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Comparison of HCV TRI-DOT with RNA PCR for confirmation of ELISA positive results and evaluation of a combination of ELISA & HCV TRI-DOT assay for diagnosis of HCV infection

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Abstract: *Hepatitis C virus, a flavivirus, undergoes frequent mutation and establishes chronic infection in majority of the patients. Anti HCV antibody appears during acute infection but may be delayed by 3-6 weeks in some patients. ELISA for detection of anti HCV antibody may show false positive results. The study was undertaken to compare a 4th generation Rapid antibody detection test with RNA PCR for confirming ELISA positive results. The study finds that a combination of ELISA with 4th generation Rapid antibody detection test achieves specificity as high as HCV RNA detection and sensitivity higher than RNA PCR.*

Keywords: *HCV infection, HCV diagnosis, 3rd generation ELISA, HCV TRI-DOT*

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I. Introduction

Hepatitis C virus, a flavivirus is a major cause of post-transfusion hepatitis. The virus encodes in its single stranded RNA genome four structural proteins and six non structural proteins. The Nucleocapsid core protein C is highly conserved among genotypes but the envelope proteins , against which neutralising antibodies are produced, are encoded by hypervariable region. Frequent mutations in this region cause structural changes in the envelope proteins, helping the virus to escape the effects of neutralising antibodies. The high mutation rate of HCV has led to emergence of six genotypes and more than 50 subtypes.[1,2,3] Mutation of the virus within a single host results in formation of quasispecies, a reason why the virus can cause persistent infection in many of the infected patients.

Diagnosis of HCV infection depends on demonstration of Anti HCV antibodies or HCV RNA in the serum of the patients. Anti HCV antibodies can be found in 50-70% of the patients during acute disease, however appearance of antibody may be delayed in others by 3-6 weeks.[2] Antibodies develop against the core, envelope, NS3 and NS4 proteins but are relatively low in titer.[1,2,3,4,5]

The first generation ELISA for detection of anti HCV antibody incorporated the recombinant c100-3 epitope from the NS4 region, were replaced by second generation assays, which incorporated additional epitopes c22-3 and c33c from the HCV core and NS3 regions. The third generation assays contained core and NS3 antigens and incorporated a new antigen from the NS5 region. [6,7,8] Moreover antibody detection tests may give false positive results in persons with circulating rheumatoid factor, in autoimmune hepatitis and in hyperglobulinemia, especially in persons with a low prior probability of infection.[7,8]

In our institution we used a third generation ELISA which uses peptide from core, NS3, NS4 and NS5 regions for anti HCV antibody detection, which we found to be associated with high false positive results, and needed RNA-PCR for confirmation and involved a high cost burden for the patient. For these reason we undertook this study for comparing a 4th Generation antibody detection test using multiple modified HCV antigens with RNA-PCR for confirmation of ELISA positive cases.

II. Aims And Objectives

The study was undertaken for

1. Comparing a 4th generation antibody detection test with RNA-PCR which is a gold standard test for HCV diagnosis.
2. To find out the frequency of false positive results in the 3rd generation ELISA.
3. To formulate a strategy for confirmation of HCV positive results by ELISA & TRI-DOT on a cost effective manner.

III. Materials And Methods

In this study a number of 64 patients who attended the in-patient and out-patient departments of Medical College, Kolkata between February, 2018 and November, 2018, and were tested positive by Anti HCV antibodies by ELISA were included after proper written consent. For control group 39 ELISA negative patients were also included for comparison.

Study Area: Department of Microbiology, Medical College, Kolkata and ICMR Virus Unit, NICED, Kolkata.

Inclusion Criteria: Patients attending in-patient and out-patient departments of Medical College, Kolkata between February, 2018 and November, 2018 for Anti HCV antibody detection.

Exclusion Criteria: Patient infected with other hepatitis viruses like HBV, HAV or HEV.

ELISA test

All samples were previously tested by a commercially available 3rd generation ELISA, HEPA-SCAN HCV ELISA, from Bhat-Biotech, using manufacturer's instruction and internal quality control sera. Before interpretation of ELISA results the quality control sera was plotted in Levey-Jenning's chart and validated.

