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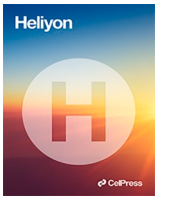

Study on genotyping and coinfection rate of human parvovirus 4 among the HTLV-I/II infected blood donors in Khorasan Razavi, Iran

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ABSTRACT

Background: Human Parvovirus 4 (PARV4) is an emerging virus infecting individuals with other blood-borne diseases. This study aimed to determine the prevalence of PARV4 in confirmed HTLVI/II positive samples from blood donors, assessing PARV4 viral load (DNA) and genotyping. Methods: A novel qReal-Time PCR, based on a plasmid construct, was developed to simultaneously detect all three PARV4 genotypes using in-house primers and probes. Positive qPCR samples were subjected to nested PCR amplification and subsequent sequencing. Phylogenetic trees were constructed using the Neighbor-joining (N.J.) method. Results: The coinfection rate of PARV4-DNA in HTLVI/II confirmed infected donors, who were previously deferred, was 14.4 % (13 out of 90), with no observed association with donation status ($p = 1.0$).
Phylogenetic analysis indicated that PARV4-positive samples closely resembled genotype 2 in Iran.qPCR quantification demonstrated significant PARV4 viral loads in positive samples, ranging between 10^4 and 10^6 DNA copies/mL of serum. Conclusion: This study presents the first evaluation of HTLVI/II and PARV4coinfection rates among blood donors. Notably, elevated PARV4-DNA titers were detected in HTLVI/II-positive donors. Given PARV's resistance to standard plasma refinery inactivation methods and the absence of its targeted inactivation, its potential impact remains a concern.

1. Introduction

Human parvovirus 4 (PARV4) emerged in 2005 as a newly identified virus within the Parvoviridae family. This non-enveloped icosahedral virus (18–26 nm in diameter) carries linear single-stranded DNA encompassing approximately 5200 bp. It comprises capsids, nonstructural genes, and two terminal hairpins [1]. Initial PARV4 isolation was associated with intravenous drug users (IVDUs) displaying viral infection symptoms [1,2]. While its potential as a human pathogen is plausible [3], several factors, including transmission pathways and clinical manifestations, remain elusive [3,4].

From 2005 to 2008, three PARV4 genotypes were identified, each linked to distinct transmission modes [5]. Genotypes 1 and 2, exhibiting less than 8 % sequence variation, have a blood-borne transmission pattern, primarily reported in the USA and Western

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Europe, predominantly spread through blood-borne routes and have been reported primarily in high-risk populations in the USA and Western Europe (immune-compromised people, HIV and HBV, hemophiliacs patients) [6–8]. Genotype 3, prevalent in Africa, demonstrates non-parenteral transmission pathways [9–12].

Research in North America, Europe, Asia, and Africa has consistently associated parvovirus 4 with blood-borne viruses (HBV, HCV, HIV) and respiratory infections, especially among persons who inject drugs (PWIDs) and individuals with hemophilia [14–16]. Recent evidence suggests heightened susceptibility to PARV4 infection among those with HIV, HBV, HCV, and severe acute respiratory infections (SARI), potentially exacerbating disease progression [9,14–17]. Molecular studies have identified substantial PARV4 levels in plasma products administered to patients with clotting factor deficiencies, originating from ostensibly healthy blood donors [8,18–20].

Flu-like syndrome, skin rash, and flare of hepatitis are common clinical manifestations in cases infected by PARV4 [6]. It is strongly linked to encephalitis and fetal hydrops [4], comparable to Herpes simplex virus clinical effects, yet PARV4 has the same behavior as chronic herpes viruses [8,13–16].

Given the shared transmission routes with blood-borne viruses, studies have reported PARV4 infection in HTLV-I/II positive cases [5,9,13]. Human T-cell leukemia/lymphoma virus (HTLV-I/II) primarily spreads through parenteral, vertical, and sexual contact, akin to HIV, HBV, and HCV. HTLV-I virus is linked to diseases including adult T cell Leukemia/Lymphoma (ATLL) and various progressive nervous system conditions [25,26]. While limited clinical evidence links PARV4 to human T-lymphocyte virus-I/II infection [13], comprehensive understanding remains lacking.

Global understanding of PARV4 prevalence is limited, particularly among blood donors. Therefore, this study's primary objective is to estimate and compare PARV4 occurrence in healthy blood donors and deferred donors suspected of HTLV-I/II infections in Khorasan Razavi, Iran. Additionally, the study aims to determine PARV4 genotypes within infected samples, contributing to broader phylogenetic analyses.

