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Hepatitis D virus infection in Slovenian patients with chronic hepatitis B virus infection: a national prevalence study and literature review

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Abstract

Introduction: Of the 350 million individuals chronically infected with hepatitis B virus (HBV) worldwide, approximately 15 to 20 million have been exposed to hepatitis D virus (HDV). This study determined for the first time the HDV prevalence in Slovenian patients with chronic HBV infection. In addition, a literature search was performed to identify all HDV prevalence studies from European countries.

Methods: A total of 1,305 HBsAg-positive serum samples, obtained from the same number of patients, were randomly selected from 2,337 patients referred to the Slovenian national reference laboratory for viral hepatitis between 1998 and 2015. All samples were retrospectively tested for the presence of total anti-HDV antibodies. Anti-HDV-positive patients were additionally tested for the presence of anti-HDV IgM antibodies, HDV antigen, and HDV RNA.

Results: Total anti-HDV antibodies were detected in three of the 1,305 patients tested (0.23%; 95% CI: 0.08–0.67%), of whom one patient had recovered from the past HDV infection and two patients had an ongoing chronic HDV infection. The literature search identified 36 peer-reviewed HDV prevalence studies published between 1983 and 2016 and originating from 21 European countries.

Conclusion: The observed prevalence of HDV infection in Slovenia was among the lowest reported in Europe and worldwide. Due to the observed low prevalence of HDV infection, routine diagnostic testing for HDV should not be considered in differential diagnosis of exacerbation of liver disease in Slovenian patients with chronic HBV infection.

Keywords: hepatitis D virus, prevalence, acute HDV infection, chronic HDV infection, Slovenia, Europe

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Introduction

Hepatitis D virus (HDV), the only representative of the family *Deltaviridae*, is one of the five primary etiological agents of viral hepatitis. HDV is an incomplete satellite virus that can replicate only in cells concomitantly infected with hepatitis B virus (HBV) because its envelope consists of HBV surface antigens (HBsAg) and lipids from the host cell (1). HDV is mostly transmitted parenterally, with the highest transmission rates in injecting drug users and individuals exposed to infected blood or blood products (2–4). In addition, intra-familiar transmission in hyper-endemic regions (4) and sexual transmission are also described (5, 6). Upon co-infection with HBV and HDV, acute hepatitis is usually followed by a resolution of both HBV and HDV infections. However, super-infection with HDV usually results in chronic hepatitis caused by both viruses. In comparison to patients with chronic hepatitis B, patients with chronic hepatitis B and D are significantly more likely to develop cirrhosis, liver decompensation, and hepatocellular carcinoma (4, 7, 8).

Of the 350 million individuals chronically infected with HBV worldwide, approximately 15 to 20 million have been exposed to HDV infection (9, 10). Interestingly, the prevalence of HDV infection varies greatly across different geographic regions and does not exactly match the distribution of patients with chronic HBV infection (11, 12). As a result of vaccination against HBV, mandatory testing of blood donors, improvements in sanitation, and behavioral changes, HDV prevalence has decreased in the last 20 years in the majority of European countries, especially in southern Europe (13). However, it has recently begun rising again in some European countries, such as France, Germany, Spain, and the United Kingdom, due to immigration from endemic areas (mainly from Africa, eastern Europe, and Turkey) (2, 14–19).

Slovenia is a country with a population of approximately two million and an estimated HBV prevalence of less than 5%. To the best of our knowledge, no reports on HDV prevalence in Slovenia have been published to date in the peer-reviewed literature. Thus, the main aim of our study was to determine the HDV prevalence in Slovenian patients with chronic HBV infection. In addition, to compare our results with existing published data, a literature search was performed to identify all HDV prevalence studies originating from European countries. A literature search was performed on June 10th, 2016. Eligible peer-reviewed studies, with no bias toward articles written in English, published between 1983 and 2016 were searched through the MEDLINE/PubMed, Web of Science, Scopus, and Google Scholar databases using a combination of the following terms: *hepatitis D virus*, *HDV*, *HDV antibodies*, *prevalence*, and *Europe*.

