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HSV -1 & HSV-2 DETECTION BY OPTIMIZED REAL TIME TRIPLEX PCR ASSAY WITH INBUILT HUMAN SAMPLE QUALITY CHECK

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ABSTRACT **SUMMARY** Multiplex real-time (RT)-PCR assays have been widely used tools for detection of human herpesvirus (HSV) pathogens. Due to genome similarity among herpes group especially HSV-1 & HSV-2, most of the assay are cross-reactive or less sensitive. While the other assay available for detection of HSV-1 and HSV-2 virus doesn't provide any check over the quality of the sample. The present study was designed to develop a single-step triplex real-time polymerase chain reaction (PCR) assay for detection of HSV-1, HSV-2 separately along with sample quality check, using human β-actin as a housekeeping gene. The primers and probes for the target genes of HSV-1, HSV-2, and β-actin were designed and an RT triplex PCR assay was standardized modulating variables including annealing temperature, extension temperature, primers, probes, and other reagent concentrations. The assay was validated and sensitivity, a specificity of the assay was determined by various experiments. This novel assay was found to be sensitive, specific, and reproducible for the detection of HSV-1 and HSV-2 in patients sample. The technology was able to detect and quantify all genotypes of HSV-1 and HSV-2. The detection limit for different HSV viral genomes was found to be 100% for viral copies ≥100 copies/mL in a single-tube assay system. The present diagnostic assay can be routinely used in the diagnostic and prognostic assay of HSV-1 and HSV-2 in clinical samples; which is a major advantage in managing seriously/critically ill patients.

KEYWORDS

HSV-1, HSV-2, Real time PCR.

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INTRODUCTION:

Herpes simplex virus (HSV) 1 and 2 are DNA viruses of family Herpesviridae, the viral genome is a linear, double stranded DNA molecule of ~152261 to ~154746 nucleotides.[1]Both the herpes viruses are major public health problem.HSV-1 has been associated with oro-labial disease, with most infections occurring during childhood, and HSV-2 with genital disease with infection following sexual debut. Consequences of HSV infection can range from inconsequential (cold sores in otherwise healthy patients) to highly morbid and fatal (in neonates).[2]

Early detection of circulating HSV-1 and HSV-2 play an important role in managing such infections as well as assessing response to therapy. The current diagnostic assay available for HSV like ELISA, viral culture and PCR are ridden with various disadvantages viz. cross reactivity, expensive, time consuming, technically challenging and low sensitivity.[3]Detection of serum markers such as IgM and IgG specific to HSV1&2 takes a week or more to become detectable in body fluids. Crude antigen tests could detect antibody to herpes simplex in general but were poor at differentiating accurately between types 1 and 2.[4] Viral culture is expensive, time consuming, technically challenging and less sensitive. HSV PCR, with its consistently and substantially higher rate of HSV detection, has replaced other tests for diagnosis. There are few assays for HSV-1 & HSV-2 detection in uniplex reaction. There are few multiplex real time(RT) PCR assays available to detect these viruses simultaneously.[6] However, development process of these assays has to face few challenges like cross reactivity and lower reproducibility.[7]Clinically HSV DNA titers vary greatly, from levels as high as 10^{10} copies/ml during acute HSV infection, to very low levels in patients undergoing antiviral therapy. Assays should have sufficient amount of reagents to detect both low & high viral counts. Variable viral load at different phases of infection is a big hurdle. Genome variability also poses a technical challenge for developing an assay for HSV-1 and HSV-2 detection. HSV structure has wide range of HSV-1 and HSV-2 genome variability. Due to genetic diversity many of the assays have limitations in picking up all the existing genotypes.[8] Available HSV-1&2 PCR assays show cross reactivity among HSV-1 & HSV-2 due to genome similarity among HSV-1 & HSV-2.[7,8] Another challenge to the assay in developing countries is to maintain quality of samples as it deteriorates during transportation from the collection centre to central laboratory for testing due to unavailability to maintain cold chain.

