Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(6): 183
https://doi.org/10.31083/j.fbl2706183

Original Research

Preliminary Study on the Possibility to Detect Virus Nucleic Acids in Post-Mortem Blood Samples

Stefania Turrina[1,*,†], Davide Gibellini[2,*,†], Giacomo Giannini[1], Anna Lagni[2], Erica Diani[2], Virginia Lotti[2], Giulia Soldati[1], Filippo Gibelli[1], Dario Raniero[1], Domenico De Leo[1]

[1]Department of Diagnostics and Public Health, Legal Medicine Section, University of Verona, 37134 Verona, Italy
[2]Department of Diagnostics and Public Health, Microbiology Section, University of Verona, 37134 Verona, Italy
*Correspondence: stefania.turrina@univr.it (Stefania Turrina); davide.gibellini@univr.it (Davide Gibellini)
† These authors contributed equally.
Academic Editor: Cristoforo Pomara
Submitted: 23 December 2021 Revised: 9 May 2022 Accepted: 20 May 2022 Published: 7 June 2022

Abstract

Background: In many forensic cases, the medical records of the deceased are not available at the time of the autopsy; therefore, no information about the deceased's state of health, including any infectious diseases contracted during life, is accessible. The detection of some of the principal viral infections, such as hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus type 1 (HIV-1), could contribute to determining causes of death and interesting applications could be found in medico-legal practice, such as occupational risk assessment. To date, accurate and sensitive serological and molecular assays capable of detecting these viruses have been validated on biological samples taken from living beings, while their efficiency on forensic post-mortem biological samples has yet to be thoroughly assessed. To further this aim, this study evaluated whether the nucleic acid amplification techniques (NAATs) for the detection of viral genomes that are applied in clinical settings can be used, with the same success rate, for these latter samples. **Methods**: Manual viral nucleic acid extraction processes and fully-automated amplification-based detection techniques developed in-house were evaluated on blood samples taken during the routine autopsies of 21 cadavers performed 2 to 9 days after death. Information on HBV, HCV, and HIV-1 seropositive status was previously known for only four of these cadavers. **Results**: Using automated quantitative real-time PCR (qPCR) and qualitative PCR (end-point) analyses, it was possible to confirm the presence of viral genomes in the four post-mortem whole blood samples with previously reported specific serological positivity. In addition, the genomes of HCV and/or HIV-1 genomes were detected in three other blood samples with unknown serological status at the time of autopsy. **Conclusions**: Therefore, our findings suggest that molecular assays may detect the presence of viral genomes in forensic post-mortem blood samples up to five days after death. This provides an additional means of investigation that can contribute to the determination of the deceased's cause of death.

Keywords: virus nucleic acids; nucleic acid amplification techniques; post-mortem blood samples; causes of death

1. Introduction

In forensic medicine, one of the main goals of post-mortem examination is to establish, to the greatest extent possible, the causes of death. Usually, to contribute to this objective, organs, tissues, and body fluids samples are collected during the autopsy to be submitted subsequently for in-depth toxicological, histopathological, microbiological, and genetic analyses.

In most cases, however, information on the deceased's health status, including any infectious diseases contracted during life, is not available at the time of the autopsy.

Consequently, analytical methods capable of directly detecting viral genomes in forensic biological samples would support the establishment of the cause of death. At the same time, they could be used as a tool to monitor the potential risk of unreported viral infections to which both forensic autopsy staff and those carrying out subsequent laboratory investigations may be exposed through direct contact with infected blood samples or indirect contact with infected autopsy equipment and devices (e.g., needles, scalpels, or saws) [1–9].

This study focuses on several clinically relevant blood-borne viral agents, including hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus type 1 (HIV-1), that may be present in forensic biological samples collected from cadavers (e.g., of those whose deaths were due to intravenous drug use).

Detection of HBV, HCV, and HIV-1 genomes or specific immune responses in biological samples collected from living subjects is typically performed using well-validated molecular methodologies and serological assays. Since forensic biological samples are collected after death, they exhibit different features from those obtained ante-mortem, which could potentially result in the failure of serological and molecular procedures developed for clinical purposes [10]. The progressive processes of post-mortem putrefactive alteration are responsible for more or less extensive damage to tissues and cellular structures; for ex-

ample, the processes of autolysis, haemolysis, and bacterial contamination of blood samples occur in the early post-mortem phases [11] with significant alterations of biological specimens. These post-mortem phenomena may adversely affect the analytical sensitivity, specificity, and repeatability of diagnostic methodologies used in clinical settings to detect the presence of viral agents, giving rise to misleading results and representing a limitation to their application in the forensic medicine field [12,13].

Serological testing is currently the most extensively used screening approach for diagnosing viral infections, but it displays some analytical drawbacks when applied to forensic post-mortem biological materials [12]. Autolysis, hemolysis, and blood clotting occur in blood samples acquired post-mortem and may negatively affect serum and plasma samples and alter detectable cell structures, thus affecting the analytical sensitivity and detection of antibodies to viruses by conventional serological tests [14].

Furthermore, the data provided by serology may represent either the immune signature of a past viral infection or the consequence of a vaccination procedure, and not necessarily an ongoing viral infection at the time of death [15–19]. Hence, the availability of alternative screening methodologies which are able to detect viral genomes in post-mortem forensic biological samples could provide a useful tool, especially if these methodologies can provide substantially more informative results [20,21].

Nucleic acid amplification techniques (NAATs) [22–25] represent an effective approach for this purpose. However, *ad hoc* analytical methods should be developed for their forensic field applications, particularly for the phase of pre-treatment, extraction, and purification of nucleic acids from biological samples to obtain the removal of any PCR inhibitors [26,27]. Although some authors have proposed molecular methods to detect viral genomes in forensic samples, there are currently few studies documenting their usefulness in this field [11,28–32].

Therefore, this study, which was performed on a small cohort of cadaveric biological samples representative of unselected routinely performed autopsies, aims to contribute to the development of analytical protocols for detecting HBV, HCV, and HIV-1 genomes through the use of specific quantitative and qualitative end-point real-time PCR or RT-PCR.

