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Authors: *Nemanja Borovcanin*, †*Elizabeta Ristanovic*, ‡*Milena Todorovic*,§*Milica Borovcanin*,‖‖*Mirjana Jovanovic*,‖§*Bela Balint*; Vojnosanitetski pregled (2017); Online First September, 2017.

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THE USE OF COMPLEMENTARY SEROLOGICAL AND MOLECULAR TESTING FOR BLOOD-BORNE PATHOGENS AND EVALUATION OF SOCIO-DEMOGRAPHIC CHARACTERISTICS OF INTRAVENOUS DRUG USERS ON SUBSTITUTION THERAPY FROM SHUMADIA DISTRICT OF SERBIA

Nemanja Borovcanin*, Elizabeta Ristanovic†, Milena Todorovic*,†§, Milica Borovcanin†¶.
Mirjana Jovanovic‖§, Bela Balint*††,**

*Institute for Transfusiology and Hemobiology, Military Medical Academy, Belgrade, Serbia;
†Institute for Microbiology, Military Medical Academy, Belgrade, Serbia;
‡Clinic for Hematology, Clinical Center of Serbia, Belgrade, Serbia;
§Medical faculty, University of Belgrade, Belgrade, Serbia;
‖Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia;
¶Psychiatric Clinic, Clinical Centre Kragujevac, Kragujevac, Serbia
††Serbian Academy of Sciences and Arts;
**Faculty of Medicine of Military Medical Academy, University of Defence, Belgrade, Serbia.

*Correspondence to:
Nemanja Borovcanin MD,
Institute of Transfusiology and Hemobiology, Military Medical Academy,
Crnotravska 17,
11 000 Belgrade, Serbia.
Tel.: +381 11 3609135; E-mail address: drsky2@gmail.com.

Running title: Serological/molecular pathogen testing and socio-demographic characteristics of intravenous drug users
Abstract

**Background/Aim.** Intravenous drug users (IDUs) are still a high risk-group for cross-reacting blood-borne infections, for vertical pathogen transmission, as well as for potentially blood/plasma donation (especially as "paid" donors). The aim of our study was to establish the profile of opiate addict and prevalence of blood-borne pathogens – Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Human Immunodeficiency Virus (HIV) among 99 patients on substitution therapy with methadone and buprenorphine from Shumadia District. **Methods.** The Treatment Demand Indicator (TDI) of Pompidou-questionnaire was used to assess the history of drug abuse and risk behavior. All blood samples were tested for HBV surface antigen (HBsAg), anti-HCV antibody (anti-HCV) and HIV antigen/antibody (HIV-Ag/Ab) by Enzyme-Linked ImmunoSorbent Assay (ELISA) or Chemiluminescent Immuno-Assay (CIA). Investigations were also performed for HBV, HCV and HIV by molecular testing – Polymerase Chain Reaction (PCR) method.

**Results.** The majority of patients were male (81.8%), median age 32 (19–57) years, lived in a city (99%), unemployed (58.6%), with finished secondary school (67.7%), unsafe injecting practices (34.3%) and never previously tested for HBV (39.4%), HCV (36.4%) nor HIV (28.3%); only four percentage of them previously got HBV-vaccine. Complementary testing resulted with following results: HBV ELISA/CIA and PCR negativity for 66 patients and positive results (by ELISA/CIA and PCR) for 19 patients. However, a difference was observed in ELISA/CIA-negative / PCR-positive result for 12 and ELISA/CIA-positive / PCR-negative for two patients, respectively. Further, negative results for HCV (ELISA/CIA and PCR testing) were found in 15 IDUs and positive results (using both methods) were found in 58 patients. Different results for ELISA/CIA-negative / PCR-positive results were found in 11 IDUs and ELISA/CIA-positive / PCR-
negative results were found in 15 patients. All investigated IDUs were negative for HIV (ELISA/CIA and PCR testing) and for pathogens of opportunistic infection (Cryptococcus neoformans; Pneumocystis carini; PCR testing), as well as for West Nile Virus (PCR testing). Just one IDU was positive for syphilis (ELISA and confirmatory testing).

**Conclusion.** This investigation undoubtedly confirmed the effectiveness and improved safety of originally designed complementary (ELISA/CIA and PCR) pathogen monitoring system. Our study demonstrated that the positivity for HBV and HCV is still very high (33.4% and 84.8%, respectively) in IDUs. Thus, we suggest that drug users have to be periodically screened using a complementary serological/molecular testing – concerning differences/discrepancies in results obtained using these methods.

