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DEVELOPME NT AND VALIDATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR DETECTION OF HUMAN HCV GENOME

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ABSTRACT.

Hepatitis C virus is considered a major reason of liver damaging, within estimated 180 million effected peoples worldwide. This study was conducted to diagnose the RNA of hepatitis C virus through isothermal amplification method (LAMP). Total genome of HCV different genotypes was aligned by MEGA CLUSTAL-W and different primers were designed by explorer version 4.0 software. All the extracted RNA of hepatitis C virus was collected from different laboratories located at different areas of Lahore, Pakistan. Extracted DNA of hepatitis B virus and other species was also included in this study to check the specificity of this technique. Persistence of hepatitis C virus was diagnosed by and isothermal amplification technique (LAMP) on dry heat block, incubator and thermo-cycler respectively. Ladder like pattern were obtained during this isothermal amplification for positive extracted RNA and no pattern was obtained for both positive and negative control samples in the population of Lahore, Pakistan. Significant difference was also observed in time consuming between LAMP and general PCR diagnosing technique. These results suggested that special measures should be adopted to diagnose the RNA of hepatitis C virus to reduce the incidence of complications of hepatitis C which increase the mortality among hepatitis C patients.

Key Words: Isothermal, Amplifications, LAMP, Diagnosing, Complications

INTRODUCTION

Knowledge about liver diseases has grown rapidly. Repair mechanism with chronic activation lead to form highly complex basic structure. Recognition of liver fibrosis as a conversely is an important event (Gutierrez et al., 2002). Largest organ of the body is liver which is about the weight of 1200-1500g (Sherlock and Dooley, 2002).

Predominance of hepatitis C virus may be different in different areas within the alike country (Idrees et al., 2008). Studies based on hospital disclosed the 5.31% persistence in Islamabad, 4-6% (Karachi), 2.45% (Rawalpindi), 20.89% (Faisalabad), 4.06% (Multan), 9% (Mardan), 25.7% (Northern Areas) and 5% (Buner, NWFP) (Jehangir et al., 2006; Farhana et al., 2009; Chaudhary et al., 2007; Khan et al., 2004; Hashmie et al., 1999; Kazmi, 2001; Muhammad and Jan, 2005; Tariq et al., 1999). It is a major cause of metabolic diseases including, obesity, insulin resistance, hyperlipidemia and hypertension. With the increasing components of the metabolic disorders are being connected to different category of cancers. HCV induced insulin resistance impairs antiviral effect of interferon (Zayadi and Anis, 2012). Gene expression is regulated by non-coding region of micro RNA with a class of 18-22 nucleotides base pairs. Deregulation of miRNA occurs with the activation of different liver related diseases (Roberts et al., 2011).

Recently, improved methods of genotyping technology have made possible to detect HCV and its other types with different molecular techniques. Non-coding segments of HCV isolates genotyping assay (5'NCR), with the particular initiator it was established in Pakistan (Idrees, 2008).

In a recent time, a fresh nucleic acid amplification assay, which is termed loop-mediated isothermal amplification (LAMP) was organized (Notomi et al., 2000). Most important advancement of this technique for particular DNA sequencing is isothermal condition which is adjust between 63^0-65^0C. This technique had been profitably applied to diagnose the other human related viruses which include hepatitis A, B and E type (Yoneyama et al., 2007; Lan et al., 2009; Moslemi et al., 2009).Target region is defined through the addition of primers outer and inner respectively, and the primers which is termed as loop primers also could be contributed to enhance the sensibility of product. Last product is consisting of DNA molecule which shows the cauliflower like appearance, and the production can be visualized by the addition of some illuminating dye through the resulting of formation of magnesium pyrophosphate precipitate during the amplification of targeted DNA. Last results can be seen with natural light or with ultra violet radiation or by the method gel electrophoresis (Roux et al., 2009; Mori et al., 2004; Mori et al., 2001). Gaining of RNA as a multiplied template was completed through RT-LAMP technique by applying reverse transcriptase method to run the process reverse transcription as an additional step with the B*st* DNA polymerase. Isothermal amplification assay can form the strand of cDNA as a template strand for further multiplication. RT-LAMP assay has been concerned successfully for the detection of some RNA viruses (Chen et al., 2008; Hong et al., 2004; Parida et al., 2004; Pham et al.,2005; Poon et al., 2005).

