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RESEARCH ARTICLE

Detection of HCV-RNA by real time PCR using SYBRGREEN DYE I

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Abstract

Hepatitis C is an infectious disease affecting primarily the liver, with about 3–4 million people are infected yearly, and more than 350,000 people die per year from hepatitis C-related diseases .There is a number of diagnostic tests for hepatitis C including: recombinant immunoblot assay, HCV antibody enzyme immunoassay or ELISA and quantitative HCV RNA polymerase chain reaction (PCR)**.** Several approaches have been employed to detect PCR products. The most popular using TaqMan probe and the other using fluorescent dye SYBR GREEN I. Unlike TaqMan florescent probes, SYBR GREEN I technology method does not require a probe for the qualitative detection and the PCR product is monitored continuously by SYBR GREEN I dye during one step PCR. **Aim of work:** Quantitative detection of HCV RNA by a simple, rapid, low cost and sensitive real time PCR using SYBR GREEN I dye and compare it with TaqMan technology. **Subjects & Methods:** The study recruited 150 HCV antibody positive patients. They were (92 males &58 females) with their ages ranging from (21:59 years). Quantitative HCV RNA was performed by both the ordinary TaqMan method as well as SYBR GREEN based method. **Results:** HCV RNA was detected by Taqman probe in 138 (92%) of total cases with a median of 3.98×10^5 while, it was detected by SYBR GREEN probe in 134 (89.3%) with median of 3.1×10^5. In addition to this, there was a substantial (good) agreement between both methods regarding qualitative assessment (Weighted κ=0.642). Furthermore, stratification of expression results into negative, Low, moderate and high quantities, revealed moderate agreement between both methods as regard quantitative assessment (Weighted κ=0.503). In addition, At <12 IU/mL level, sensitivity of SYBR GREEN I method was 94.4%, specificity was 66.7%, PPV was 97%, NPV was 50% and accuracy was 92%. **Conclusion:** detection of HCV-RNA by syber green method could be used as a good screening test for qualitative detection especially in blood donor screening.

INTRODUCTION

Hepatitis C is an infectious disease affecting the liver, caused by the hepatitis C virus (HCV)which is a ribonucleic acid (RNA) virus ***(Kim and Changk, 2013)***. It is estimated that 130–170 million people, or 3% of the world's population lives with chronic hepatitis C. About 3–4 million people are infected yearly, and more than 350,000 persons die yearly from hepatitis C-related diseases .The infection is usually asymptomatic in early stage ,but later

74%to 86%of infected persons progress to chronicity ***(WHO, 2013).*** Countries with particularly high rates of infection include Egypt (22%), Pakistan (4.8%) and China (3.2%) ***(WHO, 2011).***

There is a number of diagnostic tests for hepatitis C including: recombinant immunoblot assay, HCV antibody enzyme immunoassay or ELISA and quantitative HCV RNA polymerase chain reaction (PCR) ***(Wilkins et al. , 2010).*** HCV RNA can be detected by PCR typically after one to two weeks of infection, while antibodies can take longer to form and thus to be detected ***(Ozaras et al., 2009).***

Conventional PCR using gel electrophoresis for identification of the amplification takes several hours. Contamination of specimens may occur from one specimen to another ***(Ratge et al., 2002).***

Real time PCR has been described for viral load monitoring both in serum and liver samples ***(Enomoto et al., 2002)*** and it was shown that this technology could be used as a very reliable and highly sensitive method for quantification of the viral load of HCV in serum ***(Komurian-pradel et al., 2001).***

Several approaches have been employed to detect PCR products. The most popular using TaqMan probe and the other using fluorescent dye SYBR GREEN I. ***(Albertoni et al, 2014)***

SYBR GREEN technology method does not require a probe, unlike TaqMan florescent probes, for the detection and the PCR product is monitored continuously by SYBR GREEN I dye during one step PCR. This assay is simple, reliable, rapid, economic and minimizes risk of contamination ***(Schroter et al., 2001).***

So the aim of our study is to quantitate HCV RNA by a simple, rapid, low cost and sensitive real time PCR using SYBR GREEN dye and compare it with TaqMan technology.

