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Herpes simplex virus PCR in 2230 explanted corneal buttons

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ABSTRACT.

Purpose: **To determine herpes simplex virus (HSV) DNA prevalence and mean cycle threshold of polymerase chain reaction (PCR) in corneal tissue of patients with penetrating keratoplasty (PKP), with (HSK+) and without (HSK−) previous clinical herpetic keratitis history.**

Methods: **Retrospective review of recipient corneal buttons which were explanted through PKP between March 2010 and September 2018 at the Department of Ophthalmology, Saarland University Medical Center in Homburg/Saar, Germany. Corneal tissue samples were analysed by real-time PCR for the presence of HSV DNA. For each subject, clinical data, including patients' demographics and clinical diagnoses, were collected.**

Results: **In total, 2230 corneal samples (age at the time of the surgery 57.3 ± 19.2 years) of 1860 patients were analysed. HSV PCR was positive in 137 (6.1%) corneal samples, with a 30.57 ± 6.01 (range 14–39) mean cycle threshold (Ct) value. Two hundred ninety-eight (13.4%) corneas of 266 patients were clinically HSK+, and 1932 (86.6%) corneas of 1600 patients were clinically HSK−. HSV DNA was detected significantly more frequently ($p < 0.0001$) in HSK+ corneal samples (108 corneal samples; 36.2%), than in HSK− corneal samples (29 corneal samples; 1.5%). Ct value was significantly lower in HSK+ than in HSK- corneal samples (29.8 ± 5.8 versus 32.6 ± 5.9; $p = 0.008$).**

Conclusion: **Our data demonstrate that a positive clinical history of HSK is related to HSV PCR positivity in about every 2.8th patient. In addition, about every 66th explanted corneal tissue is HSV PCR-positive despite the lack of clinical suspicion. These patients may need additional local/systemic antiviral treatment to avoid newly acquired HSK following penetrating keratoplasty.**

Key words: herpetic keratitis – prevalence – herpes simplex virus – keratoplasty – recurrent

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Introduction

Worldwide, most people go through a herpes simplex virus (HSV) infection during their life. Herpes simplex virus keratitis (HSK) is a leading cause of corneal scarring and blindness in developed countries. After an initial infection with HSV, which typically affects the conjunctiva, the virus remains dormant in the trigeminal ganglion (Seitz & Heiligenhaus 2011). Most clinically observed infections occur from reactivation of the latent virus and appear as epithelial keratitis, stromal keratitis or endotheliitis. HSK is a clinically relevant (5%–10%) indication for penetrating keratoplasty (PKP) (Remeijer et al. 2009).

Recurrent HSK with history of clinical HSK may originate from the reactivation of the virus in the sensory nerve ganglia or in the corneal tissue. Reactivation of HSV may be caused following PKP by emotional or surgical stress as well as perioperative immune suppression and may result in HSK (Higaki et al. 2015). However, HSK may also occur as newly acquired (0.9%) after PKP in patients without a clinical history of HSK (Remeijer et al. 1997). The origin of a 'newly acquired HSK' after PKP is discussed controversially. Nevertheless, most researchers agree that HSV reactivation may occur with viral shedding from sensory nerve ganglia or from the recipient corneal tissue (Kaye et al 1991; Borderie et al 2004). In addition, a few cases

of HSV donor-to-host transmission through PKP have also been reported (Reimejer et al. 2001; Stradivis et al. 2012).

Determination of HSV DNA prevalence in explanted recipient corneal buttons of subjects without a clinical history of HSK may be important in order to evaluate the risk of a newly acquired HSK on the graft and the potentially related allograft failure. Prophylactic local and oral antiviral therapy should reduce the risk of HSK recurrence and graft failure after PKP in such cases. Therefore, eyes without a clinical history of HSK but in case of HSV polymerase chain reaction (PCR) positivity may require timely local and/or systemic antiviral treatment (Seitz & Heiligenhaus 2015).

The aim of our study was to determine HSV DNA prevalence in corneal tissue of patients after PKP, with (HSK+) and without (HSK−) previous clinical HSK history, and to compare our results with previous findings (Kaye el al. 2000; Remeijer et al. 2009).