Material and methods

This study included 1,305 HBsAg-positive serum samples obtained from the same number of patients randomly selected from all 2,337 patients with chronic hepatitis B referred to the Slovenian national reference laboratory for viral hepatitis at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, between February 1998 and December 2015 (Fig. 1). Information on sex, age, and place of residence was available for all 1,305 patients tested; our study group comprised 792 men (60.7%)

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and 513 women (39.3%). The mean age for men and women at diagnosis of chronic hepatitis B was 45.5 and 43.3 years, respectively. Considering the 95% confidence interval and 2.5% margin of error, our sample size was representative for all patients with chronic HBV infection in Slovenia.

The selected HBsAg-positive serum samples were retrospectively tested for the presence of total anti-HDV antibodies using the commercially available enzyme-linked immunosorbent assay ETI-AB-DELTAK-2 (DiaSorin, Saluggia, Italy) with 99.0% (95% confidence interval; CI: 97.8–99.6%) clinical specificity and 99.4% (95% CI: 96.8–100%) clinical sensitivity for detection of total anti-HDV antibodies, as declared by the manufacturer. Additional serum samples were retrieved from all anti-HDV-positive individuals and retrospectively tested for the presence of anti-HDV IgM and HDV-Ag using the commercially available enzyme immunoassays ETI-DELTA-IGMK-2 (DiaSorin) and ETI-DELTAK-2 (DiaSorin), respectively. According to the manufacturer, ETI-DELTA-IGMK-2 (DiaSorin) and ETI-DELTAK-2 (DiaSorin) have a clinical specificity of 99.0% (95% CI: 97.9–99.6%) and 99.0% (95% CI: 98.0–99.6%), respectively, and a clinical sensitivity of 99.5% (95% CI: 97.4–100%) and 100% (95% CI: 87.2–100%), respectively. All serology-based methods were performed strictly following the manufacturer's instructions.

For detecting HDV viremia, total nucleic acids were extracted from all anti-HDV-positive serum samples using the commercially available MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) on the MagNA Pure Compact Instrument (Roche Diagnostics), following our in-house protocol. Briefly, prior to automatic extraction of nucleic acids using a Total_NA_Plasma_external_lysis protocol, an external lysis of each serum sample (200 µl) was performed using 300 µl of MagNA Pure LC Total Nucleic Acid Isolation Kit – Lysis/Binding Buffer Refill (Roche Diagnostics) and 30-minute incubation at 25 °C and 350 rpm. Total nucleic acids were eluted in 100 µl of elution buffer and stored (in 10 µl aliquots) at –20 °C. In all samples, the quality/integrity of the extracted RNA was verified by amplification of the 85-bp fragment of human ribosomal RNA (rRNA), encoding the S9 protein, using primers (RibPS9-F and RibPS9-R) and a probe (RibPS9-Probe) previously published (20). The RibPS9 reverse-transcription real-time PCR was conducted using LightCycler 480 RNA Master Hydrolysis Probes (Roche Diagnostics) in a 25 µl reaction mixture, consisting of 5 µl of template RNA, 7.4 µl of 2.7 × LightCycler 480 RNA Master Hydrolysis Probes, 1.3 µl of Activator (50 mM), 1 µl of 20 × Enhancer, 0.5 µM of each primer, 0.2 µM of the TaqMan probe, and water. The test was performed on the LightCycler 480 II RT-PCR Instrument (Roche Diagnostics) under the following conditions: 3 min at 63 °C (4.4 °C/s), 30 s at 95 °C (4.4 °C/s), followed by 45 cycles of 15 s at 95 °C (4.4 °C/s), 1 min at 60 °C (2.2 °C/s), and 1 s at 72 °C (4.4 °C/s), and a final 10 s cooling of the reaction mixture at 40 °C (2.2 °C/s). Only RibPS9-positive samples were used in downstream determination of the presence of HDV RNA. The in-house HDV reverse-transcription real-time PCR (HDV rt-RT-PCR), enabling amplification of a 71-bp fragment of the conserved genomic region encoding HDV-Ag, was performed using primers (HDV-F1, HDV-F2, and HDV-R) and a probe (HDV-probe) (21, 22) previously published, following the same protocol as described above, with the exception of annealing temperature, which was set to 55 °C. Based on the testing of HDV-RNA-positive clinical samples with known concentrations of viral RNA (300-11,000,000 viral copies/ml), the analytical sensitivity of the HDV rt-RT-PCR was estimated to be at least 300 viral copies/ml.

