Journal of Clinical Microbiology

Clinical Microbiology | Full-Length Text

Diagnostic performance of real-time quantitative PCR in tear samples in various subtypes of herpes simplex keratitis

Gautier Hoarau,[1] Oscar Haigh,[2] Christelle Vauloup-Fellous,[2,3] Rafaël Boucher,[1,2] Alexandra Rouquette,[4] Pascale Faure,[1] Lamia Limam,[3] Marc Labetoulle,[1,2] Antoine Rousseau[1,2]

AUTHOR AFFILIATIONS See affiliation list on p. 9.

ABSTRACT Diagnosis of herpes simplex keratitis (HSK) is mostly based on clinical findings, yet biological confirmation supports management of challenging cases. This study evaluated the place of real-time quantitative PCR (RT-qPCR) on tear samplings in the management of HSK. Clinical records of patients who underwent tear sampling tested by RT-qPCR for herpes simplex virus type 1 for an acute episode of corneal inflammation or defect between January 2013 and December 2021 were retrospectively reviewed, and results were compared to clinical diagnosis (i.e., HSK or not) based on biomicroscopic findings and medical history. Of 465 tested tear samples from 364 patients, a clinical diagnosis of active (ongoing) HSK was recorded in 240 cases, among which 76 were RT-qPCR positive (global sensitivity of 31.6%, specificity of 99.5%). Sensitivity of RT-qPCR was higher in epithelial (97.4%) and stromal keratitis with ulceration (48.7%), compared to other types of HSK (23.5% in keratouveitis, 13.6% in endotheliitis, 11.1% in postherpetic neurotrophic keratopathy, and 8.1% in stromal keratitis without ulceration). The highest viral loads were detected from epithelial and stromal keratitis with ulceration, while in HSK with no epithelial involvement, the viral load detected was 196-fold lower, on average. The proportion of clinically characterized HSK patients with negative tear samples was higher in patients receiving antiviral treatment ($P < 0.0001$). RT-qPCR, performed on tear samples, can help in confirming diagnosis in case of presumed HSK, including clinical forms with no obvious epithelial involvement. The sensitivity of tear sampling is much higher whenever epithelial keratitis is present.

KEYWORDS HSV, keratitis, cornea, PCR

Herpes simplex keratitis (HSK) is a broad term that encompasses a range of corneal diseases caused by herpes simplex virus type 1 (HSV1). These diseases involve varying combinations of viral replication, immune reactions, and trophic alterations. With an estimated prevalence of 150/100,000 in the general population, HSK is considered as a leading cause of infectious blindness in industrialized countries (1–3). Diagnosis is based on clinical findings and medical history, although it can be challenging in atypical presentations (4, 5). Furthermore, it is advisable to have biological confirmation of this chronic and potentially recurring disease in order to enhance the comprehensive medical and surgical treatment options that may be necessary to optimize outcomes.

Due to a very high sero-prevalence in the general population—up to 60% in subjects above 40 years in industrialized countries (6)—serology is rarely helpful, except if negative (3). For these reasons, microbiological analysis of ocular surface (OS) samples may be of utmost importance in challenging cases and in cases of clinical failure to anti-herpetic drugs, in order to assess potential antiviral resistance (7). In this context, detection and quantification of the viral genome by polymerase chain reaction (qPCR) on

Editor Alexander J. McAdam, Boston Children's Hospital, Boston, Massachusetts, USA

Address correspondence to Antoine Rousseau, antoine.rousseau@aphp.fr.

The authors declare no conflict of interest.

Received 14 July 2023
Accepted 20 October 2023
Published 1 December 2023

OS samples have largely supplanted other techniques such as immunofluorescence or viral titration on cell culture (8).

Two main techniques are commonly used to sample HSK: corneal scraping and tear sampling, and there are no existing recommendations regarding the preferable technique, especially according to the clinical form of HSK (5, 8, 9). Additionally, aqueous tap can be considered in HSK cases involving viral replication within the anterior chamber, such as endotheliitis and keratouveitis (10–12). Although corneal scraping may logically offer a higher sensitivity (due to a broader harvest of infectious particles) than tear samples (8), it is not appropriate for HSK subtypes with no spontaneous epithelial involvement, since the additional epithelial defect induced by this invasive procedure may be detrimental for the time of HSK healing, especially in these patients who often present with altered corneal sensitivity and trophicity (10). Using a Schirmer strip for tear sampling provides a convenient, painless, and non-invasive alternative to evaluate the presence of viral genetic material in tears and to identify genetic variants of HSV1, when applicable.

In this study, we evaluated the diagnostic performances of real-time quantitative PCR (RT-qPCR) in tear samples from Schirmer strip for diagnosis of various clinical presentations of HSK, and we discuss the advantages of this method compared to other sampling techniques in the management of HSK.

MATERIALS AND METHODS

Patients

In this retrospective study conducted at Bicêtre Paris-Saclay University Hospital labeled as the French Reference Center for recurrent HSK, we reviewed all the medical charts of patients who underwent tear sampling for HSV1 RT-qPCR for an acute episode of corneal inflammation or defect between January 2013 and December 2021, and we compared the results to clinical diagnosis (i.e., HSK or not), based on biomicroscopic findings (slit lamp examination) and medical history. The results of this study were reported according to the CONSORT guidelines.

Patient data collected included age, gender, clinical features of HSK, and any ongoing antiviral treatment at the time of sampling (i.e., antiviral prophylaxis in patients with multiple previous recurrences). Only cases of active corneal inflammation or corneal defect at time of sampling were included in the analysis. Relapses were differentiated from first episodes and reported as well as keratitis with or without epithelial involvement. The study adhered to the principles of the Declaration of Helsinki and received approval from the Ethics Committee of the French Society of Ophthalmology (IRB 00008855 Société Française d'Ophtalmologie IRB#1).

