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Molecular detection of HCV infection in suspected liver disease patients of District Mardan, Khyber PuhktoonKhwa

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Abstract

Chronic hepatitis is a common cause of liver related problem due to different viruses concerned with liver, where Hepatitis C (HCV) has been documented as the major cause and lead to many complications. A total of 1500 (54% Male and 46% Female) suspected liver disease patients were selected for the study. Confirmation was carried out by ICT, ELISA and q-PCR. Age wise and gender wise and Tehsil wise distribution of the data was carried by statistical analysis. HCV RNA was detected in 438 (29.2%) samples out of total 1500 samples of suspected liver disease patients via q-PCR. Out of 438 patients, 210 (47.94%) were male and 228 (52.06 %) were female patients. In age group 41-60 years there were 210 (47.94%) HCV RNA positive patients. In age group 21-40 years it was 104 (23.74%), in >60 years it was 95 (21.68%) and in <20 years 29 (6.62%) was observed. In Tehsil wise distribution it was noted that 228 (52.05%) HCV RNA positive samples were from Tehsil Mardan and 115 (26.25%) and 95 (21.68%) from Takhtbhai and Kattlang respectively. This study concludes that the most specific, sensitive and reliable test use for detection of HCV is q-PCR. Female infectivity rate with HCV is relatively higher then male in District Mardan. Age group 41-60 years was found the most susceptible group to HCV infection in this study. Among the three Tehsil of District Mardan, the highest number of HCV infected patients was found in Tehsil Mardan with 228 (52.05%) patients.

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Introduction

Chronic hepatitis is a frequent cause of liver related morbidity due to different hepatic viruses, where Hepatitis C (HCV) has been identified as the main cause (Khokhar *et al.,* 2004) and lead to many complications. HCV also leads to many complications including HCC in 32% of infected patients (Gaeta et al., 1990). HCV was reported in 1989 by Choo and his colleagues as a major cause of chronic liver disease and various metabolic disorders (Sangiovanni *et al.,* 2006).

Hepatitis C virus has six most common genotypes and hundreds of subtypes were identified throughout the world, Genotype distribution of HCV is different among different geographical areas (Choo *et al.,* 1989). Different types of HCV genotypes are related to epidemiological studies, vaccine development, response rates to anti-viral treatment, and clinical management of the infection.

As, people are not habitual to adopt proper hygienic measures and it is for the reason that non implementation of international standards in following procedures like blood transfusion, reuse of injections, intravenous drug users, tattooing, shaving from barbers, unsterilized dental reuse of needles for ear and nose piercing and surgical instruments are the key factors of HCV transmission in Pakistan (Zein *et al.,* 1996).

HCV transmission may occur due to contaminated blood transfusion, surgical instrument, dental surgery, excessive dental consultation, sexual contacts and drug abuses. It is also transmitted through sharing of razors, toothbrushes and shaving from the barber (Chan *et al.,* 1992). Blood transfusion is major source of HCV transmission in Pakistan. It is due to lack of resources, weak infrastructure, ill-equipped resources, poorly trained staff and ineffective screening of blood donors for anti-HCV antibody (Delisse *et al.,* 1991).

About 170 million individual were infected by Hepatitis C virus and every year three to four million new individuals are infected (Okamoto *et al.,* 1991). Due to limited resources and facilities prevalence of HCV in developing countries were high as compared to developed world (Simmonds *et al.,* 1994). Pakistan a developing country with a population of 170 million people has alarming rate of outbreaks of hepatitis C virus (Hijikata *et al.,* 1991). HCV prevalence in Pakistani population is nearly 5%. Its prevalence in injection drug users (IDUs) and the multi-transfused population is high, suggesting that the reuse of syringes is common among the injecting drug users, and that blood transfusions are not properly screened here in Pakistan (Chan *et al.,* 1992).

Different serological test are done for detecting antibodies to HCV that include agglutination, immunofilteration, Immunochromatographic test. ELISA is most widely used screening tests for HCV antibodies detection and q-PCR for nucleic acid (RNA) detection (Wilkins *et al.,* 2010).