HCV TRI-DOT test

The samples were then tested using a 4th generation rapid antibody detection test, HCV TRI-DOT test from J. Mitra & Co, again following manufacturer's instruction and quality control sera.

RNA PCR

The RNA-PCR was performed in collaboration with Virology unit, NICED, Kolkata using the ABI 7500 from Applied Biosystems. Viral RNA was converted to a complimentary DNA copy (cDNA) prior to enzymatic DNA amplification by the use of reverse transcriptase. Reverse Transcription was carried out in thermocycler at 42°C for 1 hour. Subsequent Taq polymerase amplification was performed on the cDNA transcripts using primer set 1. 5′-TCCACCATGAATCACTCCC-3′ and 2. 5′-CGGAACCGGTGAGTACACC-3′.[9] The reactions were allowed to proceed with 94°C for 2 minutes for initial denaturation followed by 35 cycles of denaturation (94°C for 30 sec), primer annealing (50°C for 30 sec), and primer extension (72°C for 1min) along with final extension (72°C for 10 min).

The data was analysed by GraphPad online and SPSS software.

Results and Analysis:

A total of 103 samples were tested by ELISA, Tri-Dot and RNA-PCR.

The age wise distribution of the patients is given in table-1.

Table 1: Distribution of patients according to age (n=103)

Age	Male		Female		Total	
	No.	%	No.	%	No.	%
≤10	4	3.9	6	5.8	10	9.7
11-20	12	11.7	8	7.8	20	19.4
21-30	4	3.9	2	1.9	6	5.8
31-40	4	3.9	8	7.8	12	11.7
41-50	16	15.5	7	6.8	23	22.3
51-60	15	14.6	5	4.9	20	19.4
>60	6	5.8	6	5.8	12	11.7
Total	**61**	**59.2**	**42**	**40.8**	**103**	**100**

Among the selected patients 25 patients had thalassemia and received blood transfusion several times in the past. A total 42 patients had received blood transfusion in the past for various reasons. Previous history of surgical procedures was found in 27 patients. However we had not been include any patient who is intravenous drug user.

The Sex wise distribution of patients is given in Table-2.

Table 2: Distribution of patients according to sex (n=103)

Sex	Number	Percentage
Male	61	59.2
Female	42	40.8
Total	**103**	**100.0**

The risk factor wise distribution of patients is given in table-3

Table 3: Distribution of patients according to risk factor (n=103)

Risk factor	Present		Absent	
Thalassemia	**25**	**24.3%**	**78**	**75.7%**
Blood transfusion	**42**	**40.8%**	**61**	**59.2%**
H/o- Surgery	**27**	**26.2%**	**76**	**73.8%**

Among the 64 patients who were ELISA positive, 31 were found to be TRI-DOT positive also and the rest were negative. Among the 64 patients HCV RNA could be detected only in 24 patients.

Among the 31 patients who were both ELISA and TRI-DOT positive HCV RNA could be detected only in 21 patients and in 10 patients RNA could not be detected. However among the 33 patients who were ELISA positive but TRI-DOT negative HCV RNA could be detected only in 3 patients and in the rest (n=30), RNA could not be detected.

Among the control group of 39 ELISA negative patients, none were found to be positive by TRI-DOT, and in none of them HCV RNA could be detected. Considering the high specificity of 4th generation Rapid kit and RNA PCR all ELISA positive cases who were reactive either in the TRI DOT or where RNA could be detected, were considered to be infected with HCV (true positive). ELISA positive cases where antibody or RNA could not be detected by either TRI-DOT or RNA PCR were considered not harbouring the virus.

Among the Thalassemia patients (n=25), 24 patients were positive by ELISA and the rest are negative (n=1). Among these ELISA positive patients (n=24), 19(n=19) tested positive by TRI-DOT, and 5 (n=5) were negative.

