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Original Article

Microbiology Section

Comparative Evaluation of Three Diagnostic Tools for the Detection of Hepatitis C Virus among High-risk Individuals in a Tertiary Care Centre of Northeast India

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ABSTRACT

Introduction: Hepatitis C virus (HCV) has posed a major public health problem globally. Since majority of HCV infected patients are asymptomatic, diagnosis of HCV infection is mainly based on the detection of anti-HCV antibodies by the Enzyme Linked Immunosorbent Assay (ELISA) or Rapid Diagnostic Tests (RDTs) and HCV Ribonucleic Acid (RNA) by real time Polymerase Chain Reaction (PCR) of serum or plasma samples.

Aim: To assess the performance of RDTs and fourth generation ELISA against real time reverse transcriptase PCR for the detection of HCV.

Materials and Methods: A hospital-based cross-sectional study was carried out in the Virology Section, Department of Microbiology, Jawaharlal Nehru Institute of Medical Sciences (JNIMS), Imphal, Manipur, India, for a period of two years from June 2019 to May 2021. The study included 3,254 plasma samples from suspected cases of HCV monoinfection, and HCV/HIV co-infection. The plasma samples were subjected to anti-HCV antibodies by RDTs (SD BIOLINE, South Korea) and fourth generation ELISA (Monolisa™ HCV Ag-Ab ULTRA, Bio-Rad, France), and HCV RNA by real time PCR. Data analysis was done using descriptive statistics, and performance of the assays was evaluated by using Cohen kappa test (κ) and Receiver Operating Characteristic (ROC) curve.

Results: PCR is considered as the gold standard test. HCV was detected by RDTs in 453 (13.92%), ELISA in 413 (12.69%) and RT-PCR in 367 (11.28%) samples. The present study demonstrated sensitivity of 97.55% and specificity of 96.71% with Positive Predictive Value (PPV) of 79.03% by HCV-RDT. The fourth generation ELISA showed high sensitivity of 99.46% and specificity of 98.34%. Using ROC curve, the area under the curve was 81% for ELISA with diagnostic accuracy of 98.46%.

Conclusion: Fourth generation ELISA is more sensitive and specific than RDTs for the detection of HCV infection. Confirmatory HCV-RNA assay could be performed to clear doubts related to false-positive and false-negative findings of the primary screening assays.

Keywords: Enzyme linked immunosorbent assay, Rapid diagnostic test, Real time polymerase chain reaction

INTRODUCTION

The HCV infection is an important health problem with an approximate prevalence of 3% across the globe [1]. There are more than 170 million carriers worldwide who are at risk of developing chronic liver disease, cirrhosis and ultimately progressing to hepatocellular carcinoma [2]. World Health Organisation (WHO) has projected that 10-24 million people are living with active HCV infection in India and seroprevalence among healthy population ranged from 0.09-2.02% in India [3,4]. On the contrary, high occurrence of HCV ranging from 3.5-44.7% has been observed in high-risk individuals such as intravenous drug users, HIV, haemodialysis patients, individuals with high-risk sexual behaviour or requiring multiple blood transfusion [5].

Diagnosis and management of HCV infection depends on serological tests to detect anti-HCV antibodies using RDTs or ELISA and molecular tests to detect and quantitate HCV-RNA or to detect genotypes employing Nucleic Acid Testing (NAT) in the serum or plasma of the patients [6]. The anti-HCV antibodies can be demonstrated in seven to eight weeks after infection and generally persist lifelong [7]. The seroconversion of HCV is very often delayed in immunocompromised patients like HIV, chronic renal failure or even on haemodialysis patients leading to false-negative results [8]. ELISA has undergone modifications over the years to improve their diagnostic capability and to increase sensitivity and specificity of the assay. Four generations of ELISA for detecting anti-HCV antibodies using recombinant proteins or synthetic peptides have been developed till date [9]. The first generation HCV ELISA, which is no longer used, was developed employing recombinant protein derived from the NS4 region (C100-3 the polypeptide), with a sensitivity of 70%-80% and a poor specificity, and false positive of 70% [10,11]. The second generation ELISA has included recombinant/synthetic antigens from non-structural NS3 and NS4 (c33c and C100-3) and core (c22-3) regions improving sensitivity to about 95% and reducing false-positive results also [10,11]. Third generation HCV ELISA was developed using recombinant antigens from the core region, NS3, NS4 and NS5 regions of the viral genome and it allowed anti-HCV detection about eight weeks after infection with sensitivity and specificity greater than 99% [10]. The most recent fourth generation assay is based on the simultaneous detection of HCV core antigen and anti-HCV antibodies, and found useful for the detection of recent HCV infections [12]. For many Low- and Middle-Income Countries (LMICs), where equipped laboratories and trained staff are limited, RDTs may be most appropriate because they are quick and easy to perform without the need for laboratory equipment's [13]. But the lack of quality-assured RDTs for HCV testing has been identified as a major barrier to large-scale access in LMICs and in addition, data on the quality and performance of test kits are limited in those countries [13]. WHO has recommended employing RDTs with a sensitivity of ≥98% and a specificity of ≥97% for HCV serology in plasma or serum specimens [14]. Hence, detection of HCV using the most accurate and sensitive NAT assay like real time RT-PCR will reduce the risk of transmission of HCV and help in the early detection even during serological window period as it can detect the HCV RNA in one to three weeks after infection [15].

