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Research Article

COMPARING HBV VIRAL LOAD IN SERUM, CERUMEN, AND SALIVA AND CORRELATION WITH HBEAG SERUM STATUS IN PATIENTS WITH CHRONIC HEPATITIS-B INFECTION

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ABSTRACT

Background: Hepatitis B is a disease that is prevalent worldwide and is responsible for 10% of the deaths that occur every year. The virus persists in 5% of infected adults and 90% of infected children and can cause chronic hepatitis. In addition to blood, the virus may also be present in other secretions; transmission through saliva, sexual fluids, and urine has been confirmed.
Objective: The main aim of this study is to compare viral DNA copies in the serum, cerumen, and saliva of patients by evaluating serum HBsAg and HBeAg levels.
Materials and Methods: Serum, cerumen, and saliva samples were collected from 50 patients whose disease was diagnosed about a year prior to the study. ELISA was performed in order to determine the presence of HBsAg and HBeAg in the gathered specimens. Viral DNA was extracted from the aforementioned specimen using kit(Qiagene) Subsequent to extraction, the number of viral DNA copies was determined using a real-time polymerase chain reaction (PCR) assay.
Results: Twenty-eight percent of the patients were HBeAg positive. The average number of viral copies in serum, cerumen, and saliva was higher in women than in men, and a meaningful correlation was observed between gender and average viral copies ($P<0.012$). However, no meaningful correlation was observed between viral copies present in the serum and cerumen and age and gender. In addition, no correlation was observed between serum HBeAg and viral copies present in serum, cerumen, and saliva. A Pearson's correlation test confirmed a direct and definite correlation between viral DNA loads in the patients' serum and cerumen (Pearson correlation= 0.97).
Discussion and Conclusion: A significant direct correlation was observed between viral DNA copies present in patients' cerumen and serum. However, the correlation between saliva viral load and serum and cerumen viral load was very slight and inverse. These findings suggest that the presence of HBV in non-invasive specimens(such as cerumen and saliva)should also be evaluated when monitoring patients to determine the course of infection and disease.
Keywords: Serum, Cerumen, Saliva, HBeAg, HBV-DNA.

INTRODUCTION

In spite of major vaccinations performed in most developed and even developing countries, hepatitis B is an infectious disease that is endemic in many regions of the world, especially in Asia and Oceania [1-4].Thirty percent of the world's population shows serologic evidence of present or past infection; about 400 million people worldwide suffer from chronic hepatitis [2-5].Hepatitis B virus (HBV) infection is considered to be the main global cause of hepatic disorders and hepatocellular carcinoma. According to the World Health Organisation, 50 million people throughout the world become infected every year. Of these, about 5-10% of adults and 90% of children experience persistent disease that leads to chronic hepatitis [1, 4, 5].With annual deaths

totalling 786000 patients, HBV is currently the tenth-leading cause of death globally [6].

The genome of HBV, a hepadnaviridae, is a double-strand DNA, found mainly in the blood and other secretions of the infected individual. Tears, bile, sexual fluids, sweat, milk, urine, feces, saliva, and cerumen may contain HBV. However, the viral load varies in different secretions, with the highest viral load being found in the blood. Since the required viral load for transmission is105cop/ml, any fluid containing equal to or more than this amount may effectively lead to transmission [7-9].

Studies performed between 1980 to 2004 determined that the presence of HBV may be proven through molecular methods in patients who have tested negative for HBsAg. Patients who have tested negative for HBsAg may carry mutations in the S gene region, incapacitating the ability to produce HBsAg [10-13]. Hepatitis B is amongst the most resistant viruses, giving it the ability to better thrive in its environment through mutations. One of these mutations occurs in the precore region of the viral genome, in which the C genome is no longer able to produce HBeAg and only HBsAg is produced. The occurrence of such a mutation has been repeatedly reported throughout Asian and Eastern European countries [14-16]. In addition, the viral load in various bodily secretions such as serum and cerumen is an indication of the high transmission rate of such secretions [11, 12, 17,18]. Such information is vital in follow-up appointments with patients, viral tracking, and treatment management. In addition, increasing benefit from non-invasive methods and increasing the sensitivity of diagnostic tests are other primary aims in caring for patients with HBV.

