

Reliable Detection and Quantitation of Viral Nucleic Acids in Oral Fluid: Liquid Phase-Based Sample Collection in Conjunction With Automated and Standardized Molecular Assays

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Oral fluid has been used widely as sample matrix for the detection and quantitation of viral nucleic acids. However, in the vast majority of previous studies, various methods for collection of oral fluid and molecular assays lacking automation and standardization were used. In this study, a new standardized liquid phase-based saliva collection system was employed followed by a fully automated viral nucleic acid extraction and real-time PCR using commercially available in vitro diagnostics (IVD)/Conformité Européene (CE) labeled molecular assays. When the lower limit of detection of herpes simplex virus (HSV)-1/2 DNA, varicella zoster virus (VZV) DNA, and hepatitis C virus (HCV) RNA in spiked oral fluid was tested, the results were found to be comparable to those with defined sample materials recommended by the assay manufacturers. When clinical specimens were investigated, 21 of 25 (84%) oral fluids obtained from patients with clinically apparent herpetic lesions tested positive for HSV DNA, 7 of 10 (70%) oral fluids obtained from patients with Ramsay Hunt Syndrome tested positive for VZV DNA, and 19 of 40 (48%) oral fluids collected from patients with chronic HCV infection tested positive for HCV RNA. The automated extraction instruments completed all extractions without malfunction and no inhibitions were observed throughout the entire study. Liquid phase-based saliva collection in conjunction with automated and standardized commercially available molecular assays allows reliable quantitation of viral nucleic acids in oral fluid samples and may contribute to improved comparable and interpretable test results. *J. Med. Virol.* 80:1684–1688, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: herpes viruses; hepatitis C virus (HCV); PCR; oral fluid; standardization

INTRODUCTION

Testing oral fluid for the presence of viral nucleic acids including herpes simplex virus (HSV), varicella zoster virus (VZV), and hepatitis C virus (HCV) has been reported recently [Furuta et al., 2001, 2005; Rey et al., 2001; Hermida et al., 2002; Dios et al., 2005; Eirea et al., 2005; Goncalves et al., 2005; Kaufman et al., 2005; Lins et al., 2005; Miller et al., 2005; Lock et al., 2006; Pastore et al., 2006]. In the vast majority of those studies, non-standardized sampling methods were carried out including spitting saliva in various collection containers or by using absorption phase-based saliva collection devices. In addition, non-standardized and non-automated home-brewed molecular assays lacking internal controls were employed. Detection rates differed significantly among those studies (0–100%) and thus are not comparable which may lead to false interpretation of test results [Ferreiro et al., 2005].

In this study, oral fluid was collected with a new standardized liquid phase-based saliva collection system. Viral nucleic acids were detected and quantified by commercially available in vitro diagnostic (IVD)/Conformité Européene (CE) labeled molecular assays

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based on fully automated sample preparation and real-time PCR. The lower limit of detection of HSV-1/2 DNA, VZV DNA, and HCV RNA was tested using spiked oral fluid samples followed by investigation of specimens of clinical oral fluid.

METHODS

Study Population and Collection of Oral Fluid Samples

For investigation of the lower limit of detection of HSV-1/2 DNA, VZV DNA, and HCV RNA, oral fluid was collected from four healthy volunteers. Clinical specimens were collected from 25 patients with clinically apparent herpetic lesions, 10 patients with Ramsay Hunt Syndrome, and 40 patients with chronic HCV infection and HCV RNA in serum (HCV genotype 1; no anti-HCV therapy; no concomitant infections; no visible lesions of the oral mucosa). All participants gave written informed consent. The study was approved by the local Ethics Committee, Medical University of Graz.

Oral fluid was collected with the Saliva Collection System (SCS; Greiner Bio-One GmbH, Kremsmünster, Austria). This system includes one tube containing 6 ml of rinsing solution, one tube containing 4 ml of saliva extraction solution (a buffer solution containing tartrazine, a synthetic, water-soluble yellow food dye), one collection beaker, and two 3.5 ml scaled vacuum sample-transfer tubes containing sodium azide in crystalline form as preservative. Immediately before sample collection, the oral cavity was cleaned with the rinsing solution. After emptying the oral cavity, it was rinsed for 2 min with 4 ml of the saliva extraction solution providing a mixture of oral fluid and saliva extraction solution. This mixture was collected by spitting it into the collection beaker and filled under vacuum into the scaled sample-transfer tubes, referred to as "oral fluid sample." The total collection volumes (ml) were recorded by reading off the scales. Sample-transfer tubes containing the oral fluid sample were then centrifuged at 2,200g for 10 min.

