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Designing of a novel rapid immunoassay for early detection of HCV infection

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Abstract

HCV is the main cause of non-A, non-B hepatitis and causing cirrhosis, Hepatocellular carcinoma (HCC), liver failure and death. An rapid diagnostic assay for early diagnosis of HCV (Hepatitis C virus) infection by detecting HCV core antigen was designed and performance evaluation of developed assay was carried out in comparison with routinely used antibody detection rapid immunoassay and ELISA. Total 79 anti-HCV antibody positive serum samples including 10 serum samples which were very weak anti-HCV antibody positive in Elisa (Ortho Diagnostics) and negative in rapid immunoassay (Standard Diagnostics) for the presence of anti-HCV antibody, 19 weak anti-HCV antibody positive serum samples, 23 moderate anti-HCV antibody positive serum samples, 27 anti-HCV antibody strong positive serum samples and 11 NAT (Nucleic acid amplification test) positive (negative for anti-HCV antibody) serum samples were tested. In addition to above mentioned anti-HCV antibody positive serum samples, two seroconversion panels from BBI Diagnostic were also tested. Total 509 anti-HCV antibody negative serum samples including 200 normal healthy donors serum samples, 54 other diseases positive serum samples, 105 interfering substances containing serum samples and 150 clinical serum samples were tested. HCV core antigen was detected in all very weak anti-HCV antibody positive serum samples, which were found negative in rapid immunoassay and positive in anti-HCV antibody detection Elisa. However HCV core antigen was detected in 17 serum samples out of 23 moderate anti-HCV antibody positive serum samples and 01 sample out of 27 nos. of strong anti-HCV antibody positive serum samples. In seroconversion panel testing HCV core antigen detected approximately 12 days earlier than the detection of anti-HCV antibodies. In testing of anti-HCV antibody negative samples, no false positive reaction was observed and hence developed HCV core antigen detection assay was found 100% specific. HCV core antigen detection rapid test has good diagnostic potential for the early detection of HCV infection and could be a promising tool for seroscreening of blood during transfusion, counseling and early diagnosis of HCV.

Key words: Cirrhosis, Elisa, HCV, HCC, NAT.

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1.0 Introduction

Hepatitis C virus (HCV) is positive RNA strand containing enveloped virus, classified within the genus Hepacivirus in the Flaviviridae family [1, 2]. HCV is considered as the main cause of liver diseases in both developed and developing countries and contributes to the increasing risk of liver failure and hepatocellular carcinoma (HCC) [3, 4, 5]. Total global HCV prevalence is 2.5% (177.5 million of HCV infected adults) [6]. HCV primarily transmitted via the parentral route which includes injection drug use, blood transfusion, unsafe injection practices and other healthcare related procedures. HCV causes subclinical acute hepatitis which gradually developed into chronic hepatitis in 80% cases of HCV infected persons which has a long time course, often extending for decades [2, 7]. HCV is considered as silent killer virus as most HCV infected people are unaware of the HCV infection inside their body even decades after infection [8].

Chronic HCV infection is often associated with the development of liver cirrhosis (causes 27% cirrhosis worldwide), hepatocellular cancer (HCC) [causes 25% HCC worldwide], liver failure, and death [3, 9]. HCV infection is characterized by ongoing changes in viral sequences that enable the virus to persist and evade immune surveillance or antiviral therapies [10].

Genome of HCV is made up of 9600 nucleotides which encodes a poly protein of about 3000 aminoacids. This poly protein is proteolytically cleaved to produce structural proteins (Core, E1, E2 and p7) from one-third of poly protein and non structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) from remaining two-third of poly protein [11,12]. Concentration of antibodies to NS3 antigen correlates with virus load as it decreases significantly with the decrease in viral load. HCV core and non-structural components; NS3 and NS5A proteins causes liver damage. Structural envelope proteins are very low immunogenic. Highest antibody response is observed against NS4 antigen of HCV, whereas responses to NS5 were generally the lowest and the most likely to be missing. HCV Core antigen is most conserved highly antigenic protein, inducing cellular and humoral response in host and has main roll in pathogenesis of HCV infection. In several studies indicates that HCV core antigen detection can be used for recent infection of HCV detection. Conversely, NS3 and NS5 show high sequence diversity among HCV subtypes and genotypes [1, 8, 13].

