Original Article

Quantitative analysis of herpes simplex virus-1 transcript in suspected viral keratitis corneal buttons and its clinical significance

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Purpose: The evaluation of Herpes Simplex virus-1 (HSV-1) transcript by different investigative methods (qPCR, PCR and IHC) in corneal buttons from suspected viral keratitis patients and the comparison of results with histopathological findings and clinical diagnosis. **Methods:** Sixty corneal buttons, 30 suspected viral keratitis, and 30 controls (keratoconus and bullous keratopathy) obtained after primary penetrating keratoplasty, were included in the study. All the corneal buttons were subjected to reverse transcriptase quantitative PCR (qPCR) for the detection of latency-associated transcript (*LAT*) gene, conventional PCR for polymerase (*pol*) gene, and immunohistochemistry (IHC) for HSV-1 antigen respectively. After obtaining baseline preoperative clinical data, all the patients were followed up for three years. The results obtained were correlated with clinicopathological features and follow-up data. **Results:** Of the 30 suspected viral keratitis patients there were 6 females and 24 males with mean age 46.5 ± 24.62 years (3-80 yrs). There was a marked male preponderance (80%). HSV-1 LAT transcript was detected in 23% (7/30) corneal buttons by qPCR, HSV-1 DNA in 6.7% (2/30) and HSV-1 antigen in 30% (9/30) cases by conventional PCR and IHC respectively. A statistically significant association was found between qPCR and DNA PCR ($P = 0.04$). All the 30 control corneas were negative for HSV-1 *LAT* gene, DNA and antigen. **Conclusion:** Detection of HSV-1 LAT transcript by qPCR may be superior to HSV-1 DNA PCR (conventional) and IHC, which has low sensitivity. However, the utility of HSV-1 LAT mRNA analysis as a diagnostic modality by qPCR needs to be validated on a larger patient cohort.

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Herpes Simplex virus-1 (HSV-1) has a known association with ocular diseases and is the most common infectious cause of blindness worldwide. The incidence of herpetic keratitis in developing countries is estimated to be 1 to 1.5 million per year.[1] Clinical manifestations of active HSV keratitis include dendritic/geographic ulcer, necrotizing stromal keratitis, endothelitis, and keratouveitis, encountered singly or in combinations. However, despite being a common occurrence, primary HSV-1 infection becomes clinically manifest only in 1- 6% of cases. The frequent episodes of reactivation (94-95%) of HSV-1 leads to profound visual loss.[1] As a consequence, corneal transplantation or penetrating keratoplasty (PKP) may be needed for visual rehabilitation, apart from antiviral treatment (topical and/or systemic) for control of infection.

The diagnosis of herpes simplex keratitis (HSK) is mainly suspected by clinical features. Laboratory methods including immunohistochemistry (IHC), immunofluorescence assays (IFA) and *in situ* hybridization (ISH) help in achieving a diagnosis but may be cumbersome and time-consuming.[2] These techniques detect different regions of HSV-1 and their results with respect to the status of HSV-1 in the cornea may be variable. This makes viral culture the present gold standard for detection of HSV-1. However, results of viral culture are time-consuming, of low sensitivity and depend on viable infection in the cornea.

Several studies have used molecular techniques to detect HSV-1 DNA in human cornea in herpetic keratitis and have revealed that cornea may support a neuronal type of HSV-1 latency.[3-8] During the clinical dormant period, almost all the genes of the HSV-1 virus get transcriptionally regressed except the overtly expressed set of LAT (Latency-Associated Transcript), which is important for efficient viral reactivation. Although few studies have detected LAT transcripts in animals as well as human cornea, very few have quantified this transcript till date.[9-13]

The aim of this study was to detect HSV-1 transcripts in corneal buttons from suspected viral keratitis patients subjected to primary PKP and to correlate with clinical and histopathological features.

Methods

Patients

This study was approved by the Medical Ethics Committee of the All India Institute of Medical Science, New Delhi (Ethics no.: IEC/

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NP-79/2012) and written informed consent was obtained from all patients. The study confirms adherence to the Declaration of Helsinki. A total of 60 corneal buttons obtained from patients undergoing primary penetrating keratoplasty (PKP) were included in the study. Of these, 30 buttons were obtained from patients clinically diagnosed with suspected viral keratitis and 30 from the patients operated for non-infective keratitis such as keratoconus and bullous keratopathy (BKP).

