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ORIGINAL INVESTIGATION

Quadruplex real-time PCR for rapid detection of human alphaherpesviruses

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Abstract

Infections with the herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) as well as with the varicella-zoster virus (VZV) may take a serious course. Thus, rapid and reliable detection of these alphaherpesviruses is urgently needed. For this, we established a qualitative quadruplex real-time polymerase chain reaction (PCR) covering HSV-1, HSV-2, VZV and endogenous human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR was validated with quality assessment samples and pre-characterized clinical samples including swabs, blood and cerebrospinal as well as respiratory fluids. For comparison, nucleic acids (NA) of selected samples were extracted manually and automatically. The protocol takes approx. 90 min, starting with the preparation of NA until the report of results. The oligonucleotide and hydrolysis probe sequences specifically detect and distinguish HSV-1 (530 nm), HSV-2 (705 nm) and VZV (560 nm) DNA. The detection limit was estimated with 100–500 copies/ml HSV-1 and HSV-2/VZV, respectively. All quality assessment samples as well as all the patient samples were classified correctly. Parallel detection of GAPDH (670 nm) DNA was implemented to demonstrate correct sampling, but was uncertain in case of swabs. To this end, alphaherpesvirus-free human DNA was also added directly into the mastermix to exclude PCR inhibition. The established protocol for parallel detection and differentiation of alphaherpesviruses is fast, highly specific as well as rather sensitive. It will facilitate HSV-1/2 and VZV diagnostics and may be further improved by opening the 670 nm channel for a combined extraction and PCR inhibition control.

Keywords Human herpesvirus · DNA · Simultaneous detection

Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are considered as two separate species within the genus *Simplex virus* of the *Alphaherpesvirinae* subfamily. Furthermore, the varicella-zoster virus (VZV) belongs to the genus *Varicellovirus* within this subfamily. All three enveloped double-stranded DNA viruses are members of the *Herpesviridae* family (Virus Taxonomy: 2017 Release, https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsdna-viruses-2011/w/dsdna_viruses/89/herpesvirales). Infection with these highly contagious viruses starts in early childhood and leads to the establishment of life-long latency [1, 2]. In Europe, seroprevalence data in adults range from 15% (HSV-2) to nearly 100% (HSV-1/VZV) [3, 4]. Alterations in cellular immunity can cause viral reactivation out from latency [5]. Both primary and recurrent infections may result in diseases accompanied with serious complications not only in immunocompromised patients, as these alphaherpesviruses may cause encephalitis as well as congenital/neonatal

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infections [2, 6–8]. Particularly under immunosuppression, the clinical picture may take a severe course, and other pathogens are to be considered in differential diagnosis [9]. Due to the availability of effective antiviral therapy regimes, prompt and correct diagnosis is highly urgent, particularly in case of neurological infections, disseminated disease, or clinical treatment failure [10]. This aim can only be achieved by diagnostic application of fast multiplex real-time PCR protocols. For this, a thermal cycler with an air-driven temperature profile and equipped with a carousel as well as glass capillaries [11] may represent a suitable technical platform. To our knowledge, commercial kits for simultaneous detection and differentiation of HSV-1/2 and VZV are not available for this type of cycler. A previous in-house protocol that matches this specific profile [10] does not include an internal control. Therefore, it cannot exclude PCR inhibition.

We aimed to develop a fast and sensitive quadruplex real-time PCR protocol for the specific detection of HSV-1, HSV-2 and VZV in various clinical specimens on such a thermal cycler. Thus, the present study extends the work of Wong et al. [10] by inclusion of a fourth primer and hydrolysis probe set, which was used here for detection of endogenous DNA. It may also be open for amplification of a combined extraction and inhibition control.

