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Detection of HCV RNA and NS5A Protein in Peripheral Blood Mononuclear Cells After Sustained Virological Response May Cause Viral Relapse

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Abstract.- Chronic hepatitis C infection is the main causative agents for public health problem. Hepatitis C virus (HCV) is generally considered as hepatotropic virus but it can also infect the peripheral blood mononuclear cells (PBMCs). The aim of present study was to detect HCV RNA and non-structural protein 5 (NS5A) in PBMCs of the patients who achieved sustained virological response (SVR) and to explore their role in relapse. We investigated HCV RNA of genotype 3a in PBMCs of SVR responders Pakistani patients. A total of 62 patients who achieved SVR treated with interferon and ribavirin for 24 weeks were included in the study. PBMCs from blood were isolated by ficoll gradient centrifugation method using Ficoll reagent. Viral RNA in PBMCs was detected by quantitative real time PCR and HCV NS5A protein in was detected by immunofluorescence assay. Seven (11.3%) patients were relapsers. A positive association between viral RNA in the PBMCs and relapse was observed. Relapse occurred significantly more often in patients with HCV RNA in their PBMCs at SVR stage than the patients who did not have the RNA (25 % Vs 4.8 %, P=*0.03* OR=6.66 CI%=1.16-38.13). HCV protein expression in PBMCs have showed the significant association with relapse (31.6% Vs 2.3%, OR=19.39; P=0.00). The study concludes that patients having HCV RNA and NS5A protein in PBMCs after achieving the SVR are more likely to go in relapse as compared to those negative for HCV RNA and protein in PBMCs.

Key words: SVR, HCV RNA, PBMCs, NS5A, qRT-PCR, immunofluorescence assay, relapse.

INTRODUCTION

Hepatitis C virus (HCV) is the major human pathogen responsible for acute and chronic infections and one of the serious global health problems. Approximately 200 million (3%) people are infected worldwide including about 10 million in Pakistan only (Waheed *et al*., 2009). Very high rate of HCV prevalence (17%) is present in Rawalpindi city (Satti *et al*.,2012). The rates of morbidity and mortality are also very high in chronic HCV infection. It is estimated that the cirrhosis rate will increase up to 30.5% than the current rate until 2030 (Davis *et al*., 2010). About 20-30% of chronic HCV patients who remain untreated for a long period of time become cirrhotic and about 2-3% become victims of hepatocellular carcinoma (Hoofnagle, 2002; Shepard *et al*., 2005). HCV infection is also one of the major causes of liver transplantation all over the world (Tang and Grise, 2009). Hepatitis C virus was first time discovered in 1989 (Choo *et al.*, 1989) and is an enveloped positive sense single stranded RNA virus (ssRNA). The virus belongs to family *Flaviviridae* (Agnello *et al*., 2002) and has six major genotypes numbered from 1 to 6 with several sub-genotypes (Messina *et al.*, 2015). The viral genome comprises of nearly 9600 nucleotides that encode a single open reading frame (ORF). This ORF consists of 3000 amino acids which produces only one large poly-protein. The poly-protein processing is carried out by various cellular and viral enzymes and produces both structural and nonstructural proteins. Structural proteins include core protein, envelope glycoproteins (E1 and E2) and an ion channel protein (p7). The outer envelope consists of both E1 and E2 proteins. The inner envelop surrounds the nucleocapsid which is composed of core protein containing RNA genome. The ORF also encodes

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nonstructural (NS) proteins such as NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Clarke, 1997).

Interferon-alpha (IFN-α) is a cytokine that has antiviral and immuno-modulatory activities. Persons infected with genotype 2 or 3 showed even better response (70-80 %) to antiviral treatment as compared to other genotypes. A few treatment options are available for HCV infected individuals that consists of antiviral therapeutic regimens i.e. standard interferon (IFN), pegylated IFN (peg IFN) or consensus IFN along with nucleoside analogue ribavirin (Hartwell and Shepherd, 2009). Standard interferon therapy (SIT) is carried out by injecting 3 million international units (MIU) of standard IFN three times a week for six months, whereas peg IFN regimen is administered by injecting 180 µg of peg IFN once a week for 6 months. Peg IFN therapy is considered more efficient because it contains PEG moiety that increases its half-life in the body and gives increased retention time for anti-viral action against HCV (Fargion *et al.*, 2004).