The inclusion criteria of the study consisted of unilaterally affected patients with central corneal lesion attributed to viral keratitis, either active or healed, on clinical grounds (characteristic history suggestive of recurrences, decreased corneal sensations, and present/past sign of infection). Along with demographic data, we also recorded baseline visual acuity (whenever possible), size of corneal lesion, presence of superficial/deep vascularization, intraocular pressure (IOP) and posterior segment status was made at the time of inclusion in the study. The keratitis was labeled active when there was an epithelial defect (dendritic or geographic) with stromal infiltrate and/or endothelial involvement, conjunctival congestion or anterior chamber reaction. The keratitis was considered healed when the eye was quiet (no epithelial defect, keratic precipitates and anterior chamber reaction) with corneal scarring/opacification and/or stromal thinning/ghost vessels. All active keratitis patients were microbiologically negative for other organisms on corneal scraping and received therapeutic dose of topical and oral Acyclovir (according to body weight) for 2-3 weeks before undergoing penetrating keratoplasty (PKP) for non-responsive ulcer. All healed keratitis patients had received prophylactic dose of oral Acyclovir for at least one year (in the recent past) and were off the antiviral medications at the time of inclusion in the study. All surgeries were performed by a single surgeon (NS) and the size of donor tissue ranged from 7.75-8.5 mm in diameter with graft-host disparity ranging from 0.25-0.75 mm depending on the intraoperative findings. The host corneal buttons were sent for pathological evaluation.

Patients were followed on day 1, 7 and month 1, 3, 6, 12, 24 and 36 after surgery based on the discretion of the surgeon. All patients were given topical moxifloxacin 0.5% 4 times a day and prednisolone phosphate 1% 6t/d immediately after surgery which was tapered according to the discretion of the surgeon and prophylactic oral Acyclovir for at least one year. At each follow-up, a noting of visual acuity, graft clarity, suture status, IOP, and posterior segment were made and sutures were removed routinely after one year or when found loose/broken/tight/infiltrated/vascularized. Graft was considered failed when it had either recurrence of active infection (previously mentioned signs of active keratitis were present both in the host and the graft; treated with therapeutic dose of antiviral agents tapered according to clinical response) and or rejection (acute onset endothelial rejection suggested by keratitis precipitates without any signs of infection or host involvement; treated with therapeutic dose of antiviral agents along with topical and intravenous steroids). Other causes of graft failure such as glaucoma (raised intraocular pressure with no evidence of rejection or infection) and chronic endothelial loss (no evidence of glaucoma, rejection or infection) were not included in recurrence.

Samples collection

Corneal buttons were removed during PKP, transferred to RNA later and transported immediately to the laboratory. Half of the corneal button was fixed in 10% formalin and processed for formalin-fixed paraffin embedding (FFPE) for hematoxylin and eosin staining, histological evaluation and immunohistochemistry, ¼ of the samples were used for total DNA extraction and remaining were put in RNA Later solution (Invitrogen, thermo fisher scientific) and stored at -20°C for RNA extraction and further analysis.

Immunohistochemistry

Sections of 3–4 µm thickness were cut on poly-L-lysine-coated slides from formalin-fixed paraffin-embedded blocks and stained immunohistochemically using avidin-biotin indirect method. After deparaffinization and rehydration, antigen retrieval was performed in citrate buffer solution (pH 6.0) at 100°C for 20–30 min. Thereafter endogenous peroxidase activity was blocked by treating the slides with 4% hydrogen peroxide in absolute methanol for 10 min. The sections were then incubated with a monoclonal primary antibody against HSV-1antigen (Clone ab49553, 1:50 dilution Abcam, Cambridge, UK). Subsequent incubations were performed using biotinylated secondary antibody and peroxidase-labeled streptavidin (Ultravision Polymer Detection System). Immunoreactivity was visualized using 3, 3′-diaminobenzidine substrate for 3–4 min, counter stained with hematoxylin and visualized by light microscopy. All tests were carried out using appropriate positive and negative controls. Vero cell line was used as positive control and sections with the omission of the primary antibody, used as negative controls.

