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Comparison of third generation ELISA and conventional nested RT-PCR for detection of HCV among hemodialysis patients

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ABSTRACT

Hepatitis C virus is one of the main cause of chronic hepatitis in developing countries. The current study was done to evaluate the efficacy of the third generation ELISA compared to nested RT- PCR for establishing the diagnosis of hepatitis C virus (HCV) in patients on hemodialysis. This descriptive, cross-sectional study was carried out on 237 Hemodialysis patients in Lucknow, Uttar Pradesh. The retrospective demographic data of the subjects was collected and the patient's serum samples were analyzed by ELISA & RT-PCR for HCV. In the present study, of total 21 HCV positive either by ELISA or PCR 12 (57.14%) were positive for both RT-nested PCR and ELISA. Total four (19.05%) patients were positive for HCV by RT-nested PCR and negative by ELISA while five (23.81%) patients were negative for RT-nested PCR and positive for ELISA. PCR method is accredited as a specific and reliable method suitable for screening of HCV and is recommended for establishing exact and final diagnosis of these patients. However third generation ELISA assays have many advantages in the diagnostic setting including ease of automation, ease of use, relative cost-effectiveness, and low variability.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, with an estimated global prevalence of 3%, occurring in about 180 million carriers and approximately 4 million people are newly infected annually (Umar *et al.,* 2010). The prevalence of HCV infection among dialysis patients is generally much higher than healthy blood donors (Al-Jamal *et al.*, 2009) and the general population (Hosseini *et al.,* 2006). Studies held in dialysis centers from different countries revealed that HCV prevalence ranges from 1-84.6% (Al-Jamal *et al.,* 2009; Rahnavardi *et al.,* 2008). HCV is of special concern in patients undergoing hemodialysis (HD) because chronic infection causes significant morbidity and mortality among these patients (Rahnavardi *et al.,* 2008). HCV infection was more frequent at dialysis centers with higher anti-HCV prevalence and failure in infection control measures. In some developing countries a rapidly growing HD population is treated using limited resources available thereby increasing the chances of nosocomial transmission, as indicated by high HCV prevalence and incidence. (Alavian *et al.*, 2009).

Laboratory assays that are available for the diagnosis and management of HCV infection includes, serological tests to detect HCV antibodies [enzyme-linked immunosorbent assay (ELISA)], molecular tests to detect and quantitate HCV RNA, and genotyping (Tashkandy *et al.*, 2007; Ali *et al.*, 2010). Serological assays for detecting anti-HCV antibody cannot distinguish between patients with active infection and those who have cleared the virus. Due to the absence of an efficient in vitro culture system for HCV or assays capable of detecting viral antigens, direct detection of HCV has depended on nucleic acid amplification technology (NAT) techniques (Fabrizi *et al.*, 2005). NAT tests are based on nucleic acid amplification (PCR and transcription-mediated amplification) and are currently used to detect viremia (Berry *et al.*, 2005). Among immunocompromised populations (e.g., hemodialysis patients) average rate of false-positive result is approximately 15 %.

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False positive anti-HCV results obtained from enzyme immuno assay (EIA) can be explained by the fact that till date no structural antigens and proteins have been derived from HCV (Wu *et al.*, 2008).

The purpose of this study was to compare the efficacy of third generation ELISA and reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) for the diagnosis of hepatitis C virus in patients on hemodialysis.

METHODS

A total of 237 HD patients were enrolled from two of the largest nephrology and dialysis units in Lucknow, Uttar Pradesh; the dialysis unit of the Department of Internal Medicine, King George's Medical University and the dialysis unit of the Department of Nephrology, Vivekanand Polyclinic. All patients were briefed about the study and proper informed consent was signed. All patients were interviewed for demographic data and risk factors to HCV infections including history of number of blood transfusion, intravenous drug use (IDU), surgical interventions, dental treatment, multiple sexual partners, exposure to known HCV-positive persons, number of years on dialysis and change of the dialysis center. Blood samples (3 ml) were collected from HD patients; sera was separated in two aliquots and frozen at -70°C for HCV ELISA and HCV RNA detection.

