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Comparison of collection methods for molecular detection of α-herpes viruses and *Treponema pallidum,* including evaluation of critical transportation conditions

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Abstract

The detection of herpes simplex viruses and *Treponemal pallidum* from genital lesions requires efficient sampling of genetic material for a reliable molecular diagnosis. From 460 patients attending the Public Health clinic, two swabs (dry cotton swabs and Eswabs) per patient were collected in alternating order from the same lesion. Additionally, three storage conditions of Eswabs up to 28 days were evaluated to assess the stability of DNA over time. Out of the 830 PCRs performed, 20 (2.4%) PCRs were discordant between the two swabs. No significant differences were observed between the two sample types. HSV1 and HSV2 could be reliably detected from Eswabs up to 28 days when kept at room temperature. A single swab from a genital lesion is sufficient for reliable diagnosis of α-herpes viruses and *Treponemal pallidum*, for which both a dry cotton swab or Eswab could be used.

Keywords: Molecular biology, Infectious disease, Public health

1. Introduction

The majority of genital ulcer diseases are caused by sexually transmitted infections such as herpes simplex viruses type 1 (HSV1), type 2 (HSV2), or *Treponema pallidum* (TP), whereas skin lesions on other parts of the body such as mouth and anus, may also be caused by varicella zoster virus (VZV) [1]. The diagnosis relies on laboratory confirmation due to a range of other potential causative infectious diseases and non-infectious aetiologies [2]. Laboratory confirmation makes use of swabs collected from mucous membrane lesions for PCR based diagnosis, which is common practise in the Netherlands [3].

For a valid diagnosis, efficient sampling is crucial [4]. Several types of swabs have been validated for PCR based diagnosis of genital ulcer diseases, among which dry cotton swabs and plastic swabs stored in media (amies) after sample collection for cultivation-based diagnostics.

Dry cotton swabs are reliable during transportation from patient to laboratory, as little to no break-down of nucleic acids occurs [5]. A sample collection method commonly used for cultivation-based diagnostics, the Eswab, can also be used for infectious disease molecular diagnostics. This potentially simplifies sample collection and downstream laboratory processes as only a single collection method for various diagnostic tests can be used. It was stated by the company (Copan diagnostics) that this swab would improve detection for various pathogens, but published data on this claim is scarce. The Eswab contains a nylon-flocked brush with an unknown efficient uptake of pathogenic material from lesions. Additionally, the brush is stored in amies medium, potentially affecting DNA stability and integrity during transportation at ambient (i.e. uncontrolled) temperatures.

In this study we compared the performance of two collection methods for the diagnosis of HSV, VZV and TP by PCR and in addition we assessed the stability of both herpes simplex types 1 and 2 in Eswab medium in time after collection and at different storage temperatures to mimic transport conditions before arrival in the laboratory.

2. Materials & methods

2.1. Clinical setting

Routine samples from patients attending the Sexually Transmitted Infections (STI) Public Health service of Amsterdam (GGD Amsterdam) with clinical suspicion of lesions caused by infectious pathogens were collected from October 2017 till June 2018. Sample collection was divided in three periods of 3 months each, with intermittent evaluations to ensure high quality diagnostic performance. The routine

clinical practise at the Public Health institute was to collect two swabs (dry cotton swabs) per patient for the diagnosis of lesions caused by α-herpes viruses and TP (study period 1). During period 2 and 3, an alternative swab (Eswab, Copan Diagnostics, California, USA) was introduced containing Amies buffer (modified Stuart buffer). As this study was a methodological assessment and part of an on-going effort to improve our diagnostic services, ethical approval was not necessary. No inclusion criteria based on patient characteristics was applied in this study.

2.2. Sampling technique

During STI consultation in case of ulcerative diseases, a dermatologist collected material directly from the lesion for dark-field microscopy for detection of *Treponema pallidum,* and for Tzanck smears for the detection of herpes simplex 1 and 2. In addition two swabs were collected from the same lesion. During the study four dermatologists and two supervisors were instructed to register the order of collection according to the study protocol for that period. Swabs were kept at room temperature until transportation (within 4 hours) to the laboratory (situated in the same building) for analyses the next working day.

2.3. Laboratory methods

All labelled swabs collected for routine diagnostics at the laboratory were processed for two PCR reactions, a TP PCR and a multiplex α-herpes virus PCR, detecting HSV-1, HSV-2 and VZV [6]. Both PCR's used Phocine herpes virus (PhHv) as internal control. A patient was positively diagnosed when at least one of the two swabs were positive by PCR. Discrepant results were not further analysed by repeat testing.

