

RESEARCH ARTICLE

SEN virus genotype H distribution in β -thalassemic patients and in healthy donors in Iraq: Molecular and physiological study

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Abstract

The SEN virus (SENV) has been linked to transfusion-associated non-A-E hepatitis; however, information regarding SENV infections in patients with thalassemia, particularly in those with hepatitis virus co-infections, remains limited. This study investigated the frequency of SENV (genotypes D and H) infections in Iraqi patients with thalassemic patients infected and not infected with hepatitis C virus. The study involved 150 β -thalassemia patients (75 with HCV infections and 75 without) and 75 healthy blood donors. Patient levels of vitamins C and E, liver function markers, and glutathione peroxidase (GPX) were determined. Recovered viral nucleic acids were amplified using the conventional polymerase chain reaction (SENV DNA) or the real-time polymerase chain reaction (HCV RNA) techniques. Only 10% of healthy donors had evidence of SENV infection. Among patients with thalassemia, 80% and 77% of patients with and without concurrent HCV infections, respectively, had SENV infections. DNA sequencing analyses were performed on blood samples obtained from 29 patients. Patients with thalassemia, particularly those with SENV infections, had higher levels of several enzymatic liver function markers and total serum bilirubin ($P < 0.05$) than did healthy blood donors. Among the examined liver function markers, only gamma-glutamyl transferase demonstrated significantly higher levels in HCV-negative patients infected with SENV-H than in those infected with SENV-D ($P = 0.01$). There were significantly lower vitamin C, vitamin E, and glutathione peroxidase levels in patients than in healthy donors ($P < 0.05$), but only glutathione peroxidase levels were significantly lower in HCV-negative thalassemia patients infected with SENV than in those without SENV infections ($P = 0.04$). The SENV-H genotype sequences were similar to the global standard genes in GenBank. These results broaden our understanding the nature of the SENV-H genotype and the differential role of SENV-H infections, compared to SENV-D infections, in patients with thalassemia, in Iraq.



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Author summary

In patients with β -thalassemia, regular blood transfusions increase patient survival but increase the risk of acquiring blood-borne viral infections, especially viral hepatitis. The SEN virus (SENV) is associated with non-A-E hepatitis but its exact role in the pathogenesis of liver disease remains unknown. This study investigated the frequency of SENV infections among Iraqi patients with thalassemia, with and without hepatitis C infections. The study revealed that the prevalence of SENV infections is significantly higher in patients with β -thalassemia, regardless of their hepatitis C infection status, than in a healthy population of blood donors. The two most common genotypes of the virus (D and H) have generally similar physiological impacts as both increase the levels of markers of hepatic dysfunction in thalassemia patients. However, SENV-H infections were associated with significantly higher levels of gamma-glutamyl transferase in HCV-negative patients with thalassemia, potentially predicting hepatocellular carcinoma development. Although thalassemia patients demonstrated significantly lower levels of the antioxidants vitamins C and E, compared with healthy donors, only the levels of glutathione peroxidase (another antioxidant) were significantly lower in SENV-infected patients than in non-SENV-infected patients. This study aids our understanding of the differential effects of SENV-D and SENV-H infections in β -thalassemia patients.

Introduction

Thalassemia, a common hereditary hemoglobinopathy, is characterized by the abnormally low production of hemoglobin. The low levels of hemoglobin result in anemia, which necessitates treatment with frequent blood transfusions. In Iraq, the prevalence of thalassemia increased slightly between 2010 and 2015, from 33.5/100,000 to 37.1/100,000, whereas the incidence of the disease decreased, from 72.4/100,000 live births to 34.6/100,000 live births, during the same period [1]. As a result of the regular transfusions required by individuals with thalassemia, many of these individuals acquire blood-borne infections. In Iraq, the same study that investigated the incidence and prevalence of thalassemia determined that patients were often infected with hepatitis C virus (HCV, 13.5%) or hepatitis B virus (0.4%) at some point during their lives [1].

Viral hepatitis is a significant cause of morbidity and mortality, worldwide, particularly in the tropics. It is caused by at least five distinct viruses, each with unique molecular characteristics and replication cycles but sharing a common tropism for the liver and causing overlapping clinical patterns of disease [2]. The hepatitis viruses are the most common chronic blood-borne viruses associated with this disease, but other infectious agents have been suggested to cause viral hepatitis that are not directly attributed to the hepatitis viruses (non-A–E hepatitis). The latest virus suggested to have a role in non-A–E hepatitis is the SEN virus (SENV). This virus, discovered in 1999 in the blood of a human immunodeficiency virus-infected patient, is a 26-nm, single-stranded DNA virus that is distantly related to the TT virus [3]. Phylogenetic analyses identified eight different SENV strains belonging to the *Circoviridae* family, a group of small DNA viruses that includes the TT virus [4]. Of the 9 SENV genotypes identified, to date, two (SENV-D and SENV-H) have been extensively studied and are present in approximately 2% of blood donors in the USA, 2% of donors in Italy, and 10% of donors in Japan; they appear to be readily transmitted by blood transfusions and other common parenteral routes [1].

