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Postmortem Detection of Hepatitis B, C, and Human Immunodeficiency Virus Genomes in Blood Samples from Drug-Related Deaths in Denmark*

ABSTRACT: Blood-borne viral infections are widespread among injecting drug users; however, it is difficult to include these patients in serological surveys. Therefore, we developed a national surveillance program based on postmortem testing of persons whose deaths were drug related. Blood collected at autopsy was tested for anti-HBc, anti-HBs, anti-hepatits C virus (HCV), or anti-human immunodeficiency virus (HIV) antibodies using commercial kits. Subsets of seropositive samples were screened for viral genomes using sensitive in-house and commercial polymerase chain reaction (PCR) assays. Hepatitis B virus (HBV) DNA was detected in 20% (3/15) of anti-HBc-positive/anti-HBs-negative samples, HCV RNA was found in 64% (16/25) of anti-HCV-positive samples, and HIV RNA was detected in 40% (6/15) of anti-HIV-positive samples. The postmortem and antemortem prevalences of HBV DNA and HCV RNA were similar. Postmortem HIV RNA testing was less sensitive than antemortem testing. Thus, postmortem PCR analysis for HBV and HBC infection is feasible and relevant for demonstrating ongoing infections at death or for transmission analysis during outbreaks.

KEYWORDS: forensic sciences, drug-related deaths, viral hepatitis, HIV infection, nucleic acid testing, postmortem testing

Viral infections, including hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV), are more prevalent among drug users than among the general population (1). Surveillance of viral infections among drug users is difficult because of their rare contact with the health system. Denmark has an estimated drug-using population of 19,500 persons (3.6/1000 population), excluding persons dependent on cannabis only. The rate of drug-related deaths is 250–300 per year and has been stable for the last decade (2). As required by Danish law since 1970, all persons suspected of dying from drug-related causes undergo a medicolegal autopsy, including toxicology examinations. In order to monitor the prevalences of HBV, HCV, and HIV infections among Danish drug users, the Danish Board of Health established a surveillance initiative to be conducted from 2004 to 2009.

In this national prospective cohort study, postmortem blood samples were tested for anti-HIV, anti-HCV, and anti-HBV core antigen (HBc) antibodies. The 2004 prevalences among 233 drug-related deaths were 4%, 51%, and 35%, respectively. Initially, a test for the HBV surface antigen (HBsAg) was included to identify ongoing HBV infection; however, testing for HBsAg was abandoned due to high levels of false positive results, presumably due to interference from postmortem degradation substances (1).

In order to test for chronic infections, we assayed for the presence of viral genomes by polymerase chain reaction (PCR). PCR analysis had been established to screen donor organs but blood samples from persons who died of drug-related causes were of lesser quality relative to organ donor samples and the PCR assay had not been validated for this type of analysis (3,4). Thus, the aim of this study was to investigate whether testing seropositive samples for the corresponding viral genome by PCR could be used to monitor the prevalences of ongoing viral infections.

Materials and Methods

Serological Analyses of Blood Samples Collected Postmortem

During 2004–2006, blood samples were collected from persons who died of drug-related causes in Denmark. At autopsy, 10 mL of blood was collected in RNase-free 10-mL plastic tubes and sent by ordinary mail to a central laboratory (Department of Clinical Immunology, Odense, Denmark) where all analyses were performed. Upon arrival, the samples were centrifuged at room temperature (4000×*g* for 10 min) and serum was withdrawn if possible. In most cases, serum withdrawal was impossible due to hemolysis and clotting. Hemolysed and clotted blood samples were diluted 1:2 or 1:4 in phosphate buffered saline (GIBCO®, Invitrogen Ltd., Paisley, UK). The blood samples were centrifuged again at 16,000×*g* for 10 min and the supernatants were used for analysis.