The objective of this study was to develop a triplex assay for differential diagnosis of HSV-1 and HSV-2 alongside an exogenous internal control (Human **β**-actin). The incorporation of an exogenous internal control increases the reliability of the RT-PCR results by permitting discrimination of true negative results from false negatives which may occur due to poor quality of samples/ improper nucleic acid extraction/the presence of PCR inhibitors in the sample. Human **β**-actin gene is expressed at relatively constant levels in all individuals.

METHODS:

All the primer and probes sequences were *optimally designed* for respective viruses i.e. HSV-1 and HSV-2. Sequences from various genotypes were downloaded from the GenBank nucleotide database and aligned using the program MEGA 5 Details of sequences and primer and probes are mentioned in table 1 and fig 1a-c.

Table 1: List of primers and probes design used for HSV-1 & 2 detection.

Sequence ID	Oligonucleotide sequence 5'-3'	Length	Target gene	Product size	Fluorescent tagging of Probe
HSV-1 Fwd	CCCAAGTTCCCAACAAAGAC	20	Ul26	143-151bp	FAM
HSV-1 Rvs	CTTTCTTCCCACACACACA	19			
HSV-1 Probe	/5FAM/AGATGCACATGCGGTTTAACACCCGT/3 IABkFQ /	26			
HSV-2 Fwd	GCTCACCACCAAGGAACTC	19	UL27	141bp	Cy5
HSV-2 Rvs	GCCGACACCAAAGCCATA	18			
HSV-2 Probe	5'Cy5/CCCGCCCCCCTCCGCGCCT/3IAbRQSp /-3'	19			

VOLUME - 4 | ISSUE - 4 |October - 2020 | ISSN (O) : 2663-2888 | ISSN (P) : 2663-287X

β-actin Fwd	ACCGAGCGCGGCTACAG	17	β-actin [14]	60	HEX
β-actin Rvs	CTTAATGTCACGCACGATTTCC	22			
β-actin Probe	5'/5HEX/TTCACCACC/ZEN/ACGGCCGAGC/3IABkFQ/-3'	19			
Condition:	· Sequences representing different genotypes of HSV-1 & HSV-2 were downloaded from the GenBank nucleotide database and aligned using the program MEGA 5.. · All the primers and probes were optimally designed to prevent primer dimer or cross dimer formation which may have later resulted in unwanted fluorescence. · No degeneracies are done as the primers and probes were binding completely to amplify all genotypes of HSV-1. The cross reactivity with the HSV-2 genome was ruled out. · The designed primers and probes were synthesized with HPLC purification via Integrated DNA Technology (IDT, Coralville, USA).				

Figures

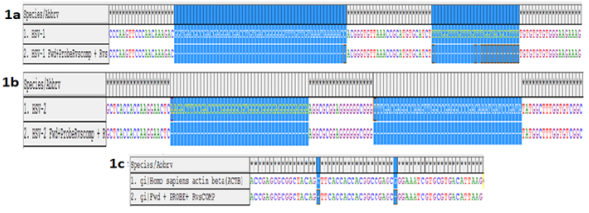

Fig. 1.(a): Alignment of HSV-1 primers and probe with UL-26 region (b) Alignment of HSV-2 primers and probe with UL27& (c) Alignment of internal control primers and probe with Human β-actin gene.