Materials and methods

This retrospective study was conducted at the Department of Ophthalmology, Saarland University Medical Center in Homburg/Saar, Germany. The study was approved by the local Ethics Committee (Number 200/19) and followed the tenets of the Declaration of Helsinki. Informed written consent was obtained from all subjects.

We analysed 2230 eyes of 1860 patients (age at the time of surgery 57.3 ± 19.2 years; sex ratio of males to females was 1308:922) who underwent PKP between March 2010 and September 2018 in Homburg/Saar. Patients with (HSK+) and without (HSK−) a clinical history of HSK before PKP were also analysed as separate groups. For each subject, clinical data, including patients' demographics and clinical diagnoses, were reviewed.

In case of a positive HSV PCR, or in case of a preoperative clinical history of HSK, all subjects received 5× ganciclovir gel daily and systemically 5 × 400 mg aciclovir for 6 weeks and then 2 × 400 mg aciclovir at least until 12 months after PKP as an additional standard treatment to their initial prednisolone-acetate eye drops 5× daily.

All explanted corneal buttons were divided into four equal quadrants, and one of these quadrants was sent for PCR testing. HSV DNA was detected by PCR using a laboratory-developed real-time PCR test. DNA was extracted from corneal tissue using the QIAamp DNA Mini Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The sample input of the cornea was at least 50 mg to ensure sufficient material for extraction and subsequent real-time PCR. The rest of the material was preserved as a reserve sample for potential clinical retesting. HSV DNA was amplified with the LightCycler 1.5 instrument (Roche, Basel, Switzerland) using primers targeting a type-specific DNA sequence within the HSV glycoprotein G gene 5′-AAgATCCCCATAAACT gggAgT-3′ and 5′-TCAgAACTACAC ggAgggCAT-3′ and the hybridization probes (5′-CCgTACAAgTTCAAggC CACCAT-3′--FL and 5′-LC640-CTA CAAAgACgTgACCgTgTCgCAgg-3′--PH) (BioSpring GmbH, Frankfurt, Germany). Preincubation at 95°C for 10 min was followed by 45 PCR cycles with denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds and elongation at 72°C for 10 seconds.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific PCR was performed to control the presence of cellular DNA using the primers 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATT TC-3′ and the hybridization probes (5′-AGGGGTCATTGATGGCAACAAT ATCCA-3′--FL and 5′- LC705-TTTA CCAGAGTTAAAAGCAGCCCTGG TG-3′--PH). For the samples which were confirmed as positive by the multicomponent view but did not cross the threshold, a mean cycle threshold (Ct) value of 40 was assigned.

Ct values were determined, and melting curve analysis was performed in order to differentiate between HSV type 1 (HSV-1) and HSV type 2 (HSV-2). Melting curves have been available in our laboratory since August 2013 in 99 PCR-positive cases (72.3%).

Statistica 11.0 (StatSoft Inc., Tulsa, OK, USA) was used for detailed analysis of data. The chi-square test was used to evaluate differences among groups, and the Pearson rank correlation test was used for correlation analysis. An univariate multivariable linear regression model was derived to determine the effect of preoperative clinical HSK history (dichotomous factor: 0 = no previous clinical HSK history, 1 = previous clinical HSK history) on HSV_{Ct}. The linear regression model was qualified with the omnibus test and Wald chi-square. p values < 0.05 were considered statistically significant.

Results

Among 2230 corneal samples of 1860 patients, HSV PCR was positive in 137 (6.1%) tissue samples, with a 30.57 ± 6.01 (range 14–39) mean Ct value. The age of the patients at the time of the surgery did not correlate with the Ct value ($r = -0.156$; $p = 0.068$).

Details of HSK+ and HSK− corneal samples are summarized in Tables 1 and 2. Our sample consisted of 298 (13.4%) HSK+ corneal samples of 266 patients (Table 1) and 1932 (86.6%) HSK− corneal samples of 1600 patients (Table 2). HSV DNA was detected significantly more frequent ($p < 0.0001$) in HSK+ corneal samples (108 corneal samples; 36.2%), in comparison to HSK- corneal samples (29 corneal samples; 1.5%). Ct value was significantly lower in HSK+ corneal samples than in HSK-s (29.8 ± 5.8 versus 32.6 ± 5.9; $p = 0.008$) (Figure 1). In subjects with HSV PCR-positive samples, the age at the time of surgery did not differ significantly between HSK+ and HSK− (59.3 ± 18.4 years versus 65.5 ± 13.4 years; $p = 0.103$) (Figure 2). The age of the patients at the time of the surgery did not correlate with the Ct value neither in HSK+ ($r = -0.105$; $p = 0.278$), nor in HSK− ($r = -0.192$; $p = 0.320$) samples.