Subjects and Methods:

Out of 225 hepatitis patients visiting the outpatient clinic of Hepatology Unit ,Specialized Medical Hospital, Mansoura University from October 2012 to June 2014, the study enrolled only150patients who were HCV antibodies positive (we excluded 75 patients who either infected with hepatitis B or co-infected with both hepatitis C and B or other causes of hepatitis as NASH, autoimmune, etc). They were (92 males &58 females) with their ages ranging (21:59 years). They were subjected to thorough history taking, full clinical examination and laboratory investigations including liver profile, CBC, hepatitis markers (HCV antibodies and HbsAg) and HCV-RNA detection using RT-PCR by both Taqman and SYBR GREEN I methods. This study was approved by the Ethical Committee of Mansoura University and all patients provided written informed consent before participation in any protocol specific procedure.

Specimen:

Venous blood sample of 6 ml were drawn under aseptic condition, 1 ml was collected into EDTA containing tube of CBC analysis, 5 ml were collected into vaccutainer tube with no additive, centrifuged at 3000 rpm and the serum were collected and separated into two aliquots, one used for liver profile and the was stored at -70ºC for detection of HCV RNA by real–time PCR.(The procedure is optimized for use with 140 µl samples).

Methods of assays:

1- CBC was analyzed using CELL-DYN Emerald cell counter, ABBOTT, Germany.
2- Liver profile assayed using **CobasIntergra 400** chemistry autoanalyzer (Roche Diagnostics, Basel, Switzerland)
3- HCV antibodies and HbsAg was analyzed by ADVIA Centaur CP Immunoassay System (Siemens, USA)
4- HCV Real time –PCR (TAQMAN , SYBR GREEN) as follow

Viral RNA extractionwas performed for all samples and standards by the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions**.** Viral RNA was stored at -20 ºC

HCV quantitation by a taqman probe based techniquewas performed by Artus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All tests were performed on Step one real time PCR.

Reagent preparation

The reaction mix contains all of the components needed for PCR except the sample.

Procedure

1. PCR tubes were placed into the adapters of the cooling block.
2. Thirty µl of the master mix was added into each PCR tube.

3. Then 20 µl of the eluted sample RNA was added. Correspondingly, 20 µl of one of the quantitation standards was used as a positive control and 20 µl of water (Water, PCR grade) as a negative control.
4. The PCR tubes was carefully

HCV quantitation by SYBR technique was performed by Verso SYBR GREEN 1-Step 1 qRT-PCR Kit. The primers used were:

5'-AGC GTC TAG CCA TGG CGT-3'and 5'-GCA CGG TCT ACG. The length of the PCR product was 266 bp.

Procedures:

i. For each reaction the following reagents are added to give a final volume of 25µl

Verso Enzyme Mix	0.25 µl
1-Step QPCR SYBR Mix	12.5 µl
RT Enhancer	1.25 µl
Forward primer (diluted 1/10)	1.75 µl
Reverse primer(diluted 1/10)	1.75 µl
Water	4,5 µl
Template (RNA)	3.0 µl
Total	25 µl

ii. The amplification was carried out using **1-Step QRT-PCR thermal cycling program**:

	Temp.	**time**	**No. of cycles**
cDNA Synthesis d	50°C	15 min	1 cycle
Thermo-Start activation	95°C	15 min	1 cycle
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