DNA extraction and Polymerase Chain Reaction (PCR)

DNA from all the 60 corneal buttons (30 suspected viral keratitis and 30 controls) and Vero cell line (HSV-1 positive control) were isolated using the QIAamp Tissue Kit (Qiagen, cat no. 60504) as per manufacturer's instructions. The quantity and quantity of extracted DNA was analyzed by spectrophotometer. The average concentration of the DNA yield per sample was 70 ng/µl. Primer for HSV-1 was selected and checked for correctness by computer-assisted analysis (BLAST searches on European Molecular Biology Laboratory (EMBL), GenBank databases). Primer for HSV-1, forward 5′ATCCGAACGCAGCCCCGCTG3′ reverse 5′TCTCCGTCCAGTCGTTTATCTTC3′ was optimized to amplify 142bp highly conserved region of DNA polymerase gene.[13]

For the detection of HSV-1 DNA, semi-quantitative PCR was performed in a 25 µl reaction mixture containing 10 pmol each of forward and reverse primers, 1.5 U of Taq polymerase, 2.0 mm $MgCl_2$, 200 mM of dNTPs in 10x PCR buffer (Fermentas) and 3 µl of template DNA in a thermocycler (AppliedBiosystems) with initial denaturation for 10 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a final extension for 10 min. DNA extracted from the Vero cell line used as positive control and distilled water was used as negative controls. The amplified product was electrophoresed in a 2% agarose gel (Sigma, StLouis, Missouri), stained with Ethidium bromide and visualized under a gel documentation system.

RNA extraction and complementary DNA (cDNA) synthesis

After measuring the weight (5–10 mg) total RNA was extracted from all 60 corneal buttons (30 suspected viral keratitis and 30 controls) and Vero cell line (HSV-1 positive control) using the QIAAmpRNeasy Mini kit (Qiagen, Germany, catalog no. 74104) according to the manufacturer's protocol. The quality and quantity of extracted RNA was assessed using Nanodrop Spectrophotometer. The average concentration of the RNA yield per sample was 35 ng/µl. 1 µg of total RNA converted into cDNA by using SuperScript III First-Strand Synthesis System (catalog no. 18080-051, Invitrogen, Carlsbad, California,

USA), according to manufacturer's protocol. Random hexamer primers were used for the cDNA conversion.

Quantitative Real Time PCR (qPCR)

Quantitative real-time PCR (qRT-PCR) assay was optimized to amplify the 198bp region of *LAT* gene on all corneal buttons using Power SYBR Green master mix (catalog no. 4309155, Applied Biosystems). Primers for HSV-1 *LAT* gene, forward 5′GACAGCAAAAATCCCCTGAG 3′ reverse 5′ACGAGGGAAAACAATAAGGG 3′ was selected from published sequences.[14] The amplification reactions were carried out in total volume of 20 µl master mixture, containing 3 µl of total RNA, 10 µl of Power SYBR® Green PCR Master Mix, 1 µl each of (0.8 µM) forward and reverse primer, and 5 µl of sterile water. The amplification conditions consisted of preincubation at 95°for 10 min followed by 35 cycles of denaturation at 95° for 15 sec, annealing at 55° for 1 min and extension at 72° for 30 sec. The quantification cycle (Ct) was calculated at which the concentration increase became exponential. The specific target amplification was analyzed by melt curve analysis of the Applied Biosystem StepOne Real-Time PCR system (catalog no. 4376357). All reactions were performed in duplicate. For quality assurance, no-template control was applied in each run.

Standard curve

To quantify LAT transcript, a standard curve was obtained for each experiment by co-amplification of a known amount of HSV-1 LAT transcript (determined spectrophotometrically). Seven consecutive dilutions (dilution factor 1:10) were prepared containing 10^7 to 10^1 copies/reaction. The amount of LAT transcript in cases and controls were obtained by plotting Ct values on to the standard curve.

Statistical analysis

The data were analyzed using STATA statistical software (version 9). Fisher exact test was used to correlate the clinicopathological parameters. A value of $P < 0.05$ was considered statistically significant.

