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A detailed report on the specific area based percentage and diagnostic methods of hepatitis C in Khyber Pakhtunkhwa, Pakistan

Syed Farhan Ahmad[1*], Zubair Anwar[2], Shahid Hussain[3], Maryam Jehangir[4], Irum Jehangir[5], Anwar Jamal[6], Jabar Zaman Khan Khattak[1], Ayaz Ali Khan[7]

1. Department of Biotechnology and Bioinformatics, Islamic International University Islamabad, Pakistan
2. Department of Virology and Immunology, NIH, Islamabad, Pakistan
3. Department of Bioinformatics, Capital University of Science & Technology, Islamabad, Pakistan
4. Department of Bioinformatics, Shaheed Benazir Bhutto Woman University, Peshawer, Pakistan
5. Department of Microbiology, Khyber Medical University, Peshawar, Pakistan
6. Department of Medicine, Khyber Medical College, Peshawar, Pakistan
7. Department of Biotechnology, University of Malakand, Chakdara, Lower Dir, Pakistan

Abstract

Background: The prevalence of hepatitis C virus (HCV) varies tremendously in different parts of the world. This study reviews the percentage and molecular diagnosis of Hepatitis C in the persons from Khyber Pakhtunkhwa, Pakistan that visited to a particular laboratory.

Methods: The method includes the diagnostic procedure steps by Real Time PCR. A Total numbers of 1050 Persons were screened during four months i.e. January-April, 2014. The collected data was evaluated for prevalence rate, age wise prevalence, gender wise prevalence and comparison of RT-PCR and ICT.

Results: Overall percentage was 64.85 which is an overestimation of a true prevalence because of the specific sampling method applied to current study. Middle age persons were more affected. The percentage was higher in male (56.9) as compared to female (43.02). The RT-PCR diagnostic test was found to be more sensitive for the detection of HCV comparative to ICT.

Conclusion: It is recommended that government should establish such laboratories equipped with RT-PCR for timely and accurate detection of HCV. Moreover, awareness programs are required to decrease the burden of HCV in the Pakistani population.

Keywords: Khyber Pakhtunkhwa, Hepatitis C, RT-PCR

Introduction

Hepatitis C virus (HCV) is one of the major etiological agents for parenterally acquired hepatitis. It is asymptomatic in large proportion of cases (65-75%) and revealed accidentally by abnormal liver function tests or anti-HCV positivity. The long term morbidity and mortality is far greater than its counterpart Hepatitis B virus in terms of chronic hepatitis 70%, cirrhosis 20-30%, hepatocellular carcinoma and liver failure (Ericksen, 1999). Infection with hepatitis C virus (HCV) has been identified as the major cause of post-transfusion

*Corresponding author. Syed Farhan Ahmad, MSc
Department of Biotechnology and Bioinformatics, Islamic International University Islamabad, Pakistan
Tel. +923453976540
Email: zaid_khan68@yahoo.com

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non-A, non-B hepatitis (Abdulkarim et al., 1998). The exact sero-prevalence rate in Pakistan is not known, however in various studies it has been reported to be 3-7% (Akhtar et al., 2002).

HCV is a tremendous health problem not only in Pakistan but also worldwide. The global epidemiology of viral hepatitis A and hepatitis B is well established, although HCV data remain limited, particularly in Pakistan. Despite the employment of modern laboratory apparatus for the screening of blood, blood transfusion remains the main mode of transmission of HCV infection, since unscreened blood and blood products are still used in many developing countries. As a result, HCV is one of the most common blood-borne infections (Lore and Kostman, 2005).

The World Health Organization estimates that approximately 3% of the world population has been infected with HCV thus far. There are about 170 million patients with HCV in the world, and three to four million individuals are diagnosed as new cases every year (Lore and Kostman, 2005 and Ray, 2002). Approximately two million cases are in Japan, 2.7 million are in United States, 5 million are in Europe, and around 10 million are in Pakistan, whereas, highest prevalence rate has been reported in Egypt (Frank et al., 2000).

Recently, HCV prevalence studies have come out of Pakistan in the Middle East. 751 out of 16,400 patients (4.57%) were found to +HCV Ab from 1998-2002 with the largest age group from 41-50 (Muhammad and Jan, 2005). Among male blood donors in Karachi, Pakistan, the seroprevalence of HCV was 1.8% with a trend of increasing proportion of positive donors from 1998-2002 (Aktar et al., 2004).