PCR examination showed HSV-1 in 71 cases (71.7%), and HSV-2 was not detectable in any of the cases. However, determination between HSV-1 and HSV-2 was not possible in 28 corneal samples (28.3%) and a melting curve was not available in 38 cases.

The univariate multivariable (or multiple) linear regression model for an estimate of HSV_{Ct} yielded the following:

$$HSV_{Ct} = 29.8 + \left\langle \begin{matrix} 2.79 \text{ for HSK+} \\ 0 \text{ for HSK−} \end{matrix} \right\rangle$$

29.8 refers to the intercept value which means that the HSV_{Ct} for HSK+ is on average 2.79 higher compared to

Table 1. Clinical diagnoses, descriptive parameters and HSV PCR analysis results of corneal buttons with previous clinical history of herpes simplex virus keratitis

HSK+ (n = 298)	Subjects, n (%)	Sex ratio of males to females	Age at the time of surgery (years; mean ± SD)	Corneas with HSV DNA, n (%)	Cycle threshold (Ct) value (mean ± SD)	HSV-1, n	Not determinable between HSV-1 and HSV-2, n	Not available, n
Infectious epithelial keratitis	6 (2.0)	4:2	72.0 ± 14.2	3 (50.0)	25.3 ± 7.4	2	0	1
Ulcerative necrotizing stromal keratitis	75 (25.2)	44:31	64.8 ± 15.9	39 (52.0)	28.9 ± 5.8	25	5	9
Non-necrotizing stromal immune keratitis	80 (26.8)	47:33	57.3 ± 20.3	23 (28.8)	32.5 ± 4.2	12	4	7
Endotheliitis	49 (16.4)	23:26	67.5 ± 12.3	18 (36.7)	29.6 ± 4.9	8	3	7
Neurotrophic keratitis	15 (5.0)	6:9	64.5 ± 12.3	5 (33.3)	28.4 ± 11.5	2	3	0
Herpetic stromal scar	41 (13.8)	27:14	58.4 ± 17.1	9 (22.0)	31.1 ± 4.5	5	2	2
Allograft failure with previous HSK	13 (4.4)	6:7	64.1 ± 14.4	6 (46.2)	31.0 ± 5.9	0	2	4
Other HSKs (detailed clinical data not available)	19 (6.4)	11:8	61.3 ± 14.5	5 (26.3)	27.5 ± 9.6	2	2	1
Total	298 (100)	168:130	62.2 ± 16.9	108 (36.2)	29.8 ± 5.8	56	21	31

HSK+, Clinical diagnosis with positive herpes simplex virus keratitis history; HSK, herpes simplex virus keratitis.

Table 2. Clinical diagnoses, descriptive parameters and HSV PCR analysis results of corneal buttons without previous clinical history of herpes simplex virus keratitis