Results

Clinical results

A total of 60 eyes from 30 suspected viral keratitis patients (6 females; 24 males) and 30 control patients (15 Keratoconus and 15 bullous keratopathy) (14 females; 16 males) with no history/evidence of viral keratitis were included in this study. There was a marked male preponderance (80%). The average age in the viral keratitis group and in the controls was 46.5 ± 24.62 years (3-80 yrs) and 30.4 ± 26.08 years (3–75 yrs) respectively. Of the 30 suspected viral keratitis patients, four had an underlying systemic comorbidity including Diabetes Mellitus (3) and chronic obstructive pulmonary disease (1). The average size of the corneal lesion was 6.92 ± 1.8 mm and all the patients had corneal involvement in the visual axis [Fig. 1a]. The lesions were clinically active in 4/30 eyes (13.33%) and inactive in the rest. Vascularization, either deep or superficial, and extending 2 mm into corneal lesions was seen in 12/30 (40%) eyes. The best-corrected visual acuity (BCVA) was less than 2/60 in all except three patients. These were children, whose visual acuity could not be assessed at the time of presentation. Five patients (16.66%) were lost to follow up. At 3 years follow up, BCVA was ≥6/18 in 10/30 patients (33.33%), 6/60 to 6/24 in 6/30 patients (20%), <6/60 in 9/30 patients (30%), in one child postoperative visual acuity could not be assessed (the rest two children were lost to follow up). The recurrence of the disease was noted in 7/30 grafts (23.33%) and 2/30 grafts (6.66%) failed because of glaucoma. Recurrence included both endothelial rejection (5/30, 16.66%) and re-infection (2/30, 6.67%) and all episodes of recurrence occurred during the first year of primary PKP. Retrospectively, 22.22% (4/18) eyes with clear grafts at final follow-up showed positive viral load at presentation. This difference was not statistically significant and could be attributed to the low number of subjects and high attrition rate in our study. The patients presented with clinically active disease and corneal vascularization developed earlier recurrence than the patients without clinically active disease ($P = 0.030$) and corneal vascularization ($p = 0.008$). In the control group, all grafts remained clear at 3 years follow-up with no episode of infection or endothelial rejection.

Histopathological and Immunohistochemistry (IHC) results

On light microscopy analysis of all 30 suspected viral keratitis test corneal buttons, corneal epithelial edema, stromal edema, inflammation and vascularization was present in 56.6%, 60%, 27%, and 60% corneal buttons. Spheroidal degeneration and retro-corneal membrane was detected in 16.6% and 10% of corneal buttons. The histopathological findings are summarized in [Table 1].

Immunohistochemical staining revealed the presence of HSV-1 antigen in 9/30 (30%) corneal buttons [Table 2]. Both cytoplasmic and nuclear positivity of HSV-1 antigen was observed in epithelial as well as stromal cells [Fig. 1b]. The patients who presented with corneal epithelial edema and stromal inflammation showed statistically significant associated with positive HSV-1 antigen ($P = 0.04$ and 0.03) [Table 1]. All control corneal buttons were negative for HSV-1 antigen.

DNA PCR results

The DNA extracted from all the 30 excised corneal buttons of suspected viral keratitis patients were subjected to PCR amplification for HSV-1 *pol* gene. Two out of 30 samples (6.7%) were positive for HSV-1 DNA and showed amplification for *pol* gene [Fig. 1b and c]. Of these two positive cases recurrence occurred in one patient only. All the 30 control corneal buttons were negative for the *pol* gene.

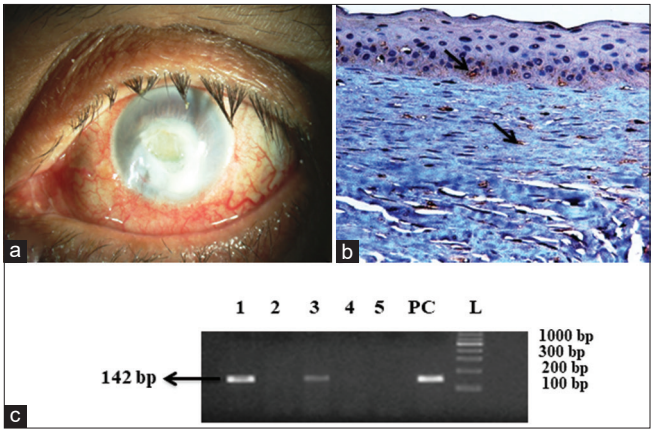

Figure 1: Clinical appearance and detection of HSV-1 antigen and DNA in suspected viral keratitis corneal samples. (a) Atypical appearance of a suspected viral keratitis eye. (b) IHC showing cytoplasmic and nuclear (arrow) expression of HSV-1 antigen in corneal samples. (c) Agarose gel electrophoresis showing amplification of 142bp region of the HSV-1 DNA polymerases gene. Lane 1 and 3 shows 142bp amplified HSV-1DNA in corneal samples. Lane 2 and 4 samples are negative for HSV-1 DNA polymerase gene. Lane 5 contains negative control. PC -Positive Control; L- 100bp ladder

