JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Brunner *et al.*, *Journal of Medical Microbiology* 2019;68:1324–1329
DOI 10.1099/jmm.0.001041

MICROBIOLOGY SOCIETY

Use of a corneal impression membrane and PCR for the detection of herpes simplex virus type-1

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Abstract

Purpose. To investigate the use of a corneal impression membrane (CIM) for the detection of herpes simplex virus type 1 (HSV-1) in suspected herpes simplex keratitis (HSK).

Methodology. In the laboratory study, swabs and CIMs made from polytetrafluoroethylene were spiked with different concentrations of HSV-1. DNA was extracted and real-time PCR undertaken using two sets of primers. In the clinical study, consecutive patients presenting with suspected HSK were included. For each patient, samples were collected from corneal lesions with a swab and a CIM in random order. Clinical details were collected using a standardized clinical form and patients were categorized into probable, presumed and possible HSK.

Results. There was no difference in the performance of both primer sets for all HSV-1 dilutions (P=0.83) using a CIM or between a CIM and a swab (P=0.18). In total, 110 patients were included. Overall, 73 patients (66.4 %) had probable, 20 patients (18.2%) presumed and 17 patients (15.5%) possible HSV-1 keratitis. The HSV-1 detection rate was significantly higher using a CIM (40/110, 36.4%) than a swab (28/110, 25.5%) (P=0.004). In the probable HSV keratitis group, the detection rate using a CIM was 43.8% compared to 27.4% for a swab (P=0.004). The cycle threshold values obtained for the conjunctival swabs were higher than those obtained for the CIMs (P<0.001).

Conclusions. In suspected HSK, a CIM is a useful alternative to a swab and more likely to detect the presence of HSV-1.

INTRODUCTION

Microbial keratitis due to herpes simplex virus type 1 (HSV-1) is a leading cause of visual impairment [1]. The annual incidence of HSV-1 keratitis (HSK) in the United States and France has been estimated at 8.4 and 31.5 per 100 000 [2]. HSK most commonly presents as an epithelial keratitis with virus replicating in, and destroying, epithelial cells [3]. The lesions start as punctuate vesicular eruptions in the corneal epithelium, which coalesce into dendritiform lesions and occasionally into larger geographic lesions [4]. HSK is prone to recurrence, usually manifesting as a dendritiform keratitis and or an interstitial stromal keratitis.

Microbial keratitis, however, can be caused by a variety of micro-organisms. Although the clinical features of HSK can be characteristic, there are other diseases and infections with similar features. HSV-1 is also a consideration in many other types of infection of the cornea such as acanthamoeba keratitis. Misdiagnosis has been associated with significantly worse outcomes for the patient for example, confusing HSK and acanthamoebic keratitis [5]. In addition, there has been an increase in HSV-1 resistance to topical and systemic antiviral agents [6, 7]. It is important, therefore, to identify and if possible, isolate HSV-1 for clinical management. Isolation of HSV-1 by culture has a low sensitivity but is the standard for diagnostic specificity, potential strain identification and epidemiological tracing [8]. Although a variety of other laboratory tests on ocular surface samples have been used, some of these, such as cell cytology and viral antigen detection (immunoassays), have low specificity and sensitivity [9]

Received 25 November 2018; Accepted 04 July 2019; Published 29 July 2019
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Keywords: Herpes simplex keratitis; corneal impression membrane; detection of HSV-1 DNA; real time PCR.
Abbreviations: BCVA, best-corrected visual acuity; CIM, corneal impression membrane; Ct, threshold cycle; HSK, herpes simplex keratitis; HSV-1, herpes simplex virus type-1; IC, impression cytology; PCR, polymerase chain reaction; PTFE, polytetrafluroethylene.

and have been replaced by viral DNA detection, in particular real-time PCR [8, 10, 11].