Sample collection

To minimize potential PCR inhibition by anesthetic or fluorescein eye drops, tear samples were obtained either before instilling any eye drop or at least 10 minutes after having rinsed the eyes with sterile 0.9% saline solution (13). A Schirmer strip (Schirmer-Plus, DINA-HITEX, Czech Republic) was placed in the lower conjunctival cul-de-sac for 5 minutes to collect the tears (14). Subsequently, the strip was placed in a sterile collection tube (Greiner Bio-one, Austria) with one drop of sterile 0.9% saline solution.

Nucleic acid extraction and real-time quantitative PCR

Schirmer strips were completely submerged in 250 µL of extraction buffer (which contains RNA carrier, proteinase K, maleic acid, and guanidine chlorhydrate) and incubated at 56°C for 1 h. The supernatant was purified using the QIAmp MinElute Virus Spin kit (Qiagen, Les Ulis, France) with a step of ethanol precipitation. Elution was performed in 50 µL of DNase-free buffer, and nucleic acid extracts were stored at −20°C until use. A 10-µL volume was used to perform RT-qPCR with HSV1&2 and

Varicella-Zoster Virus (VZV) R-GENE kit (bioMérieux, Marcy L'étoile, France). Quantification (copies per milliliter) was performed against calibration standard curve of the kit.

Patient groups

As clinical diagnosis is considered as the reference in typical HSK (4, 5), we chose it as the diagnostic gold standard in this study and based it on the following: (i) slit lamp examination for epithelial, stromal or endothelial abnormalities typical of HSV1 infection; (ii) the disease history (multiple relapses, in the same eye, of epithelial, stromal or endothelial keratitis or keratouveitis), including the efficacy of antiherpetic treatment (oral valacyclovir, oral famciclovir, oral or topical acyclovir, topical ganciclovir) for previous episodes; and (iii) no history of herpes zoster ophthalmicus. HSK presentations were classified into six clinical subtypes (5): (i) epithelial keratitis including dendritic and geographic ulcers, (ii) stromal keratitis without ulceration, (iii) stromal keratitis with ulceration, (iv) endothelial keratitis, (v) kerato-uveitis, and (vi) postherpetic neurotrophic keratopathy.

Four groups were included in the analysis, as depicted in the flowchart (Fig. 1): (i) true positives (TP), HSK patients with clinically confirmed diagnosis and a positive sample; (ii) false negatives (FN), HSK patients with clinically confirmed diagnosis but a negative sample; (iii) true negatives, patients with an alternative diagnosis, who had a negative qPCR sample; and (iv) false positives (FP), patients with an alternative diagnosis, who had a positive RT-qPCR sample. Patients with an alternative diagnosis were considered as a control group.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.0. Clinically diagnosed HSK patients were categorized based on their RT-qPCR results (positive or negative) and compared together using nonparametric Mann-Whitney *U* tests for continuous variables and χ^2 (or Fisher exact when necessary) tests for categorical variables. The values of qPCR (viral loads) were compared using nonparametric Mann-Whitney *U* tests and reported using scatter plots. A *P*-value of less than 0.05 was considered statistically significant. Sensitivity, specificity, and negative and positive predictive values (NPV and PPV) of the RT-qPCR test (and their 95% confidence intervals) were calculated using the clinical diagnosis of HSK as the reference standard on the entire sample and according to the keratitis subtype.

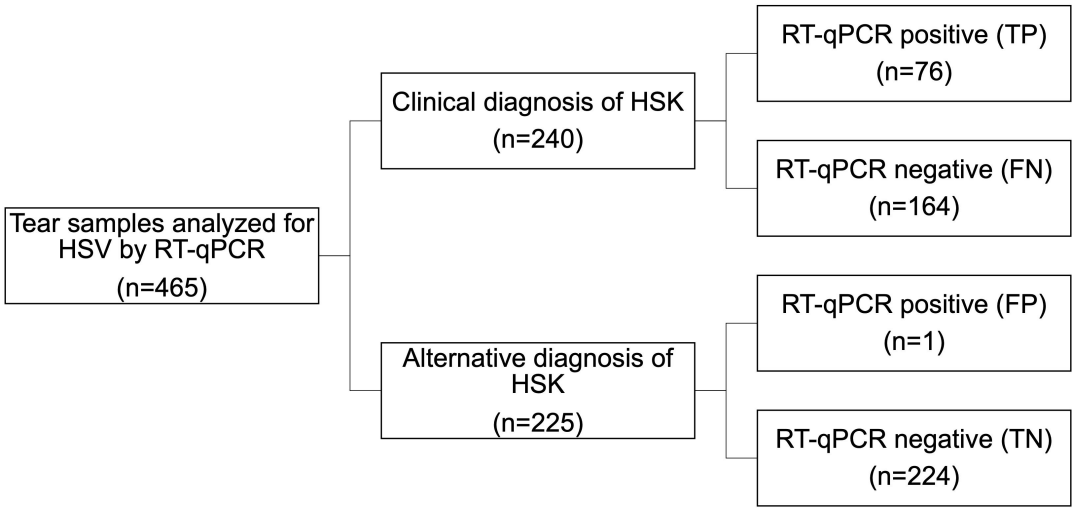

FIG 1 Flow chart of the study. RT-qPCR, real-time quantitative PCR; HSK, herpes simplex keratitis; TP, true positive; FN, false negative; FP, false positive; TN, true negative.