Serological analyses were performed by Microparticle Enzyme Immunoassay (MEIA) using the Abbott AxSym® analyzer (Abbott Laboratories, Abbott Park, IL). AUSAB® (Abbott Laboratories) was

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used to detect anti-HBs; Core™ (Abbott Laboratories) was used to detect anti-HBc; HIV Ag/Ab Combo was used to detect anti-HIV; and HCV version 3.0 was used to detect anti-HCV. Confirmatory tests were performed for anti-HIV using INNO-LIA™ HIV I/II Score and for anti-HCV using INNO-LIA™ HCV Score (Innogenetics N.V., Gent, Belgium). All analyses/tests were performed in duplicate and according to the manufacturer's guidelines.

Samples with results close to the cut-off values for anti-HCV (signal-to-cut-off ratio [S/C] = 0.5–3.0) and all samples with an anti-HIV S/C > 2.0 were tested again using confirmatory assays. Confirmatory assays were not available for anti-HBc and anti-HBs detection; the presence of both markers was considered confirmatory. If only one marker was detected, a stronger test signal was required for the sample to be classified as positive (S/C < 0.5 for anti-HBc [inhibitory assay] and >10.0 IU for anti-HBs), as previously described (1).

Selection of Samples for Nucleic Acid Testing

We analyzed all anti-HIV-positive samples for HIV RNA if sufficient material was available. To screen for HBV DNA and HCV RNA, we selected approximately the same number of strongly seropositive samples with sufficient material for testing (n = 15). Regarding HBV DNA analysis, strongly seropositive samples were defined as anti-HBc S/C < 0.5 and anti-HBs < 2 IU/L. HCV samples were defined as strongly seropositive when the anti-HCV S/C was >100. Information regarding the results of antemortem testing of the subjects was collected from The National Register of HIV and Viral Hepatitis, hospital records, and police reports available at autopsy.

Detection of HBV DNA

Hepatitis B virus DNA was extracted from the samples without dilution using Qiagen QIAamp DNA extraction kit according to manufacturer's guidelines (Qiagen GmbH, Hilden, Germany). The optimal amount of serum for HBV DNA extraction was 400 μL. If material was sparse, HBV DNA was extracted from 200 μL. Carrier RNA (Qiagen GmbH) was added in order to increase the yield of DNA.

Primers were selected within the S gene of the HBV DNA: HBV375F (forward primer, at position 375: 5′-GGATGTGTCTGC GCGTTTT-3′), HBV800R (reverse primer at position 800: 5′-AGACAAAAGAAAATTGGTAAC-3′), and Hep50 (reverse primer at position 1285: 5′-CTAGGAGTTCCGCAGTATGGA-3′) (5). The PCR was carried out as follows: 2.5 μL of PCR Buffer II ($MgCl_2$ 15 mM; Applied Biosystems, Carlsbad, CA), 2 μL of dNTP (2.5 mM), 1.25 μL of the forward primer, 1.25 μL of the reverse primer, 0.25 μL of AmpliTaq polymerase (Applied Biosystems), 1 μL of HBV DNA, and 16.75 μL of sterile H_2O. The PCR reaction was carried out at 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 2 min, with final extension at 72°C for 7 min. To exclude the possibility that hemolysis and degradation of the blood samples inhibited the PCR, a human housekeeping gene (mannan binding lectin) was amplified with primers MBL198f (5′-AGAGGTATTTAGCA-3′) and Exon 1R (5′-AGGATCCAGGCAGT-3′). The PCR was carried out as follows: 2.5 μL of PCR Buffer II ($MgCl_2$ 15 mM; Applied Biosystems), 2 μL of dNTP (2.5 mM), 1.25 μL of the forward primer, 1.25 μL of the reverse primer, 0.25 μL of AmpliTaq Gold polymerase (Applied Biosystems), 1 μL of HBV DNA, and 16.75 μL of sterile H_2O. PCR was carried out at 94°C for 12 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 1 min, 72°C for 2 min, with a final 7 min extension at 72°C. The detection limit of the HBV DNA assay was >1 IU/mL as determined by sequential dilution of a reference sample (Pelispy, AcroMetrix, Benicia, CA).

