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Medicine

CLINICAL UTILITY OF VITAMIN D LEVEL IN MANAGEMENT OF CHRONIC HBV INFECTION

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ABSTRACT **Aims&Objectives:** The aim of this study is to evaluate the correlation of vitamin D levels with virological parameters in patients with treatment naive chronic HBV infections.

Methods and Material: Hospital based prospective study was conducted,after set inclusion and exclusion crieteria We screened 81 patients for the study and a total of 55 patients were included in this study as shown in figure 1.Statistical analysis used: All data are expressed as means ± SD and percentage. All the parametric variables were analysed by student -t test where as non parametric variables were compared by Mann-Whitney *U* test Statistical significance was accepted for p values < 0.05.Software used were SPSS 11.5, and MS excel 2007**.** Associations between dichotomic (e.g., hepatitis B early antigen [HBeAg]-positive versus negative) was assessed by logistic regression models and linear (e.g., HBV DNA serum concentration) variables and 25(OH)D_3 serum levels were assessed by linear regression models. After univariate analyses, multivariate analyses was performed for significant associations. Multivariate models was obtained by backward selection, using a *P* value >0.15 for removal from the model. Group differences were assessed by means of chi-square contingency tables or Wilcoxon-Mann-Whitney's U tests, as appropriate.

Conclusions: Infection by hepatitis B virus causes complicated immunological, biochemical and histological changes in host immune response which can be specific or non specific. The non specific response occurs via cytokines or other substance. Vitamin D is known to suppress pro-inflammatory cytokines and increase interleukin 10.Thus it could be suggested that vitamin D deficiency may be related to increased viral replication and viral load.

KEYWORDS : Hepatitis B virus (HBV), Cholecalciferol, Hydroxylated Cholecalcifero 25(OH)D_3

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problem worldwide. Hepatitis B is an infectious disease, affecting an estimated 350 million chronically infected patients[1,2]. Despite the availability of potent vaccines, infection with hepatitis B virus still represents one of the most significant infectious diseases worldwide[3]. Hepatitis B virus is associated with a significant risk of developing severe liver disease including liver failure, cirrhosis, and hepatocellular carcinoma (HCC).

Although HBV is not directly cytopathogenic towards hepatocytes, the interaction between the virus and the host immune response plays a significant role in the pathogenesis of liver disorders[4-6]. These immune responses occur as complex interferences between the inborn immune and adaptive immune response[7].

The natural course of chronic HBV infection has five phases including the immune tolerant phase, immune reactive hepatitis B e antigen (HBeAg) positive phase, inactive HBV carrier state, HBeAg-negative chronic HBV and hepatitis B surface antigen (HBsAg) negative phase[8]. Patients with chronic hepatitis B who have HBV DNA levels >2,000 IU/mL, elevated serum alanine aminotransferase (ALT) levels, and at least moderate fibrosis or necroinflammation in liver biopsies require antiviral therapy to avoid progression of liver diseases to cirrhosis and its complications[8-10]. Though these criteria for treatment initiation are generally accepted, current knowledge on the natural history of CHB is incomplete, for example, with respect to the long-term prognosis and risk of hepatocellular carcinoma development in immune-tolerant patients with high viral load[8,9].

However, it was estimated that most patients will depend on life-long therapy with NAs, a strategy of thus far unknown safety and efficacy[9-11]. In addition, long-term treatment with NAs usually does not lead to clearance of HBV-infected hepatocytes, indicating that this treatment modality may not necessarily eliminate risk of HCC development. In view of these facts, the development of novel therapeutic strategies, especially of novel immunomodulatory approaches to control HBV infection, appears to be highly relevant.