RESULTS

Between January 2013 and December 2021, a total of 465 eyes from 364 patients underwent tear sampling with HSV1-specific qPCR testing and were included in this study. Among them, a clinical diagnosis of HSK had been established for 240 eyes, in all cases for a unilateral herpetic disease. The remaining 225 samples were assigned alternative diagnoses and served as negative controls. These alternative diagnoses included microbial keratitis (34.4%), catarrhal infiltrates (15.2%), severe keratoconjunctivitis sicca (8.5%), non-herpetic neurotrophic keratopathy (8.5%), recurrent corneal erosion syndrome (6.1%), endothelial decompensation (4.4%), and Thygeson keratitis (4%). Other diagnoses (18.9%) comprised various keratopathies such as exposure keratitis, corneal dellen, post-surgical keratitis, and mechanical ulcers. Presumed VZV-induced keratitis cases were excluded from either group.

Clinically characterized HSK

In this group, RT-qPCR was positive in 76 samples (TP) and negative in 164 samples (FN). The clinical features of these HSK are summarized in Table 1. Among clinically characterized HSK patients, 160 (67%) received antiviral prophylaxis at the time of sampling (Table S1). Significant differences were observed, with a higher proportion of treated patients in the stromal keratitis without ulceration subgroup (P = 0.048, 35% vs 22.5%) and a higher proportion of untreated patients in the epithelial keratitis subgroup (P = 0.0002, 28.7% vs 10%). HSV1 viral loads ranged from 1 to 10^7 copies/mL (mean: $1.8 \times 10^5 \pm 1.4 \times 10^5$ copies/mL) (Fig. 2A; Table 2). An epithelial defect was found in 75% of positive samples and significantly associated with a higher viral load (Fig. 2B; $2.3 \times 10^5 \pm 1.7 \times 10^5$ vs 327 ± 907 copies/mL, P < 0.0001). Otherwise, epithelial keratitis and stromal keratitis

TABLE 1 Clinical characteristics of patients and controls[a]

	Clinically characterized HSK patients			Alternative diagnosis	
	RT-qPCR positive, *n* = 76 (31.5%)	RT-qPCR negative, *n* = 164 (68.5%)	*P*-value	RT-qPCR positive (*n* = 1)	RT-qPCR negative (*n* = 224)
Age (years, median)	56.5	54.6	0.5	56	51.8
Male	46 (60%)	96 (58.5%)	0.9	0	107 (48%)
Female	30 (40%)	68 (41.5%)	0.9	1 (100%)	117 (52%)
Keratitis subtype					
Epithelial	38 (50%)	1 (0.6%)	<0.0001	0	66 (29.5%)
Stromal without ulceration	6 (7.9%)	68 (41.5%)	<0.0001	1 (100%)	56 (25%)
Stromal with ulceration	19 (25%)	20 (12.2%)	0.01	0	8 (3.6%)
Endotheliitis	6 (7.9%)	38 (23.1%)	0.004	0	62 (27.7%)
Kerato-uveitis	4 (5.3%)	13 (8%)	0.6	0	13 (5.8%)
Postherpetic neurotrophic keratopathy	3 (4%)	24 (14.6%)	0.009	0	19 (8.5%)
Epithelial defect	60 (79%)	44 (26.8%)	<0.0001	0	149 (66%)
First episode (vs recurrence)	32 (42.1%)	26 (15.8%)	<0.0001	Non relevant	Non relevant
Antiviral intake at the time of sampling	39 (51.3%)	121 (73.8%)	<0.0001	0	35 (15.7%)
Epithelial	16 (21.1%)	0	<0.0001		6 (2.7%)
Stromal without ulceration	4 (5.2%)	52 (31.7%)	<0.0001		12 (5.4%)
Stromal with ulceration	12 (15.8%)	15 (9.1%)	0.13		5 (2.2%)
Endotheliitis	4 (5.2%)	26 (15.8%)	0.02		2 (0.9%)
Kerato-uveitis	2 (2.6%)	9 (5.5%)	0.5		4 (1.8%)
Postherpetic neurotrophic keratopathy	1 (1.3%)	19(11.5%)	0.005		6 (2.7%)
Treatments at the time of sampling					
Valacyclovir	29 (38.2%)	94 (56.7%)	0.008	0	33 (14.8%)
Acyclovir	3 (3.9%)	10 (6%)	0.75	0	0
Famciclovir	6 (7.9%)	17 (10.3%)	0.64	0	2 (0.9%)
Topical ganciclovir	2 (2.6%)	0	0.09	0	0

[a]HSK, herpes simplex keratitis; RT-qPCR, real-time quantitative PCR.

with ulceration were significantly more prevalent (respectively; $P < 0.0001$ and $P = 0.01$) in clinically characterized HSK patients with positive tear sample (Table 1). In the latter group, viral loads were similar regardless of whether patients were receiving antiviral treatment at the time of sampling or not (Fig. 2C). However, the proportion of clinically characterized HSK patients with negative tear sample was higher in patients receiving antiviral treatment at the time of sampling ($P < 0.0001$ Table 1). Finally, the viral load was not different between first episodes and relapses of HSK (Fig. 2D).