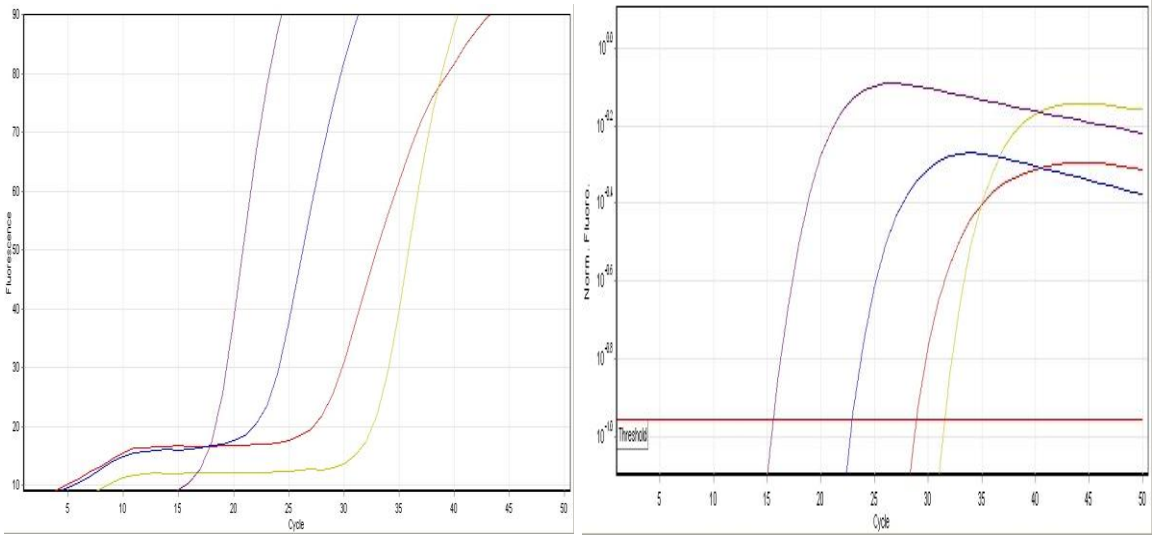
iii. A melt curve program was performed to confirm the specificity of the reaction as follow.

Denaturation	95°C	30 sec	1 cycle
Starting temp.	60°C	30 sec	1 cycle
Melting step	60°C	10 sec	80 cycle

HCV reference standards

HCV reference preparations were obtained from NIBSC (Hertfordshire, United Kingdom). Each vial was reconstituted with 0.5 mL of distilled water .This concentration is equivalent to 260000 IU/ml which was used to construct a standard curve.

Standard curve of the SYBR GREEN the



threshold line and cut off value of real time PCR (Amplification plot) standard curve sample

Sensitivity detection

In order to determine the analytical sensitivity of our test, RT-PCR was performed on serial dilutions of NIBSC standard RNA, each in 3 replicates. The sensitivity of the test was 100 copies/ml

Specificity detection

The specificity was validated with 20 HCV negative plasma used in the study samples. Melting peaks analysis on the PCR products showed that there were no primer-dimers and non-specific products and only a single peak was visible.

Validation of reproducibility

To assess the intra-assay and the inter-assay variability, RNA standards from 1 × 105 to 1 × 102 copies/ml and titrated viruses at different dilutions were tested by one step SYBR GREEN I RT-PCR assay in triplicate in a single assay (intra-assay) and at three different days (inter-assay).The coefficient of variation (CV) of threshold cycle (Ct) was determined.

Statistical Analysis

The statistical analysis of data was done by using Microsoft ***excel*** program 2010 (Microsoft Corp., Redmond, Wash.) and ***SPSS***(statistical package for social science) program (SPSS, Inc, Chicago, IL) version 16.

Results:

Table (1): Some demographic, clinical and laboratory data of the studied patients

Parameter		**Patients (n=150)**
Age (years)mean ± SD		41.2 ±10.28
Sex	**Males**	92 (61.3 %)
	Females	58 (38.7 %)
Clinical presentation	**asymptomatic**	72(48%)
	fatiguability	52(34.7%)
	Non specific symptoms	26(17.3%)
Liver profile mean ± SD • **ALT (U/L)** • **AST (U/L)** • **Total bilirubin (mg/dl)** • **Albumin (gm/dl)**		 54.97± 31.8 66.91± 34.6 1.027± 0.57 3.917± 0.46
CBC mean ± SD • **Hb (gm/dl)** • **WBCs (10^3/cmm)** • **PLT (10^3/cmm)**		 11.52 ± 2.22 5.52 ± 2.72 147.6 ± 72.3

Table (2) represents serum HCV RNA analysis by Taqman real time PCR in all studied patients.

		HCV RNA	
		N.	**%**

Negative	**<12 IU/ml**	12	8
Positive (>10 IU/ml)	**Low (12-10^5 IU/ml)**	34	22.66
	Moderate10^5-10^6 IU/ml)	94	62.66
	High (>10^6 IU/ml)	10	5.26
	Total positive	138	92
	Mean ± SD	$4.49X10^5 \pm 3.98X10^5$	
	Median (range)	$3.98\ X10^5$ ($7X10^3$-$2.1X10^6$)	

Figure (1) shows comparison of Taqman RT-PCR and SYBR GREEN real-time RT-PCR for diagnosis of HCV. HCV RNA was detected by Taqman probe in 138 (92%) of total cases with median of $3.98X10^5$. While it was detected by Syber Green probe in 134 (89.3%) with median of $3.1X10^5$.

