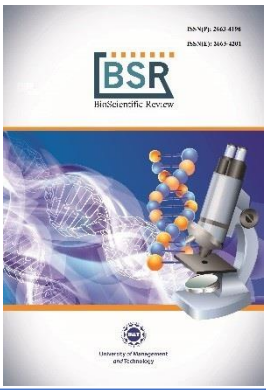

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Comparative Analysis of PCR and LIPA Method for HCV Genotypes Screening

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Abstract

Hepatitis C virus (HCV) is a major health problem worldwide. About 6% of the population of Pakistan is suffering from HCV infection. HCV has a high mutation rate and consists of seven genotypes and sixty-seven subtypes. Genotype information of patients infected with HCV is significant for its treatment. In this study, 416 HCV serum samples were collected and HCV prevalence rate was studied in different districts of Punjab, Pakistan. Nested PCR and INNO LIPA HCV-II were used for HCV genotyping and their respective performance was evaluated. This study was conducted by the approval of Lahore Clinical Laboratory and Research Centre situated at Shadman, Lahore. The highest prevalence of HCV was found in Shekhupura district followed by Bhakkar, Narowal and Okara districts, respectively. In Punjab, the most prevalent genotype was 3a (70.29%), followed by genotype 1 (5.47%), untypable genotypes (5.44%) and genotype 3a/3b (4.64%). Nested PCR was found to be more reliable than INNO LIPA-II. Nested PCR results were more accurate and only 5 samples remained untypable whereas 33 samples could not be typed by LIPA method. This study was focused on the comparative analysis of Nested PCR and LIPA method for screening HCV genotypes and their prevalence in different districts of Punjab, Pakistan. HCV genotyping is important since different genotypes require different therapeutic treatments. In Punjab, 3a is the most prevalent genotype followed by non-typable genotypes. LIPA is the most commonly used HCV genotype assay but this study found Nested PCR to be a highly sensitive and cost-effective method in this regard. This study can lead to the better selection of genotyping methods and treatment.

Keywords: HCV, genotyping, LIPA, Nested PCR, untypable genotypes

1. Introduction

Hepatitis C virus is a human pathogen that usually spreads through the contamination of blood and via other bodily fluids. HCV is the leading cause of chronic hepatitis, which further develops into cirrhosis and hepatocellular carcinoma [1]. The transmission route of HCV varies from parenteral to sexual transmission in both developed and developing countries [2]. HCV is a negative sense single stranded RNA virus that is small and enveloped by lipid bilayer. It belongs to genus *Hepacivirus* and family *Flaviviridae* [3, 4]. The virus has 5' and 3' untranslated regions which consist of 9030-9099 nucleotide based open reading frames. It encodes 3010-3033 amino acids based single polyproteins [5, 6]. There are two types of proteins, that is, structural (C, E1 and E2) and non-structural (P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Non-structural proteins perform the function of genome replication and polyprotein

translation [7]. According to WHO, approximately 150 million people are infected with HCV infection, worldwide [8]. The epidemiology rate of HCV is highest in Egypt with 15-20% prevalence rate and lowest in Scandinavia and England with 0.01% and 0.1% prevalence rate, respectively. The prevalence rate of HCV is 2.9% in Africa, [9] (Petruzziello et al., 2016), 2.8% in Asia, and 1.8% in Europe (Paper 6). In South Asia, Pakistan has a 4.8% to 6.2% HCV prevalence rate [10].

