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Genotyping and Phylogenetic Analysis of Human Parvovirus B19 in Beta Thalassemia Major Patients

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Abstract: Beta thalassemia major (βTM) is a hereditary condition that has previously been connected to blood-borne virus infections due to repeated transfusions of blood, Human parvovirus B19 (B19V) viral infection was proposed as a risk factor due to recurrent hemotherapy. Thus, this study aimed to investigate the prevalence, genotyping, and phylogenetic analysisof B19V in βTM patients. The study included 60 control volunteers and 120 βTM patients throughout November 2023 and March 2024. Blood samples were subjected to genomic DNA extraction, then nested PCR (nPCR) targeting fragments (VP1-VP2 region, 288 nt) was followed. Furthermore, genotyping and phylogenetic analysis were performed on DNA-positive samples by direct sequencing method. The study findings revealed that age was a risk factor, meanwhile, sex was not. The B19V was detected by nPCR represented 4.1% (5 out of 120), while none were found in the control group. B19V genotyping showed a predominance of Genotype 1a (100%). A unique accession number PP747257 has been deposited in GenBank to represent B19V for this study. It deserves to be noted that the investigated isolate was suited in the vicinity of three reference sequences deposited from the Netherlands (GenBank acc. no. JN211139.1, JN211160.1, and JN211182.1). In conclusion, despite the low rate of B19V prevalence among the βTM patients, this does not eliminate its potential risk in such a vulnerable group which may lead to severe consequences as a transient plastic crisis. Therefore, blood banks in Baghdad might need to consider screening for B19V especially when transfusion is intended for high-risk populations.

Keywords: Human Parvovirus,nPCR, Genotyping, Phylogenetic Analysis, Thalassemia

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Introduction

Human Parvovirus B19 (B19V) is a tiny naked icosahedral single stranded DNA virus (nearly 5.5 kb) belonging to genus *Erythovirus* (*Parvovidae* Family) (1). Due to its strong affinity for erythroid progenitor cells, B19V can cause a short-term infection of the bone marrow. This might eventually exacerbate anaemia and temporarily stop bone marrow erythropoiesis, which is indicative of a transitory aplasia crisis(2). In children with sickle-cell disease, B19V has been identified as the causative agent of severe aplastic crisis, which can progress into a number of potentially fatal illnesses (3). Patients, especially children with inherited hemolytic diseases like beta thalassemia major (βTM) caused by inherited β-haemoglobin disorder leading to monogenic blood defect, resultant to chronic anaemia are particularly vulnerable to B19V(4,5). Patients with

βTMalso termed as transfusion-dependent thalassemia (TDT) are generally rely on a hyper-transfusion regimen (hemotherapy) usually before the age of 2 years (6).

In Iraq, the thalassemia prevalence escalated between 2010 and 2015 registered 33.5 to 37.1 per 100,000 (7). The thalassemia prevalence over the last 5 years in Iraq have been raised from 12106 cases in 2018 to 13390 cases in 2022 (8). Both males and females are equally affected by βTM(9). Many local studies have focused on different risk factors and complications with βTM(10–12), meanwhile other recent researchers conducted on Iraqi βTM patients to evaluate the serum immunological markers like Toll Like Receptor- (3 and 4) and Interleukin-(7 and 37)(13), evaluatedA1pha hemoglobin stabilizing proteingeneexpression and its serum level (14), and the relevance of rs3459829 single nucleotide polymorphism of human beta globin gene(15)

TheTDT patients mainly acquire transfusion-transmitted infections, including viruses that infect the liver “hepatitis C virus HCV or hepatitis B virus (HBV)” (16). Nevertheless, the majority of haematological units across the globe do not routinely screen the βTM patients or donors of blood for B19V. Therefore, this study aimed to investigate the prevalence, genotyping and phylogenetic analysis B19V among patients with βTM.

