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Clinical Research

HIV RNA VIRAL LOAD BY REAL TIME PCR: A PRELIMINARY INVESTIGATION FORM INFECTED POPULATION.

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ABSTRACT Acquired immunodeficiency Syndrome (AIDS) is the most prevalent disease all over the world. It is caused by an infective retrovirus HIV (Human Immunodeficiency Virus). It was first isolated from South Africa. HIV disease also characterized by a gradual deterioration of an individual's defense system as infection spreads. This may lead to the final stage termed "acquired immunodeficiency syndrome" (AIDS). **"Syndrome"** is used to describe the cluster of medical conditions that can batter an HIV-infected person's immune system. Crucial immune cells called CD4 cells (also known as T-helper cells) are disabled and killed during the typical course of infection. The dynamic range of Real-time RT-PCR assay makes it particularly useful for quantifying full range of virus load observed in treated and untreated patients with HIV infection. In our study out of 37 samples were collected from HIV infected population who were attended to department of DVL and Surgery department, KGH, Visakhapatnam between February 2016 to January 2017 for the regular check up. Out of 37 samples, 32 were showed standard curve means positive results where as CD4 count shows <200/mL. Results revealed that the Ct values are ranging from 25.39- 34.87. The dynamic range of Real-time RT-PCR assay makes it particularly useful for quantifying full range of virus load observed in treated and untreated patients with HIV infection.

KEYWORDS : HIV, AIDS, Syndrome, CD4, RT-PCR, T - helper cells

I.Introduction

Human immunodeficiency virus (commonly known as HIV, and formerly known as HTLV III and lymphadenopathy-associated virus) is a retrovirus that is the cause of the disease known as AIDS (Acquired Immunodeficiency Syndrome), a syndrome where the immune system begins to fail, leading to many life-threatening opportunistic infections (1, 3). HIV is transmitted through direct contact of a mucous membrane with a bodily fluid containing HIV, such as blood, semen, vaginal fluid, preseminal fluid or breast milk (2). This transmission can come in the form of: penetrative (anal or vaginal) sex; oral sex; blood transfusion; contaminated needles; exchange between mother and infant during pregnancy, childbirth, or breastfeeding; or other exposure to one of the above bodily fluids. Infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognized on December 1, 1981, making it one of the most destructive pandemics in recorded history. In 2005 alone, AIDS claimed an estimated 2.4—3.3 million lives, of which more than 570,000 were children. Progressive HIV infection causes an increase in plasma HIV-RNA levels accompanied by a corresponding decline in CD4+ T cell count. Absolute CD4+ T cell count has been used as a criterion to initiate antiretroviral therapy (ART) (4). However, CD4+ T cell count alone is inadequate predictor for initiating antiretroviral treatment since T cell is subjected to enormous variations due to age, sex, various socio-economic and biological factors (5). On the other hand plasma HIV-RNA levels, depicting the actively replicating circulating virus, contribute more important information particularly in early HIV infection, guiding individual treatment decisions, evaluating prognosis, determining the rates of mother to child transmission and assessing the growing trend of antiretroviral drug resistance(7). Recent studies have shown that changes in plasma HIV virus load precede changes in CD4+ T cell counts by 8 to 12 weeks(6). Thus, it is considered to be the best predictor of the disease progression as well as effectiveness of ART.

Development of new molecular techniques designed to detect circulating virion- associated HIV RNA in plasma has created an opportunity to study viral dynamics and HIV pathogenesis in substantial detail (8). Of the three quantitation assays namely Real-time Reverse-transcriptase Polymerase Chain Reaction (Real-time RT-PCR) assay, Branched DNA assay and Nucleic Acid Sequence-based amplification assay Real Time RT-PCR being a kinetic assay provides better quantification of the initial copy numbers than end-point measurements employed by the other assays(9). The dynamic range of Real-time RT-PCR assay makes it particularly useful for quantifying full range of virus load observed in treated and untreated patients with HIV infection(10).

II.Materials and Methods

In our study specimens were collected from HIV infected population who were attended to department of DVL and Surgery department, KGH, Visakhapatnam between February 2016 to January 2017 for the regular check up. In brief, EDTA blood samples were collected from 37 patients who were clinically, serologically posititve and their CD4 count shows very low(<200mL)(21). The samples were centrifuged and separated plasma and were processed, for RNA extraction.