2. Materials and methods

2.1. Sample collection, definitions, and ethics approval

A total of one hundred and fifteen blood samples were gathered from healthy blood donors between the winter of 2019 and the early spring of 2020 at the Khorasan Razavi Blood Transfusion Center in Iran. The selection of healthy blood donors involved a stringent process of physical examination and questionnaire-based interviews, following the established protocols of the IBTO. Subsequently, all prospective blood donors underwent laboratory screening to detect blood-borne diseases, including HBV, HCV, HIV, Syphilis, and HTLV I/II, conducted at the blood transfusion center.

Following IBTO's Standard Operating Procedures (SOPs), blood donors were categorized as follows: First-time donors who successfully donated blood for the first time, Repeat donors with a history of blood donation and an interval of more than a year between donations, and Regular donors, who donated blood more frequently, with a frequency of more than once a year [27]. Similarly, a group of 115 blood donor samples exhibiting suspicious HTLV-I/II ELISA test results, collected sequentially during the initial screening process, constituted the HTLVI/II infected group. Demographic data were extracted from the integrated donor database of the Iranian Blood Transfusion Organization (IBTO) in Khorasan Razavi.

The collected samples were processed further to assess the coinfection rate of Parvovirus 4 among seropositive HTLVI/II blood donors. All aliquoted donor samples were promptly preserved and stored at −80 °C until viral DNA extraction commenced. Before participation, informed consent was obtained from all blood donors. This study received ethical approval from the institutional ethics committee of the High Institute for Education and Research in Transfusion Medicine, Tehran, Iran (IR.TMI.REC.1397.034, IR.TMI.REC.1397.035).

2.2. Qualitative ELISA testing of HTLV-I/II total antibodies and confirmatory immunoblotting assay

One hundred and fifteen blood donor samples with repeated reactive results for HTLV-I/II infection have been collected (HTLV I&II total Abs, Diapro co. Sesto San Giovanni, Milan, Italy). All confirmed samples by HTLV-I/II confirmatory immunoblotting (M P. Diagnostics HTLV Blot 2.4, USA) were kept for viral DNA extraction.

2.3. Viral DNA isolation

According to the manufacturer's instructions, nucleic acids were extracted from the donor's serum using a commercial QIAamp viral kit (Qiagen, Hilden, Germany). Furthermore, a cloned fragment of Brome Mosaic Virus (BMV) by TOPO TA cloning vector kit (Invitrogen, CA, USA) was integrated as an internal control (I.C.) in subjected isolated viral DNA of donors' samples to check extraction quality and inhibitor of real-time PCR. Briefly, 200 μl of the donor's sample was mixed with lysing buffer and internal control. After incubation and repeated washing steps, viral DNA quality and concentration were evaluated using a nanodrop spectrometer (Nanodrop 1000, Thermo Fisher Scientific, USA). Purified DNAs were kept at −80 °C before starting the PCR procedure.

2.4. In-silico primers and TaqMan probes designing

Two sets of primers and a TaqMan probe were designed by allele ID version 7.5 (PREMIER Biosoft, San Francisco, CA, USA) and Primer 3 software according to data used for the positive plasmid control that covered all genotypes of PARV4. These primers and

probes did not interfere with other members of the Parvoviridae family or other DNA viruses. Likewise, in silico analysis was carried out by SnapGene (GSL Biotech, San Diego, CA, USA) and Mfold web software (http://www.unafold.org/) for other thermodynamic parameters. Primers and Probes were applied in this study; PARV4F1: GTTGGTCCTGGTAATCCT (18bp, from nucleotide 2989 to 3006), PARV4R1: GTTGTTGGTCCGAAGAAG (18 bp, from nucleotide 3287 to 3304), PARV4F2: GTCCTGGTAATCCTCTG (17 bp, from nucleotide 2993 to 3009), PARV4R2: CGTGACCRTGTAAATAAG (18 bp, from nucleotide 3098 to 3115) and PARV4 TaqMan Probe: FAM-TCCTCAATCATCTCTGCGTACC-BHQ1 (22 bp, from nucleotide 3065 to 3086) of PARV4 sequence (accession number EU546211). According to experimental and in-silico analysis, the amplified region lengths were 316 bp and 123 bp for the first step of nested PCR and both tests in the second step of nested PCR and TaqMan Real-time PCR, respectively.

2.5. Construction of pUC57-PAV4 plasmid

According to the alignment of all retrieved PARV4 sequences from the GenBank, a conserved region of PARV4 (accession number EU546211) from nucleotide 2983 to 3310 (328 bp lengths) was selected and synthesized into pUC57 plasmid by Biomatik Co. (Ontario, Canada). Then, the constructed plasmid was transformed into *E. coli*. The plasmid containing the PARV4 genome was utilized as a positive control after purification by a plasmid purification kit (High Pure Plasmid Isolation Kit, Roche, Germany) to accomplish sensitivity, specificity, repeatability tests, and PCR process optimization.