Materials and methods

Quality assessment samples were provided by Instand e.V. (Düsseldorf, Germany). With respect to data of all interlaboratory test participants, Instand e.V. calculates a robust mean viral load per samples in copies/ml. These results were used here as quantitative standard for calculation of the detection limit (LOD). Furthermore, 23 swabs taken from skin, mucosa or cornea, 4 cerebrospinal fluids, 2 blood samples, 2 respiratory samples and 1 cell culture supernatant, all sent for diagnosis of a suspected HSV/VZV infection to the previous German consulting laboratory for HSV and VZV (former Institute of Virology and Antiviral Therapy, Jena University Hospital, designated here as Jena 1–32) were included. In the former German consulting laboratory for HSV and VZV, the PCR for detection of HSV-1 and −2 was performed with oligonucleotide primer pairs specific for UL 42 region of HSV-1 and thymidine kinase gene of HSV-2, respectively [12]. Varicella-zoster virus DNA was amplified using primers of VZV open reading frame 28 [13]. In addition, we used four swabs sent to our laboratory for routine HSV diagnostics (designated here as Kiel 1–4) which was previously done with a commercial kit that utilizes dual hybridization probes (LightMix® 40-0378-32 HSV EC, TIB MOLBIOL, Berlin, Germany). All human samples were used anonymized.

Nucleic acids (NA) were extracted manually by help of the Qiagen Viral RNA Mini Kit (Qiagen, Hilden, Germany). For comparison, some NA were also extracted automatically using the MagNAPure Compact system together with the MagNA Pure Compact NA Isolation Kit I (all Roche, Mannheim, Germany). The NA isolation protocols were strictly followed the recommendations of the manufacturers.

For amplification, 5 µl of NA and 1.5 µl of sterile water were pipetted together with 10 µl of the QuantiFast Multiplex PCR Master Mix (Qiagen) and oligonucleotides and hydrolysis probe mixes for detection of HSV-1, HSV-2, VZV (1 µl each) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 0.5 µl). In several cases, 1 µl of sterile water was substituted by a virus-free sample containing 10 ng human DNA. Cycling was done with the LightCycler® (LC) 2.0 system using 20 µl capillaries (both Roche) [11]. Primer and hydrolysis probe sequences which were taken from previous publications [14–17] are listed in Table 1. Notably, the GAPDH hydrolysis probe sequence was slightly modified by deletion of one nucleotide at the 5′-end. Furthermore, chemistry of dyes and quenchers had to be adapted to allow their parallel detection on the LC 2.0. All oligonucleotides and hydrolysis probes were purchased from biomers.net GmbH (Ulm, Germany). The composition of the master mix and the cycling conditions were in strict accordance to the recommendations of Qiagen. In brief, an initial denaturation step (95 °C, 5 min) was followed by 45 cycles of denaturation (95 °C, 10 s) and annealing/extension (60 °C, 30 s). The fluorescence signal was measured in this combined annealing and extension step. Then, PCR was finished by a cooling step (40 °C, 30 s). A separate color compensation file was generated before the first run as recommended by Qiagen (Color compensation file for LightCycler® 2.0 (PCR81 Aug-08)). This file was then used for data analysis in each case. The channels 530 nm (HSV-1), 560 nm (VZV), 670 nm (GAPDH), and 705 nm (HSV-2), respectively, were checked for the appearance of specific signals given as threshold cycle (CT) values.

Statistical analyses were done with GraphPad (https://www.graphpad.com).

Results

The suitability of mixed primer and hydrolysis probe sequences and chemistry as well as of the general protocol was examined by usage of 39 quality assessment samples, and further by inclusion of four own routine patient samples pretested with the LightMix® 40-0378-32 HSV kit as well as of 32 pre-characterized patient samples provided by the former German consulting laboratory for HSV and VZV (Tables 2, 3, 4). First, analytical specificity was demonstrated by testing of seven HSV-1/2 and VZV-free quality

