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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 also been confirmed.
Objectives: The main aim of this study was to compare viral DNA copies in the serum, cerumen, and saliva of patients with HBeAg levels in their sera.
Patients and Methods: This was a cross-sectional study and subjects were selected by non-randomized methods. Serum, cerumen, and saliva samples were collected from 50 patients who were diagnosed with chronic hepatitis B about a year prior to the study. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the presence of HBsAg and HBeAg in the gathered specimens. Viral DNA was extracted from specimens by using a Qiagen kit. The number of viral DNA copies was determined using a real-time polymerase chain reaction (PCR) assay. The study was performed in Ilam province in western Iran.
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 significant correlation was observed between the gender and average viral copies. However, no significant correlation was observed between viral copies present in the serum and cerumen with the age and gender of patients. In addition, no correlation was observed between serum HBeAg and viral copies present in serum, cerumen, and saliva. The correlation analysis confirmed a direct and definite correlation between viral DNA loads in the patients' serum and cerumen.
Conclusions: A significant direct correlation was observed between the viral DNA copies present in patients' cerumen and serum. However, the correlation between saliva viral load with serum and cerumen viral load was very low and inverse. These findings suggest that the presence of the hepatitis B virus (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

1. Background

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 and 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 organization, 50 million people throughout the world become infected with the virus every year. Of these, about 5% -10% of adults and 90% of children experience a persistent disease that leads to chronic hepatitis (1, 4, 5). With annual deaths totaling 786,000 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 infected individuals. Tears, bile, sexual fluids, sweat, milk, urine, faces, 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 is 10^5 cop/mL, any fluid containing equal to or more than this amount may effec-

tively lead to transmission (7-9).

Studies performed between 1980 and 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 among 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 the benefit from non-invasive methods and increasing the sensitivity of diagnostic tests are other primary aims in caring for patients with HBV.

2. Objectives

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

3. Patients and Methods

Out of 1,740 patients with chronic hepatitis B who had some medical record in the referral polyclinic in Nourozabad, Ilam, Iran, only 163 subjects met the inclusion criteria. Inclusion criteria included having an infection at least for a year without taking any antiretroviral drugs. Those who did not meet these criteria were excluded. Sampling was done using a non-random, targeted method. Among those who met the inclusion criteria, only 50 people were HBsAg positive and refused to participate in the study. Informed consent was obtained from the patients and a specific questionnaire was used to collect demographic data. The ages of the patients ranged from 20 to 40 years; 29 were men and 21 were women. To determine the antibody titer and viral load in serum, cerumen, and saliva, samples were collected.

3.1. Sample Collection

For each participant, 5 mL of blood was collected and its serum was separated. In order to collect saliva samples, the patients were told to collect approximately 3 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 eppendorf 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 clinical microbiology research center of Ilam University of Medical Sciences, Ilam, Iran.

3.2. Serological Tests

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

3.3. Molecular Tests

The main aim of molecular analysis was 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). The diagnostic kit used SYBR Green. Heat cycles were programmed as follows. First, national denaturation was set to 95°C for two minutes. Denaturation was also performed at 95°C, but for 30 seconds. Annealing was performed at 61°C for 45 seconds. Extension took place at 72°C for 30 seconds and final extension was carried out at 72°C for seven minutes. The aforementioned cycle was repeated 42 times (each cycle begins with denaturation and ends in extension). The confidence limit of the results of diagnostic kit was 95 percent. The amount of viral copies was expressed as an exponent of 10(e).

3.4. Statistical Analysis

Data was entered into SPSS software Version 16 (IBM; Armonk, New York, USA) and assessed with the Kolmogorov-Smirnov test for their normality. Then, nonparametric tests such as Mann-Whitney U test and Spearman's rank-order correlation were employed.

4. Results

Of our total population sample of 50 patients, 58% (29 patients) were men with an average age of 34.21 ± 6.8 years.

The other 42% (21 patients) were women with an average age of 30.48 $\pm$ 5.57 years. All subjects tested positive for HBsAg and HBV-DNA in their blood. For HBeAg, 14 of the 50 subjects (28%) were positive. Of these, six were women (43% of the total) and eight were men (57% of the total). For HBV viral particles, 100% of the serum specimens, 42% of the cerumen specimens, and 68% of the saliva specimens were positive.

More viral DNA copies were present in samples obtained from the women than the men. Using the Mann-Whitney U test, it was determined that a significant correlation existed between gender and average viral DNA copies in saliva samples. However, no correlation was observed between viral DNA copies in serum and cerumen samples with the gender and/or age of patients.