Anti-HCV antibody appears late upto 1 year after infection in HIV coinfection. Presence of HCV core antigen indicates current infection where as anti-HCV antibody remains present even after clearance of HCV infection and thus HCV core antigen detection is also significant from treatment point of view. In several studies it indicated that HCV core antigen detection is very useful in low-resource settings, where PCR is unavailable as compared to anti-HCV antibody detection test because by detecting HCV core antigen HCV window period can be shorten [14, 15, 16, 17, 18].

2. Materials and methods

2.1 Capture and detector antibody selection

Capture and detector anti-HCV core antibody pair was selected in direct ELISA format. Commercially available anti-HCV core antibodies from four different sources were screened (Table 1). Each antibody was tested as capture and as detector in ELISA format.

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Table 1: List of anti-HCV core antibodies.

Sr. No.	Description of antibody	Host	Catalogue	Source	Type
1	Anti-HCV core antibody (IgG1)	Mouse	ANT-286	Prospec protein specialist, Israel	Monoclonal
2	Anti-HCV core antibody (IgG1)	Mouse	BECHCVS101	Fapon, China	
3	Anti-HCV core antibody (IgG2b)	Mouse	BEJHCVS202	Fapon, China	
4	Anti-Hepatitis C virus core antibody [H21] (IgG1)	Mouse	ab13829	Abcam, UK	
5	Anti-HCV core antibody	Goat	OAMA02672	Aviva systems biology, USA	Polyclonal

Biotinylation of antibodies was carried out by addition of 80µg of biotin for 1 mg of antibody. This mixture was incubated at room temperature for 30 minutes. Dialyze this mixture in 10mM Phosphate buffer saline, pH 7.2 for 16 to 20 hours. For antibody coating to ELISA wells (Biomat), 2 µg /mL antibody solution was prepared in 10mM Phosphate buffer saline, pH 7.2 and added 100µL into wells of polystyrene wells of ELISA plate. After 16 to 20 hours incubation at +2°C to +8°C, unbounded sites were blocked by 2% BSA solution for 2 hours at room temperature. After incubation blocking solution was decant. Test was carried out by addition of 1000ng/mLl to 1.37 ng/mL of recombinant HCV core antigen (Prospect protein specialist) and incubating at +37°C for 60 minutes which was followed by 5 times washing with 10mM Phosphate buffer saline, pH 7.2 having 0.05% Tween 20. After washing 100 µL of 1:10000 diluted biotin coupled ani-HCV core antibody was added and incubated for 30 minutes at room temperature which was followed by washing for 5 times and addition of 100µL TMB color reagent. After 30 minutes incubation in dark, the reaction was stopped by addition of 100 µL of 0.1N H_2SO_4. Developed color was measured spectrophotometrically at 450 and 630 nm.

2.2 Design of HCV core antigen detection rapid immunoassay

Monoclonal anti-HCV Core antibody (ANT-286) from Prospec protein specialist was used as detector antibody. Detector antibody was coupled to colloidal gold by using protocol mentioned by Lishan He et all [19]. Polyclonal anti-HCV antibody (OAMA02672) from Aviva system biology was used as a capture antibody for Test line and Goat anti-mouse antibody was used for control of assay procedure. Capture antibody was sprayed on nitrocellulose membrane (Pall lifesciences) in 10mM Carbonate buffer, pH 9.6 . After spraying of capture molecules, nitrocellulose membrane was dried at +37°C for 1.5 hours. Detector antibody coupled gold was sprayed on conjugate pad (Ahlstrom). After spraying conjugate pad was dried for 2 hours at +37°C. Sample pad was used remove particulate matter from the sample. Test strip (Immunochromatographic Test strip-Dipstick) assembly was prepared by application of absorbent pad (AP080) from MDI on the top of the sprayed and dried nitrocellulose membrane, sprayed conjugate pad was applied below nitrocellulose membrane and below conjugate pad sample pad was applied.

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Assay procedure adopted was initially add 200 µL of wash buffer (0.1% Tween 80 in 50mM Phosphate buffer saline, pH 7.2) into disposable plastic test tube. Apply 50µL of serum/plasma sample on the sample pad of Test strip. After application of sample on test strip place test strip vertically in to Test tube. Read the results after 25 minutes.