**Key words:** intravenous drug users, blood-borne pathogens, HBV, HCV, HIV

**Komplementarno serološko i molekularno testiranje krvno-prenosivih patogena i procena socio-demografskih karakteristika kod korisnika intravenskih droga na supstitucionoj terapiji u Šumadijskom okrugu Srbije**

**Apstrakt**

**Uvod.** Korisnici intravenskih droga predstavljaju visokorizičnu grupu zbog međusobnih infekcija krvno prenosivim bolestima, vertikalne transmisije patogena, kao i zbog mogućnosti da budu potencijalni donori krvi/produkata plazme (naročito kao plaćeni donori). **Cilj.** Cilj našeg istraživanja bio je utvrđivanje demografsko-sociološkog profila 99 opijatnih zavisnika Šumadijskog okruga lečenih u Kliničkom centru Kragujevac supstitucionom terapijom metadonom i buprenorfinom, kao i određivanje prevalencije infekcija krvno prenosivim bolestima: virusom hepatitisa B, virusom hepatitisa C i virusom...
stečene imunodeficijencije (HBV, HCV i HIV). **Metode:** Ispitanici su odgovarali na pitanja iz Pompidu upitnika i podaci iz ovog upitnika su korišćeni za analizu osnovnih socio-demografskih karakteristika Svi uzorci su prvo testirani ELISA (Enzyme-Linked ImmunoSorbent Assay) i CIA (Chemiluminescent Immuno-Assay) metodom, a zatim PCR-om (Polymerase Chain Reaction). **Rezultati:** Najveći broj ispitanika je bilo muškog pola (81,8%), starosti 32 (19–57) godine, 99% ispitanika je živelo u gradu, nezaposlenih je bilo 58,6 %, sa završenom srednjom školom 67,7 %, a korisnika neadekvatne primene igala bilo je 34,3%. Netestiranih na HBV je bilo 39,4 %, na HCV 36,4 %, HIV 28,3 % a samo njih 4 (4 %) je primilo vakcinu protiv HBV. Što se tiče analiza na prisustvo HBV infekcije, ELISA/CIA i PCR negativnih je bilo 66, HBV ELISA/CIA i PCR pozitivnih je bilo 19, HBV ELISA/CIA-negativnih / PCR-požitivnih 12 i HBV ELISA/CIA-požitivnih / PCR-negativnih 2 ispitanika. Testiranje na HCV infekciju je pokazalo sledeće: ELISA/CIA i PCR negativnih ispitanika je bilo 15, HCV ELISA/CIA i PCR pozitivnih je bilo 58, HCV ELISA/CIA-negativnih / PCR-požitivnih 11, a HCV ELISA/CIA-požitivnih / PCR-negativnih 15. Svi ispitanici su bili negativni na HIV (ELISA/CIA i PCR testiranje), kao i na patogene oportunističkih infekcija (Cryptococcus neoformans; Pneumocystis carini; PCR testiranje) i na prisustvo virusa zapadnog Nila (West Nile Virus, PCR testiranje). Jedan ispitanik je bio pozitivan na sifilis (ELISA testiranje). **Zaključak:** Naši rezultati su pokazali da je pozitivnost na prisustvo patogena krvno prenosivih bolesti HBV i HCV visoka u ispitivanoj grupi korisnika intravenskih droga i iznosi 33,4% i 84,8%, respektivno. Preporuka bi bila da oni budu periodično testirani na prisustvo HBV, HCV i HIV infekcije, komplementarnim ELISA/CIA testovima kao i PCR testovima, obzirom na izvestan stepen diskrepance u dobijenim rezultatima serološkog i molekularnog testiranja.

**Ključne reči:** korisnici intravenskih droga, hematogeni patogeni, HBV, HCV, HIV
Introduction

The integrative approach is the necessity in the modern treatment of addiction, especially considering early detection and additional treatment of somatic states in intravenous drug users. The "drug-use-disorder" show to be very frequent in European countries (approximately 0.5% of population or about two million people), with relatively more problem of diseases caused by "drug-use-disorder" in Western Europe and especially high rates of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection in this vulnerable population.

The intravenous drug users (IDUs) face stigma not only due to psychological and behavioral aspects of their functioning, but also because of significantly higher rates of blood-borne and/or sexually transmitted infections due to unsafe injecting practices and risky sexual behaviors. The pharmacological substitution programs of methadone and buprenorphine are "harm avoidance" programs that are also useful in prevention of blood-borne infections.

