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Short Communication

Frequency of Hepatitis D Viral Infection in Chronic Hepatitis B Patients in Pakistan

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ABSTRACT

Hepatitis Delta Virus (HDV) is a satellite virus of hepatitis B virus (HBV) as HDV relies on hepatitis B surface antigen (HBsAg) for viral development and release from hepatocytes. It causes the most severe viral hepatitis and suppresses the serological and molecular markers of HBV infections; for example, Hepatitis B envelope antigen (HBeAg), inhibition and reduction of HBV DNA titer during HDV infections. Very few studies described the clampdown of HBV-related markers during HDV replication and the current study is the only report from Pakistani perspective. The objective of this study is to find the interference of HDV in HBV replication, both in HBV chronic active and inactive state patients from four different a Pakistani provinces. Blood samples along with patient history and demographic data were collected from 100 (n=100) patients, all of these were positive for HBsAg (further characterized as acute or chronic hepatitis B). Viral titer was quantified from the HBsAg positive samples by real time-PCR, HBeAg by ELISA, and HDV RNA was detected by nested PCR and sequencing. HBV DNA was detected in 58% of the samples. The quantification of viral load revealed chronic active HBV infection at 11% and chronic inactive HBV infection as 63%. HDV RNA was present in 41.2 % of inactive chronic HBV carriers and only 18.1% in active HBV-infected patients. I has been observed that the presence of HDV infection may suppress the HBV replication and convert it into an inactive state and the overall prevalence of HDV in Pakistan is 35% in HBV-positive patients.

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Authors' Contribution
JA and IA executed the experimental work. LA and MS helped in prepration of manuscript. SA and MI finalized the outline of project.

Key words
Hepatitis delta virus, Hepatitis B virus, Hepatitis B surface antigen, Hepatitis B envelop antigen, co-infection, Super-infection

HDV is a global health concern that has infected an estimated 15 to 20 million chronic HBV patients worldwide. This animal virus was first discovered by Rizzetto *et al*. (1977), which relies on hepatitis B Virus (HBV) to infect humans. It is the only member of the genus Delta virus and acts as a co-virus for HDV by providing its envelope proteins for its infection (Taylor, 2006). A coinfection condition is the one when HDV infects an HBV patient and super infection occurs when HDV infects chronic HBV carriers. superinfection converts into chronic hepatitis D (CHD) in nearly 80% of cases within 5-10 years. HBV/HDV dual infection causes a more severe form of hepatitis, which leads to Liver Cirrhosis, Hepatocellular carcinoma, and liver failure (Stockdale *et al*., 2020). Similarly, spontaneous bacterial infections, an altered profile of oral and gut microbiota are some other effects of HBV/HDV co-infection. In this regard, the oral microbiota is responsible for the production of metabolites and active ingredients, that play a pivotal role in the regulation of host metabolic rate as well as immune responses. It is also reported that HBV-affected patients can also develop different tongue coatings due to the dysbiosis of the oral microbiota. In this, context, tongue coatings are considered one of the most complex ecological biofilm niches in the mouth, often linked to the pathological dynamics of many diseases (Bernardi *et al*., 2019; Continenza, 2013). These are the reasons which proved that HDV infection along with HBV is known to cause the most severe form of viral hepatitis (Yurdaydın *et al*., 2010).

Vaccination against HBV has contributed considerably to the decrease in HDV incidence observed in 1990s, but is still endemic in some parts of the world (Hughes *et al*., 2011). Although, the immunogenicity elicited by the HBV vaccine has been established in several studies, with benefits at different range of age (children, adolescents and adults) (Mastrodomenico *et al*., 2021) but, countries like Pakistan and Iran account for a significant

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proportion of global HDV burden which is a serious health concern (Abbas *et al*., 2010). HDV/HBV co-infection in Pakistan has been underestimated and is very high among the population. The most prevalent HDV genotype in Pakistan is HDV-1 and that of HBV is HBV-D (Butt *et al*., 2014). According to a study, the prevalence of anti-HDV antibodies in Pakistani population was 58.6% in 2009. The major reason for high prevalence of HDV as well as HBV in Pakistan may be attributed to poverty , densely populated areas, lack of education and improper HBV vaccination strategies. The interplay between hepatitis B (HBV) and delta (HDV) virus is extremely complex and not always identified, during chronic HDV infection. In clinical practice, patients infected with both HBV and HDV show a significant decrease in HBV-DNA viral load (Alfaiate *et al*., 2016). HBsAg, HBeAg and HBV DNA viral loads are three of the major HBV markers during HBV infection which are generally down-regulated or suppressed during HDV infection (Negro, 2014). Around 70 to 90% of the patients co-infected with HDV are negative to HBeAg, (Hughes *et al*., 2011) a surface antigen that is considered to be a serological marker which appears after the onset of HBV infection. If it persists the incubation period of 6 to 16 weeks after infection it gives an indication of chronic HBV infection. In chronic hepatitis B patients, positive HBeAg results shows high HBV titer that is clue to active HBV replication and high infectivity.