HCV genome consists of seven genotypes and sixty-seven subtypes. All genotypes respond differently to different medications and treatment, so it is important to know HCV genotype or subtype before its treatment. For this purpose, sequencing and genotyping is essential. There are different methods of genotyping, such as the sequencing of a definitive PCR-Amplified product of HCV genome, followed by its phylogenetic examination. It is the most definitive and standard method. Some other methods include HCV RNA PCR-Amplification, which is followed by second round of PCR-amplification with genotype specific primers or genotype-specific probes hybridization [11, 12, 13, 14] or by site-specific cleavage of PCR products [15]. Another method is serotyping which has two types i.e. Recombinant Immunoblast Assay Sialic Acids (RIBA SIA) and Murex HCV Serologic Genotyping Enzyme Immune Assay [16]. Real time PCR uses reflective probes and provides information regarding the progression of PCR reaction in real time. Nested PCR genotyping performs the amplification of the core region of HCV genome using nested primers. Different HCV genotypes are detected on the basis of varying sizes of different bands, since similar size bands do not appear on the same gel. LIPA method is a reverse hybridization Line Probe Assay in which biotinylated DNA PCR products are hybridized with immobilized oligonucleotide probes.

The objective of the current study is to determine the prevalence of HCV genotypes in different districts of Punjab and to compare the Nested PCR and LIPA method for the detection of HCV genotypes.

2. Methodology

2.1. Sampling

For this study, HCV positive serum samples were collected from Lahore, Okara, Sahiwal, Faisalabad, Layyah, Hafizabad, Toba Tek Singh, Multan, Sheikhupura, Narowal and Gujrat. This study was conducted by the approval of Lahore Clinical Laboratory and Research Centre situated at Shadman, Lahore. 416 samples were genotyped by Nested PCR and LIPA method after screening by Real Time PCR.

2.2. Nested PCR Genotyping

In Nested PCR, 416 samples were used for genotyping. HCV RNA was extracted from samples using Qiamp Viral RNA mini kit. The extracted RNA was converted into cDNA for which 5'-UTR region was reverse transcribed by Molony-Murin Leukemia Virus Reverse Transcriptase that converts RNA into cDNA besides M-MLV dNTPs. In the first round of PCR, 3µl of cDNA was used as a template with forward and reverse primers, Taq DNA polymerase, $MgSO_4$, dNTPs, PCR grade water, and all reagents and buffers from Invitrogen. PCR products of first round were observed on gel electrophoresis. In the second round of PCR, first round products were used. In this study, the detection of nine different genotypes was the target on the basis that different genotypes give different sized bands on gel. However, as far as HCV type

2a is concerned, its antisense primer also binds with genotype 4. So, genotype 2a specific primer was also added to the mix. In this method, Mix A and Mix B containing different primers were used for the observation of genotypes 1b, 2a, 2b, 3b (Mix A) and 1a, 3a, 4, 5a, 6a (Mix B), respectively. The analysis of PCR products was carried out on gel electrophoresis.

2.3. LIPA Method

Using LIPA, 416 HCV positive samples were analyzed. In this method, biotinylated DNA PCR products were hybridized with immobilized oligonucleotide probes. These probes were anchored with a nitrocellulose strip by a poly-(T) tail. Biotinylated PCR product forms a complex with alkaline phosphatase labeled as streptavidin. This complex forms brown precipitate when it is exposed to chromogen which results in a visible pattern of bands on the strip specific for distinct genotypes. Unhybridized PCR products were washed away from the strips. Each strip had twenty-two parallel DNA probe lines and three control lines containing sequences specific for each genotype. These strips were aligned with the reading card and the line patterns were compared with the patterns on the interpretation chart.

3. Results

3.1. HCV Prevalence in Punjab

A total of 416 samples of suspected HCV patients were screened, out of which 211 (50.72%) were of females and 205 (49.2%) were of males. Of these 416 samples, 400 were found positive for HCV. They were genotyped and analyzed on the basis of district, gender and age. Overall, HCV infection was found to be more frequent in males than females (Table 1). For females, the highest prevalence was recorded for age group 26-35 (52.67%), whereas for males it was recorded for age group 36-45 (49.67%). The prevalence of HCV was higher in middle aged people as compared to older and/or younger people. The highest HCV prevalence rate was found in age group 36-45 and it was 36.2%. The lowest prevalence rate was found in patients less than 15 years of age.

