Original Article

The Study of Cerumen Hepatitis B Infection in Chronic Hepatitis B Patients by Real-Time PCR

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Abstract

Introduction:

The hepatitis B is a viral infection that causes a big problem globally. About 2 billion people worldwide are infected and there are now about 400 million HBV-DNA carriers around the world. HBV infection is the ninth cause of death worldwide and infects about 350 million new cases each year in the world. HBV-DNA can be spotted in different body secretions and fluids, including serum, saliva, tears, urine, amniotic fluid index, and cerumen isolated.

Materials and Methods:

This is a case - control study on the population of 140 participants (70 patients with chronic hepatitis B as cases and 70 healthy volunteers community as a control). The presence of HBV-DNA in their serum and ears cerumen using qualitative PCR and quantitative molecular detection Real-Time PCR (BioRad-CFX system) was determined.

Results:

Copy of serum HBV were detected in 98.5 % of case group and 7 % of healthy population (control group). In case group, 61 patients (87.2%) had HBV-DNA in their cerumens and 5control subjects (about 7 %) were positive for HBV-DNA in their cerumens. All patients group and two subject (2.8%) of control group, were positive in HBsAg test.

Average HB virus genome load in cerumen and serum of chronic HBV patients (group) were 8.98×10^{6} and 3.60×10^{8} copies per ml of the sample respectively.

Conclusion:

Like other body secretions, Ear cerumen is constantly produced and is subject to a pathogen such as HBV infection. The possiblity of disease transmission seems unlikely through Cerumen, however considering the average copy of HBV genome in the cerumen, no doubt, it can be claimed that there is a potential transmission risk of HBV infection.

Keywords:

Cerumen, Chronic, HBsAg, HBV-DNA, Hepatitis B, Real-Time PCR

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Introduction

One third of the world's population has been infected with hepatitis B virus and roughly 400 million people worldwide are chronically suffering from hepatitis B (1-4). The disease is ninth cause of death in the world. Although the hepatitis B virus has the smallest genome structure among all types of DNA viruses, but in terms of protein-producing and efficiency of its genome is peerless and unique.

Often in serum of patients who are in danger of chronic hepatitis, some HBV-DNA, HBsAg and HBeAg will remain (5-7). In western countries, especially in America, HBV is a major cause of 25 percent of chronic hepatitis while in Iran 70-80 percent of chronic hepatitis is caused by this virus.

For this reason HBV is the most important factor in liver diseases and their major cause of death in Iran (8-10).

Hepatitis B virus like HIV and HCV is spread by blood and its products and also by pituitary secretion of carriers (5). The HBV-DNA exists in various fluid secretions such as saliva, urine, tear, semen, bile and excrement. Recently by molecular technique like PCR, Real-Time PCR, we are able to prove the existence of HBV-DNA in cerumen and ear discharge (3,4,11-13). Nonetheless, ear cerumen of patients who are infected with chronic hepatitis B could be one of the most potential infected sources of transmissions to other people (3,4).

Materials and Methods

This is a case-control study that was conducted from January 2009 to august 2010 in Ilam. Seventy people with chronic hepatitis B and positive HBsAg were included in Case group and 70 people of healthy population were included in Control group. (Healthy people of society have acknowledged in their questionnaire that they haven't suffered from hepatitis B as yet). Age of both Case and Control group were between 20-40 years.

The tools that used for data collection were questionnaire, E test, PCR and Real-time PCR. This project was proposed in medical ethics committee and had been given permission for serum sampling of different people. Before sampling, written consents were obtained from all healthy people and volunteers.

Samples Collection

About 5ml of blood were taken from each person in the case and control groups, then by using swaps and sterilized snappers with 0.5 cm tooth diameter, ear cerumen samples were obtained and poured in 1.5ml Eppendorf tubes which contained 0.5ml normal saline. If there were not sufficient cerumen in the ear for sampling, that patient would be excluded from the study.