MATERIALS AND METHODS

Extracted RNA of different genotypes of HCV samples were collected from global diagnostic lab and National Genetics Lab (NGL). Samples were supplied in -20^{o}C to the University of Veterinary and Animal Sciences (UVAS) department of Institute of biochemistry and biotechnology (IBBT). Samples were stored in -20^{o}C until further processing.

cDNA SYNTHESIS

All the required chemicals were centrifuged to homogenize. Each reaction tube was comprised with the volume of 20µl, contained 1µl random hexaprimer, 5 X reaction buffer, 10mM dNTPs, reverse transcriptase, nuclease free water and 10µl of template HCV RNA strand.

Firstly, all the process was run on ice to avoid the degradation of RNAs. Reaction tube containing Template RNA was run on 25^{o}C for 5 minutes, followed by 42^{o}C for 60 minutes, 70^{o}C for 5 minutes and 4^{o}C for infinity.

RT-LAMP PRIMER DESIGNING

According to the general survey studies of HCV genotypes distribution in Pakistan and all over the world full-length sequence of different genotypes were achieved from the NCBI, GenBank. Data was aligned using MEGA CLUSTAL-W. Primers were selected from the 5' NCR region of the HCV genotypes. Following Six primers were designed manually to follow the conserve region of various HCV genotypes. Forward and reverse inner primers, Forward and reverse loop primers, Outer forward and inner reverse primers. Two primers forward inner and reverse inner primers were joint by a 'TTTT' spacer. Designed primers were pasted in collective align data to check the conserve region. List of Different primers with spacer is shown in the Table I. Primers were obtained from primer explorer software version 4.0, to proceeded the LAMP amplification on MS explorer sheet.

Table I.

Primer iD	Sequence	Size
F3	CGGGAGAGCCATAGTGGT	18
FIP	GGCATTGAGCGGGTTGATCCAATTTTTGCGGAACCGGTGAGTAC	44
RIP	CGCGAGACTGCTAGCCGAGTTTTTACCCTATCAGGCAGTACCAC	44
B3	CACGGTCTACGAGACCTCC	19
FLP	GAAAGGACCCGGTCGTCC	18
RLP	GTTGGGTCGCGAAAGGC	18

RT-LAMP Reaction

This isothermal amplification was performed in a simple incubator at 65^{o}C. There was no need of thermo-cycler. Total mixture of a reaction was consisted of 25µl. And the total mixture was consisting of: 0.5 µl of forward loop (FLP) and forward inner primer (FIP), 0.5 µl of reverse loop (RLP) and reverse inner primer (RIP), and 0.3 µl of forward (F3) and reverse (R3) primer, with supplied buffer, 1.8 µl of $MgSO_4$, 0.25 µl of Bst DNA polymerase, 9.85 µl of nuclease free water, 2 µl of dNTPS, and 2 µl stored cDNA as a template. Betaine was also added. Negative control samples tubes were also added without cDNA template to check the specificity.

In last for the detection of LAMP outcomes, trisborate buffer 5X was used to prepare 2% agarose gel with 0.5 µg/ml ethidium bromide. LAMP product can also have been seen with naked eye or by gel electrophoresis. 5 µl of LAMP product was mixed with methylene blue and loaded on the gel. Gel was run in the gel tank after applying voltage for 15 minutes. Amplified products after the running of gel were apprehended using UV transluminator light at 302 nm. We could also add SYBR GREEN I for the detection of amplified product, for positive samples, in which orange color changes into yellow luminance which can be seen by human naked eye.

Mean ± S.D of general PCR and LAMP method with time consuming in minutes were calculated in table. Statistical analysis of the data was carried out by employing Student 't' test.

RESULTS

Distinction and specificity of the selected primers was tested by an isothermal amplification (LAMP) technique, with the extracted DNAs from being including, chicken, cow, buffalo, sheep, goat, camel and Hepatitis B virus. Extracted DNA samples of all the positive control samples were not detected by LAMP method (Fig I).Total extracted RNA of different HCV genotypes were first set on the incubator for iso-thermal amplification. Electrophoretic analysis of LAMP products demonstrated positive results with ladder like banding patterns (Fig II). Total extracted RNA of different HCV genotypes were second time set on the dry heat block for isothermal amplification. Analysis through electrophoresis determined LAMP products with positive results among ladder like banding patterns (Fig III). Total extracted RNA of different HCV genotypes were third time set on the PCR thermo-cycler for iso-thermal amplification. Electrophoretic analysis of LAMP products demonstrated positive results with ladder like banding patterns (Fig IV). Selected primers showed

ladder like banding patterns which were conventional among HCV genotypes. While the ladder like pattern was absent in the positive control samples. Negative control tubes without template were also run to check the specificity. No banding pattern was seen into the negative control samples. Ladder was run to find the difference between banding pattern as shown in figure. Space and size of the bands among each group of pattern was highly demonstrated in the results.