Objective

This study has been designed to determine the level of viral copies of HBV in the serum, cerumen, and saliva of infected patients through use of a polymerase chain-reaction assay (PCR assay) and by comparing the results in each specimen with their HBeAg levels.

MATERIALS AND METHODS

After obtaining the required licenses from the ethical committee of Ilam University of Medical Sciences, the study began in 2012 in 50 patients with active hepatitis B infection who were admitted to the Nourooz-Abad polyclinic. All patients had received a diagnosis of hepatitis B about one year before the beginning of the study. The ages of the patients ranged from20 and 40 years; 29 were men and 21 were women.

Specimen collection

5 ml of blood was collected from the patients and the serum was separated. In order to collect saliva samples, the patients were told to collect approximately3 ml of their saliva in sterile plastic containers prior to using any form of mouthwash. Cerumen was collected using sterile spoons and sterile swabs from both ears and placed and homogenized in 1.5-ml eppendrof containers that contained 0.5 ml of normal saline. Saliva and cerumen specimens were tested in order to determine the presence of blood using Meyer reagent base. The samples were stored at–20°C and transferred to the lab.

With respect to serologic testing, an enzyme linked immune sorbent assay (ELISA) (Diaplus Inc; USA) was performed on the samples in order to determine HBsAg and HBeAg status.

Molecular tests

The main aim of molecular analysis is to determine the quantity of the specimen. In order to do so, viral DNA was extracted from all three specimens using QIAamp DNA Kit (Qiagene; Venlo, Limburg, The Netherlands). Extraction was performed in accordance with the guidelines and instructions provided by the manufacturer. The quantity of the samples was evaluated using a real-time PCR specific assay kit (AJ Roboscreen GmbH/Analytik Jena GROUP; Leipzig, Germany) and the Bio Rad-CFX detection system (Bio-Rad Laboratories; Hercules, California, USA). Heat cycles were programmed as follows. First, national denaturation was set to 95°C for 2 minutes. Denaturation was also performed in 95°C, but for 30 seconds. Annealing was performed in 61°C for 45 seconds. Extension took place at 72°C for 30 seconds and final extension was carried out at 72°C for 7 minutes. The aforementioned cycle was repeated 42 times (each cycle begins with denaturation and ends in extension).

Statistical analysis

Data was entered in SPSS software Version 16 (IBM; Armonk, New York, USA) and quantitative and qualitative parameters were assessed by chi-square test, Pearson's correlation test, and t-test. P values less than 0.05 were statistically significant.

Table 1: Comparing average viral DNA copies present in cerumen and saliva in accordance with serum group and serum HBsAg

Variable statistic		Copy mean cp/ml		
serum Group	No	HBV-DNA of Serum	HBV-DNA of Cerumen	HBV-DNA of Saliva
$<10^5$	27	1.28E 4	5.29E3	2.95E10
10^5-10^7	15	9.37E 6	4E5	9.95E7
$>10^7$	8	2.42E9	5.71E7	3.8E4

Table 2: Comparing average viral load in serum and saliva in accordance with cerumen group and serum HBsAg

Variable statistic		Copy mean cp/ml		
cerumen Group	No	HBV-DNA of cerumen	HBV-DNA of Serum	HBV-DNA of Saliva
$<10^5$	44	2.65E3	2.23E8	1.73E10
10^5-10^7	4	6.21E6	3.95E8	6.30E4
$>10^7$	2	2.07E8	7.25E9	-

Table 3: Comparing average viral DNA copies in the serum and cerumen, in accordance with saliva group and serum HBsAg