Although the performance of various RDTs, ELISA and molecular assays for the detection of HCV infection has been evaluated in different parts of India or world [1,16-20], such study is still unexplored in Manipur, India. Hence, this study was undertaken to evaluate the diagnostic accuracy of rapid antibody test and fourth generation ELISA by comparing with the gold standard test, reverse transcriptase real time PCR, for the detection of HCV.

MATERIALS AND METHODS

This study was a hospital-based cross-sectional conducted in the Virology Section, Department of Microbiology, Jawaharlal Nehru Institute of Medical Sciences (JNIMS), Imphal, Manipur, India, for a period of two years from June 2019 to May 2021. This study was approved by the Institutional Ethics Committee (IEC), JNIMS, Imphal, Manipur, India [No. Ac/03/IEC/JNIMS/2018-(R), dated 15th March 2018]. Participants were informed about the objectives and the importance of the study and their participation was voluntary assuring the confidentiality of the collected information through anonymity of the participant, and that, study results would be used only for the purpose of research. Those who agreed to participate signed an informed consent and accent in case of minors. This study included 3,254 plasma samples from suspected cases of HCV monoinfection, and HCV/HIV co-infection.

Inclusion criteria: It included patients of both sexes and all age groups attending out-patient and in-patient department with one or more risk factors for HCV, HIV infected clients/individuals of both sexes and all age groups, and HCV mono-infected or HIV/HCV co-infected clients referred from Non Governmental Organisations (NGOs).

Exclusion criteria: Individuals or patients who refused to participate were excluded in the study.

Sample size calculation: Considering 95% confidence interval, 5% margin of error, Z score of 1.96 (rounding off to 2) and prevalence rate of 29% for HIV/HCV co-infection, and 74% for HCV mono-infection only [21], the calculated sample size for the study was 638 (330 for HIV/HCV co-infection and 308 for HCV infection only) using the formula-Sample size(n)=Z^2pq/l^2, Where Z=Z-score, p=prevalence rate, q=(1-p), l=absolute allowable error. However, the final sample size during the study period was 367.

Sample Collection and Processing

Following data collection through a structured preform such as personal information, risk factors associated with HCV (Tattoo/Acupuncture, blood transfusion, intravenous drug users, blood transfusion, history of surgical procedures, dental procedures, dialysis and case of HCV within family) and obtaining informed consent or an accent in case of minors, a blood sample of 5 mL was collected from each participant using a Ethylenediamine Tetra Acetic Acid (EDTA) vacutioner. The plasma was separated by centrifugation at 2000 rpm for 10 minutes and placed in cryo vials at -80°C until use. All the participants were subjected to rapid anti-HCV antibody test, fourth generation ELISA for HCV Ag-Ab and HCV RNA detection by reverse transcription (RT) real-time PCR.

I. **Anti-HCV rapid diagnostic test:** Detection of anti-HCV antibody was carried out in plasma samples using a commercially available immunochromatographic rapid test from SD BIOLINE (Standard Diagnostics, South Korea) following manufacturer's guidelines. A reddish-purple line develops within 20 minutes in the presence of HCV-specific antibodies.

II. **Fourth generation ELISA:** Then the samples were subjected to fourth generation ELISA for the detection of capsid antigen and antibodies (against recombinant antigens from NS3 and NS4 regions of the viral genome) to HCV using Monolisa™ HCV Ag-Ab ULTRA, Bio-Rad, France as per manufacturer's guidelines.