Antibodies Screening

All the subjects were screened for anti HCV antibodies by third generation enzyme linked immunosorbent assay (ELISA) test according to the manufacturer's instructions (ERBA Transasia, India). The kit has a sensitivity of 100% and specificity of ≥99% according to manufacturer.

RNA extraction

HCV RNA was extracted from 200µl of patient's serum using viral total nucleic acid extraction kit as per manufacturer's instructions (Invitrogen, Carlsbad, California, USA). The eluted RNA was stored at -70°C until use.

cDNA synthesis & Nested PCR for 5'NC region

c-DNA was transcribed using cDNA synthesis kit (Applied Biosystems, Foster City, California, USA) using random primers. Reverse transcription process was carried out using manufacturer's instructions at 37 ^{o}C for 2 h. Direct PCR was performed with the cDNA in the reaction mixture containing PCR buffer with MgCl2 (10 X) 2.5µl, 100 µmol/µl dNTPs, 1 U Taq DNA polymerase (Finzymes, Thermo), and 10 pmol primers (IDT, USA) outer primer pair for 5'-NC region, and 5µl of cDNA in a total reaction volume of 25 µl. The primers were selected from most conserved region of HCV having least variability among types and subtypes of HCV i.e. 5' NC region. The outer primer (round one) sequence was sense ACTGTCTTCACGCAGAAAGCGTCTAGCCAT (nucleotides 285 to 256) & antisense CGAGACCTCCCGGGGCACT CGCAAGCACCC (nucleotides 14 to 43). The inner primer were sense ACGCAGAAAGCGTCTAGCCATGGCGTTAGT (nucleotides 276 to 247) & antisense TCCCGGGGCACTCGCAAGCACCCTATCAGG (nucleotides 21to 50) (Bukh *et al.*, 1992). Nested PCR was performed in the similar reaction mixture except outer primer inner primer (round 2) were used and 0.3 µl of first round PCR product was used as template, in a total reaction volume of 25 µl. The 1st and 2nd rounds of PCR were composed of the following conditions: 94 ^{o}C for 5 min followed by 35 cycles at 94 ^{o}C (denaturation), 54.1 ^{o}C & 51.9 ^{o}C (annealing) for round one and round two respectively, 72 ^{o}C (elongation), each for 60 sec, with final extension at 72 ^{o}C for 7 min. The amplified PCR product was electrophoresed in ethidium bromide-stained 2 % agarose gel (Sigma-Aldrich, USA) and visualized in a Gel-Doc System (Alpha Innotech, San Leandro, USA) for identifying desired 256 bp fragment using molecular weight marker (Fig. 1). Positive and negative controls were also included.

Statistical Analysis

Statistical Analysis was done using Graph Pad version 3. P value < 0.05 was considered statistically significant.

RESULT AND DISCUSSION

Among 237 samples collected from hemodialysis patients, 153(64.56%) were males and the remaining 84 (35.44%) were females. The age group varied from 13 to 83 years (mean age: 45.72 years, SD: 15.43 years). The number of hemodialysis sittings received by these patients ranged from 12 to 87 per patient. Biochemical laboratory tests were significantly higher in hepatitis C patients when compared with non infected patients. Aspartate transaminase (AST) and Alkaline Phosphatase (ALP) levels were significantly higher in HCV positive patients ($p< 0.05$). Positive history of blood transfusion (receiving >10 blood units) was found to be a significant ($p<0.05$) risk factor in patients infected with HCV. The average number of blood transfusion units received was 13 (Standard deviation: ±9). Duration of dialysis was significantly associated with risk of the infection (Table 1).

Significant difference was detected between the study population and the two techniques of the study. Of total 237 patients, 21 were positive for HCV either by ELISA or nested PCR. In the present study, 57.14% (n=12) of HD patients were positive for HCV, both by RT-nested PCR and ELISA. However, of total positive individuals 19.05% (n=4) patients were positive for HCV by RT-nested PCR and negative by ELISA, while 23.81% (n=5) cases of total positives were positive for HCV by ELISA but not by PCR (table 2). The specificity and sensitivity of third generation ELISA as compared to RT-nested PCR for the detection of HCV was 97.78% and 80% respectively. The positive predictive value (PPV) was 76.19% while negative predictive value was 98.21% for ELISA. Hepatitis C virus (HCV) infection has reached an epidemic proportion globally and is associated with many extra hepatic manifestations. The prevalence of anti-HCV