SENV infections, particularly those caused by the D and H genotypes, are frequently associated with non-A–E hepatitis, giving rise to the suggestion that the virus may be the causative agent. However, there is no firm evidence of the virus causing hepatitis or worsening existing disease [5,6]. Co-infections involving SENV and the hepatitis C virus (HCV) or hepatitis B virus (HBV) are also common because the viruses share similar transmission routes (e.g., blood transfusions) [7]. Our previous study [8], demonstrated the presence of SENV viremia in Iraqi patients with β -thalassemia who were and were not infected with HCV. Further, the study also demonstrated that both the D and H SENV genotypes were present in Iraqi patients and noted the impact of the viral infection on liver function indicators in infected individuals. As the previous study was relatively limited, this study investigate the potential for differential effects caused by the SENV D and H genotypes on patients with β -thalassemia.

Materials and methods

A total of 150 patients with β -thalassemia were referred to the Hereditary Blood Disease Center (Baghdad, Iraq) and included in this retrospective study. These individuals were divided into two groups, according to their HCV infection status; equal numbers ($n = 75$) were HCV-positive and HCV-negative. Another 75 individuals were randomly recruited into the study among the healthy blood donors attending the Iraqi National Center of Blood Transfusion. The healthy donors were afebrile, not jaundiced, did not demonstrate any signs of chronic liver disease; further, none had any known contact with individuals with jaundice. The ages of included participants ranged from 5 to 44 (mean, 18 ± 7.8) years and all received regular blood transfusions or donated blood between January and May 2018. All procedures performed in this study involving human participants were in accordance with the University of Anbar Ethical Approval Committee (authentication no. 31, December 6, 2017).

Serology

Liver function markers. A blood chemistry analyzer (Celercare M1, MNCHIP, Tianjin, China) and lyophilized liver function panel kits (MNCHIP) were used to determine levels of alanine transaminase (ALT), aspartate aminotransaminase (ASP), alkaline phosphatase (ALP), total serum bilirubin (TSB), and gamma-glutamyl transferase (GGT).

Detection of anti-HCV-antibodies. All serum samples were examined for the presence of anti-HCV antibodies using enzyme-linked immunosorbent assay (ELISA) kits (Human Gesellschaft fur Biochemica und Diagnostica, Wiesbaden, Germany).

Vitamins C and E and glutathione peroxidase (GPX) levels. ELISA-based double antibody sandwich kits (Shanghai Yehua Biological Technology, Shanghai, China) were used to assay levels of human vitamin C (catalog no. YHB3202Hu), vitamin E (catalog no. YHB3208Hu), and GPX (catalog no. YHB1369Hu) in patient serum samples.

Molecular study

Nucleic acid extraction. Viral nucleic acid extraction kits (SaMag Viral Nucleic Acids Extraction Kit, Sacace Biotechnologies, Como, Italy) were used to extract SENV DNA and HCV RNA from participant plasma specimens. Briefly, the extraction process involved lysing, binding, washing, and eluting the nucleic acids. Frozen plasma samples were thawed at room temperature ($15\text{--}25^\circ\text{C}$) and processed in an automated instrument (SaMag-12, Sacace Biotechnologies), after equilibrating to room temperature [8].

Nucleic acid concentration and purity. A quantum fluorometer (Promega, Madison, WI, USA) was utilized to measure the concentration of extracted nucleic acids; preprogrammed settings were available for quantitating DNA (QuantiFluor ds DNA, Promega) and

RNA (QuantiFluor RNA, Promega). A volume (100 μ L) of diluted nucleic acid sample (1 μ L of nucleic acid + 99 μ L buffer) was mixed with the appropriate QuantiFluor dye. After 5 minutes of incubation at room temperature, the DNA or RNA concentrations were determined in the fluorometer. The nucleic acid samples were also read in a spectrophotometer, equipped with Nanodrop software (ThermoFisher, Waltham, MA, USA), at 260 nm and 280 nm. If the results were between 1.7 and 1.9, the samples were considered to be contamination free and adequate for further analyses.

HCV real-time polymerase chain reaction (PCR) quantification. HCV RealTM Quant kits (Sacace Biotechnologies) were used for the quantitative detection of HCV in human plasma. Briefly, HCV RNA was extracted from plasma samples, amplified, and detected using fluorescent reporter dye probes specific for either HCV or immune complexed HCV. An internal control served as the extraction and amplification control for each individually processed specimen, allowing identification of possible inhibitions. Immune complexes were detected in a specifically labeled channel other than that used for HCV RNA. Real-time monitoring of fluorescence intensities allowed detection and quantification of the accumulating product without opening the reaction tube after real-time amplification. The internal control was detected on the FAM channel and HCV RNA on the CY3 channel. For each control and patient specimen, the concentration of HCV RNA was calculated using the following formula: HCV RNA copies/specimen (CY3 channel)/immune complexed RNA copies/specimen (FAM channel) \times coefficient = IU HCV/mL. These results were also expressed as copies/mL by multiplying by 4 [9, 10].

