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HEPATITIS C VIRUS;

DETECTION OF HEPATITIS C VIRUS RNA ALONG WITH ITS GENOTYPE IN THE SALIVA OF TREATMENT NAIVE HEPATITIS C VIRUS INFECTED PATIENTS.

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ABSTRACT… Introduction: The current study aims to detect the presence of Hepatitis C virus Ribonucleic Acid in the saliva of confirmed infected male patients. At present there are a lot of genotypes of Hepatitis C virus have been reported worldwide but in our country genotype 3 and 2 are more common than the rest. That is why the objective of this study was not only to detect this virus in the saliva of known infected patients but also to determine their genotypes in positive secretions as well. **Study Design:** Descriptive cross sectional study. **Setting:** A clinic located in Lahore and The PCR LAB, Jail Road, Lahore, Pakistan. **Period:** 13 Months. **Material and Methods:** A sample size of 100 hepatitis C positive patients (statistically calculated), in which the anti-viral therapy was not started, were selected with regard to inclusion and exclusion criteria by non-probability, convenient sampling. Saliva (2 ml) was taken in a sterile container, to detect the occurrence of Hepatitis C virus by Reverse Transcriptase- Polymerase Chain Reaction and subsequent genotyping by multiplex Polymerase Chain Reaction. **Results:** Out of 100 patients only 7% patients had virus in their saliva. Out of 100, the total saliva positive samples were 7 in number. The subsequent genotyping of the positive samples showed that out of 7 positive cases, 4 cases were of Genotype 2a, 2 cases were of genotype 3a, and only 1 case was of genotype 4. **Conclusion:** There was very less percentage of Hepatitis C virus Ribonucleic Acid found in the salivary secretions obtained from the patients. Moreover the patients who had positive secretions were mostly of genotype 2a whereas genotype 3a is known to be most significantly present among the population of Pakistan. This study may lead to better planning and management in the transmission of the disease, control and its spread.

Key words: Hepatitis C Virus, Genotype, Ribonucleic Acid, Saliva,

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INTRODUCTION

Hepatitis is inflammation of the liver, and the main cause is viral infection. There are 6 different viruses that may lead to the development of hepatitis, that are A, B, C, D, E, and G. Among them, the types, hepatitis A and hepatitis E spread through the feco-oral route, while B, C, D, and E Hepatitis mainly passes through blood or blood products. Other sources have also been noted for the spread of sperm, vaginal secretions, saliva and tears. Hepatitis C virus infection in Pakistan is commonly referred to as Kala Yarqan.[1] The prevalence of hepatitis C virus and its genetic distribution has been widely dispersed across the world; around 170 million people worldwide are known to have been infected with the virus.[2]

The Centers for Disease Control and Prevention (CDC) showed that infection of humans with hepatitis C virus accounted for about 60% of the sources of injecting drugs, 15% through sexual contact, 10% through transfusions, and 10% via other occupying the roads to develop the disease are unknown.[3,4] The presence of HCV in the saliva of positive hepatitis C patients has also been confirmed from a study.[5-7] The presence of HCV (originally referred to as “hepatitis A, non-B”) was assumed in 1970 and confirmed in 1989.[8-10] Viral hepatitis is a contagious disease caused by the hepatitis C virus, which mainly affects the liver. At first, they remain without symptoms, but when they are chronic, they can lead to scars and cirrhosis of the liver after several years. Liver failure and carcinoma may occur in late events

of liver cirrhosis.[11,12] Various studies have been conducted to determine the genotype prevalence and it has been found that genotype 3 is the commonly prevailing genotype in Pakistan. This type shows an improved response to combination therapy with the interferon and ribavirin.[13-19]

According to the Australian Government Health Department, the concentration of the virus (viral load) must be high enough to pose a threat to infection with HCV. The common method of spreading the hepatitis virus is blood and blood, which means that the infected blood must enter the blood of the uninfected persons. Although body fluids have shown evidence of HCV, low viral load is an obstacle to proliferation from one person to another. Slots or openings in the skin are potential sites for HCV entry.[20,21] Usually, in young people or women, acute infections do not spread to adults and are automatically resolved in 10% to 50% of cases.[22] Most patients (80%) have a chronic infection with the virus. During the first few decades, the signs and symptoms of liver cirrhosis and stroke are still silent. Several years later, the rate ranges from 10% to 30% in 30 years. If the patient is infected with HIV or hepatitis B, the risk of cirrhosis is greater and more common among patients with alcohol abuse and male patients, and the risk of developing hepatocellular carcinoma.[23,24]