2.6. Amplification of PARV4 VP2 region by polymerase chain reactions

2.6.1. Quantitative real-time-PCR

Real-time PCR was done by QuantiTect probe PCR kit (Qiagen-Germany) by using a master mix, the combination of each primer (10 μM)-probe (0.5 μM) set, and I.C. (2×10^5 copy/μl).

The concentration of the extracted pUC-PAV4 plasmid was determined by nanodrop at 260 nm (Thermo Fisher Scientific, USA). Then, T.E. buffer containing glycogen prepared a serial dilution ($2x10^1$ - $2x10^9$ copies/μl) to preserve its uniformity. A standard curve was drawn by dilution series in real-time PCR.

The real-time PCR was performed on the Rotor gene Q platform (Qiagen, Hilden, Germany). The thermal profile included activation at 95 °C for 10 min followed by 40 cycles, 15 s at 95 °C, 35 s at 60 °C, and 72 °C for 20 s. Quantitative data analysis was performed by Rotor-Gene v2.3.5 software (Qiagen, Hilden, Germany). Also, positive and negative controls for each pathogen were included for every run.

According to molecular diagnostic GLP (good laboratory practices), preventive measures were also considered to prevent false positives and contamination.

2.6.2. Analytical sensitivity

The plasmid construct of pUC57-PARV4 was serially diluted from $2x10^1$ copies/μL to $2x10^9$ copies/μL and used as a reproducibility, sensitivity, and specificity test template.

2.6.3. Limit of detection (LOD)

A triplicate serial dilution of the lowest detectable concentration of pUC57-PARV4 plasmid was run for Real-time PCR to determine the LOD of tests.

2.6.4. Analytical specificity

Analytical specificity assay was carried out by a viral panel including HIV, HBV, HCV, HTLV-I/II, CMV, and SARS-COV2. Likewise, we checked primers and probes with pooled genomic DNA.

2.7. Nested-PCR

The nested PCR protocol was set up using specific primers, as mentioned in 2.4. The first run of nested PCR inclusion at 20 μL consists of 10 x PCR buffer, 2.2 mM MgCl2, l μL of each primer (10 μM), and 2 mM dNTP. In the second run, a 20 μL master mix containing 2 mM Mgcl 2 plus l μL of second-round primer (10 μM) was used. The detail of the thermal profile was as follows: 5 min of an initial denaturation step at 95 °C, followed by 25 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 60 °C, and 35 s at 72 °C for the extension. The conditions for the second PCR round were the same as the first round, except for the template volume (1 μL), followed by 30 cycles. Both rounds included final extensions at 72 °C for 5 min and cooled for 4 min. The amplicons were analyzed by electrophoresis on 2.0 % agarose gels.

2.8. Sequencing of PARV4 ORF2-VP2 region

To confirm PARV4-DNA, three positive samples were amplified by Nested PCR. Then, positive products were purified and sequenced using Sanger dideoxynucleotide sequencing technology by Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems GmbH, Waltham, MA, USA). The sequences were also analyzed and aligned by the Basic Local Alignment Search Tool (BLAST) in the NCBI GenBank database.

2.9. Phylogenetic analysis

MEGA v.7 software (Molecular Evolutionary Genetics Analysis, Penn State University, Pennsylvania, USA) was used to align the sequences by Clustal W. The neighbor-joining method was used to construct the phylogenetic tree based on a maximum likelihood estimate. The phylogenetic tree was built using 1000 bootstrap replicates to meet the confidence level of the phylogeny test. By comparing the sequences with the retrieved references from the GenBank database, the sequence homology of the ORF2-VP2 region of PARV4 was determined.

2.10. Statistical analysis

SPSS version 25 (SPSS Inc., Chicago, IL, USA) and RStudio 1.1 (RStudio PBC, Boston, Massachusetts, USA) were used to analyze the results. Preason's chi-square test evaluated the association between PARV4 viremia and HTLV-I/II infection. The minimal threshold of significance was set at $P < 0.05$.

3. Results

3.1. Donors' demographic data

The demographic profile of participants in this study revealed that the mean age and standard deviation for all participants was 38.48 ± 9.74 years, ranging from 20 to 63 years old. Among the healthy blood donors, male participants constituted 46.1 %, with an average age of 38 (20–63 years). Correspondingly, female donors comprised 3.9 % of the sample, with an average age of 45 (Range: 24–60 years). The comparison presented in Table 1 indicates no statistically significant distinction between the mean ages and the HTLV-I/II infection rates determined by both ELISA and Western Blot tests ($p = 0.185$, 0.608, respectively).