Information on HBV viral load was retrieved from the database of the Slovenian national reference laboratory for viral hepatitis.

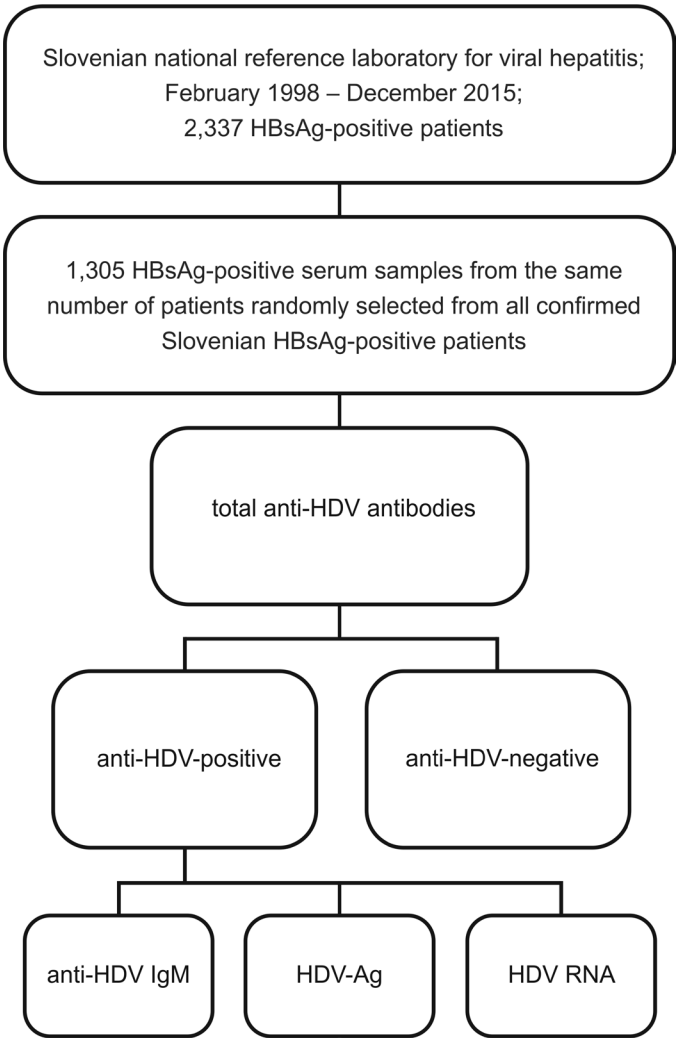

Figure 1 | Overview of the study protocol. Between February 1998 and December 2015, 2,337 HBsAg-positive patients were followed by the Slovenian reference laboratory for viral hepatitis. Among all HBsAg-positive patients, 1,305 patients were randomly selected for total anti-HDV antibody testing. All anti-HDV-positive patients were further tested for the presence of anti-HDV IgM antibodies (anti-HDV IgM), hepatitis D antigen (HDV-Ag) and HDV RNA.