Variable statistic		Copy mean cp/ml		
Saliva Group	No	HBV-DNA of Saliva	HBV-DNA of Cerumen	HBV-DNA of Serum
$<10^5$	41	4.43E3	1.12E7	4.33E8
10^5-10^7	6	4.17E5	3.82E4	2.90E8
$>10^7$	3	2.66E11	3E4	4.69E5

Table 4: Comparing viral DNA copies present in the cerumen and saliva, in accordance with serum groups and serum HBeAg

Variable statistic			Copy mean cp/ml					
Serum group	No		HBV-DNA of Serum		HBV-DNA of Cerumen		HBV-DNA of Saliva	
	HBeAg +	HBeAg -						
			HBeAg +	HBeAg -	HBeAg +	HBeAg -	HBeAg +	HBeAg -
$<10^5$	3	24	3.44E4	1.01E4	1.62E4	4.43E3	3.90E1	3.32E10
10^5-10^7	7	8	2.08E6	1.57E7	1.33E4	7.38E5	1.38E8	6.55E7
$>10^7$	4	4	2.58E9	2.26E9	5.84E7	5.58E7	2.9E4	4.7E4

Table 5: Comparing viral DNA copies present in the serum and saliva in accordance with cerumen group and serum HBeAg

Variable statistic			Copy mean cp/ml					
Cerumn group	No		HBV-DNA of Cerumen		HBV-DNA of Saliva		HBV-DNA of Serum	
	HBeAg +	HBeAg -						
			HBeAg +	HBeAg -	HBeAg +	HBeAg -	HBeAg +	HBeAg -
$<10^5$	11	33	2.66E4	2.30E3	7.54E7	2.41E10	6.79E8	4.3E7
10^5-10^7	2	2	2.52E5	3.7E7	1.16E5	3.65E4	5.51E9	2.4E7
$>10^7$	1	1	2.3E8	2.07E8	-	-	6.9E9	7.6E9

Table 6: Comparing viral DNA copies present in the serum and cerumen in accordance with saliva group and serum HBeAg

Variable statistic saliva group	No		Copy mean cp/ml HBV-DNA of saliva		HBV-DNA of cerumen		HBV-DNA of Serum I	
	HBeAg+	HBeAg -						
			HBeAg +	HBeAg -	HBeAg +	HBeAg -	HBeAg +	HBeAg -
$<10^5$	11	30	6.64E3	3.62E3	2.12E7	7.64E6	8.02E8	2.98E8
10^5-10^7	2	4	3.46E5	4.53E5	7.82E4	1.82E4	7.56E8	5.72E7
$>10^7$	2	1	9.69E7	3.9E11	9.42E3	4.03E4	9.47E5	2.31E5

RESULTS

Of our total population (N=50), 58% (29 patients) were men, with an average age of 34.21 years. The other 42% (21 patients) were women, with an average age of 30.48 years. All subjects tested positive for HBsAg and HBV-DNA in their blood. Fourteen of the 50 subjects (28%) were HBeAg positive,6 women (43% of the total) and 8 men (57% of the total). One hundred percent of the serum specimens, 42% of the cerumen specimens, and 68% of the saliva specimens were positive for HBV viral particles.

The presence of viral particles was determined by employing real-time PCR; 82% of the cerumen samples and 68% of the saliva samples contained DNA particles.

