



## Evaluation of Chronic Hepatitis B Infection in Patients with Seronegative HbsAg

*E Gholami Parizad<sup>1</sup>, \*A Khosravi<sup>2</sup>, E Gholami Parizad<sup>3</sup>, N Sadeghifard<sup>4</sup>, S Ghafourian<sup>5</sup>*

<sup>1</sup>Dept. of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran

<sup>2</sup>Dept. of Immunology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran

<sup>3</sup>Dept. of Public Health, Faculty of Health, Ilam University of Medical Sciences, Ilam, Iran

<sup>4</sup>Clinical Microbiology Research Centre, Ilam university of Medical Sciences, Ilam, Iran

<sup>5</sup>Clinical Dept., Ilam University of Medical Sciences, Ilam, Iran

(Received 18 July 2011; accepted 11 Jan 2011)

### Abstract

**Background:** It is estimated that about 370 million people are chronic carriers of HBV worldwide. Apparently 3% of Iranian populations are chronic carriers of this virus. We aimed to evaluate the viral DNA in biological fluids of chronic hepatitis patients compared to a control group.

**Methods:** The current case-control study was designed to evaluate the viral DNA in biological fluids of 70 chronic hepatitis patients compared to a control group using ELISA, PCR and Real Time.

**Results:** All individuals (100%) in case group were HBsAg positive while in control group only 2 individuals (2.8%) were HBsAg positive. Three individuals, in control group were positive using PCR and Real Time PCR indicating that about 7% of those in control group were chronic carriers of HBV. The interesting point was the copy of viral DNA; ( $5.49 \times 10^4$ ,  $2.162 \times 10^3$  and  $7.26 \times 10^6$ ) for 3 chronic carriers using sera while it was about ( $5.71 \times 10^3$ ,  $1.45 \times 10^2$  and  $2.56 \times 10^5$ ) using ear cerumen confirming the necessity of investigating for the carriers of HBV in different biological fluid and by different methods.

**Conclusion:** It can be concluded chronic carriers of hepatitis B are much more than what is diagnosed by routine diagnostic tests. On the other hand ELISA alone can not be relied on as a complete test for screening of chronic carriers in hepatitis B. PCR and Real Time PCR are more reliable tests for this purpose.

**Keywords:** Hepatitis B, HBsAg, Serum, Carrier, Ear cerumen

### Introduction

Hepatitis infection is one of the major global health problems involving more than 2 billion people throughout the world. To date more than 370 million people are chronically infected with HBV worldwide, leading to chronic liver disease and development of Hepatocellular carcinoma (HCC) in many cases. Chronically carriers of this disease are living throughout the world mostly in the South East of Asia (1-4). It is estimated that more than 3% of Iranian population

to be the chronic carriers of the viruses (5, 6). Approximately 40 million out of 370 million chronic carriers are died due to hepatic cirrhosis while up to 60 million are passing out due to hepatocellular carcinoma (3-5). Recent studies have shown that some HBsAg negative individuals may develop some sort of chronic hepatitis B which is detectable employing some new laboratory diagnosis techniques such as molecular tests (7-9). To date, 8 genotypes (called A-

H) that differ by definition in at least 8% of their complete genome have been found. The genotypes can be further divided into 24 sub-genotypes that differ by at least 4% from each other (10). In general the infection with HBV can lead to a wide spectrum of clinical manifestations, e.g. self-limited acute or fulminant hepatitis, asymptomatic infection, or chronic hepatitis with progression to liver cirrhosis that can lead to hepatocellular carcinoma (10).

Although HBV is a DNA virus, it is highly variable under immunity or drug induced selection pressure, resulting in vaccine-related escape mutants and drug resistance. In human during decades HBV has had many mutations by which this agent has been able to escape from the immune system reactions so that HBV by far has changed its life style. One of the well know mutation for HBV has been occurred in Pre core region by which C gene is unable to produce HBsAg but producing HBcAg instead. This special mutation has been reported mostly in Asia and East European countries. Chronic hepatitis B with negative HBsAg is usually seen with mutation in S gene (11). Considering such mutations into account, it seems necessary to employ the new molecular techniques to analyze the diagnosis of HBV DNA in chronic carriers and healthy individuals whose sera HBsAg was negative with ELISA either qualitatively or quantitatively. We aimed to evaluate the viral DNA in biological fluids of chronic hepatitis patients compared to a control group.

## Materials and Methods

Seventy patients with chronic hepatitis B as case and 70 individuals from healthy people living in Ilam as the control group were randomly selected to participate in this study. PCR and Real Time PCR were applied to test the samples for HBV. ELISA is the standard test for diagnosis of many diseases including HBV which is rou-

tinely used in medical Laboratories using HBsAg (12-14). The limitation of this test is that usually 50-60 days post infection, the HBsAg can be detected, though when the viremia is low, detection may not have happened, this is why the quantitative PCR is recommended (15) and is considerably being used increasingly as it is used for evaluation of treatment and for progress analysis of the disease.

The quantity of viral DNA in sera is one of the most important parameters used for predicting the liver cancer and also for analyzing the anti-viral treatment efficacy. One of the best reliable methods in diagnosing the HBV is Real Time PCR which needs a standard in order to determine the quantity of HBV DNA comparing to standard curve (16-18).

The PCR and Real Time PCR were used in this study as briefly describes here:

Serum viral DNA was extracted using Gel – based filter tube including silica gel.