Results

Total anti-HDV antibodies were detected in three of 1,305 patients with chronic HBV infection (0.23%; 95% CI: 0.08–0.67%). In a 48-year-old male patient, except for total anti-HDV antibodies, no other HDV infection markers were detected in any of the samples tested, suggesting resolved HDV infection in this patient (patient 1, Table 1). In contrast, anti-HDV IgM antibodies and HDV RNA were detected in all samples tested obtained from a 28-year-old female patient and a 60-year-old male patient (patients 2 and 3, Table 1) suggesting ongoing chronic HDV infection in both patients. Interestingly, HDV-Ag could not be detected in any serum samples from both patients with ongoing HDV infection. All three anti-HDV antibody-positive patients tested negative for the presence of human immunodeficiency virus (HIV) infection and were generally considered to be immunocompetent.

Discussion

A literature search on HDV prevalence studies from European countries, performed on July 10th, 2016, resulted in 36 peer-reviewed studies from 21 European countries (Table 2). According to this search, Slovenia was one of the few remaining European

countries with no reports on HDV prevalence published in the peer-reviewed literature. In this study, total anti-HDV antibodies were detected in 3/1,305 (0.23%; 95% CI: 0.08–0.67%) Slovenian patients with chronic HBV infection. Our results could be considered representative for all patients with chronic HBV infection in Slovenia, considering the 95% confidence interval and 2.5% margin of error. Even though information on sex, age, and place of residence was available for all patients in the study, the low rate of HDV positivity prevented the analysis of a possible relationship between anti-HDV positivity and patients' characteristics.

As a result of vaccination against HBV, mandatory testing of blood donors, improvements in sanitation, and behavioral changes, HDV prevalence has decreased in the last 20 years in the majority of European countries, especially in southern Europe (13). In several cross-sectional studies, which included hundreds of HBsAg-positive individuals with liver disease, the HDV prevalence in Italy was estimated at 24.7% in 1981, 23.4% in 1987, 14.4% in 1992, 8.3% in 1997, and 8.1% in 2007 (23–27). However, since 2007 no further decrease in HDV prevalence was recorded in Italy, with steady prevalence rates of 8.4% among HBsAg carriers (28). Of note, the reported prevalence of HDV infection was lower, ranging from 1.3 to 5.0% in Italians and from 4.7 to 7.9% in immigrants, in studies providing data from limited geographic regions (29, 30). Unlike several HDV prevalence studies in Italy, the data on HDV prevalence in the other three countries bordering Slovenia are scant and outdated because they date back to 1985, 1993, and 1994, respectively (31–33). Thus, the prevalence of HDV infection in Austria, Croatia, and Hungary was estimated at 2.9% (HBsAg carriers), 19.0% (chronic HBsAg carriers), and 13.6% (chronic HBsAg carriers), respectively (31–33). Even though a more thorough comparison of HDV prevalence is hampered due to outdated information from the majority of countries bordering Slovenia and the use of HDV diagnostic tests with different analytical characteristics in prevalence surveys in different countries, it seems that the HDV prevalence in Slovenian patients with chronic HBV infection is among the lowest in southern Europe. The low prevalence of HDV infection may be attributable to the low HBV burden in Slovenia, which reflects broad HBV vaccination coverage and mandatory testing of blood donors, pregnant women, and family members and partners of HBsAg-positive individuals. It is noteworthy that in Slovenia the national HBV vaccination program started in 1983 and continued to expand until 1998, when HBV vaccination of preschool children was added to the national vaccination schedule. Interestingly, Italy's incidence of acute HBV (1 per 100,000) is similar to that of Slovenia (0.8 per 100,000) and Italy established a HBV vaccination program for neonates and 12-year-olds in 1991 (34, 35); however, in comparison to Slovenia, the prevalence of HDV infection in still significantly higher in Italy. It appears that in Europe HDV is sustained by two different residual pools of HDV-infected patients: (i) young individuals that are migrating from HDV endemic areas and (ii) older individuals that were infected with HDV during the 1980s epidemics (also referred to as the domestic pool) (2, 8, 36). Without accurate epidemiologic data, one can only speculate that the observed difference in HDV prevalence between Slovenia and Italy is a consequence of an existing reservoir of HDV-positive individuals that were infected during the 1980s epidemics in Italy. Moreover, the higher HDV prevalence in Italy could also be attributable to differences in risky behavior among HDV-positive individuals in both countries, such as drug abuse or promiscuous sexual practices.

