Research Article

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Prevalence of Hepatitis C Virus and Human Pegivirus Type 1 Co-Infection in Patients Infected with Human Immunodeficiency Virus Type-1

İnsan İmmün Yetmezlik Virüsü Tip 1 ile Enfekte Hastalarda Hepatit C Virüsü ve İnsan Pegivirüs Tip 1 Ko-Enfeksiyonlarının Prevalansı

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ABSTRACT

Objectives: Human immunodeficiency virus (HIV) infection remains a global health concern. As individuals infected with HIV struggle with the complexities of their condition, the coexistence of additional pathogens can significantly alter the course of the disease. This study aimed to determine the prevalence of hepatitis C virus (HCV) and human pegivirus type 1 (HPgV-1) co-infection in patients with HIV-1 infection using an in-house developed multiplex real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) assay.

Materials and Methods: This cross-sectional study included 113 HIV-1-positive patients. The HIV-1 load was evaluated using the Artus HI Virus-1 RG RT-PCR Kit in serum samples. Subsequent to the assessment of optimal annealing temperature, primer-probe concentration, analytical sensitivity, and endpoint sensitivity, selected primer-probe sets for HCV, HPgV-1, and ribonuclease P were recruited to identify co-infections.

Results: Of the 113 HIV-1-positive individuals, 24% were female and 76% were male. Interestingly, 74% of the patients had no history of addiction. Optimization of the in-house developed RT-

ÖZ

Amaç: İnsan immün yetmezlik virüsü (HIV) enfeksiyonu küresel bir sağlık sorunu olmaya devam etmektedir. HIV ile enfekte kişiler, durumlarının karmaşıklığıyla mücadele ederken, ek patojenlerin bir arada bulunması, hastalığın seyrini önemli ölçüde değiştirebilir. Bu çalışma, şirket içinde geliştirilen multipleks kantitatif gerçek zamanlı polimeraz zincir reaksiyonu testi (RT-qPCR) kullanılarak HIV-1 ile enfekte hastalarda hepatit C virüsü (HCV) ve insan pegivirüs tip 1 (HPgV-1) ko-enfeksiyonlarının prevalansını incelemeyi amaçladı.

Gereç ve Yöntemler: Bu kesitsel çalışmaya 113 HIV-1-pozitif hasta dahil edilmiştir. HIV-1 yükü serum örneklerinde Artus HI Virus-1 RG RT-PCR Kiti kullanılarak değerlendirildi. Optimum tavlama sıcaklığı, primer-prob konsantrasyonu, analitik hassasiyet ve uç nokta hassasiyetinin değerlendirilmesinin ardından, ortak enfeksiyonları tanımlamak için HCV, HPgV-1 ve ribonükleaz P için seçilen primer-prob setleri kullanıldı.

Bulgular: HIV-1 pozitif olan 113 kişiden %24'ü kadın, %76'sı erkekti. İlginçtir ki hastaların %74'ünde bağımlılık öyküsü yoktu. Şirket içinde geliştirilen RT-qPCR testinin optimizasyonu, kabul edilebilir bir verimlilik ve 287 kopya/µL tespit sınır ile doğrusal bir

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qPCR test revealed an acceptable efficiency and a linear dynamic range with a limit of detection of 287 copy/µL. HCV was detected in five patients (4.43%), whereas no HPgV-1 was detected.

Conclusion: More than 74% of the participants had no history of addiction, which may explain the differences in the reported prevalence of HCV/HIV co-infection worldwide and in Iran. Findings of the present study are consistent with the prevalence reported for the general population (4%). In the present study, HPgV-1 was not detected in the collected samples, which is consistent with reports from Iran (a range of 0-26%).

Keywords: HIV, HCV, HPgV-1, viral load, RT-qPCR

dinamik aralık ortaya çıkardı. Beş hastada (%4,43) HCV saptanırken, HPgV-1 saptanmadı.