2. Materials and Methods

2.1 Sample Collection

Post-mortem peripheral blood samples were collected from the femoral or subclavian arteries of 21 cadavers (7 females and 14 males) who underwent autopsy examination between 2 and 9 days after death. The causes of death ascertained through the autopsies, supplemented by available circumstantial data, were: road accidents (4 subjects); self-induced dynamics (4 subjects); endogenous pathological processes (4 subjects); and overdose of illicit substances (9 subjects). At the time of autopsy, information regarding seropositivity for HBV, HCV, and HIV-1 were only available for four of the corpses: one was positive for HBV (even though the documentation in possession disclosed no specific indication for the detection of HBsAg or HBsAb), one for HCV, one for HIV-1, and one for all three viruses. No data were available regarding any possible antiviral treatment received during the patients' lifetimes. All data regarding the cohort are summarized in Table 1. Blood samples collected post-mortem were identified by progressive numbering from 1 to 21 to ensure complete anonymity. The samples were stored at –20 °C until analysis.

2.2 Nucleic Acids Extraction Procedures

Viral genomes of HBV, HCV, and HIV-1 were extracted from post-mortem whole blood samples by manual protocols and fully automated procedures.

Manual DNA and RNA extraction from whole blood samples were carried out using a solid-phase extraction approach, without any sample dilution. Generally, viral nucleic acid extraction protocols are validated on patients' serum or plasma samples [33–40].

For DNA extraction, we used the NucleoSpin® Blood kit (Macherey-Nagel) and slightly adjusted the manufacturer's instructions to increase DNA purity and recovery [41].

More specifically, the incubation time with Proteinase K was prolonged to 1 hour, vortexing several times to improve cell lysis and DNA recovery from the post-mortem blood samples. Additionally, to enhance the final DNA concentration, this was recovered in a final volume of elution buffer which was half of that recommended. Briefly, 200 μL of buffer B3 and 25 μL of proteinase K were added to 200 μL of each post-mortem whole blood sample. After incubation at 70 °C for 1 hour, 210 μL of ethanol (96–100%) was added to each sample and vortexed for a few seconds. The lysate was then loaded into the NucleoSpin Blood Column placed in a collection tube to allow DNA absorption to the spin silica membrane column during the centrifugation at 11000 $\times$ g for 1 minute. The spin column was washed twice, first with 500 μL of buffer BW and subsequently with 600 μL of buffer B5. The flow-through collected into the collection tube was discarded at each step. After 1 minute of room temperature incubation of 50 μL of preheated (70 °C) elution buffer BE, the purified DNA was eluted from the spin column by centrifuging at room temperature for 1 minute at 11000 $\times$ g.

For RNA extraction, we used the NucleoSpin® RNA Blood kit (Macherey-Nagel) with minor modifications to the procedure specified in the manufacturer's instruction to increase the RNA yield [42].

In particular, since the proteinase K-mediated lysis was carried out at room temperature, it was extended to 30 minutes to improve blood cell lysis and, therefore, to enhance RNA recovery from the post-mortem blood samples.

Table 1. Demographic and clinical characteristics of cohort.

Subject ID	Sex	Age	Nationality	Time elapsed between death and autopsy	Causes of death	Medical record; serological tests
1	F	48	Italian	2	Overdose of illicit substances	HCV RNA positive serology
2	M	32	Italian	5	Overdose of illicit substances	HBV DNA, HCV RNA, and HIV-1 proviral DNA positive serology
3	M	58	Italian	2	Overdose of illicit substances	HIV-1 proviral DNA positive serology
4	M	19	American	3	Overdose of illicit substances	HBV DNA positive serology
5	M	82	Italian	5	Self-induced dynamics	*
6	M	78	Italian	2	Road accident	*
7	M	56	Italian	4	Overdose of illicit substances	*
8	M	60	Italian	4	Self-induced dynamics	*
9	F	59	Italian	5	Self-induced dynamics	*
10	M	70	Italian	5	Endogenous pathological processes	*
11	M	60	Nigerian	9	Endogenous pathological processes	*
12	F	83	Italian	5	Endogenous pathological processes	*
13	F	80	Italian	9	Road accident	*
14	F	67	Moroccan	2	Endogenous pathological processes	*
15	F	45	Italian	2	Overdose of illicit substances	*
16	M	28	Italian	2	Overdose of illicit substances	*
17	M	30	Italian	2	Overdose of illicit substances	*
18	M	44	Italian	2	Road accident	*
19	M	52	Italian	3	Self-induced dynamics	*
20	M	24	Moldavian	3	Endogenous pathological processes	*
21	F	43	Italian	4	Overdose of illicit substances	*

*Incomplete clinical and/or laboratory history.

In addition, the elution time of the RNA from the column was prolonged and the final volume of RNA recovery was adjusted to achieve a higher concentration of RNA.

In brief, 200 μL of lysis buffer DL and 5 μL of Proteinase K were added to 200 μL of each post-mortem whole blood sample. The samples were placed on a shaker (TS-100 Thermo-Shaker Biosan (LV)) at 1400 rpm for 30 minutes at room temperature. Then, 200 μL of 70% ethanol was added to the lysate of each sample and, after accurate mixing, was transferred to the NucleoSpin RNA blood column placed in a collection tube, and centrifuged at 11000 $\times$ g for 30 seconds. Membrane desalting buffer MDB (350 μL) was added to each spin column and centrifuged at 11000 $\times$ g for 30 seconds. After this step, 95 μL of DNase was added to each spin column and kept at room temperature for 30 minutes to achieve complete DNA digestion. The spin column was washed three times: once with 200 μL of buffer RB2 to inactivate the DNase, and twice with 600 μL and 250 μL of RB3 buffer, respectively. At each phase, the flow-through gathered in the collecting tube was discarded. The three washes were repeated twice to ensure the elimination of any hemolysis products. To eluate the RNA, 30 μL of RNase-free water was added to the spin column and centrifuged at room temperature for 30 seconds at 11000 $\times$ g.

After manual extraction of viral nucleic acids, the DNA or RNA extracted from each sample was assessed by spectrophotometric measurements using the NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo Fischer, Waltham, USA). The total DNA and RNA amounts in the samples were determined as between 78.4 and 621.4 ng/μL and 24.9 and 648.7 ng/μL, respectively.

Automated viral nucleic acids extraction was performed using the COBAS system. This system is a fully automated platform that simultaneously performs the extraction and purification of total nucleic acids from the specimens within the sample processing module, amplification, and the detection of extracted nucleic acids and their quantification by real-time polymerase chain reaction (PCR) assay. By using two different fluorescent dyes, one labeled to the specific viral genome probe and the other to the internal control (IC) probe, the COBAS system allows the simultaneous detection of the viral genomes and verification that the entire process has been well performed. Because the COBAS method had not yet been used on whole blood samples collected from cadavers, before sample processing, dilutions varying from 1:5 to 1:10 with phosphate-buffered saline (PBS), optimized for each post-mortem blood sample (Table 2) according to their degree of hemolysis [13], were performed to reduce possible interference with inhibitors of the PCR process due to post-mortem modification sample phenomena [13,26,27]. All the samples were tested once (n = 1). In case of inconclusive results, the test was repeated (n = 2).