PCR amplification of SENV DNA. The primers used in the study were SENV-AI-IF (5'TACTCCAACGACCAGCTAGACCT3'), SENV-AI-IR (5'GTTTGTGGTGAGCAGAA CGGA3') for the first step of PCR, SENV-D-1148 F (5'GCAGTTGACCGCAAAGTTACAAG AG3'), and SENV-D-1341 R (5'GCAGTTGACCGCAAAGTTACAAGAG3') for the secondly step of PCR (AlphaDNA, Montreal, QC, Canada). The template DNA and primers were added to a PCR tube, along with nuclease-free water, to a total volume of 50 μ L. The PCR reaction was carried out in a 50- μ L mixture containing *Taq* DNA polymerase, dATP, dGTP, dCTP, dTTP, 1.5 mM MgCl₂, reaction buffer (pH 9), loading dye buffer (yellow and blue dyes), amplification primers (2 μ L, each), target DNA (10 μ L), and nuclease-free water [8].

The SENV DNA was amplified for all study samples including healthy blood donors using nested conventional PCR, as follows. The first-round amplification was: 94° C for 4 min, 1 cycle for initial denaturation of the template; 35 cycles at 94° C for 40 s to denature the DNA; 55° C for 50 s, 35 cycles for annealing; 72° C for 50 s, 35 cycles for extension; finally, 72° C for 10 min, 1 cycle for final extension. The second PCR run was conducted as follows: 94° C for 4 min, 1 cycle for initial denaturation of the template; 35 cycles at 94° C for 30 s to denature the nucleic acid; 55° C for 50 s, 35 cycles for annealing; 72° C for 50 s, 35 cycles for extension; and 72° C for 10 min, 1 cycle for final extension.

Upon PCR completion, 10 μ L of amplified DNA was mixed with 4 μ L of Redsafe nucleic acid stain and loaded on to 2% agarose gels. After electrophoresis was complete, the gel was placed on an ultraviolet transilluminator and digitally documented.

Sequencing. The National Instrumentation Center for Environmental Management (Seoul, Korea) sequenced the PCR products. Sanger dideoxynucleotide sequencing technique was dependent in this study. The sequences were then run through the standard gene BLAST program (<http://www.ncbi.nlm.nih.gov>) and sequences were aligned using the BioEdit program (Ibis Therapeutics, Carlsbad, CA, USA). An evolutionary analysis was conducted using Molecular Evolutionary Genetics Analysis software (version 6, Pennsylvania State University, State College, PA, USA) [11].

Statistical analysis

Data are presented as percentages, means, standard deviations, and ranges. Significant differences between means (quantitative data) were examined using Students *t*-test for differences between two independent means, paired *t*-tests for differences between paired observations (or two dependent means), or analyses of variance for differences among more than two independent means. Significant differences between percentages (qualitative data) were determined using the Chi-square test, applying Yate's correction or Fisher's exact test when applicable. All statistical analyses were performed using SPSS (ver. 22, IBM, Armonk, NY, USA); a *P*-value ≤ 0.05 was considered significant.

Results

The patients with β -thalassemia and strongly suspected of having HCV infections were confirmed to be HCV-positive; the other 75 patients with thalassemia and the 75 healthy donors were confirmed to be HCV-negative. Nested conventional PCR was used to identify SENV infections, with the results revealing a significant occurrence of SENV infections among patients with thalassemia than among healthy donors. Agarose gel electrophoresis effectively differentiated between the 193-bp SENV-D and 118-bp SENV-H genotypes as well as demonstrated their co-occurrence in some individuals (Figs 1 and 2).

Table 1 shows that the frequency of SENV infections among patients with thalassemia (78%) was significantly higher than those observed in healthy blood donors (10%; $P < 0.05$). Table 1 also emphasizes that the prevalence of SENV infections in patients with thalassemia was not dependent on their HCV infection status. Interestingly, among the healthy blood donors with SENV infections, more SENV-positive individuals living in urban environments (9/51, 17.6%) than those are living in rural environments (1/24, 4.1%); this was a statistically significant ($P = 0.003$).

SENV infection distribution, according to patient age, sex, and liver function indicators

Among patients with β -thalassemia, the highest frequencies of SENV-D (49.3%), SENV-H (37%), and combined SENV-D and SENV-H (13.7%) infections were recorded in patients 15–26 years old (Table 2). Interestingly, SENV infections were not observed among any of the youngest donors.

The sex-based distribution of SENV genotypes was also determined (Table 3). Among those with β -thalassemia, a high percentage of males (52%) were infected with SENV-D, whereas SENV-H was more predominant among females (40.3%). Co-infections with both SENV-D and SENV-H were most commonly observed in males (18.0%). However, a statistically significant sex-based distribution was not observed for either SENV genotype (Table 3).

Significant differences were found in the activities of key liver function indicators in HCV-negative patients with SENV infections, relative to the healthy donors (Table 4). In the patients with SENV infections, most of the measured liver function indicators were also higher than the normal levels associated with healthy liver function. Moreover, the HCV-negative patients with thalassemia also showed liver function indicator (ALT, AST, ALP, TSB) levels that were significantly elevated compared with similar patients without SENV infections. Among the healthy donors, significant differences were not noted for any of the liver function indicators, regardless of SENV infection status.