Blood serum samples and collected cerumen were kept in plastic tubes at 20 degrees below zero. Then they were transported to the laboratory. Serological tests and serum samples from group of case-control were evaluated by Elisa test. By Elisa kit using, the anti HBe, anti HBC, HBeAg total anti HBc, (IgG-IgM) were measured and finally the results were read by an Elisa Reader.

Molecular Tests

The aim of molecular analysis is to determine the quantity of sample and for this purpose the Real-Time PCR method is chosen. First we attempted to isolate and extract DNA of hepatitis B virus from serum and cerumen samples.

Serum: A DNA Extraction method using the kit. The extraction method was based on serological tubes filters method. In this method, 100 ml of serum is taken then especial Lysis buffer, proteinase K, washing buffer and isopropanol alcohol are used according to the kit manual in several phases. Finally the DNA virus is collected from the bottom of the sterile eppendorf.

Cerumen: Since the cerumen samples are usually fatty and waxy, for isolating the

virus some pre-extraction steps are necessary. By using NaOH and PH meter, we brought the above solution to Ph 9. The above solution is a type of lysis buffer. Cerumen samples were centrifuged at a 10000 rounds per minute and at the bottom of eppendorf 30 ml was deposited then we added 150 ml of lysis buffer and 20 ml of proteinase K.

After the period mentioned above, extraction process like serum case, was also performed on cerumen. After extraction of DNA virus from serum and cerumen samples of case-control group, samples were quantified by devices such as BioRad-CFX detection system Real Time PCR. Especial Real-time PCR kit which used in this study was a commercial kit. (aj Roboscreen, German).The heating protocol that used in Real time method is given in table 1. The Standard changes which applied in this study were based on the respective kit. (10^1-10^8) (per copy in milliliter) (Table 1).

Table 1: Heat protocol of Real-Time PCR

Step	Temp	Time	Repeat
Taq activation	95ć	4:00min	1
Synthesis	57ć	1:00min	
Melting	95 ć	0:30sec	
Fluorscence detection	45 ć	0:30sec	45

Results

Hepatitis B virus was isolated from cerumen in 61 (87.2%) cases of chronic HBV patients. Cerumen of 5 person of control group (7%) had HBV-DNA. Average virus copies per milliliter in serum and cerumen samples were as follow (Table 2): patient serum of case-control groups (3.40×10^8), patient cerumen (8.9×10^6), control serum (1.6×10^6) and in control cerumen (5.64×10^5) (Table 2)

The changes of copy per ml in serum and cerumen of case-control group were as follow: patient serum (1.2×10^6-7.56×10^9), patient cerumen (1.53×10^2-2.9×10^8), control's serum (1.3×10^3-5.5×10^5) and in control cerumen (1.3×10^2-2.6×10^5). Table1

In 11 out of 70 cases (15.7%) HBeAg and Anti-HBe were negative simultaneously by Elisa test and 22 out of 70 cases (31.3%) were HBeAg positive and about 37 people (53%) were HBeAg negative.

69 people from control group (98.5%) and from Anti HBe and HBeAg simultaneously were negative, and one person from positive HBeAg (1.5%) and negative HBeAg from this group were 98.5%. The positive HBV-DNA from patient group was 98.5 and from control group was 7% (Table 3).

The results of studies have shown that 25.71% of serum's samples and 11.42% of patient's cerumen have virus copies more than 10^5 ml from each sample. Table 4

Table 2: Changes and average of virus copy per millimeter serum and cerumen in Ilam studies-2010

Studies group	Serum average copy/ml	Cerumen average copy/ml	Serum changes copy/ml	Cerumen changes copy/ml
Patient	$3.6*10^8$	$8.98*10^6$	$1.2*10^2$-$7.56*10^9$	$1.53*10^2$-$2.9*10^8$
Control	$1.6*10^6$	$5.64*10^5$	$1.3*10^3$-$5.5*10^5$	$1.3*10^2$-$2.6*10^5$

Discussion

Structure of hepatitis B virus genome is small and has unique antigen producing efficiency. The virus has four major genes (C X P S) for producing antigen and polymerase action (14,15). Studies have shown that HBsAg test becomes positive about 50-60 days after virus infection (16,17).