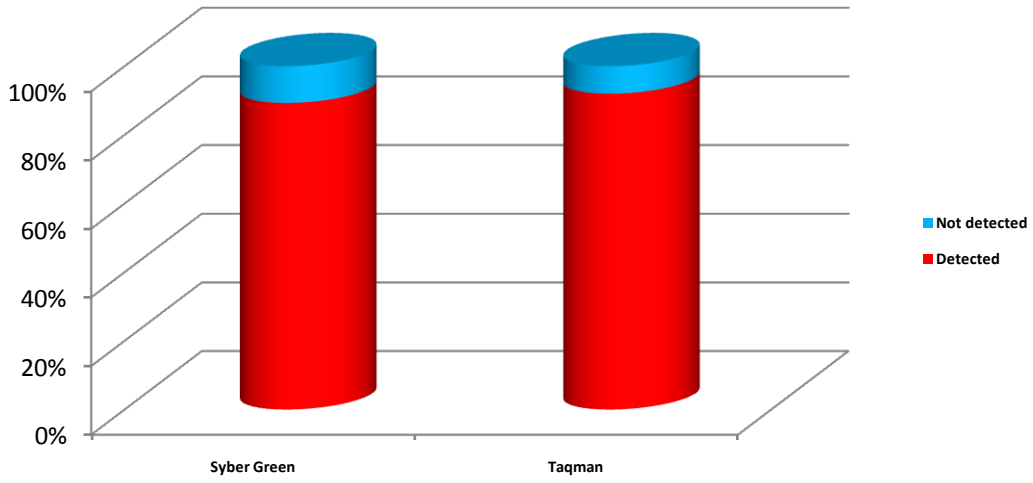


Table (3) Qualitative and Quantitative relationship between TaqmanRT-PCR and

Repetition	Result 1	Result 2	Result 3	Mean	SD	% CV
Sample 1	181,000	87,000	109,000	125666.7	49166.38	39.12444
Sample 2	35,000	9,500	15,000	19833.33	13419.51	67.66141
Sample 3	1,500	1,900	2,500	1966.667	503.3223	25.59266
Sample 4	150	45	110	101.6667	52.99371	52.12496

Sample 5	153,000	170,000	89,000	137333.3	42712.22	31.10113
Sample 6	41,000	17,000	9,500	22500	16454.48	73.13103
Sample 7	3,500	6,000	1,200	3566.667	2400.694	67.30919
Sample 8	90	95	180	121.6667	50.57997	41.57258
Sample 9	87,000	119,000	130,000	112000	22338.31	19.94492
Sample 10	14,800	16,000	9,000	13266.67	3743.439	28.21687
Sample 11	500	850	1,500	950	507.4446	53.41522
Sample 12	250	300	90	213.3333	109.6966	51.42026

Intra- assay CV (n=12) = average %CV= 45.88%

	Repetition	Result 1	Result 2	Result 3	Mean	SD	Mean of means	SD of means	%CV
Sample 1	**Run 1**	109000	87000	181000	125666.7	22338.31	125000	12679.82	10.14
	Run 2	89000	170000	153000	137333.3	22338.31			
	Run 3	130000	119000	87000	112000	22338.31			
Sample 2	**Run 1**	15000	9500	35000	19833.33	13419.51	18533.33	4751.959	25.64
	Run 2	9500	17000	41000	22500	16454.48			
	Run 3	9000	16000	14800	13266.67	3743.439			
Sample 3	**Run 1**	2500	1900	1500	1966.667	503.3223	2161.111	1319.126	61.04
	Run 2	1200	6000	3500	3566.667	2400.694			
	Run 3	1500	850	500	950	507.4446			
Sample 4	**Run 1**	110	45	150	101.6667	52.99371	145.5556	59.54301	40.91
	Run 2	180	95	90	121.6667	50.57997			
	Run 3	90	300	250	213.3333	109.6966			

Inter- assay CV= 34.43%

Table (4) illustrates qualitative and quantitative relationship between TaqmanRT-PCR and SYBR GREEN real-time RT-PCR for diagnosis of HCV.