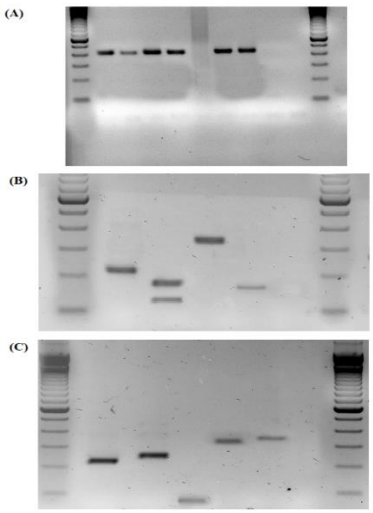

Figure 1. Detection of HCV genotypes. Gel electrophoresis of the PCR products

The frequency of HCV in different districts of Punjab is described in Figure 1. In the studied districts, Sheikhupura was found to be the most infected with 20.43% HCV prevalence rate. It was followed by Layyah with 20.19% prevalence rate. Bhakkar, Narowal and Okara were found to have the lowest HCV prevalence rates. HCV genotypes were classified by Nested PCR and LIPA method (Table 2. Figure 2), along with the number of untypable samples and mixed genotypes. Genotypes 1, 2, 3, 4, 5 and 10 were typed by both Nested PCR and LIPA. Nested PCR yeilded 5 (1.20%) untypable samples. Whereas 33 (7.93%) samples were untypable by LIPA. HCV genotypes and

subtypes classified after Nested PCR and LIPA analysis are given in Table 3.

Table 1. Gender Wise HCV Prevalence in Punjab (N= 400)

Age	HCV Prevalence	No. of Patients	Gender	
			Male	Female
<15	1.75%	7	71.4%	28.5%
15-25	8.5%	34	58.82%	41.17%
26-35	34.25%	137	47.32%	52.67%
36-45	37.25%	149	49.67%	50.33%
46-55	12.5%	50	49.01%	50.98%
>55	5.75%	23	52.94%	47.05%

Table 2. Number of Patients having Different Genotypes, Classified by Nested PCR and LIPA

Genotypes	Nested PCR	LIPA
	No. of Patients (%)	
1	16.82	16.82
2	0.96	0.96
3	73.55	66.82
4	1.68	1.68
5	0.48	0.48
10	0.48	0.48
Mixed Genotype	4.80	4.80
Untypable	1.20	7.93
Total Number of Patients	416 (100%)	416 (100%)