Materials and methods

Patients and control groups

This is a prospective study involves 120 βTM patients who received treatment and blood transfusion at the Ibn Al Balady Children and Maternity Hospital in Bagdad, Iraq, between November 2023 and March 2024. Sixty healthy blood donors who attended the Iraqi National Center of Blood Transfusion in Baghdad, Iraq, served as the study's control group. The healthy donors were tested to be free of viruses (HCV, HBV, and HIV). Every participant, or their parents, gave their written consent. The study protocol was approved by the Ethics Committee of the College of Science, University of Baghdad (CSEC/1223/0136) and in accordance with Iraqi Ministry of Health approval (No. 62751Dated 22 October 2023). Patients over 40 yearswere excluded and a specific age range of controls were included in the inclusion criteria. Furthermore, individuals with chronic or autoimmune disorders were considered not eligible.

Molecular detection of parvovirus

A commercial extraction kit from Taiwan's Geneaid Company was used for genomic DNA extraction based onmanufacturer's instruction. According to a previous reference, two DNA fragments of B19V in the overlapping area shared by the major (VP2) and minor (VP1) capsid protein genes were amplified using the nested PCR (nPCR) procedure (17). In the first round, 398 bp of the target area were amplified using the outer primer sequences 5'-CAAAAGCATGTGGAGTGAGG-3' (nt 3187–3206) and 5'-CTACTAACATGCATAGGCGC-3' (nt 3584–3565). The 25 µl total PCR mixture consisted of 5.0 µl DNA templet, 1.0 µl of each primer (Forward and Reverse), 12.5 µl master mix (Promega, USA), and 5.5 µl of nuclease-free water to finish the volume. In the second cycle, 288 bp of the initial product were amplified using inner primers (sequences 5'-CCCAGAGCACCATTATAAGG-3' (nt 3271–3290) and 5'-GTGCTGTCAGTAACCTGTAC-3' (nt 3558–3539). One cycle of 94°C for 5minas (initial denaturation), 30 cycles of 94°C for 1 minas (denaturation step),

then for first round 55°C for 2 min or 57°C for 2 min(second round)as (annealing step), followed by 30 cycles of 72°C for 3 min as (extension step), lastly 1 cycle of 72°C for 5 min as (final extension)were the thermal conditions for the nPCR protocol. A (2%) agarose gel was used to visualize the amplified products.

Genotyping and phylogenetic analysis

One genetic fragment, covering the PV1 within this virus was selected in this study. The amplified fragment was made up to 288 bp and was subsequently exposed to Sanger sequencing to resolve the pattern of its genetic diversity for the analyzedhuman parvovirus isolate (assigned D1). By using the reference-based genotyping, the NCBI BLASTn analysis was conducted for the DNA sequences alignment of the VP1/VP2 locus of the analyzed samples.

The BioEditver, 7.1. (DNASTAR, Madison) was used to demonstrate the nucleic acid variationscomparing DNA chromatograms with the deposited viral DNA sequences. Then, SnapGene Viewer ver. 4.0.4 was used to detected each variant within the B19V genes.

A specific tree for the observed variants was constructed to resolve the effects of the PV1-based variations on the phylogenetic positioning. This tree was based on the incorporation of three viral reference genotypes from Netherlands (GenBank acc. no. JN211139.1, JN211160.1, and JN211182.1).

Statistical analysis

Using SPSS version 25, a statistical program, data were coded and entered. Descriptive statistics, such as mean, standard deviation, median, minimum and maximum values for quantitative variables, and number and percentage for qualitative values, were used to summarize the data. Categorical variables were represented by numbers and percentages, while the Kolmogorov Smirnov test was used to assess the normal distribution of numeric variables. Statistical differences between groups were tested using Chi Square test for qualitative variables, independent sample test and ANOVA (analysis of variance) test for quantitative normally distributed variables. P-values less than or equal to 0.05 were considered statistically significant.

Results and discussion

Demographic parameters

The ages of included patients ranged from 5 to 40 (mean, 18 ± 7.8) years, there were 14 (12%) βTM patients were below 10 years. The βTM patients aged between 11 to 20 years were 39 (32%), and only 3 (5.0%) for the controls. Meanwhile, the βTM patients in the age group (> 20 years) were 67 (56%), for controls were 57 (95.0%) (P-value <0.001). Both the βTM patients and controls were comparable in mean age and sex distribution (P > 0.05). Regarding sex distribution, the males were more predominant among βTM patients 67 (56%) compared to the control group 28 (47%). The females were less predominant in patients with 53 (44%) compared to controls were 32 (53%) (P value 0.075) (Table 1).