Subjects were divided into two groups in accordance with the age range that was considered high-risk (groups of 20 - 30 and 31 - 40 years old). Correlation analysis tests showed that a definite and significantly correlation was present between the viral DNA copies in the patients' serum and their corresponding age group (sig-2tailed = 0.019). However, such a correlation was not observed in other specimens (cerumen and serum) and the corresponding age group of patients.

In accordance with the direct correlation between viral load and transmission possibility, and also given that transmission becomes possible with viral loads $\geq 10^5$ cp/mL, three patient groups were stratified based on viral load, namely less than 10^5 cp/mL, between 10^5 and 10^7 cp/mL, and more than 10^7 cp/mL. 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 the other groups was more than 10^7 cp/mL (2.42e9 cp/mL). In this group, the average viral loads in the saliva and cerumen were 3.8e4 and 5.7e7 cp/mL, respectively (Table 1).

When comparing the results of viral DNA copies present in the cerumen of HBsAg-positive patients (100%) with the average viral load in any of the three specimens, the viral load was higher than 10^7 cp/mL in the cerumen (2.07e8 cp/mL) in only one of the 50 subjects. The highest viral load in the serum was detected in this patient as well (7.25e9 cp/mL). The average viral load of their saliva was not determined (Table 2).

When comparing the 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 4.59e11 cp/mL. Further comparison revealed that the group for which the viral load in the saliva was less than 10^7 cp/mL additionally had a lower viral load in the serum and cerumen than did the group with $\geq 10^7$ cp/mL (Table 3).

Moreover, analysis showed no significant difference between the viral copies in different groups with positive or negative HBeAg.

The average viral DNA copies in HBeAg positive patients were higher than 10^7 cp/mL (2.86e9 cp/mL). The average load in the serum and cerumen was higher in this group than in other groups, with the viral load less than 10^5 cp/mL. In addition, an increase in average viral DNA copies in HBeAg-negative patients was found to be associated with an increase in average viral copies in the cerumen and decreases in the viral copies shown in the saliva (Table 4).

Comparing cerumen viral copies with the average viral copy of other specimens showed no evident variation between viral copies according to whether patients were negative or positive for HBeAg. As shown in Table 5, 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: $>10^7$ 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 10^5 cp/mL, the average viral DNA copies in the saliva did not vary according to whether the subjects were positive or negative for HBeAg. The highest average viral DNA copies corresponding for viral copies in the saliva were seen in the serum (Table 6).

5. Discussion

The level of the biomarker HBsAg in serum is an indication of the 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 diseases. Viral proliferation is closely related to HBsAg levels and asymptomatic liver diseases (19, 20). Also, various studies indicate that the presence of the "e" antigen of hepatitis B increases the chances and risk of disease progress 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 (10^5 cp/mL or higher) may be an indication of immune tolerance. Therefore, HBsAg screening may provide valuable data considering the differentiation of immune tolerance when ALT levels are normal and HBV-DNA is high (22). The presence or absence of HBeAg and its antibody, accompanied with the HBsAg biomarker and HBV-DNA level, may be used to identify the progress towards the three stages of HBV infection (8, 21).

Table 1. Comparing Average Viral DNA Copies Present in Cerumen and Saliva in Accordance With Serum Group

Variable Statistic	No.	Copy Mean, cp/mL		
Serum Group		HBV-DNA of Serum	HBV-DNA of Cerumen	HBV-DNA of Saliva
$< 10^5$	27	1.28E4 ± 1.97E4	5.29E3 ± 1.10E4	2.95E10 ± 1.53E11
10^5 - 10^7	15	9.37E6 ± 2.078E7	4E5 ± 1.47E6	9.95E7 ± 2.75E8
$> 10^7$	8	2.42E9 ± 3.02E9	5.71E7 ± 9.93E7	3.81E4 ± 5.42E4

Table 2. Comparing Average Viral Load in Serum and Saliva in Accordance With Cerumen Group

Variable Statistic	No.	Copy Mean, cp/mL		
Cerumen Group		HBV-DNA of Cerumen	HBV-DNA of Serum	HBV-DNA of Saliva
$< 10^5$	46	5.08E6 ± 3.39E7	2.23E8 ± 1.038E9	1.73E10 ± 1.175E11
10^5 - 10^7	3	7.43E6 ± 8.25E6	5.54E8 ± 8.30E8	6.30E4 ± 5.86E4
$> 10^7$	1	2.07E8	7.6E9	-

Table 3. Comparing Average Viral DNA Copies in the Serum and Cerumen in Accordance With Saliva Group