More viral DNA copies were present in samples obtained from women than from men. Using a t-test, it was determined that a meaningful correlation existed between gender and average viral DNA copies in saliva samples ($P<0.012$). However, no correlation was observed between viral DNA copies in serum and cerumen samples with gender and/or age (cerumen,$P>0.494$, serum $P>0.7$) Subjects were divided two groups, in accordance with the age range that was considered high-risk(groups: 20-30 and 31-40 year). T-tests examination show that a definite and meaningful correlation is present between viral DNA copies in the patients' serum with their corresponding age group ($P <0.03$). However, such a correlation was not observed between viral copies present in other specimens-cerumen and serum-with the corresponding age group of the patients (cerumen,$P>0.355$; serum ,$P>0.175$)

In accordance with the direct correlation between viral load and transmission possibility, and also given that transmission becomes possible with viral loads≥105cp/ml, three patient groups were stratified based on viral load: less than 105 cp/ml, between 105and 107cp/ml, and more than 107cp/ml. Along these lines, the average viral load was studied for the aforementioned secretions.

According to the serum groups and the presence of HBsAg in the patients' serum, the highest viral load average when compared to other groups in the serum was more than 107cp/ml (2.42e9 cp/ml). In this group, the average viral load in the saliva and cerumen, respectively, was 3.8e4 and 5.7e7 cp/ml (Table I).

When comparing the results for measurement of viral DNA copies present in the cerumen of HBsAg-positive patients (100%) with the average viral load in any of the three specimens, in only 2 of the 50 subjects was the viral load higher than 107 cp/ml in their cerumen (2.07e8 cp/ml). These patients had the highest viral load in their serum as well (7.25e9 cp/ml). The average viral load of their saliva was not determined (Table II).

When comparing viral DNA copies in the saliva with the average viral load present in the serum, cerumen, and saliva, the highest average copies in the saliva was determined to be 2.66e11 cp/ml. Further comparison revealed that the groupfor whichthe viral load in the saliva was less than 107cp/ml additionally had a lower viral load in the serum and cerumen than did the group with ≥107 cp/ml (Table III).

Additionally, comparison using a t-test showed no significant difference between viral copies present in specimens gathered from patients divided into groups according to whether they were positive or negative for HBeAg.

The average viral DNA copies in HBeAg positive patients was higher than 107cp/ml (2.258e9 cp/ml). The average load in the serum and cerumen was higher in this group than in other groups, with the viral load being lowest in the group

with less than 105 cp/ml. In addition, in HBeAg-negative patients an increase in average viral DNA copies is associated with an increase in average viral copies in the cerumen and decreases in the viral copies shown in the saliva (Table IV).

Analyzing cerumen viral copies and comparing them to the average viral copy rate for specimens collected at all three sites showed no evident variation between viral copies according to whether patients were negative or positive for HBeAg. As shown in Table V, increases in cerumen viral copies are mostly seen in subjects with a high number of serum viral particles. The lowest viral load was seen in the same cerumen group (group: >107 cp/ml).

By comparing saliva groups with average viral DNA copies in serum and cerumen, it was concluded that in patients whose viral load was less than 105cp/ml, the average for viral DNA copies in the saliva did not vary according to whether subjects were positive or negative for HBeAg. The highest average viral DNA copies corresponding with viral copies in the saliva was seen in the serum (Table VI).

DISCUSSION

The level of the biomarker HBsAg in serum is an indication ofthe transcriptional activity of the viral genome. In addition to evaluating the level of viral copies, HBsAg screening may also be used in follow-up exams and in predicting the progress of HBV-related disease. Viral proliferation is closely related with HBsAg levels and asymptomatic liver diseases [19,20]. In addition, various studies indicate that the presence of the "e" antigen of hepatitis B increases the chances and risk of disease progression and even the possibility of infection, inadvertently leading to chronic and active hepatitis, liver cirrhosis, and hepatocellular carcinoma [8-21].

The presence of high levels of HBsAg (105cp/ml or higher) may be an indication of immune tolerance. Therefore, HBsAg screening may be able to provide valuable data considering the differentiation of immune tolerance, when ALT levels are normal and HBV-DNA is high. The presence or absence of HBeAg and its antibody, accompanied with the HBsAg biomarker and HBV-DNA level, may be used to identify which of the three disease stages of HBV any given instance of HBV infection has progressed to [8, 21]. Differentiating active from passive transmitters of HBV and the presence or absence of HBeAg is of vital importance; the first case responds better to long-term treatments and leaves behind fewer complications than does the latter. The hepatic complications caused in the second case are usually severe and life threatening [8, 18].