Opiate addiction treatment in Serbia is conducted in four clinical centers by supervision of the Ministry of Health, Republic of Serbia – including more than 4000 patients on substitution therapy, but objective, precise and longitudinal data about opiate addiction and infectious disease "co-occurrence" is still lacking.

Serological testing, like routine screening of blood donors are performed by anti-HCV Enzyme Linked ImmunoSorbent Assay (ELISA) or Chemiluminescent Immuno-Assays (CIA) methods. In the last years of the 20th century, two more tests were initiated to detect the presence of HCV: HCV Ag/Ab, and HCV Nucleic Acid Testing (NAT) or Polymerase Chain Reaction (PCR) assays.
The period of the "window" (the time from entering the virus in the body until the moment when it is detectable by the available techniques) before the introduction of PCR was about 70 days. By introducing PCR individual testing this period is reduced to 15 days, while the window period for HCV Ag/Ab is 40 days. The window period is 16 days for HIV Ag/Ab, while in HIV PCR it is reduced to 9 days. The actuality of comparisons of test results by ELISA and PCR methods lies in determining the infectivity of the samples or the phase of infection in which there are dependents on psychoactive substances in this case. It is necessary to determine the infectivity of the tested addicts and in terms of delineation of the test results: they did not come into contact with these viruses, the start of the infection – the "window" period, an infection, a past active infection.

The study aim was to evaluate the profile of opiate addict and above all the prevalence of blood-borne infections such as HBV, HCV and HIV among IDUs on substitution therapy with methadone and buprenorphine in Shumadia District of Serbia. The results of complementary ELISA or CIA and PCR testing were also compared in attempt to improve of pathogen monitoring system, as well as the diagnostic algorithm for this vulnerable population.

Methods

Patients/Subjects

In this study patients on substitution therapy with methadone or buprenorphine at Department of addictions, Psychiatric Clinic, Clinical Centre Kragujevac – as a regional addiction treatment center – were included. This centre is managing the drug dependence
treatment using the Treatment Demand Indicator (TDI) approach. The TDI was formulated in 2000 by the European Monitoring Centre for Drugs and Drug Addiction – EMCDDA/Pompidou Group, aiming to collect comparable and reliable data about the number and characteristics of drug addicts in EU countries.\(^9\)

TDI is evaluating treatment needs and assessing the history of drug abuse and risk behavior. Collected data from this questionnaire was used for the socio-demographic and injection practice analysis. Diagnoses of opioid related disorders (F11) or other psychoactive substance related disorders (F19) were established using International Statistical Classification of Diseases and Related Health Problems.

The protocol of this "cross-sectional" study was approved by the Ethic Committee of Clinical Centre Kragujevac and conducted in accordance with all the ethical principles of the Declaration of Helsinki. Informed consent was obtained from all of the patients before starting any study procedure.

**Pathogen investigation methods**

Total of 99 IDUs were tested using complementary serological and molecular testing. All samples were taken into 9 ml tubes with K2EDTA (Bio-One Vacuette, Greiner), than the tubes were centrifuged at 3500 rpm, 30 minutes and plasma samples were analyzed. Samples were initially tested by ELISA or CIA systems (Evolis, Biorad; Architect i2000 SR, Abbott) and afterward with s201 system (COBAS Ampliprep/ COBAS Taqman, Roche). Preliminary positive specimens were analyzed using ID (Individual Donation) PCR. Preliminary negative samples were tested by mini-pool (MP) PCR technique (6 samples).

HCV ELISA/CIA-negative / PCR-positive samples were tested using confirmatory test (n=11; Innolia Innogenetics)\(^7\). In addition, all 99 IDUs were tested on pathogens of
opportune infections, such as Cryptococcus neoformans and Pneumocystis carini. Finally, patients were also investigated for syphilis by ELISA or CIA method and West Nile Virus (WNW) by PCR testing.

The pathogen investigations were performed in the Institute of Transfusiology and Hemobiology of Military Medical Academy (MMA) and in the Institute of Microbiology of MMA in Belgrade – during the period from July to August 2015.

**Statistical analysis**

The data was presented as absolute numbers, median and percentages. The tables were used to present the socio-demographic characteristics of IDUs, as well as complementary serological and molecular investigations. The 2×2 contingency table was used to compare the results of serological and molecular testing. The statistical analyses were performed using SPSS 20.0 software. Differences were considered as statistically significant if the p value was less than 0.05.