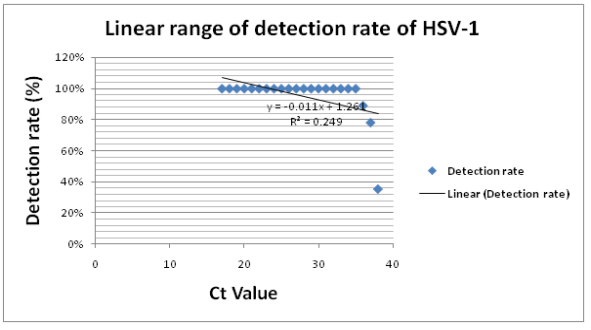

Fig.2. (a): Linear range of HSV-1 detection

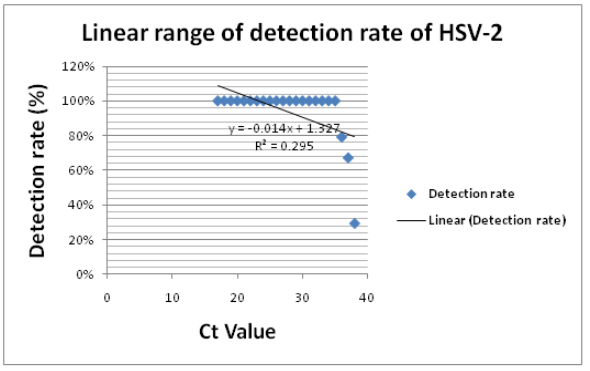

Fig 2 (b) Linear range of HSV-2 detection.

A testing panel of 20 clinical samples from clinical bank store(-80°C) were subjected to real time PCR. The testing panel includes positives for HSV-1(5), HSV-2(5), co-positives (5) and negatives (5) for both HSV-1 and HSV-2. PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA) according to the manufacturer's guidelines, while FastStart Essential DNA Probe Master (Roche, Germany) mastermix were used for real time PCR. Details of real time PCR is mentioned in table 2.

The PCR reaction was optimized keeping in mind the concept of 'limiting concentration' to combat the challenge of variable viral load. A series of dilution of primers and probes were made using a dilution matrix for optimizing the concentration of both. Amplification reactions were performed with a RTthermocycler (light cycler 480, Roche) program consisting of a denaturation step of 10 minutes at 95°C followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing for 20 seconds at 55°C and extension at 72°C for 15 seconds. The fluorescence was acquired at the time of annealing. All the primers and probes were designed in such a way that there was no primer dimer or cross dimer formation which later results in unwanted fluorescence. After optimization of the multiplex reaction, all the above tested 20 samples were retested using the triplex format (table 2).

Table 2: Comparative results of 20 known samples tested with our primers & probes insingleplex& multiplex format

S.No.	Singleplex Reaction		Multiplex /Triplex Reaction	
	HSV-1 (Copy No.)	HSV-2 (Copy No.)	HSV-1 (Copy No.)	HSV-2 (Copy No.)
1.	+ (10^8)	-	+ (10^8)	-
2.	+ (10^3)	-	+ (10^3)	-
3.	+ (10^7)	-	+ (10^7)	-
4.	+ (10^5)	-	+ (10^5)	-
5.	+ (10^5)	-	+ (10^5)	-
6.	-	+ (10^2)	-	+ (10^2)
7.	-	+ (10^4)	-	+ (10^4)
8.	-	+ (10^7)	-	+ (10^7)
9.	-	+ (10^4)	-	+ (10^4)
10.	-	+ (10^5)	-	+ (10^5)
11.	+ (10^6)	+ (10^3)	+ (10^6)	+ (10^3)
12.	+ (10^3)	+ (10^6)	+ (10^3)	+ (10^6)
13.	+ (10^5)	+ (10^7)	+ (10^5)	+ (10^7)
14.	+(10^8)	+ (10^5)	+(10^8)	+ (10^5)
15.	+ (10^4)	+ (10^3)	+ (10^4)	+ (10^3)
16.	-	-	-	-
17.	-	-	-	-
18.	-	-	-	-
19.	-	-	-	-
20.	-	-	-	-
Primer & Probes	·1µl of forward and reverse primer (10pm) ·0.5µl probe(5pm) ·Finally diluted to 10picomole/microlitre		·0.35µl of forward and reverse primer (10pm) ·0.175µl probe ·2.3 µl of primer & probe cocktail ·Finally diluted to 10picomole/microlitre ·β actin: 0.2µl primer/ 0.15µl probe	
Extraction	**TNA by Invitrogen**		**TNA by Invitrogen**	
Reaction mixture	·12.5µlReal Time Mastermix (2x) ·2.5µl of primer & probe of each target in separate reaction ·5µl H_2O (DNAse and RNAse free) ·5µl extracted total nucleic acid was added to 20 µl of prepared mastermix		·12.5µlReal Time Mastermix (2x) ·2.3 µl of primer & probe cocktail ·5.2µl H_2O (DNAse and RNAse free) ·5µl extracted total nucleic acid was added to 20 µl of prepared mastermix	
Real time PCR condition by LightCycler 480 II system (Roche, Germany).	·Initial Denaturation: 10 min at 95°C ·Denaturation: 45 cycles at z5°C for 15sec ·Annealing: 20sec at 55°C ·Extension: 15sec at 72°C ·Cooling: 30sec at 40°C			