Material and method

Present study was conducted at District Head Quarter Hospital, Mardan (DHQ) in collaboration with Provincial program for Hepatitis Control and Prevention.

In present study, a total of 1500 patients with liver disease were included who were referred to Head Quarter Hospital, Mardan (DHQ) from three Tehsil of District Mardan during August 2012 to October 2012 in the Provincial Program for Prevention and Control of Hepatitis which provides molecular based diagnostic facility that general public sensitive, specific and more reliable diagnostic tests on the basis of utilizing PCR and real-time PCR methods. Mardan is the most populous District of KPK. All sera were stored in aliquots at -70 °C till was used for nucleic acid (RNA) isolation. All the patients are liver disease patients visiting during that project. Diagnosis was done first on ICT method. ICT positive patients are subjected to ELISA then ELISA positive patients forwarded for RT-PCR. All Serological and biochemical data of these patients were available. These samples belonged to different

Tehsil/geographical regions of Mardan District. Which include Tehsil Mardan, Tehsil Kattlang, and Tehsil Tkhatbhai. There was no need for separate written informed consent from subjects for this study, since this analysis was a part of the original protocol in a routine workup of Molecular Diagnosis.

ICT

The sera of HCV samples were tested for infection of HCV. To analyze the HCV infection, strip device (ACON, USA) was used. To use the test device, it was brought to room temperature before opening and placed on a cleaned and leveled surface. With the help of dropper, 3 drops (~100 µl) of serum sample was added vertically to the specimen well of the test device. The device was left for fifteen minutes till the sample rose in the strip and the results were appeared. These results were recorded.

ELISA

Preparation steps

Solution-1: The antigen-conjugate complex was prepared by added conjugate and conjugate diluents with 1:10

Solution-2: Substrate solution was prepared by added substrate and substrate diluents with 1:10

Procedure

All the patient's sera positive for ICT were checked for Anti-HCV Antibodies using 3rd generation ELISA assay kit (Glibe Diagnostics, ITALY) using the methodology described in the manufacturer's protocol. In brief, incubator was set to 37 ±1 ºC. All the reagents were brought to room temperature before use (approximately 1 hour), without removing the plate from the bag. All components were shaken well. Then the plate was removed from the package. Firstly added 100 µl of two negative controls and one positive control and first was leave blank. Then 30 µl sample diluents were added to rest of wells and then 70 µl patients serum were added. Plate was covered with a sealing sheet and incubated to 37 ±1 ºC for 60 min. After incubation The seal was removed and aspirate liquid from all wells and wash five times with 0.3 ml of washing solution per well. Then 100 µl of solution-1 was added into each well, and then Plate was covered with a sealing sheet and incubated to 37 ±1 ºC for 50 min. After incubation The seal was removed and aspirate liquid from all wells and wash five times with 0.3 ml of washing solution per well. Then 100 µl of solution-2 was added into each well and then Plate was covered with a sealing sheet and incubated at room temperature for 25 min, protected from light and then immediately 50 µl of stopping solution was added into all wells. Finally, Read with ELISA Plate Reader at 450 nm within one hour of stopping and cut off value was determined by following formula.

Cut off value = negative control + negative control / 2 × 0.181 (Kit constant)

q-PCR

HCV RNA Extraction

Sera of the patients were subjected to viral RNA extraction by using viral RNA extraction kit (Nucleospine® RNA virus), according to the manufacturer protocol.

Preparation steps

1) Added carrier RNA one vial Lysis buffer and after use store the Lysis buffer at 2-8 C.
2) Added 100 ml ethanol into wash buffer 2.