Molecular virological techniques play a key role in diagnosis and monitoring of treatment. Because it is difficult to culture the virus, molecular techniques were instrumental in first identifying HCV, making it one of the first pathogens to be identified purely by molecular diagnostics (Choo et al., 1989). HCV RNA can be detected in the blood using amplification techniques such as polymerase chain reaction (PCR). (Pawlotsky, 2002). The implementation of PCR in routine diagnostic laboratories, however, requires a level of standardization that was not originally provided by in-house methods. Additionally, by inhouse methods the occurrence of false-positive results due to amplified product contamination is not always preventable and false-negative results due to inhibition are not always easily detectable (Albadalejo et al., 1998).

The present study was designed to check the percentage of occurrence of hepatitis C in the residents of Khyber Pukhtoonkhwa who approached to the Human molecular genetics (HMG) Laboratory Islamic International University Islamabad. The aims and objectives

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of the present study were;

To review the currently available molecular diagnostic tests for HCV and their clinical applications.

To determine the percentage of HCV infection in the people who came to HMG Laboratory Islamic International University. during the months of January-April 2014 referred by physicians or on their own wish.

To give awareness in the public about Hepatitis C.

To provide data to public health department regarding the percentage of Hepatitis C, so that proper preventive & treatment programs/projects may be launched.

Materials and Methods

This was an institutional based study carried out in a research laboratory to observe the percentage of hepatitis C and to determine the best approach for its diagnosis. Several parameters were sorted out such as Age wise percentage, Gender wise percentage, percentage based on diagnostic tests and comparison of percentage of hepatitis C in different districts of Pakistan.

The area selected for the present study was Khyber Pakhtunkhwa, Pakistan. The total estimated population of the area is 2,0215,000 (World Gazetteer 2009). The area was selected due to the reason that hepatitis C is known to be an emerging disease in this area.

Those people were selected who visited Human Molecular Genetics Laboratory (HMG) Islamic International University Laboratory, belonging to different regions of Khyber Pakhtunkhwa for diagnosis of hepatitis C referred by physicians or on their own wish. Most of the patients were assumed to be infected with acute hepatitis C. The data of the patients was recorded on printed questionnaire. The questionnaire included questions about Age of patient, gender, reference of physicians, marital status, socioeconomic status and diagnostic tests etc.

Blood (5ml) was collected from the patient's radial vein by using disposable Syringe under strict aseptic conditions and was transferred to Effandorf tube. Serum was isolated from blood samples by keeping the samples bottles in slant position. After coagulation of blood, serum oozed out and was transferred to Effandorf tubes. Serum was stored at -20 ˚C in refrigerator and was used for further analysis. HCV RNA was extracted and purified using QIAamp DSP Virus Kit (QIAGEN, cat. no. 60704). Isolation of HCV RNA was performed according to the standard procedure given in QIAamp DSP Virus Kit Handbook (2007). Each step was carefully followed to ensure that all samples are properly processed. QIAGEN kit handbook and user manual is available at www.qiagen.com

Applied Biosystems 7700 Real Time PCR System by ThermoFisher Scientific company was used as an instrument for polymerse chain reaction of RNA. Pathogen detection by the

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polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR the amplified product is detected via fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. The reagents and enzymes for the specific amplification of a 240 bp region of the HCV genome were applied. Amplification of HCV RNA was done by Preparing 25x reagent mix. A final maste rmix was prepared with calculated values of each reagent. These reagents include PCR grade water, Mg-sulfate (50Mm), 2x Reaction mix (buffer containing RT-PCR buffer, 6 mM Mg-sulfate and dNTP), 25x reagent mix (containing primers and probe) and 10x passive reference dye solution. Appropriate aliquots of the master mix were added to 0.2 ml optical PCR tubes or strips considering the volume of sample to be added. After manual or automated dispensing of the master mix, equal aliquots of extracted RNA samples were added to the tubes. One negative control was always run with the samples. To prepare a negative control, the template RNA sample was replaced with equal volume PCR grade water. An optical cover was tailored according to the number of used wells and the tubes were sealed carefully. Centrifugation was done at 200 x g for 1 min (1000 rpm) in a standard benchtop centrifuge. To avoid leakage during amplification, after placing on the wells the cover was tightly pressed and a compression pad (Applied Biosystems) was used following the instructions of the manufacturer! The wells were selected containing non-target controls ("Blank") and samples ("Unknown") from the sample type pop-up menu. The dye "JOE/VIC" was Chosen from the dye layer pop-up menu. The amplification conditions were setup.