Table 2. COBAS samples: PBS dilution.

Subject ID	HBV DNA	HCV RNA	HIV-1 RNA
1	1:8	1:10	1:8
2	1:10	1:10	1:10
3	1:8	1:8	1:8
4	1:10	1:10	1:10
5	1:5	1:5	1:5
6	1:5	1:5	1:5
7	1:5	1:5	1:5
8	1:5	1:5	1:5
9	1:8	1:10	1:8
10	1:8	1:8	1:8
11	1:5	1:5	1:5
12	1:8	1:8	1:8
13	1:5	1:5	1:5
14	1:5	1:5	1:5
15	1:5	1:5	1:5
16	1:10	1:8	1:10
17	1:5	1:5	1:5
18	1:8	1:8	1:8
19	1:5	1:5	1:5
20	1:5	1:5	1:5
21	1:8	1:10	1:10

The extraction of viral nucleic acids is based on the use of magnetic bead technology: after cell lysis has been carried out with a lysis buffer containing Proteinase K and chaotropic salts, the nucleic acids bind to the surface of magnetic glass beads, and cell debris is effectively removed from the lysate by several washing steps.

2.3 Quantitative Analysis of HBV DNA, HCV RNA, and HIV-1 RNA in Post-Mortem Whole Blood Samples

Quantitative analysis of HBV DNA, HCV RNA, and HIV-1 RNA was carried out using the COBAS 6800 system (Roche, Switzerland), an accurate *in vitro* viral nucleic acid quantification platform validated on plasma and serum samples. The COBAS system provided for the fully automated extraction, amplification, and detection of the viral nucleic acid target, and was able to simultaneously detect viral genomes and verify the analytical performance of the entire process by means of probes using the TaqMan technology and the use of an IC probe [43]. The quantitative result indicated the degree of active viral load and was expressed in standardized international units per milliliter (IU/mL) or copies/mL.

Three specific kits were employed to amplify and quantify these three viruses by following the manufacturers' instructions: COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 kit [44], COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test, v2.0 kit [45] and COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 [46].

The COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 kit can detect all eight genotypes of HBV (A-H) with a limit of detection (LoD) of 20 international units (IU)/mL (1 IU = 5.26). The COBAS®AmpliPrep/COBAS® TaqMan® HCV Quantitative Test, v2.0 kit is characterized by a LoD of 15.3 IU/mL, where 1 IU generally corresponds to 2.7 copies/mL and can detect genotypes 1 to 6. The COBAS HIV-1 v2.0 assay using two TaqMan probes simultaneously amplifies and detects two highly conserved regions (gag gene and LTR region) in the HIV-1 genome. The LoD was determined at 20 copies/mL.

Every kit displayed one negative sample and two specific positive samples, characterized by a high and a low number of copies respectively. In all samples, a non-viral Quantitation Standard (QS) was added, in known copy numbers, in the preparation step as an internal control to monitor the whole procedure from the extraction of the nucleic acid to its quantification.

2.4 Qualitative End-Point Real-Time PCR for Detection of HBV DNA in Post-Mortem Whole Blood Samples

Hepatitis B virus (HBV) DNA was revealed by qualitative endpoint real-time PCR using a fluorescent DNA-binding dye called BRYT (Promega, Madison, WI, USA) and a set of forward and reverse primers designed to target positions 379 to 476 in the highly conserved region of the HBV S gene encoding the surface antigen (HBsAg), as previously described [47]. The primer sequences were: HBVF 5′-GTGTCTGCGGCGTTTTATCA-3′ and HBVR 5′-GACAAACGGCAACATACCTT-3′. After the amplification process, an amplicon of 98 base pairs (bp) was obtained. The amplification mix consisted of 100 ng DNA/reaction, 12.5 μL of 2X GoTaq® qPCR Master Mix (Promega), 0.5 μL of each primer (10 μM), and distilled water to achieve a final volume of 25 μL. The amplification was carried out at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 30 seconds, and 72 °C for 5 seconds, with a final extension at 72 °C for 7 minutes in a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Melting curve analysis was performed at the end of the qPCR amplification by raising the temperature from 60 °C to 95 °C, in increments of 0.5 °C/s. The software used for amplification acquisition was CFX Manager (BioRad).

In parallel, the amplification of a highly conserved region of the C-C chemokine receptor type 5 (CCR5) gene located on chromosome 3 was performed for each sample using the following set of primers: CCR5F: 5′-ATGATTCCTGGGAGACGC-3′ and CCR5R: 5′-AGCCAGGACGGTCACCTT-3′ [48]. The CCR5 amplicon size was 81 bp. The amplification mix was the same as reported above. The amplification was carried out at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 30 seconds, 72 °C for 5 seconds, and 76 °C for 5 seconds, with a final extension at 72 °C for 7 minutes,

in a CFX96 Real-Time PCR Detection System (BioRad). Single fluorescence detection was performed at 76 °C to reveal the samples' positivity and rule out eventual interference (i.e., primer-dimer artifacts). Melting curve analysis was performed at the end of the qPCR amplification by raising the temperature from 60 °C to 95 °C, in increments of 0.5 °C/s. CCR5 gene amplification was used as an internal control to exclude any interference with or inhibition of the PCR process due to haemolysis or degradation phenomena resulting from post-mortem blood samples and, at the same time, to allow the evaluation of the number of nucleic acids present in the sample.

HBV-negative and HBV-positive blood samples were used as negative and positive HBV controls, respectively.

2.5 Qualitative End-Point Real-Time PCR for Detection of HCV RNA in Post-Mortem Whole Blood Samples

The total RNA purified from post-mortem whole blood samples was reverse-transcribed to generate complementary DNA (cDNA) by using the ImProm-II™ Reverse Transcription System kit (Promega) [49], according to the manufacturer's instructions.

Briefly, reverse transcription (RT) reaction mix was prepared for each sample by adding ImProm-II™ 5X reaction buffer, 2.5 mM $MgCl_2$, dNTP (0.5 mM for each deoxynucleotide), 5U of ImProm-II™ Reverse Transcriptase and nuclease-free water to a final volume of 15 μL. An aliquot of extracted RNA (4 μL) from each sample and random primers (0.5 μg/reaction) were mixed with the RT mix for a final reaction volume of 20 μL per reaction. The reverse transcription reaction was started with the annealing step at 25 °C for 5 minutes, then the cDNA synthesis continued at an extension temperature of 55 °C for 60 minutes and the reaction vessel was finally incubated at 70 °C for 15 minutes to heat inactivate the ImProm-II™ Reverse Transcriptase.