According to the studies performed, HBs and HBe antigens are present both in the serum and in the saliva of the patients, while HBV-DNA may be found in their serum, cerumen, and saliva [22, 23, 26,27]. Zhevachevsky and colleagues(2000) performed a study on saliva gathered from 505 patients known to be infected with HBV. The results of this study indicated that the stage of HBV-related disease is closely related with the levels of HBs and HBe antigen present in serum and saliva. HBsAg levels were evidently related to the level of HBeAg in the saliva of the patients whose disease was in its acute stages. After about a month, the levels of these antigens declined, rendering them undetectable in the blood. However, in 66% of the patients who either had acute hepatitis or were in the first stages of convalescence, HBeAg levels were higher in their saliva than in their serum. In 95% of the cases, although HBsAg was cleared from the blood after a month, HBeA remained positive in their saliva [22].

In this study, the highest viral copies present in serum, cerumen, and saliva samples were seen amongst women (3.98e10, 4.2e8,and 1.17e7, respectively). The reason may be the lower age average for the female subjects. However, t-test evaluation revealed no definitive correlation between viral copies per ml of serum ($P>0.70$) and cerumen ($P>0.49$) and gender, while such a correlation was evident between gender and viral copies per ml of saliva ($P<0.012$). Additionally, no relationship was observed between age group and viral copies in serum and cerumen, whilst the correlation between viral copies present in the saliva and age group was considered statistically significant($P<0.03$).

The highest viral load present in the saliva among the study cohort was in a 26-year-old woman who had tested negative for HBeAg (7.9e11 cp/ml); her serum viral load and cerumen viral load were reported to be 6.2e4 and 4.2e4 cp/ml, respectively. As was previously stated, the association between average viral copies present in the saliva and age group is meaningful. Therefore, it may be concluded that the viral load in women's saliva is higher than

that of men. However, further studies are required for verification.

All subjects had tested positive for HBsAg (50 subjects); 14 subjects (28%) were HBeAg positive. The highest viral DNA cp/ml of serum was reported in a female who was reported to be HBeAg positive (6.9e9 cp/ml); viral DNA cp/ml of cerumen was also evaluated in this subject and were also high (2.3e8 cp/ml), while viral load in her saliva was not reported. In 7 of 14HBeAg-positive subjects (50% of the HBeAg-positive patients), salivary viral load was not reported. However, viral DNA cp/ml in serum was equal too higher than 105for each of these subjects.

The highest viral DNA cp/ml of serum was seen in an HBeAg-negative patient (1.6e7). Test results show that his serum and saliva contained, respectively, 1.5e8 and7.3e4 cp/ml in the obtained samples.

Cerumen viral DNA count was not reported for 9 HBV-positive subjects (18%); all of these patients had fewer than 103cp/ml viral DNA in their serum and saliva. Also, in 6 subjects (12% of the study group), viral DNA was >105 cp/ml in their cerumen. In research performed by Kaciogluin a group of 70 subjects, only 2 patients (2.8%) had cerumen viral load (ie, >105 cp/ml viral load) [23].

In all patients who were HBsAg positive and had tested either negative or positive for HBeAg, increases in cerumen viral load were associated with an increase in serum viral loads and a decrease in saliva viral loads (Tables II and V). An inverse correlation appears to exist between viral load present in the serum and cerumen and that present in the saliva. The importance of this is that, with higher serum viral loads, the higher the viral load in the cerumen, thereby increasing the possibility of HBV transmission via this secretion.

In a study performed in zhang and colleagues (2008) in 200 patients who were infected with HBV, it was observed that in the group whose subjects had greater than 105 viral DNA copies present in their serum and saliva a significant and meaningful difference was seen between viral DNA copies present in their serum and saliva; a correlation in which an increase in serum viral copies was associated with a decline in the average copies present in their saliva [24].