HSK- (n = 1932)	Subjects, n (%)	Sex ratio of males to females	Age at the time of surgery (years; mean ± SD)	Corneas with HSV DNA, n (%)	Cycle threshold (Ct) value (mean ± SD)	HSV-1, n	Not determinable between HSV-1 and HSV-2, n	Not available, n
Mycotic keratitis	63 (3.3)	31:32	56.5 ± 22.8	3 (4.8)	30.0 ± 8.7	2	0	1
Pellucide marginal degeneration	22 (1.1)	17:5	55.1 ± 13.3	1 (4.5)	37	1	0	0
Graft failure without allograft reaction	180 (9.3)	100:80	63.6 ± 14.5	6 (3.3)	34.3 ± 8.2	1	3	2
Bacterial keratitis	221 (11.4)	128:93	66.5 ± 18.1	6 (2.7)	29.0 ± 5.7	4	0	2
Other corneal dystrophies	60 (3.1)	33:27	50.6 ± 19.4	1 (1.7)	35	1	0	0
Fuchs' endothelial dystrophy	206 (10.7)	102:104	70.7 ± 9.1	3 (1.5)	33.3 ± 4.0	1	1	1
Keratoconus	477 (24.7)	327:150	40.8 ± 14.8	7 (1.5)	34.1 ± 4.8	4	3	0
Allograft reaction	82 (4.2)	55:27	62.5 ± 13.2	1 (1.2)	33	0	0	1
Corneal scars	182 (9.4)	98:84	56.3 ± 18.7	1 (0.6)	32	1	0	0
Bullous keratopathy	260 (13.5)	150:110	65.3 ± 16.9	0 (0)	n/a	0	0	0
Acanthamoeba keratitis	53 (2.7)	23:30	38.7 ± 13.1	0 (0)	n/a	0	0	0
High corneal astigmatism after PKP	49 (2.5)	34:15	56.0 ± 13.6	0 (0)	n/a	0	0	0
Chemical injury	25 (1.3)	13:12	62.0 ± 14.7	0 (0)	n/a	0	0	0
Keratoglobus	14 (0.7)	8:6	50.6 ± 12.0	0 (0)	n/a	0	0	0
Other diseases	38 (2.0)	21:17	50.4 ± 26.1	0 (0)	n/a	0	0	0
Total	1932 (100)	1140:792	56.6 ± 19.4	29 (1.5)	32.6 ± 5.9	15	7	7

HSK−, Clinical diagnosis with negative herpes simplex virus keratitis history; HSK, herpes simplex virus keratitis; PKP, penetrating keratoplasty.

HK− on HSV_{Ct}. The overall performance of this generalized linear model is $p = 0.023$ ($\chi^2 = 7.5$ Omnibus test).

Among HSK+ corneal samples, PCR examination showed HSV-1 in 56 cases (72.7%), determination between HSV-1 and HSV-2 was not possible for 21 corneal samples (27.3%), and melting curve was not available in 31 cases. In 108 (36.2%) HSV PCR-positive HSK+ corneas, prevalence of PCR positivity was the highest following a history of ulcerative necrotizing stromal keratitis (52.0%), epithelial keratitis (50.0%), in allograft failure with previous HSK (46.2%) and in herpetic endotheliitis (36.7%) (Table 1).

Among HSK− corneal samples, PCR examination showed HSV-1 in 15 cases (68.2%), determination between HSV-1 and HSV-2 was not possible for 7 corneal samples (31.9%), and melting curve was not available in

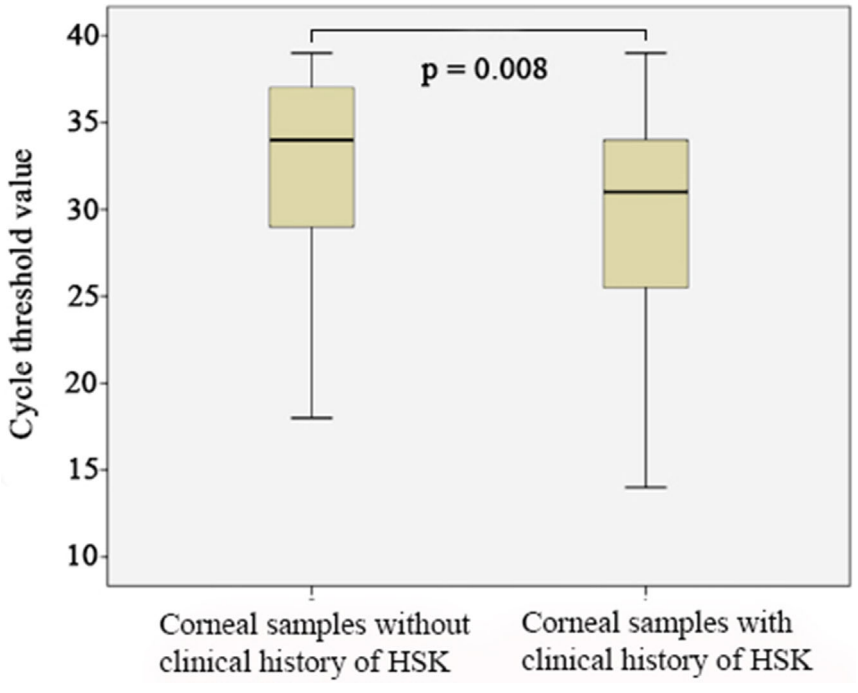

Fig. 1. Cycle threshold value of HSV PCR-positive corneal tissues (median ± interquartile) with and without a clinical history of HSK (HSK, herpetickeratitis).