Alternative diagnosis

Out of 225 eyes with an alternative diagnosis, RT-qPCR on tear samples showed negative results in 224 cases (specificity of 99.5%, Table 1). The clinical characteristics of HSK patients and patients with an alternative diagnosis (control group) are summarized in Table S2. Notably, the rate of RT-qPCR positivity was significantly lower within the control group ($P < 0.0001$; Table S2). Indeed, we detected only one positive HSV1 sample among the control group, coincidentally with a clinical presentation typical of adenoviral (ADV)

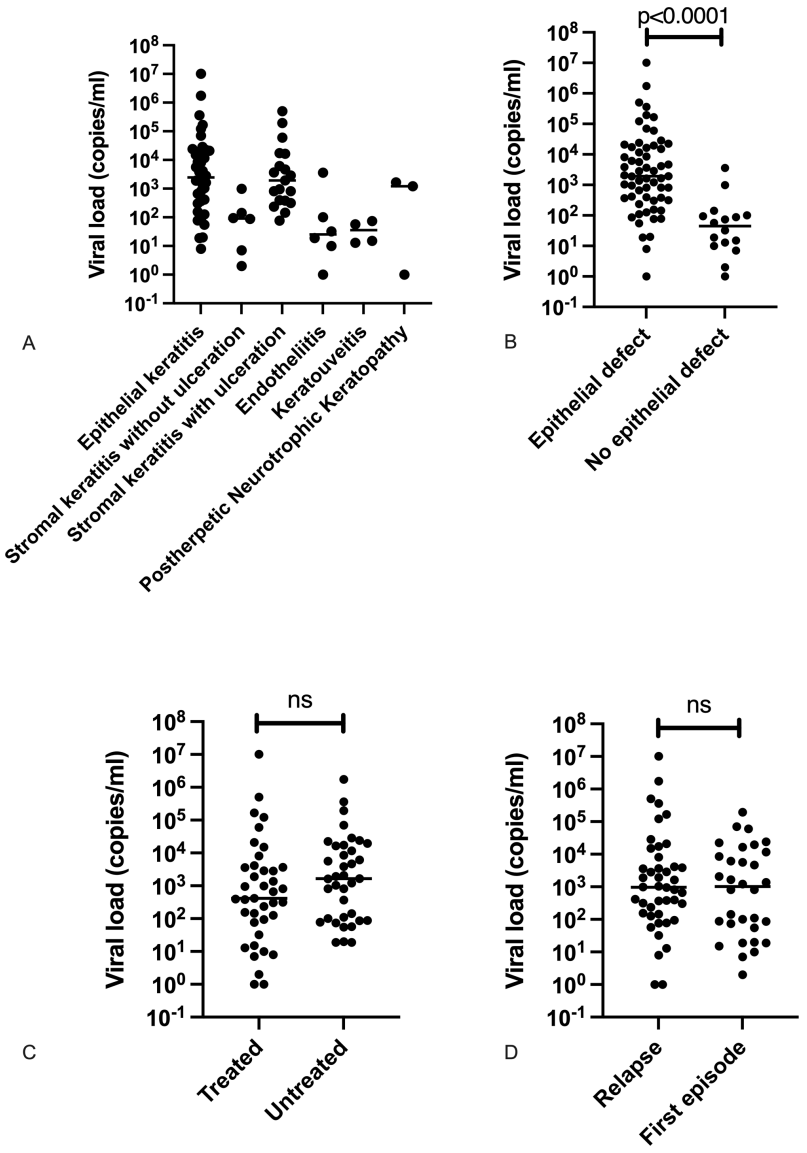

FIG 2 (A) Concentration of HSV1 DNA in tear samples according to the keratitis group. Scatter plots of viral loads according to (B) presence versus absence of an epithelial defect ($P < 0.0001$). (C) Treated versus untreated with antivirals at sampling (non-significant). (D) Relapse versus first episode (non-significant).

TABLE 2 HSV1 viral loads in tear samples (mean, standard deviation of mean)

	Specimens	HSV1 DNA, copies/mL
Epithelial keratitis	38	$3.3 \times 10^5 \pm 2.7 \times 10^5$
Stromal keratitis without ulceration	6	219 ± 153
Stromal keratitis with ulceration	19	$4.3 \times 10^4 \pm 2.7 \times 10^4$
Endotheliitis	6	629 ± 596
Kerato-uveitis	4	40 ± 15
Postherpetic neurotrophic keratopathy	3	954 ± 493

keratoconjunctivitis and a positive ADV PCR in tears. The presence of HSV1 DNA in this case was considered due to incidental shedding, as the complete medical records were indicative of ADV epidemic keratoconjunctivitis and the ocular condition improved without specific antiviral HSV therapy. This case was thus classified as a false positive.

Diagnostic accuracy of tear samples

When compared to the clinical reference standard, the overall sensitivity, specificity, PPV, and NPV of HSV1 RT-qPCR in tears for diagnosing HSK were 31.6%, 99.5%, 98.7%, and 57.7%, respectively. The sensitivity was higher for epithelial and stromal keratitis with ulceration (97.4% and 48.7%, respectively) compared to keratouveitis, endotheliitis, postherpetic neurotrophic keratopathy, and stromal keratitis without ulceration (23.5%, 13.6%, 11.1%, and 8.1%, respectively). The characteristics of the test, including sensitivity, specificity, and predictive values, are summarized in Fig. 3.

The sensitivity of the test was globally better in treatment naïve versus antiviral treated patients (Tables S3 and S4).

DISCUSSION

Our results confirm the usefulness of RT-qPCR on tear samples to obtain microbiological confirmation in various clinical subtypes of HSK.

Pros and cons of tear sampling

Tear sampling is a non-invasive and painless method which is useful in HSK subtypes where scraping is not possible or could be deleterious, i.e., when the corneal epithelium is intact. In the present study, tear samples yielded positive results in HSK clinical subtypes with predominant intraocular inflammation such as endotheliitis, where aqueous tap may be proposed. The overall rate of viral DNA detection in active HSK (regardless of the sampling method) has been reported in the literature to range from 20% to 37% (14–17), which is consistent with our series (31.8%). Previous studies have reported a higher detection rate of HSV1 DNA in epithelial keratitis, ranging from 80% to 100% (18–20), compared to 97.4% in our study.