Comparing only HCV-negative patients with thalassemia to the healthy donors (Table 5), all liver function parameters, except GGT, were similarly elevated regardless of the genotype of

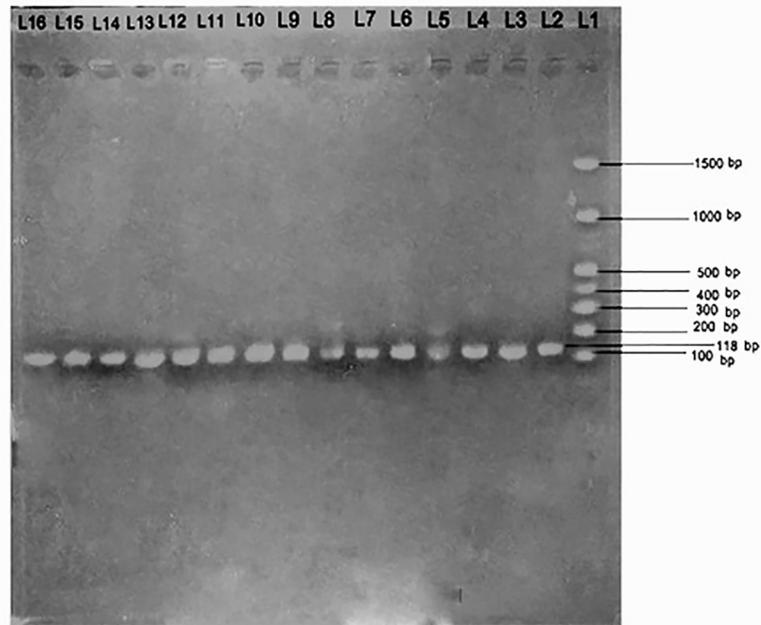


Fig 1. Agarose gel electrophoresis of amplified SEN virus H-gene DNA. Bands showing the amplified SEN virus-H gene (118 bp) are shown in lanes L2–L15. DNA molecular weight markers (100–1500 bp) are present in lane L1.

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the infecting SENV. This analysis revealed that GGT levels were significantly higher in patients with SENV-H infections compared with those observed in patients infected with SENV-D, combined SENV-D and H infections, or in healthy donors (all, $P = 0.01$). A notable increases in other liver function parameter levels were also observed in the patients with SENV-H

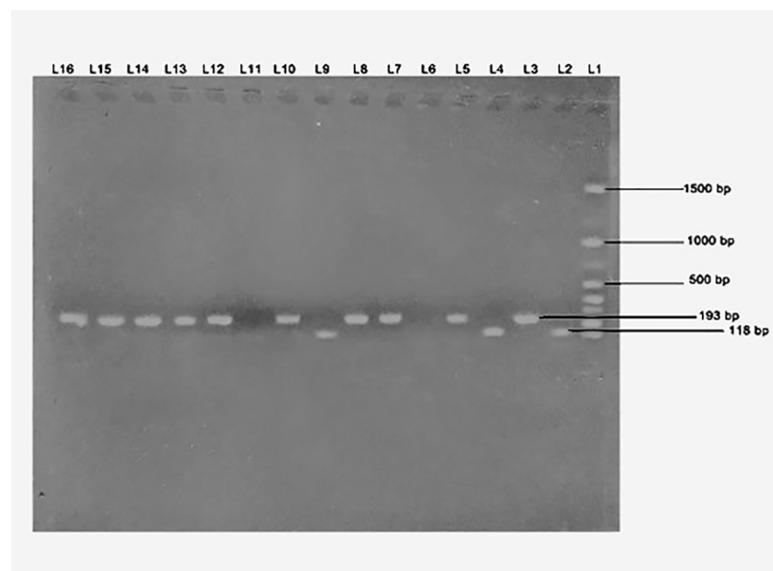


Fig 2. Agarose gel electrophoresis of amplified SEN virus D- and H-gene DNA. Bands showing the presence of SEN virus D (193 bp) and H (118 bp) genes in lanes L5, L10, and L15. DNA molecular weight markers (100–1500 bp) are present in lane L1.

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Table 1. SEN virus infection rates among healthy blood donors and among patients with β -thalassemia, with and without evidence of hepatitis C virus (HCV) infection.

SEN virus status	Patients with β -thalassemia		Healthy donors (n = 75)
	HCV RNA-positive (n = 75)	HCV RNA-negative (n = 75)	
Positive	60 (80%)	58 (77%)	10 (13%)
Negative	15 (20%)	17 (23%)	65 (87%)

*Significant difference between proportions using the Pearson Chi-square test

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infections, in comparison with those to those with SENV-D infections (Table 5), but these differences were not statistically significant.

This study also showed significant differences in serum vitamin C, vitamin E, and GPX levels between healthy blood donors and SENV-positive, HCV-negative patients with thalassemia (Table 6). However, there was no significant difference in the levels of vitamins C and E between SENV-positive and SENV-negative patients. Interestingly, the level of GPX was significantly lower in SENV-positive patients than in SENV-negative patients.

Although infection with SENV resulted in lower levels of these three antioxidants in the SENV-positive, HCV-negative thalassemia patients, compared with controls, only the GPX level appeared to be significantly lower in patients infected with SENV-H ($P = 0.04$). The distribution of antioxidants, including vitamins C and E and GPX, in patients with thalassemia and infected with the two SENV genotypes showed that the GPX levels were significantly lower in patients infected with SENV-D or SENV-H, than for healthy donors (Table 7).