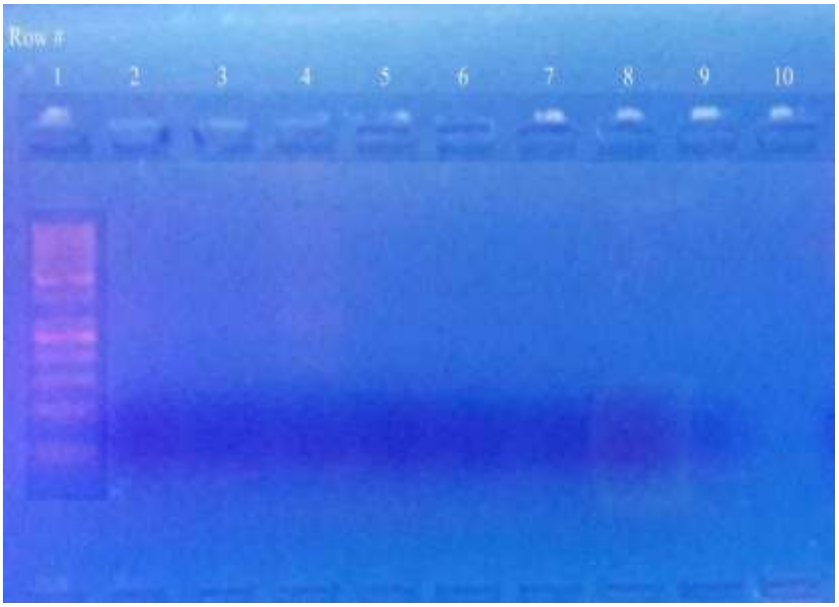

Figure I: Electrophoretic analysis of the RT-LAMP. Rows: 1: 50-bp DNA ladder (Fermentas); Positive Controls Rows 2: Chicken; 3: Cow; 4: Buffalo; 5; Sheep; 6: Goat; 7; Camel: 8: Hepatitis B Virus and Negative Controls Lanes 9 & 10.

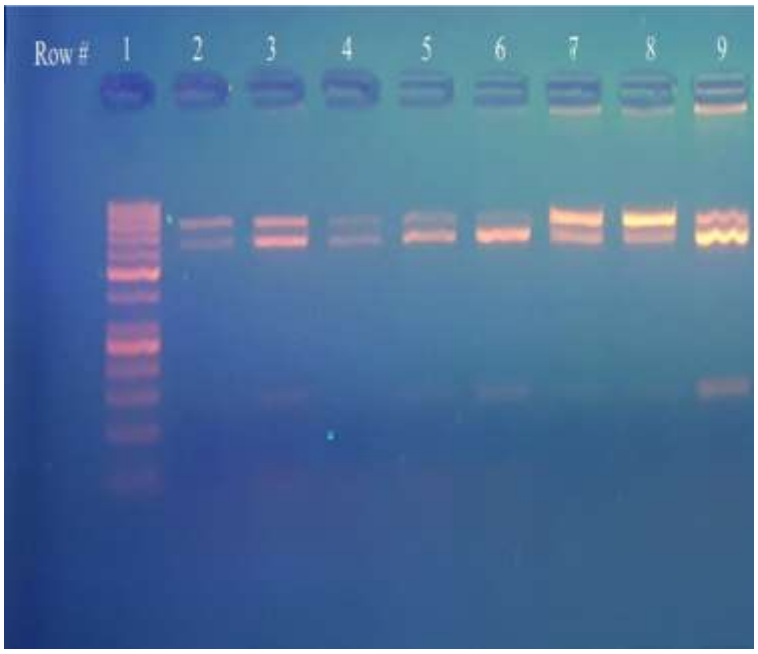

Figure II: Electrophoretic analysis after incubation of the RT-LAMP product of the 5'NCR region of hepatic virus genome. Rows: 1: 50 bp DNA ladder (Fermentas); Rows 2, 3, 4, 5, 6, 7 & 8 Ladder-like pattern product of Hepatitis C Virus of genotype 3 and Lane 9 Ladder Like pattern Hepatitis C virus Genotype 1. RT-LAMP.