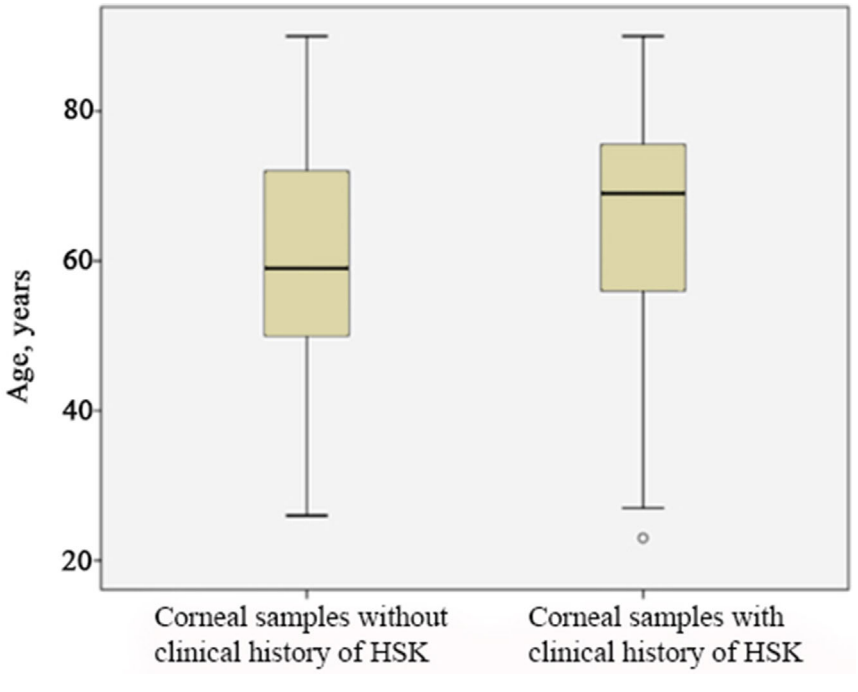

Fig. 2. Patient age at the time of surgery of HSV PCR-positive samples (median ± interquartile) with and without a clinical history of HSK (p = 0.103) (HSK, herpetic keratitis).

7 cases. Among 29 (1.5%) HSV PCR-positive HSK- corneas, HSV DNA was most often detectable in corneas with mycotic keratitis (4.8%), pellucide marginal degeneration (4.5%), graft failure without allograft reaction (3.3%) and bacterial keratitis (2.7%) (Table 2).

Among 2230 corneal samples of 1860 patients, there were 245 repeat PKPs, 32 in HSK+ corneas and 213 in HSK− eyes. Regarding the prevalence of repeat PKPs, there was no significant difference between HSK+ and HSK- tissue samples (11.0% versus 10.7%; p = 0.903).

Among 1932 HSK− corneal buttons, in four eyes (0.02%) of four patients (two cases with the diagnosis of keratoconus and two with microbial corneal ulcer), recipient HSV PCR was negative after PKP, but later on graft failure occurred (in three cases) or persistent epithelial defect was observed (in one eye). After repeat PKPs, HSV PCR was positive in all of these four cases.

In another four eyes of four patients among 1932 HSK− corneal buttons (2 with keratoconus, one with pellucide marginal degeneration and one with Fuchs' endothelial dystrophy), HSV PCR was only positive for one eye.

Among 298 HSK+ corneal buttons, in three eyes of three patients, the primarily explanted corneal tissue was HSV PCR-positive, but after initiation of antiviral treatment and repeat PKP, the repeat HSV PCR examination became negative. Nevertheless, in four eyes of four patients, HSV PCR remained positive after initiation of antiviral treatment and repeat PKP, and in one case also after a second repeat PKP.

Discussion

In our study, we determined the prevalence of HSV and the diagnostic importance of DNA detection in human recipient corneal buttons after PKP in HSK+ and HSK− subjects.