positivity among dialysis patients varies in different countries (5%-85% worldwide), but may exceed 95% in some hemodialysis units (Al Traif *et al.,* 2000). Hepatitis is considered as one of the important causes of death in chronic renal failure patients undergoing hemodialysis or renal transplantation. A variety of diagnostic tests in serum samples have been developed since the identification and molecular characterization of hepatitis C virus in 1989 (Kuo *et al.,* 1989). Documentation of past infection and identification of infecting HCV genotype is necessary for clinical and therapeutic decisions for HCV infection. (Alberti *et al.,* 2002). Mainly two diagnostic blood tests are available for HCV infection; the antibody tests (ELISA) and the polymerase chain reaction for RNA detection (Prince *et al.,* 1997) that aid in decision making. The presence of HCV antibodies suggests prior exposure to HCV while PCR-RNA detection indicates that the patient has detectable levels of HCV in his blood (Australian-Hepatitis Council., 1999).

The three generations of serodiagnostic anti-HCV antibody tests are available in the market, with each new generation providing incremental improvements in the sensitivity to anti-HCV antibodies. The third-generation ELISAs can detect antibodies to four recombinant HCV proteins, and it was claimed to be more specific than their predecessors (Wu *et al.,* 2008). Recently, a variety of in-house PCR assays to test for the presence of HCV RNA in serum are available. Direct molecular qualitative detection of HCV RNA by reverse transcription (RT) and PCR is considered as the gold standard by many researchers for the diagnosis of HCV infection (Ayesh *et al.,* 2009; WHO. 2002). The current study was carried out to compare between the two techniques (i.e., ELISA and RT-nested PCR) for the better diagnosis of HCV infection. The findings indicate that both methods are satisfactory for the diagnosis of HCV infection in clinical laboratories though PCR is better. In this study, a small number of cases falsely negative by ELISA, were detected using RT-nested PCR (4, 1.81%). This indicates that PCR-based assays are able to ascertain minute amounts of HCV RNA in serum, as previously reported (Tashkandy *et al.,* 2007). PCR specially helps to resolve weakly positive ELISA results in presence of HCV infection compatible clinical signs and/or risk factors. However, the results of the two techniques should be interpreted with caution as, detection of HCV RNA usually precedes the detection of antibody reactivity in serum after acute exposure.; HCV RNA can be identified as early as 2 weeks following exposure, whereas anti-HCV antibodies are generally not detected before 8–12 weeks (Ghany *et al.,* 2009). Conversely, during the course of infection when the virus is cleaned up, only the antibody remains positive, and the nucleic acids are generally not detected. Hence, the detection rate of PCR was lower when ELISA was used as a gold standard (Wang *et al.,* 2004). In the present study, 57.14% of HCV patients were positive for HCV by both RT-nested PCR and ELISA indicating that the HCV infection is acute or chronic depending on the clinical context. In 19.05% of HCV samples, results were positive by RT-nested PCR and negative by ELISA; this might indicate early acute HCV infection, chronic HCV in chronic immunosuppressed patients or false positive HCV RNA test. Negative RT-nested PCR results and positive ELISA was reported in five (23.81%) HCV cases (1.3%) which might indicate the resolution of HCV, acute HCV during the period of low-viremia, or false anti-HCV positive.

In a study performed by Baheti *et al.,* in 2000, the seroprevalence of anti-HCV antibodies was studied among healthy blood donors and high-risk individuals. They found that, out of 99 health-care workers from various departments, a total of 9.09% were positive for anti HCV antibody. They included dental surgeons (20%), nursing staff (12.9%), surgeons (11.1%) and physicians (4.7%), who were infected in descending order of frequency. They did not find any infection in pathologists and laboratory workers. The prevalence of anti-HCV antibodies in patients on HD was 38.09% and among healthy blood donors was 2.4%. These values suggest that there is relatively low prevalence of HCV infectivity in Lucknow among HD patients.

The sensitivity and the specificity of ELISA in the current study were 80% and 97.78%, respectively; these are good enough for a diagnostic assay. Similarly, the specificity of RTPCR was absolute at high sensitivity (100%) indicating that it is not only suitable for clinical diagnosis but also suitable for the screening of HCV to prevent the transmission of this disease.