Hepatitis B viral DNA was sequenced using the ABI Prism® 3130 XL Genetic Analyzer (Applied Biosystems) and the ABI Prism® Big Dye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed using SeqScape (Applied Biosystems). BLAST (basic local alignment search tool) was used to align the test sequences with HBV DNA sequences deposited in Genebank.

Detection of HCV RNA

To prevent the inhibition of PCR by components in the hemolysed blood, as described in other studies (6), samples were diluted according to the degree of hemolysis (1:2, 1:4, 1:8, 1:16, or 1:20). Pure serum samples were left undiluted.

Hepatitis C viral RNA was detected with the Cobas Amplicor™ Hepatitis C Virus Test v2.0 (Roche Diagnostics, Rotkreuz, Switzerland) using a Cobas Amplicor Analyzer according to the manufacturer's guidelines. This assay was not validated for postmortem use. Analysis was performed with 200-μL sample material and the detection limit of the assay was 50 IU/mL. Only anti-HCV-positive blood samples were analyzed and information about the antemortem HCV RNA status was not available. The test included an internal control that served to determine whether RNA was successfully amplified.

Detection of HIV RNA

Blood samples analyzed for the presence of HIV RNA were diluted as described above. HIV RNA was detected using the COBAS AmpliPrep/TaqMan HIV-1 Test (Roche Diagnostics) and COBAS AmpliPrep Analyzer. The assay was not validated for postmortem use. Analysis was performed with 1000 μL of sample material and the detection limit of the assay was 50 copies/mL. Only blood samples that had tested positive for anti-HIV were analyzed. In four cases, the subjects were known to have been HIV-positive before death, but their treatment status and HIV RNA viral load *in vivo* were not known.

Cost of Analyses

The prices of the commercial PCR assay kits were $44 U.S. for HCV RNA and $89 U.S. for HIV RNA per test. These costs were covered by project funds. The cost of the reagents for the in-house HBV DNA test was $20 U.S. per test, and a laboratory technician spent about 0.5 h to perform each test. The cost of the serological tests ranged from $5 to $15 U.S. per test.

Results

Serological Analysis

Among the 875 drug-related deaths recorded from 2004 to 2006, 668 persons (76%) had blood samples withdrawn at autopsy with a volume large enough for one or more analyses. Serological analyses produced the following results: 3% (18/622) were anti-HIV positive, 57% (347/613) were anti-HCV positive, and 36% (211/588) were anti-HBc positive. Of the latter, 98 were anti-HBc positive but anti-HBs negative.

Detection of HBV DNA

Among 98 samples positive for only for anti-HBc, 31% (30/98) fulfilled our serological inclusion criteria; of these, only 50%

(15/30) contained enough material to perform HBV DNA analysis (Table 1). The human gene encoding mannan-binding lectin was detected in all samples analysed for HBV DNA, indicating the presence of intact DNA in the samples. HBV DNA was detected by PCR in 40% (6/15) of the cases. In 20% (3/15; 95% confidence interval [CI], 4–48%) of the subjects, the sequence of the amplified HBV DNA could be confirmed by alignment with known HBV DNA S gene sequences (B2004-4, B2004-5, and B2006-1). In the remaining three PCR-positive samples, we were unable to produce sequences of a quality that permitted confirmation of HBV DNA.

Detection of HCV RNA

Among 347 subjects who tested positive for anti-HCV, 8% (27/347) were strongly positive (S/C > 100), and in 25 subjects enough sample was available for HCV RNA analysis. Sixty-four percent (16/25; 95% CI, 43–82%) were positive for HCV RNA (Table 2). In three cases (2005-1, 2005-13, and 2005-20), the results were inconclusive (negative internal control and no material left for further testing). The mean interval (MI) between death and autopsy was 3.5 days. For subjects with positive samples, the MI was 3.25 days and for subjects with negative samples the MI was 4 days.