In contrast to southern Europe, HDV prevalence has recently begun to rise again in other European countries, such as France, Germany, Spain, and the United Kingdom, due to immigration from endemic areas (mainly from Africa, eastern Europe, and Turkey) (2, 14–19). With on-going globalization and the influx of immigrants from less-developed endemic countries, where HBV is not controlled through vaccination and screening, regular epidemiological surveillance of HDV prevalence in Slovenia is also recommended in the near future.

To evaluate whether differences in HDV prevalence across European countries could be at least partially attributed to the use of anti-HDV antibody tests with different analytical characteristics (mainly different analytical specificity), the material and methods sections of published studies (Table 2) were carefully analyzed. Out of 36 eligible studies, the commercially available enzyme-linked immunosorbent assay ETI-AB-DELTAK-2 (DiaSorin) was used in nine studies (14, 16, 19, 30, 37–41) and the Abbott anti-HD radioimmunoassay (Abbott Laboratories, Chicago, IL) and Radim anti-HDVAb (Radim Iberica, Barcelona, Spain) were used in one study each (17, 29). Unfortunately, tests used for detecting anti-HDV antibodies were not specified in the majority of eligible studies. In addition, due to the wide timespan of eligible studies (published from 1983 to 2016), we were unable to associate reported HDV prevalence rates with the use of specific diagnostic test(s) for detecting anti-HDV antibodies.

Table 1 | Characteristics of three anti-HDV-positive patients: one patient with past HDV infection (patient 1) and two patients with ongoing chronic HDV infection (patients 2 and 3).

					HBV infection markers			HDV infection markers		
Patient no.	Sex	Age at diagnosis	Place of residence	Sample no. (date: mm/dd/yyyy)	HBsAg	HBV DNA (IU/ml)	Total anti-HDV Ab	HDV-Ag	anti-HDV IgM	HDV RNA
1	M	48	Ljubljana	1 (03/14/2000)	positive	N/A	positive	negative	negative	negative
				2 (03/23/2000)	positive	N/A	positive	negative	negative	negative
				3 (12/17/2001)	positive	353	positive	negative	negative	negative
2	F	28	Kranj	1 (11/07/2008)	positive	N/A	positive	negative	positive	positive
				2 (11/11/2008)	positive	232	positive	negative	positive	positive
				3 (02/03/2009)	positive	1,020	positive	negative	positive	positive
				4 (12/08/2009)	positive	‹ 6	positive	negative	positive	positive
3	M	60	Postojna	1 (10/10/2007)	positive	65	positive	negative	positive	positive
				2 (09/17/2008)	positive	34	positive	negative	positive	positive
				3 (10/20/2009)	positive	58	positive	negative	positive	positive
				4 (02/01/2011)	positive	370	positive	negative	positive	positive
				5 (05/24/2011)	positive	68,452	positive	negative	positive	positive
				6 (09/05/2011)	positive	646	positive	negative	positive	positive
				7 (12/06/2011)	positive	‹ 15	positive	negative	positive	positive

Note: total anti-HDV Ab = total anti-HDV antibodies, anti-HDV IgM = anti-HDV IgM antibodies, HDV-Ag = hepatitis D antigen, N/A = not analyzed

Table 2 | Prevalence of HDV infection in European countries (1983–2016) according to a literature search performed on July 10th, 2016.