Peg IFN-α is a better option than conventional interferon for treatment of hepatitis C infection. Due to huge difference in cost, standard interferon combination therapy is still the preferred therapy for genotype 2 and 3, especially in under developed countries like Pakistan (Hamid *et al.*, 2004). Though HCV is considered to hepatotropic virus, some investigations suggest that the virus infects peripheral blood mononuclear cells (PBMCs) that might support to viral resistance in the course of anti-viral therapy (Cavalheiro *et al*., 2007). Re-treatment of non-responders with combination of Peg-IFN α-2b and ribavirin was found to improve the response rate of chronic HCV patients (Goncales *et al*., 2010). HCV RNA in PBMCs is also considered as the main cause of re-infection after liver transplantation. Early treatment of these patients can prevent or reduce the chances of re-infection (Powers *et al.*, 2006).

As no study on the topic is available from Pakistani patients infected predominantly with HCV genotype 3a, the present study was designed to detect HCV RNA and NS5A protein in PBMCs of hepatitis C responder patients and to find out their influence on relapse after one year of completion of therapy.

MATERIALS AND METHODS

Study population

The present study was conducted from August 2011 to July 2013 at Holy Family Hospital, Rawalpindi, Pakistan. A written informed consent was obtained from each patient and the study was approved by the Institutional ethics committee of PMAS-Arid Agriculture University, Rawalpindi. The data of patients were recorded using questionnaire. HCV genotype 3a infected patients were selected for the study. The patients infected with hepatitis B virus (HBV), hepatitis D virus (HDV), human immunodeficiency virus (HIV) or having chronic alcoholism were excluded from the study. Female with confirmed pregnancy were also excluded from the study.

Collection of blood samples

Three ml of whole blood in CP vacutainer was collected from all selected at the end of week 48 and 72. Plamsa and PBCMs were separated for detection of HCV RNA in PBMCs and plasma This study includes a total of 62 patients who achieved sustained virological response after receiving 3MU of interferon-α 2b thrice weekly with ribavirin (800-1200 mg daily) orally for 24 weeks. Average age of the patients was 34.8 ± 9.4 years, ranging from 18 to 55 years with 30 males and 32 females.

Plasma RNA extraction

The RNA was extracted from plasma following the protocol of instant virus RNA kit (AJ Roboscreen, Analytikjena, Germany).

HCV RNA quantification

Master Mix was prepared by combining 2 × reaction mixture (buffer containing reverse transcription- polymerase chain reaction, 6 mmol/L Mg sulfate and dNTPs), Mg-sulfate (50 mmol/L), 25 × reaction mixture (containing primers and probes) and RT-PCR enzyme mixture. Quantification of HCV RNA was carried out in Rotor-Gene 3000 (Corbett research) detection system using RoboGene HCV Quantification kit (AJ Roboscreen, Analytikjena, Germany). Thermal cycle program were used as hold 1, 59°C for 60

min, hold 2, 95°C for 120 min, 45 cycles for amplification 95°C for 15 s, 57°C for 60 s.

Separation of PBMCs

PBMCs from whole blood were separated by density gradient centrifugation following the method described by Panda *et al.* (2012) with some amendments. Three ml of Ficoll-histopaque solution was taken in tubes and equal volume of whole blood was layered on the top of Ficoll solution. The tubes were centrifuged at 400 × g (2200 rpm) for 30 min at 4ºC. After centrifugation PBMCs layer was carefully separated and washed with 10 ml of fresh 1X PBS (pH 7.4). The cells were washed three times and centrifuged at 2200 rpm for 10 min and the supernatant was discarded. Separated PBMCs were preserved in RPMI 1640 medium (Gibco) with FBS (HyClone) penicillin, streptomycin and gentamycin (PSG) and nonessential amino acids (Sigma). The cells were counted and cells viability was checked with the help of countess machine using trypan blue dye.

Total RNA extraction from PBMCs and qRT-PCR

Total RNA from PBMCs cell suspension was extracted following the protocol developed by Chomczynski and Sacchi (2006) using Tri-Reagent (Molecular Research Center, Cincinnati, OH) with some changes.

The following primers were designed for amplification of HCV 5' UTR by Oligo Primer D using the gene bank sequence (Accession No. D10749).