qPCR results

Quantitative real-time PCR was performed to detect the HSV-1 *LAT* gene transcript in all RNA samples extracted from both suspected viral keratitis and control corneal buttons. *LAT* gene transcript was detected in 7 out of 30 samples (23%) of suspected viral keratitis corneal samples [Table 2]. The number of LAT transcripts detected in the 7 corneal samples varied from 4.44 x 10^3 to 1.46 x 10^7 [Table 3]. Of these seven LAT transcript positive cases, recurrence developed in two patients and one of these two patients was diagnosed with clinically active disease at presentation. The same patient had a high viral load in the corneal button (1.46 x 10^7 copies/mg) and also experienced reinfection after 1 year post PKP. The patients who presented with stromal inflammation displayed higher positive rates of qPCR than the patients with no stromal inflammation (p = 0.0067) [Table 1]. The limit of detection of qPCR was found to be 10 copies of HSV-1 LAT transcript. The standard curve had a slope of -3.695 with a regression coefficient (R^2) value of 0.995 and an efficiency 97.1%. No amplification was detected in the no-template control reaction containing sterile water instead of target template. The melt curve analysis of the real-time PCR assay showed a Tm of 86-86.5°whenever there was the presence of HSV-1 LAT transcript [Fig. 2a-c]. LAT transcript positive titers did not show a statistical correlation with clinical features due to small sample size as well as low positivity rate. All the 30 control corneal buttons were negative for *LAT* gene transcript.

Comparison between qPCR, DNA PCR and IHC

The relation between HSV-1 *pol* gene DNA, IHC and real-time PCR results revealed that two cases were positive by IHC, PCR and real-time PCR. The sensitivity and specificity of each of the tests were evaluated [Table 2]. IHC and conventional PCR showed lower sensitivity 57.8% and 28.6% than real-time PCR, respectively. A statistically significant association was found between qPCR and conventional PCR (P = 0.04) [Table 4].

Discussion

In this study, LATtranscript was detected in 23% cases of suspected viral keratitis with copy numbers ranging from 1.46 x 10^7 to 2.35 x 10^5 copies/µl by qPCR. Although many of the earlier published studies detected the LAT transcript in human corneal buttons, very few have quantified it. Higaki *et al.*, detected and quantified the LAT transcripts in 3/6 corneal buttons with a history of herpetic keratitis and also suggested the possibility of HSV-1 being latently present in the cornea.[8] In 1991 Cook *et al.* were the first to detect infected cell protein O (*ICPO*), *LAT* and thymidine kinase (*TK*) gene fragments by both DNA-PCR and RNA-PCR in the cornea and trigeminal ganglia of latently infected rabbit and found LAT RNA in 9% of corneas and 100% of corresponding trigeminal ganglia.[14] Kaye *et al.* detected *LAT* gene as the only RNA viral transcript present in 8 of 10 recipient corneal buttons of herpetic keratitis cases by RNA-PCR. In 1993 Shimomura *et al.* identified the HSV-1 latency in 4 of 8 (50%) corneas obtained after PKP from the patients of herpetic stromal keratitis. They detected a latent

Table 1: Association of clinicopathological features with HSV-1 genome in suspected viral keratitis patients

