia
h4	CHG3 (PP920018)	-	Acute lymphocytic leukemia
h5	BEB6 (PP920019)	-	Acute myeloid leukemia
h6	D (PP920020)	-	Acute lymphocytic leukemia
h7	D (PP920021)	-	Acute lymphocytic leukemia
h8	BEB6 (PP920022)	-	Sarcoma
h9	CHG3 (PP920023)	-	Acute lymphocytic leukemia
h10	BEB6 (PP920024)	-	Acute lymphocytic leukemia
h11	D (PP920025)	-	Non-Hodgkin lymphoma
h12	BEB6 (PP920026)	-	Acute myeloid leukemia
h13	CHG3 (PP920027)	-	Acute lymphocytic leukemia
h14	BEB6 (PP920028)	-	Ewing sarcoma
h15	CHG3 (PP920029)	IeA11G3 T3 (PP921519)	Acute lymphocytic leukemia
h16	I (PP920030)	-	Abdominal tumor

* Amplification of the *gp60* gene for this sample was failed

A case–control study involving 132 children undergoing chemotherapy and 132 healthy controls reported a 3.8% prevalence of *Cryptosporidium* spp. among the cancer patients, using Ziehl–Neelsen staining and PCR. Although prevalence was higher in the cancer group, the difference was not statistically significant (Li et al. 2019b). Similarly, Jiménez-González et al. (2012) detected microsporidia in six of ten pediatric leukemia and lymphoma patients, with *E. bieneusi* being the predominant species.

Opportunistic parasitic infections are often self-limiting in immunocompetent individuals, frequently causing mild or no symptoms. However, in immunocompromised hosts, these infections can lead to severe disease and even mortality. The increasing number of individuals with compromised immunity due to conditions such as cancer or HIV has heightened the clinical importance of these infections. In tropical and subtropical regions, where transmission is more common, ongoing public health interventions aimed at controlling these pathogens remain critical (Bairami 2023).

In a study by Ghoyounchi et al. (2019), microsporidia were identified in 10.6% of pediatric cancer patients, with *E. bieneusi* accounting for 71.4% of microsporidia-positive cases. Another study reported lower prevalence rates of microsporidia (0.5%) and *Cryptosporidium* spp. (2.2%) using staining techniques. The use of chromotrope 2R staining, while established, may underestimate true prevalence due to the inherent limitations of microscopy for microsporidia detection (Hawash et al. 2022). Although chromotrope 2R is a validated method, molecular techniques are generally more sensitive and reliable.

In this study, genotyping of *E. bieneusi* based on ITS sequencing revealed four genotypes: BEB6, CHG3, D, and I—with BEB6 being the most common. Previous studies from Iran have frequently reported genotype D as predominant in human samples (Ghoyounchi et al. 2019; Mirjalali et al. 2015; Tavalla et al. 2017). However, recent investigations in the same region have identified BEB6 and CHG3 as dominant genotypes in domesticated animals (Mohammad Rahimi et al. 2021). These genotypes have also been reported in children from China (Yu et al. 2019; Zhang et al. 2022). Notably, BEB6, CHG3, and I belong to Group 2 genotypes, which are associated with high zoonotic potential (Li et al. 2019a, 2019b). These findings suggest a shift in *E. bieneusi* transmission patterns in southwest Iran, with increasing evidence pointing toward zoonotic transmission from domestic animals.

In addition, *C. hominis* was identified in two children. Subtyping of one sample revealed the subtype IeA11G3T3. This subtype has previously been reported in children from Kuwait, a neighboring country to Iran's Khuzestan province (Sulaiman et al. 2005). It has also been found in children in Peru, where subtypes Ie and Ib were the most common among symptomatic cases (Cama et al. 2008). Moreover, IeA11G3T3 was sporadically reported during outbreak investigations in Scotland between 2012–2013 (Deshpande et al. 2015), and has been identified as the dominant subtype in HIV-infected patients in Thailand (Sannella et al. 2019). Its presence in wastewater in China further highlights its environmental distribution (Feng et al. 2009). Reports of this subtype in both animals and children across multiple countries