II.1. RNA Extraction

A total number of 37 cases were taken, which were proved clinically and symptomatically as HIV positive by ELISA and other methods. Each specimen was used for RNA extraction. RNA extraction was done by TRI solution reagent (13, 14).

In detail, 300 µL of TRI solution was added to each sample. After mixed well and the reaction mix was incubated at room temperature for 5 minutes. To the mix 80 µL of chloroform was added and mixed well. The reaction mix was incubated at room temperature for 5 minutes and followed by centrifugation at 11,200 g for 15 minutes at 4°C. The aqueous phase was collected into a 1.5mL micro centrifuge tube and added 150 µL of Isopropyl alcohol, mixed well. The reaction mix was incubated at room temperature for 10 minutes followed by centrifugation at 13,400 rpm for 10 minutes at 4°C. Was the pellet with 75% absolute alcohol and centrifuged at 9,300g for 10 minutes at 4°C. Airs dries the pellet and resuspend the pellet in 10 µL of RNase free water.

II.2. cDNA synthesis

Reverse transcription PCR is used for detection of viral RNA. This method involves conversion of RNA to cDNA is amplified and values are compared in Real Time PCR against a set of known standards. This assay is used to monitor effect of antiretroviral treatment. cDNA was synthesized with I script cDNA synthesis kit (Bio-Rad) according to the maufacturers instructions.

II. 3. Real Time PCR Taqman assay

HIV RNA RT PCR Mater mix preparation:10.0µL of 2X master mix, 1.0µL HIV primer probe, 4.0 µL of Rnase free water and finally added 5.0 µLof extracted cDNA. The above mix was run for RT PCR using the following cycles. 95^0 C for 3 minutes, 95^0 C for15 seconds, 50^0 C for 15 minutes and 60^0 C for 1 minute repeated for 45 cycles.

A pathogen specific primer and probe mix is provided and this can be detected through the FAM channel (15). The primer and probe mix provided exploits the so-called Taq Man principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe is included in the same reaction

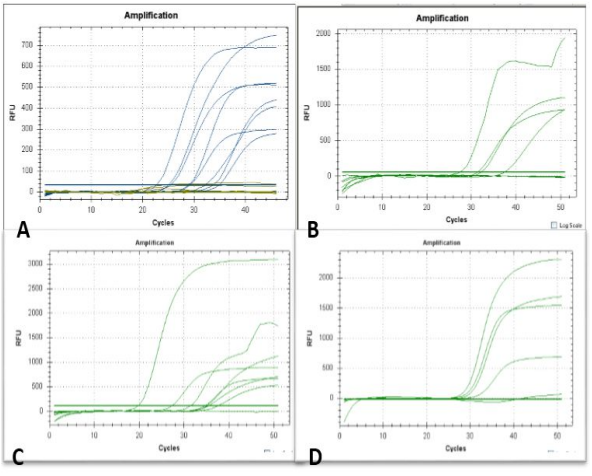
mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms(16-18). CT values represent the viral copy number.

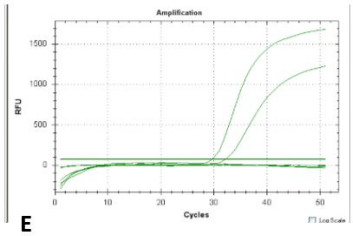
III. Results

In the present study we processed for viral load or quantification of viral copies. Total 37 HIV patients samples were collected, who were clinically, serologically posititve and their CD4 count shows very low (18).

Table – 1 shows the Ct values and viral copy number of HIV infected population.