To determine the analytical sensitivity: the plasmids were used. A log-10 serial dilution of each standard plasmid from 10^8-10copies/ml was

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prepared. Serial dilutions were then assayed in triplicate using the optimised singleplex and triplex RT-PCRs. For analysis CT values were compared. Each test was performed on three occasions to verify repeatability and regression analysis and one-way ANOVA was performed on the CT values (the limit of detection [LOD] of the assay) (GraphPad Prism 6).

Table 3: Comparative results of real time PCR with claimed primers and probes, in singleplex and multiplex format with published primers & probes (n=300)

	Singleplex PCR with our newly designed Probes and Primers	Multiplex PCR with our newly designed Probes and Primers
HSV-1(positives/ number tested)	9/300	9/300
HSV-2(positives/ number tested)	2/300	2/300
Human β-actin(positives/ number tested)	294/300	294/300

For analytical specificity determination: To determine specificity, a testing panel of 28 samples were prepared for two groups of viruses; first Herpes groups which includes Cytomegalovirus, Epstein Bar virus, Varicella Zoster virus and another non herpes group like Human parvovirus B19, Japanese encephalitis virus, Dengue, hepatitis B virus, hepatitis C virus, Human Immunodeficiency Virus, Enterovirus, Influenza virus, Respiratory Syncytial virus, Metapneumovirus, Mumps virus and Measles virus. The panel was tested with designed primers and probes by RT PCR to check cross reactivity with other viruses.

Designed primer and probes were evaluated diagnostic specificity and sensitivity for respective viruses i.e. HSV-1 and HSV-2 on routine clinical samples like CSF, serum, skin vesicles and genital swabs. A total of 300 routinely received clinical samples were tested for HSV-1 and HSV-2 by designed real time PCR reaction in both singleplex and triplex formats. The primer-probe set was also evaluated for viral load sensitivity. The experiment was conducted to see the high *viral load* of one virus over the amplification of low copy number of the other virus in multiplex reaction. Sample with high viral load of HSV-1 (10^8copies/ml) was mixed with 10 fold dilution of HSV-2 sample starting with viral load (10^8copies/ml) and vice versa.

RESULTS:

The self designed primers and probes sequences (table 1 and fig 1a-c) targeting HSV-1 and HSV-2 were bioinformatically checked, with all the genome submitted to NCBI. They were suitable to amplify all the types and subtypes of their respective viruses and did not show any complementarity with human genome or any other viruses genome.

The self designed primer and probes had high sensitivity as standard curves (CT or Crossing Points versus log10 DNA or RNA copies) demonstrated linear amplification for both HSV-1 and HSV-2 DNA. The detection limits of primer-probes set were 100% for copies ≥100 copies/ml viral genome copies, respectively, in singleplex and triplex reactions. CT values obtained with singleplex and triplex assays for detection of HSV-1 and HSV-2 targets was significantly indifferent. Regression analysis of standard curves confirmed linearity. Singleplex RT-PCR for HSV-1 had correlation co-efficient (R2) of 0.989 and 101.91% amplification efficiency(E), whereas the singleplex RT-PCR for HSV-2 had R2 = 0.991 with 101.32% E. Internal control yielded CT values between 18 and 35. The lower limit of the test is 100 copies/ml for both HSV-1 & HSV-2, respectively with 100% sensitivity. For copies≤100 copies/mL sensitivity was 80% and 72% respectively. Real time assay was negative for copies < 100 copies/ml.Real time PCR was performed in triplex format in triplicate on each standard and on two different days to graph the limit of detection (LOD) of the assay. The linear range of detection of HSV-1 and HSV-2 both were from 10^8 to 100 copies/mL (Figure 2a and 2b).