Secondary prevention of HCV infection, i.e. early diagnosis and immediate treatment can help to reduce disease and prevent the spread of the disease to others. Accurate screening methods should be used to determine the most appropriate treatment for patients. To diagnose HCV, enzymatic immunosuppression was used to identify the existing antibodies against HCV in the blood. If this test result is positive, a confirmation test is performed to validate the assay and determining the viral load. The viral load has been usually determined by utilizing the technique of polymerase chain reaction (PCR). Antibodies to the virus take longer to appear in the blood whereas through PCR, the RNA of HCV can be detected in only one to two weeks after the infection is contracted.[25,26]

The following study aims to detect HCV RNA in saliva of infected patients by RT-PCR confirmatory test followed by performing molecular determination of HCV genotype in saliva secretions of patients by multiplex PCR.

MATERIALS AND METHODS

Study Design
Descriptive cross sectional study was the basic design of this study.

Study Setting
This research was conducted at a clinic located in Lahore and The PCR LAB, Jail Road, Lahore, Pakistan. This clinic is a primary care facility, which deals with all general medical and surgical cases as outpatient cases. The PCR LAB is one of the renowned private laboratory of the town which provides the facility of all basic and special tests under one roof.

Study Duration
13 months.

Sample Size
This study included 100 patients who already had confirmed HCV infection. The most of the patients for study were taken from catchment area. The sample size was calculated by the following formula, keeping the confidence interval 95% and margin of error 10 % (as margin of error can be from 1 to 10%). The sample size should be 96 that is 100.

$$n = \frac{Z_1 - \frac{\alpha}{2} \times P(1-P)}{d^2}$$

$[Z_1 - \alpha/2] = 1.96$ (for 95% confidence level)
P = Studied population of HCV in Saliva i.e.± 52.4% [21]
d = margin of error i.e. 10% (it can be from 1 to 10%)
n = Sample size i.e. 96 = 100

Sampling Technique
100 male Hepatitis C virus patients, already confirmed by PCR, were selected by non-probability convenient sampling.

Sample Collection

After informed consent, drug and clinical history was obtained from the patients by using questionnaires. Most of the study patients were from Bedian road Lahore cantt due to the feasibility regarding patients' samples collection. It was very difficult to select already confirmed (RT PCR) Hepatitis C virus RNA patients especially the ones who never had any antiviral therapy before. For this purpose, free screening camps were placed as well to gain the said number of sample size. All those people who turned to be positive in screening were requested to go for RT PCR Qualitative test. Out of those who were confirmed positive were given the consent forms and with the help of inclusion and exclusion criterion the selected patients were requested to provide their saliva samples in sterile containers respectively at clinic. Weekly, 2 to 3 samples used to be collected on only 2 specific days a week and subjected further for RNA isolation, optimizing of RT-PCR and genotyping accordingly at laboratory. The screening of the people leading to RT-PCR qualitative test of the suspected individuals was conducted simultaneously with the sample collection from these patients. Hence no specific time duration was recorded for the patients when they were first detected.

Saliva Sample

Under the roof of Clinic the saliva samples were obtained from the patients who were enrolled for this study program. Before collection of saliva sample the patients were requested to rinse their mouth with tap water to avoid any food particle or anything, which could hamper the saliva. To ensure saliva stimulation they were asked to think about any citrus fruit like lemon / oranges or any tasty meal instead of chewing to avoid any pressure on the teeth. Patients were asked to spit into a sterile container, which was already provided to them to obtain saliva samples. The samples were macroscopically observed to be free of blood right on the spot before sending it to the laboratory. Whole saliva samples (approx. 2 ml) were then kept into a cold chain box and then transferred to the laboratory for testing.

Inclusion and Exclusion Criterion for HCV Patients

Inclusion Criterion

- Gender: Male
- Age: 18 to 60 years
- Patients who were HCV RNA positive as already determined by RT-PCR.
- Patients who were not on any antiviral therapy.

Exclusion Criterion

- None of the patients had any disease / diseases like Sjogren's syndrome, which could affect the secretions of the body
- None of the patients had any dental problems like gingivitis, bleeding gums, oral fissures or oral ulcers.
- Not willing to provide their body secretions i.e. saliva.

Clinical history profile of HCV patients by filling the designed proforma

The clinical history of each patient was obtained to fulfill the selection criterion. The only confirmed hepatitis C virus cases that had never taken any antiviral therapy before were selected. From those selected patients the careful dental, bleeding and drug history, which could hamper the body secretions, was inquired in order to select an appropriate sample for the study. All the previous known reports if present, like ultrasound abdomen, genotype were also recorded.

SAMPLE PROCESSING

Storage of Samples

One ml of sample (saliva) was mixed with equal volume of Trizol reagent and stored at -20 ^{0}C for subsequent RNA isolation.