Table 1 Primer and hydrolysis probes used for detection of alphaherpesviruses and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Target (LC 2.0 channel)	Name	Sequences (5′-3′) of oligonucleotides or probes	Final concentration (µM)	References
HSV-1 (530 nm)	HSV-1f	GGCCTGGCTATCCGGAGA	0.5	Sequences taken from [14, 16]
	HSV-1r	GCGCAGAGACATCGCGA	0.5	
	HSV-1-probe	FAM-CAGCACACGACTTGGCGTTCTGTGT-BHQ1	0.2	
HSV-2 (705 nm)	HSV-2f	AGATATCCTCTTTATCATCAGCACCA	0.5	Sequences taken from [14, 16]
	HSV-2r	TTGTGCTGCCAAGGCGA	0.5	
	HSV-2-probe	FAM-Cy5.5-CAGACAAACGAACGCCGCCG-BMN-Q620	0.2	
VZV (560 nm)	VZV-f	TCTTGTCGAGGAGGCTTCTG	0.5	Sequences taken from [14, 17]
	VZV-r	TGTGTGTCCACCGGATGAT	0.5	
	VZV-probe	YAK-TCTCGACTGGCTGGGACTTGCG-BHQ1	0.2	
GAPDH (670 nm)	GAPDH-f	GGACTGAGGCTCCCACCTTT	0.25	Sequences taken from [15]
	GAPDH-r	GCATGGACTGTGGTCTGCAA	0.25	
	GAPDH-probe	Atto490LS-ATCCAAGACTGGCTCCTCCCTGC-BMN-Q620	0.1	

assessment samples as well as by testing of quality assessment samples containing relevant DNA loads of human cytomegalovirus (CMV), Epstein–Barr virus (EBV), adenovirus as well as of human polyomaviruses BK (BKV) and JC (JCV), respectively. In addition, all HSV-1/2 and VZV quality assessment samples were generally tested in parallel for all three alphaherpesviruses. Since none of these samples were found to be false positive, cross-reactivity could be excluded and primer/hydrolysis probes analytical specificities were estimated with 100% (Tables 2, 3, 4).

Testing of pre-characterized patient samples resulted in 100% agreement to the results of the LightMix® Kit or of the German consulting laboratory; results of both were considered here as gold standard/reference consensus standard (Tables 3, 4). Thus, the protocol developed in this study was found to be highly suitable for alphaherpesvirus diagnostics in clinical settings. The direct comparison of HSV-1/2-specific CTs (i.e., the threshold cycle when the specific signal clearly exceeds the background level) of quadruplex real-time PCR to the LightMix® Kit revealed a mean gain ± standard deviation of 1.99 ± 0.60 cycles (Table 3, supplementary Fig. 1).

Eight quality assessment samples positive for alphaherpesviruses were also pre-mixed with human DNA to demonstrate that parallel GAPDH detection does not have any influence on the detection of alphaherpesvirus species (supplementary Fig. 2).

The linear dynamic range [18] was exemplarily demonstrated over three to four logarithmic dilutions steps using quality assessment samples with defined viral loads (supplementary Fig. 3). These samples were also diluted in a GAPDH-containing NA background and results were similar (data not shown). Furthermore, we also tested dilutions containing a DNA mixture of all three alphaherpesviruses in this matrix. Thus, even in the rather unlikely event of multiple alphaherpesvirus infections, the assay was able to specifically detect and differentiate low genome equivalents (9371–15,154 copies/ml) of these pathogens (supplementary Table 1). Next, the LOD was determined by repeated testing of NA dilutions representing 1500, 1000, 500, 100 and 50 copies/ml HSV-1, HSV-2, or VZV, respectively. In this experiment, 1 µl alphaherpesvirus-free human DNA was directly added to the master mix. Under these conditions, 100 HSV-1 copies/ml were repeatedly (5/5) detectable as well as 500 copies/ml HSV-2 or VZV, respectively (Fig. 1, supplementary Table 2). Since only 140 µl out of 1000 µl sample input was used for NA extraction by the Qiagen kit, and 5 µl out of 60 µl elution volume are tested, the detection limit ranged from 1.2 to 5.8 viral copies/test. The results are close to the most sensitive LOD of three copies/PCR [18]. Such data just represent a rough estimation, and have to be interpreted with caution. Furthermore, these pathogen-specific CTs are probably outside of the linear dynamic range. To be closer to the clinical situation, one sample each of plasma, respiratory fluid and smear was prepared to contain 1000 copies/ml HSV-1, HSV-2 or VZV, respectively. These artificial samples were used for NA extraction, and repeatedly tested in quadruplex real-time PCR. Thereby, HSV-1, HSV-2 or VZV-DNA was detected at mean CTs 32.3–33.8, and a relevant matrix effect was largely excluded (supplementary Table 3).

