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RESEARCH ARTICLE

Study of HCV Genotypes and viral Blood Load at Thalassaemic Children in Mid-Euphrates Area, Iraq

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Abstract

Background: Hepatitis C virus infection is a cosmopolitan infection that has been considered a serious health problem to whom at risk like thalassaemic patients.
Patients and Methods: Serum samples from 563 of thalassaemic children from four Iraqi governorates were collected and subjected to investigation for the presence of HCV infection and genotyping through by use of reverse transcription PCR (RT-PCR), positive samples were further examined by RT-qPCR for estimation of viral blood load.
Results: percentage of infection at thalassaemic children was 17.4% (98 /563), genotype 4 was the dominant type that appeared in 8.16% of infected cases followed by genotypes 1b, 2b, 3a, and 6a which found in percentages of 8.16%, 2%, 1% and 4.1%, respectively; The total mean of viral load at the study groups was 5840976.9 (5.8 x 10^6) IU /ml of blood.
Conclusions: The present study emphasized the public health importance of HCV infection in thalassemic children at the study area , genotype 4 is dominant type.

Recommendations: Further studies at different areas, peoples and age groups are required to investigate the molecular epidemiology of HCV in our community.

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INTRODUCTION

Hepatitis C virus infection is one of the major public health problem in both developed and developing countries, it considered as the first cause of post-transfusion and community acquired non A, non-B hepatitis. It account for more than (20%) of acute viral hepatitis , (60% - 70%) of chronic hepatitis, (30%) of liver cirrhosis and end stage liver disease, and cause the major cases of liver cancer, certain conditions were at high risk like those whom continuously blood receivers especially thalassemic children [1,2].

According to the International Committee on Taxonomy of Viruses (ICTV) at 2011, HCV was classified under *Flaviviridae* family; has linear, single stranded, positive sense (positive polarity) RNA genome, approximately 9400 bases in length; this single and large open reading frame (ORF) encodes a polyprotein of more than 3000 of amino acids that represent 98% of all the nucleotides of the viral genome[3].

The virus exhibit substantial genetic variation so there are at least eleven distinct viral genotypes : 1 through 11 , and variable number of sub- types found usually in specific geographical regions and display significant difference in their aggressiveness and in response to antiviral therapy [4]. Genotypes 1, 2 and 3 are widely distributed throughout USA, Europe, Australia and East Asia; genotype 4 is largely confined to the Middle East, Egypt and

central Africa; genotype 5 and 6 are found predominantly in South Africa and South East Asia; HCV genotypes 7, 8, and 9 have been identified mainly in Vietnamese patients; while genotypes 10 and 11 have been reported from Indonesia [5].

There are an increasing evidences that HCV genotypes possess different biological potentials, certain genotypes are more frequently associated with severe forms of liver disease and more amenable to interferon treatment make the genotyping of infecting virus is one of the prime predictors of the disease progression and the response to antiviral therapy. Consequently, typing of HCV isolates becomes an additional tool in diagnosis of the infection [6].

The distribution of HCV types and viral blood load at thalassaemic children in our country is still unclear because of the marked paucity in the studies about them, the purpose of this study was to estimate the prevalence of HCV genotypes and their blood load among children with thalassaemia from different regions of Iraq.

The most efficient transmission route of HCV is through large or repeated direct percutaneous exposures to blood and blood products, e.g., blood transfusions, organs or tissues transplantation from infectious donors, it has been estimated that approximately 2 million HCV infections are acquired annually worldwide from contaminated health care injections, blood reception and haemodialysis process [7,8]. Thalassaemic children are considered as high risk group because they are continuously blood recipients[9].

viral load is the amount of specific viruses found in a given volume of blood from infected patient, the viral load in the HCV infected subjects range from 10^2 to 10^9 genomes per ml. A high viral load is considered greater than 800 000 IU/ml whilst a low viral load is defined as less than 800 000 IU/ml.

There is a direct association between viral blood load and the transmission rate of the virus besides that the clinical studies had found that patients with low HCV RNA levels had a 15% to 39% better response rate than those with high HCV RNA levels, a finding that is consistent across trials using different formulations and dosages of interferon, the pretreatment viral load serves as a baseline for comparison with subsequent measurements during treatment[10, 11].

In addition there is marked proportional correlation between viral blood load and viral shedding in semen and saliva and thereby increasing of viral infectivity [12,13].