Determination of the Saliva Content in Oral Fluid Samples

The saliva extraction solution contains the yellow dye tartrazine which allows determination of the saliva content in oral fluid samples using photometrically the Greiner Bio-One Saliva Quantification Kit (SQK) according to the manufacturer's instructions. As reported recently, 200 μ l of sample were taken and its optical density was determined with a Hitachi 912 (Roche Diagnostics, Mannheim, Germany) analyzer [Raggam et al., 2008]. The saliva content of the oral fluid sample was expressed in vol.-% and was determined prior to viral nucleic acid extraction and real-time PCR.

Preparation of Spiked Oral Fluid Samples

The four oral fluid samples obtained from healthy donors were spiked with HSV DNA Type 1 (HSV-1;

4.1×10^5 copies/ml; Panel Code HSV06-05), HSV DNA Type 2 (HSV-2; 2.8×10^6 copies/ml; Panel Code HSV06-09), VZV DNA (7.7×10^5 copies/ml; Panel Code VZV06REF06) and HCV RNA (4.4×10^6 IU/ml; Panel Code HCV06-02) taken from the Quality Control for Molecular Diagnostics (QCMD) 2006 Proficiency Program panel (<http://www.qcmd.org>) prior to nucleic acid extraction. Dilution series (1.0 log steps for HSV-1, HSV-2, and VZV; 0.5 log steps for HCV) were prepared. Each dilution was analyzed three times on different days.

Molecular Assays

An automated standardized DNA extraction protocol with the MagNA Pure Compact instrument (Roche Applied Science, Mannheim, Germany) and real-time PCR on the IVD-LightCycler 2.0 instrument (Roche Applied Science) were used for quantitation of HSV and VZV DNAs in oral fluid samples. For automated DNA extraction, the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Applied Science) was employed. On the MagNA Pure Compact instrument, the Total NA Plasma 100 400 V 3.1 protocol was chosen; the input volume was 350 μ l of sample plus 5 μ l of the internal control (IC). The elution volume was set to be 50 μ l. For quantitative real-time PCR, CE-labeled assays available commercially, the artus[®] HSV-1/2 LC PCR Kit, and the artus[®] VZV LC PCR Kit (Qiagen, Hilden, Germany) were employed according to the instructions of the manufacturer. Fluorescence curves were analyzed with the LC software (version 4.05). The lower limit of detection of both the HSV-1/2 and the VZV assay is 250 copies/ml.

For quantitation of HCV RNA in oral fluid samples, the IVD/CE-labeled COBAS AmpliPrep/COBAS TaqMan HCV Test (Roche Molecular Systems, Inc., Branchburg, NJ) available commercially was used according to the manufacturer's instructions. This assay is based on automated RNA extraction (1.1 ml input volume) on the COBAS AmpliPrep (Roche Molecular Systems, Inc.) instrument followed by quantitative real-time PCR on the COBAS TaqMan (Roche Molecular Systems, Inc.) analyzer. The lower limit of detection is 15 IU/ml.

RESULTS

Oral fluid sample collection volumes ranged from 6.2 to 7.5 ml, median 6.8 ml, with saliva contents ranging from 44 to 67 vol.-%, median 54 vol.-%. When the lower limit of detection of HSV-1/2 DNA, VZV DNA, and HCV RNA in oral fluid was evaluated, 4.1×10^2 copies/ml for HSV-1 DNA, 2.8×10^2 copies/ml for HSV-2 DNA, 7.7×10^2 copies/ml for VZV DNA, and 1.3×10^2 IU/ml for HCV RNA, could consistently be detected with the molecular assays (Fig. 1a–d). With dilutions containing lower concentrations, the molecular assays produced inconsistent negative and positive results.

A total of 75 clinical samples were tested with the molecular assays. When oral fluid samples obtained from 25 patients with clinically apparent herpetic