Variable Statistic	No.	Copy Mean, cp/mL		
Saliva Group		HBV-DNA of Saliva	HBV-DNA of Cerumen	HBV-DNA of Serum
$< 10^5$	41	4.43E3 ± 1.63E4	1.12E7 ± 4.76E7	4.33E8 ± 1.58E9
10^5 - 10^7	6	4.17E5 ± 5.03E5	3.82E4 ± 5.86E4	2.90E8 ± 6E8
$> 10^7$	3	2.66E11 ± 4.59E11	3E4 ± 1.8E4	4.69E5 ± 4.46E5

Table 4. Comparing Viral DNA Copies Present in the Cerumen and Saliva in Accordance With Serum Groups

Variable Statistic	No.		Copy Mean, cp/mL					
Serum group	HBeAg+	HBeAg-	HBV-DNA of Serum		HBV-DNA of Cerumen		HBV-DNA of Saliva	
			HBeAg+	HBeAg -	HBeAg+	HBeAg -	HBeAg+	HBeAg -
$< 10^5$	3	24	3.44E4 ± 3.14E4	1.01E4 ± 1.69E4	1.62E4 ± 1.42E4	4.63E3 ± 1.02E4	3.90E1 ± 6.75E0	3.32E10 ± 1.62E11
10^5 - 10^7	7	8	2.08E6 ± 3.06E6	1.57E7 ± 2.74E7	1.33E4 ± 5.95E3	7.38E5 ± 2.02E6	1.38E8 ± 3.66E8	6.55E7 ± 1.85E8
$> 10^7$	4	4	2.58E9 ± 2.86E9	2.26E9 ± 3.58E9	5.84E7 ± 1.14E8	5.58E7 ± 1.01E8	2.9E4 ± 5.79E4	4.72E4 ± 5.73E4

Table 5. Comparing Viral DNA Copies Present in the Serum and Saliva in Accordance With Cerumen Group and Serum HBeAg

Variable Statistic	No		Copy mean, cp/mL					
Cerumen group	HBeAg+	HBeAg-	HBV-DNA of Cerumen		HBV-DNA of Saliva		HBV-DNA of Serum	
			HBeAg+	HBeAg-	HBeAg+	HBeAg-	HBeAg+	HBeAg-
$< 10^5$	13	33	1.79E7 ± 6.37E7	9.97E3 ± 1.64E4	7.45E7 ± 2.68E8	2.41E10 ± 1.38E11	6.79E8 ± 1.90E9	4.30E7 ± 2E8
105 - 10^7	1	2	1.55E5	1.10E7 ± 7.53E6	1.16E5	3.65E4 ± 5.16E4	1.51E9	7.66E7 ± 1.03E8
$> 10^7$	0	1	-	2.07E8	-	-	-	7.6E9

Differentiating the active from the 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 (18, 23).

Table 6. Comparing Viral DNA Copies Present in the Serum and Cerumen in Accordance With Saliva Group and Serum HBeAg

Variable Statistic	No		Copy Mean, cp/mL					
Saliva group	HBeAg+	HBeAg-	HBV-DNA of Saliva		HBV-DNA of Cerumen		HBV-DNA of Serum l	
			HBeAg-	HBeAg+	HBeAg+	HBeAg-	HBeAg+	HBeAg-
$< 10^5$	11	30	6.64E3 ± 2.20E4	3.62E3 ± 1.41E4	2.12E7 ± 6.92E7	7.64E6 ±3.77E7	8.02E8 ± 2.05E9	2.98E8 ± 1.39E8
10^5 - 10^7	2	4	3.46E5 ± 3.25E5	4.53E5 ± 6.18E5	7.82E4 ± 1.08E5	1.82E4 ± 1.43E4	7.56E8 ± 1.06E9	5.72E7 ± 7.18E7
$> 10^7$	1	2	9.69E7	3.9E11 ± 5.63	9.42E3	4.03E4 ± 3.32E3	9.47E5	2.31E5 ± 2.38E5

According to prior studies, 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 (24-27). Zhevachevsky et al. (2000) performed a study on saliva gathered from 505 patients known to be infected with HBV. The results of their study indicated that the stage of HBV-related disease was closely related to 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 one 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, HBeAg remained positive in their saliva (24).

