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Virology & Immunology Journal
ISSN: 2577-4379

Prevalence of Hepatitis Delta Virus in Hemodialysis Patients with Hepatitis B Virus in Benghazi, Libya as Starting Point

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Research Article

Volume 4 Issue 3

Received Date: August 14, 2020

Published Date: September 08, 2020

DOI: 10.23880/vij-16000250

Abstract

Hepatitis delta virus (HDV) is a subviral satellite RNA virus. It is consisting of approximately 1700 nucleotides long genomic circular RNA with one open reading frame encoding for two proteins (small delta antigens (HDAg-S) and large delta antigens (HDAg-L) coated with Hepatitis B virus (HBV) surface antigen (HBsAg). HDV was discovered in 1977 in an Italian patient who had chronic HBV infection. HDV infection is of particular concern in hemodialysis (HD) units due to HD patient's segregation into groups, lack of HDV diagnosis, irreversible complications of the infection, and controversial treatments. Several different studies were conducted in different parts of the world to explore the prevalence HDV in HD patients. The prevalence was ranging from zero to 58%. In France HDV infection in chronic HD and kidney transplant patients was zero. HDV antibody was detected in 1.7% of HD and renal transplantation patients in Brazil. In Khartoum State, Sudan prevalence of HDV among hepatitis B patients on HD was 13.3%. The prevalence of HDV in HD patients in Iran was 44.5%. HDV antibody was positive in 58% of the HD patients in Menoufiya, Egypt there is no study so far conducted on the prevalence of HDV in HD patients in Libya. The aim of this study was to call for medical personal managing HD units to apply new restriction rolls in managing their work environments for the wellbeing of their patients.

Keywords: Hepatitis B Virus; Chronic Hemodailysis; Blood; Patients

Introduction

HDV is transmitted through transfusions of unscreened or poorly screened blood and blood products [1-10]. It also spreads at lower frequencies among drug users and from an infected mother to her infant during delivery [11]. With risk factors that are similar to those of HBV infections, it spreads in areas where HBV is common. This was demonstrated by examining HBsAg-positive individuals from various parts of the world for the presence of the hepatitis delta antibody [12]. The antibody was found to be prevalent in unselected HBsAg-positive Italian individuals, despite those individuals residing in various countries around the world [12]. In the same study, the antibody was detected in drug addicts and poly transfused HBsAg patients from all over the world. The data indicated that the infection was associated with HBV, and spread among Italians through parental contact. It also spread among Italians and others via parenteral routes. The parenteral transmission was confirmed in a separate study using polytransfused patients with chronic blood disorders: a higher prevalence of HDV antibody was found among haemophiliac HBsAg-positive patients than the general HBsAg-positive population in Italy, Germany, and the U.S.A. Another study showed 8.5% HDV prevalence among HBsAg-positive patients in three villages in a rural area of Gabon, Africa [13]. In a different study HDV prevalence among HBsAg-positive pregnant women in Gabon was found to be

15.6% [14]. The prevalence of HDV among HBV infected patients in Benghazi city was 2.5% [15].

HDV is a quasi-species virus with a worldwide distribution [16]. Quasi-species is phenomenon resulting from the lack of 3’ to 5’ exonuclease activity of DNA-dependent RNA polymerases acting on RNA templates, due to the absence of the proofreading ability to correct mistakes, populations of the replicating virus are obtained [17]. These mutational changes have important consequences on the viral evolution and pathogenicity [18]. Based on genetic diversity, HDV is classified into eight clades [19]. Clade I is the most distributed among the eight clades, and is present throughout Europe mainly in Italy, North America, Africa, and some regions of Asia [20,21]. Clade II is mostly present in Taiwan, Japan, and parts of Russia [16-22]; Clade III is the most-prevalent genotype in South America (Venezuela, Peru, and Colombia) [16,21]. The presence of five other genetically variable clades was discovered in HDV samples from West and Central regions of Africa [22]. These highly divergent sequences resulted from extensive mutational changes that occur in highly closed population [23]. The identification of these different clades pointed to the epidemiology of this virus in different parts of the world.

Materials and Methods

A total of 16 blood samples were collected from venous of hepatitis B chronically infected chronic hemodailysis patients from Benghazi nephrology centre. These samples were collected into two different tubes.

- EDTA tubes for CBC examination.
- Plane tubes (noanticoagulant) for liver function test (LFT) and serology tests.

