Detection of herpes simplex-1 and -2 and varicella zoster virus by quantitative real-time polymerase chain reaction in corneas from patients with bacterial keratitis

Detecção de vírus herpes simplex tipo 1 e 2 e varicella zoster por reação em cadeia de polimerase em tempo real em córneas de pacientes com ceratites bacterianas

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ABSTRACT

Objective: Bacterial keratitis occurs worldwide, and despite recent developments, it remains a potentially blinding condition. This study assesses the presence of herpes simplex virus (HSV-1 and -2) and varicella zoster virus (VZV) by quantitative real-time polymerase chain reaction (qPCR) in corneal scrapings from patients with bacterial keratitis.

Methods: A total of 65 patients with clinical diagnoses of infectious corneal ulcers prospectively underwent clinical eye examinations. Corneal scrapings were investigated by Gram staining, Giemsa staining, culture, and qPCR (the study group). Risk factors and epidemiological data were recorded. The control group comprising 25 eyes with typical herpes dendritic keratitis was also analyzed by qPCR.

Results: From the study group (n=65), nine patients (13.8%) had negative smears, cultures, and qPCR findings. Fifty-six (86.2%) patients had positive cultures: 51 for bacteria, 4 for fungi, and 1 for amoebae. Of the patients who had positive bacterial cultures, qPCR identified 10 patients who were also positive for virus: one for VZV and nine for HSV-1. Of the 25 patients in the control group, 21 tested positive for HSV-1 by qPCR analysis.

Conclusions: Herpes may be present in patients with bacterial corneal ulcers, and qPCR may be useful in its detection.

Keywords: Herpes simplex; herpesviridae; Keratitis, herpetic; Polymerase chain reaction

RESUMO

Objetivo: *Ceratites bacterianas ocorrem mundialmente e apesar dos novos desenvolvimentos permanece como uma condição que pode levar à cegueira. Avaliar a presença de herpes simples (-1 e -2) e vírus varicella zoster (VZV) por reação em cadeia quantitativa de polimerase em tempo real (qPCR) em raspados corneanos de pacientes com ceratite bacteriana.*

Métodos: *Sessenta e cinco pacientes com ceratite infecciosa foram submetidos a raspados corneanos estudados para gram, Giemsa, cultura e qPCR (grupo de estudo). Foram avaliados fatores de risco e epidemiológicos. O grupo controle foi composto por 25 casos de úlcera dendrítica típica por herpes analisados por qPCR.*

Resultados: *Do grupo de estudo (n=65), nove pacientes (13,8%) apresentaram cultura, qPCR e raspado negativos. Cinquenta e seis (86,2%) pacientes apresentaram cultura positiva, 51 para bacteria, 4 para fungo e 1 para ameba. A qPCR identificou 10 pacientes do grupo de cultura positiva para bactéria que também foram positivos para vírus, um VZV e 9 para HSV-1. Dos 25 pacientes que compunham o grupo controle, 21 apresentaram qPCR positivo para HSV-1.*

Conclusão: *Herpes pode estar presente em pacientes com úlceras de córnea bacterianas e a qPCR pode ser útil na sua detecção.*

Descritores: *Herpes simples; Infecções por herpesviridae; Ceratite herpética; Reação em cadeia da polimerase*

INTRODUCTION

Infectious keratitis occurs worldwide, and despite recent developments, it remains a potentially blinding condition[1]. Often, a specific diagnosis is not clinically possible. Early diagnosis and immediate appropriate treatment can reduce disease morbidity[2,3]. Laboratory identification is important because different etiological agents can manifest themselves with similar clinical profiles, such as *Acanthamoeba* spp., herpes viruses, bacteria, and fungi[4-6].

The development of nucleic acid amplification tests, such as the polymerase chain reaction (PCR), has provided more specific and sensitive methods for diagnosis[7-9]. Quantitative real-time PCR (qPCR) has advantages over conventional methods because it is quicker and more sensitive[10]. It can also detect genes that are expressed only in the replication state and can relatively quantify the DNA sample[11,12].

The current study assesses the presence of herpes simplex virus-1 and -2 (HSV-1 and -2) and varicella zoster virus (VZV) by qPCR in corneal scrapings from patients with infectious keratitis. Risk factors are also studied.

METHODS

Patients with clinical diagnoses of infectious corneal ulcers referred for diagnostic corneal scrapings were included in this study (the study group). Risk factors and epidemiological information, as well as the duration of disease and its clinical evolution, were recorded for each patient. All patients signed an informed consent form after approval by the Ethics Committee (1422/06).