Clinicopathological features (N=30)	HSV-1 DNA PCR (DNA *pol* gene)		*P*	Real Time PCR (*LAT* transcript)		*P*	IHC (HSV-1 antigen)		*P*
	Positive (*n*=2)	Negative (*n*=28)		Positive (*n*=7)	Negative (*n*=23)		Positive (*n*=9)	Negative (*n*=21)	
Sex									
Male (24)	2	22	1.00	7	17	0.29	7	17	1.00
Female (6)	0	6		0	6		2	4	
Age									
<45 (14)	0	14	0.48	3	11	1.00	5	9	0.69
>45 (16)	2	14		4	12		4	12	
BCVA at presentation									
FCCF or worse (27)	2	25	1.00	6	21	1.00	8	19	1.00
Better than FCCF (3)	0	3		1	2		1	2	
Infiltration/opacity									
Opacity (4)	1	3	0.25	1	3	1.00	1	3	1.00
Infiltration (26)	1	25		6	20		8	18	
Corneal vascularization									
Yes (18)	1	17	1.00	5	13	0.66	5	13	1.00
No (12)	1	11		2	10		4	8	
Clinically active disease									
Present (4)	1	3	0.25	1	3	1.00	1	3	1.00
Absent (26)	1	25		6	20		8	18	
Recurrence									
Present (7)	1	6	0.41	2	5	1.00	3	4	0.64
Absent (23)	1	22		5	18		6	17	
Corneal Epithelium Edema (17)	2	15	0.49	4	13	1.00	8	9	*0.04
Stromal edema (18)	0	18	0.15	3	15	0.39	4	14	0.42
Stromal vascularization (18)	0	18	0.15	4	14	1.00	3	15	0.10
Stromal inflammation (8)	1	7	0.46	5	3	*0.006	5	3	*0.03

BCVA- Best corrected visual acuity, FCCF - Finger counting close to face, LAT - Latency Associated Transcript, pol - Polymerase, PCR - Polymerase chain reaction, IHC - Immunohistochemistry *Statistically significant

Table 2: Results of qPCR versus IHC, DNA PCR and DNA PCR versus IHC

	IHC			PCR (*pol* gene)		
	Positive (%)	Negative (%)	Total (%)	Positive (%)	Negative (%)	Total (%)
qPCR (*LAT* gene) +ve	4 (13.3)	3 (10)	7 (23.3)	2 (6.7)	5 (16.7)	7 (23)
qPCR (*LAT* gene) -ve	5 (16.7)	18 (60)	23 (76.6)	0 (0)	23 (76.7)	23 (76.6)
Total	9 (30)	21 (70)	30	2 (6.6%)	28 (93.4)	30
	Sensitivity	57.1% (95% CI: 18.4-90.1)		Sensitivity	28.6% (95% CI: 3.67-71)	
	Specificity	78.3% (95% CI: 56.3-92.5)		Specificity	100% (95% CI: 85.2-100)	
	PPV	44.4% (95% CI: 13.7-78.8)		PPV	100% (95% CI: 15.8-100)	
	NPV	85.7% (95% CI: 63.7-97)		NPV	82.1% (95% CI: 63.1-93.9)	

	IHC		
	Positive (%)	Negative (%)	Total (%)
PCR (*pol* gene) +ve	2 (6.7)	0 (0)	2 (6.6)
PCR (*pol* gene) -ve	7 (23.3)	21 (70)	28 (93.3)
Total	9 (30)	21 (70)	30
	Sensitivity	100% (95% CI: 15.8-100)	
	Specificity	75% (95% CI: 55.1-89.3)	
	PPV	22.2% (95% CI: 2.81-60)	
	NPV	100% (95% CI: 83.9-100)	

qPCR - Quantitative polymerase chain reaction, PCR- Polymerase chain reaction, IHC - Immunohistochemistry, PPV- Positive predictive value, NPV- Negative predictive value, CI- Confidence interval

Table 3: Copy numbers of LAT gene transcript in suspected viral keratitis patients

Patients No.	Age (years)	Sex	HSV-1 LAT transcript (copies/mg) in corneal tissue
1	53	M	4.44×10^3
2	67	M	1.46×10^7
3	20	M	4.61×10^4
4	50	M	1.19×10^4
5	35	M	8.44×10^3
6	72	M	2.35×10^5
7	19	M	2.35×10^6

LAT - Latency associated transcript

Table 4: Comparison between IHC, DNA PCR and qPCR in suspected viral keratitis cases

qPCR (*n*=30)	HSV-1 DNA PCR (*n*=30)		IHC (*n*=30)	
	Positive (*n*=2)	Negative (*n*=28)	Positive (*n*=9)	Negative (*n*=21)
Positive (*n*=7)	2	5	4	3
Negative (*n*=23)	0	23	5	18
P	0.048*		0.153	

*Statistically significant

virus from the culture supernatant of a section of the cornea.[15] In our study conventional PCR (28.6%) and IHC (57.1%) showed lower sensitivity than real-time PCR. Sensitivity and specificity of real-time PCR for detecting HSV-1 by qPCR have been reported as 98% and 100% respectively.[29] Due to the lack of adequate samples, culture of corneal tissue could not be done in this study.