Table 4: Comparison between ELISA, TRI-DOT & RNA-PCR

Type of test	PCR Positive		PCR Negative		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**
ELISA & TRI-DOT Positive	21	87.5	10	12.66	31	30.10
ELISA positive TRI-DOT Negative	3	12.5	30	37.97	33	32.04
ELISA Negative TRI-DOT Positive	0	0	0	0.00	0	0.00
ELISA & TRI-DOT Negative	0	0	39	49.37	39	37.86
Total	**24**	**100**	**79**	**100.00**	**103**	**100.00**

Among the thalassemia patients HCV RNA could be detected only in 13 patients, all of whom belong to those group of patients (n=19) where anti HCV antibody could be detected by both ELISA and TRI-DOT, and in none of the patients (n=5) who were ELISA positive and TRI-DOT negative, RNA could be detected. In 6 patients (n=6) RNA could not be detected in spite of them being both ELISA and TRI-DOT positive.

Table 5: Comparison table between ELISA, ICT & PCR among thalassemia positive patients

	PCR Positive		PCR Negative		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**
ELISA & TRI-DOT Positive	13	100	6	54.55	19	79.17
ELISA POSITIVE TRI-DOT Negative	0	0	5	45.45	5	20.83
ELISA NEGATIVE TRI-DOT Positive	0	0	0	0.00	0	0.00
ELISA & TRI-DOT Negative	0		0	0.00	0	0.00
Total	**13**	**100**	**11**	**100.00**	**24**	**100.00**
Pvalue	Chi-Square – 19.068, df = 1, P value = 0.000 (**Significant**)		Chi-Square – 49.008, df = 1, P value = 0.000 (**Significant**)			

IV. Discussion

Analysing the sera of 64 ELISA positive cases with a 4th generation rapid TRI-DOT test and RNA-PCR, it was found that the specificity of the 3rd generation ELISA under evaluation was merely 56.52% with a high number of false positive results. The positive predictive value for ELISA was 53.12% and negative predictive value is 100%. The low specificity may be due to poor quality of ELISA kit that was being used at the time of the study as in some studies the false positivity of 3rd generation EIA was found to be as low as 0.01%.[10] On the other hand the specificity of the 4th generation rapid antibody detection test (TRI-DOT) and RNA PCR were 100% with no false positive reaction was found in the control group. The positive predictive value and negative predictive value of TRI-DOT is 100% and 95.83% respectively, which was similar to similar studies. [11]

It was found that the sensitivity of TRI-DOT was about 91.17% as in three cases HCV RNA could be detected where TRI-DOT test was negative.

It was also found that sensitivity for RNA PCR in our study was only 70.58%, as in 10 cases RNA could not be detected where antibody against HCV could be found both by ELISA and TRI-DOT assay. The reason for this non detection of RNA could be the delay that occurred in processing the cDNA transcription from the RNA, which in some cases went to several weeks owing to logistical problems. In our experience detection of RNA were considerably low after a delay of 48 hours. This probably resulted in the abnormally low sensitivity of RNA PCR in our study.The few cases (n=3) where TRI-DOT were negative but RNA could be detected by RNA PCR, may be explained by the fact that in some early cases of HCV infection the appearance of Anti HCV antibody may be delayed by 3-6 weeks.Among the thalassemia patients specificity of a combination of ELISA and TRI-DOT was again 100% where the specificity of only ELISA was 16.66%. The Positive and negative predictive value of combination of ELISA and TRI-DOT is 100%.The concordance between the TRI-DOT assay and RNA PCR is about 61.76 which is lower than that was found by Caudai. C et al.[12]

V. Conclusion

In view of the low specificity of the 3rd generation ELISA in our study it cannot be recommended alone for the diagnosis of HCV infection in patients. However it can still be used as a screening test, with all positive cases need to be confirmed by an alternative antibody detection test using a different antigen set like HCV TRI-DOT assay or by RNA PCR. TRI-DOT test may be the preferred test as it is less costly, is easy to perform and is rapid. It also has a high sensitivity and specificity, and it can be used as a single test for diagnosis except in early infection. In early infection RNA PCR may be a better option as appearance of antibody may be delayed, and also for determination of genotypes for treatment purposes. However a combination of ELISA and TRI-DOT using different set of antigens (recombinant and modified) may be the best possible alternative to RNA-PCR with specificity, positive predictive value and negative predictive value each approaching 100%.

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