Diagnosis of HDV is very important for the proper management of chronic HBV patients. If this infection occurs in chronic HBV patients, it leads to chronic hepatitis D in almost 80% of the patients. Once chronic hepatitis D develops, it leads to cirrhosis in most of the cases. This worsens the disease and majority of the patients die because of hepatic failure. The present study was planned to find replication interference between HDV and HBV co-infection in HBV chronic active and chronic inactive patients from Pakistani population.

Materials and Methods

A total of 200 blood samples of patients with suspected HBV infection were collected from Genome Centre for Molecular Diagnostics (GCMD) Lahore, Pakistan from December 2017 to April 2018. A written consent was taken from patients participating in the study. Samples were received from four different provinces of the country (i.e. Punjab, Sindh, Khyber Pakhtunkhwa and Baluchistan). Patient history including alanine aminotransferase level reports, were documented. 100 samples, positive for HBsAg with complete demographic data were included in the study. Patients infected with other hepatitis virus like HCV were excluded from the study.

Hepatitis B surface antigen (HBsAg) and hepatitis B envelope antigen (HBeAg) were detected in the serum of all samples with antigen capture ELISA (AccuDiag™). Protocol and the results were interpreted by measurement of the absorbance of sample wells at 450nm wavelength. Results were calculated by using the following formula: Cut off value = N.C x 2.1 (N.C is the mean absorbance value of 3 negative controls). Specimens giving absorbance less than the cut off value were considered negative whereas those giving absorbance higher than the cut off value were considered positive for HBsAg.

For HBV DNA quantification HBV DNA was extracted from 200µl of the serum sample using Ribo-virus DNA extraction kit (Sacace) following the manufacturer's instructions. HBV quantification was performed by real time PCR using HBV Real-TM Quant kit (Sacace Biotechnologies, Italy).

For PCR amplification of *HDAg* gene was extracted from 200µl serum sample according to the manufacturer's standard protocol with slight modifications using GeneJET RNA Purification Kit (ThermoFisher Scientific, USA) in bio-safety cabinet class II A2.

Complementary DNA (cDNA) was synthesized from extracted RNA by reverse-transcription using Reverse Transcriptase (M-MLV RTase) (Invitrogen). Outer antisense primer HD-2 was used for cDNA synthesis.

HDV Delta antigen gene was amplified by nested PCR using two sets of primer pairs. Two rounds of PCR were performed using product of first round as the template for second round PCR. Outer forward primer (5′GCCCAG-GTCGGACCGCGAGGA-3′) and outer reverse primers(5′-ACAAGGAGAGGCAGGATCACCGAC-3′) (HD1 and HD2, respectively) were used to amplify broader regions of HDAg gene during first round PCR in a thermal cycler (Applied Biosystems).

While inner forward primer (5′-GAGATGC-CATGCCGACCCGAAGAG-3′) and inner reverse primers(5′-GAAGGAAGGCCCTCGAGAACAAGA-3′) (HD3 and HD4 respectively) were used in second round PCR in a thermal cycler. The amplified gene was detected by 1.2% (w/v) agarose gel stained with ethidium bromide. The expected size of HDAg gene was 400bp, which was identified by comparing with 100bp and 1kb DNA ladder (Fermentas Technologies USA).

The purified DNA product of HDAg gene was sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) according to the manufacturer's instructions. Primers HD3 and HD4 were used in the sequencing reaction.

Results

A total of 200 serum samples were screened for the presence of hepatitis B surface antigen. Out of 200 samples,

120 were found positive (60%) for HBsAg. 100 of these HBsAg positive samples with complete demographic data were included in the study. ELISA was performed to detect HBeAg inHBsAg positive patients. 63% of samples had undetectable or low HBV titer <2000IU/ml with negative HBeAg and history of normal ALT level indicating an inactive HBV chronic infection. 11% of the samples had high HBV titer >2000IU/ml, positive HBeAg reaction and irregular ALT enzyme levels history indicating an active HBV chronic infection.

Overall, HBV DNA was detectable in 58% of the HBsAg positive patients out of which 29.3% had HBV titer above 2000IU/ml whereas, 70.7% had HBV DNA undetectable or titer below 2000IU/ml.