Regarding gender-based analysis, a notable association between gender and HTLV-I/II infection is evident in the ELISA Test ($p = 0.019$), while such a significant connection is not observable in the context of the Western blot analysis ($p = 0.559$). Notably, the risk of infection is considerably higher among females than males (70 % vs. 47 %; $p = 0.019$).

Significant associations between donation status and HTLV-I/II seropositivity rates are evident through ELISA and Western blot tests ($p < 0.001$). Among the seropositive cases, a substantial proportion (76.9 % and 87.1 % as determined by ELISA and Western blot tests, respectively) were identified among first-time donors. This information is presented comprehensively in Table 1. It is noteworthy that HTLV-I/II seropositivity rates were observed as follows: 90 % (81 out of 90) for first-time donors, 6.6 % (6 out of 90) for repeat donors, and 3.3 % (3 out of 90) for regular donors.

In total, the count of donors suspected of HTLV-I/II infection was 115 out of 230 participants, representing a 50 % prevalence rate. Of the 115 donors who exhibited repeat reactivity in HTLV-I/II ELISA tests, 78.3 % (90 out of 115) tested positive upon confirmation through Western blot analysis.

3.2. Bioinformatics studies for supplying PARV4 positive control

To find a conserved region that covers all genotypes of PARV4, we checked and aligned about thirty sequences related to PARV4 open reading frame 2 by omega online software (https://www.ebi.ac.uk/Tools/msa) that retrieved their sequences from GenBank.

Table 1
HTLV-I/II seropositivity rates based on age, gender, and the history of donation among healthy blood donors and suspected HTLVI/II infected donors, by both ELISA test (n = 230) and confirmatory test of Western blot (n = 115).

Characteristics	Test					
	Repeated HTLV-I/II ELISA test			HTLV-I/II Confirmatory Western Blot Test		
	Test result	N (%) 230(100)	*P*-value*	Test Result	N (%) 115 (100)	*P*-value*
Age (mean ± SD)	Suspicious	39.3 ± 10.2	0.185	Positive	39.6 ± 10.6	0.608
	Healthy	37.6 ± 9.3		Negative	38.5 ± 8.8	
Sex	Suspicious	21(70)	0.019	Positive	18(85.7)	0.559
Female	Healthy	9(30)		Negative	3(14.3)	
Male	Suspicious	94(47)		Positive	72(76.6)	
	Healthy	106(53)		Negative	22(23.4)	
Donation Status	Suspicious	93(76.9)	<0.001	Positive	81(87.1)	<0.001
First time	Healthy	28(23.1)		Negative	12(12.9)	
Repeat	Suspicious	13(48.1)		Positive	6(46.2)	
Regular	Healthy	14(51.9)		Negative	7(53.8)	
	Suspicious	9(11)		Positive	3(33.3)	
	Healthy	73(89)		Negative	6(66.7)	
Total	Suspicious	115(50)	–	Positive	90(78.3)	–
	Healthy	115(50)		Negative	25(21.7)	

*Chi-square test.

These sequences include (DQ873387.1, EU546204.1, EU546211.1, EU546210.1, AY622946.1, AY622949.1, DQ873386.1, GU120197.1, (AY622951.1) for genotype 1; for genotype 2: (KM390024.1, EU175872.1, EU175856.1, KJ5411120.1, KJ541119.1, KJ541121.1, HQ593532.1, EU546206.1, HQ593531.1 EU546205.1) and for genotype 3: (KU871315.1, KU871314.1, JN798200.1, JN798199.1, JN798197.1, JN798196.1, JN798195.1, JN183936.1, JN183935.1, JN798194.1). The conserved region (2800–3500 nucleotides) was more suitable for all available genotypes. Finally, a 328 bp conserved region covering the VP2 gene was selected for gene synthesis as a template for downstream application.

3.3. Analytical performance of PCR testing for detecting human PARV4

Also, Real-time PCR was launched with the protocol described in the materials and methods. A standard curve with serial dilution (10^1-10^9 copies/μL) of cloned PARV4 was established. The linearity test was determined to be 10^2-10^6 copies/μL by four replicates for each dilution; the results are depicted. The standard plasmid curve had the R2-value = 0.97 and a slope of −3.006, performing inter- and inter-assays of 5.8 % and 6.03 % as part of the validation assay, respectively (Tables 2 and 3). PARV4 Viral load quantification was determined by real-time PCR. All PARV4-positive Samples showed a viral load between 4.22×10^4 - 1.26×10^6 DNA copies/mL of serum. The limit of detection (LOD) was evaluated by probit analysis. It was 1.5×10^1 copies/μl, demonstrating a valid assay for PARV4 detection, just as most investigations in this field revealed that the probable detection limit for PARV4 using PCR and qPCR techniques is 1–10 copies/mL [17–19].