HCV cDNA was then used for the qualitative endpoint RT-PCR assay using two specific 5′-non-coding regions (5′-NCR) HCV primers: 5′-GTCTAGCCATGGCGTTAGTATGAG-3′ (77–100; HCVP1) and 5′-ACCCTATCAGGCAGTACCACAAG-3′ (302–280; HCVP2) [33], which allowed the amplification of a specific fragment of 226 bp.

The amplification mixture was prepared with 12.5 μL of 2X GoTaq® qPCR Master Mix (Promega), 0.5 μL of each primer (10 μM), 5 μL of cDNA, and distilled water for a 25 μL final volume. The amplification program was set up as previously described [33,50] in a CFX96 Real-Time PCR Detection System (BioRad). Melting curve analysis was performed at the end of the qPCR amplification. HCV-negative and HCV-positive blood samples were used as negative and positive HCV controls, respectively.

2.6 Qualitative End-Point Real-Time PCR for Detection of HIV-1 Proviral DNA in Post-Mortem Whole Blood Samples

HIV-1 belongs to the *Retroviridae* family and exhibits a peculiar replication cycle in which its genomic RNA is retro-transcribed into proviral DNA by a reverse transcriptase enzyme and then integrated into the host cell genome [51–53]. This particular feature indicates two distinct approaches to recognizing the presence of the HIV genome in clinical samples: the first is the detection of HIV-1 virions with RNA genome in plasma (described above) and the second is the detection of HIV-1 proviral DNA integrated into the genome of infected cells (usually $CD4^+$T lymphocytes and monocytes/macrophages) in peripheral blood [52].

To determine the presence of HIV-1 proviral DNA, we selected forward and reverse primers that anneal to complementary sequences within the long terminal repeat (LTR) region of HIV-1, LTR1-F: 5′-TACTGACGCTCGCACC-3′ and LTR2-R: 5′-TCTCGACGCAGGACTCG-3′. These oligonucleotides have been previously described [48,54] and generate an amplicon of 127 bp in size. The amplification mix consisted of 100 ng DNA/reaction, 12.5 μL of 2X GoTaq® qPCR Master Mix (Promega), 0.5 μL of each primer (10 μM), and distilled water to achieve a final volume of 25 μL. The amplification was carried out at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 30 seconds, 72 °C for 5 seconds, and 80 °C for 5 seconds with a final extension at 72 °C for 7 minutes, in a CFX96 Real-Time PCR Detection System (BioRad). Single fluorescence detection was performed at 80 °C to reveal the sample positivity and rule out eventual interference (i.e., primer-dimer artifacts). Melting curve analysis was performed at the end of the qPCR amplification by raising the temperature from 60 °C to 95 °C in increments of 0.5 °C/s. CCR5 internal control, as described above, and negative and positive HIV-1 controls were used.

3. Results

The main goal of this study was to detect and quantify the HBV, HCV and HIV-1 genome sequences in forensic post-mortem blood samples using a large array of quantitative and qualitative PCR/RT-PCR assays. To further this purpose, whole blood samples collected during autopsy examinations from the femoral or subclavian arteries of 21 cadavers, between 2 and 9 days after death, were analyzed.

3.1 Quantitative Detection of HBV, HCV, and HIV-1 Genomes in Forensic Post-Mortem Blood Samples

The quantitative evaluation of HBV DNA was carried out using the COBAS®AmpliPrep/COBAS® TaqMan® HBV Test, v2.0, which amplifies the pre-core/core (PreC/C) region of the HBV genome. This region is one of the four HBV open reading frames (ORFs) encoding for both the hepatitis B e antigen (HBeAg) and the core protein (HBcAg). Unfortunately, no amplification of the PreC/C

Table 3. HBV DNA, HCV RNA, and HIV-1 proviral DNA detection in post-mortem blood samples by qualitative endpoint real-time PCR/RT-PCR and by quantitative COBAS platform.

Subject ID	HBV DNA		HCV RNA		HIV-1 proviral DNA	HIV-1 RNA
	Qualitative endpoint real-time PCR/RT-PCR	Quantitative COBAS platform (copies/mL)	Qualitative endpoint real-time PCR/RT-PCR	Quantitative COBAS platform (IU/mL)	Qualitative endpoint real-time PCR/RT-PCR	Quantitative COBAS platform (copies/mL)
1	-	-	+	180	-	-
2	-	-	-	-	+	1290
3	-	-	-	-	+	904
4	-	-	-	-	+	3130
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	inconclusive	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	-	-	-	-	+	1215
21	-	-	+	6780	+	780

region of HBV was observed in 20 out of 21 samples (Table 3), including two samples with ante-mortem HBV positive serology. Even after adequate dilution of the samples, inconclusive results were obtained for sample 16, which did not exceed the amplification of the COBAS system's internal control.

HCV viral RNA quantification was performed using the COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test, v2.0 kit. HCV positivity was detected in samples 1 (with positive serology already known during life) and 21 (180 and 6780 IU/mL respectively), as shown in Table 3. In contrast, sample 2 (with a known positive serology) resulted in a negative result for HCV viral RNA quantification in the post-mortem sample.

To determine HIV-1 genomic RNA, the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 was used in properly diluted post-mortem blood samples. This assay detected genomic HIV-1 RNA in 5 out of 21 specimens (numbers 2, 3, 4, 20, and 21) ranging from 780 to 3130 copies/mL (Table 3), for two of which (number 2 and 3) the patients were known to be HIV-1 seropositive during life.

3.2 Qualitative Detection of HBV, HCV, and HIV-1 Genomes in Forensic Post-Mortem Blood Samples

Viral nucleic acid was extracted from post-mortem whole blood samples after minor modifications were made to manufacturers' instructions and were analyzed using the qualitative end-point real-time PCR method, based on the BRYT molecule that binds dsDNA with the subsequent fluorescence emission.

Detection of HBV DNA in post-mortem blood samples based on qualitative PCR analysis was negative for all 21 specimens, including the two samples with HBV-positive ante-mortem serological status (Table 3). Amplification curve analyses were negative for all samples, indicating the absence of detectable HBV DNA in the blood samples even though the CCR5 amplification control was positive in all specimens, excluding the presence of PCR artifacts.

Positive amplification curves for the HCV RNA genome were detected by qualitative assay in 2 out of 21 blood samples (numbers 1 and 21; as shown in Fig. 1 and Table 3). These results were also confirmed by quantitative COBAS data.