Molecular and phylogenetic analyses

As part of this study, 14 samples of amplified SENV DNA were sent for sequencing and phylogenetic analyses, including 2 (14.3%) from healthy blood donors (nos. 15 and 17) infected with SENV-H, 9 (64.3%) from patients with thalassemia (nos. 1, 5–7, 10, 19, 20, 22, and 23) infected with SENV-H, and 3 (21.4%) from patients with thalassemia (nos. 2, 3, and 4) co-infected with SENV-D and SENV-H (Fig 3). An alignment study of SENV-H samples recovered from patients with thalassemia revealed a closely related genotype that is unique from samples isolated from patients in Iran, China, Japan, and France (GenBank accession numbers are documented in Table 8). Further, the sequencing of these genes revealed 85–97% compatibility with the global standard genes in GenBank.

In the SENV-H gene sequencing study of isolates no. 1–7, 10, 15, 17, 19, 20, 22, and 23, the sequences corresponded to sequences already present in GenBank, specifically accession numbers: AY183662.1, KM593803.2, AY264849.1, AY206683.1, and AB059353.1. The sequencing results also revealed that after product alignment, the amplified SENV-H gene showed two types of substitutions (transition and transversion) compared with those in GenBank. Additionally, isolates 1 and 7 showed 100% identity with the sequence for AY183662.1. The remaining isolates yielded 92–99% identity; the low percentage of non-identity was due to transitions and transversions. The sequencing and BLAST analyses of the partial SENV-H genes and the types of gene polymorphisms are shown in Table 9.

Discussion

The present study was a follow-up study from an earlier investigation of SENV infections in patients with β -thalassemia, in Iraq [8]. In addition to support the results from the earlier study, this study also sought to explore, in greater detail, after investigating the potential

Table 2. Age distribution of SEN virus infections in patients with β -thalassemia and healthy blood donors.

	Age group (years)	SENV Virus genotype			Total
		D	H	D & H	
Patients	3–14	15 (60.0%)	8 (32.0%)	2 (8.0%)	25 (21.2%)
	15–26	36 (49.3%)	27 (37.0%)	10 (13.7%)	73 (61.9%)
	27–38	8 (40.0%)	6 (30.0%)	6 (30%)	20 (16.9%)
	Total	59 (50.0%)	41 (34.7%)	18 (15.3%)	118 (100.0%)
Healthy donors	3–14 (n = 8)	0.00	0.00	0.00	0.00
	15–26 (n = 38)	2 (50.0%)	2 (50.0%)	0.00	4 (40.0%)
	27–38 (n = 29)	4 (67.0%)	2 (33.0%)	0	6 (60.0%)
	Total	6 (60.0%)	4 (40.0%)	0	10 (100.0%)

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impact of SENV-H infections in patients with thalassemia. The study showed that the distribution of SENV-H is similar to that of SENV-D and that, generally, the physiological impacts of both genotypes of the virus are similar. However, the study also demonstrated that there are some differentiating hepatic effects, particularly related to GGT levels, in patients infected with SENV-H that are not observed in patients with SENV-D infections. SENV infections also appear to impact levels of some antioxidants in patients with adequate nutrition.

The present study confirmed that, in Iraq, SENV infections are more common in patients with thalassemia than in healthy donors. Furthermore, patients with thalassemia demonstrated similar frequencies of SENV infection, regardless of their HCV infection status. The high rate of SENV infections among these patients reflects the similarly high rate of HCV infections typically observed in these patients [12]. The high rates of infection for SENV and HCV in this patient population may be due similar transmission risk factors, e.g., blood transfusions. Blood-borne infections, such as HCV, pose significant risks for patients with transfusion-dependent thalassemia [8]. Further, the significantly greater occurrence of SENV infections among patients with thalassemia than among healthy donors remains suggestive of blood transfusions being the primary source of SENV infections in patients with thalassemia [13]. Sani et al. [14] reported SENV viremia in 90.0% of patients with thalassemia (regardless of their HCV infection status) and in 76.7% of patients with HCV infections. These results are similar to the SENV infection rates reported in Taiwan [15]. It seems SENV infections in patients with thalassemia, regardless of their HCV status, have been suggested to further increase in patients need for blood transfusions [16]. This may be of particular concern since

Table 3. Sex-based distribution of SEN virus infections in patients with β -thalassemia and healthy blood donors.

	Sex	SENV Virus genotypes			Total
		D	H	D and H	
Patients	Females	27 (47.4%)	23 (40.3%)	7 (12.3%)	57
	Males	32 (52.5%)	18 (29.5%)	11 (18.0%)	61
	Total	59 (50.0%)	41 (34.7%)	18 (15.3%)	118
Healthy donors	Females	2 (66.7%)	1 (33.3%)	0	3
	Males	4 (57.1%)	3 (42.8%)	0	7
	Total	6 (60.0%)	4 (40.0%)	0	10

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Table 4. Liver function indicators (mean \pm SD) in hepatitis C virus-negative patients with β -thalassemia with and without SEN virus infections and in healthy blood donors.