1. **RNA Isolation from Saliva Samples**
   By using commercial viral isolation kit, RNA extraction was done.
2. **Quantification of RNA**
3. **Molecular Detection of HCV in Patients Secretions**

o **cDNA Synthesis**
Single stranded cDNA of each sample was synthesized from the extracted RNA sample

by reverse transcriptase method.

- **RT-PCR Analysis**
  A nested RT-PCR was then performed for the confirmation of HCV presence or absence in body secretion (saliva) as described earlier.[27]

HCV Genotyping of the Positive Cases

The genotype of HCV was done in only positive samples of saliva, in which the HCV was detected by RT-PCR. The genotyping was done by sensitive multiplex PCR strategy as described earlier.[28]

Data Analysis

Data Analysis was done with the help of SPSS 22.0 and the results were written and tabulated accordingly. Frequencies and percentages were used to formulate the result.

RESULTS

Patient's status according to their Age.

Age of Patients	Frequency	Percentage
20-29	35	35%
30-39	42	42%
40-49	18	18%
50-59	05	05%
Total	100	100%

Table-I. Frequency distribution of patients according to their age

The total number of male patients in this study was 100 with the mean age of 33.86 years and standard deviation ± 7.9. The range of the age distribution was 36 years with maximum age 56 and minimum was 20 years. The maximum number of patients included for this study were 42 which were of 30 to 39 years of age group, whereas the minimum number of patients for this study were from 50 to 59 years of age and the total number of patients for this group was just 5. The rest of the two groups, one was of 20 to 29 years age, which had 35 patients and the group of 40 to 49 years, was consist of 18 patients.

Patient's saliva HCV RNA status according to lab test results.

HCV RNA LAB Status	Percentage (%)				Total
	Positive	%	Negative	%	
HCV RNA IN Saliva	07	7	93	93	100

Table-II. Frequency distribution of patients saliva HCV RNA status according to lab test results

The reverse transcriptase polymerase chain reaction test was performed on saliva secretions of 100 known HCV positive patients, to detect the HCV RNA. Out of 100 samples, the frequency of saliva samples, which were obtained from the positive patients were 7 i.e. 7 %, whereas the 93 saliva samples i.e. 93% did not have HCV RNA.

PCR Analysis of HCV-RNA on Agarose Gel Electrophoresis

Figure-1 shows the positive or negative results according to the presence or absence of HCV RNA in saliva secretions, of confirmed hepatitis C virus infected patients by the help of RT-PCR. For this the agarose gel electrophoresis process is used to separate DNA or proteins in a matrix of agarose. It is the first step for analysis of specific DNA and RNA fragments.

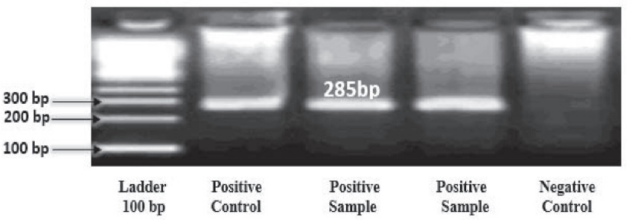


Figure-1. PCR analysis of HCV-RNA on agarose gel electrophoresis

Genotypes Status of HCV RNA Positive Samples

The subsequent genotyping of the positive samples were done by the help of multiplex PCR as shown in the Figure-2.

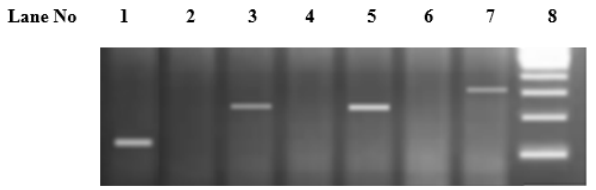
The results showed that out of 7 positive cases, 4 cases were of Genotype 2a i.e. 57.1%, which is the maximum number among all the detected genotypes. Then next comes genotype 3a, which accounts only 2 in number or 28.5%, and only 1 case i.e. 14.4 % was of genotype 4. This can clearly be observed in Table-III.

Genotype	Frequency	Percentage (%)
2a	04	57.1
3a	02	28.5
4	01	14.4
Total	07	100

Table-III. Frequency distribution of genotypes of HCV RNA positive samples according to Lab results

Genotype of HCV-RNA by Multiplex PCR

Figure-2 shows the key of HCV genotyping. Multiplex PCR is a method, which is used for the detection of specific genotype of hepatitis C virus.