HIV-1 proviral DNA genome was determined by qualitative end-point real-time PCR in post-mortem blood samples, using the same total DNA extraction protocol employed for HBV DNA detection. A positive amplification curve was detected in 5 out of 21 samples (numbers 2, 3, 4, 20, and 21; as shown in Fig. 1 and Table 3), even though HIV-1 ante-mortem seropositivity was only reported for 2 out of 5 samples (numbers 2 and 3).

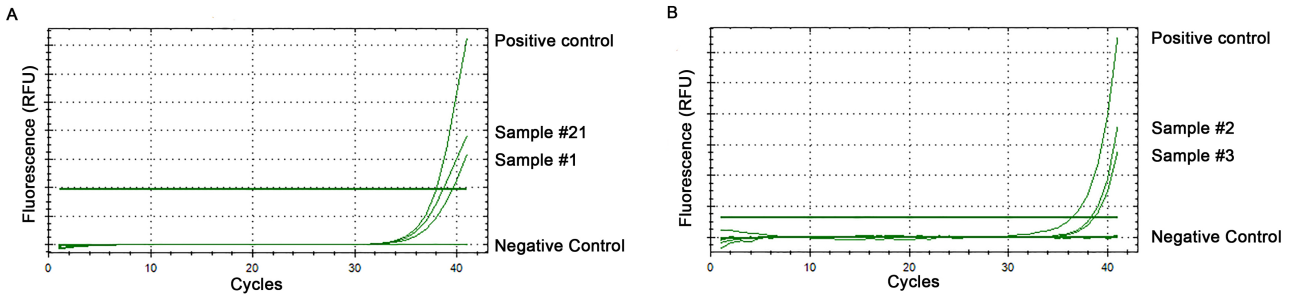

Fig. 1. Real-time detection of HCV RNA and HIV-1 proviral DNA positive controls and samples by RT-qPCR. (A) HCV RNA amplification curves of the positive control, negative control, and samples 21 and 1. (B) HIV-1 proviral DNA amplification curves of the positive control, negative control, and samples 2 and 3.

4. Discussion

HBV, HCV and HIV-1 infection diagnosis in cadavers is currently performed through serological tests which are able to detect the specific antibodies and/or viral antigens. However, these procedures have some drawbacks when applied to forensic post-mortem biological materials [12]. In particular, serological data may represent either the immune signature of past viral infection or the consequence of a vaccination, and not necessarily an ongoing viral infection at the time of death [15–19]. It is noteworthy that the blood collected from cadavers shows putrefactive and transformative phenomena due to the cause of death, the environmental conditions, and the elapsed time. These phenomena lead to the degradation of the proteins and macromolecules, affecting the analytical sensitivity and detection of antibodies to viruses [14].

Since the efficiency of the nucleic acid amplification technique as applied to forensic post-mortem biological samples had yet to be thoroughly assessed, in this study we investigated whether the qualitative and quantitative real-time PCR/RT-PCR approaches used routinely in clinical settings to detect HBV, HCV, and HIV-1 genomes in blood samples taken from patients and the blood, organ, and tissue of living or deceased donors (transplantation), may be used for the same purpose when applied to forensic blood samples taken from a cadaver.

The quantitative determination of the HBV, HCV, and HIV-1 genomes in the post-mortem whole blood samples was carried out by quantitative real-time PCR using the COBAS platform. Initially, this analytical platform showed inhibited amplification due to the haemolysis of the tested blood samples. All blood samples were therefore variably diluted with buffer PBS to overcome the failure of internal control amplification. These data confirm several previous studies indicating that the dilution of post-mortem blood samples by PBS is mandatory for successful amplification [13,26,27,30,32,55]. Unfortunately, some samples displayed a failure of internal control amplification, and we thus carried out qualitative PCR/RT-PCR procedures used in research and clinical study for the detection of HIV-1 proviral DNA, HBV DNA, and HCV RNA genomes. A substantial agreement between the qualitative and quantitative data acquired with the qualitative endpoint real-time PCR and the quantitative real-time PCR methods was observed.

Interestingly, we observed a discrepancy between ante-mortem serological analysis, as reported in medical records, and molecular methods with regard to HBV and HCV detection. Since the serological analysis in HBV infection diagnoses is related to chronic infection only when specific combinations of HBV serological markers are revealed, the detection of HBV DNA in blood by qualitative/quantitative molecular approaches expresses the definite presence of the viral genome in the blood sample and, therefore, an actual condition of active viral infection. Given that acute HBV infection resolves spontaneously in most adults (about 95%) [56], it is not surprising that a difference may exist between a positive HBV ante-mortem serological report and the failure of post-mortem HBV genome detection. To explain the discrepancy between HCV ante-mortem serology and post-mortem molecular results, it is important to mention that positive HCV-specific serological detection is not necessarily linked to chronic HCV disease and the presence of the HCV genome in blood samples [57]. In two samples we detected the HCV genome using the quantitative and qualitative HCV-specific real-time RT-PCR and classical RT-PCR methods, respectively. Interestingly, RNA molecules undergo a consistent and relatively rapid decay after death [13,26,27], with a clear decrease of RNA in blood samples after only 24 hours [23], because cellular RNAse elicits the fast degradation of this nucleic acid. The ability of molecular procedures to detect some positive samples may be related to viral structure; in fact, the capsid protein shell may prevent RNA degradation for a relatively long time by isolating the nucleic acid from the external environment. This possibility has been confirmed in some studies in which several viral RNAs were preserved in the virion protein structure and detected using the RT-PCR for some days [58–63].

HIV-1 genome detection can be performed either

through the detection of the RNA genome in plasma or through the detection of the proviral DNA in peripheral blood mononuclear cells (PBMC). The choice of qualitative PCR to detect HIV-1 proviral DNA rather than genome RNA in forensic blood samples collected from cadavers was a result of several considerations: firstly, DNA molecules are less susceptible than RNA to post-mortem alterations, resulting in a better chance of detecting HIV-1; secondly, HIV-1 infected patients are generally treated with combined antiretroviral therapy (cART), and as a result, the blood HIV-1 RNA load may be below the detection limits of current RT-PCR techniques [49], while HIV-1 proviral DNA is still detectable in the integrated viral DNA of infected cells ($CD4^+$T lymphocytes and monocytes/macrophages) [58,64,65].

It is noteworthy that even the detection of HIV-1 proviral DNA and viral RNA using qualitative end-point PCR and the COBAS technology, respectively, on the same post-mortem blood samples resulted in complete concordance. Interestingly, the elapsed time between death and the collection of post-mortem blood samples did not affect the possibility of detecting viral genomes by different molecular methods, for at least up to 5 days after death (Table 1). It should be underlined that HIV-1 as RNA and proviral DNA was detected in blood samples taken from cadavers 2 to 16.5 days after death [66,67].