Data obtained was statistically analyzed by using online statistical program, prism, demoversion 05 (www.graphpad.com). Mean and percentage frequency were analyzed for every parameter and has been presented in the form of tables.

Results

A total of 1050 Patients having different ages, both male and female referred by different physicians, were diagnosed through Real Time PCR for hepatitis C. On the prescribed proforma, each patient's history (reason of diagnostic tests, HCV positivity or negativity prior to diagnostic test, treatment and other relevant clinical data) was recorded.

Data was collected from HMG Islamic International University Laboratory. The Study was conducted during the months of September, October, November and December of 2014.

The present study was intended to determine the institutional based percentage of hepatitis C in Khyber Pakhtoonkhwa during the four months of 2014 through real time PCR. For this purpose the whole project was splitted

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down into the following seven parameters, which are:

1. Month wise prevalence of Hepatitis C (as a whole)
2. Age wise percentage of Hepatitis C
3. Gender wise Hepatitis C percentage
4. Comparison of RT-PCR and ICT ratio
5. Quantitative Tests range
6. ALT range
7. Comparison of HCV p of different districts of Pakistan

Month wise Prevalence of Hepatitis C

Prevalence is the proportion of individual in a population having a disease (On line medical dictionary). Prevalence is commonly measured in new cases per 100,000 of population at risk per year. Prevalence was measured by the following formula

$$\text{Prevalence} = \frac{\text{no of patients} \times 100{,}000}{\text{Total population of the area}}$$

Data regarding as a whole is given in table 05. During January the prevalence was 0.999, in February prevalence was 0.945, in March prevalence was 0.555 and in April, 2014 prevalence was 0.87 per 100,000 as given in table 01.

Table 01 Hepatitis C Prevalence during a Four month period from January-April 2014

S. No	Month	No. of persons who visited HMG Lab Islamic International University Laboratory	No. of patients	Prevalence*
1	January	324	202	0.999
2	February	251	191	0.945
3	March	248	112	0.555
4	April	227	176	0.87
		1050**	681	3.36

*Prevalence was calculated as per 100,000 populations
**Total Number of persons visited HMG Lab Islamic International University (Islamabad) from March 2014 to April 2014
***Average prevalence/month

Table 02 Age wise Hepatitis C percentage from January 2014 to April 2014

S. No	Age Range (years)	No of Patients	Percentage %
1	0-10	0	0
2	11-20	92	13.5
3	21-30	169	24.8
4	31-40	216	31.71
5	41-50	186	27.3
6	51-60	15	2.2
7	60 plus	03	0.44

* Percentage was taken out of total number of patients (i. e 681)

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Table 03 Age wise Gender Distribution of Patients

S. No	Age range (years)	Female* (%)	Male** (%)
1	0-10	0	0
2	11-20	12.96	13.9
3	21-30	28.3	22.16
4	31-40	26.96	35.30
5	41-50	29	26
6	51-60	2.38	2
7	60 plus	0.34	0.51

*Female were 293 out of 681 (43.02%)
**Male were 388 out of 681 (56.9%)

Comparison of Real time Polymerase Chain Reaction (RT-PCR) and Immuno Chromatographic Technique (ICT) ratio

Two types of diagnostic tests including PCR and ICT were conducted for Hepatitis C detection in the people visited to HMG Lab Islamic International University Laboratory. The patient's positivity ratio for each test was determined which marked the difference among their results. The RT PCR based tests showed that total 681 (64.8%) patients out of 1050 were positive. Through ICT (Immuno Chromatographic Technique) 698 (66.4%) patients were positive. The RT PCR is more authentic than ICT due to its high sensitivity and specificity. HCV is usually detectable in the blood by PCR within one to three weeks after infection and antibodies to the virus are generally detectable within three to 15 weeks. Periodically, ICT might show normal results but may become abnormal, once antibodies remained even after the elimination of virus from the patients body. RT PCR Ratio (negativity and positivity) and ICT Ratio (negativity and positivity) were considered during comparative analysis.