Some of the major causes of graft failure after primary keratoplasty are irreversible rejection, infection, primary donor failure, and late endothelial failure. Herpetic infection is also a risk factor for endothelial rejection.[16,17] In our study, clinical recurrence of the disease occurred in seven patients, of these, endothelial rejection was seen in five and re-infection in two grafts. A series of studies have also reported that HSV infection caused severe endothelial cell loss in corneal organ culture and has been implicated in some cases of primary graft failure.[18,19] LAT transcript was detected in two of these patients.

Low positivity for LAT transcript (23%) in our study could be because the patients received oral antiviral acyclovir preoperatively. It is known that viral replication is effectively inhibited by acyclovir.[20,21] Acyclovir is not capable of preventing recurrences but may prolong the recurrence-free survival and hence reduce the duration of herpetic disease. This could also explain detectable HSV-1 in one patient despite clinically active disease at presentation in 4 patients in our study. The same patient also experienced re-infection after surgery. Additionally, the level of *LAT* gene expression, degree of genome repression & immunosurveillance may also influence the efficacy, copy numbers of LAT transcript and reactivation.

However, in 23.07% (6/26 eyes) of clinically inactive cases, viral transcripts were detected. Thereby highlighting the role of LAT gene transcripts in clinically dormant cases of HSV keratitis. Moreover, due to small patient cohort the status of qPCR as a diagnostic tool needs to be validated on a larger patient cohort.

The reported prevalence rate of HSV DNA in corneal buttons is between 7.8% to 40% in different studies.[22,24] We detected HSV-1 DNA in 2 (6.7%) cases of suspected viral keratitis by DNA PCR. This variation in results could be due to patient's selection criteria, patients received antiviral therapy preoperatively and differences in detection methods used. The selection of the region of the HSV-1 genome to be amplified influences the sensitivity of the PCR. In earlier studies the UL-42 gene was detected in 14 out of