All patients underwent a clinical examination that included slit lamp biomicroscopy and photography. Diseased corneas were scraped and specimens were sent for direct examination (Gram and Giemsa staining techniques) and microbiology investigation for bacteria, fungi, and *Acanthamoeba* as well as qPCR for viruses. At the time of exa-

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mination, the following information was recorded for each patient: gender, age, occupation, history of ocular trauma, previous intraocular surgery, presence of local or systemic risk factors (such as contact lens use, diabetes, HIV, advanced age, and corneal exposition), current medications, and symptom duration.

Corneal scrapings were obtained from the edge of the ulcer after application of topical anesthesia and the ocular surface was washed with physiologic saline to avoid false-positive results during qPCR[9]. A portion of the sample was quickly frozen (-80°C) and sent for molecular diagnostic techniques. Cultures were considered positive when there was heavy growth on stria blood or chocolate agar and a positive correlation with the results of smears.

Total DNA extraction was performed using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol.

Molecular detection was carried out using TaqMan-based technology, and reactions for HSV-1 and -2 and VZV (sequences of primers and probes are listed in Table 1) were tested for analytical specificity using positive (known viral DNA from HSV-1 and -2, and VZV) and negative (molecular grade water) controls[13]. The reaction was standardized with primers and probes for HSV-1/2 in a duplex format and a VZV target in a separate reaction. Briefly, 5 mL of DNA was added to a final reaction volume of 25.0 mL (12.5 mL of the 2X TaqMan™ universal PCR master mix, 0.3 mM of each primer, 0.3 mM of each probe, and 5.7 mL of molecular grade sterile water), and 45 PCR cycles were run: denaturation step (20 sec at 95°C), primer annealing and extension (1 min at 60°C). The beta-globin gene was used as the endogenous control[14] (10.0 mL of 2X TaqMan™ universal PCR mix, 0.4 mM of each primer, 0.2 mM of the probe, 7.0 mL molecular grade sterile water, and 2.0 mL of DNA sample to a final volume of 20.0 mL) and the same PCR conditions described above were used. All reactions were carried out in an ABI Prism 7500 device (Applied Biosystems, Carlsbad, CA, USA). The clinical samples were then analyzed for the presence of HSV-1 and -2 and VZV DNA. The molecular test was designed to detect only actively replicating virus, through collection of corneal scrapings only from symptomatic patients. The alpha subfamily of the herpes viruses replicates efficiently in skin and mucosa, in which the mucosa sore is a known site of active replication[14]. The control group comprised 25 eyes with typical herpes dendritic keratitis that were also analyzed by qPCR.

RESULTS

Sixty-five eyes of 65 patients (38 females; mean age 44.9 years, range 8-93 years) who had been clinically diagnosed with bacterial keratitis (the study group) and 25 eyes of 25 patients presenting with typical herpes dendritic keratitis (the control group) were tested from May 2008 to December 2010 (Table 2).

From the study group, 9 of 65 eyes (13.8%) had negative smears, cultures, and PCR findings. From these nine patients, eight had corneal ulcers <2 mm and one had a 3.5 × 3 mm ulcer. Six patients were contact lens wearers and two reported previous ocular trauma. All eyes with negative cultures were also negative for viruses by qPCR analysis.

The most frequent etiologic agent was coagulase-negative *Staphylococcus* spp., in 21 of 56 culture-positive eyes (37.5%). From the culture-positive eyes, gram-positive microorganisms were responsible for the infections in 37 eyes (66.1%). Gram-negative microorganisms were present in 13 eyes (23.2%) and fungi were present in four eyes (7.1%). One eye (1.8%) presented *Acanthamoeba* growth on cultures. Most of the patients with positive cultures had ulcers >2 mm with variable clinical aspects. Risk factors for keratitis were present in 21 eyes (37.5%) and included trauma, previous intraocular surgery, bullous keratopathy, contact lens wear, and Steven-Johnson syndrome.

In 5 of 56 eyes with positive cultures, more than one etiological agent was detected: two eyes presented two Gram-positive organisms and three presented one Gram-positive and one Gram-negative agent. One of the patients with two Gram-positive organisms was also positive for HSV-1 by qPCR analysis. From these five patients, four were contact lens wearers and one had a history of several previous intraocular surgeries. All had ulcers >2 mm.

Four eyes harbored fungi and one eye presented *Acanthamoeba* growth in cultures. Although these eyes were clinically diagnosed as being potential bacterial corneal ulcers, microbiology studies were able to reveal the causative microorganisms and guide appropriate management. None of those patients were positive for herpes viruses by qPCR analysis.

Ten of 65 eyes (15.3%) were positive for viruses in the qPCR analysis. Of those, VZV was identified in one eye of one patient (a 93-year-old female), and HSV-1 was identified in nine eyes. All had ulcers >2 mm. Ocular comorbidities or severe systemic diseases were present in 7 of the 65 eyes (70%) (Table 3).