This comparison has been shown in figure 1.

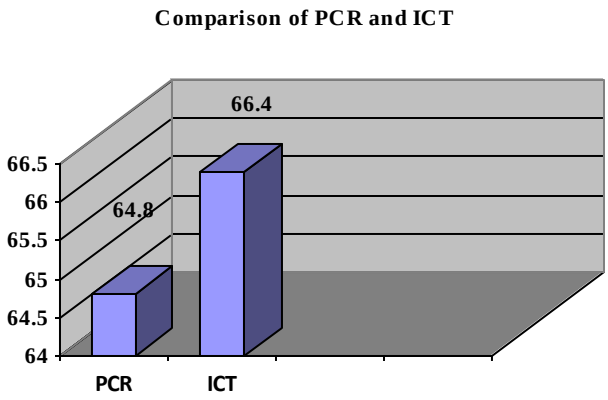

Figure 1 Camparison of PCR & ICT positivity ratio among the patients

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Quantitative Tests Range

Out of 1050 persons, 129 patients were referred for quantative PCR tests. The number of RNA copies per dl of blood was detected. The minimum value recorded was 1.5×103 no. of copies per dl and maximum value was 6.9×106 no. of copies per dl. Range was calculated by the following formula;

$$\text{Range} = \frac{\text{maximum value} - \text{minimum value}}{2}$$

Range = 3.4×10^4 no of copies per dl.

Alanine Amino Transferase Value

The ALT value of each patient was also checked out. The range among the different age groups and also in male female was sorted out by using the following formula.

$$\text{Range} = \frac{\text{maximum value} - \text{minimum value}}{2}$$

The ALT range in male & female of the age group 11-20 years was 96 IU/L and 101 IU/L. It was 120IU/L in male and 152 IU/L in female of age group 21-30 years, 173 IU/L in male and 145 IU/L in female of age group 31-40 years, 139 IU/L in male and 121 IU/L in female of age group 41-50 years. 49 IU/L in male and 74 IU/L in female of age group 51-60 years, 29 IU/L in male and 16 IU/L in female of age group 60 plus years. Finally mean (average) range was also found by the statistical formula given as:

$$\bar{X} = \frac{\Sigma X}{N}$$

Where:

$\bar{X}$ (sometimes call the X-bar) is the symbol for the mean.

Σ (the Greek letter sigma) is the symbol for summation.

X is the symbol for the range values.

N is the symbol for the number of patients

Table 04 Age and Gender wise ALT Range

S. No	Age range (years)	Female (IU/L)*	Male (IU/L)*
1	11-20	96	101
2	21-30	152	120
3	31-40	145	173
4	41-50	121	139
5	51-60	74	49
6	60 plus	16	29
Mean Range		100.66	101.33

IU/L*= International Unit per Liter

Comparison of Percent Prevalence of Hepatitis C in Different Districts of Pakistan

Percentage of Hepatitis C determined in the persons who visited HMG Lab Islamic International University Laboratory was 64.8% during January-April 2014 belonging to

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different districts of Khyber Pakhtunkhwa. Where percent prevalence of Hepatitis C was also reported by different research workers in different districts of Pakistan. Prevalence rate in these districts is as; 4.57% in Buner, NWFP (Muhammad and Jan 2005), 9% in Mardan NWFP (Khan et.al., 2004) 3.2%-11.26% in Rawalpindi (Shah et.al 2002 and 2005), 5.3% in Islamabad (Khokher et.al., 2004) 5.78%-13.5% from Lahore(Shah et.al 2002), 9% from Jamshoro (Almani et.al 2002), 2.2%-4.6% from Karachi (Zakria et.al., 2003). The seroprevalence of Hepatitis C in different parts of the country reported in last 5 years is from 2.2% to 13.5%. The highest seroprevalence of Hepatitis C is reported from Lahore (13.3%), Jamshoro (9%) and Mardan (9%).