Parameter	Patients with β -thalassemia		P-value	Healthy donors	P-value
	SENV-positive Mean \pm SD (n = 58)	SENV-negative Mean \pm SD (n = 17)			
ALT (U/L) Normal: <40	50.7 \pm 13.6	30.7 \pm 18.0	<0.001	27.3 \pm 7.02	<0.001
AST (U/L) Normal: <40	50.8 \pm 10.7	27.4 \pm 10.5	0.001	24.2 \pm 4.63	<0.001
ALP (U/L) Normal: <125	135.9 \pm 46.8	89.6 \pm 17.1	<0.001	71.1 \pm 13.8	<0.001
GGT (U/L) Normal: <50	50.1 \pm 34.5	29.0 \pm 6.5	0.229	27.6 \pm 12.0	<0.001
TSB (mg/dL) Normal: <1.46	2.7 \pm 1.34	1.98 \pm 1.07	0.001	0.99 \pm 0.461	<0.001

ALT, alanine transaminase; ASP, aspartate aminotransaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; TSB, total serum bilirubin

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SENV transmission may occur primarily via parenteral routes, e.g., blood transfusion, intravenous drug use, or hemodialysis [17].

In this study, we also examined the age- and sex-based distributions of SENV infections. Among healthy donors, approximately 10.5% of 15–26-year and 20.7% of 27–38-year-old donors respectively were observed to have an evidence of SENV infections. There was no evidence of a significant sex-based predominance. The prevalence and age distribution of SENV infections in this Iraqi population differs somewhat from an analysis of SENV infections in Taiwan, where 25–30% of adolescents were observed to have SENV infections [18]. Similar to the Taiwan study, however, there was a slight predominance of SENV-D infections compared with SENV-H infections. One major difference that was observed in the present study, compared with the Taiwan study, was that significantly more of the SENV infections in healthy Iraqi donors dwelt in urban areas. In the study of healthy adolescents in Taiwan [18], there were significantly higher rates of SEN viremia among those residing in rural or mountainous locales than for those living in urban environments. Thus, SENV infections appear to occur in

Table 5. Liver function marker levels (mean \pm SD) in hepatitis C virus-negative patients with β -thalassemia infected with SEN virus and in healthy donors.

Liver function marker	SENV Virus genotype				P-value
	D (n = 19)	H (n = 31)	D and H (n = 8)	Healthy donors (n = 75)	
ALT (IU/L)	55.57 \pm 18.5	60.44 \pm 35.7	64.54 \pm 16.4	38.7 \pm 18.0	<0.01
AST (IU/L)	52.21 \pm 30.9	57.0 \pm 17.6	54.3 \pm 17.1	37.4 \pm 15.5	0.03
ALP (IU/L)	134.4 \pm 47.5	143.2 \pm 54.0	135 \pm 39.5	103.5 \pm 57.1	<0.01
TSB (mg/dL)	2.61 \pm 1.32	2.87 \pm 1.26	3.24 \pm 1.38	1.7 \pm 1.07	0.01
GGT (IU/L)	47.23 \pm 28.6	85.2 \pm 51.2	58.62 \pm 18.3	37.6 \pm 32.5	0.01

ALT, alanine transaminase; ASP, aspartate aminotransaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; TSB, total serum bilirubin

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Table 6. Vitamin C, vitamin E, and glutathione peroxidase levels in healthy blood donors and in SEN virus (SENV)-positive and -negative patients with β -thalassemia without hepatitis C virus infections.

Parameter	Patients with β -thalassemia		P-value	Healthy donors (n = 75) Mean \pm SD	P-value
	SENV-positive Mean \pm SD (n = 58)	SENV-negative Mean \pm SD (n = 17)			
Vitamin C (μ mol/L)	58.9 \pm 37.9	63.8 \pm 40.1	0.871	73.0 \pm 18.0	0.002
Vitamin E (μ mol/L)	32.4 \pm 13.9	39.6 \pm 15.1	0.628	44.6 \pm 15.5	<0.001
Glutathione peroxidase (U/L)	238.2 \pm 121.7	312.2 \pm 127.3	0.049	353.5 \pm 59.3	<0.001

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relatively young, healthy individuals, suggesting that the infection is unlikely to occur via a par-enteral route in these individuals.

The SENV-H DNA samples recovered during the present study, from both patients with thalassemia and healthy donors, showed 97% compatibility with Iranian samples and 93% compatibility with samples from China. Moreover, the results showed that 93% and 92% compatibility with samples from Japan and France, respectively. Samples in each branch of the SENV-H phylogenetic tree have corresponding DNA sequences, most probably indicating that the virus has been transmitted via blood transfusions. This is likely because of the DNA sequence compatibility between blood donors and patients with thalassemia and because the H sequences in isolates from individuals co-infected with both SENV-D and SENV-H are the same as the H sequences in isolates from individuals SENV-H infections. Karimi and Bouzari found a 90.8% frequency of SENV infections among healthy blood donors as well as high nucleotide homology in the sequenced amplicons of isolates from patients with thalassemia and healthy donors [4]. It is suggested that SENV-infected healthy blood donors act as partial sources of SENV transmission to patients with thalassemia and possibly to other individuals receiving transfusions. Karimi-Ratehkenari and Bouzari [16] found that a 90.8% frequency of SENV infection among healthy blood donors as well as high nucleotide homology of sequenced amplicons between isolates from patients with thalassemia. These results suggest that SENV-infected healthy blood donors act as partial sources of SENV transmission to patients with thalassemia and possibly to other individuals receiving transfusions. Therefore, our study suggested that the most probable mode of transmission to patients with thalassemia was through blood transfusions, based on the sequence homology between donors and recipients.