Sonuç: Katılımcıların %74'ünden fazlasının bağımlılık geçmişi olmadığından, bu durum dünyadan ve İran'dan bildirilen HCV/HIV ko-enfeksiyonu prevalansındaki farklılıkları açıklayabilir. Bu çalışmanın bulguları genel nüfus için bildirilen yaygınlık (%4) ile tutarlıdır. Bu çalışmada, İran'dan gelen raporlarla tutarlı olarak (%0-26 aralığı) toplanan örneklerde HPgV-1 tespit edilmemiştir.

Anahtar Kelimeler: HIV, HCV, HPgV-1, viral yük, RT-qPCR

Introduction

Human immunodeficiency virus (HIV) infection remains a global health concern, challenging scientists from all aspects of virology, immunology, molecular biology, medicine, pharmacology, and socioeconomic fields are challenging. First identified in the early 1980s, despite remarkable efforts in research and medical interventions, the HIV pandemic has continued, and no promising short-term or long-term solutions are expected in the near future (1). As a lentivirus belonging to the Retroviridae family, HIV primarily targets macrophages and CD4+ T cells of the immune system, progressively compromising the immune system's ability to develop an effective response against infections and malignancies. From the initial acute infection to the chronic stages and, potentially, the development of acquired immunodeficiency syndrome, the disease spectrum poses diverse challenges to affected individuals and healthcare providers. The most outstanding progress in disease management has been the advent of antiretroviral therapy, which has transformed HIV infection from a notorious incurable infectious disease into a manageable chronic condition with an approximate normal life expectancy (2).

The dynamic interplay between HIV and co-infections has been a critical aspect in understanding the multifaceted nature of the disease and its impact on affected individuals. Co-infections introduce a mixture of challenges that involve the already compromised immune system of HIV-infected patients. As individuals infected with HIV struggle with the complexities of their condition, the coexistence of additional pathogens, such as bacteria, viruses, and parasites, can significantly alter the course of disease progression, treatment outcomes, and overall health. Understanding the interplay between HIV and these co-pathogens is critical not only for comprehensive patient care but also for developing effective prevention and treatment strategies (3).

The coexistence of HIV and hepatitis C virus (HCV) in a patient represents a more complicated medical intersection. As a member of the hepacivirus genus from the Flaviviridae family, HCV is a small spherical enveloped virus that contains positive-sense single-stranded genomic RNA. The virus has a specific tropism for hepatocytes, and its replication and pathogenesis may lead to liver dysfunction, fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC). Although the advent of direct-acting antiviral agents has revolutionized HCV treatment and has offered new hope for improved outcomes in this complex population, understanding the specific challenges posed by HCV in the context of HIV-infected individuals requires a comprehensive exploration of the clinical manifestations, treatment paradigms, and intertwined epidemiology (4). HIV and HCV, both blood-borne pathogens, share common routes of transmission, creating a substantial overlap in the populations affected by these viruses. The synergy between these two viruses not only puts more pressure on the immune system but also increases the risk of severe liver disease progression to cirrhosis and/or HCC (5,6).

High coinfection rates have also been reported for human pegivirus type 1 (HPgV-1) in patients with HIV and HCV infection. This member of the pegivirus genus within the Flaviviridae family has similar virion and genomic characteristics as HCV. The virus can induce persistent infection that is not associated with hepatitis or other obvious clinical symptoms or diseases in healthy individuals (7). Several studies have indicated that persistent infection with HPgV-1 is associated with slower disease progression in not only HIV-infected patients but also other viral infections (8,9). The beneficial effect of persistent HPgV-1 infection may be associated with the inhibition or reduction of abnormal/excessive immune activation, especially in T lymphocytes (8). Understanding the interplay between HPgV-1 and HIV via the same transmission route also requires comprehensive investigations of the disease course, treatment outcomes, and molecular epidemiology.

Therefore, the aim of the present study was to determine the prevalence of HCV and HPgV-1 co-infection in patients with HIV-1 infection using an in-house developed multiplex real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) assay. HCV co-infection was found in less than 5% of the samples, whereas no HPgV1 positivity was identified in this group.