Qualitative relation		**Taqman**		**Total**	**P value**	**Weighted κ**	**95% CI**
		Not detected	**Detected**				
Syber Green	**Not detected**	8	8	16	<0.001	0.642	0.487-0.757
	Detected	4	130	134			
Total		12	138	150			

Quantitative relation		**Taqman**				**Total**			
		Negative	Low	Moderate	High				
Syber Green	Negative	8	6	2	0	16	0.002	0.503	0.314-0.654
	Low	4	10	21	2	36			
	Moderate	0	16	62	6	84			
	High	0	2	10	2	14			
Total		12	34	94	10	150			

Figure (2) ROC curve for negative results by SYBR GREEN I, taking Taqman as a reference method.

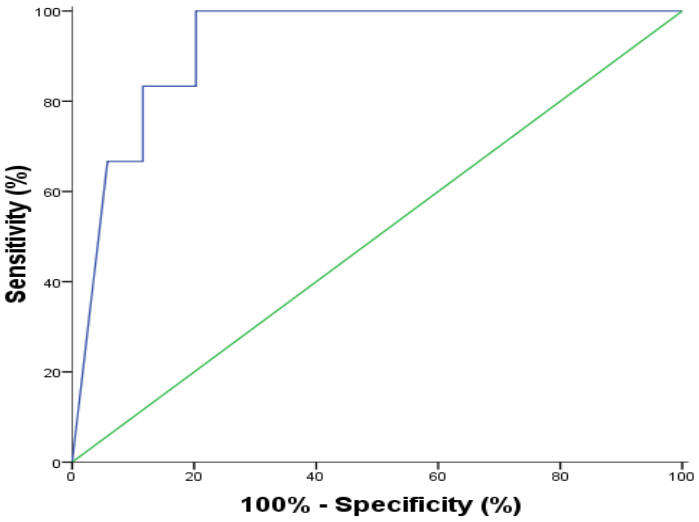


Table (5) Sensitivity, specificity, PPV, NPV and accuracy of HCV RNA detection levels by SYBR GREEN I method with reference to Taqman method.

SYBR GREEN					
IU/ml	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Accuracy (%)**
<12	94.2	66.7	97	50	92.0
105	71.0	100	100	23	73.3
106	10.1	100	100	8.8	17.3

Figure (3): Comparison of values of Taqman RT-PCR and SYBR GREEN 1 real timeRT-PCR for diagnosis of HCV. No significant differences were detected in the values of samples' populations tested with both methods (p =0.192).

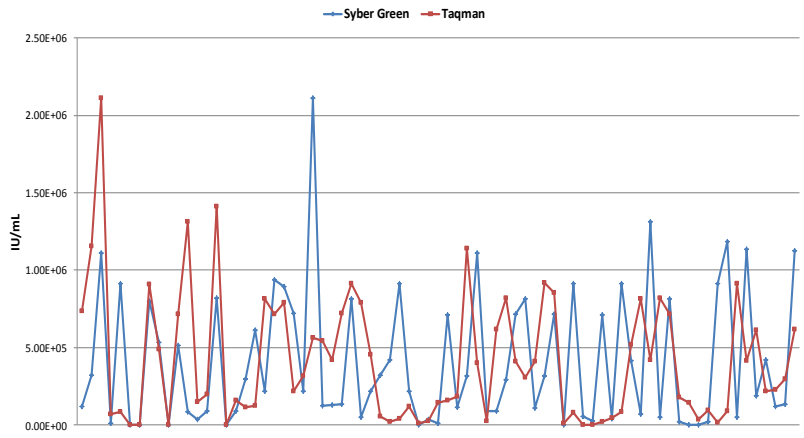


Table (6) represents the evaluation of reliability and repeatability measurement.

Evaluation of reliability measurement			
Correlation		**ICC**	
R	***P***	**Estimate**	**95% CI**
0.100	0.427	0.108	-0.137-0.340

Evaluation of repeatability measurement.				
	Mean of means	**differences mean (Bias)**	**Differences SD**	**Differences 95% CI**
IU/mL	$4.37X10^5$	2295.8	546356.8	-1068563.6-1073155

Discussion:

In the present study, the studied patients were 92 males and 58 females with a mean age of 41.2 ±10.28 years. The predominance of HCV infection in males was also reported by study of ***Saad et al., (2013)*** who studied the predictors of response to therapy in Egyptian patients with chronic hepatitis C virus genotype 4. In their study, the male to female ratio was 68/32.