3.4. PARV4 PCR validation test

3.4.1. Specificity of the Realtime-PCR

To estimate the specificity of the designed primers, probes, and the whole procedure, real-time PCR was done on some blood-borne viruses such as HBV, HCV, HIV, HTLV-I/II, and HCOV-SARS genomes. No positive signal in amplification with the above-mentioned viral genome was seen. In addition, as our clinical sample was human sources, we checked our real-time PCR with a pooled human genomic DNA. No specific signal at the end of the reaction was detectable.

3.4.2. Sensitivity of the Realtime-PCR

The assay's sensitivity was determined by serial dilution of a known concentration of pUC57-PARV4 plasmid stock. Three replicates for each concentration (2×10^1 to 2×10^3) were implemented. The standard deviation of the test was 0.83, and the coefficient of variation was 2.8%. Regression analysis showed R2:0.93 with an efficiency of 99%.

3.5. The coinfection rate of PARV4 and HTLV-I/II among suspected and confirmed HTLVI/II infected donors

The DNA extracted from both healthy blood donors (N = 115) and HTLV-I/II infected blood donors (N = 115) was subjected to amplification using the established real-time PCR method outlined in the materials and methods section. As illustrated in Table 4, the coinfection rate of PARV4-DNA among the suspected HTLV-I/II infected donors amounted to 13 % (15 out of 115), revealing a significant association with the donors' status ($p = 0.038$). Likewise, the coinfection rate of PARV4-DNA within the subset of HTLV-I/II confirmed infected donors stood at 14.4 % (13 out of 90), with no observed association with donation status ($p = 1.0$).

Remarkably, a complete absence of PARV4 viremia was detected within 115 healthy blood donors.

3.6. Sequencing and phylogenetic analysis

Three sequences of PARV4 were aligned using the BLAST tool (GenBank, NCBI). The results revealed high similarity (up to 98.9 %) with human parvovirus 4 isolate T609 from India with accession number KM390024. The results of the phylogenetic tree showed genotype 2 of PARV4. The sequences of three isolates were submitted to GenBank and accession numbers (MZ681469.1, MZ681470.1, and MZ681471.1). The partial phylogenetic trees are depicted in Fig. 1.

Table 2
Cycle Threshold (C.T.) Values of pUC57-PARV4 Plasmid by real-time PCR in four replicates for each concentration.

pUC57-PARV4 plasmid	Replicates CT Value	
	Observed CT of Run (Mean ± S.D.)	CV (%)
Negative	0	0
STD2	29.02 ± 0.97	3.36
STD3	24.22 ± 0.44	1.82
STD4	21.18 ± 0.60	2.86
STD5	19.58 ± 0.63	3.21
STD6	16.31 ± 0.95	5.82

Table 3
Inter- and Intra-assay and Reproducibility experiments of the pUC57-PARV4 plasmid.

pUC57-PARV4 plasmid	Inter-assay CT values		Intra-assay CT values	
	C.T. of Run (Mean ± S.D.)	CV (%)	C.T. of Run (Mean ± S.D.)	CV (%)
STD2	29.25 ± 1.89	6.46	28.78 ± 1.88	6.55
STD3	26.24 ± 1.69	6.44	25.14 ± 1.37	5.45
STD4	23.62 ± 2.86	5.42	21.45 ± 1.43	6.66
STD5	18.67 ± 0.54	2.89	17.89 ± 1.66	5.30
STD6	16.49 ± 1.99	5.06	15.76 ± 1.04	6.62
STD7	12.70 ± 0.75	5.90	12.53 ± 0.70	5.59

Inter- and Intra-assay and Reproducibility experiments of pUC57-PARV4 plasmid (10^2-10^7 copies/μL) were performed in three replicates.

Table 4
The coinfection rate of PARV4 and HTLV-I/II, based on gender and donor status among the HTLV-I/II suspicious individuals (N = 115), and HTLV-I/II positive by confirmatory Western blot (N = 90).