2.3 Performance evaluation of HCV core antigen detection rapid immunoassay

Anti-HCV antibody positive, anti-HCV antibody negative and HCV NAT positive serum samples were collected from clinical laboratories and blood bank of Surat, Gujarat, India. In order to determine the status of the sample, all samples were tested in anti-HCV antibody detection test Elisa (Ortho Diagnostics) and HCV rapid immunoassay (Standard Diagnostics). In order to determine the role of HCV core antigen detection for the early diagnosis of HCV infection strong, moderate, weak, and very weak anti-HCV antibody positive serum samples were tested. In present study, very weak anti-HCV antibody positive samples which were found positive in anti-HCV antibody detection Elisa but found negative in anti-HCV antibody detection rapid immune assay due to sensitivity limitations of rapid test were also tested in HCV core antigen detection rapid test in order to determine that by detecting HCV core antigen those samples can be detected in rapid immunoassay test format or not. Total 79 nos. of anti-HCV antibody positive serum samples including 10 nos. of serum samples which were very weak anti-HCV antibody positive in Elisa and negative in rapid immunoassay for the presence of anti-HCV antibody, 19 nos. of weak anti-HCV antibody positive serum samples, 23 nos. of moderate anti-HCV antibody positive serum samples and 27 nos. of anti-HCV antibody strong positive serum samples were tested.

In addition to above mentioned anti-HCV antibody positive serum/ plasma samples, 02 nos. of Nucleic Acid Test (NAT) positive (negative for anti-HCV antibody) serum samples and two seroconversion panels from BBI Diagnostic were also tested to determine the role of HCV core antigen detection in preseroconversion phase of HCV infection.

In order to determine the specificity of the developed HCV core antigen detection rapid immunoassay, total 509 nos. of anti-HCV antibody negative serum samples including 200 nos. of normal healthy donors serum samples, 54 nos. of other diseases positive serum samples in order to determine the cross reactivity with other diaseases, 105 nos. of interfering substances containing serum samples for determination of interference caused by interfering substances and 150 nos. of clinical serum samples were tested.

3. Results and Discussion

3.1 Capture and detector antibody selection

In antibody pair selection in direct ELISA format anti-HCV antibody pair ANT-286 – OAMA02672 showed better reactivity as compared to other anti-HCV core antibody pairs (Figure 1) hence this pair was selected as capture and detector antibody for designing a rapid immunoassay, detecting HCV core antigen.

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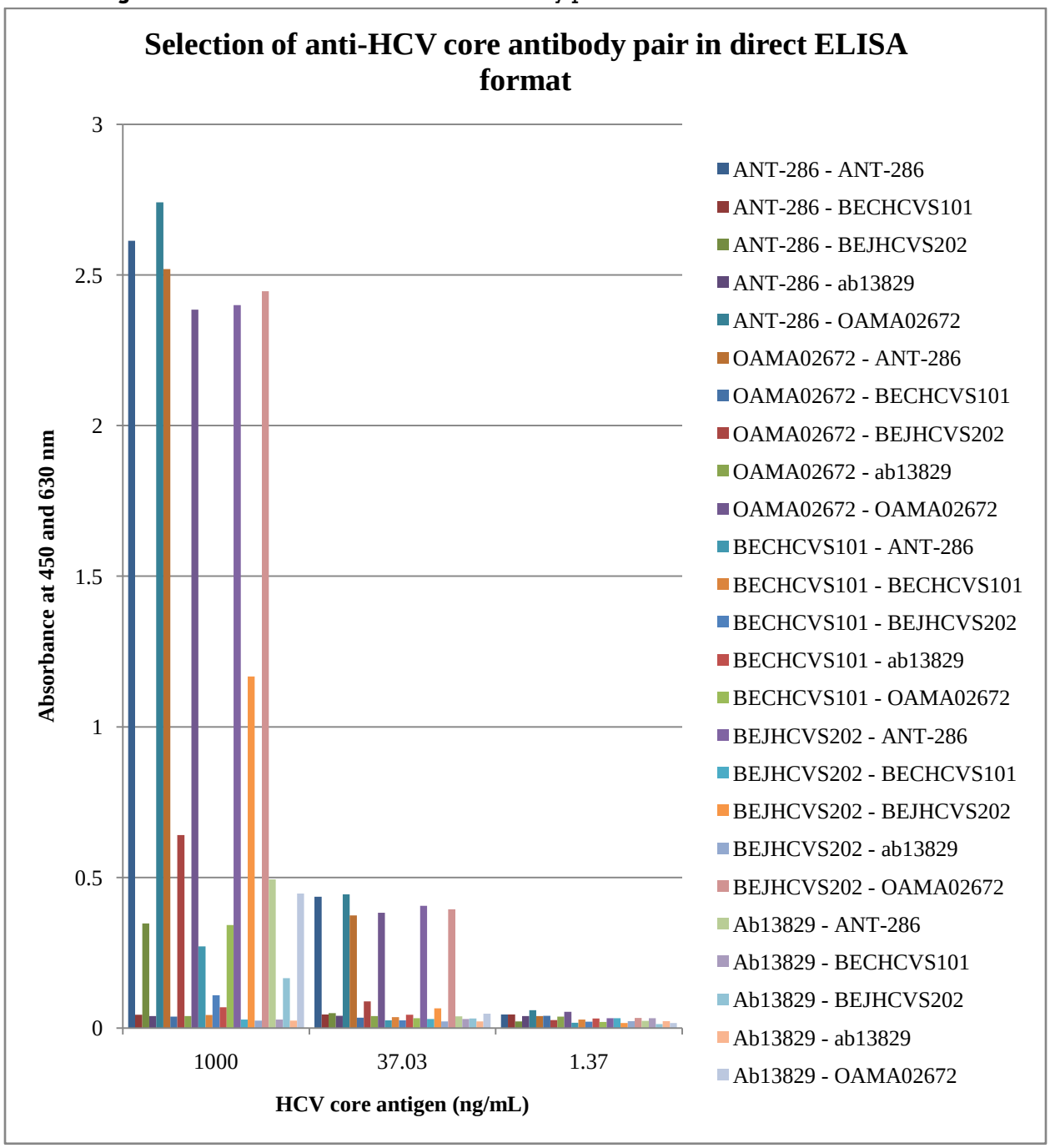
Figure 1: Selection of anti-HCV core antibody pair selection in Direct ELISA format.