III. **Real time PCR:** RNA was extracted from 140 µL of plasma using QIAcube extraction system (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Then Taqman real-time PCR was implemented using artus® HCV RG RT-PCR Kit with a lower limit of sensitivity of 34 IU/mL (Qiagen, Hilden, Germany). Procedures were done according to the manufacturer's instructions using Rotor gene Q 5Plex HRM from Qiagen, Germany.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS), version 20.0 was used for statistical analyses. Descriptive statistics such as percent and proportion were determined. The performance of ELISA as screening test compared to PCR as a gold standard test was evaluated using the area under ROC curve. Sensitivity, specificity and diagnostic accuracy were calculated using 2×2 (two-by-two) table as sensitivity=true positive/true positive+false negative, specificity=true negative/true negative+false positive and accuracy=true positive+true negative/true positive+true negative+false positive+false negative. Cohen kappa test (κ) was also assessed to determine agreement with over 0.75 as excellent, 0.40 to 0.75 as fair to good and below 0.40 as poor. Chi-square test was used to observe the association between the rapid antibody test or ELISA and the gold standard PCR using SPSS version 20.0. The p-value ≤0.05 was considered as significant.

RESULTS

Of the 3254 samples, 1009 (31%) belonged to age group of 41-50 years and male 3034 (93.24%) outnumbered female 220 (6.76%). Majority of the participants were married 3018 (92.75%) and literate 3073 (94.44%) as shown in [Table/Fig-1].

During the study period of two years, 3254 samples were tested for HCV by the three diagnostic tools where rapid antibody test detected HCV in 453 (13.92%), ELISA in 413 (12.69%) and PCR in 367 (11.28%) specimens as shown in [Table/Fig-2]. The 2455 samples (75.44%) were collected from outpatient and 799 (24.55%) from inpatient department. Of the total 3254 samples, 344 (10.57%) were HIV infected individuals (57 from ICTC, 185 from ART, 79 from

Age groups (years)	No. of sample	Gender#		Marital status$		Education*		
		M (%)	F (%)	S (%)	M (%)	Illiterate (%)	≤Pri. School (%)	>Pri. School (%)
0-10	1	1 (100)	0	1 (100)	0	0	1 (100)	0
11-20	130	127 (97.69)	3 (2.31)	110 (84.62)	20 (15.38)	12 (9.23)	21 (16.15)	97 (74.62)
21-30	651	626 (96.16)	25 (3.84)	31 (4.76)	620 (95.24)	51 (7.83)	26 (3.99)	574 (88.17)
31-40	586	545 (93)	41 (7)	39 (6.66)	547 (93.34)	36 (6.14)	11 (1.88)	539 (91.98)
41-50	1009	946 (93.76)	63 (6.24)	35 (3.47)	974 (96.53)	45 (4.46)	17 (1.68)	947 (93.86)
51-60	651	591 (90.78)	60 (9.22)	12 (1.84)	639 (98.16)	19 (2.92)	9 (1.38)	623 (95.7)
61-70	163	144 (88.34)	19 (11.66)	7 (4.29)	156 (95.71)	11 (6.75)	5 (3.07)	147 (90.18)
71-80	63	54 (85.71)	9 (14.29)	1 (1.59)	62 (98.41)	7 (11.11)	5 (7.94)	51 (80.95)
Total	3254	3034 (93.24)	220 (6.76)	236 (7.25)	3018 (92.75)	181 (5.56)	95 (2.92)	2978 (91.52)

[Table/Fig-1]: Demographic distribution of participants.
#M: Male; F: Female; $S: Single; M: Marital; *Pri.: Primary

Study population	Total sample tested	HCV				
		Screening + ve				
		Rapid test (%)	Fourth Gen* ELISA (%)	HCV RNA +ve (%)	HCV Moninfection (%)	HCV+HIV (%)
ICTC	57	27 (47.37)	22 (38.6)	15 (26.32)	0	15 (26.32)
ART Centre	185	61 (32.97)	53 (28.65)	45 (24.32)	0	45 (24.32)
Medicine department	2330	295 (12.66)	281 (12.06)	265 (11.37)	260 (11.16)	5 (0.21)
Surgery department	214	16 (7.48)	11 (5.14)	7 (3.27)	7 (3.27)	0
Orthopedics dept.	166	15 (9.03)	13 (7.83)	8 (4.82)	8 (4.82)	0
Obstetrics and Gynecology dept.	131	7 (5.34)	5 (3.82)	3 (2.29)	3 (2.29)	0
Pediatrics dept.	18	0	1 (5.56)	1 (5.56)	1 (5.56)	0
Psychiatry dept.	88	32 (36.36)	27 (30.68)	23 (26.14)	21 (23.86)	2 (2.27)
Blood Bank	65	0	0	0	0	0
Total	3254	453 (13.92)	413 (12.69)	367 (11.28)	300 (9.22)	67 (2.06)

