

Sero-prevalence of Hepatitis C Virus by using different diagnostic techniques in Hazara Division of Pakistan

¹. Kamal Abbasi [Abbasi K],

². Dr. Tamer Yilmaz [Yilmaz T],

³. Dr. Hatica Bebis [Bebis H],

⁴. Dr. Nighat Sultana [Sultana N]

^{1,2,3} Faculty of Health sciences department of Biochemistry Near East University North Cyrus

Faculty of Health sciences department of Biochemistry Near East University North Cyrus

⁴ Faculty of Health Sciences department of Biochemistry Hazara University Mansehra, Pakistan.

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Abstract

Current study was focused on RT-PCR based prevalence of HCV among different areas i.e. Battagram, Mansehra, Abbottabad and Haripur and age groups i.e. (≤ 25 yrs), (25-50 yrs) and (≥ 50 yrs) of Hazara division of Pakistan. RT-PCR is considered to be a most reliable diagnostic technique. In this study total 1000 patients ($n=1000$) who were confirmed HCV positive by rapid Device test and ELISA method, which were further evaluated by RT-PCR. HCV RNA was isolated from patient sample serum which was further quantified and amplified by RT-PCR. The highest infection rate was found in Mansehra (21.2%), Abbottabad (15.5%), Battagram (6.8%) and Haripur (3.8%) respectively. Similarly, the highest infection rate was found in age group 25-50 years (33%) while lowest infected age group was less than 25 years (7.4%). It was thus concluded that it is very crucial to screen HCV patients by using RT-PCR to avoid necessary false positive values.

Keywords: Hepatitis C Virus, RT-PCR, ELISA, Rapid device test, Immunity, Infectious diseases, Quantification, Amplification.

1. Introduction

Viral hepatitis is a great public health dilemma afflicting the globe. Five different types of hepatitis viruses have been documented up till now, named hepatitis A, B, C, D and E viruses (Wasley *et al.*, 2008). Though some hepatitis viral infections are acute yet hepatitis B, C and D can accordingly manifest into chronic infections (Mujeeb *et al.*, 1997; Shah and Dar, 2004). In humans, the hepatitis C infection, a life threatening disease is caused by hepatitis C virus (HCV) which belongs to family *Flaviviridae* and genus *hepacivirus* affecting the people all over the world especially in developing countries like Africa, Indonesia and Pakistan (Choo *et al.*, 1989; Ryan and Ray, 2004). HCV infection is a chronic liver disease that may be symptom less at beginning culminating in liver cirrhosis (Sy and

Jamal, 2006). Hepatitis C is not manifested by a single virus but a group of viruses are accountable for hepatitis all over the world known as hepatitis viruses. In pregnancy, it may also pass on from infected mother to a child (Hunt *et al.*, 1997).

HCV has a positive sense single-stranded RNA (messenger RNA) genome that consists of a single open reading frame which is about 9.6 kb in size and about 55-60 nm in diameter (Kato, 2000; Kapoor *et al.*, 2011). There are seven HCV genotypes so far reported (Nakano *et al.*, 2011), Symptoms of hepatitis C include mild fever, headache, fatigue, muscle aches, nausea, joints pain and weight loss, loss of appetite, vomiting and diarrhea (Ryder and Beckingham, 2001). Diagnosis of the disease has developed remarkably over the passage of time, showing improvement from the simple detection of anti-HCV antibodies by ELISA to molecular methods. With early serological assays, especially when applied to Hepatitis and immune depressed

patients, false-negative results were a major hurdle obtained for a significant number of patients (Simmonds *et al.*, 1993).

Previous research was mainly focused on the prevalence of anti-HCV antibodies among the general population from KPK province (erstwhile N.W. F.P) using immuno-chromatographic tests while active infection has never been explored appropriately. By addressing this issue, study on prevalence of active HCV infection 1000 study subject blood samples were examined for anti-HCV or HCV RNA by Rapid Device Test, ELISA and Real-time PCR. Our results indicated that prevalence of anti-HCV antibodies, as detected by ELISA, and the prevalence of active HCV infection, as detected by RT-PCR, was comparatively higher than earlier investigations which were solely based on only antibody-based methods (Khan *et al.*, 2004; Ahmad *et al.*, 2009).

Numerous attempts have been made to conduct studies regarding prevalence of active HCV in Pakistan but the main crux in these studies was that these were performed mainly through random sampling across different populations. Use of one diagnostic tool i.e. ELISA was another limiting factor in these epidemiological studies. Our research has the advantage that it is not based on random sampling. All patients were at first subjected to Rapid device Test and ELISA and upon confirmatory positive results the screened subjects were assayed by RT-PCR.

2. Methodology

The study was carried out at District Head Quarter Hospital (DHQ) Abbottabad on 1000 subject patients (n=1000) belonging to four different districts of Hazara Division of Khyber Pukhtunkhwa, Pakistan. Informed consent was taken in written form from each patient. Other useful information regarding demographic characteristics, age and district with complete address and phone number of patients were documented.