Materials and Methods

Study Population

This cross-sectional study included 113 HIV-1-positive patients who were referred to the Reference Laboratory of the Iran University of Medical Sciences, Tehran. The study was approved by the Research Ethics Committee of the Faculty of Medicine at the Iran University of Medical Sciences (approval number: IR.IUMS.FMD.REC.1401.517, date: 21.01.2013). A consent form was signed by all participants or their legal representative.

Sample Collection

Five microliters of fresh blood were collected from each HIV-1-positive individual in anti-coagulant tri-potassium ethylenediaminetetraacetic acid-containing tubes. Samples were centrifuged at 7000 g for 10 min, and the serum was stored at 20 ℃ until use.

Nucleic Acid Extraction

HIV-RNA was extracted from serum samples using a Zybio Semi-Automatic Nucleic Acid Extraction Kit (Zybio, Shenzhen, China) according to the manufacturer's instructions. Two hundred microliters of the serum samples were extracted, and RNA was eluted into a volume of ~50 µl. The extracted RNA was stored at 80 ℃ until further analysis.

HIV Load Assessment

The HIV-1 RNA load was detected using the Artus HI virus-1 RG RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, RT-PCR was performed on 10 µL extracted nucleic acid in a 25 µl reaction volume. The following thermal profile was programed for Rotor gene Q (Qiagen; Germany) software: First hold for reverse transcription at 50 ℃ for 30 min, second hold for DNA polymerase activation at 95 ℃ for 15 min and 50 amplification cycles of 30 s at 95 ℃, 60 s at 50 ℃ and 30 s at 72 ℃.

Assessment of the Multiplex Assay

Primer-Probe Sets

Primer-probe sequences for the detection of HCV were adapted from Chen et al. (10), for the detection of HPgV-1 was adapted from Schlueter et al. (11) and for the internal control ribonuclease P (RNase P) was adapted from our previous study (12). Selected primer-probe sets (Table 1) were further validated by a Basic Local alignment search tool analysis for analytical specificity (in Silico testing) and the OligoAnalyser Tool (Integrated DNA Technologies; USA) for secondary structures and primer dimer formation.

Annealing Temperature Optimization

To determine the most appropriate annealing temperature, a gradient test was performed for each primer set over a temperature range of 55 ℃-62 ℃. The reaction mixture contained 10 µL of SYBR green master mix (Ampliqon; Denmark), 1 µL of each forward and reverse primer (10 nM), and 6 µL of distilled water. Two microliters of the control plasmid were added to each reaction, resulting in a final volume of 20 µL. The following thermal profile was applied on a QIAquant real-time PCR thermal cycler: 10 min at 95 ℃ and 40 amplification cycles of both 15 s at 95 ℃ and 45 s at 55-62 ℃. Data acquisition was programed for the green channel [fluorescein amidite (FAM)] at the end of each annealing/extension step. The results were analyzed for lower Cq, higher signal-to-noise ratio, normal amplification plot, and the absence of unintended PCR amplicons (through a melt curve analysis at the end of the amplification cycles).

Optimization of Primer-Probe Concentration

For each primer-probe set, a concentration matrix test with 500, 250, and 125 nM concentrations of each primer and 500, 400, 300, 200, and 100 nM concentrations of each probe were investigated for lower Cq, higher signal-to-noise ratio, and normal amplification plots. The test was performed by 5 µL of 4X capital 1-step RT quantitative reverse transcription PCR (qRT-PCR) probe master mix (biotechrabbit, Germany), 1 µL RTase with RNase inhibitor (RT-RI), 1 µL of each primer dilution, 1 µL of probe dilution, and 9 µL of distilled water. Two microliters of the control plasmid were added to each reaction, resulting in a final volume of 20 µL. Duplicate reactions were performed in a QIAquant real-time PCR thermal cycler with the following cycling conditions: 10 min at 95 ℃ and 40 cycles of both 15 s at 95 ℃ and 45 s at 55 ℃. At the end of the annealing/extension step, data acquisition was programed on channel green (FAM), orange [carboxy-X-rhodamine (ROX)], and red [cyanine 5 (Cy-5)].