Detection of HIV RNA

Among the 18 samples confirmed positive for anti-HIV, sufficient material was available for further analysis in 78% (14/18) of cases. All cases were highly reactive in MEIA tests (S/C > 10.0) and positive in confirmatory tests. HIV RNA was detected among 40% (6/14; 95% CI, 18–71%; Table 3). In two cases (I2004-2 and I2004-3), the results were inconclusive (negative internal control and no material left for further analysis). The MI between death and autopsy was 4 days. The MI for positive test results was 3.6 days (range, 2–5 days) and for negative test results the MI was 5 days (range, 3–7 days; $p = 0.60$).

Eleven subjects had tested anti-HIV positive antemortem; no information was available for the remaining three cases. In five of 11 cases diagnosed antemortem, the subjects were positive for HIV RNA. However, it was not known whether these anti-HIV-positive drug users had received antiretroviral treatment prior to death.

TABLE 1—*HBV-DNA detection in anti-HBc-positive/anti-HBs-negative samples from drug-related deaths, 2004–2006.*

Case	HBV DNA Detected	HBV DNA Sequenced
B2004-1	+*	–
B2004-2	–	–
B2004-3	–	–
B2004-4	+*	+
B2004-5	+*	+
B2005-1	–	–
B2005-2	–	–
B2005-3	–	–
B2005-4	–	–
B2005-5	–	–
B2005-6	–	–
B2005-7	–	–
B2006-1	+	+
B2006-2	+	–
B2006-3	+	–
Total 15	6 positives	3 positives

Drug-related death ID refers to the year of death of the subjects.
*DNA was amplified by PCR but the poor quality of the sequence precluded confirmation as HBV DNA.

TABLE 2—*HCV-RNA detection among anti-HCV positive samples from drug-related deaths, 2004–2006.*

Case	Dilution	HCV RNA Detection	Time Between Death and Autopsy (days)
C2004-1	1:4	+	3
C2004-2	1:4	Inconclusive	2
C2005-1	—	Inconclusive	5
C2005-2	1:4	–	2
C2005-3	1:2	+	4
C2005-4	1:2	+	2
C2005-5	—	–	5
C2005-6	1:2	+	4
C2005-7	1:16	–	6
C2005-8	1:20	–	2
C2005-9	1:2	+	3
C2005-10	1:2	+	2
C2005-11	1:2	+	3
C2005-12	1:4	+	3
C2005-13	—	Inconclusive*	3
C2005-14	1:2	+	5
C2005-15	1:4	+	2
C2005-16	-	Inconclusive*	5
C2006-17	1:2	+	3
C2005-18	1:4	+	5
C2005-19	1:8	–	5
C2005-20	1:4	+	4
C2006-1	1:2	+	3
C2006-2	1:4	+	1
C2006-3	1:2	+	4
Total tested: 25		16 positive, 5 negative, 4 inconclusive	

HCV, hepatitis C virus.
*Inconclusive: presence of HCV RNA could not be confirmed nor denied.

TABLE 3—*HIV-RNA detection among anti-HIV-positive samples from drug-related deaths, 2004–2006.*

Case	Dilution	HIV-RNA	Time Between Death and Sampling	Anti-HIV Test Prior to Death
I2004-1	1:4	–	4 days	+
I2004-2	1:4	Inconclusive*	4 days	+
I2004-3	1:4	Inconclusive*	4 days	+
I2004-4	1:4	–	3 days	+
I2004-5	1:4	+	Not reported	Not reported
I2004-6	1:4	–	5 days	+
I2005-1	1:4	–	7 days	Not reported
I2005-2	1:4	+	2 days	+
I2006-1	1:10	–	3 days	Not reported
I2006-2	1:10	–	Not reported	Not reported
I2006-3	1:10	–	4 days	+
I2006-4	1:10	+	5 days	+
I2006-5	1:10	+	2 days	+
I2006-6	1:10	+	5 days	+
Total tested: 14		6 Positive 6 Negative 2 Inconclusive		10 Positive 4 Not reported

*Inconclusive: presence of HCV RNA could not be confirmed nor denied.