The collection of samples from corneal lesions in HSK is conventionally undertaken using a swab, or less commonly a blade or a needle to scrape the edges of the ulcer. Swabs, however, are cumbersome, may be difficult to localize to the ulcer using slit lamp biomicroscopy and, may come into contact with the conjunctiva and or the eyelids. This is important as asymptomatic shedding of HSV-1 into the tear film over the conjunctiva has been reported so that it is necessary to sample the corneal lesion itself [12, 13]. It is unclear, however, whether in clinical practice the majority of specimens are collected from the conjunctiva and tear film rather than from the cornea. Sharp instruments, such as a blade or needle, more commonly used in suspected bacterial or fungal ulcers, are seldom used for the detection of HSV-1, particularly because they require expertise and may lead to further corneal injury.

Corneal impression membranes (CIMs) made for example, from cellulose acetate or polytetrafluoroethylene (PTFE) have been used to collect samples from the cornea and or the conjunctiva. This method, called impression cytology (IC), has been shown to reliably remove epithelial surface cells from the ocular surface for diagnostic purposes in a variety of infectious and non-infectious corneal conditions, including viral [14], fungal [15], acanthamoeba [16] and bacterial keratitis [17], ocular surface neoplasia [18], keratoconjunctivitis sicca [19], vitamin A deficiency [20] and atopic keratoconjunctitivits [21]. The removal of human epithelial cells using a CIM enables amplification of human DNA as an internal control and an indication of sample quality. In contrast, it is not known whether and to what extent a swab collects corneal epithelial cells, which is important for intracellular infections such as HSV-1. As has been shown in cases of suspected bacterial, acanthamoeba and fungal keratitis, use of a CIM has several practical advantages over conventional methods using swabs or sharp instruments, with good isolation rates [17]. This technique is easy to perform, less traumatic and invasive for the patient and if needed, can be sized to cover the entire ulcer [17]. In addition, it has recently been shown that HSV-1, acanthamoeba and pseudomonas aeruginosa DNA is stable for prolonged time periods on a CIM [22].

To date, however, there are no clinical data available on the comparison of detection rates for HSV-1 using the above mentioned collection techniques. In addition, a variety of different sets of PCR primers have been used for the detection of HSV-1 in cases of suspected HSK. Bennett *et al.* [8] demonstrated that the primers designed and used by Ryncarz *et al.* [10] on CSF, have good sensitivity and specificity when used in a multiplex PCR for the detection of HSV-1 from eye swabs. Dupuis primers have been shown to have good sensitivity and specificity on CSF (Cerebrospinal fluid) samples and have also been used to demonstrate the stability of HSV-1 DNA on a CIM [11, 22]. Comparison between primer sets is useful in cases where the result may be equivocal. The aim of this study, therefore, was to compare *in vitro* and *in vivo*, the detection of HSV-1 using a CIM and a swab and for the former, whether this was dependent on the set of primers used for the PCR. Two sets of primers were used to compare *in vitro*, spiked swabs and CIMs and then a clinical study was undertaken to compare the results of a swab and CIM in cases of suspected HSK.

METHODS

HSV-1 PCR

The performance of two HSV-1 primer sets in PCRs was evaluated, those from Ryncarz *et al.* [10] and Dupuis *et al.* [11]. PCRs for both primer sets comprised Roche Lightcycler 480 Probes Master Mix (Roche, Risch-Rotkreuz, Switzerland) and oligonucleotides (Eurogentec) with amplicons detected using an FAM-labelled fluorescent probe (Eurogentec). Human RNaseP gene and GAPDH oligonucleotides (Eurogentec, Liège, Belgium) were used as internal amplification controls [23]. In total, 10µl aliquot of eluted nucleic acid was added to 15 µl master mix in a 96-well reaction plate. The parameters using a real-time PCR LC480 analyser (Roche) were 95 °C for 5 mins, 45 cycles of 95 °C for 10 s, 60 °C for 45 s and 72 °C for 1 s and a final cooling step of 40 °C for 30 s. Based on the work of Bennett *et al.* a cycle threshold (Ct) of less than or equal to 38.7 was set as the cut-off for a positive result for the HSV-1 DNA amplification [8].