Country	Tested population (time period)	HDV infection marker(s) tested	HDV prevalence; number of positives / samples tested (% positives)	Reference
Albania	patients with chronic viral and/or alcohol-induced liver disease (1995 and 2005)	total anti-HDV Ab	1995: 10/106 (9.4%); 2005: 7/99 (7.1%)	Kondili et al., 2010 (43)
Austria	HBsAg carriers (N/a)	total anti-HDV Ab	4/138 (2.9%)	Frisch-Niggemeyer and Kunz, 1985 (31)
Belgium	chronic HBsAg carriers (2008–2009)	total anti-HDV Ab	44/800 (5.5%)	Ho et al., 2013 (39)
Bulgaria	chronic HBsAg carriers (N/a)	HDV-Ag	9/105 (8.6%)	Naoumov et al., 1986 (44)
Croatia	chronic HBsAg carriers (N/a)	N/a	19/100 (19.0%)	Jelić and Jelić, 1994 (33)
Denmark	chronic HBV patients (1970–1985)	N/a	29/100 (29.0%)	Krogsgaard et al., 1988 (45)
Finland	HBsAg carriers (1983–1984)	total anti-HDV Ab	1/121 (0.8%)	Pohjanpelto et al., 1985 (46)
France	HBsAg-positive blood donors (1997–2011)	total anti-HDV Ab, HDV RNA	1997–2011: 89/4,492 (2.0%); 1997–2005: 33/2,831 (1.2%); 2010: 13/200 (6.5%); 2011: 2/234 (0.9%)	Servant-Delmas et al., 2014 (14)
Germany	HBsAg carriers in Hannover (1992–2006)	total anti-HDV Ab	266/2,354 (11.3%)	Wedemeyer et al., 2007 (15)
	chronic HBsAg carriers in Frankfurt (2000–2011)	total anti-HDV Ab	210/2,844 (7.4%)	Rehnheimer et al., 2012 (16)
Greece	HBsAg carriers (1997–2010)	total anti-HDV Ab	1997–2010: 90/2,137 (4.2%); 1997–2003: 1,280/2,244 (57.0%); 2004–2010: 857/2,429 (35.3%)	Manesis et al., 2013 (47)
Hungary	chronic HBsAg carriers (N/a)	N/a	16/118 (13.6%)	Horváth et al., 1992–1993 (32)
Italy	HBV-infected patients (1978–1981)	total anti-HDV Ab	494/2,001 (24.7%)	Smedile et al., 1983 (23)
	chronic HBsAg carriers (1987 onward)	total anti-HDV Ab	364/1,556 (23.4%)	Sagnelli et al., 1992 (24)
	chronic HBsAg carriers (1992)	total anti-HDV Ab	143/996 (14.4%)	Sagnelli et al., 1997 (25)
	HBsAg carriers (1997)	total anti-HDV Ab	69/834 (8.3%)	Gaeta et al., 2000 (26)
	chronic HBsAg carriers (2006–2007)	total anti-HDV Ab	112/1,386 (8.1%)	Stroffolini et al., 2009 (27)
	chronic HBsAg carriers in Ferrara (1997–2009)	total anti-HDV Ab	Italians: 1/78 (1.3%); immigrants: 6/76 (7.9%)	Contini et al., 2012 (29)
	HBsAg carriers in Milan (2007–2008)	total anti-HDV Ab	Italians: 19/381 (5.0%); immigrants: 5/107 (4.7%)	De Paschale et al., 2012 (30)
	HBsAg carriers (N/a)	total anti-HDV Ab	Italians: 53/716 (7.4%); immigrants: 34/295 (11.5%)	Brancaccio et al., 2014 (28)
Kosovo	general population (healthcare workers, pregnant women, blood donors, patients included in routine blood testing) (2005)	anti-HDV IgG	1/1,287 (0.08%)	Quaglio et al., 2008 (38)
Poland	chronic HBV patients (N/a)	total anti-HDV Ab	4/102 (3.9%)	Chlabicz et al., 2003 (48)
	chronic HBsAg carriers in the northern part of the country (2002–2004)	total anti-HDV Ab, HDV RNA	total anti-HDV Ab: 3/63 (4.8%); HDV RNA: 5/63 (7.9%)	Bielawski et al., 2006 (37)
Portugal	chronic HBsAg carriers (N/a)	N/a	N/a (17.3%)	Ramalho et al., 1987 (49)
Romania	chronic HBsAg carriers (2005)	anti-HDV IgG, HDV RNA	223/1,094 (20.4%)	Popescu et al., 2013 (50)
Serbia and Montenegro	HBsAg carriers (N/a)	total anti-HDV Ab	69/614 (11.2%)	Delić et al., 1993 (51)
Spain	immigrants (HBsAg carriers) from Equatorial Guinea (2002–2008)	total anti-HDV Ab	249/1,220 (20.4%)	Rivas et al., 2013 (17)
	HIV-positive patients (2004 onward)	total anti-HDV Ab	17/1,147 (1.5%)	Fernández-Montero et al., 2014 (52)
	African immigrants (HBsAg carriers) treated by specialists (N/a)	total anti-HDV Ab	1,984/2,518 (78.8%)	Cuenza-Gómez et al., 2016 (18)
Sweden	chronic HBsAg carriers (1997–2008)	N/a	650/9,160 (7.1%)	Ji et al., 2012 (53)
Switzerland	chronic HBV patients (mostly immigrants) (N/a)	total anti-HDV Ab/ anti-HDV IgM/ anti-HDV IgG/ HDV-Ag/ HDV RNA	101/1,699 (5.9%)	Genné and Rossi, 2011 (3)
	HBsAg carriers (2002–2013)	total anti-HDV Ab	15/338 (4.4%)	Hirzel et al., 2015 (41)
United Kingdom	HBsAg carriers (1970–1989) in Northern Ireland	total anti-HDV Ab	9/401 (2.2%)	Curran et al., 1989 (54)
	chronic HBV patients (mostly immigrants) (2000–2006) in London	total anti-HDV Ab	82/962 (8.5%)	Cross et al., 2008 (2)
	HBsAg carriers (2008–2012) in London	total anti-HDV Ab, anti-HDV IgM, HDV RNA	22/1,048 (2.1%)	William Tong et al., 2013 (40)
	HBsAg carriers (mostly immigrants) (2005–2012) in London	total anti-HDV Ab	162/3,610 (4.5%)	El Bouzidi et al., 2015 (19)