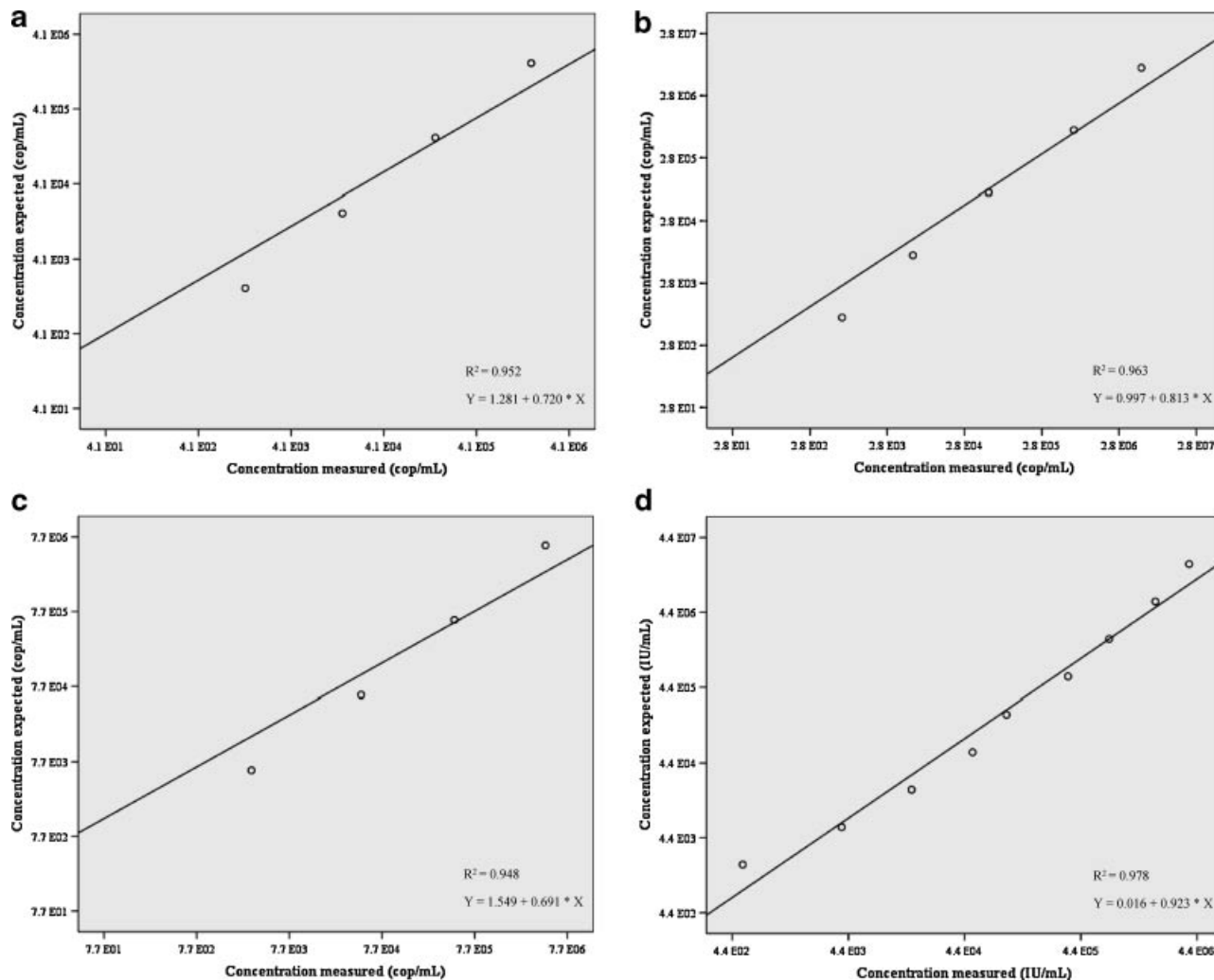


Fig. 1. Dilution series (1.0 log steps for HSV-1 DNA, HSV-2 DNA, and VZV DNA; 0.5 log steps for HCV RNA) were prepared by using four different oral fluid samples obtained from four healthy donors and spiked with known concentrations of HSV-1 DNA (a), HSV-2 DNA (b), VZV DNA (c), and HCV RNA (d) secondary standards. Each dilution of the corresponding virus was analyzed three times on different days; values are expressed in means.

lesions were tested, 21 samples (84%) were found to be positive for HSV DNA; 19 positive for HSV-1 DNA (range, 1.2×10^4 to 2.1×10^5 copies/ml) and 2 positive for HSV-2 DNA (range, 1.4×10^3 to 2.2×10^4 copies/ml). When oral fluid samples were obtained from 10 patients suffering from Ramsay Hunt Syndrome, 7 samples (70%) were found to be positive for VZV (range, 3.3×10^4 to 5.8×10^5 copies/ml). Results were compared to those reported in previous studies (Table I).

Oral fluid samples were collected from 40 patients with chronic HCV infection. Serum HCV RNA levels ranged from 2.7×10^4 to 4.4×10^7 IU/ml. In 19 patients (48%), oral fluid samples tested positive with HCV RNA levels ranging from 7.5×10^2 to 1.8×10^3 IU/ml. No correlation was found between serum HCV RNA levels and those of corresponding oral fluid samples.