The suitability of the quadruplex real-time PCR for routine diagnostics was further demonstrated by testing 14 NAs which were extracted manually and automatically in parallel. The resulting CT values indicate an almost perfect

Table 2 Results of quadruplex real-time PCR for defined quality assessment samples

Sample name	Qualitative value	Mean viral load in cop/ml (Instand e.V.)	CT-value				Assessment
			HSV-1	HSV-2	VZV	GAPDH	
Quality assessment samples containing other DNA viruses as well as defined samples being negative for HSV-1/-2 and VZV (Alpha)							
364035/18	BKV pos.	BKV: 1,677,041	neg.*	neg.*	neg.*	28.9*	RN
365129/18	CMV pos.	CMV: 785,201	neg.*	neg.*	neg.*	28.7*	RN
371066/18	AdV pos.	AdV A31: n.a.	neg.*	neg.*	neg.*	29.1*	RN
394028/18	JCV pos.	JCV: 43,662	neg.*	neg.*	neg.*	28.8*	RN
376067/18	EBV pos.	EBV: 34,166	neg.*	neg.*	neg.*	29.5*	RN
363099/18	Alpha neg.	0	neg./neg.*	neg./neg.*	neg./neg.*	29.0/28.9*	RN
366066/18	Alpha neg.	0	neg./neg.*	neg./neg.*	neg./neg.*	30.4/29.3*	RN
366059/17	Alpha neg.	0	neg.	neg.	neg.	30.1	RN
366064/17	Alpha neg.	0	neg.	neg.	neg.	30.3	RN
363092/17	Alpha neg.	0	neg.	neg.	neg.	28.7	RN
363094/17	Alpha neg.	0	neg.	neg.	neg.	28.6	RN
366046/15	Alpha neg.	0	neg.	neg.	neg.	31.5	RN
Quality assessment samples containing HSV-1 DNA							
363102/18	HSV-1 pos.	169,708	27.6/26.9*	neg./neg.*	neg./neg.*	neg./29.4*	RP
363074/16	HSV-1 pos.	151,544	26.7	neg.	neg.	neg.	RP
363085/17	HSV-1 pos.	51,276	29.6	neg.	neg.	neg.	RP
363100/18	HSV-1 pos.	45,234	29.8/28.4*	neg./neg.*	neg./neg.*	neg./29.2*	RP
363093/17	HSV-1 pos.	43,712	29.4	neg.	neg.	neg.	RP
363097/18	HSV-1 pos.	12,865	31.7/29.3*	neg./neg.*	neg./neg.*	neg./29.3*	RP
363089/17	HSV-1 pos.	12,342	30.9	neg.	neg.	neg.	RP
363095/17	HSV-1 pos.	10,560	30.8	neg.	neg.	neg.	RP
363086/17	HSV-1 pos.	3544	32.6	neg.	neg.	neg.	RP
Quality assessment samples containing HSV-2 DNA							
363098/18	HSV-2 pos.	176,483	neg./neg.*	28.1/27.6*	neg./neg.*	30.1/28.2*	RP
363075/16	HSV-2 pos.	93,706	neg.	27.7	neg.	neg.	RP
363101/18	HSV-2 pos.	69,577	neg./neg.*	29.5/29.0*	neg./neg.*	neg./29.0*	RP
363087/17	HSV-2 pos.	32,171	neg.	30.5	neg.	neg.	RP
363091/17	HSV-2 pos.	31,110	neg.	30.6	neg.	neg.	RP
363096/17	HSV-2 pos.	8007	neg.	32.3	neg.	neg.	RP
363090/17	HSV-2 pos.	7422	neg.	32.2	neg.	neg.	RP
363088/17	HSV-2 pos.	2662	neg.	33.6	neg.	neg.	RP
Quality assessment samples containing VZV-DNA							
366063/17	VZV pos.	1,702,634	neg.	neg.	22.6	neg.	RP
366061/17	VZV pos.	162,907	neg.	neg.	26.1	neg.	RP
366065/18	VZV pos.	114,974	neg./neg.*	neg./neg.*	27.1/27.3*	neg./28.1*	RP
366048/15	VZV pos.	59,000	neg.	neg.	27.4	neg.	RP
366067/18	VZV pos.	23,362	neg./neg.*	neg./neg.*	28.6/28.5*	neg./28.4*	RP
366068/18	VZV pos.	16,109	neg./neg.*	neg./neg.*	30.0/29.5*	neg./28.9*	RP
366045/15	VZV pos.	13,410	neg.	neg.	29.0	neg.	RP
366047/15	VZV pos.	7230	neg.	neg.	29.8	neg.	RP
366053/16	VZV pos.	5187	neg.	neg.	30.7	neg.	RP
366062/17	VZV pos.	1307	neg.	neg.	32.6	neg.	RP