Forward (nt 98-116):
5' GAGTGTCGTGCAGCCTGGA 3',

Reverse (313-294):
5' CACTCGCAAGCACCCTATCA 3'
and Probe (nt238-267):

5' (FAM)
CCCGCAAGACTGCTAGCCGAGTAGTGTTGG
(TAMRA) 3'.

About 19 µl of master mix was prepared according to the kit protocol of TaqMan EZ RT-PCR Kit (Applied Biosystems, USA) for quantification of one µl of viral RNA in PBMCs. RNA was denatured at 50ºC for 1 min, reverse transcription was carried out at 60ºC for 50 min, cDNA synthesis at 95ºC for 5 min, denaturation at 94ºC for 15 s, annealing at 55ºC for 10 s and final extension was performed at 69ºC for 60 s. A total of 50 cycles was completed for the process (Zaman *et al*., 2014).

Detection of HCV NS5A proteins in PBMCs by using indirect immunofluorescence assay

PBMCs suspension volume was adjusted to 1×10^6 cells/ml with RPMI-1640 medium. HCV NS5A proteins were detected in PBMC by indirect immunofluorescence method described by Gong *et al.* (2003) with some changes. Briefly, PBMCs were fixed in 4% paraformaldehyde (PFA) at room temperature for 15 min and washed three times with wash buffer at 300× g (2500rpm). The fixed cells were incubated with HCV virus NS5A-specific primary antibodies (MAB8694 Merck Millipore), for 25 minutes, washed and incubated with secondary antibodies (Rhodamine wheat Germ agglutinin RL-1022, Vector laboratories) for 20 minutes. The cells were washed to remove unbound secondary antibodies and cell nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI). Cells were observed for immunofluorescence staining using a Zeiss LSM 510 laser confocal microscopy system.

Statistical analysis

The data were analyzed in SPSS 16.0 for windows (SPSS Inc. Chicago, IL).The results obtained were shown in the form of rate (%) and standard deviation (SD). Chi-Square ($\chi2$) test and Fischer's Exact test were used where appropriate. A P-value less than 0.05 was considered as statistically significant.

RESULTS

Relapse after one year of cessation of therapy

The baseline characteristics of the patients are presented in Table I. Out of the total 62 patients, 20 (32.3%) were positive for HCV RNA in PBMCs at SVR stage while 42 (67.7%) were negative. Relapse occurred in a total of 7 (11.3%) out of 62 patients. An association was found between viral RNA in the PBMCs at the stage of SVR and the incident of relapse. Significantly higher rate of relapse was

observed in patients with HCV RNA in the PBMCs at SVR stage than the patients who did not have HCV RNA in the PBMCs at SVR stage (P=*0.030)* as 25% of the patients with HCV RNA in PBMCs showed relapse compared with 4.8% of the patients who were negative for HCV RNA in PBMCs. The patients having viral RNA in PBMCs at SVR stage have 6.66 odds (95%CI= 1.16-38.13) more likely to go in relapse than the patients negative for viral RNA in PBMCs at the same stage (Table II).

Table I.- Baseline characteristics of treated patients (n=62).

Variable	Mean	Range	SD
Age (years)	34.84	18-55	9.4
Body weight (Kg)	68.56	57-78	5.5
Haemoglobin (g/dl)	13.6	9.3-18	2.0
TLC (count /mm^3)	7.5×10^3	4.4×10^3-11.4×10^3	1.6×10^3
Platelet(count/mm)	220.4×10^3	88×10^3-474×10^3	74.6×10^3
Bilirubin (mg/dl)	0.83	0.3-2.3	0.34
ALT (U/L)	111.8	13-444	80.8

HCV proteins detection

Blood samples of all 62 SVR responders were used for detection of HCV NS5A protein expression by using indirect immunofluorescence method. HCV NS5A signals were observed in positive PBMCs (Fig. 1). This protein was mostly localized to the cytoplasm of infected cells. Among 62 responder patients, 07 were relapsers after one year of treatment. Out of these 62 responders, HCV NS5A protein was detected in 19 patients while 43 patients were HCV NS5A protein negative in their PBMCs. Among 43 patients with negative protein, 42 (97.7%) showed response and 1 (2.3%) showed relapse. Total 19 patients were positive for HCV NS5A protein in PBMCs. Out of these 19 patients, 13 (68.4%) were responders while 6 (31.6%) were relapsers (Table III). A significant difference was observed in response achieved by both groups of HCV NS5A protein positive and negative in PBMCs (31.6% Vs 2.3%, OR=19.39, 95 % CI=2.13–176.08 P=0.000). These results indicate that PBMCs are the main extrahepatic reservoirs for HCV proteins translation.