Characteristics		HTLV-I/II-ELISA-Test Result	PARV4 qPCR			HTLV-I/II Confirmatory Western Blot Tests Result	PARV4 qPCR		
			Positive	Negative	*P-value**		Positive	Negative	*P-value**
Sex	**Female**	Suspicious	6(28.6)	15(71.4)	0.141	Positive	5(27.8)	13(72.2)	1.00
	Male	Healthy	0(0.0)	9(100)		Negative	1(33.3)	2(66.7)	0.680
		Suspicious healthy	9(9.6)	85(90.4)		Positive	8(11.1)	64(88.9)	
			0(0.0)	106(100)		Negative	1(4.5)	21(95.5)	
Donor status	**First time**	Suspicious healthy	13(14)	80(86)	0.038	Positive	12	69(85.2)	1.00
			0(0.0)	28(100)		Negative	(14.8)	11(9.6)	
							1(2.5)		
	Repeated	Suspicious healthy	1(0.4)	12(5.2)	0.481	Positive	0(0.0)	6(100)	1.00
			0(0.0)	14(6.1)		Negative	1(14.3)	6(85.7)	
	Regular	Suspicious healthy	1(0.4)	8(3.5)	0.110	Positive	1(0.9)	2(1.7)	0.333
			0(0.0)	73(31.7)		Negative	0(0.0)	6(5.2)	
Total		Suspicious healthy	15	100	<0.001	Positive	13	77(85.6)	0.518
			(13.0)	(87.0)		Negative	(14.4)	23(92)	
			0(0.0)	115(100)			2(8)		

*Chi-square test or, if needed, Fisher exact test.

4. Discussion

HTLV-I/II is an endemic virus with a high frequency in the province of Khorasan Razavi in Iran. Multiple studies indicate that the prevalence of HTLV-I/II in Khorasan provinces is significantly greater than in the other regions of Iran, with an average rate of 2–3 %. HTLV-I/II infection is still a public health concern in this province [20–24].

According to IBTO guidelines, all donated blood samples undergo lab testing after strict donor selection to screen blood-borne diseases such as HBV, HCV, HIV, Syphilis, and HTLV I/II (only in 7 provinces) at each blood center. An HTLV I&II total Abs primarily evaluates the screening of anti-HTLV-I/II antibodies in BTCs. The Western blot technique (MP. Biomedical Asia Pacific Pte. Ltd., Singapore) is recommended by the FDA as a confirmatory test for all initially reactive results of the HTLV I&II Elisa test [25]. In 1995, all donated blood samples were regularly screened for antibodies against HTLV-I/II and then confirmatory tests; Western blot analysis was launched in northeastern Iran. Then, these tests extended to 7 endemic areas out of 31 provinces in the north and northeast of Iran, including Khorasan Razavi, our studied site [26,27]. Recent reports have shown that the prevalence of HTLV I/II in blood donors is about 7.5-fold less than in the Khorasan general population. Based on this study, the HTLV seroprevalence in blood donors is 0.28 %. This rate represents the low-risk blood donors following the improvement of the donor selection procedure and launching a software system for temporary or permanent deferral reactive blood donors in these endemic areas [27,28]. Highly qualified physicians with deep knowledge of the routes of HTLV I/II transmission can significantly impact a safe donor selection process in this area. The leukocyte reduction procedures, established long ago in IBTO, with the significant improvement of technical methods in IBTO's lab, are another reason for this success. So far, there are no documented cases of transfusion-transmitted HTLV-I/II anywhere in the country; IBTO regularly assesses the remaining risk of infection in the blood donation pool [27].

In the present study, for the first time, the presence of PARV4-DNA was screened in HTLV-I/II infected donors. The results indicated that 13 out of 90 HTLV-I/II confirmed infected donors (14.4 %) were positive for PARV4 by qReal-Time PCR. These 13 coinfected donors were deferred already. The coinfection rate of PARV4-DNA in the suspected HTLVI/II infected donors was 13 %, significantly associated with first-time donors($(p = 0.038)$. It is necessary to explain that the primary diagnosis of HTLV-I/II infection is based on detecting specific antibodies in serum by the ELISA method. Due to the cross-reactivity and a high false positive rate in these assays, confirmatory assays with high specificity are needed for samples that show repeatedly reactive results in the ELISA method. Therefore, the real coinfection rate of RAV4 and HTLV-I/II can be reported based on the results of HTLV-I/II positive Western blot and PARV4-DNA (14.4 %). It is well understood that PARV4 has transient viremia; anti-PARV4 was found in a significant percentage of HIV and