Samples were initially tested for HBsAg using HBsAg ELISA kit (HBsAg ELISA 2.0; Ortho Diagnostics, Raritan, N.J) to confirm their infections. Samples are then tested for the presence of HDV antibody and *delta* antigen using ELISA method (HDV antibody (Ab) competitive Enzyme Immunoassay for the determination of antibodies to Hepatitis Delta Virus in human serum and plasma, HDV Ag Enzyme Immunoassay for the determination of Hepatitis Delta Virus in human serum and plasma DIA. PRO Diagnostic Bioprobes SrI Milano Italy). All samples were tested for CBC and LFT as follows:

Complete Blood Count (CBC)

CBC measurements were done using Sysmex system (type K1000, made in Japan). The specific parameters measured are hemoglobin concentration (Hb), total number of white Blood Cells (W.B.C.s), total number of red Blood Corpuscles (R.B.C.s) and platelets counts. CBC determination was performed as follows: Blood samples collected in EDTA test tubes were aspirated into CBC machine. Blood cells were measured and differentiated based on cell assortment using flow cytometry technique.

Liver Function Test

Total Bilirubin: Serum samples were separated from sold blood components by centrifugation. The collected serum samples were used in the diagnostic of LFT and serology tests. Diazotized sulfanilic acid is formed by combining sodium nitrite and sulfanilic acid at low PH. Bilirubin (unconjugated) in the sample is solubilized by dilution in a mixture of caffeine\benzoate\acetate\EDTA. Upon addition of the diazotized sulfanilic acid, the solubilized Bilirubin including conjugated bilirubins (mono and diglucoronides) and the *delta* form (biliprotein-Bilirubin covalently bound to albumin) is converted to diazo-Bilirubin which is detected using a bichromatic (540,700 nm) end point technique.

Alkaline Phosphatase (ALP): Alkaline Phosphatase catalyzes the transphosphorylation of p-nitrophenylphosphate (p-NPP) to p-nitrophenol (p-NP) in the presence of the transphosphorylation buffer, 2 amino-2-methyl-1-propanol (AMP). The reaction is enhanced through the use of magnesium and zinc ions. The change in absorbance at 405 nm due to the formation of p-NP is directly proportional to the ALP activity.

Alanine Aminotransferase (ALT): Alanine aminotransferase catalyzes the transamination of L-alanine to α-ketoglutarate (α-KG), Forming L-glutamate and pyruvate. The pyruvate formed is reduced to lactate by lactate dehydrogenase (LDH) with simultaneous oxidation of reduced nicotinamide – adenine dinuceotide (NADH). The change in absorbance is directly proportional to the ALT activity and is measured using a bichromatic (340,700 nm) rate technique.

Aspartate Aminotransferase (AST): Aspartate aminotransferase catalyzes the transamination from L-Aspartate to a-ketoglutarate. Forming L-glutamate and oxalacetate. The oxalacetate formed is reduced to malate by malate dehydrogenase (MDH) with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH). The change in absorbance with time due to the conversion of NADH to NAD is directly proportional to the AST activity and is measured using a bichromatic (340,700 nm) rate technique.

HDV Ag ELISA Test

HDV Ag, if present in the sample, is captured by a specific monoclonal antibody, in the 1st incubation. A detergent is added to the sample in order to dissolve the specific antigen from HDV participle. In the 2nd incubation, after washing, a tracer, composed of a second anti HDV Ag antibody, labelled with horse reddish peroxidase (HRP), is added

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to the microplate and binds to the captured HDV Ag. The concentration of the bound enzyme on the solid phase is proportional to the amount of HDV Ag in the sample and its activity is detected by adding the chromogen/substrate in the 3rd incubation. The presence of HDV Ag in the sample is determined by means of a cut-off value that allows for the semi quantitative detection of the antigen.

Assay Procedure

The assay has been performed according to the manufacturer protocol.

1. The required number of strips was placed in the plastic holder. Controls, calibrator and samples were carefully tested.
2. The first well was left as blank.
3. 100 ml of the negative control were placed for testing in triplicate, 100 ml of the calibrator were placed in duplicate, 100 ml of the positive control in single test and 100 ml of the samples.
4. 100 ml specimen diluents were then add to all the wells, except for A1.
5. Samples were then incubated for 120 min at +37°C.
6. All sample wells were washed three times.
7. 100 ml of the Enzyme conjugate were then added to all wells, except for A1 and incubated for 60 min at 37°C.
8. The sample wells were then washed three times.
9. 100 ml of chromogen/substrate were added into each of the wells. A1 included.
10. Samples were then incubated and protected from light at room temperature (18-24°C) for 20 min.
11. 100 ml of sulphuric acid were then added into all the wells to stop the enzymatic reaction. Addition of the stop solution turned the positive control and positive samples from blue to yellow.
12. 14. The colour intensity was measure in each well, using a 450nm filter.