Storage conditions of Eswab samples at -80 °C (reference temperature at which nucleic acid degradation is minimal), 4 °C (4^0C- 8 °C), and 21 °C (18^0C-22 °C) were assessed by selecting HSV1 and HSV2 positive Eswabs. These were aliquoted for five timepoints (Day 0, 3, 7, 14, and day 28) at three different temperatures (15 tubes each per original sample).

For the extraction of nucleic acids, a quick protocol was used. In brief, dry cotton swabs were hydrated in 600 μl PBS and shaken for 30 minutes (plate shaker). Next, the patient sample was disintegrated by incubation of 30 μl of the eluted PBS at 95 °C for 15 minutes. Eswabs were briefly vortexed, 30 μl pipetted and disintegrated at 95 °C for 15 minutes. Both Eswab and cotton swab sample liquids were briefly centrifuged ($\sim$ 10 seconds, 5.000 RPM) and 3 μl (α-herpes) or 4 μl (TP) was used per PCR reaction.

Primers and probes were used as described previously by van Doornum *et al.* for the α-herpes PCR assay (HSV-1, HSV-2 and VZV multiplex) [6]. For the TP assay

primers and probe was used as described by Heymans *et al* [7]. A real-time multiplex PCR assay was performed using 2X Platinum Q-PCR supermix (Invitrogen, USA) on a RotorGene (Qiagen, Hilden, Germany) PCR platform. A 20 µl PCR reaction with 3 µl isolated DNA sample, 5 ng/µl per primer, 2.5 ng/µl probe, 1 µl 50 mM $MgCl_2$, and 2.5 µl water was used. The same conditions and mastermix were also used for TP PCR except for a larger sample input of 4 µl.

2.4. Statistics

Mean grouped comparisons of Ct values were performed using Wilcoxon rank sum test. Paired nominal data (# of matching results between swabs) were tested using McNemar test with $p < 0.05$ as significant. All analyses were done using R (version 3.3.3).

2.5. Ethics

According to the Dutch Medical Research Involving Human Subjects Act no additional ethical approval was required for this study, as the samples used here were collected as part of routine procedures and only tested for clinically relevant targets.

3. Results

3.1. Storage conditions of clinical samples

To assess the stability of nucleic acids in Eswab medium over prolonged periods of time, 14 HSV1 and 14 HSV2 positive swabs were aliquoted and stored at -80 °C, 4 °C, and 21 °C and tested up to 28 days after collection (see methods). Large variance in Ct values for HSV1 and HSV2 was observed at all time points and all storage conditions (Fig. 1). Large variance was even observed when Eswabs were stored at -80 °C, which we assumed to be the most stable storage condition available in most laboratories. No significant trends in DNA presence (both in terms of increase or decrease) was observed at any storage condition over time, suggesting limited influence of time and temperature on HSV-1 and HSV-2 stability in the Eswab samples (Fig. 1).