A final aspect of this research is related to the prevalence of viral genome detection among drug users. Viral genomes related to HCV and HIV-1 were identified in 5 of 9 blood samples taken from deceased drug users, suggesting that there is a real risk of contracting a viral infection for operators during autopsies.

In conclusion, the molecular procedures may be useful for the detection of viral infection in forensic sciences as an additional method with respect to serological analysis. The automatic quantitative COBAS platform may be considered to be a valuable technique for the determination of the presence of viral infections such as HIV-1, HCV and HBV. However, a second approach represented by qualitative PCR/RT-PCR may be useful both to analyze samples with inconclusive COBAS results due to the failure of internal control, and as an alternative method which can be used to reinforce and confirm COBAS results. Moreover, the unexpected detection of viral genomes in our sample cohort, highlighted by the use of these molecular platforms, emphasizes the importance of molecular analysis techniques as support for analytical investigation in this specific context.

5. Conclusions

In this preliminary study, attention was focused on evaluating the use of molecular technologies to detect viral genomes in forensic samples. Substantially, the manual and fully-automated methods used in this study to reveal HBV, HCV, and HIV-1 in forensic post-mortem blood samples represent a valuable approach for use in forensic sciences.

Undoubtedly, our results are derived from a low number of samples and, therefore, further investigation should be performed on a broader set of samples.

However, these findings suggest that by developing appropriate viral nucleic acid extraction and forensic biological sample pre-treatment protocols (such as dilution for the COBAS platform), the nucleic acid amplification techniques, calibrated to specific viral genomic targets, can be successfully used, proving to be reliable for the detection of viral genomes in forensic post-mortem blood samples up to 5 days after death.

These molecular techniques could therefore play a pivotal role in forensic science and contribute to the monitoring of potential infection risks for forensic operators, thus improving their safety at work.

Abbreviations

HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; NAATs, nucleic acid amplification techniques; q-PCR, quantitative real-time PCR; PCR, polymerase chain reaction; RT-PCR, real time PCR; HBsAg, Hepatitis B surface antigen; HBsAb, Hepatitis B surface antibody; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; MDB, membrane desalting buffer; UV-Vis, Ultraviolet–visible; IC, internal control; PBS, phosphate-buffered saline; HBVF, HBV forward; HBVR, HBV reverse; bp, base pairs; CCR5, C-C chemokine receptor type 5; CCR5F, CCR5 forward; CCR5R, CCR5 reverse; QS, quantitation standard; cDNA, complementary DNA; RT, reverse transcription; dNTP, deoxyribose nucleotide triphosphate; NCR, non-coding region; LoD, limit of detection; IU, international unit; LTR, long terminal repeat; LTR1-F, LTR1 forward; LTR2-R, LTR-2 reverse; ds-DNA, double stranded DNA; PreC/C, precore/core; ORF, open reading frame; HBeAg, Hepatitis B e antigen; HBcAg, hepatitis B core antigen; anti-HBc antibodies, anti-hepatitis B antibodies; PBMC, peripheral blood mononuclear cells; ART, antiretroviral therapy.

Author Contributions

ST, DG and DDL designed the research study. ST, DG, GG, AL, ED, VL and GS performed the research. GG, FG and DR provided help on the study. ST, DG and DDL provided advice on the study. ST, DG, GG, AL, ED, VL, GS, FG, DR and DDL analyzed the data. ST, DG, GG, GS and DDL wrote the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the University of Verona's Approval Committee for Research on the Person (CARP) (number 02 R.1/2021).

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Weston J, Locker G. Frequency of glove puncture in the post mortem room. Journal of Clinical Pathology. 1992; 45:177–178.

[2] Li L, Zhang X, Constantine NT, Smialek JE. Seroprevalence of Parenterally Transmitted Viruses (HIV-1, HBV, HCV, and HTLV-I/II) in Forensic Autopsy Cases. Journal of Forensic Sciences. 1993; 38: 13510J.

[3] Butts JD. Forensic pathology automatically exposure-prone. North Carolina Medical Journal. 1994; 55: 210.

[4] du Plessis R, Webber L, Saayman G. Bloodborne Viruses in Forensic Medical Practice in South Africa. The American Journal of Forensic Medicine and Pathology. 1999; 20: 364–368.

[5] Wetli CV. Autopsy Safety. Laboratory Medicine. 2001; 32: 451–453.

[6] Nolte KB, Taylor DG, Richmond JY. Biosafety Considerations for Autopsy. The American Journal of Forensic Medicine and Pathology. 2002; 23: 107–122.

[7] Nolte KB, Yoon SS. Theoretical Risk for Occupational Blood-Borne Infections in Forensic Pathologists. Infection Control & Hospital Epidemiology. 2003; 24: 772–773.

[8] Burton JL. Health and safety at necropsy. Journal of Clinical Pathology. 2003; 56: 254–260.

[9] Wilson ML. Infectious diseases and the autopsy. Clinical Infectious Diseases. 2006; 43: 602–603.

[10] Victer TNDF, dos Santos CSR, Báo SN, Sampaio TL. Deceased tissue donor serology and molecular testing for HIV, hepatitis B and hepatitis C viruses: a lack of cadaveric validated tests. Cell and Tissue Banking. 2016; 17: 543–553.

[11] Christensen PB, Kringsholm B, Banner J, Thomsen JL, Cowan S, Stein GF, *et al*. Surveillance of HIV and Viral Hepatitis by Analysis of Samples from Drug Related Deaths. European Journal of Epidemiology. 2006; 21: 383–387.

[12] Zhang X, Constantine NT, Li L, Abdel-Hamid M, Bansal J, Smialek JE. Application of commercial assays to detect IgG antibodies to hepatitis C virus in urine: a pilot study from autopsy cases. Journal of Medical Virology. 1994; 44: 187–191.

[13] Padley DJ, Lucas SB, Saldanha J. Elimination of false-negative hepatitis C virus RNA results by removal of inhibitors in cadaver-organ donor blood specimens. Transplantation. 2003; 76: 432–434.

[14] Bakri FG, Al-Abdallat IM, Ababneh N, Al Ali R, Idhair AKF, Mahafzah A. Prevalence of blood-borne viral infections among autopsy cases in Jordan. Qatar Medical Journal. 2016; 2016: 14.