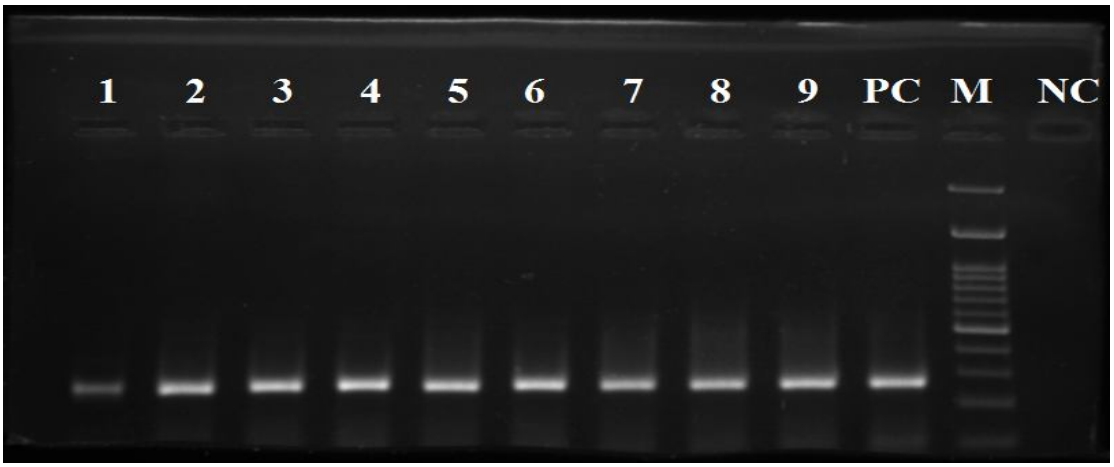

Fig. 1: Agarose gel electrophoresis patterns of reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) products of hepatitis C patients. Lane 1 to 9 HCV positive samples (256 bp): Lane 10 positive control (PC): Lane 11 DNA size marker 100 bp; Lane 12 Negative control (NC).

Table. 1: Comparison of demographic features and risk factors in patients on hemodialysis with and without HCV Infection.

	HCV infected cases (n=21)	HCV non-infected cases (n=216)	p value
Male 153 (64.56%)	17	136	0.15
Female 84 (35.44%)	4	80	
Age (in yrs)			
<14 (n=1)	0	1	0.41
≥14-60 (n=193)	15	178	
>60 (n=43)	6	37	
Total serum Bilirubin (mean±SD)	0.41±0.23 mg/dl	0.47±0.46 mg/dl	0.55
Serum Alkaline Phosphatase (mean±SD)	365±185.1 IU/L	166.55±133.69 IU/L	<0.0001*
Serum Aspartate transaminase (mean±SD)	54.3±45.2 IU/L	33.67±24.85 IU/L	<0.001*
Serum Alanine transaminase (mean±SD)	42±58.59 IU/L	34.65±28.67 IU/L	0.32
Duration of dialysis in months (mean±SD)	17±8	12±7.53	0.004*
Positive history of blood transfusion (n=179)	18	161	0.31
History of ≥10 units of blood transfusion received (n=45)	16/18	29/161	<0.0001*

*significant

Table. 2: Percentage of false negativity & positivity of HCV-Antibody ELISA test compared with nested RT-PCR.

	Number of ELISA Negative	PCR Positive cases from Negative ELISA		Number of ELISA Positive	PCR Negative cases from Positive ELISA
Total Patients n=237	220	4	Total Patients n=237	17	5
% false negativity of ELISA		1.81%	% false positivity of ELISA		29.41%
Negative Predictive Value		98.21%	Positive Predictive Value		76.19%

CONCLUSION

In conclusion, ELISA assays have many advantages in the diagnostic setting including ease of automation, ease of use, relative cost-effectiveness, and low variability. However, as with all enzyme immunoassays, false-positive results are occasionally a problem with third generation ELISA, additional or confirmatory testing like RT-nested PCR is often helpful. Moreover, further studies are recommended to study hepatitis C genotypes to facilitate improved clinical outcomes and epidemiologic studies and to provide information that has major implications for clinical management of hepatitis C and possible HCV vaccine development.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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