Adverse side effects

The incidence of adverse effects was observed in some patients with interferon and ribavirin treatment. At initial stages, the rate of adverse effects was very high but it gradually decreased in later stages. Some patients discontinued treatment for some time because of these early side effects. The main adverse effects observed during the treatment were chills, 74%; fever, 90%; muscle pain, 80%; poor appetite, 50%; headache, 30%; joint pain, 40%; anemia, 11%; weakness, 45%; low mood, 27%; malaise, 6%; sleepiness, 3%; weight loss (<10 %), 4%; nausea, 40%; injection site reaction, 3%; aggression, 1%.

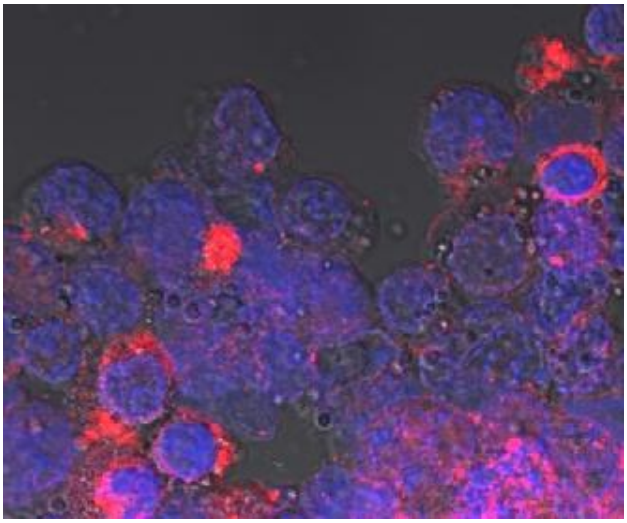

Fig.1. Detection of HCV NS5A protein infected PBMCs.
PBMCs sample used for the detection of HCV NS5A protein expressed in the cytoplasm of cells. NS5A has detected with by immunofluorescence. Red fluorescence signals indicating NS5A proteins in cells. Blue signal shows DAPI and it is used only for nuclei of cells. The cells were observed under Confocal Microscope with x63 objective.

DISCUSSION

Combination therapy of interferon alfa and oral ribavirin is given to chronic HCV patients in order to achieve SVR. Although liver cells are considered to be the main targets of HCV, research studies show that this virus can also replicate in other tissues or cells especially the immune cells

Table II.- HCV RNA detection in PBMCs at SVR stage is associated with relapse one year after treatment.

Category	Relapsers	Non-Relapsers	χ^2	Odds Ratio	95%CI	P-Value
HCV RNA Positive BMCs (n=20)	5 (25 %)	15(75 %)	5.54	6.66	1.16-38.13	0.03
HCV RNA negative BMCs (n=42)	2(4.8%)	40(95.2 %)				
Total (n=62)	7(11.3%)	55(88.7%)				

Table III.- Association between NS5A protein expression in patients PBMCs at SVR and relapse rate after 24 weeks.

Category	Non-Relapser	Relapsers	χ2	P –Value	Odds Ratio with CI (95%)
NS5A Negative in PBMCs (n=43)	42 (97.7 %)	1 (2.3 %)	11.25	0.00	19.39 (2.13 – 176.08)
NS5A Positive PBMCs (n=19)	13 (68.4 %)	6 (31.6 %)			
Total (n=62)	55	7			

(Radkowski *et al*., 2002). This study focused the presence of HCV RNA and NS5a protein in PBMCs of patients during SVR and their influence on relapse. As HCV genotype 3a is predominant in Pakistan (Attaullah *et al.* (2011), the study was confined only to the patients infected with genotype 3a.