Our previous study showed that liver enzyme levels were significantly increased in thalassemia patients infected with HCV and/or SENV and hence, there appeared to be some additional increase associated in liver marker levels in patients co-infected with both viruses as opposed with just one or the other [8]. In the present study, we focused on the impacts of SENV

Table 7. Glutathione peroxidase and vitamin C and E levels (mean \pm SD) hepatitis C virus-negative patients infected with SEN virus and in healthy donors.

Parameter	SENV Genotypes			Healthy donors (n = 75)	P-value
	D (n = 19)	H (n = 31)	D and H (n = 8)		
Vitamin C (μ mol/L)	56.1 \pm 36.9	58.6 \pm 40.4	57.571 \pm 38.6	62.85 \pm 40.1	0.871
Vitamin E (μ mol/L)	31.7 \pm 11.9	30.2 \pm 14.4	37.7 \pm 7.0	33.6 \pm 15.1	0.628
Glutathione peroxidase (U/L)	227.0 \pm 121.1	217.1 \pm 116.8	221.8 \pm 127.7	282.6 \pm 127.3	0.04

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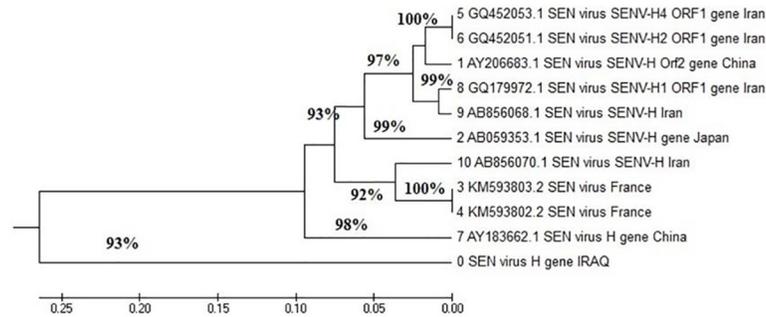


Fig 3. Phylogenetic tree analysis of the genetic distance between the Iraqi SEN virus H genotype and other sequences deposited in GenBank.

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infections in patients with thalassemia but who were not infected with HCV. The results of the current study showed and the liver function markers (ALT, ASP, AST, and TB) were elevated above normal values in HCV-negative patients with SENV infections which were significantly higher than in patients without either HCV or SENV infections or in healthy donors; the levels of the markers were similar between thalassemia patients not infected with either virus and healthy donors. Regardless, for most of the indicators of liver health, the elevations in marker levels were similar between patients infected with SENV-D and SENV-H. However, this was not the case for GGT. Among all HCV-negative patients with thalassemia, GGT levels significantly elevated compared to the donors but were not significantly above normal values. However, when GGT levels were examined in patients infected with different genotypes of SENV, the levels were significantly higher in patients infected with SENV-H than in those infected with SENV-D. Moreover, the GGT levels were considerably above the normal range. Individuals infected with both SENV-D and SENV-H had GGT levels that were intermediate to the levels in patients infected with only SENV-D or SENV-H.

GGT is a known marker of oxidative stress because of its role in the catabolism of extracellular GPX (representative of intracellular antioxidants). A previous study showed that serum GGT levels are associated with clinical outcomes, even after adjusting for the presence of liver disease and liver function test results. Specifically, GGT levels are reportedly associated with the presence of HCC in patients with HCV infections [19]. Therefore, GGT levels might be predictive of HCC development in patients without cirrhosis, even after successful HCV eradication [20]. These observations suggest that a similar correlation might exist for patients with elevated GGT levels associated with SENV-H infections.

Table 8. BLAST results of SEN virus genotype H DNA in GenBank, and DNA sequence compatibility with the global standard genes.

	Accession No.	Country	Source	Compatibility
1.	AY206683.1	China	SEN virus strain SENV-H Orf2 gene	93%
2.	AB059353.1	Japan	SEN virus SENV-H gene	93%
3.	KM593803.2	France	SEN virus	92%
4.	KM593802.2	France	SEN virus	92%
5.	GQ452053.1	Iran	SEN virus Guilan SENV-H4 ORF1 gene	97%
6.	GQ452051.1	Iran	SEN virus Guilan SENV-H2 ORF1 gene	97%
7.	AY183662.1	China	SEN virus H gene	85%
8.	GQ179972.1	Iran	SEN virus Guilan SENV-H1 ORF1 gene,	94%
9.	AB856068.1	Iran	SEN virus SENV-H	91%
10.	AB856070.1	Iran	SEN SENV-H	90%

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Table 9. Characteristics of the clinical samples of SEN virus, genotype H.