Compared to tear specimens, corneal scrapings have been suggested to yield higher viral loads. Satpathy et al. demonstrated a twofold higher rate of positive PCR in corneal scrapings (36.6% vs 18.3% in tear sampling) among 153 clinically suspected HSK cases, in which both techniques were employed (14). The rate of detection in tear specimens was much lower in non-epithelial keratitis, especially stromal keratitis (18–20). In our study, the sensitivity of tear sample RT-qPCR in cases of stromal keratitis without ulceration was low (8.1%). Kakimaru-Hasegawa et al. and Fukuda et al. found a sensitivity of tear sampling for stromal HSK of 43% (12/28) and 57% (8/14), respectively, but their studies included a limited number of cases (19, 20). In a larger cohort of 146 clinically suspected stromal HSK patients, Qiu et al. reported a 10.96% rate of positive PCR in tears (21), similar to our findings.

Anterior chamber tapping is often considered as a good option for diagnosis of HSV1 endotheliitis or keratouveitis while tear sample testing is generally not mentioned in literature in such cases. Kakimaru-Hasegawa et al. found 25% of positive PCR (2/8) in the

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
All HSK (n=240)	31.6% (25.8-37.9)	99.5% (97.5-99.9)	98.7% (92.3-99.9)	57.7% (52.6-62.7)
Epithelial keratitis (n=39)	97.4% (86.5-99.9)	100% (94.5-100)	100% (90.7-100)	98.5% (91.9-99.9)
Stromal keratitis without ulceration (n=74)	8.1% (3-16.8%)	98.2% (90.6-99.9)	85.7% (42.1-99.6)	45.1% (36.2-54.3)
Stromal keratitis with ulceration (n=39)	48.7% (32.4-65.2)	100% (63-100)	100% (82.3-100)	28.5% (13.2-48.7)
Endotheliitis (n=44)	13.6% (5.2-27.3)	100% (94.2-100)	100% (54-100)	62% (51.7-71.5)
Kerato-uveitis (n=17)	23.5% (6.8-49.9)	100% (75.3-100)	100% (39.7-100)	50% (29.9-70)
Postherpetic neurotrophic keratopathy (n=27)	11.1% (2.3-29.1)	100% (82.3-100)	100% (29.2-100)	44.1% (29-60.1)

FIG 3 Diagnostic testing accuracy. PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval. Color code: red shading, 0%–25%; orange shading, 25%–75%; green shading, 75%–100%.

aqueous humor in cases of endotheliitis (20), a rate that is of the same order of magnitude than our results from tears (13.6%), in contrast to other studies in which HSV DNA was not detected in tears from endotheliitis patients (18, 19). Table 3 summarizes the findings from various studies on the detection rate of HSK, which, although diverse in nature, were considered relevant.

In addition to its non-invasiveness nature, tear sampling from Schirmer strip offers the possibility to perform genotyping for the detection of resistance to antiviral drugs (7, 23). This technique, a convenient alternative to phenotypic testing, relies on sequencing both viral thymidine kinase and viral DNA polymerase genes to ascertain whether genetic mutations may account for the diminished sensitivity of the isolated viral strains to antiviral drugs (24, 25). Although obtaining a lower viral load in tears compared to corneal scrapings (16, 20) can sometimes hinder the effectiveness of genotyping (7, 10, 23), we recently published a series of HSK cases caused by acyclovir-resistant strains, in which RT-qPCR on tear samples unveiled resistance to antiviral drugs in a significant number of cases (26). Finally, the main drawback of tear sampling appears related to its non-invasive nature, as it does not encompass a step of epithelial debridement, which accelerates the healing process in dendritic and geographic epithelial keratitis (27).

Why is diagnostic accuracy so different in the different clinical HSK subtypes?

There are some important anatomical and pathophysiological mechanism differences between HSK clinical subtypes that may influence effectiveness of tests on tear samples (5, 10). Epithelial HSK is mainly caused by viral replication in corneal epithelium (directly in contact with tears). Similarly, stromal HSK with ulceration is also associated with high levels of viral replication on the OS, including epithelium and stroma (5, 10, 28). At the opposite, stromal HSK without ulceration is mainly immune mediated, induced by the immune conflict in response to HSV stromal antigens, and associated

TABLE 3 HSV PCR detection rates according to clinical subtypes and sampling techniques in the literature[a]

Reference	HSK subtypes	Type of sampling	Detection rate	Viral load	Sensitivity	Specificity
Fukuda et al. (19)	Epithelial: *n* = 27	Tears	100%	6.4×10^5 (1)	NA	NA
	Stromal: *n* = 14	Tears	57%	1.4×10^5 (1)		
Kakimaru-Hasegawa et al. (20)	Epithelial: *n* = 15	Tears/scraping	100%	$3.5 \times 10^5/10^7$ (1)	NA	NA
	Stromal: *n* = 10	Tears/scraping	50%	$4.7 \times 10^2/2.8 \times 10^5$ (1)		
	Endothelial: *n* = 8	Aqueous humor	25%	2.9×10^2 (1)		
Fukuda et al. (18)	Epithelial: *n* = 37	Tears	81%	3.9×10^5 (2)	NA	NA
	Stromal: *n* = 22	Tears	59%	8.9×10^5 (2)		
	Uveitis: *n* = 7	Aqueous humor	14.30%	3.8×10^4 (2)		
Satpathy et al. (14)	Not specified	Tears	18.30%	No quantifications	100% (3)	90.7% (3)
	n = 153	Scraping	36.60%		100% (3)	71.3% (3)
Shoji et al.(22)	Epithelial: *n* = 23	Tears	78.30%	2.3×10^4 (1)	55.8% (4)	100% (4)
	Stromal: *n* = 9	Tears	33.30%	3.1×10^2 (1)		
Qiu et al. (21)	Stromal: *n* = 146	Tears	11%	NA	11% (4)	100% (4)
Present study	Epithelial: *n* = 39	Tears	97.40%	3.3×10^5 (2)	97.4% (4)	100% (4)
	Stromal without ulceration: *n* = 74	Tears	8.10%	219 (2)	8.1% (4)	98.1% (4)
	Stromal with ulceration: *n* = 39	Tears	48.70%	4.3×10^4 (2)	48.7% (4)	100% (4)
	Endotheliitis: *n* = 44	Tears	13.60%	629 (2)	13.6% (4)	100% (4)
	Keratouveitis: *n* = 17	Tears	23.50%	40 (2)	23.5% (4)	100% (4)
	Neurotrophic: *n* = 27	Tears	11.10%	954 (2)	11.1% (4)	100% (4)