Results highlighted with an asterisk (*) were obtained by spiking the sample with virus-free human DNA

AdV, adenovirus; Alpha, human alphaherpesviruses (HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus); BKV, human polyomavirus BK; CMV, human cytomegalovirus; CT, threshold cycle; EBV, Epstein–Barr virus; GAPDH, human glyceraldehyde 3-phosphate dehydrogenase; JCV, human polyomavirus JC; n.a., not available; neg., negative; pos., positive; RN, right negative; RP, right positive

Table 3 Comparison of quadruplex real-time PCR with the LightMix (LM) kit

Sample name	Qualitative value	Mean viral load in cop/ml (Instand e.V.)	CT-values (LM/quadruplex)				Assessment
			HSV-1	HSV-2	VZV	GAPDH	
363092/17	Alpha neg.	0	neg./neg.	neg./neg.	n.a./neg.	n.a./28.7	RN
363094/17	Alpha neg.	0	neg./neg.	neg./neg.	n.a./neg.	n.a./28.6	RN
Kiel 1 (sw)	HSV-1 pos.	n.a.	19.0/16.6	neg./neg.	n.a./neg.	n.a./28.1	RP
Kiel 2 (sw)	HSV-1 pos.	n.a.	24.5/21.8	neg./neg.	n.a./neg.	n.a./31.9	RP
363074/16	HSV-1 pos.	151,544	29.8/26.7	neg./neg.	n.a./neg.	n.a./neg.	RP
363085/17	HSV-1 pos.	51,276	31.1/29.6	neg./neg.	n.a./neg.	n.a./neg.	RP
363093/17	HSV-1 pos.	43,712	31.6/29.4	neg./neg.	n.a./neg.	n.a./neg.	RP
363089/17	HSV-1 pos.	12,342	32.8/30.9	neg./neg.	n.a./neg.	n.a./neg.	RP
363095/17	HSV-1 pos.	10,560	33.2/30.8	neg./neg.	n.a./neg.	n.a./neg.	RP
363086/17	HSV-1 pos.	3544	33.9/32.6	neg./neg.	n.a./neg.	n.a./neg.	RP
Kiel 3 (sw)	HSV-2 pos.	n.a.	neg./neg.	18.0/16.8	n.a./neg.	n.a./neg.	RP
Kiel 4 (sw)	HSV-2 pos.	n.a.	neg./neg.	27.2/24.5	n.a./neg.	n.a./23.8	RP
363075/16	HSV-2 pos.	93,706	neg./neg.	30.3/27.7	n.a./neg.	n.a./neg.	RP
363087/17	HSV-2 pos.	32,171	neg./neg.	31.9/30.5	n.a./neg.	n.a./neg.	RP
363091/17	HSV-2 pos.	31,110	neg./neg.	32.6/30.6	n.a./neg.	n.a./neg.	RP
363096/17	HSV-2 pos.	8007	neg./neg.	33.8/32.3	n.a./neg.	n.a./neg.	RP
363090/17	HSV-2 pos.	7422	neg./neg.	33.9/32.2	n.a./neg.	n.a./neg.	RP
363088/17	HSV-2 pos.	2662	neg./neg.	34.9/33.6	n.a./neg.	n.a./neg.	RP