Cholecalciferol is the precursor of the bioactive vitamin D metabolite, calcitriol[13]. Nutritional sources of cholecalciferol are rare and its largest proportion in humans is synthesized in the skin during exposure to ultraviolet light[12]. To get bioactivated, cholecalciferol is hydroxylated to 25(OH)D_3 at position 25 in the liver and subsequently at position 1 in the kidneys. The resulting bioactive vitamin D metabolite, 1,25(OH)$_2D_3$, which is also called calcitriol, exerts its biological functions predominantly by signaling through a nuclear vitamin D receptor (VDR), which serves as a ligand-activated transcription factor[13]. Importantly, clinical assays to quantify calcitriol are generally characterized by poor reliability[12].

Therefore, the stable, easy-to-quantify metabolite, 25(OH)D_3, is usually measured in clinical routine to assess a patient's vitamin D status[12]. By induction or repression of expression of hundreds of genes, calcitriol serves as an important modulator of numerous signaling pathways related to both innate and adaptive immunity[12,14-16].

The main purpose of this study is to evaluate the correlation of vitamin D levels with virological parameters in patients with treatment naive chronic HBV infections.

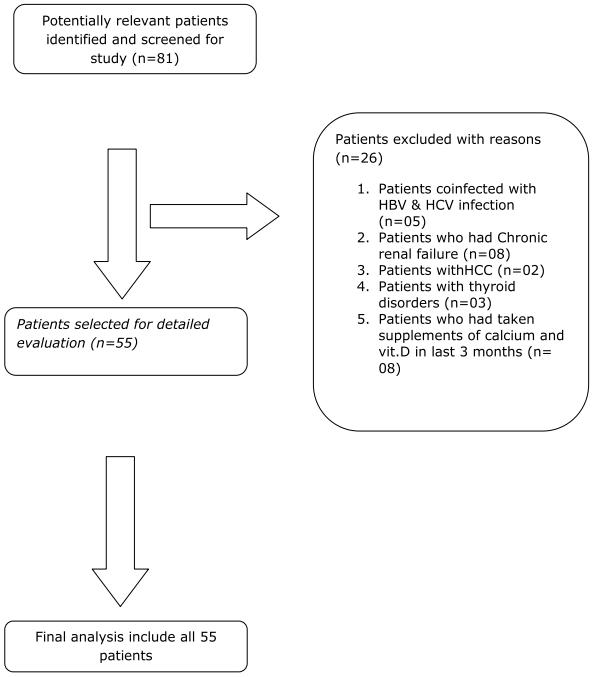
Study was conducted in the department of Gastroenterology, Yashoda Hospital, Hyderabad. It was a prospective study. Informed consent of the study participants was obtained in all cases. The study had approval of local Ethical Committee.

Treatment naïve chronic hepatitis B (CHB) defined as detectable hepatitis B surface antigen (HBsAg) , HBV DNA $\geq$ 6 months and aged >18years were included in the study. Patients with Coinfection with HCV/HBV, human immunodeficiency virus (HIV),excessive alcohol consumption (>40 g/day) malignancy including HCC, Chronic renal failure (serum creatinine > 1.25mg/dl),thyroid disorders,history of calcium or Vitamin supplements within previous 3 months or liver allograft were excluded from the study.

To the best of our knowledge this could be the first Indian study to test these results in our population.

We screened 81 patients for the study and a total of 55 patients were included in this study as shown in figure 1.

Figure 1. Flow chart of the study



METHODOLOGY:

Data was collected prospectively from consecutive patients from both outdoor and indoor patients based on clinical interview and review of records.

1. Detailed history with special emphasis on presenting illness and past history of any systemic disease.
2. Patients were subjected to a detailed general and systemic examination.
3. Blood samples were obtained for complete blood count, renal function test, liver function test, Thyroid function tests, serum calcium levels etc.
4. Blood samples were taken for vitamin D levels, quantitative HBV DNA, HBeAg for CHB patients.