Laboratory study

Recovery of HSV-1 DNA from a CIM and a swab

Sterile CIM (Biopore filter paper, diameter 4 mm, pore size 0.4 µm; Millicell-CM 0.4 µm PICM 01250, Millipore, Bedford, MA, USA) and swabs (Sigma) were used. A 4 mm diameter membrane was used based on previous work [17]. HSV-1 virus stocks of 10^4, 10^3, 10^2 and 10 genome copies ml^{-1} were made by diluting cultured virus from a clinical isolate in buffer containing detergent (Hologic Apitima**,** Hologic, MA, USA). The number of virus genomes (copies ml^{-1}) was determined using a commercial quantitative HSV-1/2 PCR kit (QIAGEN, Hilden, Germany). The assay was only used for this purpose and not testing CIM or swabs as currently only the presence or absence of HSV-1 DNA is clinically required. To mimic clinical samples, human genomic DNA (Roche Diagnostics, Burgess Hill, UK) was diluted 10 000-fold and 1 µl added to each CIM and swab. This dilution resulted in a Ct value of 29 to 31, which was comparable to that obtained from a clinical sample.

Altogether, 5 µl of titrated HSV-1 cultured virus stock was applied to CIMs and allowed to soak into the material. Then, 400 µl buffer containing detergent (Hologic) was added to one set of each duplicated sample and vortexed for 5 s before transfer of the liquid into a secondary tube for automated DNA extraction using the Roche MagNA Pure Compact and the Nucleic Acid Isolation Kit I DNA (Roche) with an elution volume of 50 µl. The CIM was left in the primary tube as it would have blocked the pipette tip on the extraction instrument if transferred. The second set of each duplicant was stored at ambient temperature for 24 h before the addition of

400 µl buffer containing detergent and DNA extraction. For comparison, simulated corneal swabs were similarly inoculated. Next, 5 µl HSV-1 at each dilution was applied to a swab, which was then added to a tube of 3 ml Sigma Virocult viral transport medium (MWE, Wiltshire, UK) and vortexed for 5 s. A 400 µl aliquot of viral transport medium was transferred to a secondary tube before nucleic acid extraction as before. These extraction procedures were also used for the CIM and the swab in the clinical study below.

Topical anaesthetic is usually applied to the eye prior to collection of samples from the cornea and as such it was necessary to investigate a possible inhibitory effect on the PCR and HSV-1 recovery [24]. After adding 5 µl HSV-1 at 10^2 or 10 virus copies ml^{-1} to each CIM, 1 µl of undiluted and diluted (1 in 10^2 and 10^3) proxymetacaine (Bausch and Lomb UK, Kingston-upon-Thamas, Surrey, UK) was added to each CIM before the addition of buffer containing detergent (Hologic) and DNA extraction.

Clinical study

Patient selection

Consecutive patients presenting to The Royal Liverpool University Hospital with suspected epithelial HSK were prospectively recruited between June 2016 and December 2017. Patient demographics and clinical details including previous ophthalmic history, best-corrected visual acuity (BCVA), characteristics of lesions, extraocular manifestations and treatment, were collected using a standardized clinical form. Patients were categorized into probable, presumed and possible HSK by two independent observers. Probable HSK was defined as the presence of a dendritic or geographic ulcer with or without an associated corneal stromal keratitis. Presumed HSK was defined as an atypical keratitis (non-dendritiform or non-geographic ulcers) with or without stromal lesions in a patient with a history of a previous and or recurrent HSK. Possible HSK was defined as clinical microbial keratitis in which HSV-1 was a consideration, but for which there were no typical HSK features and no history of HSK. Patients below age 18 years, with incomplete data either clinical or samples were excluded. All included patients provided informed consent. The study received Institutional Review Board approval from the ethical committee of The Royal Liverpool and Broadgreen University Hospital and was conducted according to the ethical standards set out in the 1964 Declaration of Helsinki, as revised in 2000. There were no conflicts of interest.

Sample collection

Two samples (a corneal swab and a CIM) were collected from the corneal lesion at presentation. The order of collection was randomized. Following instillation of a topical anaesthetic (one drop of 0.5% proxymetacaine) to the lower conjunctival fornix, a sample was collected. The swab was rolled across the corneal lesion and placed in 3 ml Sigma Virocult viral transport medium. This was followed or preceded by application of a CIM (4 mm diameter millipore filter paper, pore size 0.4 µm), to the surface of the lesion for 5 s using sterilized forceps. The filter paper was then transferred to a sterile tube without medium and transported dry to the laboratory for DNA extraction and PCR as described above using the Roche MagNA Pure Compact and the Nucleic Acid Isolation Kit I DNA (Roche). Corneal swabs were processed using the HSV-1 Dupuis primers as described above. CIM samples were processed using both the Ryncarz and Dupuis primers as described above.