Discussion

In this study, we demonstrated the feasibility of detecting viral genomes in blood samples collected during routine autopsy from persons who died from drug-related causes. The samples were taken a median of 4 days after death and forwarded for examination without precautions to preserve nucleic acids, e.g., the samples were transported at ambient temperature for more than 24 h. Still, we performed PCR on all samples and detected a human gene in

all samples tested, indicating the presence of intact DNA. HBV DNA was detected and confirmed by sequencing in 20% of samples analyzed (Table 1), which agreed with data from our previous study among living Danish drug users; among 58 anti-HBs-negative and anti-HBc-positive drug users, the prevalence of HBV DNA was 16% (7).

Schleicher et al. (5) detected HBV DNA in 6/12 of HBsAg-positive subjects, similar to the prevalence in live drug users. Thus, the lower prevalence of HBV DNA among the subjects in our study reflected the low prevalence of HBV DNA among patients positive for anti-HBc only when compared with those positive for HBsAg. According to the literature, only 5–10% of "anti-HBc only" individuals have been found positive for HBV DNA (8). Because our HBsAg assay did not work on the postmortem samples assayed in this study, we do not know the prevalence of HBsAg positivity. However, our prevalence of HBV DNA among anti-HBc positive/anti-HBs negative drug users was comparable with those from a previous Danish study among living drug users in which the prevalence of HBsAg was 22% (13/58) among anti-HBc positive/anti-HBs negative and 9% (13/140) among all drug users (7). Therefore, we assumed that the prevalence of HBsAg-positives in our population would be similar.

The very high prevalence of HCV among injecting drug users (57%) found in this study coincided with the 57% anti-HCV-positives among injecting drug users reported in the 2002 U.S. national survey (9). The prevalence of HCV among drug users could vary considerably and was not stable over time (10). Continued surveillance among persons who die from drug-related causes will enable us to follow changes in HCV prevalence over time and to monitor the effectiveness of future preventive measures.

The detection rate for HCV RNA among the subjects in this study was 64% (Table 2), consistent with previous findings that 65% of anti-HCV-positive Danish drug users were HCV RNA positive (1,11). In a study by Kato et al. (12), HCV RNA was detected in only 16.7% (2/12) of the subjects. No anti-HCV screening had been performed prior to HCV RNA detection. This prevalence seems low; in our study, random testing would have yielded 33% HCV RNA-positive subjects (64% of 51% of anti-HCV-positive subjects). However, the sensitivity of the assay and the prevalence of HCV infection in their drug-using population were not reported.

Among anti-HIV positive subjects, we would expect all to be HIV RNA positive; however, 57% were negative (Table 3). Among living anti-HIV-positive subjects, all were HIV RNA positive if they had not received antiretroviral therapy. We had no information regarding treatment at the time of death for these subjects, but if some had received antiviral treatment, this could explain the low prevalence of HIV RNA-positive results. However, one of the anti-HIV-positive/HIV RNA-negative subjects was the first diagnosed postmortem, and it is unlikely that this person had received antiretroviral treatment prior to his death. Thus, our results might reflect a high false-negative result rate. The commercial assays for HIV and HCV RNA were of almost equal sensitivity, but the mean viral load among untreated patients was 1–2 log lower for HIV patients when compared with HCV patients (13–16). Therefore, the HIV RNA assay might be more sensitive to postmortem degradation of RNA than the HCV RNA assay. Burtonboy and Delloye (3) found that the HIV RNA detection rate fell from 96% antemortem to 55% at 24 h or more postmortem. The time interval from death to sampling ranged from 2 to 5 days in this study and was longest among subjects who tested negative for HIV RNA.

We concluded that ongoing HBV and HCV infections could be detected reliably postmortem by viral nucleic acid testing. HIV RNA detection was feasible but was probably less sensitive than anti-HIV serological analysis. In addition, the cost of the PCR testing was 10 times greater than that of serological testing. The advantage of PCR is that it detects ongoing viral infections at death and enables sequence analysis that can be used to investigate routes of transmission. Therefore, we suggest that nucleic acid testing is a useful supplement to postmortem serological testing; however, it cannot replace antibody testing to determine the prevalence of past infections or the proportion protected by HBV vaccine.

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