Quantitative HBV DNA levels were done by ABI real time PCR using Taqman Chemistry**.** Real time PCR is a sensitive, accurate and efficient method for detection and quantification of Hepatitis B viral DNA in specimen over wide dynamic range. HBV DNA from specimen is isolated and subjected HBV DNA specific amplification using primers and probe based on Taqman Chemistry. An internal control is also used along with sample DNA. The fluorescent signal based on quantity of the viral DNA expressed as Ct (Threshold Cycle) which is compared to the Cts of standards to derive the quantity of viral copies in specimen. The viral load is expressed both as copies/mL as well as IU/mL (VIRAL LOAD 1IU = ~5.82 COPIES), linearity (17.1 – 34364262 IU/mL or 100 – 200000000 copies /mL). Hepatitis B Virus 'e' Antigen was done by enzyme immunoassay test (EIA) and was reported as 'Positive or Negative with a cut off value of 0.100. Samples reading >0.100 were taken as positive and below 0.100 were taken as negative.

25-Hydroxy Vitamin D 25(OH)D levels were done by EIA and values were measured in ng/L. Patients were classified into three groups as per their vitamin D levels as vitamin D deficient (≤10 ng/ml),insufficient (11-20 ng/ml) and optimal (>20 ng/ml).

STATISTICAL METHODS:

HBV DNA and vitamin D levels are numeric variables, so the mean and standard deviation was calculated. After analyzing normal or non-normal distribution of the continuous variables, continuous data was examined using the student t test (if normally distributed), Mann–Whitney test (if non-normally distributed), and categorical variables were examined by chi square test. The relationship and comparison between viral loads and vitamin D levels was assessed using Pearson correlation coefficient.

Associations between dichotomic (e.g., hepatitis B early antigen [HBeAg]-positive versus negative) was assessed by logistic regression models and linear (e.g., HBV DNA serum concentration) variables and 25(OH)D_3 serum levels were assessed by linear regression models. After univariate analyses, multivariate analyses was performed for significant associations. Multivariate models was obtained by backward selection, using a *P* value >0.15 for removal from the model. Group differences were assessed by means of chi-square contingency tables or Wilcoxon-Mann-Whitney's U tests, as appropriate.

Results

Table1: Demographic, Clinical And Biochemical Features Of Hbv Patients

Parameters (N=55)	Total Patients
Age(yrs)	43.7 (14)
Sex(M/F)	36/19
BMI(kg/m2)	23.8(1.7)
Hb(g/dl)	13.3 (1.7)
TLC (/µl)	6.8 (2.3)
Platelet(10^9/L)	2.3 (0.8)
Bilirubin (mg/dl)	0.9 (0.5)
SGOT (U/L)	41.8 (24.6)
SGPT (U/L	43.1 (33)
ALP (U/L)	91.9 (18.2)
Total Protein (g/dl)	6.9 (0.5)
Albumin (g/dl)	3.7 (0.4)
Prothrombin Time (seconds)	14.8 (1.6)
Serum Creatinine (mg/dl)	1.0 (1.1)
Serum Calcium (mg/dl)	9.6 (0.7)
TSH (µIU/ml)	2.6 (1.4)
25(OH)D (ng/ml)	20.4 (15.7)
HBV DNA log10(IU/ml)	3.04(2.08)
HBeAg (Positive/Negative)	18/37

Values are shown as Mean (SD)

A total off 55 patients were selected according to the inclusion criteria. Out of 55 patients 36 were males and 19 were females with mean age of 43 (±14) years . The majority of the patients were HBeAg negative. Mean serum vitamin D concentrations of entire cohort was 20.4±15.7) ng/ml.

Table 2: Demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to 25(OH)D levels