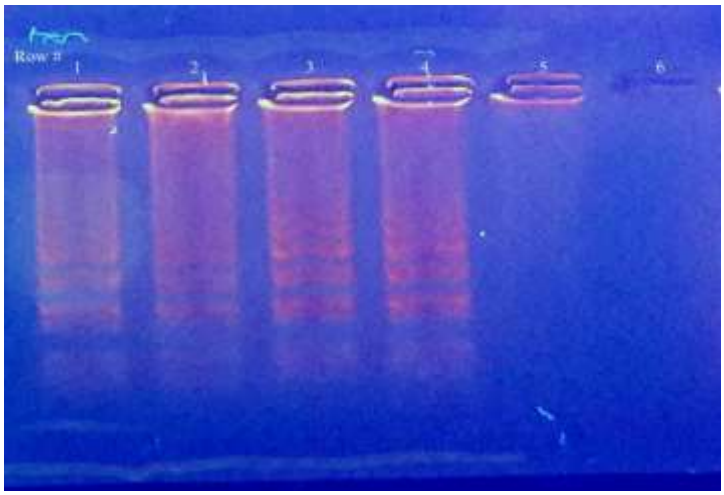

Figure III. Electrophoretic analysis of the RT-LAMP product after dry heat block of the 5NCR region of hepatic virus genome. Rows: 1, 2, 3, 4 & 5 Ladder like pattern of Hepatitis C Virus Genotype 3; Lane: 6 Negative Control.

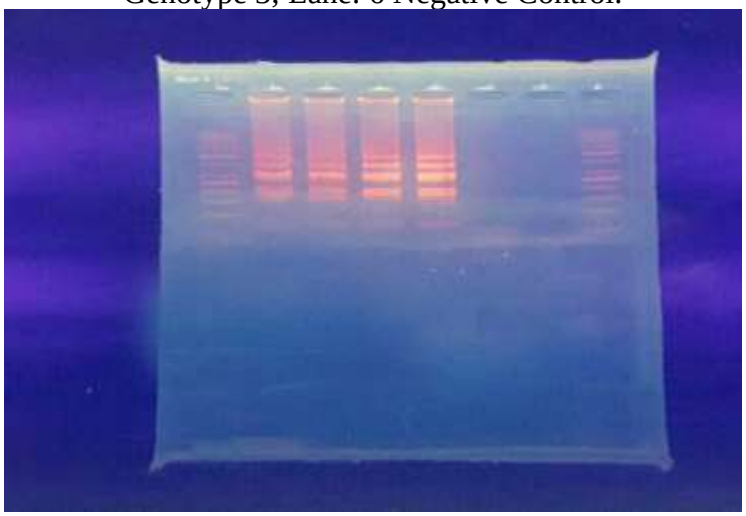

Figure IV. Agarose gel electrophoresis reults after PCR thermocyler. Targeted the 5NCR region of Hepatitis C virus. Rows: 1 & 8: 50-bp DNA ladder (Fermentas); Rows 2, 3, 4 & 5 Ladder like pattern of Hepatitis C Virus Genotype 3, Negative Control Lane: 6 & 7.

Time duration in minutes was higher for general PCR method as compared to LAMP method. In general PCR time duration was for handling the blood samples and proceeding were 120 ± 6.29 min. While for LAMP method time duration in minutes was calculated as 60 ± 3.1min. The observed difference was significantly higher for general PCR method. Table II.

Table II: Mean Time Value in Minutes For General PCR and LAMP Method Technique**.**

Category	**No. of Samples**	**Mean Time Value in Minutes (Mean ±SEM)**	**SD**
LAMP method Timing	18	60 ± 3.1	13.35
General PCR Timing	18	120 ± 6.29	26.71