Analytical Sensitivity (Efficiency, Linearity) and Endpoint Sensitivity Limit of Detection (LOD)

Eight dilutions of the synthesized control plasmid were tested in quadruplicate over two runs to determine the efficiency, linearity, and LOD (13). The slope of the log-linear portion of the calibration curve was used to assess the amplification efficiency. The highest to lowest quantifiable copy numbers were analyzed for the linear dynamic range. The test LOD was defined as success in at least three out of four amplification reactions with the lowest dilution of the control plasmid.

Multiplex RT-qPCR

Multiplex RT-qPCR reaction mixture was prepared by including 6.25 µL of 4x capital 1-step qRT-PCR probe master mix

Table 1. Sequences of the primer-probe sets used for target detection

Virus	5′-UTR	bp 62	F	GCCTTGTGGTACTGCCTGAT
			R	TGCACGGTCTACGAGAC
			P	FAM-CCGGGGCACTCGCAAGCACCC-BHQ1
HCV	5′-NCR	bp 186	F	CGGCCAAAAGGTGGTGGATG
			R	ACGACGAGCCTGACGTCGG
			P	ROX-TGGTAGCCACTATAGGTGGGTC-BHQ2
Internal control	RNase P	bp 65	F	AGATTTGGACCTGCGAGCG
			R	GAGCGGCTGTCTCCACAAGT
			P	Cy5-TTCTGACCTGAAGGCTCTGCGCG-BHQ2

HCV: Hepatitis C virus, RNase P: Ribonuclease P, 5′UTR : 5′ untranslated region, 5′-NRC: Non-coding region

(biotechrabbit, Germany), 1.25 µL RT-RI, 6 µL of 1:4:1 primer-probe mixture (HCV: HPgV-1: RNase-P), and 1.5 µL of distilled water. Ten microliters of the extracted RNA were added to each reaction, resulting in a final volume of 25 µL. A temperature profile of 50 ℃ for 30 min, 95 ℃ for 10 min, and 40 amplification cycles of 15 s at 95 ℃ and 45 s at 55 ℃ was programed for the Rotorgene Q real-time PCR machine. Data acquisition was performed at the end of the annealing/extension step on channel green (FAM) for HCV, orange (ROX) for HPgV-1, and red (Cy-5) for RNAse-P.

Statistical Analysis

SPSS version 22.0 (IBM SPSS Statistics, Chicago, IL, USA) was used for all statistical analyses. To compare proportions, Fisher's exact test was applied. For variables with normal distribution and variables without normal distribution, the independent t-test and Mann-Whitney U test was selected, respectively, to compare means/medians between groups. A p-value of less than 0.05 was considered statistically significant.

Results

Patients Characteristics

Of the 113 HIV-1-positive individuals, 24% were female and 76% were male. The mean (± standard deviation) age of the participants was 41.38 (±10.14) in women and 44.51 (±12.25) in men. The majority of patients (~40%) were in the age group of 31-40 years, followed by the age group of 41-50 years (~27%). Approximately 74% of patients had no history of addiction. Most patients (82.3%) had a viral load of 500,000 international unit/mL. The characteristics of the participants are summarized in Table 2.

Multiplex Test Performance Evaluation

The optimum annealing temperature of the primers was selected at 55 ℃ by analyzing the performance of the primers using a gradient PCR assay. The combination of forward primer, reverse primer, and probe ratio was chosen as 500:500:500 nM for all targets, and the ratio of primer-probe mixture in the multiplex test was chosen as 1:4:1 for HCV, HPgV-1, and RNase P, respectively.