In spite of their importance, there was a marked paucity in the studies about HCV genotypes in our country; The aim of this study was to identify HCV genotypes and viral blood load in thalassemic children at the study regions in order to obtain a real knowledge about the genotypes epidemiology besides to getting a future predictable data about the burden of HCV caused progressive liver diseases.

Patients and Methods:

Serum samples were collected from a total of 563 thalassaemic children ranging from (2-17) years from four Iraqi governorates; Najaf, Babylon, Qadisyia and Karbala were included in this study that performed from the beginning of June 2013 to the end of August 2014. A consent were taken from all patients and/ or their families. All samples were subjected to investigation of HCV genotypes by use of reverse transcription PCR (RT-PCR).

Genotyping through Reverse Transcription PCR (RT-PCR), the protocol consists of 4 main procedures: Extraction, Reverse transcription, Amplification and Detection.

1- RNA Extraction:

It was implemented by use of Ribo Virus Columns Extraction kit, Sacace Biotechnologies, Italy, the procedure was done according to the manufacturer's instructions; at which 150 µl of serum was loaded to each Column followed by consecutive steps of washing and finally the RNA that attached to the silica membrane in the Ribo Virus columns were dissolved in nuclease-free water and aggregated at button of Eppindorff tubes by centrifugation than plugged rapidly and stored at -70°C until use.

2- Reverse Transcription: This process was done by used of Reverse Transcription System Kit, Promega Corporation, USA. The procedure have been done inside the biological cabinet and the steps conducted according to manufacturer's instructions, The frozen RNA samples directly thawed inside incubator at 70˚c for 10 minutes then added to a mixture from kit components, mixed and underwent several steps of heating cycles. The mixture hence contain cDNA , stored at -20˚C until use.

3- Amplification: Mixture sets of primers were used according to Ohno *et al*.[14] the primer solutions were prepared by dissolving the lyophilized primers, Alpha DNA company, Canada in Tris-EDTA buffer as the instructions of the manufacturer. Two rounds of amplification had implemented, the products of first round were used in the second round [14].

4- Detection**:** Reaction products were analyzed by 2% agarose gel electrophoresis, 8 µl of amplification products added in each well. The products of the two mixture of primer were separately loaded. The DNA bands were observed through viewing under UV transilluminater, and pictures took by Bio-Document Analyzer, Biometra, Turkey.

5- Measurement of Viral Blood Load:
All positive samples were analyzed by use of Quantitative Real Time PCR (RT-qPCR) to obtain viral blood load; Quantitative real time reverse transcription PCR kit from Genekam Biotechnology A.G. company, Germany.
According to the manufacturer´s instruction, About 2µl of the cDNA template of the positive samples were added by use pipette, tips with filter to each specific reagent containing micro-tubes with avoiding of touching the wall of the micro-tubes, then plugged and centrifuged at 1000 rpm for 25 second. The Real Time PCR Thermocycler: Exicycler^{TM} Quantitative Thermal Block, Version 3.0. Bioneer Company, Korea. Following programme was employed as in manufacturer´s instructions: 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Results:

The RT-PCR assay reveals that the total thalassemic children whom positive to HCV infection were 98 out of 563 with percentage of 17.4%; the highest infection rate was at Babylon governorate which was 19.76% whilst the lowest percentage was recorded in Kerbala governorate (15.38%). The statistical analysis does not found out any significant difference among these percentages of infection in the study areas at level of p- value ≤ 0.05. (Table-1).

Table (1): Percentages of HCV Infection in Thalassemic Children from Four Iraqi Governorates

Governorate	No. of Samples	No. of HCV Infected Children	Percentage of HCV Infection
Najaf	143	26	18.18%
Babylon	167	33	19.76%
Kerbala	117	18	15.38%
Qadisyia	136	21	15.44%
Total	563	98	17.4%

The genotyping by use of RT-PCR show that 83 patients were infected with genotype 4 from total 98 HCV positive cases with percentages of 84.6% which indicates that genotype 4 is the predominant type.

Genotype 1b rank below genotype 4 and appeared in 8 cases (8.16%), followed by both genotypes 6a which appeared in 4 cases and percentage of 4.1%, where as genotype 2b was found only in two cases (2%) whereas G3a found in just one case (1%) which represent the lowest rate among others (Table-1), (Figure-1).