[15] Leoni MC, Ustianowski A, Farooq H, Arends JE. HIV, HCV and HBV: a Review of Parallels and Differences. Infectious Diseases and Therapy. 2018; 7: 407–419.

[16] Razavi H. Global Epidemiology of Viral Hepatitis. Gastroenterology Clinics of North America. 2020; 49: 179–189.

[17] Roudot-Thoraval F. Epidemiology of hepatitis C virus infection. Clinics and Research in Hepatology and Gastroenterology. 2021; 45: 101596.

[18] Shukla A, Ramirez NP, D'Orso I. HIV-1 Proviral Transcription and Latency in the New Era. Viruses. 2020; 12: 555.

[19] Khanal S, Schank M, El Gazzar M, Moorman JP, Yao ZQ. HIV-1 Latency and Viral Reservoirs: Existing Reversal Approaches and Potential Technologies, Targets, and Pathways Involved in HIV Latency Studies. Cells. 2021; 10: 475.

[20] Busch MP, Kleinman SH, Jackson B, Stramer SL, Hewlett I, Preston S. Committee report. Nucleic acid amplification testing of blood donors for transfusion-transmitted infectious diseases: Report of the Interorganizational Task Force on Nucleic Acid Amplification Testing of Blood Donors. Transfusion. 2000; 40: 143–159.

[21] Pawlotsky J. Use and interpretation of virological tests for hepatitis C. Hepatology. 2002; 36: s65–s73.

[22] Webster A, McLaughlin JE, Johnson MA, Emery VC, Griffiths PD. Use of the polymerase chain reaction to detect genomes of human immunodeficiency virus and cytomegalovirus in post-mortem tissues. Journal of Medical Virology. 1995; 47: 23–28.

[23] Burtonboy G, Delloye C. Polymerase chain reaction in cadaveric blood and tissues. Transplantation Proceedings. 1996; 28: 2927–2928.

[24] Heim A, Wagner D, Rothämel T, Hartmann U, Flik J, Verhagen W. Evaluation of serological screening of cadaveric sera for donor selection for cornea transplantation. Journal of Medical Virology. 1999; 58: 291–295.

[25] Meng Q, Wong C, Rangachari A, Tamatsukuri S, Sasaki M, Fiss E, *et al*. Automated Multiplex Assay System for Simultaneous Detection of Hepatitis B Virus DNA, Hepatitis C Virus RNA, and Human Immunodeficiency Virus Type 1 RNA. Journal of Clinical Microbiology. 2001; 39: 2937–2945.

[26] Wilson IG. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology. 1997; 63: 3741–3751.

[27] King CE, Debruyne R, Kuch M, Schwarz C, Poinar HN. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques. 2009; 47: 941–949.

[28] Cattaneo C, Nuttall PA, Molendini LO, Pellegrinelli M, Grandi M, Sokol RJ. Prevalence of HIV and hepatitis C markers among a cadaver population in Milan. Journal of Clinical Pathology. 1999; 52: 267–270.

[29] Smyth BP. Retrospective cohort study examining incidence of HIV and hepatitis C infection among injecting drug users in Dublin. Journal of Epidemiology & Community Health. 2003; 57: 310–311.

[30] Eriksen MB, Jakobsen MA, Kringsholm B, Banner J, Thomsen JL, Georgsen J, *et al*. Postmortem Detection of Hepatitis B, C, and Human Immunodeficiency Virus Genomes in Blood Samples from Drug-Related Deaths in Denmark. Journal of Forensic Sciences. 2009; 54: 1085–1088.

[31] Gubbe K, Scharnagl Y, Grosch S, Tonn T, Schmidt M, Hourfar KM, *et al*. Validation of Virus NAT for HIV, HCV, HBV and HAV Using Post-Mortal Blood Samples. Transfusion Medicine and Hemotherapy. 2012; 39: 381–385.

[32] Gharehdaghi J, Abedi Khorasgani MH, Ghadiani MH, Kazemifar AM, Solhi H, Solhi S. Prevalence of HCV, HBV, and HIV Seropositivity among Cadavers Referred to Autopsy Hall of Legal Medicine Bureau of Tehran, Iran. Advances in Preventive Medicine. 2017; 2017: 1–4.

[33] Gibellini D, Gardini F, Vitone F, Schiavone P, Furlini G, Carla Re M. Simultaneous detection of HCV and HIV-1 by SYBR Green real time multiplex RT-PCR technique in plasma samples. Molecular and Cellular Probes. 2006; 20: 223–229.

[34] Zeldis JB, Lee JH, Mamish D, Finegold DJ, Sircar R, Ling Q, *et al*. Direct method for detecting small quantities of hepatitis B virus DNA in serum and plasma using the polymerase chain reaction. Journal of Clinical Investigation. 1989; 84: 1503–1508.

[35] Busch M, Lee L, Satten G, Henrard D, Farzadegan H, Nelson K, *et al*. Time course of detection of viral and serologic markers preceding human immunodeficiency virus type 1 serocon-