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3.3 Performance evaluation of HCV core antigen detection rapid immunoassay

In testing with anti-HCV antibody positive serum/plasma samples, HCV core antigen was detected in 10 Nos. of very weak anti-HCV antibody positive serum samples (positive in anti-HCV antibody Elisa), which were found negative in anti-HCV antibody detecting rapid immunoassay and weak anti-HCV antibody positive serum samples. It indicated that by detecting HCV core antigen, HCV positive samples in early phase of disease (earlier than detection of anti-HCV antibody) can be detected. By detecting HCV core antigen, early seroconversion phase samples, undetectable in rapid immunoassay for anti-HCV antibody detection due to very low amount of anti-HCV antibody in patient sample can be detected in rapid immunoassay format by detecting HCV core antigen as HCV core antigen appears in patient sample in preseroconversion and early sero conversion phase in detectable amount. HCV core antigen is also detected in weak antibody positive serum samples (19 Nos.). However HCV core antigen was detected in 17 nos. of serum samples out of 23 nos. of moderate anti-HCV antibody positive serum samples and HCV core antigen detected in 01 nos. of sample out of 27 nos. of strong anti-HCV antibody positive serum samples because of the fact that HCV Core antigen disappears from patient samples soon after appearance of anti-HCV antibody (Table 2).

Table 2: Testing of anti-HCV antibody positive serum samples.

-	Sample	HCV core antigen detection rapid immunoassay		Anti-HCV antibody detection Elisa		HCV Antibody Detection Rapid immunoassay	
		Positive	Negative	Positive	Negative	Positive	Negative
Total numbers of samples **n= 79**	Anti-HCV antibody very weak positive serum samples (Positive in Elisa but negative in Rapid test) **n = 10**	10	0	10	0	0	10
	Anti-HCV antibody weak positive serum samples (Positive in Elisa and Rapid test) **n = 19**	19	0	19	0	19	0
	Anti-HCV antibody moderate positive serum samples (Positive in Elisa and	17	6	23	0	23	0

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	Rapid test) **n = 23**						
	Anti-HCV antibody strong positive serum samples (Positive in Elisa and Rapid test) **n = 27**	1	26	27	0	27	0

From all NAT positive (negative for anti-HCV antibody) serum samples HCV core antigen was detected (found positive) in rapid immunoassay for HCV core antigen detection (Table 3).

Table 3: Testing of HCV NAT positive serum samples.