3.2. Sampling effects and validity of one sample per patient

During a period of eight months, routine diagnostic swabs from a total of 460 patients who attended the STI clinic in Amsterdam with clinical signs of genital lesions were included in the study. Of the 460 samples, 370 (80.4%) were tested for both α-herpes viruses and TP, 61 (13.2%) were only tested for α-herpes viruses (HSV1,HSV2, VZV), and 29 (6.3%) only for TP.
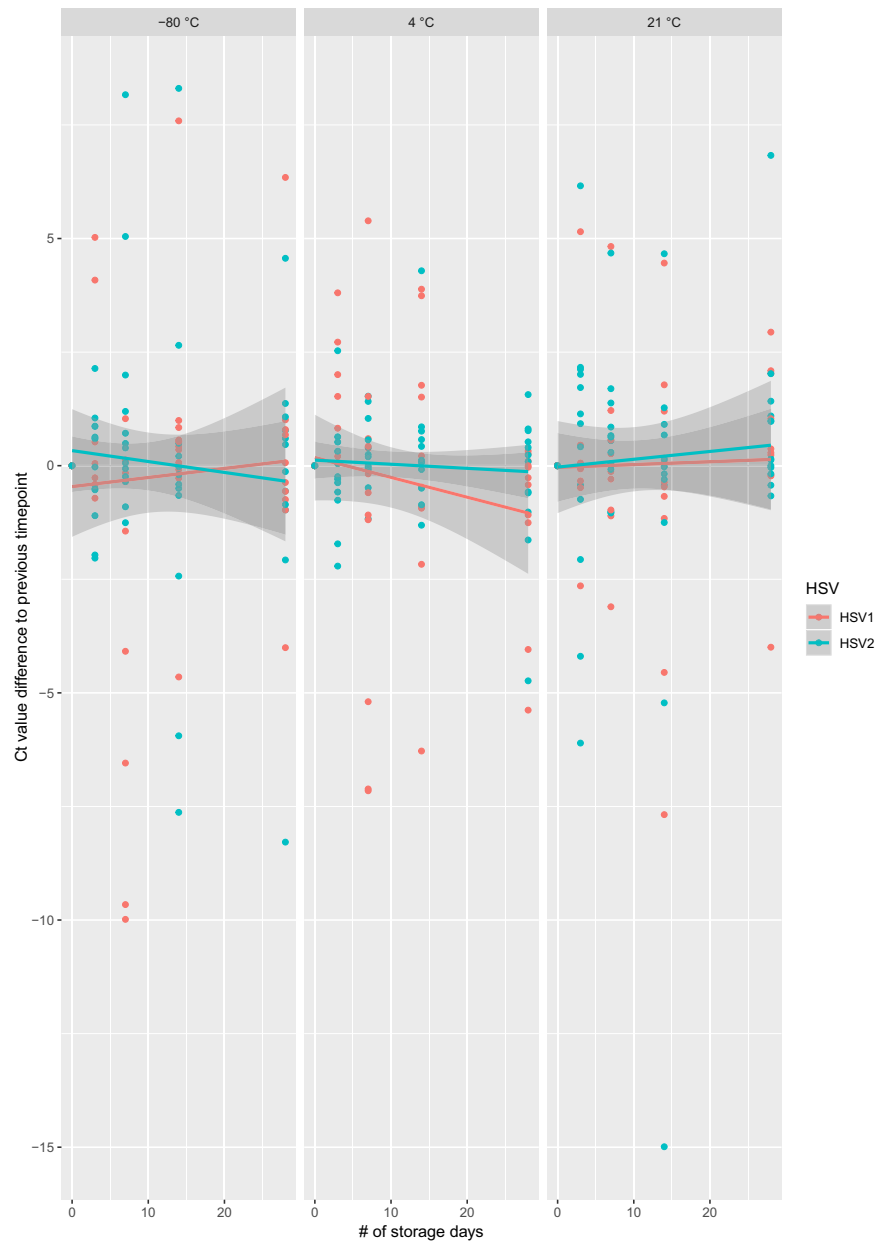
Fig. 1. HSV-1 and HSV-2 plot, fitted linear model. Data show difference in Ct value per patient to previous sampling moment. Grey zone is standard error. Declining Ct value difference means increase of DNA over time.

To assess the methodological reliability of sampling lesions for the detection of HSV, VZV and TP, two swabs from a single lesion were collected per patient. Out of the 460 samples, 93 HSV1 (20%), 115 HSV2 (25%), 4 VZV (1%) and 96 TP (24%) positives were detected. Eight double positives (2%) were noted, of which two with HSV1 and TP double positives, and six with HSV2 and TP double positives.

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Table 1. Overview of samples and number of positive (HSV1, HSV2, VZV, TP) results per study period.

Study period characteristics				First vs second swab		Positive test results (%)*				
Period	First swab	Second swab	# of samples	discordant (neg-pos)	discordant (pos-neg)	HSV1	HSV2	VZV	TP	NEG
1	Dry cotton swab	Dry cotton swab	179	3	7	37 (20.7)	43 (24.0)	1 (0.6)	39 (21.8)	61 (34.1)
2	Dry cotton swab	E-swab	154	3	1	35 (22.7)	34 (22.1)	2 (1.3)	25 (16.2)	60 (39.0)
3	E-swab	Dry cotton swab	127	1	5	21 (16.5)	38 (29.9)	1 (0.8)	32 (25.2)	39 (30.7)
Total			460		20	93	115	4	96	160

*Total numbers do not add up due to double infections.

The study was performed over three calendar periods, each with alternating combinations of swab collection methods (Table 1). The first period represented the standard diagnostic methodology.

3.3. Reliability of sampling

Out of the 830 PCR tests performed (431 α-herpes viruses + 399 TP), 810 (97.6%) had the same results for the two consecutively taken swabs from the same genital lesion. Twenty samples (2.4% of total) gave discordant results of which 13 (65%) had a negative second swab and seven a negative first swab. All 20 discordant samples were equally distributed over the three pathogens (6 or 7 discrepancies per pathogen). No statistical difference was observed between the first and second swab overall (or per individual study period), suggesting one swab was sufficient to reliably diagnose α-herpes viruses and TP in the STI clinic attending population (McNemar 0.9, p = 0.343).