Note: total anti-HDV Ab = total anti-HDV antibodies, anti-HDV IgM = anti-HDV IgM antibodies, anti-HDV IgG = anti-HDV IgG antibodies, HDV-Ag = hepatitis D antigen, N/a = not available

In this study, total anti-HDV antibodies were detected in only three patients: in one patient that recovered from a past HDV infection and in two patients with an ongoing chronic HDV infection. Interestingly, HDV-Ag could not be detected in any serum samples of both patients with ongoing HDV infection (Table 1). This is in accordance with previously published studies reporting that in immunocompetent individuals HDV-Ag is frequently neutralized by anti-HDV antibodies and thus not detectable (10, 42). In contrast, HDV-Ag is usually detected in serum samples obtained from immunocompromised patients chronically infected with HDV (10, 42).

In conclusion, in the first Slovenian national prevalence study the observed prevalence of HDV infection was among the lowest reported in Europe and worldwide. The low HDV prevalence in Slovenia is most likely a result of successful prevention of HBV infection with mandatory testing of blood donors, pregnant women, and family members and partners of HBsAg-positive individuals and universal vaccination against hepatitis B. Due to the observed low prevalence of HDV infection, routine diagnostic testing for HDV should not be considered in differential diagnosis of exacerbation of liver disease in Slovenian patients with chronic HBV infection. However, regular epidemiological surveillance of HDV prevalence in Slovenia is still recommended.

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Conflict of interest

The authors have no conflicts of interest to declare.

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