[Table/Fig-2]: Department wise distribution of HCV among the study population.
*Gen: Generation; @ ICTC: Integrated counseling and testing centre; #ART: Antiretroviral therapy

medicine and 23 from psychiatry). However, HCV monoinfection was detected in 300 (9.22%) and HCV/HIV co-infection in 67 (2.06%) specimens as depicted in [Table/Fig-2].

Majority of the participants was Injecting Drug Users (IDUs) (60.6%) and least risk behaviour was associated with vertical transmission (0.06%) as shown in [Table/Fig-3].

Risk factors	N (%)
IDU*	1972 (60.6)
Tattoo/Acupuncture	869 (26.71)
Sexual exposure	252 (7.74)
Surgery	231 (7.1)
Blood transfusion	191 (5.87)
Family with HCV infection	31 (0.95)
Dialysis	29 (0.89)
Dental procedure	8 (0.25)
Vertical transmission (Mother to child)	2 (0.06)
Unknown	101 (3.1)

[Table/Fig-3]: Distribution of risk behaviours of HCV among participants.
*Injecting drug users multiple factors

On comparing rapid antibody test against gold standard PCR in detecting HCV, 11% of the samples were diagnosed HCV positive compared to 11.28% by PCR with strong level of agreement (κ=0.855) and p-value of <0.001. Similarly, performance of ELISA was compared against PCR which resulted in detection of HCV in 11.22% by ELISA compared with 11.28% by PCR with an almost perfect level of agreement (κ=0.929) and p-value of <0.001 as depicted in [Table/Fig-4].

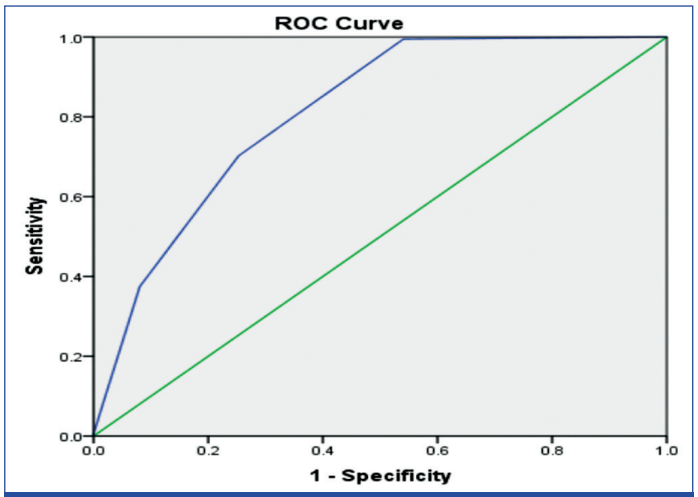
Test	PCR		Total	Cohen's kappa	p-value*
	Positive (%)	Negative (%)			
Rapid antibody test					
Positive (%)	358 (11)	95 (2.92)	453	0.855	<0.001
Negative (%)	9 (0.28)	2792 (85.80)	2801		
Total (%)	367 (11.28)	2887 (88.72)	3254		
ELISA					
Positive (%)	365 (11.22)	48 (1.48)	413	0.929	<0.001
Negative (%)	2 (0.06)	2839 (87.24)	2841		
Total (%)	367 (11.28)	2887 (88.72)	3254		

[Table/Fig-4]: Comparison of rapid antibody test and ELISA to PCR.
*p-value is calculated using Chi-square test

Using ROC curve, the area under the curve was 0.810 or 81% for ELISA giving a sensitivity of 99.46%, specificity of 98.34%, 1-specificity (false positive) of 1.66%, 1-sensitivity (false negative) of 0.54% with diagnostic accuracy of 98.46% as shown in [Table/Fig-5,6].

Assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)	Diagnostic accuracy
RDTs	97.55	96.71	79.03	99.68	3.29	2.45	96.80
ELISA	99.46	98.34	88.38	99.93	1.66	0.54	98.46

[Table/Fig-5]: Performance of RDTs and ELISA against PCR.
PPV: Positive predictive value; NPV: Negative predictive value; FPR: False positive rate; FNR: False negative rate



[Table/Fig-6]: Receiver Operating Characteristic Curve (ROC) for ELISA.
Area under the curve: 0.810, Standard error (SE):0.029, p-value of <0.001 (Chi-square test)

DISCUSSION

The diagnosis of HCV can be performed by three common assays which include anti-HCV antibody assay, HCV-RNA detection and recently introduced HCV core antigen assay. An ideal assay should be able to differentiate between those who have or those who do not have an infection and be cost-effective as well. Therefore, an optimal screening assay should be the one with high sensitivity (~100%) with an acceptable specificity to detect all true positive samples; although, some amount of wastage due to false positivity might be acceptable.

Among 3254 samples collected, 3034 (93.24%) were males and the remaining 220 (6.76%) were females. The mean age was 41.72 years. Similar findings were reported by Prakash S et al., where males constituted 64.56% with mean age of 45.72 years [1]. Most common risk behaviour associated with this study was IDUs (60.6%). This finding was consistent with that of Kermode M et al., Basu D et al., and Sood A et al., [21-23]. A systematic review of 1125 articles revealed an estimated 10 million IDUs are HCV positive, mostly in Eastern Europe, East Asia, and Southeast Asia [24]. However, Indian studies have demonstrated seroprevalence of HCV seropositivity in the range of 20-90% among the IDUs, although some parts of the country witnessed a very high seropositivity (60-90%) in IDUs [25].

The present study demonstrated sensitivity of 97.55% and specificity of 96.71% with PPV of 79.03% by HCV-RDT. Similar results have been shown by Maity S et al., (sensitivity of 95.5% and specificity of 100%, J Mitra and Co, PVT Ltd), Mane A et al., (sensitivity of 99.4% and specificity of 99.7%, SD Bioline)and Chevalier S et al., (sensitivity of 97% and specificity of 100%, SD Bio line) [26-28]. However, El-Sokkary RH et al., and Bajpai M et al., obtained lower sensitivities of 83.33% and 25% respectively [18,29]. Performance of RDTs would be considered satisfactory if it shows high PPV and lower degree of false negatives [30]. This study also revealed low PPV (79.03%) and high false negative rate (2.45%) for RDTs, therefore it is not suitable to be adopted as a good screening assay. WHO guidance on performance criteria for in-vitro diagnostics for HCV recommends a sensitivity of ≥98% and a specificity of ≥97% for HCV serology RDTs [14].

The present study revealed high sensitivity of 99.46% and specificity of 98.34% by the fourth generation ELISA (Monolisa™ HCV AgAb ULTRA, Bio-Rad) while comparing with PCR. It also showed PPV of 88.38% with false negative rate of 0.54%. Similar findings were obtained by Yang J et al., (sensitivity of 100% and specificity of 95.40% for Murex HCV AgAb, Abbott), Ahmed S et al., (sensitivity of 98.5 % and specificity of 99% for Elecsys anti-HCV 3rd generation assay), Maity S et al., (sensitivity of 100% and specificity of 88.1%, J Mitra and Co, Pvt Ltd), Brandão CP et al., (sensitivity of 97.5% and specificity of 99.71% for Monolisa™ HCV AgAb ULTRA, Bio-Rad) and Jiang X et al., (sensitivity of 99.2% for Murex HCV AgAb, Abbott) [7,19,26,31,32]. On the other hand, lower sensitivities of 80% (3rd generation ELISA, Erba, Trans Asia, India) and 87.5% (3rd generation, Hepanostika HCV Ultra; UBI Diagnostics, Beijing, China) were reported by Prakash S et al., and El-Sokkary RH et al., respectively [1,18]. False positive rate has also been observed low (1.66%) in this study as compared to 29.14% and 15.56% shown by Prakash S et al., and El-Sokkary RH et al., respectively [1,18], indicating improved performance with fourth generation ELISA. Infections like malaria, syphilis, or HIV, malnutrition, and various chronic diseases have been hypothesised to increase the false positivity of HCV antibody tests (Ortho HCV version 3.0 ELISA test, Ortho Clinical Diagnostics, New Jersey) in African populations, although these associations remain conjectural [33].