Hdv Ab

Anti-HDV antibodies, if present in the sample, compete with a virus-specific polyclonal IgG antibody, labelled with peroxidase (HRP), for a fixed amount of recombinant-HDV coated on the microplate. The test is carried out with a two steps incubation competitive system. First the sample is added to the plate and specific anti HDV antibodies bind to the adsorbed antigen. After washing, an enzyme conjugation polyclonal antibody to HDV is added and binds to the free portion of the antigen coated. After washing a chromogen/substrate mixture is dispensed. The concentration of the bound enzyme on the amount of anti-HDV antibodies in the sample and its activity is detected by the added chromogen/substrate. The concentration of HDV-specific antibodies in the sample is determined by means of a cut-off value that allows for the semi quantitative detection of anti-HDV antibodies.

Assay Procedure

1. The required number of wells has been placed in the microplate holder. A1 well was left empty and used as blank.
2. 100 ml of negative control in triplicate, 100 ml of calibrator in duplicate, 100 ml positive control in single and then 100 ml of samples were added to the wells and incubated at +37°C for 60 min.
3. Micro plate was then washed three times.
4. 100 ml enzyme conjugate were added to all the wells except A1 and incubated at +37°C for 60 min
5. After the incubation period micro plate was washed three times.
6. 100 ml TMB/H2O2 mixture in each well, the blank wells included and incubated at room temperature for 20 min.
7. 100 ml of sulphuric acid were then added into all the wells to stop the enzymatic reaction. Addition of the stop solution turned the negative control and negative samples from blue to yellow.
8. The colour intensity of each well was then measured using a 450nm filter.

Results

In an attempt to shied a light on the HDV infection situation in HD centres in Libya, HBsAg positive patients attending Benghazi Nephrology Center were tested for the prevalence of HDV infection. Sixteen (16) sera samples were tested for the presence of HDV infection. A competitive Enzyme Linked Immunosorbent Assay (ELISA) method was used to detect Immunoglobulin G (IgG) monoclonal antibodies and ELISA test was used for detection of *delta* antigen (DIA. PRO Diagnostic Bioprobes SrI Milano Italy). All sera samples used in this study were tested for the presence of HBsAg to confirm their infections, three of these 16 HBsAg-positive samples were HBsAg-negative, two of these HBsAg negative samples were HDV positive (indicating that they are HBV positive, as HDV is totally dependent on HBV present and HDV infection might suppress HBV infection), 8 of the 16 samples were HDV positive and one is negative for both viruses making the prevalence of HDV infection at (50%). Although the possibility of false-negative or false positive results of ELIZA test is present, tests used in this study have a sensitivity of >98% and specificity of >98% in both competitive ELISA test and direct ELISA, indication questionability of false results unexpected. The negativity of HDAgs in all cases is well-matched with most of the literature.

In most of the investigations carried, HDAgs are rarely detected, because ribo immuno assay (RIA) or Enzyme

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Immuno Assay (EIA) used is depending on antigen-antibody interaction, the presence of high copies of HDAg antibody in patients serum may theoretically form complex with HDAgs thus overwhelm their binding sites and leading to false negative results. Another reason for not detecting HDAgs in HDV patients serum is their localization, HDAgs are not secreted or membrane proteins, and the virus itself is not released into blood circulation, this could reduce their levels in serum samples, unless liver biopsy is used HDAgs will not be detected (Table 1).

Hemodailysis patient infected with HBV according to Benghazi Nephrology Centre results	16
HBV Positive HDV Positive	6
HBV Positive HDV Negative	7
HBV Negative HDV Positive	2
HBV Negative HDV Negative	1

Table 1: Hemodialysis Infected Nephrology.

All the 13 HBV infected haemodialysis patients and the three HBV negative haemodialysis patients were used to examine the levels of WBC, RBC, Hb and platelets counts. Only RBC and HB were relatively low. This finding could be attributed to the HD process not to the infection (Table 2).

Hemodialysis patient infected with HBV according to nephrology center results	WBC	RBC	HB	Platelets
HBV Positive HDV Positive	5.7±1.6	3.5±0.75	9.7±2.3	175±64
HBV Positive HDV Negative	6.9±2.3	3.0±0.42	9.1±1.0	139.5±35
HBV Negative HDV Negative	7.5	3.12	10.8	147
HBV Negative HDV Positive	3.42±0.63	3.7±0.47	11.5±1.6	150.5±2.1
Normal values	M 4.3 - 6.2x106/mL F 3.9 - 5.6x106/mL	M 4.3 - 6.2x106/mL F 3.9 - 5.6x106 /mL	M 13-18 g/dl F 12-18 g/dl	M 150-270 IU/L F 150-240 IU/L

Table 2: CBC Differences among Different Hemodaylsis Groups.

All the samples that were tested for CBC were used to determine the levels of the liver function (Bilirubin, AST, ALT and ALP). No difference was observed between HD patient infected with either HBV alone or HDV-HBV infection. This could be attributed to the lack of disease progress information (Table 3).