The mean age of patients who had positive qPCR results for herpes was higher than that of those who were negative for herpes: 60.4 and 42.2 years, respectively (p=0.01) (Table 4).

The control group comprised 25 patients with clinical diagnoses of herpes epithelial keratitis. All had dendritic lesions at presentation. Twenty-one of these patients were positive for HSV-1 by qPCR analysis (84%).

DISCUSSION

HSV-1 and VZV were identified in this study using qPCR of samples of infectious keratitis with bacterial growth in cultures, indicating a potential co-infection. Systemic and ocular comorbidities were found more frequently (70%) in cases that were positive for both virus and bacteria than in cases that were positive only for bacteria (32%). Neovascular glaucoma with several previous intraocular surgeries, systemic immunosuppression due to HIV infection, chronic steroid use, severe allergy, and dry eye disease were found in 7 of 10 patients with a confirmed co-infection, and 4 of the 10 patients (40%) from this group were older than 70 years. This is a large proportion compared with the general study population, in which 9 of the 65 patients (14%) were older than 70 years. Statistical analysis revealed that the mean age of patients with positive qPCR results was significantly higher than that of patients with negative results (60.4 and 42.18 years, respectively; p=0.01, Table 3). In addition, there was a tendency to a higher prevalence of positive PCR results in patients older than 70 years (p=0.07). Therefore, advanced age could be a factor for herpes infection.

A possible study limitation was the detection of latent HSV-1 and -2, as well as VZV. However, the target of the real-time primers and probes were amplified genes (the beta-globin gene in HSV-1 and -2, and the gene for the ORF29 protein in VZV), which are expressed almost exclusively during active viral replication. HSV-1 latency in the nuclei of neurons of regional ganglia is well described[2,3]. As such, when clinical samples are collected from lesions located in the periphery of an axon, in the conjunctiva or mucosal surfaces, the virus is actively replicating and not latent. Besides, there are limited genes expressed during latency, and in designing the primers for this study,

Table 1. Primers and probes targeting herpes simplex virus-1 and -2, and varicella zoster virus

Herpes virus	Sequences for primers and probes	Genetarget
HSV-1 and -2	HSV forward: CGCATCAAGACCACCTCCTC	Beta-globin
	HSV reverse: GCTCGCACCACGCGA	
	HSV1 primer: JOE-TGGCAACGCGGCCCAAC-TAMRA	
	HSV2 primer: FAM-CGGCGATGCGCCCCAG-TAMRA	
VZV	VZV forward: AACTTTTACATCCAGCCTGGCG	ORF29
	VZV reverse: GAAAACCCAAACCGTTCTCGAG	
	VZV primer: FAM-TGTCTTTCACGGAGGCAAACACGT-TAMRA	

HSV= herpes simplex virus; VZV= varicella zoster virus.

Table 2. Microbiology and quantitative real-time polymerase chain reaction results from patients with clinical diagnoses of infectious corneal ulcers