	25(OH)D (ng/ml)			
	>20	10-20	<10	p value
Number (%)	12 (21.8)	25(45.4)	17 (30.9)	
25(OH)D3 (ng/ml)	40.3(11.4)	15.9(3)	6.4(1.8)	**0.0001**
Age(yrs)	41(16)	40 (8)	45 (15)	0.405
Sex (M/F)	22/12	9/4	5/3	
BMI(kg/m^2)	23.2(3.2)	23.9(2.4)	23.6(2.5)	0.988
Hb(g/dl)	13.8(1.4)	12.7(1.6)	12.9(1.4)	0.290
TLC (/µl)	6.9(2.3)	6.4(1.4)	6.9(3.3)	0.814
Platelet(10^9/L	2.4(0.9)	2.1(0.4)	2.4(1.5)	0.488
Bilirubin (mg/dl)	0.9(0.5)	0.7(0.3)	1.0(0.8)	0.512
SGOT (U/L)	37.5(16.8)	35.6(16.3)	33.3 (11.4)	0.216
SGPT (U/L	36.7(36.4)	48(29)	59.5 (16.9)	0.177
ALP (U/L)	90.9(20.3)	93.1(13.4)	94.3 (16.8)	0.864
Total Protein (g/dl)	7.0(0.6)	6.9(0.3)	6.8(0 .4)	0.141
Albumin (g/dl)	3.7(0.4)	3.8(0 .3)	3.3(0.4)	0.021
Prothrombin Time (seconds)	14.4(1.0)	14.6(0 .9)	15.6(3.1)	0.022
Serum Creatinine (mg/dl)	0.9(0.1)	1(0.2)	0.8(0 .2)	0.184
S. Calcium (mg/dl)	9.6(0 .6)	9.6(0 .7)	9.6(1.1)	0.986
TSH (µIU/ml)	2.5(1.4)	2.4(1.0)	2.9(2.1)	0.784
HBV DNA Log10 (IU/mL)	1.07	2.37(1.07)	5.54(1.55)	**0.0001**
HBeAg (Positive/Negative)	0/13	01/24	17/0	**<0.0001**

Values are shown as Mean (SD)

The comparison of demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to 25(OH)D levels were done. No significant differences were observed

in any of these parameters in relation to 25(OH)D levels except for HBV DNA and HBeAg.

Of the 55 patients of the entire cohort, 17 (31%) had severe vitamin D deficiency [25(OH)D <10ng/ml], 25 (47%) had insufficient vitamin D levels [25(OH)D ≥10 and <20ng/ml] and 12 (22%) had optimal vitamin D levels [25(OH)D ≥ 20ng/ml] (p <0.0001). All patients with vitamin D levels ≥20ng/ml were HBeAg negative, whereas all patients with vitamin D levels <20ng/ml were HBeAg positive and this difference was statistically significant (p<0.0001). Patients with vitamin D levels below 20 ng/m had significantly high viral loads as compared to patients with vitamin D levels ≥ 20ng/ml (p<0.0001).Hence vitamin D deficiency and insufficiency was highly prevalent in CHB patients.

The comparison of demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to HBV DNA levels (**Table 3**) were also done. No significant differences were observed in any of these parameters in relation to HBV DNA levels except for 25(OH)D and HBeAg. Vitamin D concentrations were significantly low in patients who had high viral loads (p<0.0001). Similarly HBeAg positive patients had low serum vitamin D levels as compared to HBeAg negative patients (p<0.0001).

Table 3: Demographic, clinical and biochemical characteristics in patients with chronic hepatitis B according to HBV DNA levels