Sample Collection Protocol

Serum samples were collected from HCV infected male and female patients of four different districts of Hazara Division namely Haripur, Abbottabad,

Mansehra, and Battagram. 1000 HCV infected patients were registered in the study whose blood samples were collected in duplicates. For serum extraction 3 ml of clotted blood samples were centrifuged for 12-15 minutes at 8000–10,000 rpm at room temperature. Supernatant was collected in a sterile polypropylene tube, labeled and stored at -20 °C for further analysis. Patients were categorized into three age groups and sex wise into two groups. For qualitative analysis of HCV, two clinical diagnostic tests i.e. HCV rapid device test and ELISA were performed. HCV rapid *In vitro* diagnostic chromatographic assay (device) was used for the determination of hepatitis C virus antibody in patient's serum. Another qualitative analysis of HCV was done by using 96 well ELISA anti HCV AG coated strips (DSI, Italy). For the quantitative analysis of HCV from serum, RT-PCR was performed.

RNA extraction, amplification and quantification by RT-PCR

RNA was extracted from human serum using viral RNA isolation manual protocols (Macherey-Nagel, Germany). Bosphore HCV quantification kit (vol. 1) was used for HCV RNA amplification and quantification (Anatolia Gene works, Turkey). In current study, four different quantification standards were used which were Standard 1 (1×10^6 µl/ml copies of the virus), Standard 2 (1×10^5 µl/ml), Standard 3 (1×10^4 µl/ml), and Standard 4 (2×10^3 µl/ml). All four external quantification standards were added into the PCR reaction together with samples and negative controls (PCR-grade water). For hepatitis C RNA quantification maximum of 40 repeats were deemed adequate to ascertain viral load (no: of the cycles on which sample becomes positive). The quantification serum standards were calibrated against WHO International Standard (NIBSC Code 06/100).

3. Inclusion criteria

All those patients who were HCV positive by ELISA and rapid test device method during the years Jan, 2013 to June, 2015 were selected as study subjects (n=1000) for advanced RT-PCR screening.

4. Results

Out of 1000 patients 480 was confirmed positive by RT-PCR and 520 patients were found as negative. Overall results of the RT-PCR are presented in Figure 1.

Figure.1 Gender wise HCV data in different districts of Hazara Division (KPK)

The highest number of routinely diagnosed HCV positive patients belonged to district Mansehra and were 448. Out of these 448 patients, 212 patients were found positive by RT-PCR. Gender wise 249 were male out of which 121 were observed positive and in case of females 91 out of 199 were confirmed positive by RT-PCR. Second highest number of patients belonged to district Abbottabad i.e. 326. Out of which 163 were male and 163 were females. 82 males and 73 female patients were found positive by RT-PCR.

From Battagram district 146 patients were enrolled. 86 were males out of which 38 were positive and 60 were female out of which 30 patients were measured positive. The lowest numbers of patients were analyzed from district Haripur which were 80 including 40 males and 40 females. 20 positive male 18 positive females' cases were obtained by RT-PCR as represented in figure 1.

Rapid Test device and ELISA Results

Total 1000 samples were collected from males and females from four different areas and there rapid test device and ELISA results were observed positive which was further confirmed by RT-PCR to start their treatment shown in Table 1.

Table.1 % of patients with false positive screening of ELISA and Rapid Device Test

Rapid Test device and ELISA results					
Region	No of Patients	Gender		Rapid Test Device	ELISA Test
		Male	Female		
Battagram	146	86	60	+	+

Mansehra	448	249	199	+	+
Abbottabad	326	163	163	+	+
Haripur	80	40	40	+	+

Age wise results

Current study was based also based on categorizing three different age groups which are shown in Table 2.

Table.2. Distribution of samples (n=1000) on the basis of Age and Gender across all four districts of Hazara Division.

ELISA and Device Method Results Age Group and Gender Wise				
Age Groups		Below 25 Year	25 to 50 Year	Above 50 Year
No of patients		142	711	147
Gender	Male	78	372	91
	Female	64	339	56
Rapid Test Device		+	+	+
ELISA Test		+	+	+

RT-PCR Age Wise Results

Table.3. % of false positive and negative values by RT-PCR

RT-PCR Gender and Age Group Wise Results				
Age Groups		Below 25 Year	25 to 50 Year	Above 50 Year
No: of patients		142	711	147
Male	Positive	39	178	50
	Negative	39	194	41
Female	Positive	35	152	26
	Negative	29	187	30

Discussion

Hepatitis C has been considered to be the most commonly emerging viral hepatitis worldwide

with major devastating consequences universally. Every year round about four million people are infected with HCV and over 150 million people are infected into chronic stage and are at risk of developing liver cirrhosis (Averhoffet *et al.*, 2012).