Table (2): The Genotyping Results of HCV infection in Thalassemic Children by Using of RT-PCR Analysis

Genotypes	No. of Cases	Percentages
G.4	83	84.6%
G.1b	8	8.16%
G.2b	2	2%
G.3a	1	1%
G.6a	4	4.1%
Total	98	-

Viral Blood Titter:

The modern molecular quantitative technique by using RT-qPCR for measurement of the viral blood loads (viral concentrations) in the blood of HCV patients in present study was registered that the viral titers were ranged from less than 100 (1×10^2) IU / ml of blood to about 4.5×10^8 IU / ml of blood and the total mean of viral load

was 5840976.9 (5.8×10^6) IU / ml of blood, whilst, the total median of the viral blood load was 36000.000 (3.6×10^4) IU /ml of the blood.

The means and medians (mean, median) of viral load in IU / ml of blood in the study areas were: (3828286.6, 450000) respectively.

The one way ANOVA analysis reflect no significant differences in viral load within and among the study groups at level of 0.05 (Table-3), (Figure-2).

Table (3): The Viral Load in IU / ml of Blood of the Infected Patients

Patients NO.		Najaf	Babylon		Kerbala	Qadisyia
1	27	4.4×10^5	6.9×10^2	1.5×10^5	7.3×10^3	3×10^6
2	28	1×10^4	7.6×10^4	1.4×10^3	1.5×10^2	1×10^4
3	29	2.6×10^2	9×10^3	3.3×10^5	6.1×10^7	2.5×10^2
4	30	1×10^2	1×10^7	4.5×10^8	1.9×10^2	2.9×10^5
5	31	6.1×10^3	5.9×10^3	1×10^4	6×10^6	1×10^2
6	32	1.8×10^4	4.6×10^5	7.9×10^5	1×10^2	8×10^3
7	33	1.3×10^5	3×10^3	2.6×10^4	5.5×10^4	5.1×10^2
8		5.3×10^4	3.1×10^2	—	2.8×10^4	8×10^5
9		9.3×10^6	8.2×10^5	—	3.8×10^6	1×10^6
10		9×10^3	6.9×10^2	—	9.7×10^6	3.9×10^5
11		1×10^2	4.1×10^5	—	1×10^4	6.7×10^5
12		2.2×10^6	8×10^5	—	2.1×10^5	4.5×10^5
13		5×10^3	9.3×10^2	—	3.6×10^3	9×10^4
14		4.9×10^2	1×10^7	—	2.2×10^5	$6.4\ 10^{6}$
15		7.5×10^3	8.5×10^4	—	1×10^5	1×10^6
16		1×10^4	6×10^6	—	9.4×10^4	2×10^4
17		7×10^5	$<1 \times 10^2$	—	1.7×10^4	8×10^5
18		9.9×10^3	3.9×10^6	—	7.3×10^6	1×10^6
19		6.7×10^2	8.2×10^5	—	—	4.9×10^5
20		5×10^2	3.2×10^2	—	—	1.5×10^5
21		3.8×10^3	5.1×10^3	—	—	2.2×10^4
22		6.6×10^4	6.3×10^2	—	—	—
23		9.8×10^5	1.6×10^3	—	—	—
24		4.7×10^3	2.9×10^5	—	—	—
25		8.8×10^2	4.2×10^3	—	—	—
26		4.1×10^6	6.1×10^2	—	—	—
Mean		**25177765.00**	**902902.22**	145800	**3828286.666**	**472694.29**
Median		**27000.000**	**9900.000**	145800.000	**450000.000**	**8000.000**
Standard Deviation		**2331202.733**	**2266519.250**	9261.30988	**2635395.253**	**1119537.488**
Standard Error		**549469.75**	**337872.74**	655.000	**507182.05**	**423145.4**