Thirty-five out of 100 of the HBsAg positive samples that were screened for HDV RNA by nested PCR were found infected with HDV (35%) and out of which 7 samples were further confirmed by sequencing. Two sequences have been submitted in Genbank with accession numbers MK192096 and MK225575.

Among chronic inactive 63% patients, HDV RNA was present in 41.2% of the samples showing HDV super-infection in patients. On the other hand, in active chronic HBV samples (11%), HDV was detectable only in 18.1% samples. HDV/HBV co-infection, their prevalence and ratio of chronic active/inactive patients in HBsAg positive patients (based on limited samples) in different provinces of Pakistan has been summarized in Table I.

Table I. HBV and HDV infection markers in all four provinces

Study group	Punjab	KPK	Sindh	Baluchistan	Total
Number of samples (n)	60	34	3	3	100
HBV DNA positive	35	20	1	2	58
HDV RNA positive	22	12	1	0	35
HBV DNA >2000IU/ml	10	6	0	1	17
HBeAg negative & HBV DNA 0 to <2000IU/ml (Chronic inactive)	39	20	2	2	63
HBeAg positive & HBV DNA >2000IU/ml (Chronic active)	9	2	0	0	11
HDV positive in HBV chronic inactive	16	9	1	0	26
HDV Positive in HBV chronic active	2	0	0	0	2

Discussion

The present study was aimed to find the correlation and replication interference between HDV and HBV in chronic active HBV patients as well as in chronic inactive HBV patients from four provinces of Pakistan. Chronic active infection was characterized by the presence of high serum HBV titer (>2000IU/ml), positive HBsAg, and positive HBeAg representing active replication of HBV in hepatocytes. Chronic inactive HBV was characterized by the presence of undetectable or low serum HBV level/viral load (<2000IU/ml), positive HBsAg, and negative HBeAg along with persistently normal ALT levels (Liver, 2012).

Overall there are three major and interesting findings of this study. Firstly, it is interpreted that prevalence of HDV is 41.2% in patients with chronic inactive HBV patients and the prevalence of HDV is only 18.1% in chronic active HBV patients. This suppression of HBV replication by HDV which converts HBV to an inactive state from active state as described previously (Hughes *et al*., 2011; Negro, 2014) but this type of study was ill defined in Pakistan. HDV super-infection in chronic HBV carriers results in inhibition of HBV markers in liver as well as in serum during the acute stage of HDV replication, as S-HDAg exerts a strong inhibitory effect on the mRNA synthesis and stability of viral hepatitis B (Sureau and Negro, 2016). Within a few weeks of super-infection, the RNA level in HDV serum increased up to 10^{12} copies/ml and HBV viral titer was undetectable or very low (<2000IU/ml).The positive HBeAg samples show the active state of Hepatitis B and on the other hand, HDV infection leads the patient to an inactive state of HBV which results in lower viral loads of HBV as well as negative HBeAg (Volz *et al*., 2015).

Our second outcome correlates with the previous published data as there is an inverse correlation of positive HBeAg with HDV infection. This is more critical and dangerous situation for a patient if not diagnosed on time then the negative HBeAg and lower viral loads of HBV can lead the chronic HBV patient to more adverse and confused condition for decision regarding timely treatment (Coghill *et al*., 2018).This finding enhances the early and accurate detection of HDV for proper management and treatment of patient.

Third finding of this study is the higher prevalence rate of HDV in Pakistan which is found to be 35% among HBV infected individuals as compare to previous studies from Pakistan (Aftab *et al*., 2018). It is evident from this report that Pakistan is still facing the burden of HDV disease as it is prevalent in different provinces of the country.

Conclusion

It is concluded from the study that prevalence of HDV in 41.2% patients in chronic inactive HBV patients as compare to 18.1% in chronic active HBV patients. This high frequency is due to the suppression of HBV replication by HDV which converts it into an inactive

state and the HBV titer was undetectable or very low. Moreover, HDV prevalence rate was found to be 35% among Hepatitis B viral infected individuals, which is a very high as compared to earlier studies from Pakistan. The current work demonstrated that Pakistan is still facing huge burden of HDV disease. Further vaccination strategy and preventive measures are required to eradicate the burden of this viral infection from the country.

Ethical statement

Before collecting the samples from patients, a complete guidance given to the patients and a written consent has been taken which was approved from the Ethical Committee of CEMB, University of the Punjab for this study.

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Statement of conflict of interest

The authors declare there is no conflict of interest.

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