Extraction Protocol of HCV RNA

Sera of the patients were subjected to viral RNA extraction by using Nucleospine® viral RNA extraction kit (Nucleospine), according to the manufacturer protocol. 150 µl of serum sample was taken and then 500 µl of prepared Lysis buffer 5.6 µl carriers RNA was added into the 1.5 ml eppendrof tube and incubated at room temperature for 10 min. Tubes were briefly centrifuged to remove drops from inside of the lid. A 500 µl of 100% ethanol were added, vortexes for 15 seconds and briefly centrifuged the tube. Took 600 µl of previous step solution in Nucleospine Column, centrifuged at 8000 rpm for 1 min, discarded the filtrate and again this step was repeated. Then 600 µl of wash buffer 1 was added and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and again 600 µl of wash buffer 2 was

added and centrifuged at 11000 rpm for 3 min and discarded the filtrate. Then again 200 µl wash buffer 2 was added for 2 min and centrifuged at 11000 rpm discarded the filtrate. Then placed the filter column in new 1.5 ml eppendrof tube and 50 µl of elution buffer/RNA free water was added, centrifuged at 8000 rpm for 1 min, discarded the filter column and Stored the filtrate containing RNA at -20°C until further use. Now the RNA was extracted and use for further procedure or freeze it – 20 when not use at this time.

Quantitative Detection of HCV RNA

Quantitative detection of HCV RNA in all patients was performed by SMART CYCLER II real-time PCR (Cepheid, USA) with an internal RNA standard derived from the 5´ UTR. 50ng of the extracted RNA was reverse transcribed into cDNA with Molony-murin leukemia virus reverse transcriptase enzyme (Gibco BRL, Life Technologies USA). The SMART CYCLER II system is a PCR system by which amplification and detection are accomplished concurrently with *Taq* Man technology (Applied Biosystems, Foster City, Calif) using fluorescent probes to detect amplification after each replicating cycle. This assay has lower and upper detection limits of 5.0×10^2 and 5.0×10^7 IU/mL, respectively. Specimens yielding values above the upper limit were routinely dilute 100-fold and retested and obtained values were multiplied by this dilution factor to obtain the actual HCV RNA concentration in international units per ml.

Statistical analysis & graphical representation

Statistical Analysis for the calculation of percentage and distribution of the samples in different groups, Microsoft Excel 2007 was used.

Results

A total 1500 liver disease patient sample were selected for the study. These samples were received at DHQ Hospital, Mardan from various geographic region of District Mardan, Khyber PuhktoonKhwa Mainly Takhtbhai, Mardan and Kattlang. All samples were diagnosed by ICT, ELISA and RT-PCR methods in each aim of the objective of the research. All samples were categorized into four age groups, gender wise and Tehsil wise distribution. Gender wise distributions 810 (54%) were male and 690 (46%) were female.

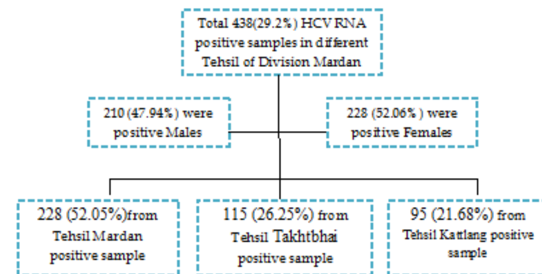

Fig. 1. Patients from various Tehsil of District Mardan, KPK having positive HCV infection and have detectable HCV RNA by qualitative q-PCR with enough viral load ≥500 IU/ml.

Sera were isolated from Blood sample and subsequently tested for the detection HCV infection by ICT, ELISA and q-PCR methods. Out of 1500 liver disease patient 438 were positive through q-PCR. In which 210 (47.94 %) were male and 228 (52.06 %) were female positive. Age group <20 out of 438 positive patients 29 (6.62 %) were positive, age group 21-40 positive sample were 104 (23.74 %), age group 41-60 positive sample were 210 (47.94 %) and age group >60 positive sample were 95 (21.68 %). Tehsil wise distribution Tehsil Mardan, Kattlang, Takhtbhai positive sample were 228 (52.5 %), 115 (26.25 %) and 95 (21.68 %) respectively.