Few studies have attended to the presence of HBV in cerumen samples. A study performed by Kacioglu and colleagues (2003)in the serum and cerumen of 70 patients who had type B hepatitis showed that the presence of HBV in the cerumen is associated with increased serum viremia[23]. This study was based on research carried out by Goh and colleagues in the cerumen and other ear-related secretions[25]. In 2013, Eftekharian and colleagues performed a study in the serum and cerumen of 30 patients who had all tested positive for HBsAg. In this study, by employing PCR, HBV-DNA was extracted from the cerumen samples gathered from two subjects (6.6%) [26]. The main difference between the current study and the aforementioned research rests in the number of subjects and also in the method of extraction. Real-time PCR is known to be more accurate than normal PCR, permitting higher differentiation and quantity evaluation.

The lowest viral presence was observed in the serum and cerumen of the patients whose saliva viral load was higher than107cp/ml (Table IV). The average viral DNA cp/ml of the set of specimens was determined to be 2.66e11 in the saliva, 4.69e5 in the serum, and 3e4 in thecerumen. These results correlate with the results obtained by Kreaseval et al. in 2013[27], who studied the saliva of 19 patients who had been receiving peg- interferon as a treatment for 3 months. The results indicated that all patients had HBV-DNA present in their serum samples; the load varied between 494 to 6.3e9 cp/ml. Additionally, HBV-DNA was also present in the saliva of all subjects, including those who had low HBV serum viremia. The number of subjects who had saliva and serum HBV-DNA levels less than 104cp/ml of sample were equal to each other, while in those whose serum viremia was higher than the 104cp/ml, viral load was reduced significantly [23]. The difference between the obtained results from the current and the latter study may be related to the intake of peg-interferon [27]. In this group, the increase in serum viral DNA load of HBeAg negative patients was accompanied by a decrease in salivary loads and an increase in cerumen loads (Table IV). This process was not observed in HBeAg-positive patients. According to t-test analysis, no correlation is present between viral presence in any of the three secretions according to whether HBeAg is positive or negative.

In this study, correlation analysis was performed using Pearson's test. The results indicate that a definite and direct correlation exists between the presence of viral DNA copies

in the serum and cerumen (Pearson correlation=0.970). A slight inverse correlation is also observed between the presence of viral copies in the serum and cerumen of the tested subjects (Pearson correlation=–0.039 and–0.031, respectively).

It was also observed that in both HBeAg positive and negative subjects, increase in saliva viral loads leads to a decrease in cerumen and serum viral DNA copies (Table VI).

CONCLUSION:

The assumption that reduction in serum and cerumen viral loads in associated with increased viral loads in the saliva needs to be further studied. The exact stage of infection must also be determined. However, it may be that decline in serum and cerumen viral loads and increases in saliva viral loads may be used in follow-up tests and examinations. When combined with the current tests, evaluation of viral loads in the saliva may be helpful in treatment management and prophylaxis. In conclusion, the proven possibility of transmission via serum and saliva, and the highly probable transmission via ear secretions such as cerumen, closer examination of biomarkers such as HBsAg, HBeAg, HBV-DNA, anti-HBS and anti-HBe present in the patients' serum, cerumen, and saliva may prove helpful in the process of treatment, prophylaxis, and patient follow up.

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Authors' Contributions

Eskandar Golami Parizad has designed the study. Elaheh Golami Parizad and Mansour Amraei performed laboratory work and statistical analysis. Afra Khosravi, Elaheh Golami Parizad and Eskanda Golami Parizad performed the study, collected important back¬ground information and drafted the manuscript. Azar Valizade and Abdoullah Davoudian conceived of this study and participated in de¬sign and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflicts Of Interest

Not Conflicts Of Interest.

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