In the first 2-4 weeks due to low viremia, HBsAg cannot be detected. 10 copies of the genome can cause infection in chimpanzees. However, sensitive tests such as HBV-DNA can be implemented to

identify people who have few copies of virus in their serum. Nowadays this goal is obtained by using a variety of Real time PCR (16-20). Our case-control study which was performed on 70 patients of hepatitis B positive and 70 people of healthy volunteers, determined that 69 patients (98.5%) and also about 7% of control group had HBV-DNA in their blood serum.

Cerumen of patients and the control group contained 87.2% and 7% HBV- DNA respectively. South Korean study (Eui-Kung Gohi et al) showed that about 66.7 percent of patients who were infected with chronic hepatitis B virus had HBV-DNA in their cerumen (4). Review results of Kolcioglu MT et al in 2004 identified that 27% of the cerumen of these patients were HBV-DNA positive (3). In addition, the average of copies in milliliter in serum and cerumen of Gholami Parizad, Kalcioglu and Gohi's studies were respectively as follow: in serum 4×10^4 and in cerumen 3.73×10^6 – in serum 2.4×10^7 and in cerumen 1.2×10^4 - in serum 3.04×10^8 and in cerumen 8.98×10^6 (3,4).

One study performed on HBV chronic patients and healthy volunteers' serum with RTD-PCR method, determined that average of four blood tests from 40 patients with hepatitis B positive was 4.3×10^8 copy per milliliter (13).

Volunteers' data obtained by questionnaire suggested that these people had not been infected with Hepatitis B, but the Real time PCR molecular results identified that 7.1% of the volunteers in their serum had had HBV-DNA. HBV-DNA virus in the serum of some volunteers who were examined in this study was negative. This might be due to infection without any clinical signs or their weakness to hepatitis in the past that probably this persons were in the occult phase and had not precognition (21).

Hepatitis B infection with negative HBsAg features was a challenging issue for years (22). Between 1980 and 2004 more than 70 articles in this field were published (21). The Study results have shown that patients with anti-HBe compared to anti-HBe and HBeAg have greater amount of virus in serum. Ilam study compared with the above studies indicates that there are significant differences between viruses isolated from the serum and cerumen and also there is a difference between the average copies of virus in a milliliter of serum and cerumen samples. The differences in the results may be related to Real Time PCR device, DNA extraction condition, method and amount of cerumen that was taken from ear cerumen and examiner's accuracy and precision.

Copy of the HBV-DNA in serum and cerumen samples in this study has shown that 25.71% of serum samples and 11.42% of cerumen samples from copy amount is more than 105 per milliliter. In other words, serum and cerumen of patients with this amount of copy virus can be considered as a potential transmission hazard of Hepatitis B. Studies of Kolcioglu MT determined that 70 percent of patients' serum and 2.8 percent of patients' cerumen have a great amount of virus copy: 105 (3). This difference could be due to disease phase, kit accuracy and the examiner differences.

Conclusion

Ear cerumen as an external secretion of human body in patients with chronic HBV often contains DNA-HBV and has a significant virus copy in milliliter. So we can claim that cerumen in these patients could be potentially contagious to other people.

Table 3: Serological status of patient's serum and control group in Ilam studies-2010

Review Group	Total	HBsAg				HBeAg				Anti HBe				Anti HBC		HBeAg and Anti HBe		HBV-DNA			
		Negative		Positive		Negative		Positive		Positive		Negative		Positive		Negative		Positive		Negative	
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Patient	70	-	-	70	100	37	53	22	31.3	37	53	22	31.3	70	100	11	15.7	69	98.5	1	1.5
Control	70	68 *	97.2	2	2.8	69	98.5	1	1.5	0	0	70	100	3 **	4.3	69	98.5	5	7	65	93

***** In 68 person of shahed, although their HBSAg was negative but, HBV-DNA of 3person of them was separated and with 2 person who have a positive HBSAg in this group that have a positive HBV-DNA, totally 5 person in this group have B hepatitis virus in their serum and cerumen.
**Three control subjects of Anti HBc were positive, one negative and two of them that have HBSAg, were positive.