***P < 0.05**

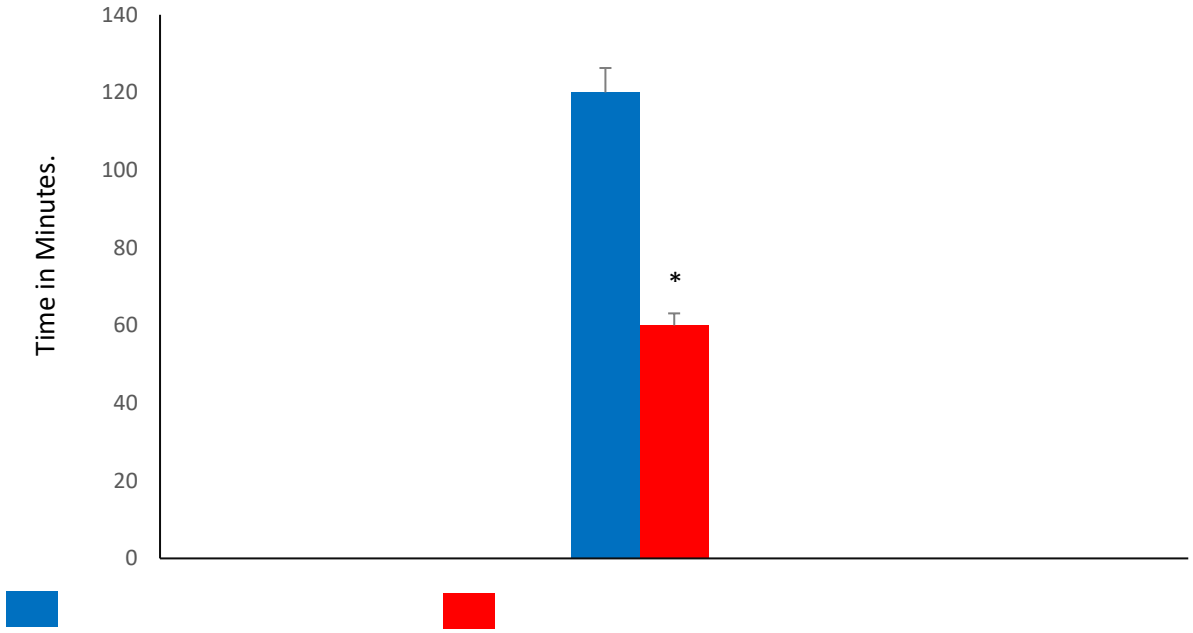

Fig V: Mean Time Value in Minutes For General PCR and LAMP Method Technique.
Values are mean ± SEM * Significant P < 0.05

DISCUSSION

This present study was conducted to check the specificity of LAMP isothermal amplification method with the selected primers. Extracted DNA samples of all the positive control samples were not detected by LAMP method. Total extracted RNA of different HCV genotypes were first run on incubator then run on dry heat block and finally was run on thermos-cycler to get amplified products on isothermal condition. Analysis of LAMP products through electrophoresis elaborated positive results with ladder like banding patterns. Selected primers created ladder like similar bands which were general to hepatic viral genome among different genotypes. While the ladder like pattern was absent in the positive control samples. Negative control tubes without template were also run to check the specificity. No banding pattern was seen into the negative control samples.

Beneficial aspects of this technique are that, they have broad expressing range as well also have low limit of detection. Most of the PCR techniques have been marketed now by different companies' brands or names these techniques increase the limits of detection with the broad expressing range and also provide straight quantification (Manns, 2001).

Resultant product size by using outer primers was in good addition with the anticipated size (256 bp) for viral RNA. Similarity between primers and coinciding nucleotide sequence was also observed 100%. Specificity of RT-LAMP was 100%, results were detected by SYBR green dye and it was correlated with the agarose gel electrophoresis outcomes (Lakshmi et al., 2016).

LAMP provided positive outcomes within 90 minutes with four selected primers and gave outcomes within 70 minutes with six selected primers. While the addition of 1.5 U of Taq DNA polymerase to enhance outcomes, and it was identified in 20 min only. Results obtained with the visual achievement were correlated with the electrophoretic analysis outcomes (Kargar et al., 2012).

In a single study obtained results were specific to hepatitis virus genotypes 1, 3, 4, 5, and 6, although resultant products were mildly positive toward genotype 3. Healthy negative control human plasma specimens were negative (Nyan and Swinson, 2016).

Developing countries have more complications as a basic indication for liver transplantation affected with hepatitis C virus (Gondeau et al., 2008; Pawlotsky, 2002). Diagnosing is not made possible due to low circulation of the virus, hence there is a need of method such as real time PCR (Lin et al., 2005; Vernet, 2004). Amplification techniques are the more basics for the diagnosing of

viral genome and also applicable in all the fields of sciences including oriented fields. Now we are facing many diagnosing related techniques. Initial studies used serum detection for positive outcomes while HCV RT-LAMP was found to be more specific and sensitive with minimum requirement of infrastructure.

Common reason for the liver and liver related disease such as hepatitis and cirrhosis, viral hepatitis is considered as a main cause for the last 20 years, 170 million peoples are affected annually calculated on worldwide level. Among this calculation 80% hepatitis C virus is responsible for liver damaging (Antonelli et al., 2008; El Serag, 2001). Above mentioned problems are not confined to these only, 20% hepatic patients further produce chronic liver related diseases such as cirrhosis and carcinoma (Chen and Morgan, 2006; Wasley and Alter, 2000). It has relative frequency of up to 4 – 5 percent/year with HCV related cirrhosis (Perz et al., 2006a; Perz et al., 2006b).

Conclusion:

In the summation, this developing and validated technique showed high sensitivity for the detection of viral genome within an hour with electrophoresis. Considering this, it made it to operate in an easy way together with its rapid and simple detection. Also provide lowest price system. Finally, the LAMP method enhances the sensitivity, than the nested real time PCR technique.

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