DISCUSSION

Recently, a novel standardized liquid phase-based system for oral fluid collection was introduced and evaluated [Raggam et al., 2008]. In contrast to absorption-based collection devices available currently, the SCS is based on liquid phase sampling without a possible matrix-dependent lower yield of nucleic acids [Rogers et al., 2007]. With the SQK, the saliva content (vol.-%) of an oral fluid sample can be calculated accurately prior to viral nucleic acid extraction. Exact recalculation of analytes' concentration in oral fluid samples is thus provided [Raggam et al., 2008]. This saliva collection system was found to provide a suitable matrix for extraction of HSV-1/2 DNA, VZV DNA, and HCV RNA. The automated extraction instruments completed all runs without malfunction. Each of the ICs included in the molecular assays was detected

TABLE I. Studies on the Detection Rates of HSV-1/2 DNA and VZV DNA in Saliva in Comparison to Results Obtained in this Study

References	Molecular assay		Collection method	n	Clinical condition(s)	HSV-1/2 DNA in saliva (%)	VZV DNA in saliva (%)
	Sample preparation	Amplification					
Furuta et al. [2001]	MEP	HBA	NS	56	RHS; ZSH	ND	52–55 ^a
Abiko et al. [2002]	MEP	HBA	SGS	16	BP	31	ND
Da Silva et al. [2005]	MEP	HBA	APB	25	HL	41	ND
Furuta et al. [2005]	MEP	HBA	NS	30	BP	ND	37–53 ^a
Kaufman et al. [2005]	MEP	HBA	APB	50	HA	47	ND
Miller et al. [2005]	MEP	HBA	SPM	125	HA	10	0
This study	FAA	FAA	LPB	35	HL; RHS	84	70

MEP, manual extraction protocol; FAA, fully automated IVD/CE-labeled test system; HBA, home-brewed assay; NS, not specified; SGS, salivary gland saliva; APB, absorption phase-based; SPM, spitting method; LPB, liquid phase-based; RHS, Ramsay Hunt Syndrome; ZSH, Zoster sine herpete; BP, Bell's palsy; HL, herpetic lesions; HA, healthy adults; ND, not determined.

^aDetection rates depending on the different stages of the disease.

throughout the entire study. Together with the lack of PCR inhibition, this indicates a negligible matrix-induced effect for the molecular assay if any.

Automation of molecular assays helps significantly to improve intra- and interassay variation and to reduce the risk of contamination in comparison to those reported for less automated molecular assays [Michelin et al., 2007; Rabenau et al., 2007]. In this study, maximum automated and standardized molecular assays useful for the routine diagnostic laboratory were employed. The inclusion of an IC which is added to the specimen prior to the start of viral nucleic acid extraction has been obligatory for those assays because PCR amplification may fail due to interference from PCR inhibitors. This ensures accurate control of the entire molecular assay avoiding false-negative test results or falsely diminished quantitation results [Rabenau et al., 2007].

Oral fluid samples obtained from healthy donors were spiked with members of QCMD proficiency panels serving as secondary standards for evaluation of the lower limit of detection of HSV-1/2 DNA, VZV DNA, and HCV RNA in oral fluid. Results obtained were found to be comparable to those with defined sample materials recommended by the manufacturers.

When oral fluid samples collected from patients with clinically apparent herpetic lesions and from patients with Ramsay Hunt Syndrome were tested on HSV-1/2 and VZV DNAs, detection rates were found to be higher to those reported recently (Table I). When oral fluid samples were collected from patients with chronic HCV infection and serum HCV RNA was analyzed, almost 50% of the samples tested were found to be positive for HCV RNA. Studies on the presence of HCV-RNA in saliva show an enormous variation of detection rates ranging from 0% to 100% (median, 42.5%) as reviewed recently (Ferreiro et al., 2005). However, in addition to the inferior quality of the molecular assays employed in those studies, results may be biased by several factors including HCV genotype, response to anti-HCV therapy, concomitant infections, and oral health status making comparisons difficult [Dios et al., 2005; Eirea et al., 2005;

Lins et al., 2005; Pastore et al., 2006]. Oral fluid was collected mainly by using non-standardized sampling methods and absorption phase-based saliva collection devices that may be responsible in addition for a decreased recovery of viral nucleic acids; however, this needs to be investigated further. To minimize those factors making comparison and interpretation of results difficult, both well defined inclusion criteria and standardized pre-analytical and analytical procedures should be applied.

In conclusion, collection of oral fluid with this liquid phase-based saliva collection system in conjunction with detection and quantitation of viral nucleic acids employing automated and standardized molecular assays available commercially allows reliable quantitation of viral nucleic acids in oral fluid samples and may contribute to improved comparable and interpretable test results.

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