version: implications for screening of blood and tissue donors. Transfusion. 1995; 35: 91–97.
[36] Gelderblom HC, Menting S, Beld MG. Clinical performance of the new rRoche COBAS TaqMan HCV Test and High Pure System for extraction, detection and quantitation of HCV RNA in plasma and serum. Antiviral Therapy. 2006; 11: 95–103.
[37] Wendel S, Levi JE, Takaoka DT, Silva IC, Castro JPD, Torezan-Filho MA, *et al*. Primary screening of blood donors by nat testing for HCV-RNA: development of an "in-house" method and results. Revista do Instituto de Medicina Tropical de SãO Paulo. 2007; 49: 177–185.
[38] Pas S, Rossen JWA, Schoener D, Thamke D, Pettersson A, Babiel R, *et al*. Performance Evaluation of the New Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 Test Version 2.0 for Quantification of Human Immunodeficiency Virus Type 1 RNA. Journal of Clinical Microbiology. 2010; 48: 1195–1200.
[39] Zitzer H, Heilek G, Truchon K, Susser S, Vermehren J, Sizmann D, *et al*. Second-Generation Cobas AmpliPrep/Cobas TaqMan HCV Quantitative Test for Viral Load Monitoring: a Novel Dual-Probe Assay Design. Journal of Clinical Microbiology. 2013; 51: 571–577.
[40] Chevaliez S, Pawlotsky J. New virological tools for screening, diagnosis and monitoring of hepatitis B and C in resource-limited settings. Journal of Hepatology. 2018; 69: 916–926.
[41] NucleoSpin Blood, Mini kit for DNA from blood, MN | MACHEREY-NAGEL. 2021. Available at: https://www.mn-net.com/nucleospin-blood-mini-kit-for-dna-from-blood-740951.50 (Accessed: 22 October 2021).
[42] NucleoSpin RNA Blood, Mini kit for RNA blood, MN | MACHEREY-NAGEL. 2021. Available at: https://www.mn-net.com/nucleospin-rna-blood-mini-kit-for-rna-from-blood-740200.50 (Accessed: 22 October 2021).
[43] COBAS® 6800 System. 2021. Available at: https://diagnostics.roche.com/global/en/products/instruments/cobas-6800.html (Accessed: 22 October 2021).
[44] COBAS®AmpliPrep/COBAS®TaqMan®HBV Test, v2.0. 2021. Available at: https://diagnostics.roche.com/global/en/products/params/cobas-ampliprep-cobas-taqman-hbv-test-v2-0.html (Accessed: 22 October 2021).
[45] COBAS®AmpliPrep/COBAS®TaqMan® HCV Test, v2.0: Qualitative and Quantitative. 2021. Available at: https://diagnostics.roche.com/global/en/products/params/cobas-ampliprep-cobas-taqman-hcv-test-v2-0-qualitative-and-quantitative.html (Accessed: 22 October 2021).
[46] COBAS®AmpliPrep/Cobas®TaqMan®HIV-1Test, v2.0. 2021. Available at: https://diagnostics.roche.com/global/en/products/params/cobas-ampliprep-cobas-taqman-hiv-1-test-v2-0.html (Accessed: 22 October 2021).
[47] Mendy ME, Kaye S, van der Sande M, Rayco-Solon P, Waight PA, Shipton D, *et al*. Application of real-time PCR to quantify hepatitis B virus DNA in chronic carriers in the Gambia. Virology Journal. 2006; 3: 23.
[48] Malnati MS, Scarlatti G, Gatto F, Salvatori F, Cassina G, Rutigliano T, *et al*. A universal real-time PCR assay for the quantification of group-M HIV-1 proviral load. Nature Protocols. 2008; 3: 1240–1248.
[49] ImProm-IITM Reverse Transcription System. 2021. Available at: https://ita.promega.com/products/pcr/rt-pcr/improm_ii-reverse-transcription-system/?catNum=A3800 (Accessed: 12 October 2021).
[50] Komurian-Pradel F, Paranhos-Baccalà G, Sodoyer M, Chevallier P, Mandrand B, Lotteau V, *et al*. Quantitation of HCV RNA using real-time PCR and fluorimetry. Journal of Virological Methods. 2001; 95: 111–119.
[51] Craigie R, Bushman FD. HIV DNA Integration. Cold Spring Harbor Perspectives in Medicine. 2012; 2: a006890.
[52] Maartens G, Celum C, Lewin SR. HIV infection: epidemiology, pathogenesis, treatment, and prevention. The Lancet. 2014; 384: 258–271.
[53] Deeks SG, Overbaugh J, Phillips A, Buchbinder S. HIV infection. Nature Reviews Disease Primers. 2015; 1: 15035.
[54] Gibellini D. Quantitative detection of human immunodeficiency virus type 1 (HIV-1) proviral DNA in peripheral blood mononuclear cells by SYBR green real-time PCR technique. Journal of Clinical Virology. 2004; 29: 282–289.
[55] Clark MA, Worrell MB, Pless JE. Postmortem Changes in Soft Tissues. In: Haglund WD and Sorg MH (eds.) Forensic Taphonomy: The Postmortem Fate of Human Remains (pp. 156–164). CRC Press: Boca Raton. 1997.
[56] Nguyen MH, Wong G, Gane E, Kao J, Dusheiko G. Hepatitis B Virus: Advances in Prevention, Diagnosis, and Therapy. Clinical Microbiology Reviews. 2020; 33: e00046-19.
[57] Manns MP, Buti M, Gane E, Pawlotsky J, Razavi H, Terrault N, *et al*. Hepatitis C virus infection. Nature Reviews Disease Primers. 2017; 3: 17006.
[58] Meyer T, Polywka S, Wulff B, Edler C, Schröder AS, Wilkemeyer I, *et al*. Virus NAT for HIV, HBV, and HCV in Post-Mortal Blood Specimens over 48 h after Death of Infected Patients – first Results. Transfusion Medicine and Hemotherapy. 2012; 39: 376–380.
[59] Bauer M, Gramlich I, Polzin S, Patzelt D. Quantification of mRNA degradation as possible indicator of postmortem interval—a pilot study. Legal Medicine. 2003; 5: 220–227.
[60] Heinrich M, Matt K, Lutz-Bonengel S, Schmidt U. Successful RNA extraction from various human postmortem tissues. International Journal of Legal Medicine. 2007; 121: 136–142.
[61] Haas C, Klesser B, Maake C, Bär W, Kratzer A. MRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. Forensic Science International: Genetics. 2009; 3: 80–88.
[62] Weinbrecht KD, Fu J, Payton M, Allen RW. Time-dependent loss of mRNA transcripts from forensic stains. Research and Reports in Forensic Medical Science. 2017; 7: 1–12.
[63] Lewis CA, Layne TR, Seashols-Williams SJ. Detection of microRNAs in DNA Extractions for Forensic Biological Source Identification. Journal of Forensic Sciences. 2019; 64: 1823–1830.
[64] Lanzafame M, Nicole S, Rizzardo S, Piacentini D, Chiesi S, Lattuada E, *et al*. Immunovirological outcome and HIV-1 DNA decay in a small cohort of HIV-1-infected patients deintensificated from Abacavir/Lamivudine/Dolutegravir to Lamivudine plus Dolutegravir. New Microbiologica. 2018; 41: 262–267.
[65] Lamers SL, Rose R, Maidji E, Agsalda-Garcia M, Nolan DJ, Fogel GB, *et al*. HIV DNA is Frequently Present within Pathologic Tissues Evaluated at Autopsy from Combined Antiretroviral Therapy-Treated Patients with Undetectable Viral Loads. Journal of Virology. 2016; 90: 8968–8983.
[66] Edler C, Wulff B, Schroder A-, Wilkemeyer I, Polywka S, Meyer T, *et al*. A prospective time-course study on serological testing for human immunodeficiency virus, hepatitis B virus and hepatitis C virus with blood samples taken up to 48 h after death. Journal of Medical Microbiology. 2011; 60: 920–926.
[67] Douceron H, Deforges L, Gherardi R, Sobel A, Chariot P. Long-lasting postmortem viability of human immunodeficiency virus: a potential risk in forensic medicine practice. Forensic Science International. 1993; 60: 61–66.