In present study, 62 patients were observed to have negative PCR testing for HCV RNA in their plasma after the completion of course of interferon ribavirin based therapy. HCV RNA was still present in freshly isolated PBMCs of 20 (32.3%) of these patients. This result is very much similar to that reported by Gallegos-Orozco *et al.* 2008. They also found that HCV RNA was present in PBMCs of 20% of individuals with clinical SVR. They proposed that viral persistence especially, the presence of HCV RNA in PBMCs may lead viral reactivation under some conditions. It is concluded from both of the studies that the absence of HCV RNA in the serum of patients by the end of treatment does not mean that there is no circulating virus in body.

Presence of HCV RNA and proteins in PBMCs may be a sign of the persisting infection. Hanno *et al*. (2014) conducted similar study in 25 Egyptian patients infected with genotypes other than 3a. HCV RNA was found in 32% of PBMCs of infected patients. They also found that patients with HCV RNA in PBMCs after therapy had significantly higher relapse rate (50%) when compared with patients negative for HCV RNA in both PBMCs and serum after finishing therapy (6%). These results are in complete agreement with our study as we also found that the patients having HCV RNA in PBMCs have significantly higher rate of viral relapse.

A study was also conducted on HCV infection in PBMCs of Iranian patients. The results of this study supported the idea that PBMCs can act as an extrahepatic reservoir of HCV RNA. Furthermore, such cells may influence the antiviral therapy outcome (Alborzi *et al*., 2013). Our results also confirm these observations as this fact is also observed in our study. Another study concluded that the absence of HCV RNA in serum of patients could not eliminate the chances of viral relapse (Zayed *et al.*, 2010). Gong *et al*. (2003) reported that HCV has capability to infect and replicate in PBMCs. Very high SVR rate (95.2%) was observed in patients who tested negatively for HCV RNA in PBMCs and serum at the end of 48 weeks. This gives us hint to the role played by HCV in mononuclear cells on the treatment outcome. On other hand, patients who tested positively for viral RNA in PBMCs at end of 48 weeks had a higher relapse rate (25%) when compared with patients who tested negatively for viral in both serum and PBMCs at the end of 48 weeks of treatment (4.8%). So this report is also supporting our finding.

Relapse of HCV infection commonly occurs after IFN therapy. Many researchers suggested that cured patients after antiviral treatment with persistent HCV response may have occult HCV infection in liver or PBMCs and they also suggested that HCV can replicate within PBMCs (Castillo *et*

al., 2005). However, HCV RNA was not found in the PBMCs of cryptogenic liver disease patients so the concept of occult infection support by Halfon and his group (Halfon *et al*., 2008) was not supported by many studies. Other authors proposed that contamination of circulating HCV RNA in plasma or passive adsorption could be the cause of HCV RNA in such cells (Meire *et al.*, 2001).

Masalova *et al.* (2012) detected HCV related structural proteins (core) and nonstructural proteins (NS4A, NS3, and NS5A and NS4B) in PBMCs by immunocytochemical staining (ICS)and reported that active HCV replication of HCV occur in PBMCs and the accumulation of viral proteins lead to immunological disorders. In agreement with previous observations, other studies also found core protein and NS3 expression in PBMCs (Sansonno *et al*., 1996). The indirect immunofluorescence assay was found to be reliable for the expression and detection of NS5A protein in PBMCs. In the present study, the expression of NS5A protein was observed in cytoplasm of PBMCs (Fig. 1). These findings are in agreement with finding of Gong *et al*. (2003) who reported that HCV NS5a protein is present in PBMCs cytoplasm.

CONCLUSION

It is concluded from the present study that HCV RNA and expression of NS5A protein in PBMCs or other extra hepatic tissues may be the cause of viral persistence and relapse after achieving the SVR. The patients having NS5a protein in their PBMCs are at a greater risk of relapse as compared to those who did not have.

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Declaration of conflict of interest

There is no conflict of interest among the authors to declare.

REFERENCES

AGNELLO, V., MECUCCI, C. AND CASATO, M., 2002. Regression of splenic lymphoma after treatment of hepatitis C virus infection. *N. Engl. J. Med*., **347**: 2168-2170.