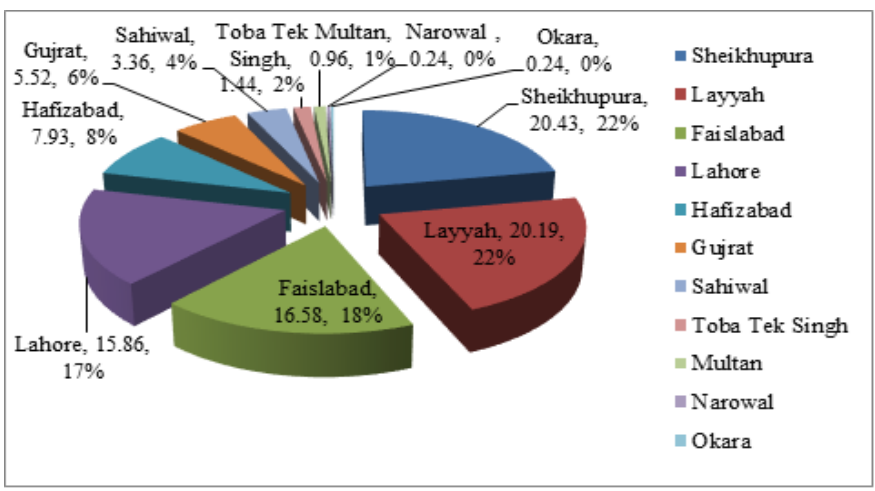

Figure 2. HCV prevalence in different districts of Punjab

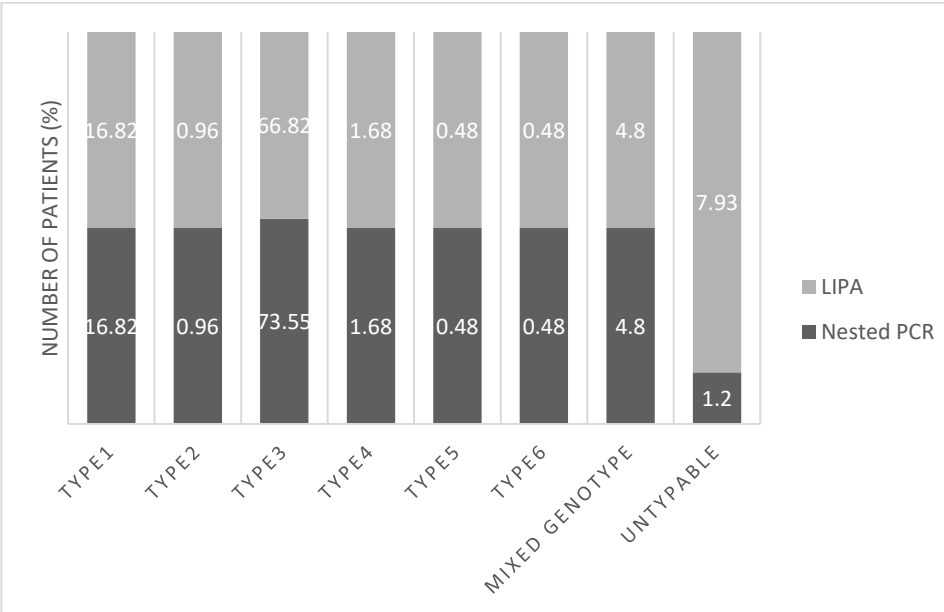

Figure 3. Number of patients having different genotypes, classified by nested PCR and LIPA

Table 3. Comparative Analysis of HCV Genotyping by Nested PCR and LIPA

Genotypes obtained by Nested PCR	Genotypes obtained by LIPA
1a	1a
1b	1b
2a	2
2b	2a
3a	2b
3b	2c
4	3a
5a	4
6a	4c
	4d
	5a
	10

4. Discussion

HCV is an infectious agent that infects human beings and causes chronic hepatitis [1]. About 6% of Pakistani population is suffering from HCV infection. Therefore, epidemiological and genotyping studies are important to estimate its prevalence and to discover its improved and effective therapeutic treatments ([17].

In this study, a total of 416 samples were genotyped by Nested PCR and LIPA method. There were 5 samples which were not typed by Nested PCR, whereas LIPA was unable to type 33 samples. Genotype 3a was found to be the most prevalent in Punjab, followed by untypable genotypes. The rate of mixed genotypes was also high in the Punjab province. People of age group 36-45 were found to be the most affected with HCV infection.

The results of the current study showed that HCV genotype 3a is the most prevalent viral genotype in the studied population. Similarly, previous studies from other cities of Punjab, Pakistan showed that 3a is the most frequent genotype [18]. In the current study, 3a was found to be the most common in age group 26-55 with the prevalence rate of 68.72%,

followed by genotype 1 with 5.24% prevalence rate. This can be compared with a previous report which showed that genotype 3a is most widespread in age group 30-57 with 89.9% prevalence rate, followed by genotype 1a with 4.19% prevalence rate [19]. In another study, genotype 3a was found to be strongly present in age group 30-55 with the prevalence rate of 92.6%, followed by genotype 1a with 3.61% prevalence rate [20]. In the current study, 3a was the most found genotype in people of ages 15 to >55 with the frequency of 67%, followed by genotype 1 with 5.5% prevalence rate. This can be compared with results from a past publication that genotype 3a is highly frequent in age group 32-58 with 77.7% prevalence rate, followed by genotype 1a with a prevalence rate of 3.45% [21]. In a previous study, the highest prevalence rate of 91.5% was found for genotype 3a, which was followed by 8.47% prevalence rate for genotype 1a in age group 29-57 [22]. Similarly, in another report genotype 3a was found to be the most prevalent with 75% prevalence rate, followed by genotype 1a with 3% prevalent rate in age group 27-55 [18].