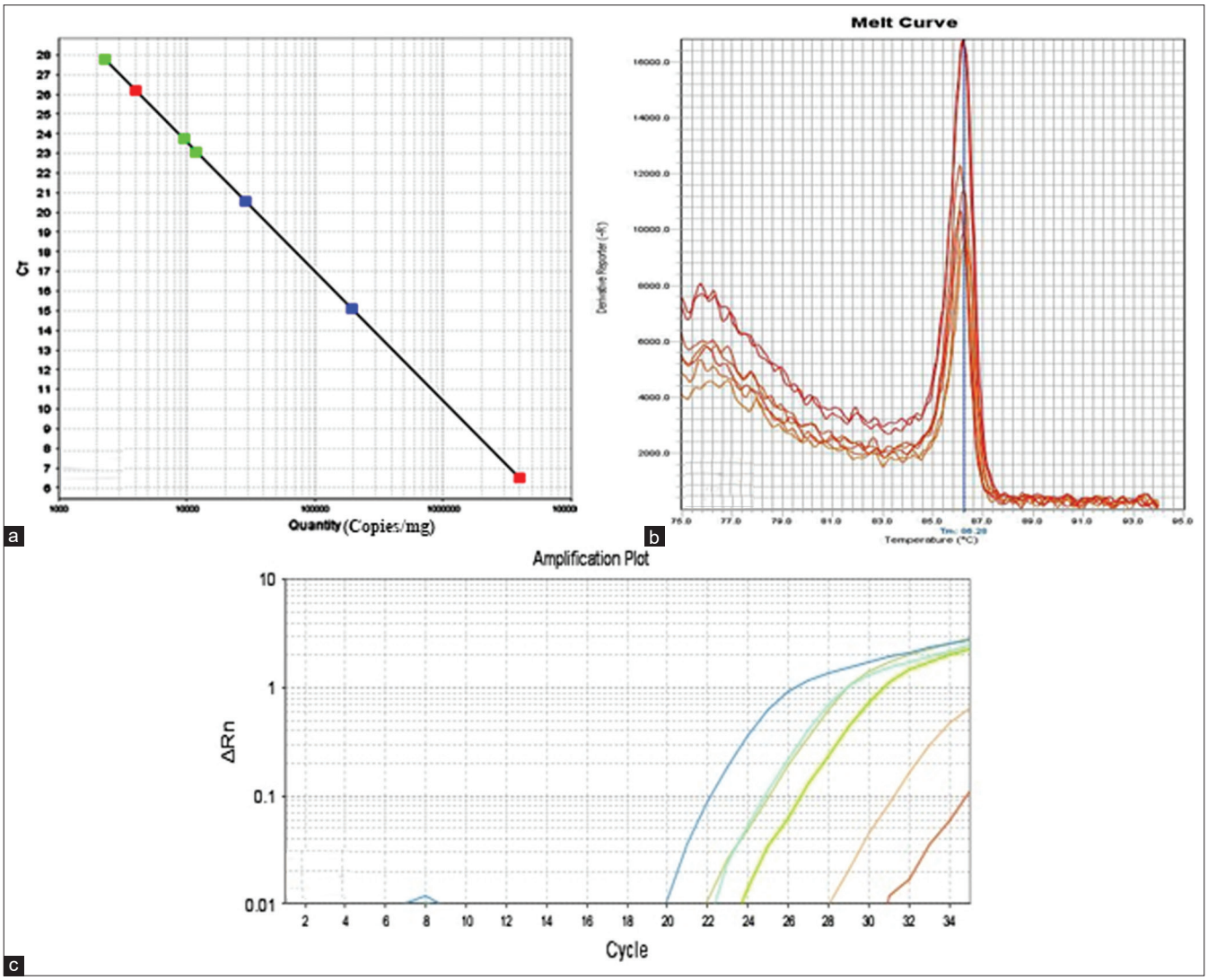

Figure 2: Evaluation of amplification and quantification of LAT transcript in suspected viral keratitis cases by qPCR. (a) Standard curve showing the quantity of LAT gene transcript in corneal samples (blue and green dots) relative to HSV-1 (red dot). (b) Melt curve of amplified product and (c) Relative amplification curve of HSV-1 LAT transcript

47 samples (30%),[26] the glycoprotein D gene in eight out of 18 (44%),[27] and DNA-polymerase gene of HSV type 1 in five out of eight (63%).[28] Since HSV-1 causes focal infection in the cornea, random sampling of the cornea could have affected the detection rate in the samples. Openshaw *et al.*[5] found HSV-1 DNA more frequently in the peripheral than the central cornea. The detection of HSV-1 DNA by PCR only provides evidence of viral DNA, it does not entail functional virus, nor does it distinguish between latent and active virus. HSV-1 DNA in the cornea has been quantified by real-time PCR and the copy number in the cornea correlated with disease severity.[29] Due to the lack of adequate samples, HSV-1 DNA load could not be quantified in this study.

The positivity for HSV-1 antigen by IHC in our study was 30%. Earlier study has shown IHC to have a sensitivity and specificity of 74% and 85%, respectively, for the detection of HSV-1 antigen.[2] However, it is not a sensitive technique for the detection of HSV-1 antigen in keratoplasty patients, due to false positivity. Moreover, IHC does not detect the latent infection and the presence of HSV-1 antigen does not necessarily indicate the presence of infectious virus.

Conclusion

To conclude, although clinical parameters are most important in the diagnosis of viral keratitis, for atypical cases laboratory tests including quantitative real-time PCR could provide an alternative to the conventional DNA PCR for detection and quantifying *LAT* gene in the cornea tissue. The limitation of this study is the small sample size and low positivity rate. Therefore the status of qPCR (LAT transcript) needs to be substantiated by studies on more number of patients with suspected herpetic keratitis, keeping the cost factor in mind.

Prospective preoperative studies of LAT gene qPCR on corneal scraping and tear samples of clinically suspected active and healed keratitis are also recommended in future to determine its clinical utility preoperatively.

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Conflicts of interest

There are no conflicts of interest.

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