Table 2. Characteristics of patients with HIV-1 infection in this study

Variables		Female	Male	Total	p-value
Number of patients		27 (24.1%)	86 (75.9%)	113	
Age ± SD		41.38 (±10.14)	44.51 (±12.25)	43.73 (±11.74)	N.S
Age groups (years)					
	0-20	1 (50%)	1 (50%)	2 (1.8%)	
	21-30	2 (50%)	2 (50%)	4 (3.5%)	
	31-40	15 (32.6%)	31 (77.4%)	46 (40.7%)	N.S
	41-50	6 (20%)	24 (80%)	30 (26.6%)	
	51-60	2 (10%)	18 (90%)	20 (17.7%)	
	>60	2 (18%)	9 (82%)	11 (9.7%)	
Education level					
	Illiterate	2 (33.3%)	4 (66.7%)	6 (5.3%)	
	Elementary school	2 (15.4%)	11 (84.6%)	13 (11.5%)	
	Middle school	9 (31%)	20 (69%)	29 (25.7%)	N.S
	High school	10 (32.3%)	21 (67.7%)	31 (27.4%)	
	University	7 (20.6%)	27 (79.4%)	34 (30.1%)	
Marital status					
	Permanent	15 (25.4%)	34 (74.6%)	59 (52.2%)	
	Single	2 (5.3%)	36 (94.7%)	38 (33.6%)	N.S
	Divorced	6 (50%)	6 (50%)	12 (10.6%)	
	Temporary	4 (100%)	0 (0%)	4 (3.6%)	
Addiction					
	No	24 (28.6%)	60 (71.4%)	84 (74.3%)	
	Yes	5 (20%)	20 (80%)	25 (22.1%)	N.S
	Unknown	0 (0%)	4 (100%)	4 (3.6%)	
Viral load (before treatment) IU/mL; mean (range)		154933 (0-1315565)	1329137 (0-26464483)	1035586 (0-26464483)	N.S
Viral load (after treatment) IU/mL; mean (range)		322 (0-3751)	381 (0-3862)	366 (0-3862)	N.S
CD4 count (cells/mm^3)		470.31 (316.98)	367.26 (±289.31)	396.17 (±297.22)	N.S

HCV: Hepatitis C virus, RNase P: Ribonuclease P, 5'UTR : 5' untranslated region, 5'-NRC: Non-coding region, bp: Blood pressure

The results revealed an acceptable efficiency and a linear dynamic range for all targets (HCV: R^2=0.98 and E=0.99, HPgV-1: R^2=0.97 and E=1.04, RNase-P: R^2=0.98 and E=1). Moreover, a scrutiny of the test LOD showed that this multiplex RT-qPCR assay is capable to detect 287 copy/µL of the target in each reaction.

Prevalence of HCV and HPgV-1 Co-Infection

Of the 113 HIV-1-positive samples, HCV was detected in five patients (4.43%). In the present study, HPgV-1 was not detected in HIV-1 infected individuals.

Discussion

HIV infection continues to pose many challenges to physicians, pharmacists, psychologists, politicians, and researchers. The virus has perfectly adapted to its host to ensure its persistence in the human population. In addition to all complexities of the virus-host interactions per se, co-infections add another level of difficult-to-manage conditions for patients and their physicians (14). In the present study, the prevalence of HCV and HPgV-1 co-infection was investigated in HIV-1-infected individuals using an in-house developed RT-qPCR. The results revealed co-infection with HCV in less than 5% of the patients, whereas HPgV-1 was not detected in the sample population.

In a study performed by Zahra et al. (15), they reported 60-80% frequency of HCV infection in HIV-1 infected patients in four different cities of Pakistan. Their sample population included only male patients with a history of intravenous drug use (IDU). Schmidbauer et al. (16) also reported a prevalence of 11.1% for HCV co-infection among patients with HIV-1 infection in Austria. More than 63% of the study population were either IDUs or men who have sex with men (16). Teimoori et al. (17) also reported 58.7% positivity for HCV/HIV coinfection in Ahvaz, Iran. They reported that the most common route of transmission (99.1%) among their patients was IDU, and 97.8% of the subjects had a history of imprisonment. In the present study, samples were collected from patients referred to counseling centers for behavioral diseases. Since more than 74% of the patients in this study had no history of addiction (including IDU) or same-sex experience, this may explain the differences in the reported prevalence of 4.43% for HCV/HIV co-infection. Accordingly, the prevalence of HCV/HIV co-infection was consistent with that reported for general population samples by Platt et al. (4%) (18). In a systematic review and meta-analysis, Bagheri Amiri et al. (19) reported a zero prevalence of HCV/HIV co-infection in both the general population and healthcare workers, whereas 10.95% was reported for IDUs in Iran.