Normal value	HBV Negative HDV Negative	HBV Negative HDV Positive	HBV Positive HDV Positive	HBV Positive HDV Negative	Measurements
Total Bilirubin	0.41± 0.20	0.48± 0.25	0.3±0.09	0.3	0.2-1.3mg/dl
Alkaline phosphates	173± 122	280±183	142± 82	317	M 150-270 IU/L F 150-240 IU/L
Alanine Aminotransferase	40 ±23	44.5± 16	41.5±20.5	25	7-40 IU/L
Aspartate Aminotransferase	36±26	28±12	28±12	18	8-37 IU/L

Table 3: LFT Differences among Different Hemodialysis Groups.

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Discussion and Recommendations

HD patients are at higher risk of been infected with HDV than other HBV infected population. Due to the ignorance of HDV examination in all HD units. Our work showed a high prevalence of HDV infections (50%) among HBV carriers in HD units in Benghazi . The prevalence of HDV infections in HD patients was 20 fold higher than ordinary HBV infected population in Libya, which is found to be 2.5% in 2017. Normally HDV infections happen either as co-infection or super-infection to HBV. In HD units' super-infections would be more expected. This could be attributed to the fact that HBV infected patients share the same HD machines without been investigated for the presence of HDV. In order to elucidate the prevalence on HDV infection, we tested for the presence of HDV antibody and HDV *delta* antigen using both competitive IgG antibody and conventional ELISA respectively.

In initial stage of HDV infection the appearance of the *delta* antigen is detected in the liver, followed by the manifestation of *delta* antigen to the blood, the existence of *delta* antigen in the blood circulation will normally present asymptomatic for a brief period of time. Accordingly, *delta* antigen cannot be detected in the blood circulation. This is in agreement with our finding that HDV antigen tested negative in all samples. Due to the dependence of HDV antibody existence on the presence of HDV *delta* antigen in the blood circulation, the appearance of anti-*delta* IgM and a low IgG titer for short period of time is expected. The same is true in the case of acute super infection, but if the acute HDV infection is sustained, as in most cases of super infection, the infection will become chronic and the appearance of *delta* IgM will be followed by high titers of Delta IgG. This scenario further supported our finding that 50% of the tested samples were HDV antibody positive.

In the case of HDV co infection, the synthesis of HDV virus is accompanied by the presence of HBsAg in the blood circulation. In some cases were HDV actively synthesized, HBV synthesis is suppressed, meaning that HBsAg will not be detected. This has been seen in two of our samples. As has been shown by others in the cases of *delta* antigen presence, only anti-*delta* IgM is detected in the serum and lasts for 2 - 6 weeks. The presence of detectable levels of Anti-*delta* IgG may be seen in some of the patients with tendency to disappearance from the blood circulation. In conclusion, the presence of HDV as a co infection may cause severe acute hepatitis B infection. An increased risk of developing fulminant hepatitis` is expected. As for liver function it is extremely difficult to differentiate between the chemical profile of HBV-HDV and HBV alone; this might be attributed to the tendency of both infections to form chronic infection. Chronic infection will cause a liver damage which results in the elevation of some liver enzymes. The complete blood picture is expected to be equally effected by haemodialysis process. There for no difference were seen between HBV-HDV and HBV alone.

Since the HDV infection can only occur as a co-infection or a super-infection to HBV, the protection from HDV infection can be achieved by vaccination against HBV, and in extreme situations, the patient may require liver transplantation. Because of these treatments and the efficacy of vaccinations, the number of HDV infections has been declining since 1970-1980 [23]. Starting from 1990 no declining was reported due to the emerging of infected migrants from HDV-endemic areas. These pools of infected immigrants were compensating for the declining number of infections by vaccination [23]. HDV should receive more attention because of the presence of lager number of illegal immigrants coming from endemic areas and to its ability to influence the course of hepatitis infection and as consequences their management thus identification of HDV infection in hemodailysis patients will lead to the protection of the rest of hemodailysis patients.

Ministry of health and Nephrology centers in Libya should pay attention to the risk factors involved in the spread of HDV infection among HD patients.
To protect patients as well as people in contact with them, the following protective measures should be implemented.

- Blood prepared for transfusions should be carefully tested for the presence of infectious agents including HDV.
- All body fluids should be considered as potentially.
- Dispose used material in proper containers and disinfects all material and surfaces in regular base.
- Medical personnel, service worker and HD machines should be assigned to serve in different areas specifically designated to serve different patients carrying different infectious agents.
- HBV carriers should be tested for the presence of HDV.
- HDV-HBV infected patients should be hemodialysis using separate hemodialysis machines.

Acknowledgement

We are grateful to our graduate students for professional experimental processing and Higger Institute of Medical Professions, Benghazi, Libya.

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