Rate of HCV infection

HCV RNA was detected in 438 (29.2%) samples out of total 1500 samples of suspected liver disease patients via q-PCR. Out of 438 patients, 210 (47.94%) were male and 228 (52.06 %) were female patients. In age group 41-60 years there were 210 (47.94%) HCV RNA positive patients. In age group 21-40 years it was 104 (23.74%), in >60 years it was 95 (21.68%) and in <20 years 29 (6.62%) was observed. In Tehsil wise distribution it was noted that 228 (52.05%) HCV RNA positive samples were from Tehsil Mardan and 115 (26.25%) and 95 (21.68%) from Takhtbhai and Kattlang respectively.

Table 1. Gender wise distribution of HCV infection through q-PCR.

Gender	HCV RNA Detected	Percentage
Male	210	47.94
Female	228	52.06
Total	438	29.2

Table 2. Rate of HCV infection in different Tehsil of District Mardan.

Tehsil	HCV RNA Detected	Percentage
Mardan	228	52.05
Takhtbhai	115	26.25
Kattlang	95	21.68
Total	**438**	**29.2**

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Table 3. Age wise distribution of HCV infection in District Mardan.

Age wise	HCV RNA Detected	Percentage
<20	29	6.62
21-40	104	23.74
41-60	210	47.94
>60	95	21.68
Total	438	29.2

Discussion

Gaeta *et al.,* (1990) and Khokhar *et al.,* (2004) showed that Chronic hepatitis is due to different hepatic viruses is a common cause of liver related morbidity. Hepatitis B (HBV) and hepatitis C (HCV) are the main causes for chronic hepatitis. It could lead to many complications. Cirrhosis, liver failure and hepatocellular carcinoma (HCC) develop in 15-40% of patients. Hepatitis C virus infection is spreading very rapidly. About 170 million individual were infected by Hepatitis C virus and every year three to four million new individuals are infected (Re and Kostman, 2005; Kim, 2002). The socio-economic burden of HCV infection globally is increasing with an urgent necessity to have better information of viral pathogenesis in order to develop new anti-HCV strategies. In Pakistan lack of proper screening facilities or expertise in screening blood and blood products for possible HCV infection in public sector hospitals is partly contributing towards the spreading of the disease.

In the present study the rate of HCV infection in liver suspected disease patients in various Tehsil of the District Mardan were determined. The samples were tested for HCV infection through ICT, ELISA and q-PCR respectively.

Benani *et al.,* (1997) has been reported that HCV infection varies with age in both male and female patients, in male was more quickly infected as compared with female. Rate of HCV infection in gender wise in (table 2) show that total detected patient is 438. In which 210 (47.94%) were male and 228 (52.06%) were female HCV RNA positive. This study showed that HCV infection in female 228 (52.06 %) were high then male 210 (47.94%), because due to lack of proper screening facilities or expertise in screening blood and blood products for possible

HCV infection in public sector hospitals is partly contributing towards the spreading of the disease. and socio-economic state of the patients.

The aim of this study was to investigate the rate of HCV infection in people of the District Mardan. The rate of HCV in general and its frequency regarding age and sex in our study is comparable to other studies (Erden *et al.,* 2002; Muhammad and Jan, 2005). Talpur *et al.,* (2006) showed that higher incidence of hepatitis C in the age group above 41 year of age is comparable. Age wise distribution is shown in table 3. In age group 41-60 years was found the most susceptible group to HCV infection in this study. In order to establish a base line for regional differences in HCV infection in District Mardan, Tehsil wise rate were examined. Table 2 among the three Tehsil of District Mardan, the highest number of HCV infected patients was found in Tehsil Mardan with 228 (52.05%) patients.

Conclusion

This study concludes that the most specific, sensitive and reliable test use for detection of HCV is q-PCR. Female infectivity rate with HCV is relatively higher then male in District Mardan. Age group 41-60 years was found the most susceptible group to HCV infection in this study. Among the three Tehsil of District Mardan, the highest number of HCV infected patients was found in Tehsil Mardan with 228 (52.05%) patients.

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