S.No	Fluor	Sample	Age	Gender	C(t)	Viral load Copies /ml
1	FAM	Case-1	68	M	29.16	1.3×10^5
2	FAM	Case-2	52	F	32.19	1.5×10^3
3	FAM	Case-3	34	F	29.32	1.3×10^4
4	FAM	Case-4	28	M	-	-
5	FAM	Case-5	36	F	25.39	9.3×10^5
6	FAM	Case-6	57	M	34.87	1×10^4
7	FAM	Case-7	31	M	33.69	1.8×10^4
8	FAM	Case-8	35	F	-	-
9	FAM	Case-9	23	F	28.95	$1.3X\ 10^6$
10	FAM	Case-10	51	M	27.42	$1.8X10^5$
11	SYBR	Case-11	25	M	24.12	6.3×10^5
12	SYBR	Case-12	34	F	-	-
13	SYBR	Case-13	41	F	28.65	$1.3X\ 10^6$
14	SYBR	Case-14	28	F	29.41	1.3×10^4
15	SYBR	Case-15	62	F	-	-
16	SYBR	Case-16	20	M	32.42	1.5×10^3
17	SYBR	Case-17	22	F	34.66	1×10^4
18	SYBR	Case-18	38	M	37.59	$0.6X\ 10^4$
19	SYBR	Case-19	34	F	27.28	$1.8X10^5$
20	SYBR	Case-20	42	F	35.50	0.9×10^4
21	SYBR	Case-21	68	F	19.71	9.3×10^5
22	SYBR	Case-22	31	F	35.12	0.9×10^5
23	SYBR	Case-23	22	M	31.40	1.2×10^4
24	SYBR	Case-24	46	F	25.14	6.3×10^5
25	SYBR	Case-25	36	F	26.84	7.3×10^5
26	SYBR	Case-26	30	M	26.35	7.3×10^5
27	SYBR	Case-27	62	F	27.21	$1.8X10^5$
28	SYBR	Case-28	29	F	29.64	1.3×10^4
29	SYBR	Case-29	34	M	32.10	1.5×10^3
30	SYBR	Case-30	38	M	32.20	1.5×10^3
31	SYBR	Case-31	40	F	-	-
32	SYBR	Case-32	56	M	29.61	1.3×10^4
33	SYBR	Case-33	28	F	32.51	1.5×10^3
34	SYBR	Case-34	54	F	29.30	1.3×10^4
35	SYBR	Case-35	59	M	27.52	$1.8X10^5$
36	SYBR	Case-36	43	F	28.14	$1.8X10^5$
37	SYBR	Case-37	61	F	32.10	1.5×10^3





Figures A-E: shows the standard curves of the Samples

IV. Discussion

Real-time PCR is a powerful advancement of the basic PCR technique. The older PCR technique is essentially qualitative in which the end point amplification products are visualized on gel-electrophoresis in a post – PCR processing step (17). This step is a major source of cross – contamination and false positive results. Moreover, conventional or basic PCR is only able to detect the 10 fold changes in the endpoint amplified product, thus increasing the chances of a false positive result. It also has a low sensitivity of detecting only 500 copies of the target nucleic acid in the sample (18). On the other hand, Real-Time PCR is essentially quantitative method, which offers dynamic range of detection of the target nucleic acid during exponential phase of amplification. Through the use of appropriate fluorescent detection strategies in conjunction with proper instrumentation, all important starting amount of nucleic acid in the reaction can be accurately quantitated (19). Quantitation is achieved by measuring an increase in fluorescence during the exponential phase of PCR. It is able to detect even two fold changes in the concentration of the product (20).

In the present study test is performed in a single tube and no post-PCR processing step is required to detect the end-point product, thus reducing the possibility of false positive results to almost negligible, thus enhancing the sensitivity of the assay multi fold (16). Taqman version of the assay is highly specific with a sensitivity of less than 50 copies of the target nucleic acid in the sample. Our assay is highly specific as HIV negative control failed to show any positive fluorescent signal (21). In addition the assay has a very high sensitivity and it can detect 30 g. eq./ml of HIV RNA. The standard curve showed linearity over eight orders of magnitude with high coefficient of correlation indicating its high sensitivity in determining viral loads over a broad range (22). Out of 37 patients 23 (62.2%) were females and the remaining 14 (37.8%)were males and the age group ranging from 23-68 yrs. In our study, 32 (86.5%) samples were showed standard curve means positive results where as CD4 count shows <200/mL. Results revealed that the Ct values are ranging from 19.71- 37.59. (Table - 1)(Figures –A-E). The use of Taqman probe contributes in measuring reporter dye emissions accurately with almost 100% specificity and sensitivity since it has already been reported that a single mismatch can lead to reduced amplification efficiencies resulting in decreased viral load measurements (23).

V. Conclusion

The dynamic range of Real-time RT-PCR assay makes it particularly useful for quantifying full range of virus load observed in treated and untreated patients with HIV infection. Applications of Real-Time PCR include measurements of viral load, gene expression studies, clinical diagnostics, and pathogen detection (23, 24).

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