[a](1) Copies/sample, (2) copies/mL, (3) compared to cell culture, (4) compared to clinical diagnosis. NA, non available.

with low-grade intrastromal viral replication with limited viral shedding in tears (5, 10, 28). Endotheliitis is linked to high-grade viral replication, but the latter occurs in the innermost corneal layer, leading to minimal viral shedding in tears. Concordantly, the present study showed better sensitivity and higher viral loads in HSK with epithelial involvement, compared to those without epitheliopathy, especially stromal keratitis without ulceration, endotheliitis, and keratouveitis. Finally, post-herpetic neurotrophic keratopathy is usually considered as a sequalae of previous episodes, not involving viral replication. However, three cases in our series were HSV1-DNA positive (11.1%), suggesting possible low-grade chronic viral shedding and/or local replication in some patients presenting with what appears to be a typical presentation of neurotrophic keratopathy.

Limitations of the study

This study was limited by its retrospective nature, although it included a large series of cases compared to those of previous publications. One possible bias in our study stemmed from using clinical aspects and disease history as the gold standard for diagnosis, instead of employing an alternative sampling method. This approach may lack objectivity, but clinical diagnosis is widely regarded as the benchmark for diagnosing typical HSK (4, 5). Additionally, it would be inconceivable to compare tear sampling with corneal scraping (and/or aqueous tap) in cases where disrupting the epithelium and/or the anterior stroma could adversely affect the clinical course of the disease. In atypical cases, slit-lamp diagnosis of HSK can be challenging and potentially lead to a biased classification of HSK. However, taking into account the patients' disease history and their response to anti-herpetic drugs provides crucial clues to correctly categorize individuals.

Moreover, it is possible that VZV keratitis might have been misidentified as HSV keratitis, given that VZV keratitis can (i) occur without the presence of shingles or herpes zoster (referred to as zoster sine herpete) (10, 29) and (ii) relapse and thus mimic HSK (30). However, we did not observe RT-qPCR positive for VZV in our group of presumed HSV keratitis, suggesting that our non-inclusion criteria were quite robust.

An additional limit in evaluating the sensitivity of PCR from tear sampling could be associated with the circumstance that a substantial number of patients were sampled due to a recurrence of HSK despite ongoing prophylactic antiviral therapy (*N* = 160, 67%), which might have affected viral load levels and detection rates. Corroborating this is the observation that patients who were undergoing preventive treatment at the time of relapse were more commonly found to test negative for RT-qPCR in tears.

The variability in tear sampling methods could potentially skew comparisons between studies. In fact, there is currently no standardized approach to tear sampling. For instance, Satpathy et al. used Schirmer strips without moistening the strip nor rinsing the OS before sampling (14), while Fukuda et al. rinsed the OS with 500 µL of saline solution and sampled non-standardized volumes using a micropipette (18). Kakimaru-Hasegawa et al. also used a micropipette to sample tears but collected a calibrated volume of 200 µL (20). Furthermore, results in the literature are presented either as copies per sample or copies per milliliter.

The interpretation of RT-qPCR results may be constrained by the inability to distinguish between pathological and normal shedding of HSV1 in the tear film (31, 32). However, viral loads associated with asymptomatic shedding are usually very low (33). Nevertheless, there is no definitive consensus threshold to distinguish between continuous asymptomatic shedding and pathogenic viral replication.

In summary, this study highlights the value of HSV1 RT-qPCR testing of tear samples as a diagnostic tool for HSK. Its diagnostic performance varies among different clinical subtypes, with notably improved accuracy when viral replication occurs in the corneal epithelium.

What is already known in the diagnostic performance of real-time quantitative PCR in tear samples?

- The viral load is higher in corneal scrapings, compared to tear specimens, and significantly yielded a twofold increased rate of detection
- Tear sampling is a non-invasive method and could be useful in HSK subtypes where scrapping is not possible
- The sensitivity is lower in non-epithelial keratitis, especially keratitis without ulceration

What this study adds

- Diagnostic testing accuracy data are scarce. This study allowed to determine the diagnostic testing accuracy in a large cohort, according to various HSK subtypes.

AUTHOR AFFILIATIONS

[1]Service d'Ophtalmologie, Hôpital Bicêtre, Université Paris-Saclay, CRMR OPHTARA, Le Kremlin Bicêtre, France
[2]Université Paris-Saclay, Inserm, CEA, Center for Immunology of Viral, Auto-Immune, Hematological and Bacterial Diseases (IMVA-HB/IDMIT), Fontenay-Aux-Roses, France
[3]Service de Virologie, Hôpital Paul Brousse, Université Paris-Saclay, Villejuif, France
[4]Service d'Epidémiologie et de Santé Publique, Université Paris-Saclay, Le Kremlin Bicêtre, France

AUTHOR ORCIDs

Gautier Hoarau http://orcid.org/0000-0002-4667-0697
Antoine Rousseau http://orcid.org/0000-0003-2355-2804

AUTHOR CONTRIBUTIONS

Gautier Hoarau, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review and editing | Oscar Haigh, Conceptualization, Validation | Christelle Vauloup-Fellous, Validation | Rafaël Boucher, Visualization | Alexandra Rouquette, Methodology, Validation | Pascale Faure, Data curation, Visualization | Lamia Limam, Data curation, Validation | Marc Labetoulle, Validation, Writing – review and editing | Antoine Rousseau, Conceptualization, Supervision, Validation, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Tables S1 to S4 (JCM00885-23-S0001.docx). Supplemental material.