Regarding PCR results, 138 out of 150 patients had HCV RNA detected in their blood by real time PCR using Taqman probe. The lowest reactive serum titer was equivalent to 7000 PCR units/mL; 34 sera (24.6 %) had more than 10 to 10^5 IU/mL (low); 94 sera (68.1 %) had 10^5 to 10^6 IU/mL (medium) and 10 sera (7.2 %) had more than 10^6 IU/ml (high). The maximal serum titer found was 2.1 x 10^6 units/mL. In comparison, the study of ***Mabrouk et al., (2013)*** analyzed retrospective data of 5,464 HCV-antibody and PCR positive Egyptian patients found that 50% had viral load <100 (x 10^3) IU/mL.

Comparison of Taqman RT-PCR and SYBR GREEN real-time RT-PCR for diagnosis of HCV, HCV RNA was detected by Taqman probe in 138 (92%) of total cases with median of $3.98X10^5$. While it was detected by Syber Green probe in 134 (89.3%) with median of $3.1X10^5$.Comparison of values of TaqmanRT-PCR and SYBR GREEN real timeRT-PCR for diagnosis of HCV. For concordance of both methods, results of the comparison of HCV RNA viral loads in serum samples obtained with repeated measures by 2 methods, tested with use of Friedman's test. No significant differences were detected in the values of samples' populations tested with both methods (p =0.192).

To evaluate the performance of SYBR GREEN method we assess both intra-assay and inter-assay precision. The mean coefficients of variation (CV) of expression values intra-assay precision (average % CV= 45.88%). While the CV inter-assay precision was =34.4%.

Reproducibility of the method was reported by ***Komurian-Pradel et al., (2001)*** through examining an HCV positive serum extracted and amplified in 10 independent experiments. The mean concentration was 5.34±0.2 Log RNA copies/ml, corresponding to a coefficient of variation of 3.7%. The intra-assay variability was also evaluated with two other HCV samples tested four times within the same experiment. The mean concentrations were 7.09±0.05 Log RNA copies/ml and 5.23±0.11 Log RNA copies/ml, with a coefficient of variation of 0.7 and 2.1% respectively

HCV RNA was detected in 130 samples and not detected in 8 samples by both methods. There was discrepancy in the results between the two methods in 12 (8%) cases. There was significant difference was found between both methods regarding detection frequency ($p<0.001$). There was substantial (good) agreement between both methods regarding qualitative assessment (Weighted κ=0.642).For discrimination between quantitations below and above detection limits of HCV RNA titers by Syber Green method, taking Taqman as a reference method. At below detection limit by Taqman method, specificity =66.7%, sensitivity=94%. PPV=97%, NPV=50%, accuracy =92%. AUC=0.928, 95% CI= 0.857-0.998, SE=0.036, p=0.001; i.e. Syber Green method can be used for detection of negative HCV RNA titers**.**

Quantitative relationship between Taqman RT-PCR and SYBR GREEN RT-PCR for diagnosis of HCV, stratification of expression results into negative, Low, moderate and high quantities, revealed moderate agreement between both methods regarding qualitative assessment (Weighted κ=0.503). Sensitivity, specificity, PPV, NPV and accuracy of HCV RNA detection levels by Syber Green method with reference to Taqman method. At <12 IU/mL level, sensitivity of Syber Green method was 94.4%, specificity was 66.7%, PPV was 97%, NPV was 50% and

accuracy was 92%. At 105 IU/mL level, sensitivity of Syber Green method was 71%, specificity was 100%, PPV was 100%, NPV was 23% and accuracy was 73.3%. At 106 IU/mL level, sensitivity of Syber Green method was 10.1%, specificity was 100%, PPV was 100%, NPV was 8.8% and accuracy was 17.3%. Notice that Syer Green method showed that at below detection limit the sensitivity was 94.2%, specificity was 66.7% due to presence of 4 false positive cases and 8 true negative cases in comparison to Taqman method. Whereas, at $\geq 5.4X10^4$ IU/mL by Syber Green method, it was the first level to have no false positive cases comparing to Taqman method.