Table 1. Effect of eye drop concentration on HSV-1 PCR detection (Dupuis primers)

HSV genome copies ml^{-1}	Eye drops concentration /dilution	HSV-1 PCR Ct	Internal control PCR Ct
10	None	35.7	31.3
10	Undiluted	35.7	31.5
10	1:100	34.9	31.6
10	1:1000	35.6	31.6
100	None	33.0	30.8
100	Undiluted	33.2	32.0
100	1:100	32.5	31.2
100	1:1000	32.5	32.0

Statistical methods

A sample size of 100 patients was based on alpha of 0.05, sensitivity 0.85, specificity 0.90, precision 0.1 and an assumed viral detection rate of 30–35% with corneal swabs [25]. Statistical analysis was performed using SPSS (version 22). Independent *t*-tests were used to compare recovery of HSV-1 DNA between CIMs extracted at 0 and 24 h and between the CIMs and swabs. Chi-square tests were used to compare the differences in HSV-1 detection rate between the CIM and conjunctival swab. One-way ANOVA was used to test for differences between the Dupuis and Ryncarz primer Ct values for the conjunctival swabs and CIMs. Post hoc analysis was carried out using the Bonferroni post hoc test.

RESULTS

There was no evidence of inhibition of the HSV-1 PCR using CIM inoculated with 10 and 100 HSV-1 copies ml^{-1} in the presence or absence of different concentrations of eye drops (P=0.91, Table 1). DNA extracted immediately after HSV-1 inoculation (wet) or 24 h after HSV-1 inoculation with dry storage yielded similar Ct values for both PCRs for all HSV-1 dilutions (Fig. 1) (P=0.83). The Ct PCR values obtained following inoculation with a CIM were approximately three PCR cycles lower than the corresponding Ct values from a swab but this was not significant (P=0.18).

Clinical study

In total, 110 consecutive patients (56 males and 54 females) were included (mean age 55.4 years, SD±17.2). As determined

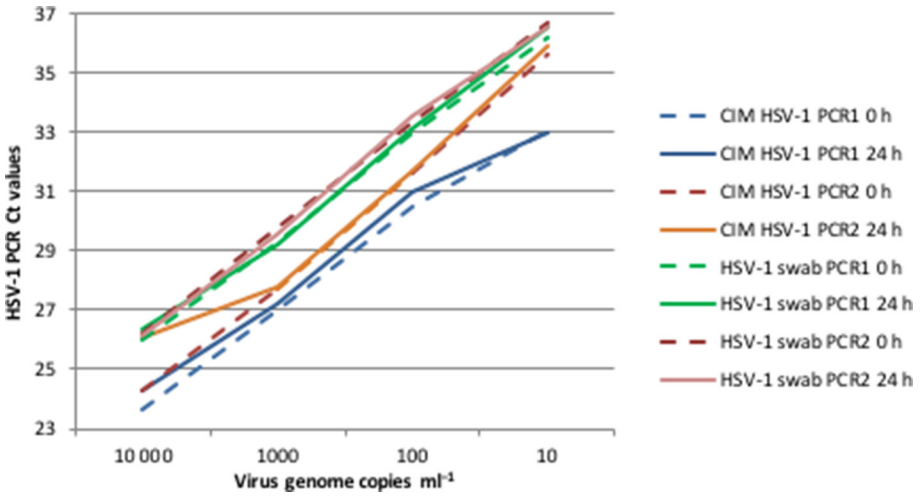

Fig. 1. Comparative sensitivities of HSV-1 PCRs using a CIM and a swab. Amplification of HSV-1 DNA from swabs and corneal impression membranes using Dupuis and Ryncarz primers. HSV-1 DNA was extracted immediately (0 h) and 24 h after inoculation. Ct values plotted for serial tenfold dilutions of virus genome copies ml^{-1}. The amount of PCR amplicon increases at a rate of one log10 every 3.32 cycles under ideal conditions.

by two independent observers; 73 patients had probable, 20 patients presumed and 17 patients possible HSV-1 keratitis. Overall, 45 patients (40.9 %) had a history of recurrent disease with previous episodes of HSK of whom 31 patients had pre-existing corneal scarring and 27 of these had associated corneal neovascularisation. Altogether, 58 patients (52.7 %) had BCVA of worse than 0.3 logMAR at presentation.