Table (1):Comparison of demographic parameters among study groups

Variables	βTM No. (%)	Control No. (%)	$\chi 2$	*P* value
Age groups				
≤ 10 years	14 (12%)	0 (0%)	43.55	<0.001**
11 – 20 years	39 (32%)	3 (5%)		
≥ 20 years	67 (56%)	57 (95%)		
Total	120 (100%)	60 (100%)		
Sex				
Male	67 (56%)	28 (47%)	3.17	0.075 NS
Female	53 (44%)	32 (53%)		
Total	120 (100%)	60 (100%)		

βTM, Beta Thalassemia Major; B19V, Human parvovirus B19; χ^2= Chi square Test; ** highly significant; NS, Non-Significant

The findings of this study are consistent with several other investigations locally conducted by many researchers regarding age and sex(18–20). Additionally, the findings of the present study come in line with an Iranian study by Arabzadeh*et al.* (2017) (4).

Molecular detection of human parvovirus (B19)

The Parvovirus B19 DNA was extracted then subjected to nPCR to detect the B19V in βTM patients and controls. There were only 5 out of 120 (4.1%) to parvovirus VP1/VP2 with PCR product size (288 bp) in βTM patients and non in the controls (Figure 1). The analysis by BLASTn tool revealed the highest similarity between the expected target and the sequenced samples, specifically the VP1/VP2 locus within human parvovirus genomic sequences indicated a 99% homology to the specified portions of the VP1/VP2 sequences with the reference strain under accession number JN211182.1 deposited in National Center for Biotechnology Information (NCBI) data base as in (Figure 2).

After placing the 288 bp amplicons' sequences within the PV1 locus of B19V sequences, the features of these sequences were highlighted, starting from the position of the forward primer to the position of the reverse primer within the same targeted sequences of B19V (Table 2).

After visualizing the chromatogram files, it was found that only one sample (**assigned D1 sample**), while the other visualized chromatograms of the other four samples did not give any clear peaks. Within the investigated D1 sample, our results indicated the presence of three nucleic acid variants;they were (37A>C, 70A>T, and 170C>T) compared with the reference sequences of Human parvovirus (GenBank acc. no. JN211182.1) (Table 3).

The accurate genotyping of the five investigated isolates were genotyped as human parvovirus genotype 1a. The investigated samples of VP1 sequences were deposited in the NCBI web server, and one unique accession number was obtained for the analyzed sequence. Accordingly, the GenBank accession number **PP747257.1**has been deposited to represent the D1 sample of the analyzedhuman parvovirus. An inclusive phylogenetic tree was generated in the present study, which was based on the observed nucleic acid sequences detected in the investigated sample. This phylogenetic tree contained the investigated isolate of Human parvovirus sequences (D1) alongside other relative reference sequences as in (Figure 3).

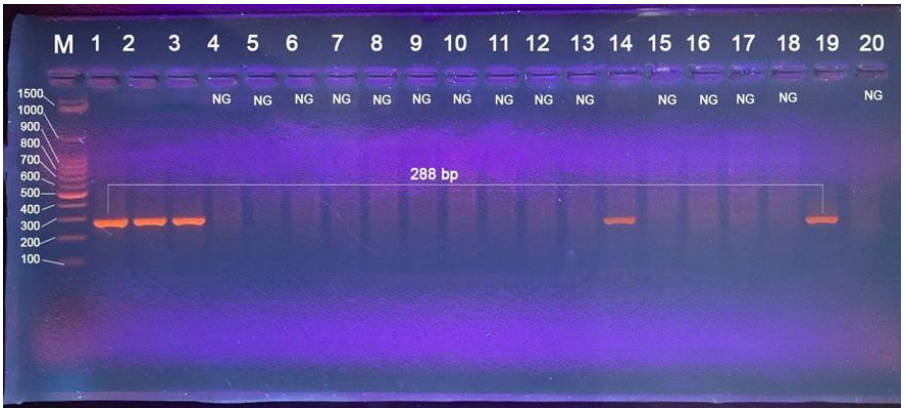

Figure (1):PCR products of human parvovirus (B19) on gel electrophoresis. Lane M: DNA marker (100-1500 bp), lanes 1 – 3, 14, and 19: positive result for amplification (288 bp), lane 4 – 13, 15 – 18, and 20: negative result for amplification.