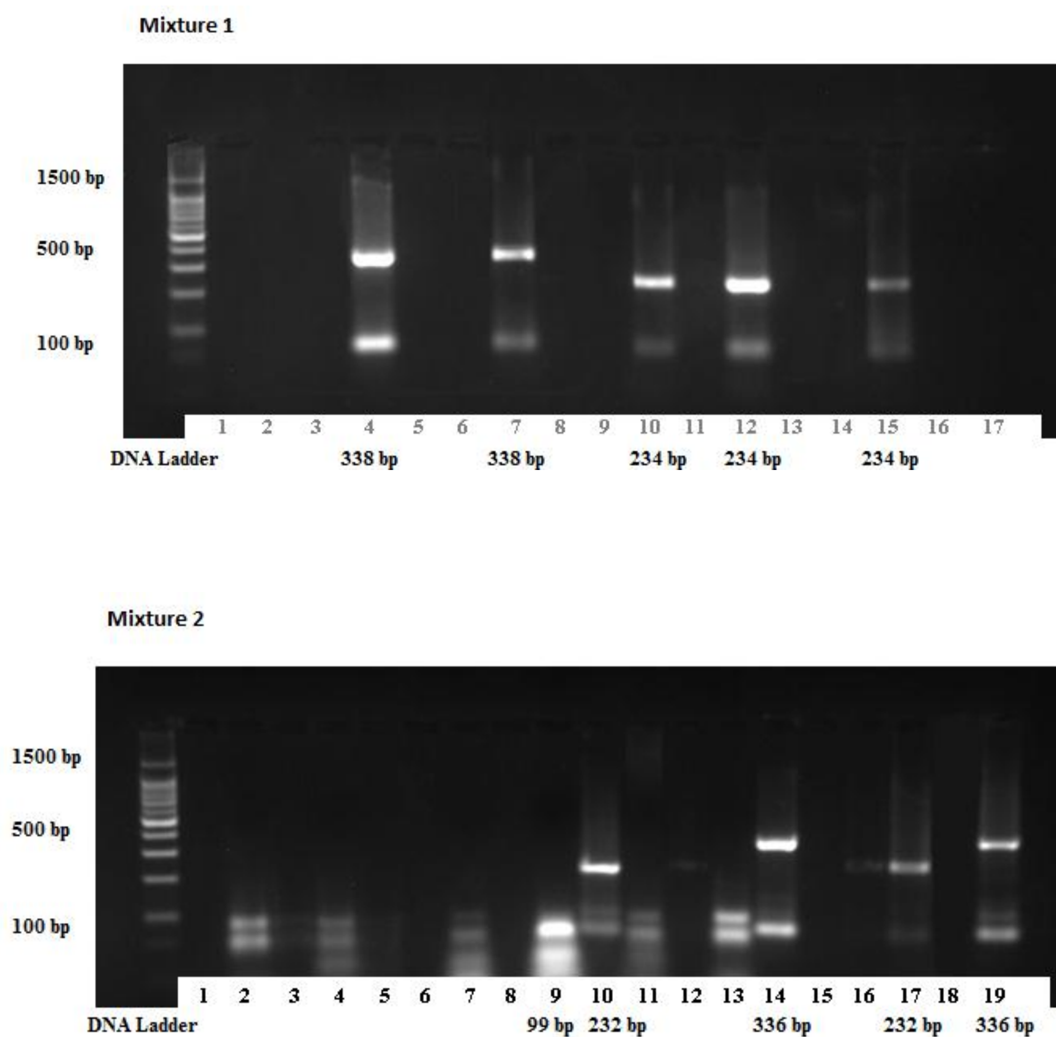
Total mean: 5840976.9 **IU /ml of blood.** **Total Median 36000.000 IU /ml of blood.**

Total SD: 2305546.949, Total SE:231716.19.

Sum of squares : between groups = 1.52E+013 , within groups = 5.06E+014 F= 0.560

Mean squares: between groups = 3.048E+012 , within groups =5.437E+012

_ : mean no infected patient.



Figure(1): Detection Bands Obtained After Application of Reverse Transcription Polymerase Chain Reaction by Using of Two Mixtures of Primers.

Mixture 1: Lanes 4 and 7 genotype 2b
Lanes 10, 12, and 15 genotype 1b

Mixture 2: Lane 9 genotype 4
Lanes 10 and 17 genotype 3a
Lanes 14 and 19 genotype 6a