ALBORZI, A., BAMDAD, T., GHADERI, M., SALIMI, H., DAVOODIAN, P., MERAT, S., HOSSAINPOR, M., JABBARI, H., SHARIFI, A. H., SHEYKHOLESLAMI, A., POURHOSSEIN, B. AND FOROUHAR-KALKHORAN, B., 2013. Comparison of HCV plus- and minus-strand RNA in PBMCs of responders and non-responders of chronically infected patients receiving ribavirin and interferon therapy. *Iranian J. Virol*., **6**: 18-25.

ATTAULLAH, S., KHAN, S. AND ALI, I., 2011. Hepatitis C virus genotypes in Pakistan: a systemic review. *Virol. J*., **8**: 433.

CASTILLO, I., RODRIGUEZ-INIGO, E., BARTOLOME, J., DE LUCAS, S., ORTIZ-MOVILLA, N., LOPEZ-ALCOROCHO, J. M., PARDO, M. AND CARRENO, V., 2005. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. *Gut*, **54**: 682-685.

CAVALHEIRO, N. P., FILGUEIRAS, T. C., MELO, C. E., MORIMITSU, S. R., ARAUJO, E. S. AND TENGAN, F. M., 2007. Detection of HCV by PCR in serum and PBMC of patients with hepatitis C after treatment. *Braz. J. Infect. Dis*., **11**: 471-474.

CHOMCZYNSKI, P. AND SACCHI, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat. Protoc*., **1**: 581-585.

CHOO, Q. L., KUO, G., WEINER, A. J., OVERBY, L. R., BRADLEY, D. W. AND HOUGHTON, M., 1989. Isolation of cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science,* **244**: 359-362.

CLARKE, B., 1997. Molecular virology of hepatitis C virus. *J. Gen. Virol.,* **78**: 2397-2410.

DAVIS, G. L., ALTER, M. J., EL–SERAG, H., POYNARD, T. AND JENNINGS, L. W., 2010. Aging of hepatitis C virus (HCV)-infected persons in the United States: a multiple cohort model of HCV prevalence and disease progression. *Gastroenterology,* **138**: 513-521.

FARGION, S., FRACANZANI, A. L. AND VALENTI, L., 2004. Treatment choices for people infected with HCV *J. Antimicrob. Chemother*., **53**: 708-712.

GALLEGOS-OROZCO, J. F., RAKELA, J., ROSATI, M. J., VARGAS, H. E. AND BALAN, V., 2008. Persistence of hepatitis C virus in peripheral blood mononuclear cells of sustained viral responders to pegylated interferon and ribavirin therapy. *Dig. Dis. Sci*., **53**: 2564-2568.

GONCALES, F.L., MOMA, C.A., VIGANI, A.G.,

ANGERAMI, A.F.C.F., GONCALES, E.S.L., TOZZO, R., PAVAN, M. H. P. N. AND GONCALES, S. L., 2010. Retreatment of hepatitis C patients with pegylated interferon combined with ribavirin in non-responders to interferon plus ribavirin. Is it different in real life?. *BMC. Infect. Dis.*, **10**: 212.

GONG, G. Z., LAI, L. Y., JIANG, Y. F., HE, Y. AND SU, X. S., 2003. HCV replication in PBMC and its influence on interferon therapy. *World. J. Gastroenterol*., **9**: 291-294.

HALFON, P., BOURLIE, R. E. M., OUZAN, D., SENE, D., SAADOUN, D., KHIRI, V., PÉNARANDA, G., MARTINEAU, A., OULÈS, V. AND CACOUB, P., 2008. Occult hepatitis C virus infection revisited with ultrasensitive real-time PCR assay. *J. clin. Microbiol.*, **46**: 2106-2108.

HAMID, S., UMAR, M., ALAM, A., SIDDIQUI, A., QURESHI, H. AND BUTT, J., 2004. Pakistan Society of Gastroenterology (PSG) consensus statement on management of hepatitis C virus infection-2003. *J. Pak. med. Assoc.*, **54**:146-150.

HANNO, A. F. F., MOHIEDEEN, K. M., ALSHAYEB, A. F. AND DEGHEDY, A., 2014. HCV RNA in peripheral blood mononuclear cells (PBMCs) as a predictor of the response to antiviral therapy in chronic hepatitis C. *Alex. J. Med*. **50**: 317-322.

HARTWELL, D. AND SHEPHERD, J., 2009. Pegylated and non-pegylated interferon-alfa and ribavirin for the treatment of mild chronic hepatitis C: A systematic review and meta-analysis. *Int. J. Technol. Assess*., **25**: 56-62.