Table 4: Abundance of serum and cermen with virus copy/ less or more than 10^5 ml

Sample	Total sample	Positive samples	Percent	People with more than 10 ml copies	Percent	People with less than 10 ml copies	Percent
Serum	70	69	98.51	18	25.71	51	72.85
Cerumen	70	61	87.14	8	11.42	53	75.71

R_2=1/000, E=%101/3 and R_2=0/984, E=%106.
Figure 1 and Figure 2 show some of cycle samples of R2 and E

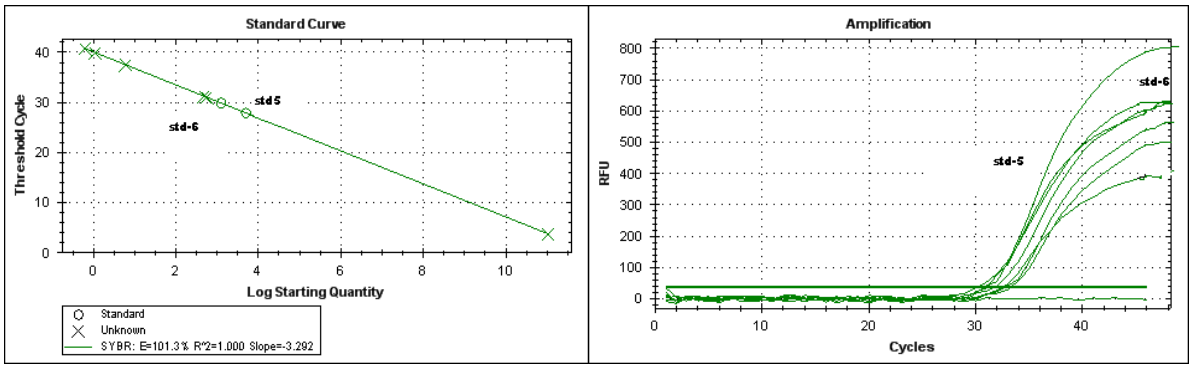

Fig 1: Some patients and control's cerumen

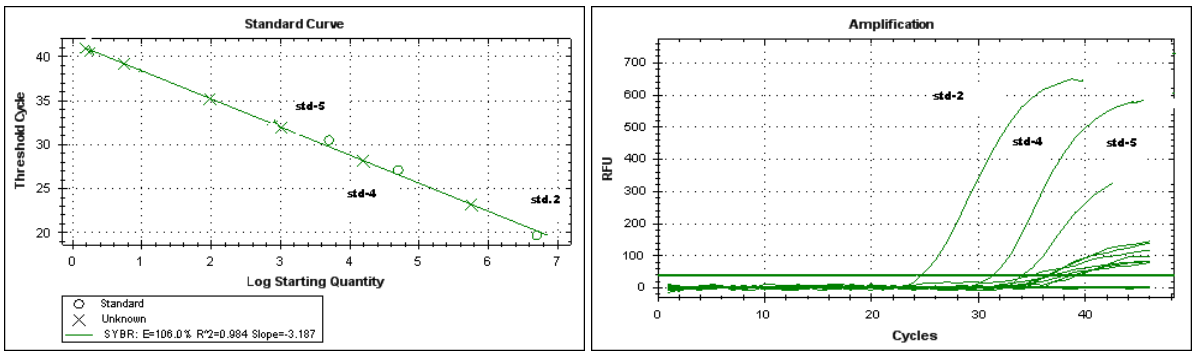

Fig 2: Some patients and control's serum

References

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden treatment, and current and emerging prevention and control measures. J Viral Hepat 2004; 11: 97-107.
2. Mancini-Bourgine M, Fontaine H, Brechot C, Pol S,Michel ML. Immunogencity of a hepatitis B DNA vaccine administered to chronic HBV carries. Vaccine 2006; 24: 4482-9.
3. Kalcioglu MT, Durmza R, Ozturan O, Bajindir Y, Direkel S. Does cerumen have a risk for transmission of hepatitis B? Laryngoscope 2004; 114: 577-80.
4. Gohi EY, Son Bo Y, Kong SK, Chon KM, Cho KS. Analysis of the hepatitis B virus in the cerumen and otorrhea of chronic HBV-infected patients. Otol Neurotol 2008; 29: 929-32.
5. Taber E, Gerety RJ. Possible role of immune response to hepatitis B core antigen in protection against hepatitis B infection. Lancet 1984; 1: 172.
6. Lemon SM, Thoimas Dl. Vaccine to prevent viral hepatitis. N Engl J Med 1997; 336: 196-204.
7. Robins WS. Hepatitis B virus and hepatitis D virus. In: Mandell, Bennett JE, Dolin R. (editors). Mandell, Douglas and Benntts principles and practice of infectious diseases. 5th ed. Philadelphia: Churchill Livingstone; 2000: 1652-84.
8. Eijk AA, Niesters HG, Gotz HM, Janssen HL, Schalm SW, Osterhaus AD, et al. Paired measurements of quantitive hepatitis B virus DNA in Saliva and serum of chronic hepatitis B patitents: Implication for saliva as infectious agent. J Clin Virol 2004; 29: 92-4.