The HSV-1 detection rate using the Dupuis primers was significantly higher using a CIM (40/110, 36.4 %) than a swab (28/110, 25.5%) (P=0.004) (Tables 2 and 3). Using the Dupuis primers, in the probable HSV keratitis group, the detection rate using a CIM was 43.8% compared to 27.4% for a swab (P=0.004). No significant difference was found between the HSV-1 detection rates between the CIM and the conjunctival swab in the presumed and possible HSK groups (Tables 2–4). The Ct values obtained for the conjunctival swabs were significantly higher than those obtained for the CIMs (P<0.001, Table 3).

There were no significant differences using a CIM between the Dupuis and Ryncarz primers for any of the three HSK groups (Table 4). There were three CIM samples out of 110, two from the probable and one from the presumed HSK group, where using the Dupuis primers, the PCR was borderline positive (35.5, 37.02 and 38.67 Ct) but negative using the Ryncarz primers. Of the swab samples from these three patients using the Dupuis primers, the CIM sample that gave a Ct of 35.5 using the Dupuis primers also produced a positive swab result. The two further CIM samples (where the CIM was borderline positive at 37.02 and 38.67 Ct), however, gave a negative swab result. Two CIM samples from patients with probable HSK, were positive using the Ryncarz but not the Dupuis primers and the corresponding swabs were also positive using the Dupuis primers. Post hoc analysis, however, demonstrated no difference of the PCR Ct values between the two sets of HSV-1 primers (Table 4).

Table 2. HSV-1 detection rates using Dupuis real-time HSV-1 PCR

HSK	*n*	Swab positive (%)	CIM positive (%)	*P*-value
Overall	110	28 (25.5)	40 (36.4)	0.004
Probable*	73	20 (27.4)	32 (43.8)	0.004
Presumed*	20	6 (30.0)	6 (30.0)	1
Possible*	17	2 (11.8)	2 (11.8)	1
		CIM positive	**CIM negative**	**Total**
Swab positive		26 (65%)	2 (2.9%)	**28**
Swab negative		14 (35%)	68 (97.1%)	**82**
Total		**40**	**70**	**110**

*As determined by two independent observers.

Table 3. HSV-1 PCR CIM versus swab using Dupuis primers

HSK	CIM positive *n*	Dupuis primers Ct mean (SD)	Swab positive *n*	Dupuis primers Ct mean (SD)	*P*-value (ANOVA between groups)
Overall	40	28.7 (4.3)	28	33.1 (4.2)	<0.001
Probable*	32	29.1 (4.0)	20	33.1 (3.9)	<0.001
Presumed*	6	27.8 (6.0)	6	33.3 (5.4)	0.037
Possible*	2	25.6 (1.5)	2	33.1 (6.6)	0.44

*As determined by two independent observers.

Table 4. HSV-1 CIM PCR Ct values using Dupuis and Ryncarz primers

HSK	CIM positive *n*	Dupuis primers Ct mean (SD)	Ryncarz primers Ct mean (SD)	*P*-value
Overall	40	28.7 (4.3)	27.1 (3.9)	0.25
Probable*	32	29.1 (4.0)	27.6 (3.5)	0.37
Presumed*	6	27.8 (6.0)	23.7† (4.9)	0.73
Possible*	2	25.6 (1.5)	27.4 (6.2)	1

*As determined by two independent observers.
†Six CIM samples tested positive using the Dupuis primers and five samples tested positive using the Ryncarz primers.