3.4. Comparison of two types of swabs for sampling the same lesion

Two swab types (dry cotton and Eswab) were compared for sampling of α-herpes viruses or TP genetic material from lesions. Ct values (as proxy for DNA load) of each paired swab per patient showed minimal variations as visualised in Fig. 2. As could be expected, when a cotton swab was used first (first and second study period), the highest amounts of DNA were detected in the first swab compared to the second swab regardless of the type of swab used as second (Wilcoxon (7204,
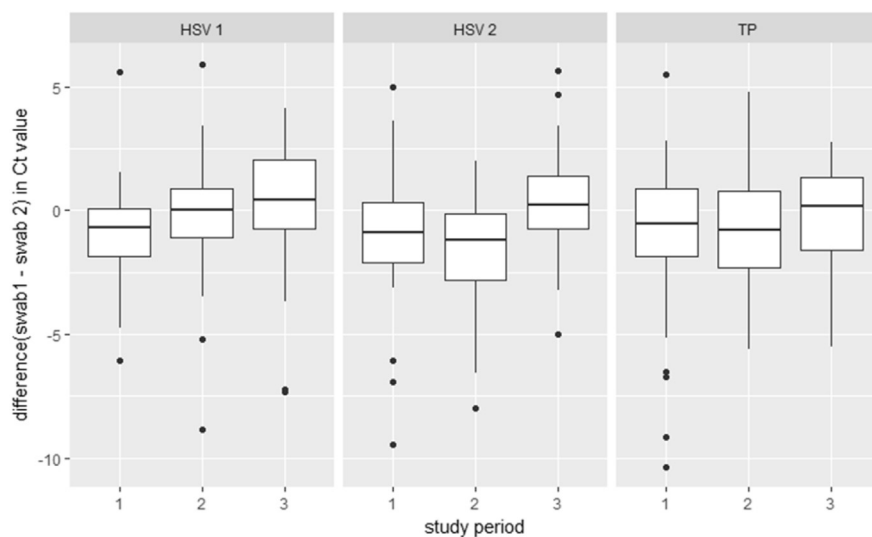

Fig. 2. Boxplots showing the median, 25th and 75th percentile of swab difference in Ct values per target (HSV,TP), and per study period. Black dots are outliers. VZV was excluded due to limited number of positives (n = 4). CS = cotton swab, ES = Eswab. Period 1: CS-CS, period 2: CS-ES, period 3: ES-CS.

p = <0.005). When E-swab was used as first swab (third study period), lower DNA loads were found in the first swab (higher Ct values) compared to the second swab (dry cotton swab), suggesting a more efficient uptake and release of pathogen DNA by dry cotton swabs (Wilcoxon rank sum (0.003 (period 1 VS 3), 0.016 (2 VS 3)). There is no statistical difference between HSV1 and HSV2 uptake between the first and second swab (Wilcoxon rank sum, 0.18).

4. Discussion

Reliable detection of STI's from genital lesions depends on the sampling methodology with sufficient genetic material uptake and release in buffer for high-quality PCR based diagnostics. In this study we compared the performance of collection methods for the diagnosis of α-herpes viruses and primary syphilis by PCR and we assessed the stability of HSV1 and HSV2 in Eswab medium.

When assessing the effects of temperature and time on DNA stability of Eswab samples, critical conditions were evaluated. Variation in Ct value over time was large, unrelated to temperature conditions nor to duration of storage. This substantiates that Eswabs are a valid sampling methodology for herpes viruses and TP PCR diagnostics. The outliers that occurred at all temperatures and time points were mostly due to high Ct values (Ct > 33) (data not shown). This variation may be due to manual nucleic acid extraction methods performed by multiple technicians using a lab developed (non-automated) test.

Our results indicate that a single swab from a genital lesion was sufficient for reliable diagnosis, in which both a dry cotton swab or Eswab could be used. Data suggest that there is a slight benefit of using dry cotton swabs over Eswabs, as uptake from lesion and release of genetic material in PBS buffer seems to be slightly better based on Ct value estimates (Fig. 2). However, in qualitative detection of herpes viruses and TP we did not observe a difference as to which swab was used since there were only few discrepant outcomes (2.4%).

A limitation of this study was that swabs were collected by trained STI clinic staff, so no self-collected samples were tested. The results can therefore not be directly translated to studies or settings where samples are self-collected by patients. In other studies concerning the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoea* from self-collected samples provided as good or even better results than clinician based collection [8]. Another limitation of this study is that only HSV1 and HSV2 stability in Eswab medium were assessed and not that of TP. Previous preliminary experiments indicated that TP DNA is also rather stable for weeks at room temperature (not shown). In addition, only the most critical parameters for degradation of nucleic acids, temperature and time, were assessed as transportation variablesIn the evaluation we assumed that transportation would take place in appropriate

medical shipping material, limiting the effects of most other external factors, such as UV light or external humidity.

To conclude, the Eswab and dry cotton swab are both valid sampling methods that can be reliably send to the laboratory at room temperature for up to one month after collection, for laboratory diagnostics of herpes viruses and primary syphilis from genital lesions.

Declarations

Author contribution statement

Pieter Smit: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Titia Heijman, Sylvia Bruisten: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Meriem el Abdallaoui: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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