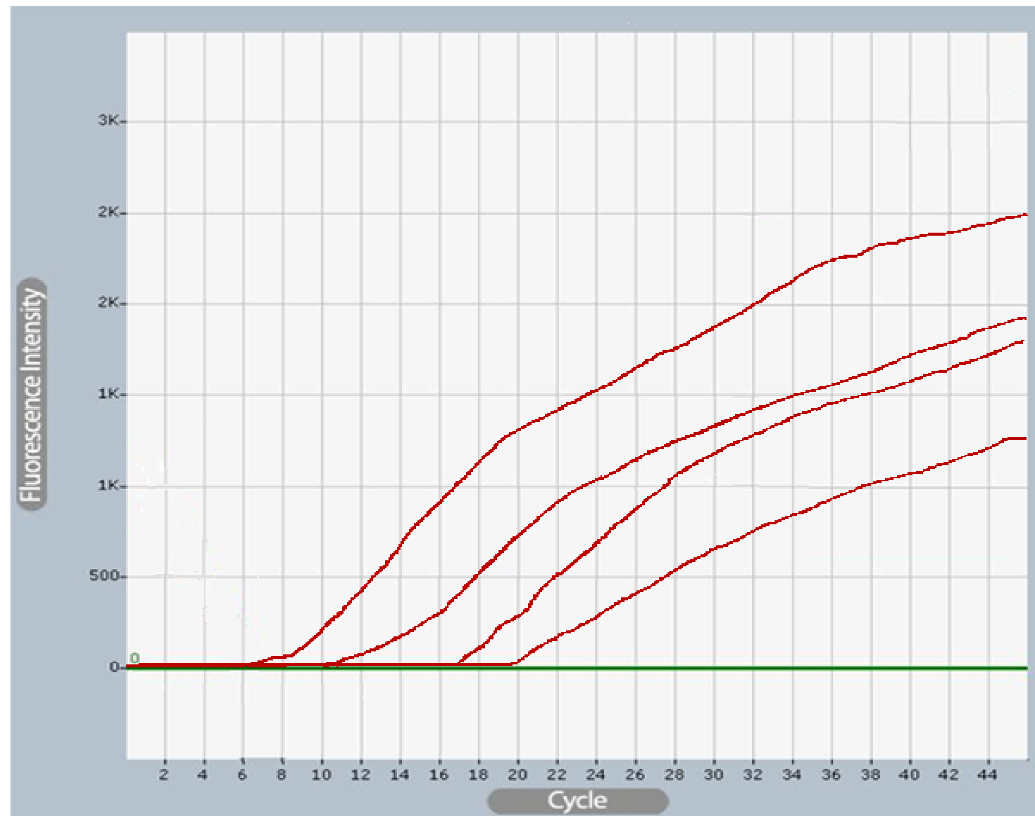


Figure (2): The Graphic Results of Positive Cases with High Viral Load that Obtained by The Real Time PCR Thermocycler: Exicycler^{TM} Quantitative Thermal Block,Version 3.0.

- X axis: cycle number, Y axis: Log. Fluorescence.

Discussion:

Hepatitis C virus infection is worldwide in distribution, the virus has been found in all regions of the world where it has been sought, but the areas of higher prevalence were include of Egypt and other Mediterranean regions followed by sub-Saharan Africa (Lavanchy, 2011)(15). A heterogeneous clinical features and sequallae with considerable morbidity and mortality mostly ensued after a long standing chronic infection initiated at childhood.

The current study showed that the infection rate at thalassemic children was 17.4% (98/563) by use of RT-PCR assay.

In Iraq, similar finding was obtained by Abdul-Sada (2011)(16) who found that HCV infection present in 27 out of 145 (18.6%) of thalassemic patients, consistent results was obtained by Kashef *et al*. (2008)(17) in Iran who found that 24 out of 131 (18.3%) of thalassemic children were harbored anti-HCV antibodies in their serum through using of ELISA test.

The obvious elevation of the infection rate at thalassemic patients was because they are continuously blood recipient and they are urgently need blood in order to normalize the sever dropping in the hemoglobin level, hence, they are prone to contract HCV infection since the blood screening at present time in our blood banks depend on serological test (ELISA-III test), that unable to detect all infected cases.

Fiadh (2006)(18) searched for HCV infection at the thalessemic patients in Al-Ramadi city (Iraq), Umar *et al.* (2010)(19) studied HCV at the Pakistani thalassemic patients, they were obtained sero-prevalences of 26% and 42% respectively, both aforementioned researches were found higher percentages than that of our study.

Fairly higher prevalences were obtained and in Iraq by Al-Kubaisy *et al*. (2006)(20) who found that HCV sero-prevalence was 67.3% (376 out of 559 thalassemic children were gave positive results for ELISA-III test that searching antibodies). Moreover, Akbar (2004)(21) in the Saudi Arabia was recorded infection rate of 70% which clearly higher than our finding.