DISCUSSION

Microbial keratitis can be caused by a variety of micro-organisms and accurate diagnosis with immediate treatment is important to optimize clinical outcome. Although the clinical features of HSK are important for the diagnosis, reliance on clinical features alone, may be misleading due to overlapping findings caused by different conditions and infections for example acanthamoeba and HSV-1, and excludes the ability to detect resistance mutations and or contact tracing.

Cell culture has been the traditional method for the detection of HSV-1, but has been largely replaced by PCR, due to its high sensitivity and shorter processing time. PCR has been optimized for the detection of HSV-1 from ophthalmic samples using swabs [8]. There is very little data, however, on the detection rates of HSV-1 from cases of HSK using swabs and although anecdotal, many ophthalmologists do not collect samples in cases of suspected HSV-1 keratitis possibly due to the low yield and cumbersome nature of a swab. It is also unclear whether in clinical practice the majority of specimens are collected from the conjunctiva and tear film rather than from the corneal ulcer itself. This carries the risk of detecting HSV-1 in cases of asymptomatic shedding into the conjunctival tear film [12, 13].

We compared two sets of HSV-1 primers as one had been used for the testing of cases of encephalitis and the other had been optimized for the testing of eye swabs but no clinical sample testing was reported [11]. Both showed good and comparable sensitivity. The CIM produced significantly higher results for patients with suspected HSK than a swab. Use of a CIM improves precision placement over the lesion and the membrane collects epithelial cells. This, in addition to the greater surface area, likely accounts for the increased detection rate observed in this study.

There were no significant differences in the detection rate between the two sets of primers, which would suggest that a specific set of primers is not peculiar to the technique. Despite the greater detection rate using a CIM compared to a swab, there were still up to 60% where the result was negative. This would suggest that either the CIM is still not sensitive enough to detect HSV-1 DNA, or that the clinical diagnosis was incorrect or, that the stage of the disease was not associated with sufficient HSV-1 proliferation to be detected in the tear film. HSV-1 production has been shown to increase 4 days prior to clinical manifestation [26] and it is possible that the time to presentation was at a stage where the amount of virus present may be declining. As the use of a CIM is not invasive, it offers the opportunity to sample the cornea at repeated time points particularly if there is no response to treatment in those cases where there has been a negative result.

Somerville *et al.* [22] recently demonstrated the stability of HSV-1 DNA recovery following inoculation of HSV-1 onto PTFE CIMs and storage at +4, −20 and −70 °C for up to 10 months using Dupuis primers. In this study, we obtained similar HSV-1 DNA Ct values to that demonstrated by Somerville *et al.*, both from CIMs extracted immediately following sample collection and those extracted 24 h after collection and storage at +4 °C [22]. This suggests that there is no significant reduction in HSV-1 DNA recovery should there be a 24 h delay in sample processing. This is reflective of clinical settings, in which samples often do not reach the laboratory until the following day after the sample has been collected.

In clinical practice, because a topical anaesthetic is applied to the eye prior to a corneal sample (either a swab or a CIM) being collected, it was important therefore to demonstrate that there were no inhibitory effects on the PCR. Our *in vitro* data demonstrated good detection of HSV-1 DNA by PCR from CIM using both sets of primers with an end point of ≤10 virus genome copies m^{-1}, which would be suitable for testing clinical samples.

A CIM is easy to use and may therefore be suitable for use by non-ophthalmologists or where less sophisticated biomicroscopes are available, such as in resource-poor settings. The CIM has the potential to be produced as a medical product either individually or as part of a sampling device. In a recently published study, we compared the microbial detection rates of a corneal scrape to that using a CIM made from PTFE [17]. The results using a CIM were significantly better than using a blade to detect bacteria and acanthamoeba from corneal ulcers in cases of suspected microbial keratitis [17]. The results of this study would suggest that a CIM may additionally be a simple and good alternative to using sharp instruments and swabs for the identification of HSV-1 in cases of suspected microbial keratitis. A CIM, therefore, offers the opportunity to investigate for bacterial, fungal, protozoan and viral causes in cases of suspected microbial keratitis, without the need for sharp instrumentation. Furthermore, a CIM has the potential to be used by non-ophthalmologists without specialized equipment.

Funding information
This work received no specific grant from any funding agency.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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