Patient	Age (years)	Sex	Microbiological result	qPCR
1	49	F	Negative	Negative
2	40	M	Coagulase-negative *Staphylococcus*	Negative
3	46	F	Gram-negative Bacillus	HSV-1
4	50	M	*Aspergillus flavus*	Negative
5	93	F	*Streptococcus pneumonia*	VZV
6	28	M	*Fusarium dimerum*	Negative
7	58	M	*Serratia nonliquefaciens*	Negative
8	32	F	Negative	Negative
9	23	F	Negative	Negative
10	70	F	*Corynebacterium* spp.	Negative
11	41	M	Negative	Negative
12	20	M	*Staphylococcus aureus*	HSV-1
13	62	M	*Streptococcus pneumonia*	Negative
14	48	M	*Moraxella nonliquefaciens* and *Corynebacterium* spp.	Negative
15	50	M	*Burkholderia cepacia*	HSV-1
16	66	F	*Enterobacter aerogenes*	Negative
17	23	M	Negative	Negative
18	62	F	*Serratia marcescens*	Negative
19	15	M	*Pseudomonas aeruginosa*	Negative
20	68	F	Beta hemolytic *Streptococcus*	Negative
21	59	M	Coagulase-negative *Staphylococcus*	Negative
22	73	F	*Staphylococcus aureus*	Negative
23	29	M	Beta hemolytic *Streptococcus*	Negative
24	26	M	*Staphylococcus aureus*	Negative
25	50	M	*Corynebacterium* spp.	Negative
26	13	M	Coagulase-negative *Staphylococcus*	Negative
27	14	F	Gram-negative cocci	Negative
28	31	F	*Acanthamoeba* spp.	Negative
29	37	F	Coagulase-negative *Staphylococcus*	Negative
30	85	F	Coagulase-negative *Staphylococcus*, *Corynebacterium* spp.	HSV-1
31	74	F	*Corynebacterium* spp.	HSV-1
32	16	F	Coagulase-negative *Staphylococcus*, *Streptococcus* group *viridians*	Negative
33	41	M	*Fusarium solani*	Negative
34	28	F	Negative	Negative
35	16	F	Coagulase-negative *Staphylococcus*	Negative
36	56	F	*Streptococcus viridians*	HSV-1
37	47	M	Coagulase-negative *Staphylococcus*	HSV-1
38	79	F	Negative	Negative
39	28	F	*Streptococcus pneumonia*	Negative
40	56	F	Coagulase-negative *Staphylococcus*	Negative
41	77	F	*Staphylococcus aureus*	Negative
42	21	M	*Serratia* SSP. and coagulase-negative *Staphylococcus*	Negative
43	27	F	*Staphylococcus aureus*	Negative
44	8	F	*Fusarium solani*	Negative
45	83	F	Gram-positive cocci	Negative
46	74	M	Gram-positive bacillus	HSV-1
47	59	M	*Streptococcus viridians*	HSV-1
48	51	M	Coagulase-negative *Staphylococcus*	Negative
49	28	F	*Pseudomonas oryzihabitans* and coagulase negative *Staphylococcus*	Negative
50	42	M	*Serratia* spp.	Negative
51	37	F	Coagulase-negative *Staphylococcus*	Negative
52	42	F	*Moraxella nonliquefaciens*	Negative
53	35	M	Negative	Negative
54	70	M	Coagulase-negative *Staphylococcus*	Negative
55	47	F	Coagulase-negative *Staphylococcus*	Negative
56	23	F	*Staphylococcus aureus*	Negative
57	76	F	Coagulase-negative *Staphylococcus*	Negative
58	70	F	*Enterobacter aerogenes*	Negative
59	24	F	*Staphylococcus aureus*	Negative
60	37	M	Coagulase-negative *Staphylococcus*	Negative
61	27	F	Negative	Negative
62	9	F	Coagulase-negative *Staphylococcus*	Negative
63	29	F	Coagulase-negative *Staphylococcus*	Negative
64	59	M	Coagulase-negative *Staphylococcus*	Negative
65	67	F	Coagulase-negative *Staphylococcus*	Negative

F= female; M= male; HSV= herpes simplex virus; qPCR= quantitative real-time polymerase chain reaction; VZV= varicella zoster virus.

Table 3. Characteristics of patients with positive bacterial cultures and positive quantitative real-time protein chain reaction results

Patient	Age (years)	Sex	Comorbidities	Microbiological results	qPCR results
1	46	F	Congenital glaucoma, several intraocular surgeries	Gram-negative bacilli	HSV-1
2	93	F	Ulcer with hypopyon	*Streptococcus pneumoniae*	VZV
3	20	M	Severe ocular allergy and chronic steroid use	*Staphylococcus aureus*	HSV-1
4	50	M	HIV-positive	*Burkholderia cepacia*	HSV-1
5	85	F	-	Coagulase-negative *Staphylococcus* and Corynebacterium spp.	HSV-1
6	74	F	-	Corynebacterium spp.	HSV-1
7	56	F	Severe dry eye	*S. viridans*	HSV-1
8	47	M	Measles sequelae (corneal thinning and opacities)	Coagulase-negative *Staphylococcus*	HSV-1
9	74	M	-	Gram-positive bacilli	HSV-1
10	59	M	Neovascular glaucoma, blind eye with several intraocular surgeries	*S. viridans*	HSV-1

F= female; M= male; HSV= herpes simplex virus; qPCR= quantitative real-time polymerase chain reaction; VZ= varicella zoster virus.

Table 4. Mean age of patients with positive and negative quantitative real-time protein chain reaction results

	Positive	Negative
Number	10	55
Mean age	60.40	42.18
Standard deviation	21.57	20.52
p value	0.01276	

sequences that are expressed only during the active phases of infection and not during latency were chosen. Therefore, it is fairly certain that replicating HSV that was causing the disease was detected rather than latent viruses[15-17].

The control group, which was composed of eyes with only active dendritic epithelial keratitis, showed a high positivity in qPCR analysis (84%). This strengthens the hypothesis of positive PCR results during active viral replication. Most of the eyes from the control group that had negative cultures and qPCR results had ulcers <2mm. The small amount of material collected could be responsible for the negative results from both culture and qPCR.

CONCLUSION

Microbiology studies are essential in infectious keratitis because different etiologic agents can manifest with similar clinical profiles. Molecular techniques show that herpes virus may be present in patients with bacterial corneal ulcers and qPCR may be useful in its detection and treatment, so as to avoid steroids, or appropriately administer antiviral drugs to patients who are positive for herpes by PCR. Cost-effective studies should be carried out to evaluate the impact of such molecular techniques.

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