REFERENCES

1. Farooq AV, Shukla D. 2012. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. Surv Ophthalmol 57:448–462. https://doi.org/10.1016/j.survophthal.2012.01.005
2. Labetoulle M, Auquier P, Conrad H, Crochard A, Daniloski M, Bouée S, El Hasnaoui A, Colin J. 2005. Incidence of herpes simplex virus keratitis in France. Ophthalmology 112:888–895. https://doi.org/10.1016/j.ophtha.2004.11.052
3. Liesegang TJ. 2001. Herpes simplex virus epidemiology and ocular importance. Cornea 20:1–13. https://doi.org/10.1097/00003226-200101000-00001
4. Kowalski RP, Gordon YJ, Romanowski EG, Araullo-Cruz T, Kinchington PR. 1993. A comparison of enzyme immunoassay and polymerase chain reaction with the clinical examination for diagnosing ocular herpetic disease. Ophthalmology 100:530–533. https://doi.org/10.1016/s0161-6420(93)31630-1
5. Lee White M, Chodosh J. 2014. Herpes simplex virus keratitis: a treatment guideline - 2014, on America Academy of Ophthalmology. Available from: https://www.aao.org/clinical-statement/herpes-simplex-virus-keratitis-treatment-guideline
6. McQuillan G, Kruszon-Moran D, Flagg EW, Paulose-Ram R. 2018. Prevalence of herpes simplex virus type 1 and type 2 in persons aged 14-49: United States, 2015-2016. NCHS Data Brief:1–8.
7. Rousseau A, Pharm SB, Gueudry J, Deback C, Haigh O, Schweitzer C, Boutolleau D, Labetoulle M. 2022. Acyclovir-resistant herpes simplex virus 1 keratitis: a concerning and emerging clinical challenge. Am J Ophthalmol 238:110–119. https://doi.org/10.1016/j.ajo.2022.01.010
8. Poon SHL, Wong WHL, Lo ACY, Yuan H, Chen CF, Jhanji V, Chan YK, Shih KC. 2021. A systematic review on advances in diagnostics for herpes simplex keratitis. Surv Ophthalmol 66:514–530. https://doi.org/10.1016/j.survophthal.2020.09.008
9. Faure P, Limam L, de Saint-Sauveur G, Da Cunha E, Best A-L, Bénichou J, Remongin PE, Vauloup-Fellous C, Eid L, Labetoulle M, Rousseau A. 2022. Virological diagnosis of anterior herpetic ocular disease on tear sample: a simple and non-invasive technique. J Fr Ophtalmol 45:735–740. https://doi.org/10.1016/j.jfo.2022.03.013
10. Labetoulle M, Boutolleau D, Burrel S, Haigh O, Rousseau A. 2023. Herpes simplex virus, varicella-zoster virus and cytomegalovirus keratitis: facts for the clinician. Ocul Surf 28:336–350. https://doi.org/10.1016/j.jtos.2021.07.002
11. Moshirfar M, Murri MS, Shah TJ, Skanchy DF, Tuckfield JQ, Ronquillo YC, Birdsong OC, Hofstedt D, Hoopes PC. 2019. A review of corneal endotheliitis and endotheliopathy: differential diagnosis, evaluation, and treatment. Ophthalmol Ther 8:195–213. https://doi.org/10.1007/s40123-019-0169-7
12. Terada Y, Kaburaki T, Takase H, Goto H, Nakano S, Inoue Y, Maruyama K, Miyata K, Namba K, Sonoda K-H, Kaneko Y, Numaga J, Fukushima M, Horiguchi N, Ide M, Ehara F, Miyazaki D, Hasegawa E, Mochizuki M. 2021. Distinguishing features of anterior uveitis caused by herpes simplex virus, varicella-zoster virus, and cytomegalovirus. Am J Ophthalmol 227:191–200. https://doi.org/10.1016/j.ajo.2021.03.020
13. Goldschmidt P, Rostane H, Saint-Jean C, Batellier L, Alouch C, Zito E, Bourcier T, Laroche L, Chaumeil C. 2006. Effects of topical anaesthetics and fluorescein on the real-time PCR used for the diagnosis of herpesviruses and acanthamoeba keratitis. Br J Ophthalmol 90:1354–1356. https://doi.org/10.1136/bjo.2006.098830
14. Satpathy G, Mishra AK, Tandon R, Sharma MK, Sharma A, Nayak N, Titiyal JS, Sharma N. 2011. Evaluation of tear samples for herpes simplex virus 1 (HSV) detection in suspected cases of viral keratitis using PCR assay and conventional laboratory diagnostic tools. Br J Ophthalmol 95:415–418. https://doi.org/10.1136/bjo.2010.191049
15. McGilligan VE, Moore JE, Tallouzi M, Atkinson SD, O'Neill H, Feeney S, Novitskaya ES, Sharma A, Sunil S, Jackson JA, Frazer DG, Moore TCB. 2014. A comparison of the clinical and molecular diagnosis of herpes simplex keratitis. OJOph 4:65–74. https://doi.org/10.4236/ojoph.2014.43011
16. Satpathy G, Behera HS, Sharma A, Mishra AK, Mishra D, Sharma N, Tandon R, Agarwal T, Titiyal JS. 2018. A 20-year experience of ocular herpes virus detection using Immunofluorescence and polymerase chain reaction. Clin Exp Optom 101:648–651. https://doi.org/10.1111/cxo.12669
17. Lee SY, Kim MJ, Kim MK, Wee WR. 2013. Comparative analysis of polymerase chain reaction assay for herpes simplex virus 1 detection in tear. Korean J Ophthalmol 27:316–321. https://doi.org/10.3341/kjo.2013.27.5.316
18. Fukuda M, Deai T, Higaki S, Hayashi K, Shimomura Y. 2008. Presence of a large amount of herpes simplex virus genome in tear fluid of herpetic stromal keratitis and persistent epithelial defect patients. Semin Ophthalmol 23:217–220. https://doi.org/10.1080/08820530802111366
19. Fukuda M, Deai T, Hibino T, Higaki S, Hayashi K, Shimomura Y. 2003. Quantitative analysis of herpes simplex virus genome in tears from patients with herpetic keratitis. Cornea 22:S55–S60. https://doi.org/10.1097/00003226-200310001-00008
20. Kakimaru-Hasegawa A, Kuo C-H, Komatsu N, Komatsu K, Miyazaki D, Inoue Y. 2008. Clinical application of real-time polymerase chain reaction for diagnosis of herpetic diseases of the anterior segment of the eye. Jpn J Ophthalmol 52:24–31. https://doi.org/10.1007/s10384-007-0485-7
21. Qiu J, Huang F, Wang Z, Xu J, Zhang C. 2017. The evaluation of diagnostic efficiency for stromal herpes simplex keratitis by the combination of tear HSV-sIgA and HSV-DNA. Graefes Arch Clin Exp Ophthalmol 255:1409–1415. https://doi.org/10.1007/s00417-017-3653-6
22. Shoji J, Sakimoto T, Inada N, Kamei Y, Matsubara M, Takamura E, Sawa M. 2016. A diagnostic method for herpes simplex keratitis by simultaneous measurement of viral DNA and virus-specific secretory IgA in tears: an evaluation. Jpn J Ophthalmol 60:294–301. https://doi.org/10.1007/s10384-016-0448-y
23. Rousseau A, Boutolleau D, Titier K, Bourcier T, Chiquet C, Weber M, Colin J, Gueudry J, M'Garrech M, Bodaghi B, Burrel S, Agut H, Deback C, Labetoulle M. 2017. Recurrent herpetic keratitis despite antiviral