9. Oon CJ, Chen WN, Goh KT, Mesenas S, Ng HS, Chaing G, et al. Molecular characterization of hepatitis B virus surface antigen mutants in Singapore patients with hepatocellular carcinoma and hepatitis B carries negative for HBsAg but positive for Anti-HBc. J Gastroentrol Hepatol 2002; 17: 5491-6.
10. Shi YH, Shi Ch. Molecular characteristic and of chronic hepatitis B virus infection. World J Gasetrol 2009; 15: 3099-105.
11. Lau STY, Everhart J, Kliner DE, Park Y, Vergalla J, Schmid P, et al. Long-term follow up of patients with chronic hepatitis B treated with interferon alpha. Gastroenterology 1997; 113: 1660-7.
12. Gish RG, Gadano AC. Chronic hepatitis B, current epidemiology in the Americas and implification for managment. J Viral Hepat 2006; 13: 187-9.
13. Alavian SM, Hajarizaddeh B, Ahmadzad AS, Kabiri A, Bagheri Lan-Karani K. Hepatitis B virus infection in Iran: A systematic review. Hepatitis 2008; 8(4): 281-94.
14. Aki AB, Inou EK, Tanaka T, Kato J, Kajijama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real time detection PCR. J Microbiol 1999; 2899-903.
15. Hatzakis A, Magiorkinis E, Haida C. HBV virological assessment. J Hepatol 2006; 44: 557-71.
16. Biswas RE, Tabor E, Hsia CC, Wrigth DJ, Laycock ME, Fiebig EW, et al. Comparative sensitivity of HBV NATs and HBsAg assay for detection of acute HBV infection. Transfusion 2003; 43: 788-98.
17. Ulrich PP, Bath RA, Seto B, Mack D, Sininsky J, Vyas GN .Enzymatic amplification of hepatitis B virus in serum compared with infectivity testing in chimpanzees. J Infect Dis 1989; 160: 37-43.

18. Hendrisk DA, Stowe BJ, Hoo BS, Kolberg J, Irvine BD, Neuwald PD, et al. Quantitation of HBV DNA in human serum using branched DNA signal amplification assay. AMJ Clin Pathol 1995; 104: 537-46.
19. Ho SK, Chan TM. An overview of assay for serum HBV DNA. Clin Lab 2000; 46: 609-14.
20. Sum SM, Wong DK, Yuen MF, Yuan HJ, Yu J, Lai CH, et al. Real time PCR assay using molecular beaconfor quantitation of hepatitis B virus DNA. J Clin Microbiol 2004; 42(8): 3438-40.
21. Brechot C. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: Old and new paradigms. Gastrointrol 2004; 127(5supp1): 56-61.
22. Chemin I, Trepo C. Clinical impact of occult HBV. J Clinical Virol 2005; 34(1): 15-21.