In the current report, out of the total 416 (100%) HCV positive patients 205 (49.2%) were male and 211 (50.72 %) were female. This can be compared to results obtained in different years such as in 2015, when out of a total of 1310 HCV patients 616 (47%) were male and 606 (46.25) were female. In 2016, out of a total of 969 patients 438 (45%) were male and 531 (54.7%) were female. In 2017, out of a total of 1101 HCV positive patients 492 (44.68%) were male and 609 (55%) were female [23]. In 2018, out of a total of 59 HCV serum samples 23 (38.9%) were collected from males and 36 (61%) were collected from females. In 2019, out of 100 infected patients 40 (40%) were male and 60 (60%) were female [18].

In the current study, Nested PCR and INNO LIPA HCV II were used to classify different viral HCV genotypes. LIPA method was found less sensitive as 33 samples remained untypable. LIPA also proved to be more expensive than Nested PCR. Nested PCR was able to identify 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a genotypes with high accuracy and had a low untypable ratio as compared to LIPA method which was able to identify genotypes 1a, 1b, 2, 2a, 2b, 2c, 3a, 4, 4c, 4d, 5a and a rare genotype 10. About 20 mixed genotypes were found by both methods. The number of untypable genotypes by Nested PCR and INNO LIPA II was 5 and 33, respectively. Verbeeck and his colleagues studied HCV genotyping using INNO LIPA HCV II. Genotypes 1, 1a, 1b, 2, 3, 4, 5a, 6 and 6c-6l were identified [24]. Out of a total of 326 samples, 302 were identified correctly, 2 were identified incorrectly and 15 were uninterpretable, while 7 samples were amplified. In another study, Shemis and others found 8 genotypes (1a, 1b, 3a, 4, 4a, 4c/4d, 4e and 4h) through INNO LIPA HCV II and only four genotypes (1a, 1b, 3a and 4) through Modified Multiplex Nested PCR [17]. The rate of non-typable genotypes for LIPA was only 1 and it was 3 for Modified Multiplex PCR but the latter was more accurate and cost-effective than LIPA. Le Pogam and others conducted a comparison between INNO LIPA HCV I, INNO LIPA II and DNA Enzyme Immunoassay (DEIA) [25]. DEIA proved to be more accurate and inexpensive than LIPA method as it accurately classified genotypes 1a, 1b, 2a, 2b, 2c, 3a, 4, 5 and 1a/3a. LIPA I identified genotypes 1a, 1b, 2a, 3, 1 (not assigned to any subtype), 4/5 1a/3a and 2a/2b. INNO LIPA II was used to classify genotypes that remained unspecified by INNO LIPA I, such as it classified unspecified genotype 1 to subtype 1b and 4/5 into

4c/4d and 5a. Genotype 2a/2b was classified as subtype 2b. Thus, in the light of these findings, more sensitive, accurate and cost-effective genotyping methods are required due to the fact that Pakistan is a developing country and has a low budget to spend in therapeutic areas.

In the current report, 3a was found to be the most prevalent genotype in Punjab followed by untypable genotypes and genotype 1a, respectively. The highest prevalence was recorded for Sheikhupura, with Bhakkar, Narowal and Okara being districts with the lowest prevalence. LIPA method proved more expensive and less specific than Nested PCR.

5. Conclusion

HCV is a growing disease in Pakistan [14]. We found a high prevalence of 3a type in different cities of Punjab but we did not cover all cities of the Punjab province. Future studies should also cover other cities of Punjab to highlight the actual burden of disease. It is important to conduct research about HCV genotypes at every level as its treatment varies with its respective genotype and subtype. In comparison to LIPA, Nested PCR was found more efficient, sensitive and cost-effective for analyzing the samples. Thus, there is a need to discover therapeutic treatments against HCV that are effective, low cost and have high SVR with minimum adverse side effects.

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