**Results**

The majority of IDUs were male, city residents and dominantly with a completed secondary school – their characteristics are presented in Table 1.

Table 1

Amongst all 99 IDUs, some of them have never been tested on virus infections – exactly, on HBV 39, on HCV 36, on HIV 28; in addition, just 4 IDUs got a vaccine against HBV.

Complementary ELISA or CIA and PCR testing of the IDUs demonstrated predominant concordance between serological and molecular analysis. Results confirmed that more than 80% of IDUs had HCV positivity, proved by both testing, comparing to more than 30%
proved HBV infection. Concordance and discordance between different methods (ELISA or CIA vs. PCR) are shown in the Table 2.

Table 2

Absolute numbers of patients – analyzed by comparative serological and molecular testing for HCV vs. HBV in this study – are summarized in Table 3 and in Figure 1.

Table 3 and Figure 1.

As presented, the number of HCV vs. HBV positive IDUs was significantly (p < 0.01) higher in our study group, using both ELISA or CIA and PCR techniques.

Regarding the opportunistic infections, all 99 IDUs were negative (PCR testing) for the most common pathogens: Cryptococcus neoformans, Pneumocystis carini and West Nile Virus. Only one patient was positive on syphilis by ELISA, VDRL and TPHA. These results are presented in Table 4.

Table 4

The presented negativity for opportunistic infection pathogens can be explain because of no severe compromised immune system – since all IDUs were negative on HIV.

Discussion

Several risk factors make IDUs vulnerable to HCV, HBV, HIV, syphilis and opportunistic infection caused by Cryptococcus neoformans, Pneumocystis carini and WNV. Hazardous behaviors include the use of non-sterilized needles and unprotected sexual activities, unsafe tattooing, cupping, blood transfusion or dental procedures in both IDUs and non-IDUs. Besides, lack of access to health services, low socio-educational level, homelessness, history of imprisonment, social exclusion, unemployment, alcohol addiction and presence of other diseases complicate the feature of infection by HCV, HBV and HIV viruses and their related outcomes in many IDUs.
The global prevalence of HCV infection among IDUs in 2010 was 46.7%, implicating that some 7.4 million of the 16 million IDUs worldwide are infected with the HCV. The HBV infection rate among IDUs is about 14.6%, that is 2.3 million IDU are infected with mentioned virus, and 18.9% or 3 million of IDUs are living with HIV worldwide. Despite the higher prevalence and "transmissibility" and the equal or higher economic costs of HCV compared to HIV infection, especially among IDUs, viral hepatitis received far less attention than HIV related disease. Worldwide, the prevalence of HIV infection amongst IDUs was calculated as 17.9% in 2009 and 18.9% in 2010.

In our study there is no HIV infection among IDUs and that is similar as prevalence in Iran (0.7%) and among blood donors tested earlier in MMA (0.005%) \(^8,12\). Prevalence of HBV (33.3%) was lower than in the Italy (where the prevalence is 60.7%), while in Mexico is 85%, similar as in Greece and Portugal, but significant higher than prevalence in Uruguay (20%), Iran (0.7%) and among blood donors (0.20%) \(^13-15\). IDUs had a much higher probability of acquiring infection than non-injectors, confirming the role of intravenous transmission. The prevalence was highest for HCV infection (58.6%) and that is lower than prevalence observed in Estonia and Latvia (about 90%), Romania and Portugal, and similar as in Russia (73%). The HCV prevalence is higher than in Hungary (23%) and among blood donors (0.12%) \(^12-16\).

The difference between results of ELISA/CIA and PCR testing can be explained on two ways. Firstly, ELISA/CIA negative, but PCR positive results show that infection with HBV or HCV is in "window" period – that mean that concentration of viral antigen or antibodies against them are to low that they can not measured by ELISA/CIA \(^8\). On the other hand, PCR negative ELISA/CIA positive results are common when we have cases of old HCV infections. The number of these results can be even 20% among blood donors, so the
prevalence of 15.1% in our study is in that range \(^{18}\). The number of two HBV PCR negative ELISA/CIA positive results show that HBV DNA levels in HBsAg-positive samples can be extremely low. About 6\% of donations would be negative by current MP HBV PCR methods. About 3\% of donations would remain undetected by sensitive single-donor PCR. These results indicate caution in any consideration of dropping HBsAg screening \(^{18}\).