The primer-probe set did not show any cross reactivity against other non-Herpes genomes that may co-exist in clinical samples. All the 20 samples with known HSV-1 and HSV-2 results tested in singleplex as well as multiplex reactions showed similar results with no significant difference in the viral load of the samples (table 2). No cross reactivity was observed with herpes and non herper group of viruses.The 28 samples panel which were tested for specificity, were found to be negative for HSV-1 or HSV-2; however human β-actin was positive in all the samples. Thus specificity of the assay was considered 100%.

For diagnostic sensitivity and specificity, a batch of 300 clinical samples was tested for HSV-1 and HSV-2 by self designed primer-probe set in both singleplex and multiples reaction (table 3). HSV-1 DNA was detected in 9 samples and HSV-2 DNA was detected in 2 samples. Human β-actin gene had mean CT value of 28.17 in 294 samples. Human β-actin gene was not amplified in six samples. All the 6 patients were asked to provide a repeat/fresh sample or repeat for extraction of viral DNA. On repeat testing of 6 samples, one tested positive for HSV-1.

DISCUSSION:

HSV-1&2 viruses are becoming major public health issues due to their widespread transmission leading to significant morbidity and mortality. Human herpesviruses infection may involve central nervous system during primary infection or following reactivation from a latent state. The infection could be life threatening in immune-compromised patients also. Rapid and reliable detection of these viruses is the key to take preventive measures timely and stop their transmission, thus infective complications in patients.[2,3] The self designed primers and probes sequences targeting HSV-1 and HSV-2 were bioinformatically checked, with all the genome submitted to NCBI. They were suitable to amplify all the types and subtypes of their respective viruses and did not show any complementarity with human genome or any other viruses genome. Our designed primer-probe set also identifies large range of conserved genome segment and reliably quantify and predict the viral load which may be modified during the treatment.

We have designed primers-probes set which can simultaneously and rapidly detect and quantify load of HSV type 1 and type 2 DNA. The triplex assay was sensitive enough to detect and quantify a very low copy number without mutual interference and cross reactivity with other viruses, thus our primers-probes set had 100% specificity and sensitivity. Our HSV DNA viral load results indicate that the triplex system described here would be a reliable tool in clinical settings as were able to detect viral copies upto 100 copies/ml with human sample quality check. Though many commercial kits are available in the market which can detect and quantify both the HSV types, but they donot have wide range of viral copies detection.[10-14] The clinical samples evaluated by available multiplex system need to be diluted and repeatedly tested in order to exceeding the upper limit of viral load quantification. Our triplex assay has inbuilt human sample quality check (by β actin detection), which none of the available kits offer.[10-14] Clinical sample quality check made this designed assay more reliable by reducing the chances of any false negative reporting. False negative reports were major concern in developing countries, where sample transport and storage is not appropriate.

The increased sensitivity and turnaround times of PCR based assays have the potential to significantly affect patient management; particularly if such assays are designed to include viruses for which antiviral drug therapy is available. When throughput is high, as is the case in our laboratory, multiplexed PCR assays are also cost-effective and provide a wider differential diagnosis than do individual assays.

Out of total 300 samples tested, 6 (2%) had poor quality. Of 6 samples, one tested positive for HSV-1 which would have been reported false negative, reflecting the advantage of present assay over other assays available.

We have established and evaluated a sensitive and cost effective in-house adaptable real-time PCR based assay for effective simultaneous detection of HSV-1 and HSV-2 in all types of samples. The lower limit of detection was less than 100 copies/ml for both HSV-1&2. Our designed assay was sensitive, specific and reproducible and could amplify various genotypes of HSV. Our assay can efficiently amplify HSV in both singleplex and triplex format.

CONCLUSION:

Present study has resulted into the development of highly specific and sensitive diagnostic assay for HSV-1 and HSV-2 detection in clinical samples. The designed primer probes set will rapidly and simultaneously detect HSV-1 and HSV-2 in triplex real time PCR format, which is a major advantage in managing seriously/critically ill patients.

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Conflict of Interest: The authors have declared that no conflict of interest exists.

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