Comparison of RT-PCR and ICT tests

Two types of diagnostic tests including RT-PCR and ICT were performed for Hepatitis C detection in the patients visited to HMG Lab Islamic International University Laboratory. RT- PCR based tests showed lower positivity rate of 64.8%. Albadalego et al., (1998) reported that RT PCR is the best for invitro detection of HCV infection. The results were compared with the results for biochemical and serological markers of HCV and it was evaluated that RT-PCR systems showed the same accuracy, with a concordance rate of 99.8%. John et al., 2007 reviewed the currently available molecular diagnostic tests for HCV and concluded that RT-PCR is the most sensitive and effective technique. Nadir, 2008 documented that detection of HCV RNA by RT-PCR is a costly procedure and requires sophisticated equipment, special environment, and technically experienced staff but it's of tremendous importance in terms of sensitivity, reliability and effectiveness. Houghton, 1991 reported that detection of HCV RNA in patient serum by highly sensitive tests such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) has become an increasingly important tool for confirming the diagnosis of hepatitis C and for assessing the antiviral response to interferon therapy. Gretch 1996 stated that being a valuable research tool, the Quantitative Assessment of HCV RNA level by RT-PCR in patients before, during, and after therapy has tremendous potential for improving the clinical management of chronic hepatitis C. All these results are in agreement with the present study and reveal that RT-PCR is the most applicable, sensitive and accurate technique for molecular diagnosis of HCV.

Discussion

HCV is a tremendous health problem not only in Pakistan but also worldwide. The global epidemiology of viral hepatitis A and hepatitis B is well established, although HCV data remain limited, particularly in Pakistan.

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Despite the employment of modern laboratory apparatus for the screening of blood, blood transfusion remains the main mode of transmission of HCV infection, since unscreened blood and blood products are still used in many developing countries. As a result, HCV is one of the most common blood-borne infections (Lore and Kostman, 2005).

Prevalence rate is occurrence of new cases in population of an area. In the present study percentage was checked in the people who visited the HMG Lab Islamic International University Laboratory in January 2014-April 2014. The results showed that overall percentage during these months was 64.8% which is quite alarming. The reported prevalence of HCV in different areas of Khyber Pakhtunkhwa (KP) ranges from 4.1 to 36%. (Waqar et al., 2014). The prevalence of HCV infection was 15.57% among the high risk groups in Khyber Pakhtunkhwa. (Ali et al., 2011). Another recent study (Khan et al., 2015) stated that prevalence of active hepatitis C virus infection in general population of Khyber Pakhtunkhwa, Pakistan was 11.64% out of total 2534 screened individuals. The recorded percentage (64.8) of our study does not correlate with these findings because other research workers have conducted their study in general population. The present study was confined to the people of high risk group who visited a specific laboratory at Islamabad due to evident hepatitis related symptoms. The major identified risk factors leading to higher prevalence in KP were usage of non-disposable syringes/needles, IV drips by untrained people at villages, shaving by barbers at villages and bazaars, dental procedures/ surgery, ear/nose piercing in females with non-sterilized needles, previous surgery and blood transfusion (Majid et al., 2010).

The disease is becoming a major health problem of developing countries, including Pakistan that has the second highest prevalence rate of hepatitis C ranging from 4.5% to 8%. (Khattak et al., 2002). Studies in Pakistan on small targeted groups including blood donors, health professionals, drug abusers and chronic liver disease patients indicate that the prevalence of hepatitis C is as high as 40%. (Abbas et al., 2008). Another study aimed to estimate HCV prevalence showed the same results as ours concluding percentage in the studied area is higher than the overall prevalence in Pakistan. Prevalence among male participants was 30 (11.8%), whereas, that among female participants was 37 (9.4%) is similar to the present findings. (Muhammad et al., 2010).

Yasir et al., (2009) documented that in Pakistan, Percentage prevalence of HCV was 4.95% ± 0.53% in the general adult population. Idrees et al., (2008) estimated the prevalence and spectrum of hepatitis C virus (HCV)