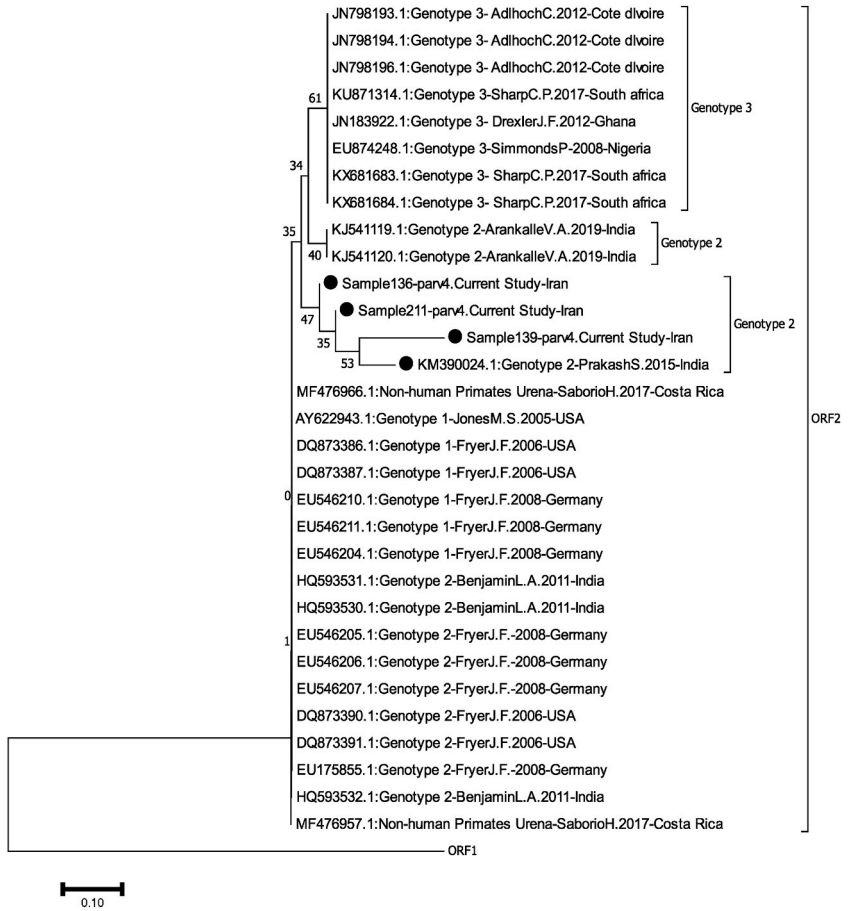

Fig. 1. Partial phylogenetic tree of human parvovirus 4 genome sequences based on ORF2-VP2 region. The phylogenetic tree of the isolates of PARV4 in the current study, in comparison to previous studies conducted in various parts of the world, based on the 123 bp PCR product, was constructed by the Neighbor-Joining method with Bootstrap 1000 using MEGA 7 software. It was genetically similar to genotype 2 of human PARV4 [29].

HCV-infected blood donors, but low viremia was reported [29]. In the current study, PARV4 causes high-titer viremia ($>10^6$ copies/mL) in blood donors, which was previously reported by other studies in individuals (10^2-10^{10} copies/mL) [7,30–32]. In a study by M. Touinssi et al., targeted blood donors' positive samples' viral load was lower than 500 copies/mL, similar to some plasma pools in the fryer study [32,33]. The PARV4 viral load among HTLV-infected individuals in Brazil was below 900 copies/mL [12]. The viremia phase can be considerable because it raises the possibility of coinfection with blood-borne viruses [19,34]. Furthermore, the PARV4 viral load is measured significantly in our study alongside a salient correlation between PARV4-DNA and HTLV-I/II ELISA-reactive samples (p-value <0.001), reinforcing this feasibility.

In the present study, the male gender had a significant relationship with the results of HTLV-I/II reactivity by ELISA test ($p = 0.019$). Still, there was no significant relationship with the results of the Western blot test ($p = 0.559$). A significant association between donation status with HTLV-I/II ELISA and Western blot tests was observed (P value < 0.001); a high percentage of seropositive individuals were first-time donors. Many IBTO reports have shown that "first-time" blood donors are more likely to be HTLV-I/II seropositive than repeat and regular donors [27,35–39]. One potential factor contributing to the higher prevalence of blood-borne infections among first-time blood donors could be the increased likelihood of engaging in risky behaviors within this group.

Additionally, there might be instances where first-time donors decide to donate blood with motivations such as assessing their health status or deriving health benefits from donation. In contrast, regular blood donors typically understand risk factors better due to their consistent participation in blood donation activities and engagement with educational programs. As a result of their enduring involvement in blood donation, these donors exhibit heightened awareness of blood safety concerns [27].

On the other hand, because of the deferral strategy for reactive HTLVI/II donors, as mentioned above, the suspicious donors are temporarily or permanently deferred from blood donations. So, in light of this evidence, the rate of reactivity in repeatdonors compared to first-time donors significantly decreased based on screening tests [40]. Indeed, this highlights the precise implementation of the donor selection guidelines within the Iranian blood transfusion organization.