IDUs in Serbia have similar social-demographic characteristics as Italian: 88\% vs. 84\% are male, median age is 32 vs. 35 years, mostly unemployed. However, IDUs in this investigation were not well educated because only 4\% of patients received HBV vaccine – while 29\% in Italy \(^{13}\). Although, this fact could be a consequence of the suboptimal medical care system in investigated region.

**Conclusion**

The majority of IDUs were male (aged 19–57 yrs), city residents and dominantly with a completed secondary school. This study undoubtedly demonstrated improved safety of originally designed complementary (ELISA/CIA and PCR) pathogen monitoring system. Our results confirmed that injecting drug practice continues to be an important risk factor for blood-borne infections; the positivity for HBV and HCV was still very high – 33.4\% and 84.8\%, respectively. Thus, drug users have to be periodically screened by complementary serological/molecular testing – concerning differences/discrepancies in results obtained using these methods. Finally, we speculate that HBV vaccination should be actively obtainable/offered to all HBV-negative IDUs.

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Figure legend

Figure 1
Comparative HCV and HBV values determined by ELISA/CIA and PCR
* and ** indicate significant differences between HBS and HCV positivity by both, serological and molecular testing (p<0.01).
Table 1

The socio-demographic characteristics of IDUs

| Parameters investigated                      | Values          |
|----------------------------------------------|-----------------|
| Age median (yrs)                             | 32              |
| Age range (yrs)                              | 19 to 57        |
| Gender Male                                  | 88 (81.8%)      |
| Gender Female                                | 11 (18.2%)      |
| Residents of the city                        | 98 (98.9%)      |
| Unemployed                                   | 58 (58.6%)      |
| Education level – secondary school           | 67 (67.7%)      |
| Unsafe injecting practices                   | 34 (34.3%)      |

IDUs = intravenous drug users.
Table 2.

Complementary ELISA or CIA and PCR investigations for viruses

| Pathogen type | ELISA or CIA and PCR testing | IDU number (percentage) |
|---------------|-----------------------------|------------------------|
| HBV           | ELISA/CIA-negative / PCR-negative | 66 (66.7%)            |
|               | ELISA/CIA-positive / PCR-positive | 19 (19.2%)            |
|               | ELISA/CIA-negative / PCR-positive | 12 (12.1%)            |
|               | ELISA/CIA-positive / PCR-negative | 2 (2.1%)              |
| HCV           | ELISA/CIA-negative / PCR-negative | 15 (15.1%)            |
|               | ELISA/CIA-positive / PCR-positive | 58 (58.6%)            |
|               | ELISA/CIA-negative / PCR-positive | 11 (11.1%)            |
|               | ELISA/CIA-positive / PCR-negative | 15 (15.1%)            |
| HIV           | ELISA/CIA-negative / PCR-negative | 99 (100%)             |

IDU = intravenous drug user.
### Table 3.

The HBV and HCV presence investigated by ELISA or CIA and PCR

| Pathogen type | ELISA or CIA testing (absolute numbers) | PCR testing (absolute numbers) |
|---------------|-----------------------------------------|--------------------------------|
|               | HCV                                     | HCV                            |
|               | negative                                | negative                        |
| HBV           | 25                                      | 18                             |
|               | positive                                | 56*                            | 50*                           |
|               | positive                                | 1*                             | 12*                           |

*significant difference in the HBV and HCV positivity (1+17 vs. 56+17; 12+19 vs. 50+19) (p < 0.01).

### Table 4.

The results of ELISA or CIA and PCR testing for other pathogens

| Pathogen type               | ELISA or CIA and PCR testing | IDU number (percentage) |
|-----------------------------|-----------------------------|-------------------------|
| Treponema pallidum          | ELISA/CIA-negative          | 98 (99%)                |
|                             | ELISA-positive              |                         |
|                             | *VDRL-positive              | 1 (1%)                  |
|                             | **TPHA-positive             |                         |
|                             | Confirmatory test-positive  |                         |
| Cryptococcus neoformans     | PCR-negative                | 99 (100%)               |
| Pneumocystis carini         | PCR-negative                | 99 (100%)               |
| West Nile Virus             | PCR-negative                | 99 (100%)               |

IDU = intravenous drug users;
VDRL = Venereal Disease Research Laboratory testing;
TPHA = Treponema pallidum Hemagglutination test.