	HBV DNA (IU/ml)				
	< 12	12-2000	2000-20,000	>20,000	p value
Number (%)	23(41.8)	12(21.8)	09(16.3)	11(20)	
Age(yrs)	44.5(13)	37.4(15)	40.3(7.0)	41 (11.8)	0.459
Sex(M/F)	14/9	07/05	08/01	07/04	
BMI(kg/m^2)	25.0(1.1)	24.8(1.2)	25.1(0.9)	24.9(1.0)	0.908
HB(g/dl)	13(1.7)	13.8(1.8)	14 (1.5)	12 (1.8)	0.068
TLC (/µl)	6.5(2.3)	8 (1.9)	6.3 (1.4)	6.6 (2.8)	0.226
Platelet(10^9/L)	2.2(0.7)	2.6 (0.8)	2.1 (0.4)	2.4 (1.2)	0.448
Bilirubin (mg/dl)	0.8(0.5)	0.9 (0.4)	0.8 (0.3)	0.9 (0.7)	0.912
SGOT (U/L)	31(15.5)	46.5(23.4	40.8(17.5	45.9(17.3)	0.178
SGPT (U/L	33.7(10.9	32.8(15.6	51.4(27.2	57.2(18.7)	0.011
ALP (U/L)	93.3(19.8	81.1(13.7	97.2(7.7)	90.4 (18)	0.013
Total Protein (g/dl)	7.1(0 .7)	6.9 (0.4)	6.9 (0.3)	6.6 (0.4)	0.105
Albumin (g/dl)	3.7(0.4)	3.7 (0.3)	3.7 (0.2)	3.5 (0.5)	0.491
Prothrombin Time (seconds)	14.3(0 .9)	14.5 (1)	14.8(0.8)	15.8 (3)	0.078
Serum Creatinine (mg/dl)	0.9(0 .1)	0.9 (0.1)	1.0 (0.1)	0.8 (0.1)	0.110
Serum Calcium (mg/dl)	9.6(0.6)	9.7 (0.6)	9.5 (0.8)	9.7 (0.9)	0.901
TSH (µIU/ml)	2.5(1.4)	3 (1.3)	1.8 (0.9)	2.8 (1.9)	0.271
HBV DNA (IU/mL)	12(0)	339.4 (338)	6275 (3866.3)	28677906 (3988 8698)	**0.000 1**
HBeAg Positive /Negative	0/19	0/10	02/05	16/03	**0.000 1**
25(OH)D (ng/ml)	44.8 (11.4	26.7(4.9)	20.3 (9)	8.2 (3.7)	**0.000 1**

Values are shown as Mean (SD)

Patients having HBV DNA levels > 20,000 IU/ml had significantly low serum vitamin D levels compared to patients with serum HBV DNA levels <20,000IU/ml (p <0.0001).

Table 4: Comparison of 25(OH)D levels according to HBeAg

HBeAg	25(OH)D3	P value
Positive (18/55)	6.65(2.36)	0.0003
Negative (37/55)	33.57(14.85)	

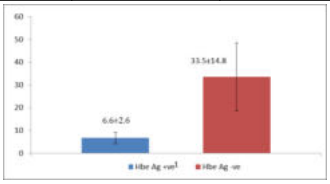


Figure 2.

Out of 55 cases 18 patients were HBeAg positive and 37 patients were HBeAg negative. The mean serum vitamin D level in HBeAg positive patients was 6.65(2.63) and in HBeAg negative patients was 33.57(14.85), which was statistically significant (p<0.0003).

To evaluate the relationship between 25(OH)D and HBV DNA serum levels, we stratified the patients according to HBV DNA serum levels <2,000 IU/ml versus >2,000IU/ml **(Table 5)**.Patients with HBV DNA viral load below this threshold, which is generally considered as relevant for clinical decision making, had substantially higher means 25(OH)D serum levels, compared to patients with HBV DNA ≥2,000IU/ml (18 versus 39 ng/ml, respectively; p<0.001).

Table 5: Comparison of 25(OH)D levels according to HBV DNA (IU/ml)

HBV DNA (IU/ml)	25(OH)D	P value
<2000 (35/55)	38.76(12.87)	0.0001
>2000 (20/55)	13.71(8.93)	

Figure 3.

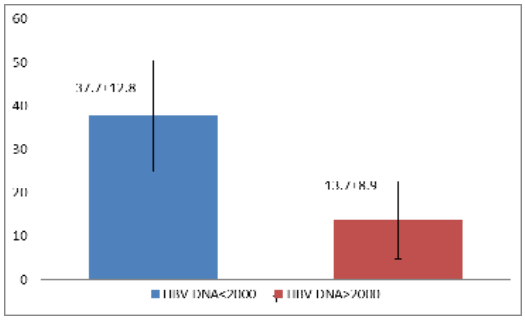


Table 6: Logistic multivariate regression analysis of determinant factors associated with 25(OH)D in CHB patients.