Alpha, human alphaherpesviruses (HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus); CT, threshold cycle; GAPDH, human glyceraldehyde 3-phosphate dehydrogenase; sw, swab/smear; n.a., not available; neg., negative; pos., positive; RN, right negative; RP, right positive

agreement of both methods (Table 4, shown in supplementary Fig. 4).

Deoxyribonucleic acid of house-keeping GAPDH was detected in 100% of respiratory fluids (2/2), blood (2/2), CSF (4/4), but only in 48.1% (13/27) of swabs. As well, GAPDH detection failed in culture supernatant Jena 15. There was no association between presence of alphaherpesvirus DNA in these clinical samples and detection of GAPDH (Fisher's exact test two-tailed P value = 0.50). Furthermore, quality assessment samples which most likely represent dilutions of inactivated viruses obtained from cell culture supernatants were frequently tested negative for GAPDH (76.5%, 26/34). For this artificial sample type, presence of alphaherpesvirus DNA was highly associated with the failure in detection of GAPDH (Fisher's exact test two-tailed P value < 0.0001).

The whole procedure takes approx. 90 min including manual or automatic nucleic acid extraction (c. 30 min), preparation of mastermix as well as set up of samples (c. 15 min), amplification and detection on the LC 2.0 (c. 45 min) and report of results.

Discussion

In immunocompromised patients, patients with central nervous system or neonatal infections, a rapid and reliable virus detection is of high significance for the prognosis of diseases caused by the alphaherpesviruses HSV-1/2 or VZV [7, 8, 19, 20]. By contrast with many other viruses, antiviral agents such as acyclovir have been proved to be highly effective in early systemic treatment [21]. In the diagnostic approach, the real-time PCR plays a crucial role for detection of HSV-1/2 and VZV DNA representing the laboratory procedure of choice to verify acute infections. In this study, a quadruplex real-time PCR protocol was established using LC 2.0 technology and hydrolysis probes. So far, most LC protocols are based on dual hybridization probes utilizing fluorescence resonance transfer technology (FRET) and, thus, require melting curve analysis [11, 22–26]. These probes have several limitations [27] and melting curve analysis needs some additional time. Furthermore, differentiation between HSV-1 and − 2 by the latter can be rather challenging and may even demand additional tests [28].

Here, we make use of a combination of FRET and TaqMan technology [27] to design a HSV-2-specific hydrolysis probe based on a published sequence. Thereby, we were able to establish a quadruplex real-time PCR on the LC 2.0

Table 4 Results of quadruplex real-time PCR for pre-characterized clinical samples obtained from the former German consulting laboratory for HSV and VZV