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infection in the general population of Pakistan and stated that HCV RNA PCR was 494 (49.50%). Mohammad and Jan (2005) assessed the frequency of hepatitis C in District Buner NWFP and found that out of 16,400 patients, 751 were found positive for anti-HCV antibodies (4.57%). The overall seroprevalence was found to be 9% in District Headquarter Hospital Mardan according to Khan et al., (2004). Luby et al., (1997) determined the prevalence and routes of transmission of hepatitis C virus (HCV) infection in Hafizabad, Pakistan and approached 504 households initially. Serum was collected from a randomly selected household member in 309 (64%). Twenty persons (6.5%) had anti-HCV antibody. A high prevalence rate of Hepatitis C (58.33%) was observed by Khan et al., (2007) at Khyber Teaching Hospital. The present study shows high percentage among those persons who visited to HMG Lab Islamic International University Laboratory and hence the results for the prevalence percentage of Hepatitis C is in correlation with findings of Khan et al., (2007) and Idrees et al., (2008). The considerable higher percentage of this study as compare to other researchers revealed that visited persons of HMG Lab Islamic International University Laboratory had more complications regarding health status.

The present data was also evaluated for age wise percentage among the people who visited HMG Lab Islamic International University Laboratory. Different groups were made according to their age. Percentage was high (31.71) in the age group of 31-40 and the lowest (0.44) was among the age group of 60 plus. No any patient was present in the age group of 0-10 years. The present results show that the percentage was high at middle age group. Wasley and Alter (2000) reported that most infections are found among persons 30-49 years old in US and Australia. Same results have also been found by Mohammad and Jan (2005), as they stated that the mean age of the patients was 37 years, the youngest was 15 years while the oldest was 65 years. A significant difference was noted in the anti-HCV prevalence rate among different age groups tested according to Idrees et al., (2008). Nadeem and Janjua (2008) reported that more than twothirds of HCV patients were 40 to 50 years old in Pakistan. Yasir et al., (2009) documented that in Pakistan, percentage prevalence of HCV was 4.95% ± 0.53% in the general adult population, 1.72% ± 0.24% in the pediatric population and 3.64% ± 0.31% in a young population. Mohammad and Junaid 2001 mentioned that a high prevalence rate (23.8%) of Hepatitis C was at the age of more than 20 years. The lowest percentage (1.2%) was among the subjects having age below 20 years. These results are in correlation with findings of the present study. After discussion

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of age wise percentage, it has been revealed that percentage is high among the middle age (10-30 years).

A total of 1050 people were enrolled in HMG Lab Islamic International University Laboratory, out of which 681 were positive for Hepatitis C through Real Time PCR. Out of 1050, total male were 557 out of which 388 (56.9%) were positive. In total 493 female were, 293 (43.02%) were positive. The present study suggests that percentage of Hepatitis C was high among the male as compared to female. The results mentioned by Mohammad and Jan (2005) are in agreement with the results of the present study. The frequency of hepatitis C has been assessed in District Buner. The frequency of hepatitis C was higher among the male, 409/751 (54.46%) as compared to female, 342/751 (45.53%). The results by Idrees et al., (2008) are also in agreement with present study. The prevalence of anti-HCV antibodies was significantly higher in males (15.09%) than in females (12.3%) in the general population of Pakistan. The findings by Khan et al., (2004) for gender wise prevalence is not in Agreement with the results of the present study. The seroprevalence of hepatitis C virus (HCV) infection and risk factors was assessed among hospital patients in District Headquarter Hospital Mardan. It has been documented that the seroprevalence in male and female was 7.8% and 12.4% respectively. This statement shows deviation from the present study due to high risks factors and more health complications regarding health in female of that area. The results of the present study and after its comparison with other research work, it has been concluded that percentage of hepatitis C is higher in male as compared to female.

Conclusion

From the present study it has been concluded that hepatitis C is an emerging disease in Khyber Pakhtoonkhwa. Real Time Polymerase Chain Reaction is considered to be the most effective and reliable test for the molecular diagnosis of HCV. A high percentage was sorted out in subjects that were studied in the present project. There is need to check prevalence in general population of Khyber Pakhtoonkhwa as no such study has been conducted on such aspect. Also it has been found that male were at high risk as compared to female. The middle age was highly affected by hepatitis C virus. Comparison of RT-PCR and ICT was also undertaken in the present study in which RT-PCR had highest sensitivity. Range of ALT and RT-PCR quantitative tests was determined statistically for information regarding HCV.

Clarification

As sampling has not been performed randomly on the normal population therefore it is stated

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that the finding in this cohort cannot be generalized to the whole population.

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Conflicts of interest: none

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