Variables	p value (Univariate)	p value (Multivaiate)
Age(yrs)	0.18	0.09
BMI (kg/m^2)	0.29	0.78
HB (g/dl)	0.07	0.17
Platelets(10$^{9/L}$)	0.28	0.27
Creat. (mg/dl)	0.47	0.99
Bilirubin (mg/dl)	0.25	0.56
AST (mg/dl)	0.03	0.99
ALT(mg/dl)	0.03	0.75
Albumin (g/dl)	0.14	0.85
Prothrombin Time (seconds)	0.37	0.88
TSH (µIU/ml)	0.24	0.52
HBeAg (Positive/Negative)	0.0001	0.614
HBV DNA log10 (IU/mL)	**0.0001**	**0.0001**

In both univariate and multivariate analyses, HBV DNA was the major determinant factor of low 25(OH)D levels , p < 0.0001.

Table 7: Pearson Correlation between HBV DNA and 25(OH)D levels:

Correlation coefficient r	-0.676
Significance level	P=0.0001

r= - 0.676, (P=0.0001)

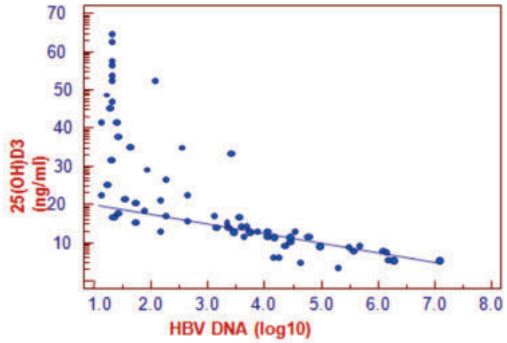
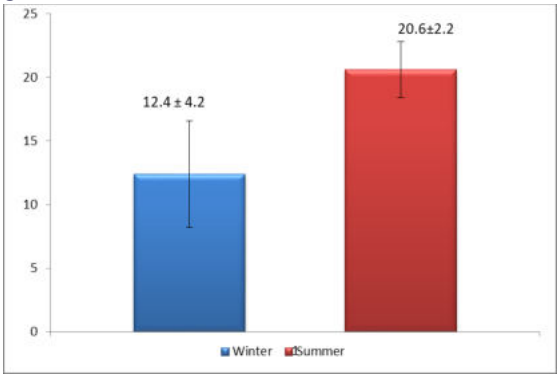


Figure 4.

25(OH)D and HBV DNA serum levels showed a significant inverse correlation, r= -0.676, (p=0.0001).

To assess the relationship between 25(OH)D serum level and season, the study period was divided into autumn-winter months (November-April; n = 25/55, 45%) and spring-summer months (May-October; n=30/55, 55%) depending on the date of vitamin D testing. The mean vitamin D serum concentrations in autumn-winter and spring-summer months were 12.4 ± 4.2 and 20.6 ± 2.2 ng/ml, respectively. The difference between seasons was statistically significant (p < 0.0001).

Bar chart depicting the seasonal variation of vitamin D levels: Figure 5.



DISCUSSION

Recently, it has been recognized that vitamin D has other functions in addition to its role in bone metabolism[22]. It has been demonstrated that vitamin D deficiency may play a role in the development of autoimmune diseases, inflammatory bowel disease, rheumatoid arthritis, psoriasis, multiple sclerosis, diabetes, certain cancer types, cardiac failure, stroke and infectious diseases such as tuberculosis and pneumonia, and that vitamin D supplementation is efficacious in these patients[23-27].

Chronicity of hepatitis B infection is also influenced by mutations in the vitamin D receptor gene, with polymorphisms being associated with higher viral load and increased disease progression and severity. Of note, the t-allele is associated with enhanced Th1 cellular immunity and promotes more efficient clearance of several viral infections, including hepatitis B and dengue virus[28,29].