Sample name	Qualitative value	CT-value quadruplex real-time PCR				Assessment
		HSV-1	HSV-2	VZV	GAPDH	
Jena 1 (csf)	Alpha neg.	neg.	neg.	neg.	29.2	RN
Jena 2 (sw)	Alpha neg.	neg.	neg.	neg.	30.0	RN
Jena 3 (sw)	HSV-1 pos.	18.7	neg.	neg.	24.8	RP
Jena 4 (ts)	HSV-1 pos.	20.6/21.2 (a)	neg./neg. (a)	neg./neg. (a)	23.6/24.9 (a)	RP
Jena 5 (sw)	HSV-1 pos.	22.2	neg.	neg.	26.9	RP
Jena 6 (sw)	HSV-1 pos.	23.6/23.2 (a)	neg./neg. (a)	neg./neg. (a)	29.1/30.0 (a)	RP
Jena 7 (sw)	HSV-1 pos.	24.2	neg.	neg.	neg.	RP
Jena 8 (csf)	HSV-1 pos.	25.9/27.7 (a)	neg./neg. (a)	neg./neg. (a)	28.0/29.8 (a)	RP
Jena 9 (bal)	HSV-1 pos.	26.5/26.6 (a)	neg./neg. (a)	neg./neg. (a)	24.5/25.6 (a)	RP
Jena 10 (sw)	HSV-1 pos.	26.7	neg.	neg.	neg.	RP
Jena 11 (bl)	HSV-1 pos.	27.5/23.6 (a)	neg./neg. (a)	neg./neg. (a)	25.6/25.2 (a)	RP
Jena 12 (bl)	HSV-1 pos.	33.3/33.8 (a)	neg./neg. (a)	neg./neg. (a)	30.4/29.9 (a)	RP
Jena 13 (sw)	HSV-2 pos.	neg.	19.0	neg.	neg.	RP
Jena 14 (sw)	HSV-2 pos.	neg.	19.1	neg.	30.9	RP
Jena 15 (cc)	HSV-2 pos.	neg./neg. (a)	21.1/21.6 (a)	neg./neg. (a)	neg./neg. (a)	RP
Jena 16 (sw)	HSV-2 pos.	neg./neg. (a)	21.7/21.6 (a)	neg./neg. (a)	neg./neg. (a)	RP
Jena 17 (sw)	HSV-2 pos.	neg.	22.7	neg.	24.6	RP
Jena 18 (sw)	HSV-2 pos.	neg.	25.6	neg.	26.4	RP
Jena 19 (sw)	HSV-2 pos.	neg.	25.8	neg.	neg.	RP
Jena 20 (sw)	HSV-2 pos.	neg.	27.8	neg.	29.7	RP
Jena 21 (csf)	HSV-2 pos.	neg./neg. (a)	33.9/33.9 (a)	neg./neg. (a)	30.6/33.0 (a)	RP
Jena 22 (csf)	HSV-2 pos.	neg./neg. (a)	34.3/34.9 (a)	neg./neg. (a)	27.9/30.7 (a)	RP
Jena 23 (sw)	VZV pos.	neg.	neg.	13.3	neg.	RP
Jena 24 (sw)	VZV pos.	neg.	neg.	14.7	neg.	RP
Jena 25 (sw)	VZV pos.	neg.	neg.	15.0	neg.	RP
Jena 26 (sw)	VZV pos.	neg./neg. (a)	neg./neg. (a)	17.7/16.3 (a)	neg./neg. (a)	RP
Jena 27 (sw)	VZV pos.	neg.	neg.	18.1	neg.	RP
Jena 28 (sw)	VZV pos.	neg./neg. (a)	neg./neg. (a)	18.6/16.9 (a)	neg./neg. (a)	RP
Jena 29 (sw)	VZV pos.	neg./neg. (a)	neg./neg. (a)	25.0/26.1 (a)	27.0/27.0 (a)	RP
Jena 30 (sw)	VZV pos.	neg.	neg.	25.1	neg.	RP
Jena 31 (sw)	VZV pos.	neg./neg. (a)	neg./neg. (a)	25.3/25.2 (a)	25.6/27.8 (a)	RP
Jena 32 (sw)	VZV pos.	neg.	neg.	31.7	neg.	RP

Results marked with (a) were obtained after automatic extraction of nucleic acids

Alpha, human alphaherpesviruses (HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus); bal, broncheoalveolar lavage; bl, blood (plasma/serum); cc, cell culture supernatant; csf, cerebrospinal fluid; CT, threshold cycle; GAPDH, human glyceraldehyde 3-phosphate dehydrogenase; sw, swab/smear; neg., negative; pos., positive; RN, right negative; RP, right positive; ts, tracheal secretion

platform which includes detection and differentiation of HSV-1 (530 nm), HSV-2 (705 nm), VZV (560 nm), as well as endogenous human GAPDH DNA (670 nm). With a total time of 1.5 h beginning from sample preparation to obtainment of final PCR results, this protocol is rather fast. The amplification time could be further shortened [11].