In an attempt to assess HPgV-1 prevalence in HIV-1-infected patients, de Miranda et al. (20) reported 17% positivity by performing conventional nested PCR. They also showed that HPgV-1 is associated with lower HIV-1 loads and higher CD4 counts. By recruiting a similar methodology, Alcalde et al. (21) reported a prevalence of 30% for HPgV-1/HIV co-infection and similar effects on viral load and CD4 count. Likewise, Li et al.(22) reported a prevalence of 9% for infection by these two viruses. Moreover, a prevalence of 2.3% was reported in healthy blood donors. In studies conducted in Iran, a range of 0-26% were reported for co-infection of HPgV-1 and HIV (23,24,25). In the present study, HPgV-1 was not detected in the samples. Considering the prevalence of HCV in the present study, which was approximately similar to that of the general population, it is plausible to expect an HPgV-1 prevalence close to that of healthy blood donors or the general population. On the other hand, the analytical sensitivity of 287 copy/µL for the in-house developed RT-qPCR assay may explain the inability of the test to detect HPgV-1. Another matter of concern is the type of samples used. Because growing evidence supports the presence/replication of hepatitis viruses as well as HPgV-1 in peripheral blood mononuclear cells (PBMCs) (8,9,26,27,28,29,30), the prevalence reported in the current study could be different if PBMCs had been subjected to nucleic acid extraction and RT-qPCR assay. However, further studies are required to validate this hypothesis.

Study Limitations

The present study had some limitations. Due to the scarcity of available financial support, the results were not re-checked using commercial kits. Therefore, no data is available for confirmed HPgV1-positive samples tested using this assay, and the power of this test for virus detection in clinical specimens is a matter of concern. Despite several reports on the high prevalence of HPgV-1 in HIV-infected patients, no positive samples were detected in the present study. An extensive literature review revealed that the prevalence of HPgV-1 is profoundly dependent on the characteristics of the studied population (21,22,23,24,25). The prevalence was highest among IDUs and lowest among the normal population. Evaluation of the demographic and epidemiological characteristics of the individuals who participated in this project revealed that the sample population resembled the general population, despite being infected with HIV-1. Therefore, the finding was explained based on this characteristic. Moreover, the LOD of the in-house developed test is another matter of concern that could be a reason for not finding HPgV-1-positive cases.

This article aims to highlight the current state of knowledge regarding the effects of coinfection on HIV-infected patients and to elucidate the intricate relationships between multiple infectious agents and the immune system. The intricate interplay of these viruses raises pivotal questions about optimal management strategies, potential synergies or antagonisms in their pathogenesis, and broader public health implications.

Conclusion

Evaluation of co-infections in the context of HIV infection is necessary for better patient management. Although most infectious agents exacerbate the condition, agents such as HPgV-1, may be beneficial for the host to combat other pathogens. However, more studies are required to support this hypothesis.

Ethics

Ethics Committee Approval: The study was approved by the Research Ethics Committee of the School of Medicine at the Iranian University of Medical Sciences (approval number: IR.IUMS.FMD.REC.1401.517 date: 21.01.2013).

Informed Consent: A consent form was signed by all participants or their legal representative.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Z.E., S.C., M.A., S.J.K., Concept: Z.E., F.B-S., M.K., S.J.M., Design: Z.E., F.B-S., M.K., S.J.M., Data Collection or Processing: Z.E., F.B., S.H.M., A.T., M.K., S.J.K., Analysis or Interpretation: Z.E., F.B., S.H.M., A.T., M.K., S.J.K., Literature Search: Z.E., Z.Y.G., S.J.K., Writing: Z.E., Z.Y.G., S.J.K.

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