The possible causal relationship between vitamin D metabolism and HBV replication, which needs to be proven by future studies, may offer attractive therapeutic opportunities for treatment of CHB. Very few studies have demonstrated an association between serum vitamin D levels and viral loads. The present study demonstrates a profound association between higher levels of HBV replication and low 25(OH)D serum levels in CHB patients.

Comparison between few recently published studies and our study is depicted in following tabular form.

Table 8.

Parameters	Farnik *et al*[17]	Demir *et al*[18]	Mohammed Amin Mohammed *et al*[19]	**Our study**
Number	203	35	75	55
Age in years (Mean)	39	32.5	49.7	43.7
Male/Female	107/96	22/13	39/36	36/19
Vitamin D Levels Ng/ml (Mean)	14.4	7.65	13.9	20.4
HBV DNA Levels (Mean)	3.47 log10 IU/ml	-	882.04 Iu/ml x10^3	3.04 log10 IU/ml
HBeAg Positive/ Negative	177/28	-	-	18/37
BMI (kg/m^2) (Mean)	22	22.9	22.3	23.8
ALT (U/L) (Mean)	40	31.25	30.07	43.8
AST(U/L) (Mean)	-	29.17	39.1	41.8
TSH (µIU/ml) (Mean)	-	1.35	1.46	2.6
Creatinine (mg/dl) (Mean)	-	0.89	1.09	1.0

In our study majority population was male (65.5%) and the mean age of population under study was 43.70 years. The mean serum vitamin D levels in our study population was 20.4 ± 15.7 ng/ml. Majority of our patients were HBeAg positive(67%).The mean HBV DNA levels of the whole cohort was 3.04 ± 2.08 log10 IU/ml which is comparable to other studies.[17,20].

Of the entire cohort, 31% had severe vitamin D deficiency [25(OH)D <10ng/ml], 47% had insufficient vitamin D levels [25(OH)D ≥10 and <20ng/ml] and 22% had optimal vitamin D levels [25(OH)D ≥ 20ng/ml] (p <0.0001) which is comparable with the other studies.

All patients with vitamin D levels ≥20ng/ml were HBeAg negative, whereas all patients with vitamin D levels <20ng/ml were HBeAg positive and this difference was statistically significant (p<0.0001) which is in contrast to the study by Farnik .

In our cohort, HBV DNA viral load appears to be the strongest determinant of low 25(OH)D serum levels which is comparable with the study by Farnik et al[17].

Patients with HBV DNA viral load <2000IU/ml, which is generally considered as relevant for clinical decision making, had substantially higher mean 25(OH)D serum levels, compared to patients with HBV DNA >2,000 IU/mL (39 versus 14 ng/mL, respectively P<0.0001) which is comparable with the study by Farnik.

Patients with vitamin D levels below 20 ng/ml had significantly high viral loads as compared to patients with vitamin D levels ≥ 20ng/ml (p<0.0001).Hence vitamin D deficiency and insufficiency was highly prevalent in CHB patients. Moreover we observed a significant inverse relationship between serum vitamin D levels and HBV DNA levels which is also comparable to the study by Demir at al[18] and Farnik et al.

Infection by hepatitis B virus causes complicated immunological, biochemical and histological changes in host immune response which can be specific or non specific. The non specific response occurs via cytokines or other substance. Vitamin is known to suppress pro-inflammatory cytokines and increase interleukin 10[30].Thus it could be suggested that vitamin D deficiency may be related to increased viral replication and viral load.

In our study inversed seasonal fluctuations of 25(OH)D and HBV DNA serum levels were seen as shown by other studies.

Of the entire cohort, 18% had severe vitamin D deficiency [25(OH)D <10ng/ml], 58% had insufficient vitamin D levels [25(OH)D ≥10 and <20ng/ml] and 24% had optimal vitamin D levels [25(OH)D ≥ 20ng/ml] (p <0.0001) which is almost comparable with the study by Gerova etal[21].

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