The importance of detecting HSV DNA in corneal buttons is debated. Sensitivity of PCR examination in corneal buttons is also puzzling, as the distribution of HSV DNA in the cornea is not homogeneous. Openshaw et al. (1995) detected HSV DNA in 41.7% of normal corneas, among those the corneal rim was PCR-positive in 80%, and the central part in 20% of the cases, whereas both central and peripheral parts in only one case. Active inflammation with immune cell infiltration can further complicate DNA detection within the corneal tissue (Biswas et al. 2005).

We found lower HSV PCR prevalence (36.2%) compared to the report of Remeijer (48%) (Remeijer et al. 2009) and Kaye (82%) (Kaye et al. 2000) in HSK+ subjects. Remeijer found no HSV-2 DNA and only one cornea with varicella-zoster virus (VZV) DNA in HSK+ subjects. In contrast, Kaye et al. found VZV DNA

in almost every fourth HSK+ corneal sample, but they found no presence of other herpesviruses (cytomegalovirus or Epstein-Barr virus) (Kaye et al. 2000). We also could not verify the presence of HSV-2 DNA in any of the HSK+ or HSK− corneal samples; thus, HSV-2 seems to play a negligible role in the aetiopathology of HSK. Nevertheless, we were not able to determine between HSV-1 and HSV-2 in 28 out of 2230 corneal samples.

Similar to Remeijer's et al. (2009) and Kaye's et al. (2000) results, the presence of HSV DNA was significantly more pronounced in HSK+ corneas than in those with HSK-. On the other hand, presence of HSV DNA did not correlate with the age at the time of surgery, neither in HSK+ nor in HSK- subjects.

Within HSK+ patients, prevalence of non-necrotizing stromal immune keratitis (26.8% and 27%) and epithelial keratitis (2.0% and 1%) was in our sample similar to Remeijer's values (Remeijer et al. 2009), but prevalence of endotheliitis was clearly higher in ours than in Remeijer's sample (16.4% versus 4%). In contrast, the prevalence of ulcerative necrotizing stromal keratitis (25.2% versus 42%) and allograft failure with previous HSK (4.4% versus 24%) was much lower in our sample compared to Remeijer.

In our and Remeijer's study, prevalence of HSV PCR positivity in HSK+ subjects was the highest in ulcerative necrotizing stromal keratitis (52.0% and 63.0%), non-necrotizing stromal immune keratitis (28.8% and 36%) and in allograft failure with previous HSK (46.2% and 45%) (Remeijer et al. 2009).

More interesting was the prevalence of HSV PCR positivity in clinically HSK- recipient corneal samples (1.5%). The development of newly acquired HSK and the origin of the infection is still speculative and determining of its origin is difficult (Qi et al. 2018). In the literature, different theories can be found. The main question is the take-off of the HSV reactivation: through donor cornea after PKP or from an endogenous reactivation (Kennedy et al. 2011).

Several case reports have been published about graft-to-host transmission of HSV (Remeijer et al. 2001; Thuret et al. 2004; Gatzioufas et al. 2011; Stavridis et al; 2012). It has also been proven that besides HSV, human immunodeficiency virus, hepatitis B and C disease are transmittable by PKP, and potential donors are screened by serology for these viruses (Biswas et al. 2000).

Tear shedding (Kaufman et al. 2005), reactivation of latent HSV in the trigeminal ganglion or in the cornea resulting in viral shedding in the donor cornea are possible origins of graft-to-host transmission (Kennedy et al. 2011). Organ culture media from corneas which had shown severe and complete endothelial necrosis (Sengler et al. 2001) were reported to be PCR-positive for HSV in 3%–43%, and therefore, keeping aseptic precautions are more important at PKP surgery (Cleator et al. 1994; Reimejer et al. 1997). Determination of the exact place of viral shedding is difficult, as 70% of 15–25-year-old and 95% of subjects over 60 years of age are seropositive for HSV-1 (Cleator et al. 1994), and annual incidence of HSK is only 11.8 per 100 000 people (Young et al. 2010). Reimejer et al. (2009) found PCR positivity in only 2% of HSK- donor corneas and concluded that screening of donor corneas for HSV is irrelevant, as only dysfunctional DNA fragments without any transmission or replicate ability can be detected (Morris et al. 1996). Postoperative external HSV infection may also happen. Thus in some cases, graft failure may be attributed to external HSV infection (De Kesel et al. 2001).