200 $\lambda$  of sample was added to 200 $\lambda$  lysing buffer, adding 50 $\lambda$  proteinase K, incubating at 65°C for 10 min. 200 $\lambda$  isopropanol was added and washing was applied few times using alcoholic buffers, yielding DNA at the end. The DNA was transferred to the sterile tubes and kept for the next steps. Serum viral DNA was extracted as follows:

A lysing buffer containing tris-HCl (500mM), ethidium bromide (20mM) and sodium chloride (10mM) was prepared. 150 $\mu$ l of the above lysing buffer was added to 30 $\lambda$  of each ear cerumen, mixed well and incubated at 65°C for 1 hour. For qualitative PCR a master mix solution including tag-polymerase, loading buffer, dNTP and two following primers was used:

F: 5' – CAC TCA CCA ACC TCT TGT CC  
-3'

R: 5' – TGA AGT TTC CGT CCG AAG GT  
-3'

The thermal reaction is summarized in Table 1.

**Table 1:** Thermal protocol for PCR

Steps	Temperature	Time	Repeat
Initial denaturation	95 °c	2min	1
Denaturation	95 °c	30sec	
Annealing	61 °c	45sec	42
Extention	72 °c	30sec	
Final Extention	72 °c	7min	

PCR was performed using Ependorf Vapa Protect (Germany). Real time PCR was performed using Aj Robo-screen kit (Germany) and BioRad CF×9<sub>b</sub><sup>Tm</sup> - Real Time Detection System, according to the manufactures instructions

## Results

Sera and cerumen of all 70 patients with chronic hepatitis B (HBsAg positive) and all 70 healthy individuals of control group who were selected from the healthy people in community were tested both quantitatively and qualitatively. Two individuals (2.8%) from the control group were HBsAg positive with ELISA. PCR and Real Time PCR were applied for all individuals in case and control group. Three out of 68 people in control group showed HBV DNA in either their sera or cerumen ( $P < 0.05$ ) while their ELISA was negative. In other words 2.8% of

participants in control group were HBsAg Positive by ELISA, while 4.3% of participants in this group had HBV DNA by PCR and real time PCR. It can be concluded that 7% of individuals in control group were the chronic carriers of HBV while ELISA could detect just 2.8% of them only. Table 1 shows the number of copies of viral DNA in either sera or cerumen for all people in case and control groups. The R<sub>2</sub> and efficiency of sera and cerumen with Real Time PCR were E=95.7 & R<sub>2</sub>=1, E=101 & R<sub>2</sub>=1 respectively.

**Table 1:** Results for the PCR & Real Time PCR comparing to ELISA

Sample	PCR		Real time PCR		HBsAg test ELISA
	Serum	Cerumen	Serum	Cerumen	
1	Positive	Positive	5.49×10 <sup>5</sup>	5.71×10 <sup>5</sup>	Negative
2	Positive	Positive	2.162×10 <sup>3</sup>	1.45×10 <sup>2</sup>	Negative
3	Positive	Positive	7.261×10 <sup>6</sup>	2.560×10 <sup>5</sup>	Negative
4	Positive	Positive	8.191×10 <sup>3</sup>	1.100×10 <sup>3</sup>	Positive
5	Positive	Positive	1.283×10 <sup>3</sup>	1.302×10 <sup>2</sup>	Positive
Mean copy /ml			1.564×10 <sup>3</sup>	5.46×10 <sup>5</sup>	-

## Discussion

HBV DNA has been detected in many human fluids including serum, saliva, tears, urine, am-

niotic fluid, seminal fluid, sweat, bile, milk, and recently cerumen and ear atorrhea (19, 20). Car-

riers of HBV in Iran have already been estimated to be about 3% (6, 21) while the results of the current study revealed that about 7% are the real chronic carriers of HBV amongst healthy people living in the community. It seems that different procedures applied in the studies previously have been done and also in current study can justify the different aspects of these studies either in the field of their focus or in the spectrum of their results. PCR and Real Time PCR are both superior to the ELISA particularly when different biological fluids are tested. The mean DNA copy/ml for cerumen & serum was  $1.56 \times 10^6$  and  $5.459 \times 10^5$  respectively. Kolciogu MT et al., who had studied 40 patients with chronic hepatitis B, reported that 12.5% of patients had HBV DNA in their cerumen while 100% showed HBV DNA in their sera using Real Time PCR. The mean copy of HBV in cerumen & serum was reported to be  $1.2 \times 10^4$  and  $2.4 \times 10^7$  respectively (22). Goh Eui et al. had studied 30 patients who showed HBV DNA in their serum, and reported that 66.7% of those patients had HBV DNA in their cerumen as well (20). As can be seen from the above studies comparing to the current study, there is a significant difference between the results of our study and the above ones. Results of the current study not only confirmed all HBsAg positive patients with ELISA showed HBV DNA in their sera and 87% of them showed HBV DNA in their cerumen but also about 7% of healthy individuals in control group had HBV DNA taking into account as chronic carriers of HBV. As we have studied the healthy people, the chronic carriers have been detected, while the others studies done so far had not shown this.

In conclusion this is the first study detecting HBV DNA from the cerumen of 5 individuals as chronic carrier of HBV amongst control group studied in Iran. After Turkey and Korea apparently this is the next study about molecular detection of ceruman HBV DNA, which is one important issue as detection of HBV DNA in cerumen is a more easy procedure to be performed and also the real time PCR is a very pre-

cise method. Another important finding of the current study is stressing on this point that HBsAg screening is not as reliable as molecular techniques such as Real time PCR in detecting HBV DNA in healthy people as chronic carriers. Real time PCR detected carriers in control group where HBsAg was not able to perform this. On the other hand it is merely important that having negative results of HBsAg in sera by ELISA does not necessarily means that there is no infection but the strength of such assay is limited. The source of infection and also the biological fluid to be tested are as important as the type of assay.

### Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

### Acknowledgments

Thanks a lot for those who have helped us during this project specially Research Department of Ilam University of Medical Sciences that funded this project. The authors declare that have no conflict of interest in this study and Ethical Committee approved the study.

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