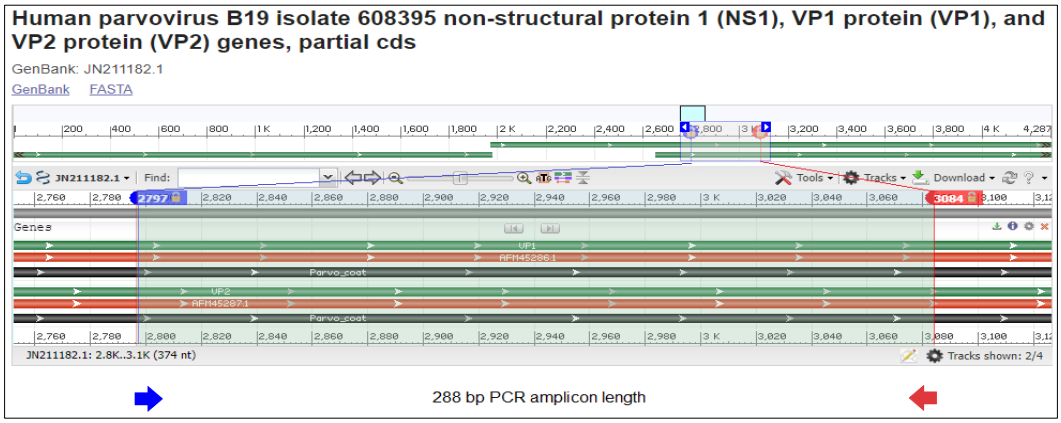

Figure (2): The exact positions of the investigated PCR amplicons that covered the PV1 locus within the Human parvovirus genomic sequences in comparison with the reference strains of Human parvovirus (GenBank acc. no. JN211182.1). The blue arrow refers to the starting point of these amplicons while the red arrow refers to their endpoints.

Table (2): The length and position of the 288 bp PCR amplicons used to the PV1 locus amplification within the Human parvovirus genomic sequences in comparison with the reference strains of Human parvovirus (GenBank acc. no. JN211182.1).

Targeted gene	Sequences (5′-3′)	Length
VP1 of Human parvovirus	*CCCAGAGCACCATTATAAGGTGTTTTCTCCCGCAGCAAGTA GCTGCCACAATGCCAGTGGAAAGGAGGCAAAGGTTTGCACT ATTAGTCCCATAATGGGATACTCAACCCCATGGAGATATTTA GATTTTAATGCTTTAAATTTATTTTTTTCACCTTTAGAGTTTCA GCACTTAATTGAAAATTATGGAAGTATAGCTCCTGATGCTTT AACTGTAACCATATCAGAAATTGCTGTTAAGGATGTTACAGA CAAAACTGGAGGGGGGGTGCAGGTTACTGACAGCAC**	288 bp
* Refers to the forward primer sequences (placed in a forward direction) ** Refers to the reverse primer sequences (placed in a reverse complement direction)		

Table (3): The identified variants in the investigated sequences that are partially covered VP1 sequences within the Human parvoviral sequences.