HOOFNAGLE, J., 2002. Course and outcome of hepatitis C. *Hepatology*, **36**: 21-29.

MASALOVA, O. V., VISHNEVSKAYA, T. V., PICHUGIN, A. V., ALKHOVSKY, S. V., SHKURKO, T. V., ATAULLAKHANOV, R. I. AND KUSHCH, A. A., 2012. Detection of hepatitis C virus proteins in peripheral blood mononuclear cells of patients with chronic hepatitis C: correlation with activity and stage of hepatitis. In: *Virology - diagnosis, epidemiology, prophylaxis and therapy*. 22nd (ECCMID) 31.03.2012 - 03.04.2012

MEIRE, V., MIHM, S., BRAN, P. W. AND RAMADORI, G., 2001. HCV-RNA positivity in peripheral blood mononuclear cells of patients with chronic HCV infection: does it really mean viral replication?. *World. J. Gastroenterol.*, **7:** 228-234.

MESSINA, J. P., HUMPREYS, I., FLAXMAN, A., BROWN, A., COOKE, G. S., PYBUS, O. G. AND BARNES, E., 2015. Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology*, **61**: 77-87.

PANDA, S.K., KUMAR, S., TUPPERWAR, N.C., VAIDYA, T., GEORGE, A., RATH, S., BAL, V. AND RAVINDRAN, B., 2012. Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks endotoxemia. *PLoS Pathog*., **8**: 1-17.

POWERS, K.A., RIBEIRO, R. M., PATEL, K., PIANKO, S., NYBERG, L., POCKROS, P., CONRAD, A.J., MCHUTCHISON, J. AND PERELSON, A. S., 2006. Kinetics of hepatitis C virus reinfection after liver transplantation. *Liver Transplant*., **12**: 207-216.

RADKOWSKI, M., WILKINSON, J., NOWICKI, M., ADAIR, D., VARGAS, H., INGUI, C., RAKELA, J. AND LASKUS, 2002. Search for hepatitis C virus negative-strand RNA sequences and analysis of viral sequences in the central nervous system: evidence of replication. *J. Virol.*, **76**: 600-608.

SANSONNO, D., IACOBELLI, A. R., CORNACCHIULO, V., IODICE, G. AND DAMMACCO, F., 1996. Detection of hepatitis C virus (HCV) proteins by immunofluorescence and HCV RNA genomic sequences by non-isotopic *in situ* hybridization in bone marrow and peripheral blood mononuclear cells of chronically HCV-infected patients. *Clin. exp. Immunol.*, **103**: 414-421.

SATTI, R., MUSTAFA, F., KHAN, M. I., HAQ, T. S., KHAN, Z. U., ZUBAIR M., RASOOL, S.T., AZAM, M., AJMAL, M. AND QAMAR, R., 2012. Prevalence of hepatitis C virus in urban Ghettos of twin cities. *Pakistan J. Zool*., **44**: 937-943.

SHEPARD, C. W., FINELLI, L. AND ALTER, M. J., 2005. Global epidemiology of hepatitis C virus infection. *Lancet Infect. Dis.*, **5**: 558-567.

TANG, H. AND GRISE, H., 2009. Cellular and molecular biology of HCV infection and hepatitis. *Clin. Sci.*, **117**: 49-65.

WAHEED, Y., SHAFI, T., ZAMAN, S. AND QADRI, I., 2009. Hepatitis C virus in Pakistan A systematic review of prevalence, genotypes and risk factors. *World J. Gastroenterol.*, **15**: 5647-5653.

ZAMAN, N., ASAD, M. J., RAZA, A., RAJA, G. K., AKHTER, S., MAHMOOD, M. AND MAHMOOD, R. T., 2014. Presence of HCV RNA in peripheral blood mononuclear cells may predict patients' response to interferon and ribavirin therapy. *Ann. Saud. Med*., **34**: 401-406.

ZAYED, R. A., RUSHDY, E. AND SALEH, D. A., 2010. Detection of HCV RNA in the peripheral blood mononuclear cells of serum HCV RNA-negative Egyptian patients under interferon treatment. *Am. J. med. Sci*., **340**: 435-438.

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