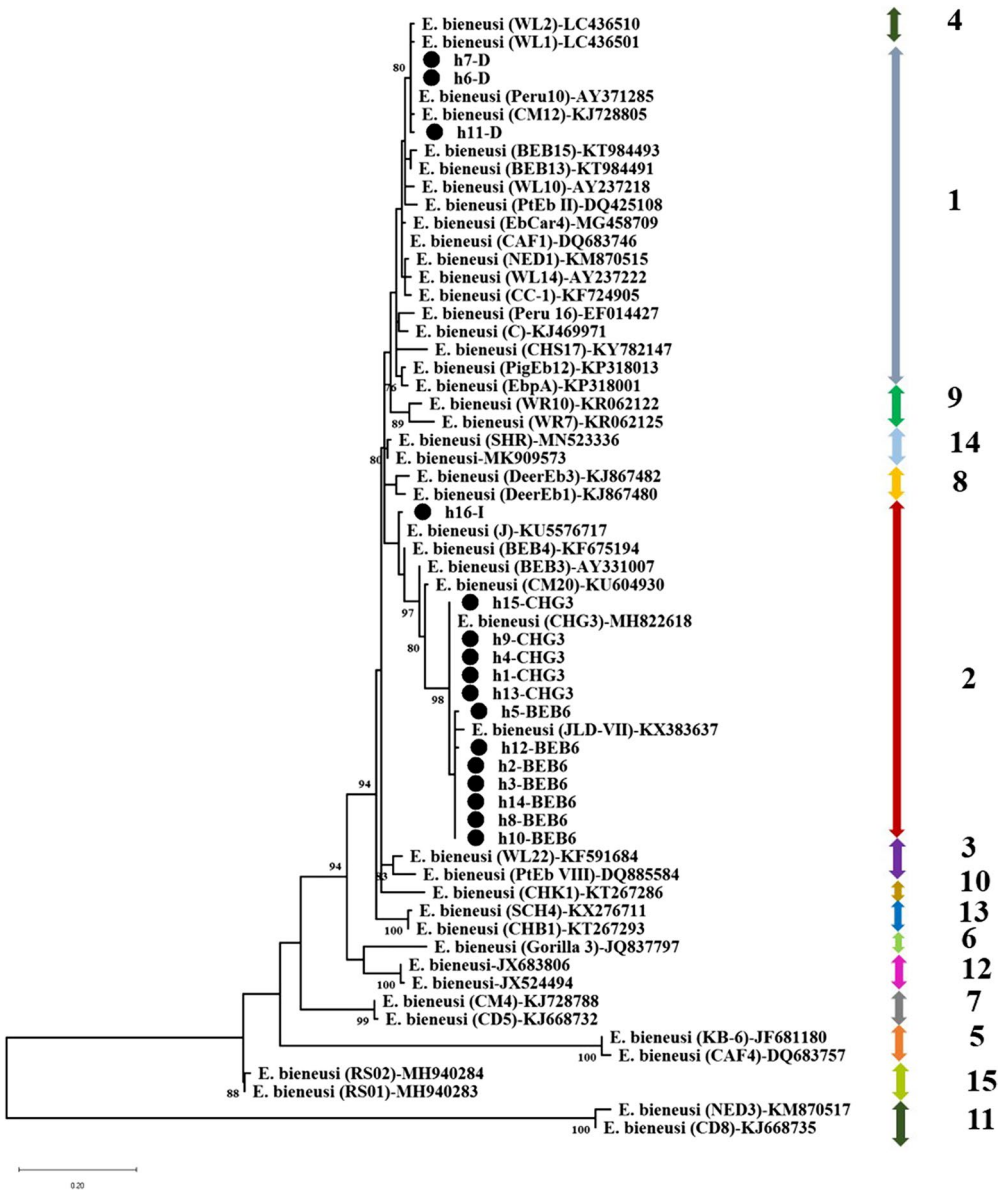

Fig. 1 Maximum Likelihood phylogenetic tree of *E. bieneusi* genotypes based on the ITS gene sequences obtained in the current study. The tree was constructed using the Tamura 3-parameter substitution model. All identified genotypes clustered within groups known to have zoonotic potential. Reference sequences representing all 15 genotype groups were included for comparison

in Sub-Saharan Africa, including evidence of zoonotic transmission in Tanzania (Krumkamp et al. 2021), emphasize the global and zoonotic significance of this lineage.

Conclusion

The molecular characterization of *E. bieneusi* and *Cryptosporidium spp.* has contributed significantly to our understanding of the transmission dynamics of these protozoan pathogens in immunocompromised individuals. Infections caused by these organisms are associated with increased morbidity and mortality, particularly among pediatric patients undergoing cancer treatment. Sequencing data confirms the existence of both anthroponotic and zoonotic transmission cycles, highlighting the importance of accurate and early laboratory diagnosis in clinical management and prevention. The identification of *E. bieneusi* genotypes with high zoonotic potential further underscores the role of animal reservoirs in the spread of these infections and the need for integrated human-animal health surveillance strategies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-025-08500-5.

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Author contributions HM and RSK designed the study. HMP and AH collected samples and data, and HMP, AFK, MAN, and MM completed the questionnaire. HMP and DD extracted DNA, and HMP performed molecular testing. Statistical analyses were performed by SB. HMP, HM, RSK, and AH contributed to the writing of this article. All authors read and approved the final version of the article.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate All experimental procedures were conducted in accordance with the ethical principles and national guidelines for medical research in Iran. The study was carried out following all relevant regulations and declarations, including the Declaration of Helsinki. Ethical approval was granted by the Thalassemia and Hemoglobinopathy Research Center, Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1402.707). Verbal informed consent was obtained from all participants or their legal guardians prior to enrollment in the study.

Consent for publication Not applicable.

Transparency statement The corresponding authors affirm that this manuscript is an honest, accurate, and transparent account of the study being reported. No important aspects of the study have been omitted, and any deviations from the original study plan (if applicable) have been clearly explained.

Competing interests The authors declare no competing interests.

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