The assay is highly specific for detection of alphaherpesviruses and can be used in the clinical setting as demonstrated here for a limited spectrum of samples and two extraction methods. Under optimal conditions, the LODs of quadruplex real-time PCR are estimated with 100 (HSV-1) to 500 (HSV-2/VZV) copies/ml, corresponding to 1.2–5.8 copies per test. Such LODs are comparable or even better than that of assays reported for separate detection of HSV-1/2 or VZV [16, 17, 25, 26, 29]. More important, low viral loads are reliably detectable under clinical conditions, as it is demonstrated exemplarily for spiked samples containing 1000 copies/ml.

The quadruplex real-time PCR has a mean gain of two cycles compared to the commercial test previously used by

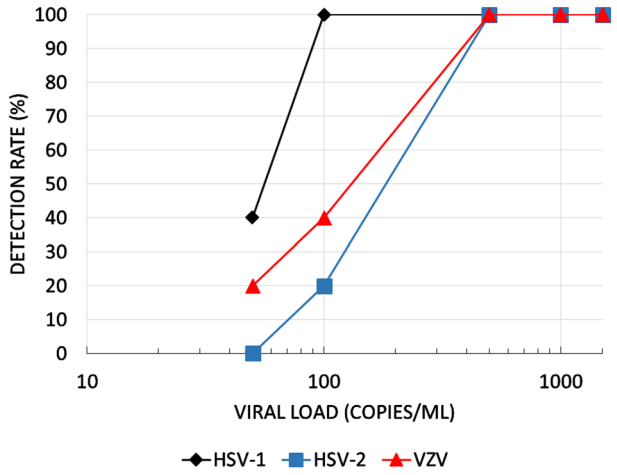

Fig. 1 Detection limit of the laboratory-developed quadruplex real-time PCR. Quality assessment samples were diluted and threshold cycle (CT) values were determined fivefold for each concentration. 100 HSV-1 copies/ml and 500 HSV-2 or VZV copies/ml, respectively, were reliably detected

us. This further indicates a high sensitivity of the laboratory-developed method.

So far, the current protocol permits only generation of qualitative results. This is, however, not a major limitation at all, since CT values can be easily compared. If desired, inclusion of standard dilutions will allow the generation of quantitative data, as it could be beneficial for testing of plasma/serum or CSF.

Endogenous GAPDH was successfully detected in both respiratory fluids which are known to contain diverse cell populations [30]. As well, its presence was demonstrated in the two serum/plasma samples as also observed by others [31]. Its detection in the four cerebrospinal fluids could result from lymphocytic pleocytosis during CNS infection [32, 33], but may represent an unreliable marker [33, 34]. Furthermore, for unknown reasons, GAPDH amplification often failed in swabs and quality assessment samples. This limitation can be overcome by addition of 10 ng virus-free human DNA directly into the mastermix as demonstrated here, or by usage of a primer and hydrolysis probe mix for alternative markers. Previously, detection of human endogenic retrovirus 3 DNA was suggested to be a suitable candidate for this [28]. Its amplification, however, could also fail in cell-free samples. Therefore, addition of defined *E. coli* T4 phage suspension directly into the patient sample might represent a better solution, and will ensure both, correct nucleic acid extraction as well as exclusion of PCR inhibition [35].

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Compliance with ethical standards

Conflict of interest Biomers, Qiagen and Roche had no influence on interpretation of data and writing of the manuscript. A.K. was an invited speaker at the "Roche Tage 2018". The authors act independently of these companies and declare no further conflict of interest.

Ethical approval This study exclusively included residual patient specimen initially sent for alphaherpesvirus diagnostics. Samples were only used anonymized. For this type of study formal consent is not required. The setting was approved by the Ethics Committee of the Christian-Albrechts-Universität zu Kiel.

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