Sample	HCV core antigen detection rapid immunoassay		Anti-HCV antibody detection Elisa		HCV Antibody Detection Rapid immunoassay	
	Positive	Negative	Positive	Negative	Positive	Negative
HCV NAT Test Positive **n = 11**	11	0	0	11	0	11

In testing of two HCV sero conversion panels PHV913 and PHV904-00-1.0 from BBI, HCV core antigen detected average 12 days earlier than the detection of anti-HCV antibodies (Table 4 and Table 5). It indicates that by detecting HCV core antigen early detection of the HCV infection can be done in preserocconversion or early seroconversion phase of the disease when anti-HCV antibody are absent in patient blood or not in detectable amount.

Table 4: Testing of HCV seroconversion panel PHV 913 testing.

Name : HCV Seroconversion Panel PHV913
Source : Boston Biomedica, Inc.

Panel Member	Bleed date	Days since 1st bleed	Elisa Results	HCV core antigen detection rapid immunoassay		HCV Antibody Detection Rapid immunoassay
				Control	Test	Results
PHV913-01	27-Feb-97	0	Negative	+	+	0
PHV913-02	01-Mar-97	2	Negative	+	+	0
PHV913-03	06-Mar-97	7	Positive	+	+	+
PHV913-04	08-Mar-97	9	Positive	+	+	+

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Table 5: Testing of HCV seroconversion panel PHV904-00-1.0 testing.

Name : HCV Seroconversion Panel PHV904-00-1.0						
Source : Boston Biomedica, Inc.						
Panel Member	Bleed date	Days since 1st bleed	Elisa Results	HCV core antigen detection rapid immunoassay		HCV Antibody Detection Rapid immunoassay
				Control	Test	Results
PHV 904-01	18-Apr-95	0	Negative	+	0	0
PHV 904-02	20-Apr-95	2	Negative	+	+	0
PHV 904-03	25-Apr-95	7	Negative	+	+	0
PHV 904-04	27-Apr-95	9	Negative	+	+	0
PHV 904-05	02-May-95	14	Positive	+	+	+
PHV 904-06	09-May-95	21	Positive	+	+	+
PHV 904-07	11-May-95	23	Positive	+	+	+

In specificity determination study, no false positive reaction was observed when tested with 509 Nos. of anti-HCV antibody negative serum samples including 200 nos. of normal healthy donors serum samples, 54 nos. of other diseases positive serum samples, 105 nos. of interfering substances containing serum samples and 150 nos. of clinical serum samples were tested (Table 6). It indicates that developed HCV core antigen detection rapid immunoassay was not cross reactive with other diseases positive samples and its results were not affected by presence of interfering substances in sample and hence in present study the specificity of the developed HCV core antigen detection rapid immunoassay was found 100%.

Table 6: Testing of HCV negative samples with HCV Core antigen detection rapid immunoassay.

Sample type			HCV core antigen detection rapid immunoassay	
			Positive	Negative
HCV Elisa negative serum	Normal Healthy donor samples n= 200		0	200
	Other diseases positive n =	HIV Positive n = 15	0	15
		Hepatitis B virus positive n = 29	0	29

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samples n= 509	**54**	**Syphilis positive n = 10**	0	10
	Interfering substances containing samples n = 105	**RA Positive samples n = 29**	0	29
		High billirubin containing samples n = 21	0	21
		High SGPT containing samples n = 3	0	3
		High cholesterol containing samples n = 38	0	38
		High hCG containing samples n = 3	0	3
		ANA positive samples n = 4	0	4
		CRP positive samples n = 7	0	7
	un-selected donors (Clinical samples) n = 150		0	150
Total numbers of samples			**0**	**509**
Specificity			**100%**	

Conclusion

A novel HCV core antigen detection rapid test has good diagnostic potential for the early detection of HCV infection as anti-HCV antibody very weak positive serum samples which were detected negative in rapid immunoassay detecting anti-HCV antibodies those samples could be detected for the presence of HCV infection by detecting HCV core antigen in rapid immunoassay. Both NAT test positive samples (negative for anti-HCV antibody) were also detected positive in Core antigen detection test. In seroconversion panel study, early detection (approx. 12 days) could be done by HCV core antigen detection. In addition to above advantages of the HCV core antigen detection is that it indicates active multiplication of virus (current disease). However HCV core antigen detection assay should be combined with anti-HCV antibody detection assay for the diagnosis of HCV infection as HCV core antigen test becomes negative after appearance of anti-HCV antibody in patient serum.

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