Another potential way for newly acquired HSK after PKP is the reactivation of the latent HSV from the recipient tissue. Surgical trauma, suture removal or postoperative high doses of topical corticosteroids may promote the endogenous reactivation. Most authors postulate that newly acquired HSK origins from a trigeminal endogenous reactivation (Holbach et al. 1998; Kennedy et al. 2011).

We found mildly lower prevalence of HSV DNA in HSK- patients compared to Remeijer (1.5% versus 4%) (Remeijer et al. 2009). Kaye found much higher HSV DNA positivity (22%) in HSK- patients using a PCR sensitivity of one genome equivalent (or 0.2 pfu/ml). However, using a sensitivity of 50 genome equivalents or 10 pfu/ml, Kaye could also not detect HSV DNA in HSK- subjects (Kaye et al. 2000). Unfortunately, our real-time PCR system was only able to give us a qualitative result (HSV-positive or HSV-negative), without finer differentiation. Remeijer et al. (2009) suggested that HSV DNA in HSK- corneas is likely to be irrelevant to the herpetic infection and represents asymptomatic tear shedding episodes.

Due to the low HSV DNA prevalence and its possible irrelevance to the herpetic infection in HSK− subjects, Remeijer et al. (2009) only offered HSV PCR screening from the explanted recipient HSK+ corneal buttons after PKP, in order to avoid a postoperative HSV recurrence. Incidence of newly acquired HSK was reported to be 0.5%–1.18% (Remeijer et al. 1997; Borderie et al. 2004, Qi et al. 2018). In total, among these cases 29 subjects had epithelial and 9 had necrotizing stromal or endothelial keratitis. In our HSK- cases, 6 corneal samples were HSV PCR-positive after graft failure; in these cases, graft failure may be caused by previous HSV reactivation. Otherwise, leading diagnoses in PCR-positive HSK- subjects were corneal ulcer (especially fungal), ectatic corneal diseases (keratoconus, keratoglobus, pellucide marginal degeneration) and graft failure. Ectatic corneal diseases and corneal ulcers (mycotic and bacterial) may be associated with atopic dermatitis, and patients with atopic dermatitis are known to have an increased risk for HSK (Hsi et al. 2019). Interestingly, no HSV PCR-positive acanthamoeba keratitis cases were found in HSK− patients. This fact contradicts previous reports, which postulated that the damage of the corneal epithelium due to HSK may predispose to the development of acanthamoeba keratitis (Mathers et al. 1997). Nevertheless, in Fuchs' endothelial dystrophy, with oxidative damage and apoptosis in the epithelium, HSV DNA positivity may be present in some cases (Zhang et al. 2015). Finally, false-positive results might also be an explanation of positive PCR results in HSK- individuals. However, internal and particularly extensive external validation of our HSV real-time PCR two times a year since 2003 had demonstrated its high specificity. This indicated that false-positive results are highly unlikely in our study.

In our sample, among HSK+ subjects, corneal samples of four patients were consequently positive for HSV

PCR after first and after repeat PKP. Besides that, we also had three cases with HSV PCR positivity after the first and negativity after repeat PKP. This could be related to the successful perioperative antiviral therapy, but also to the sampling from a HSV DNA-negative tissue part or very low viral loads below the threshold of PCR sensitivity (Seitz & Heiligenhaus 2011).

In conclusion, our data show that with clinical history of HSK, HSV PCR is 33%–52% positive in epithelial and ulcerative necrotizing stromal keratitis, allograft rejection, endotheliitis and neurotrophic keratopathy. Without a clinical history of HSK, HSV PCR is positive in 2.7%–4.5%, mainly among subjects with corneal ectatic diseases, graft failure, mycotic and bacterial keratitis. These patients may need additional local/systemic antiviral treatment to avoid newly acquired HSK following penetrating keratoplasty.

Limitation of our study is that neither appropriate information on the interval between the last clinical episode and surgery nor detailed information on the type of HSK (with its changing fashion over years) was available in the vast majority of our patients. This happened through the retrospective design of our study and as many of the patients have been treated for years or decades in several centres in- or outside Germany before PKP in our institution. Therefore, we could not include these factors in our statistical analysis. The effect of the interval between the last clinical episode and surgery and the type of HSK should be analysed in a future prospective study.

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