A molecular survey of ORF1 and ORF2 shows that ORF2 is more conserved than ORF1 in pairwise comparisons of PARV4 genotype

strains and amino acid sequences among genotypes, indicating a higher degree of similarity in ORF2 than ORF1, which makes it the better choice for developing positive control and primers [4,41]. In our study, due to the unavailability of a confirmed serum sample containing the PARV4 genome as a positive control for diagnosis, a positive control plasmid from a high-homological VP2 region in ORF2 of PARV4 (328 bp in pUC57-PARV4 Plasmid) was developed; for optimizing PCR assays, determining the minimum detectable virus (10 copies/mL); and detecting all genotypes of PARV4 simultaneously; The analytical sensitivity, specificity, LOD, and repeatability of real-time-PCR were determined to validate the standard plasmid. The standard plasmid curve had the R2-value: 0.97 and a slope of −3.006 with inter- and inter-assays of 5.8 % and 6.03 %, respectively. The LOD 1.5×10^1 copies/μl demonstrated a valid assay for PARV4 detection, same as most investigations in this field revealed that the probable limit of detection for PARV4 using PCR and qPCR techniques is 1–10 copies/mL [17–19]. Technically, molecular methods such as TaqMan Real-time PCR are more valuable than serological methods and conventional PCR assays because they show the virus's active infection and can determine the number of viral copies that have an essential role in the spread of the virus. Notably, our PCR validation assays have underpinned our sequencing and genotyping results based on phylogenetic analysis. The qReal-Time PCR based on a newly designated plasmid for the first time in Iran was developed to identify three genotypes of PARV4 concurrently using in-house designed primers and probes, which is comparable to previous studies published in 2020 and E. Väisänen's method in 2014 [42,43]. As a result, this method will reduce diagnostic costs if they are employed in the future.

Three genotypes of PARV4 have been identified worldwide. Genotype 2 was discovered alongside genotype 1, previously identified in Europe and North America and distributed throughout the world. Genotype 2 is the dominant pattern in India [2,10,18,32,42,44–46]. The nucleotide similarity is observed in the three different genotypes of human PARV4; just 8–9% of the nucleotide sequence differs between genotypes, as in the nonstructural (N.S.) of ORF1 and capsid encoding sequences of ORF2 [4]. The nucleotide sequences obtained by the sequencing method in our study were most similar to the strains found in India (KM390024) [46] (Fig. 1). As a result, the sequences of isolates of human PARV4 in this study with accession numbers (MZ681469.1, MZ681470.1, and MZ681471.1) have been recorded in GenBank and are identified as genotype 2.

Several studies, including the following: registered sequences in GenBank (HQ593530 and HQ593532), have supported our findings in GenBank [12,30]. However, the ORF1 region was the only focus of earlier Iranian studies [3,47,48] and reported that PARV4 genotype 1 was the dominant strain [3,47]. Furthermore, a recent study in China on plasma pools reported genotypes 1 and 2 in Chinese donor serums [17]. As a result, it should be emphasized that Iran can have genotypes similar to those found in Europe and the United States [32,49].

Studies show that Parvovirus 4 has the potential to persist in tissues and undergo re-infection after a while, the best-described example being papillomaviruses [1,50] considering that, according to current research, viruses such as HIV, HCV, parvovirus B19, and HTLV-I can induce persistent infection and T-cell exhaustion [51–54]. This evidence has led to the conclusion that, while not considered a direct cause, Parvovirus 4 can operate as an initiator of a compromised immune system, particularly in locations where other blood-borne viruses (HTLV-I/II) are endemic, leading to persistent infection and T-cell depletion, causing immunological modulation and making the body prone to various diseases.

5. Conclusions

For the first time, we evaluated the coinfection rate of HTLV-I/II and PARV4 viruses in Iranian blood donors. We detected a significant titer of PARV4-DNA among HTLV-I/II positive donors. This finding suggests that there may be a potential association between HTLV-I/II and PARV4 infections. Studies have shown that coinfection with human parvovirus 4 and HTLV-I/II is rare. However, it is still important to screen for both viruses in blood transfusion programs to minimize the risk of transmission, especially in the endemic area. The frequency of coinfection may vary depending on the population being screened and the prevalence of each virus in that population. PARV4 is resistant to inactivation strategies applied in blood transfusion, and its inactivation has not yet been addressed [55]. Therefore, this infection can still be a cause for concern.

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Data availability statement

The sequences of three isolates in this study were deposited in the GenBank database (MZ681469.1, MZ681470.1, and MZ681471.1). The supporting data for the findings presented in this study can be obtained from the corresponding author upon request.

CRediT authorship contribution statement

Hooman Ramezany: Investigation, Software, Writing – original draft. **Maryam Kheirandish:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Project administration. **Zohreh Sharifi:** Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Shahram Samiee:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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