Key of HCV Genotyping

Lane 1:	139bp	2a
Lane 2, 4, 6:		Negative
Lane 3:	232bp	3a
Lane 5:	232bp	3a
Lane 7:	176bp	4
Lane 8:	100bp	Ladder Marker

Figure-2. Genotype of HCV-RNA by multiplex PCR

DISCUSSION

Infections related to the hepatitis C virus has become a burden of disease in Pakistan. Despite new revolutions in the therapeutic field, the number of HCV patients has increased significantly in this region of the world during the past decade. Although the hepatitis C virus infection rate in the developed countries that include the United States is declining, developing and underdeveloped countries it is continuous to be a major health problem.[29]

In general, HCV is present in the bloodstream and can also be found in other body fluids of the infected person. Therefore, in this study, HCV RNA was studied in saliva from 100 naïve patients (untreated patients) and HCV patients whose genetic patterns were confirmed. There are many international studies that verifies the occurrence of HCV RNA in saliva, but it is difficult to find data on HCV RNA detection and subsequent genotyping.

The distribution of HCV genotypes shows geographic variation. According to researchers, in America the most common genotype found is 1, whereas is in Pakistan it is genotype-3.[30] One of the local researches clearly shows the presence of mixed variety of HCV genotype among the residents of Lahore. Out of 211 males, 24 (4.9%) had genotype 1, 168 (35.4%) had genotype 3a, 03 (0.6%) had genotype 3a, 10 (2.0%) had genotype 4, 01 (0.2%) had co-infection of genotypes 1 & 3 and un-type-able were 5 (1.02%).[31] The following study clearly depicts that the genotype 3a was the most common genotype found in the population of the city of Lahore. Another study regarding the different types of HCV genotypes in all over Pakistan was published online in October 2014 at US Library of Medicine National Institutes of Health. This clearly showed that 3a was the most common genotype i.e. 39.4% followed by genotype 2a, which was 24.93%. In the regions of Punjab and Sindh, the predominant genotype of HCV is 3 and in the area of Khyber Pakhtunkhwa the most common genotype is 2. A point of consideration in this research was the increasing incidence of genotype-2a and decreasing trend of genotype-3a in our country.[32]

The total number of patients for the current study was 100 and out of them the most number of patients were from the 30 to 39 years old age group, which accounts the total 42 patients whereas only 5 patients were from the 50 to 59 years of age group. The study shows that the total number of genotype 2a were four out of seven positive patients which had HCV RNA in their salivary secretion, whereas genotype 3a, which is the most common genotype in Pakistan, were two in number and only one genotype 4 detected in a patient. So only 7 % of the total patients had positive HCV RNA in their secretion i.e. saliva samples.

Genotype-3 comes at second number after genotype 1 according to the world statistics, whereas Genotype-3 is the most commonly present in Pakistan. According to Pastore, it is unlikely to have HCV in Saliva of known genotype 3a patients. In this study comprising of 46 HCV patients, only 36.13% saliva samples were positive for HCV. HCV has been detected in the saliva,

semen, and other human body fluids in confirmed plasma positive patients. Another research, which showed evidence of presence, HCV RNA in saliva in HCV positive patients stated that cell-free saliva might be one of the causes of low frequency of HCV RNA found in their saliva samples.[20] One of the researches stated that out of 61 saliva samples, 34 came out to be positive for HCV RNA (52.4%) concluding its presence in saliva of the patients.[22] These almost all studies, which were conducted, the data was taken from that part of the world where the usual HCV genotype is 1. The epidemiology of HCV infection in Pakistan shows greater prevalence of genotype-3a. It has been noticed in this study that genotype-2a presents a greater risk of being excreted in the secretions like saliva. Hence the health care providers should emphasis on counseling of patients with genotype-2a with greater care with regard to changes of their behavioral activities.

CONCLUSION

Out of the 100 samples of the salivary secretions of HCV positive patients' obtained, HCV RNA was detected in a total of 7 patients. Among these, it was found that the presence of HCV in the saliva was confirmed for these 7 samples. A total of 3 genotypes were observed in the study the most common being genotype 2a accounting for four of the patients. Genotype 3a was found in 2 patients and genotype 4 was detected in only one patient.

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Cowards die many times before their actual **deaths**.

"Julius Caesar"

AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	M. Usman Sheikh	Did all the main research work, especially all the sample collection, testing and its analysis. Compilation of national and international research data for literature review. All the discussion after compiling result, its conclusions and appropriate recommendations based on the results was also done.	
2	Malahat Alina Usman	The major help was in editing, scripting and helping in gathering the research data related to literature review.	
3	Saira Shabbir	Helped in planning and organizing the sample collection after getting the knowledge about the latest techniques to execute the research process.	