prophylaxis: a virological and pharmacological study. Antiviral Res 146:205–212. https://doi.org/10.1016/j.antiviral.2017.09.013
24. Burrel S, Deback C, Agut H, Boutolleau D. 2010. Genotypic characterization of UL23 thymidine kinase and UL30 DNA polymerase of clinical isolates of herpes simplex virus: natural polymorphism and mutations associated with resistance to antivirals. Antimicrob Agents Chemother 54:4833–4842. https://doi.org/10.1128/AAC.00669-10
25. Afshar B, Bibby DF, Piorkowska R, Ohemeng-Kumi N, Snoeck R, Andrei G, Gillemot S, Morfin F, Frobert E, Burrel S, Boutolleau D, Crowley B, Mbisa JL. 2017. A European multi-centre external quality assessment (EQA) study on phenotypic and genotypic methods used for herpes simplex virus (HSV) drug resistance testing. J Clin Virol 96:89–93. https://doi.org/10.1016/j.jcv.2017.10.002
26. Rousseau A, Burrel S, Gueudry J, Deback C, Haigh O, Schweitzer C, Boutolleau D, Labetoulle M. 2022. Acyclovir-resistant herpes simplex virus 1 keratitis: a concerning and emerging clinical challenge. Am J Ophthalmol 238:110–119. https://doi.org/10.1016/j.ajo.2022.01.010
27. Wilhelmus KR. 2015. Antiviral treatment and other therapeutic interventions for herpes simplex virus epithelial keratitis. Cochrane Database Syst Rev 1:CD002898. https://doi.org/10.1002/14651858.CD002898.pub5
28. Holland EJ, Schwartz GS. 1999. Classification of herpes simplex virus keratitis. Cornea 18:144–154. https://doi.org/10.1097/00003226-199903000-00002
29. Chern KC, Margolis TP. 1998. Varicella zoster virus ocular disease. Int Ophthalmol Clin 38:149–160. https://doi.org/10.1097/00004397-199803840-00014
30. Tran KD, Falcone MM, Choi DS, Goldhardt R, Karp CL, Davis JL, Galor A. 2016. Epidemiology of herpes zoster ophthalmicus: recurrence and chronicity. Ophthalmology 123:1469–1475. https://doi.org/10.1016/j.ophtha.2016.03.005
31. Kaufman HE, Azcuy AM, Varnell ED, Sloop GD, Thompson HW, Hill JM. 2005. HSV-1 DNA in tears and saliva of normal adults. Invest Ophthalmol Vis Sci 46:241–247. https://doi.org/10.1167/iovs.04-0614
32. Crouse CA, Pflugfelder SC, Pereira I, Cleary T, Rabinowitz S, Atherton SS. 1990. Detection of herpes viral genomes in normal and diseased corneal epithelium. Curr Eye Res 9:569–581. https://doi.org/10.3109/-02713689008999597
33. Kumar M, Hill JM, Clement C, Varnell ED, Thompson HW, Kaufman HE. 2009. A double-blind placebo-controlled study to evaluate valacyclovir alone and with aspirin for asymptomatic HSV-1 DNA shedding in human tears and saliva. Invest Ophthalmol Vis Sci 50:5601–5608. https://doi.org/10.1167/iovs.09-3729