The area under the ROC curve (AUC) is a criterion to measure accuracy of diagnostic test. It considers values from 0 to 1, where 0 suggests perfectly inaccurate test and 1 suggests a perfectly accurate test [34]. An AUC of 0.5 indicates no discrimination, 0.7 to 0.8 is regarded acceptable, 0.8 to 0.9 is excellent, and more than 0.9 is denoting outstanding [34]. This study demonstrated the AUC of 0.81 (standard error, 0.029) for fourth generation ELISA assay which suggested that the assay would correctly distinguish a HCV infected patient from non infected one. Similar figure of 85% for ELISA was shown by El-Sokkary RH et al., [18]. However, Rouet F et al., reported AUC of 0.90 (standard error, 0.04) for fourth generation Monolisa HCV Ag-Ab ULTRA ELISA assays [35].

Molecular detection of HCV RNA by reverse transcription (RT) real time PCR is considered gold standard for the detection of HCV infection [36]. In this study, RDTs detected HCV in 453 (13.92%) samples, ELISA in 413 (12.69%) while Real Time PCR was able to confirm the infection in 367 (11.28%) samples only. Detection of HCV infection by the most commonly employed tools has been varying among various studies [Table/Fig-7] [1,16-20,37,38].

PCR was able to detect 9 (0.28%) samples which were shown negative by RDTs. This might be due to the fact that PCR can determine minute amounts of HCV-RNA in serum or plasma and it will be positive in all acute infections with or without elevation of hepatic enzymes, as previously reported [6]. On the other hand, PCR picked up 2 (0.06%) positive samples from those seronegative samples. This is possible during early acute phase of HCV infection as ELISA can be best employed after first three weeks of infection. Again, PCR failed to detect 48 (1.48%) from ELISA positive samples. This condition might occur during the chronic phase or resolution of HCV infection and also in case of PCR false negative results or false anti-HCV positive. However both RDTs and ELISA had demonstrated strong and almost perfect agreement respectively with PCR. El-Sokkary RH et al., reported good level of agreement for RDTs (κ=0.69) and ELISA (κ=0.69) on comparing with PCR [18]. Jindal N et al., has also demonstrated good agreement (κ=0.61) between ELISA and PCR [37].

Authors	Place, Year of publication	RDTs (%)	ELISA (%)	Real time PCR (%)
Kazemi B et al.,[16]	Iran, 2004	-	29.82	19.88
Jasuja S et al., [17]	New Delhi, 2009	-	21.8	27.7
Jindal N et al., [37]	Punjab, 2015	-	30	20
Prakash S et al., [1]	Lucknow, 2014	-	7.17	6.75
El-Sokkary RH et al., [18]	Egypt, 2017	37.7	40.6	34.8
Ahmed S et al., [19]	Egypt, 2018	-	9	8
Barik G et al., [20]	Kolkata, 2019	30.1	32.04	20.39
Arora SK et al., [38]	New Delhi, 2021	1.67	2.19	-
Singh RM et al., (present study)	Manipur, 2022	13.92	12.69	11.28

[Table/Fig-7]: Comparison of RDTs, ELISA and Real Time PCR in detecting HCV [1,16-20,37,38].

The strength of this study was a large size of samples comprising mostly of high risk behaviours was included and tested using the three available diagnostic tools- RDTs, fourth generation ELISA and Real Time PCR. Present study findings have shown concordant with most previous studies.

Limitation(s)

The present study could have been much better if the study population covers good number of samples from the blood bank and fourth generation HCV tridot, having 100% sensitivity and 98.9% specificity as per WHO evaluation, could have also considered for evaluation.

CONCLUSION(S)

Serological assays are simple, reliable, user friendly specially RDTs, easily available and more cost effective for the detection of HCV infection, yet they cannot be employed in case of acute infection. RDTs has demonstrated a sensitivity of 97.55% and specificity of 96.71% whereas ELISA is highly sensitive (99.46%) and specific (98.34%) with PPV of 88.38 % and false negative rate of 0.54%. Strict implementation of infection control measures should be a part of higher system management plan in all hospitals. With the implementation of National Viral Hepatitis Control program (NVHCP) under National Health Mission since 2019, molecular detection of HCV employing CBNAAT or Truenat has reached far flung areas in India.

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