In this study, the highest viral copies present in serum, cerumen, and saliva samples were seen among women (3.98e10, 4.2e8, and 1.17e7, respectively). The reason may be the lower average age for the female subjects. However, the Mann-Whitney U test analysis revealed no definitive correlation between viral copies per mL of serum and cerumen and gender while such a correlation was evident between gender and viral copies per mL of saliva. These results are consistent with a similar study in 2015 performed by Keshvari et al. (22). Additionally, no relationship was observed between age group and viral copies in serum and cerumen while the correlation between viral copies present in the saliva and age group was statistically significant (sig.2-tail = 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 stated previously, 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 HBeAg positive (6.9e9 cp/mL). Viral DNA cp/mL of cerumen was also evaluated for this subject and was also high (2.3e8 cp/mL) while viral load in her saliva was not reported. In seven of 14 HBeAg-positive subjects (50% of the HBeAg-positive patients), salivary viral load was not reported. However, viral DNA cp/mL in serum was higher than 105 for each of these subjects.

The highest viral DNA cp/mL of serum was seen in an HBeAg-negative patient (1.6e7). Test results showed that his serum and saliva contained 1.5e8 and 7.3e4 cp/mL in the obtained samples, respectively.

Cerumen viral DNA count was not reported for nine HBV-positive subjects (18%) while the viral load DNA in serum and saliva of these patients was less than 10^3 cp/mL. Also, in six subjects (12% of the study group), viral DNA was $> 10^5$ cp/mL in their cerumen. In research performed by Kacioglu in, out of a group of 70 subjects, only two patients (2.8%) had cerumen viral load (i.e., $> 10^5$ cp/mL viral load) (25).

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 2 and 5). An inverse correlation appeared to exist between viral loads present in the serum and cerumen and in the saliva. This is crucial as higher viral loads in serum and cerumen result in a greater possibility of HBV transmission via this secretion.

In a study performed by Zhang et al. (2008) on 200 patients who were infected with HBV, it was observed that in the group whose subjects had greater than 10^5 viral DNA copies present in their serum and saliva, a significant 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 (26).

Few studies have investigated the presence of HBV in cerumen samples. A study performed by Kacioglu et al. (2003) using serum and cerumen of 70 patients who had

type B hepatitis showed that the presence of HBV in the cerumen was associated with increased serum viremia (25). Their study was based on research carried out by Goh et al. in the cerumen and other ear-related secretions (28). In 2013, Eftekharian et al. performed a study on the serum and cerumen of 30 patients who were all positive for HBsAg. In their study, HBV-DNA was extracted from the cerumen samples gathered from two subjects (6.6%) by employing PCR (29). The main difference between the current study and this mentioned 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.

Findings of this study indicated that the lowest viral presence was observed in the serum and cerumen of the patients whose saliva viral load was higher than 10^7 cp/mL (Table 4). 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 the cerumen. These results correlate with the results obtained by Krasteva et al. in 2013 (27), who studied the saliva of 19 patients who had been receiving peg-interferon as a treatment for three 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. This causes the use of a noninvasive specimen to be considered in identifying pathogens. Estakhri et al. have suggested using saliva to detect anti H. pylori IgG test (30). The number of subjects who had saliva and serum HBV-DNA levels less than 10^4 cp/mL was equal to each other. However, in those whose serum viremia was higher than the 10^4 cp/mL, the viral load was reduced significantly (25). The difference between the obtained results from the current and the latter study may be related to the intake of peg-interferon (23, 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 4). This process was not observed in HBeAg-positive patients. According to Mann-Whitney U test analysis, no correlation was present between viral presence in any of the three secretions, regardless of whether HBeAg was positive or negative.

In this study, correlation analysis was performed using Spearman's rank-order correlation test. The results indicated that a definite and direct correlation exists between the presence of viral DNA copies in the serum and cerumen. A slight inverse correlation was also observed between the presence of viral copies in the serum and cerumen of the tested subjects.

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 6).

5.1. Conclusions

This is the first article that examines the viral load of HBV on three secretions in patients with hepatitis B infections. Noninvasive methods to evaluate the disease and diagnose HBV, specifically the chronic form of the disease, have been proven by this study. Also, the proven possibility of transmission via serum and saliva; the highly probable transmission via ear secretions, such as cerumen; and 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 to be helpful in the process of treatment, prophylaxis, and patient follow up.

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Footnote

Authors' Contribution: Eskandar Gholami Parizad designed the study. Elaheh Gholami Parizad and Mansour Amraei performed laboratory work and statistical analysis. Afra Khosravi, Elaheh Gholami Parizad, and Eskandar Gholami Parizad performed the study, collected important background information, and drafted the manuscript. Azar Valizadeh and Abdoullah Davoudian conceived of this study, participated in design, and helped to draft the manuscript. All authors read and approved the final manuscript.

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