Isolate No.	Type of substitution	Location	Nucleotide	Sequence ID	Identity
1	-----			ID: AY183662.1	100%
2	Transversion	135	T>A	ID: KM593803.2	92%
	Transversion	118	A>C		
	Transition	116	T>C		
	Transversion	112	A>T		
	Transversion	97	G>C		
	Transition	96	T>C		
	Transversion	90	G>C		
3	Transition	448	G>A	ID: AY264849.1	97%
	Transversion	443	C>A		
	Transversion	441	A>C		
4	Transition	926	A>G	ID: AY206683.1	93%
	Transition	999	T>C		
	Transversion	896	G>C		
	Transition	881	A>G		
	Transition	872	A>G		
	Transition	857	T>C		
	Transversion	851	T>G		
5	Transition	1076	T>C	ID: AB059353.1	97%
	Transversion	1070	T>G		
	Transition	1061	T>C		
6	Transition	860	C>T	ID: AY183662.1	94%
	Transversion	855	G>T		
	Transversion	853	G>T		
	Transversion	852	G>C		
	Transition	851	G>A		
	Transition	850	C>T		
	7	-----			
10	Transition	829	G>A	ID: AY183662.1	99%
15	Transition	1076	T>C	ID: AB059353.1	97%
	Transversion	1070	T>G		
	Transition	1061	T>C		
17	Transition	829	G>A	ID: AY183662.1	99%
19	Transversion	868	A>T	ID: AY183662.1	93%
	Transversion	861	C>A		
	Transversion	860	C>A		
	Transversion	859	T>A		
	Transversion	855	G>C		
	Transversion	853	G>T		
	Transversion	849	T>G		

(Continued)

Table 9. (Continued)

Isolate No.	Type of substitution	Location	Nucleotide	Sequence ID	Identity
20	Transversion	868	A>T	ID: AY183662.1	92%
	Transversion	861	C>A		
	Transversion	860	C>A		
	Transversion	859	T>A		
	Transversion	855	G>C		
	Transversion	853	G>T		
	Transversion	849	T>G		
	Transition	829	G>A		
22	Transversion	868	A>T	ID: AY183662.1	92%
	Transversion	861	C>A		
	Transversion	860	C>A		
	Transversion	859	T>A		
	Transversion	855	G>C		
	Transversion	853	G>T		
	Transversion	849	T>G		
	Transition	829	G>A		
23	Transversion	868	A>T	ID: AY183662.1	92%
	Transversion	861	C>A		
	Transversion	860	C>A		
	Transversion	859	T>A		
	Transversion	855	G>C		
	Transversion	853	G>T		
	Transversion	849	T>G		
	Transition	829	G>A		

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The association of GGT with oxidative stress prompted additional evaluations of the association of certain antioxidant levels with SENV infections, in the current study. Vitamins C and E both have antioxidant properties and were two of the antioxidants examined in this study. Vitamin C is a natural, water-soluble, free radical scavenger with the ability to donate two electrons from the double bond of its 6-carbon chain. During this process, oxidized vitamin C generates a stable intermediate product, dehydroascorbic acid, which can be taken up by erythrocytes and reduced to vitamin C via endogenous glutathione reductase [21]. In plasma, a relevant role of vitamin C is to restore α -tocopherol by oxidizing it. To be an effective antioxidant, the oxidized α -tocopherol must be reduced, but this process is slower than ascorbate recycling. Therefore, α -tocopherol is likely recycled in the cell membrane by a mechanism that involves enzymatic ascorbate recycling via α -tocopheroxyl [22]. Some studies have suggested using dietary antioxidants, like vitamins C and E, to reduce liver enzyme levels in individuals with HCV infections [23]. In the present study, we found that the levels of vitamins C and E were significantly lower in thalassemia patients than in the healthy donors. However, the levels were similar between patients with and without SENV infections. Additionally, there was no difference in vitamin C/E levels between patients infected with either genotype of SENV. There were also no differences in the levels of vitamins C and E associated with SENV-D or SENV-H infections. When the levels of GPX were examined in the same individuals, the levels were significantly lower in the thalassemia patients than in the healthy donors. Examination also revealed that the levels of GPX were lower in patients infected with SENV-H than in those infected with SENV-D. A decrease in GPX concentration in hepatitis patients might cause the

effectiveness of GPX activity to be restricted, as manifested by the condensation of lipid peroxidation and the increased level of final products of their peroxidation.

This study subjected to some limitations. First, the retrospective design of the study makes it susceptible to the same type of bias that is common to all retrospective studies, particularly since consecutive patients were recruited for the study based on a clinical diagnosis that was suggestive of the presence of HCV infection. Second, the study population was relatively small. Additional studies will be needed to confirm the accuracy of the conclusions derived from the present study. Finally, since this study was limited to a specific area of Iraq, the ability to generalize the results to other geographic areas and ethnic groups of people is limited.

In conclusion, this study confirmed the results of our previous study that suggested that, in Iraq, the prevalence of SENV infections is higher in patients with β -thalassemia than in healthy blood donors, but that the presence of HCV infections does not affect the prevalence of SENV infections. Furthermore, this study was able to demonstrate the absence of a sex-based predominance of infection by the SENV D and H genotypes. The study also showed that, in the present population, the SENV-H DNA indicated a high degree of similarity with other global isolates deposited in GenBank, especially with those from Iran. SENV-H was also suggested to play a more pronounced role in raising serum levels of GGT. Finally, SENV infections were shown to depress levels of GPX to a greater extent than in patients without SENV infections.

Supporting information

S1 Checklist. STROBE checklist.
(DOC)

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