No.	Position in PCR amplicons	varia nt	SNP	Position in the reference genome	Position in protein	Consequences
1.	37	A>C	37A>C	2833	Ala44	Silent (p.Ala44=)
2.	70	A>T	70A>T	2866	Ala55	Silent (p.Ala55=)
3.	170	C>T	170C>T	2966	His89	Missense (p.89His>Tyr)
Ala, A, Adenine; C, Cytosine; T, Thiamine; Ala, Alanine; His, Histone; p., protein						

The constructed phylogenetic tree included thirty-one aligned nucleic acid sequences and revealed the presence of three major genotypes (genotype 1, 2, and 3) among which our investigated sample was classified. Insights gleaned from the PV1-based tree provided clear phylogenetic information about our investigated virus as in (Figure 3). In this study, the investigated D1 sample was incorporated within a specific position of genotype-1, subtype-a. The number of incorporated samples within this specific clade was fifteen. Within this specific clade, it deserves to be noted that our investigated isolate was suited in the vicinity of three reference sequences deposited from Netherland (GenBank acc. no.JN211139.1, JN211160.1, and JN211182.1). The observed data indicated the high biological diversity for the genotype-1 compared with the other viral genotypes. What has confirmed this observation was found in the wide geographical distributions of the clade of the unclassified genotype-1. Which was found to variable in countries that are originated from a variety of Asian, European, and South American sources.

According to a study by Abdul Sadah and Al-Marsome (2020) focused on the detection of human parvovirusin humans among beta thalassemia patients, it was reported a 37% positive to the viral infection which disagree with this study findings (21).Pichon *et al*. (2019) evaluated the performance of optimized NS1-VP1u for detecting and sequencing of B19V in clinical samples and claimed (100%) sensitivity and specificity (22). Additionally, the phylogenetic analysis confirmed that the viruses in all samples belonged to genotype 1. Another study conducted in South India by Seetha *et al*. (2021) reported a (7.2%) of human parvovirus detected by nPCR in Kerala State (23). This study's findings are agreed with Atbee*et al*. (2020) who reported (2 βTM out 208 hemoglobinopathiespatients represents 4.8%)in Basara city were detected by molecular methods using the nPCR(20). However, the current finding disagrees with Alnasser and Shallal, (2023) findings who reportedthat 7 out of 60 βTM patients represents (11.7%) in Thi-Qar Governorate (24). Another study byAl-Asi*et al*. (2017) revealed the prevalence of genotype 1 in patients suffering from renal failure (25).

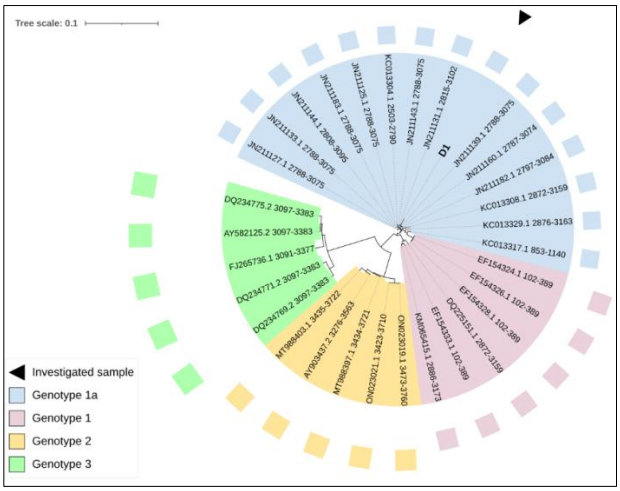

Figure (3): Rectangular phylogenetic tree of genetic variants of the PV1 locus within one viral sample of B19V. The different colored numbers refer to the specific genotypes involved. The scale on the left side of the tree represents the degree of phylogenetic position among the tree-categorized viral species. The symbol "D1" represents the code of the studied sample.

Conclusion

In this study, human parvovirus B19 was detected by nPCR which revealed low rate prevalence (4.1%) among patients with βTM. Nevertheless, this may indicate that the B19V can potentially be transmitted through recurrent blood transfusion from blood donors to βTM patients. Further research with a large sample size is needed to explore this association. There are currently no policies or procedures in place for B19V screening blood donors. Implementing preventive measures, such as screening given blood, is necessary to lower the blood-borne virus prevalence in this susceptible group (βTM patients).

Limitation of the study

The study was carried in single center in Baghdad, the patients sample size was not large, other risk factors such as Iron overload, hepcidin, and ferritin serum levels were not explored. The B19V viral load and gene expression by real time PCR may be more sensitive method for the viral prevalence in vulnerable group as βTM patients.

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