Sayhood (2008)[22] was recorded that anti-HCV antibody was found at 8.57% of thalassemic patients in AL-Qadisyia governorate (Iraq) whereas Ocak *et al*. (2006)[23] in Turkey found that the sero-prevalence of anti-HCV antibodies in the serum of the thalassemic patients was 4.51% (18/399), these two studies were recorded infection rates lower than that of present study. The variation in the prevalence of HCV infection at many countries and many centers at the same region was occurred as a result of many factors such as efficiency of blood screening and the tests used at the blood banks, age of the thalassemic children and numbers of blood units that they were received.

The current study was revealed that the genotype 4 is the predominant genotype which found in 84.6% of the infected cases followed by Genotypes 1b, 6a, 2b, 3a and that came in percentages of 8.16%, 4.1%, 2% and 1%, respectively.

This multi-genotypic finding came in alignment with the majority of the global studies, the molecular epidemiological studies have shown marked differences in geographical distribution by geographical regions and among patients groups, genotypes 1, 2 and 3 are widely distributed throughout USA, Europe, Australia and East Asia, genotype 4 is largely confined to the Middle East, Egypt and central Africa, genotype 5 and 6 are found predominantly in South Africa and South East Asia, respectively (Agha *et al.* , 2004[24] ; Simmonds *et al*[25]. , 2005 ; Argentini *et al.* , 2009[26]). In the our study we used a genotyping method through RT-PCR analysis that has been originally employed by Ohno *et al.* (1997)[14] which involved wide range of HCV genotypes: 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a.

In Iraq, Al-Kubaisy *et al.* (2006a)[20] have studied the sero-prevalence of HCV genotypes by use of genotype specific ELISA-III test searching antibodies at thalassemic Iraqi children, they were reported that genotype 4 was the most frequent type that has been found it in 35.4% of the infected cases followed by 1a in 27.1% of cases and genotype 1b that occurred in 22.9% of cases whereas mi×ed infection with genotypes 1a and 4 was set in 14.6% of the HCV among infected thalassemic children. Other study, by Al-Kubaisy *et al.* (2006b)[27] who used the same technique (genotype specific ELISA-III test searching antibodies) and observed differed result among haemophiliac patients in Baghdad and co-infected with the human immunodeficiency virus (HIV), they referred that the genotype 1a was the dominant genotype followed by genotypes 1b and 4.

The above difference in genotype patterns in Iraq may be attributed to the test that has been used in the diagnosis (ELISA test searching antibodies), number of samples, type of the patients (haemphiliac and thalassemic) and the co-infection with HIV.

The interpretation of the presence of genotypes 1b, 2b and 3a in some Iraqi patients in the current study is that these types might had been introduced to our country from Iran, Jordan and Turkey where the prevalence of these genotypes was relatively high as a result of traveling, immigration and the matrimonies.

The viral loads in the blood of HCV patients in present study were ranged from less than 100 (1×10^2) IU / ml of blood to 4.5×10^8 IU / ml of blood , whereas, the total mean and median of viral load were 5840976.9 (5.8×10^6) and 36000 (3.6×10^4) IU /ml of blood respectively,

This wide range in the viral blood titer may be set due to the variety of the patients whom were from various groups, ages and the stages of the disease and this was in agreement with what explained by Fabrizi *et al.* (2003)[28]. Similar result has been referred by Yeo *et al.* (2001)[29] in United State who found that viral loads were ranged from 10^2 to 10^7 IU / ml of blood and the mean was $10^{5.77}$ IU / ml. Also slightly similar finding was registered by Ndong-Atome *et al.* (2008)[30] in Gabon were the viral loads came in range of $6 \times 10^2 – 24.9 \times 10^6$ IU / ml and the median was 3.7×10^5 IU / ml.

Higher viral load was obtained by Fabrizi *et al.* (2000)[31] in United State which were ranged from 6.3×10^5 to 1.6×10^8 IU / ml of blood, mean and median were 8.6×10^6 IU / ml and 3×10^6 IU / ml of blood respectively.

The immune response and the current status of the patient were also have important role in the fluctuation of the viral load at the same individual (Jacobson *et al.* , 2010[32]; Mukherjee *et al.* , 2015[33]). The variation in the viral titer among the different studies may belong to many other causes like technique of the measurements, group of patients and the number of samples.

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