In a random study on 7148 patients carried out by Khan *et al.*, (2004) about prevalence of active HCV infection among the blood donors of Khyber Pukhtunkhwa and FATA region of Pakistan and their subsequent evaluation of the screening tests for anti-HCV showed that 3.13% of the volunteers were detected positive for anti-HCV antibodies that were further processed by ELISA which further manifested that 1.89 % were positive for anti-HCV antibodies among total number of volunteers deemed as positive. These samples were positive by either ICT or ELISA used for HCV RNA extraction and subsequent RT-PCR which revealed about 118 (1.65%) donors for HCV RNA in their blood. Shah and Dar (2004) demonstrated that the highest number (i.e. 67 % patients) of HCV positive cases by ELISA belonged to 20-40 years of age including 16 males and 15 females. Their analysis reported HCV positive patients by ELISA between 17-60 years of age group. Comparatively our findings show similarities to Shah and Dar (2004) i.e. 71% (711/1000) HCV positive patients belonged to 25-50 year of age group.

Another study by Ali *et al.*, (2011) including 305 HCV positive patients was comprised of 177 males and 128 female patients with pathogenesis involving different genes. Furthermore, it was conducted from the same location and through random sampling from different localities of Hazara region and found 3a genotype as the most prevalent genotype of HCV found in Hazara region of Pakistan. Contemporary study was not based on genotypes; rather it was just based on quantification by RT-PCR. Total 480 patients were found confirmed HCV positive out of 1000, out which 267 were males and 213 were females.

Anwar *et al.*, (2013) on random sampling of 4246 blood samples demonstrated that out of 4246, 210 patients were confirmed positive for HCV infection with highest prevalence among 20-29 years and 29 to 39 years of age group. Another prevalence study by Ali *et al.*, (2010) in three areas Mansehra, Balakot and Oghi of Hazara

Division regarding active hepatitis C virus (HCV) infection showed that out of 400 patients (300 male and 100 females) 3.5% of the people harbored active infection with HCV whereas 7% of the population, in general, had the presence of antibodies against HCV in their blood.

Serological testing by third generation ELISA conducted by Hinrichsen *et al.*, (2002) on 2786 patients to find out prevalence as well risk factors in the causation of Hepatitis C infection showed the overall frequency of HCV (HCV antibody and/or HCV-RNA positivity) to be 7.0% (195 patients). Antibody positivity occurred in 171 patients (6.1%). Viraemia was detectable in 111 patients (4.0%). Twenty four of 111 HCV RNA positive patients (21.6%) were negative for HCV antibodies. Thus 0.8% of the entire study population was HCV positive but could not be diagnosed by routine HCV antibody testing. Primary issues recognized by a standard questionnaire in 1717 of 2796 patients were the number of blood transfusions individuals had received and duration of dialysis, the later including patients who received no blood transfusions.

Due to lack of eminent epidemiological research on hepatitis in Pakistan, majority of investigations carried out so far have focused on groups such as blood donors and chronic liver disease patients. On the other hand population based studies have also its limitations owing to small sample size and lack of representation across the country side (Shah and Shabbeir, 2002).

Main theme of the study was to assess RT-PCR based confirmatory prevalence of hepatitis C in Hazara division among male and females belonging to different age groups. Although many studies have been conducted and their work published, but, no previous study is ever based on RT-PCR based prevalence in Hazara division of Pakistan. This evaluation was comprised of 1000 serum samples belonging to diverse age groups i.e. (≤ 25 yrs.), (25-50 yrs.) and (≥ 50 yrs.) along with different demographic features (146 from Battagram, 448 from Mansehra, 326 from Abbottabad and 80 from Haripur districts) of Hazara division of Pakistan. HCV RNA was extracted from serum samples which was further quantified and amplified by Kit method and

minimum of 40 repeats of RT-PCR was deemed adequate to ascertain viral load. The total number from Battagram patients was 146 out of which 68 (6.8%) were positive. 448 (21.2%), 326 (15.5%) and 80 (3.8%) patients from Mansehra, Abbottabad and Haripur were respectively analyzed positive by RT-PCR. Highest infection rate was found in age group 25-50 years (33%) while lowest infected age group was less than 25 years (7.4%). As can be seen from the figures these values are higher as compared to the reported literature because already the selected patients in this study were those who were found positive by clinically diagnostic test like Rapid test device and ELISA, which were then subsequently screened and their confirmatory prevalence was sanctioned by RT-PCR.

Importance of our study is the quality as well as range of sample size which is larger when compared to other HCV prevalence studies in Hazara. In addition, our study not only analyzed the extent of the problem but also assessed the general awareness about HCV which has not yet been attempted in this area. This research will, therefore, form basis for further research and provide valuable information for future strategies of health sector and policy making in Hazara division.

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