Shahzamani et al., (2011) who intend to design and validate a low-cost assay for the detection of hepatitis C virus (HCV) RNA using rapid-cycle RTPCR. A SYBR GREEN -based real-time RT-PCR for rapid detection of HCV. They found that the minimum detection level of the assay was less than 50 IU/mL. The results on 100 plasma samples were comparable with commercial assays. The authors concluded that this method is useful for rapid qualitative detection of HCV infection and particularly suitable for routine diagnostic applications.

In spite of these advantages, analysis with SYBR GREEN I remains a qualitative assay in comparison with the Taqman method with lower intra-assay and inter-assay agreement. So detection of HCV-RNA by syber green method could be used as a good screening test for qualitative detection especially in blood donor screening.

In Conclusion:

There was substantial (good) agreement between both methods regarding qualitative assessment (Weighted κ=0.642). Quantitative relationship between Taqman RT-PCR and SYBR GREEN RT-PCR for diagnosis of HCV. Stratification of expression results into negative, Low, moderate and high quantities, revealed moderate agreement between both methods (Weighted κ=0.503). Sensitivity of Syber Green method was 94.4%, specificity was 66.7%, PPV was 97%, NPV was 50% and accuracy was 92%.

References:

1. Albertoni G', Girão M, Schor N (2014): Mini review: Current molecular methods for the detection and quantification of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. International Journal of Infectious Diseases 25. 145–149
2. Enomoto M., Nishiguchi S., Shiomi S., Tanaka M., Yokogawa T., Fukuda K., Ueda T., Tamori A., Habu D., Takeda T., Yano Y. and Otani, S., (2002): Changes in serum levels of hepatitis C virus genotype1b monitored by real-time quantitative polymerase chain reaction asa predictor of long term response to interferon-alpha treatment. Am.J. Gastroenterol. 97 (2), 420–426
3. Kim CW and ChangkM. (2013):Hepatitis C virus:virology and life cycle. Clin Mol Hepatol 19:17-25.
4. Komurian-Pradel, F., Paranhos-Baccasla, G., Sodoyer, M., Chevallier, P.,Mandrand, B., Lotteau, V. and Andre, P., (2001): Quantitation of HCVRNA using real-time PCR and fluorimetry. J. Virol. Methods 95 (1–2),111–119.
5. Mabrouk M., El-Raziky M., Zayed N., Salama R., El-Akel W., Awad T., El Beshlawy M. and Esmat G. (2013): Clinical, biochemical and pathological profiles of 5464 Egyptian chronic hepatitis C-infected patients. Hepatogastroenterology ;60(127):1731-5.
6. Ozaras, R. and Tahan, V. (2009): "Acute hepatitis C: prevention and treatment". Expert review of anti-infective therapy 7 (3): 351–61.
7. Ratge D., Scheiblhuber B., Landt O., Berg J. and Knabbe C. (2002): Two-round rapid-cycle RT-PCR in single closed capillaries increases the sensitivity of HCV RNA detection and avoids amplicon carry-over. J ClinVirol. ; 24: 161 – 172.
8. Saad Y., Ahmed A., Saleh DA. and Doss W. (2013):Adipokines and insulin resistance, predictors of response to therapy in Egyptian patients with chronic hepatitis C virus genotype 4. Eur J GastroenterolHepatol. ;25(8):920-5.
9. Schröter M., Zöllner B., Schäfer P., Laufs R. and Feucht HH. (2001): Quantitative detection of hepatitis C virus RNA by light cycler PCR and comparison with two different PCR assays. J Clin Microbiol.;39(2):765-8.
10. Shahzmani K.,Sabahi F., MeratS. and Sadeghizadeh M., et al.(2011): "Rapid low cost detection of hepatitis C virus RNA in HCV-infected patients by real time PCR using SYBR GREEN I". Arch Iran med 14(6):396:400.
11. Wilkins, T.; Malcolm JK.; Raina D. and Schade RR. (2010):"Hepatitis C: diagnosis and treatment". American family physician 81 (11): 1351–7.
12. World Health Organization (WHO) (2011):"Hepatitis C"
13. World Health Organization (WHO) (2013):"Hepatitis C"