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SHORT COMMUNICATION

Efficacy of sample collection without virus transport medium in suspected enteroviral infections for molecular diagnosis

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ABSTRACT
Clinical swabs with suspected viral infection are usually transported in virus transport medium (VMT). During epidemics/pandemics, tampons without VTM would be more suitable for saving space and cost. This study was conducted to verify the applicability of throat swabs without VTM in the diagnosis/screening of enteroviral infections by polymerase chain reaction (PCR) in a volunteer study group. Three different swab types were used in 40 volunteers: swabs with two different tips (cotton- or synthetic-tipped) without VTM and standard synthetic tips with VTM. The swabs were processed immediately or after 12 days of storage at either –80°C or +4°C. The molecular analysis included viral RNA extraction, and combination of reverse transcriptase PCR and nested PCR. Enteroviral RNA was detected in 15% (6/40) of the studied volunteers. When processed immediately, the results for all three swab types were compatible. Swabs without VTM may be used for collection of clinical samples in the diagnosis of suspected enteroviral infections or as potential screening tools for enteroviruses *(Tab. 2, Ref. 15).* Text in PDF *www.elis.sk*
KEY WORDS: enterovirus infection, swab, transport medium, PCR, molecular diagnostics.

Introduction

Enteroviruses (EVs) are small non-enveloped viruses composed of single-stranded RNA. These viruses spread via the fecal-oral route. These human pathogens infect both children and adults with an increased incidence of infection in summer and early autumn. The monitoring results in the Slovak Republic (1–3) of human EVs (before and after changes of the polio vaccination strategy) show that the non-polio EVs and coxsackievirus B5 are circulating, and echovirus 3 and 11 have been endemic. Clinical manifestations of EV infections vary from asymptomatic to serious and also include chronic illnesses (4, 5). EVs have a wide range of organ tropism and may infect different organs after the viremic phase. They are also associated with severe conditions such as paralytic poliomyelitis, flaccid paralysis, aseptic meningitis, herpangina, hand-foot-and-mouth disease, acute hemorrhagic conjunctivitis, exanthemas, myocarditis and fulminant diseases of neonates (6). The diagnosis of enteroviral infections is based on direct and indirect evidence of the etiological agent. The "gold standard" of virus isolation and identification in tissue cultures with supportive serological methods is demanding and time-consuming. Currently, rapid molecular diagnostic tests are frequently used. Swab collections for virus isolations utilize swabs inserted in a virus transport medium (VTM). The VTM provides appropriate conditions for maintaining the viability of viruses during sample transport. Maintaining the virus viability is necessary for the virus isolation and identification process. Presently, the transport of clinical specimens requires VTM, with consequent transport or storage problems due to needed space and weight of the specimen tubes. To prove the presence of viral nucleic acid by molecular techniques, the stability of the genome is essential. Only a few authors have successfully tested the applicability of dry swabs in the molecular identification of several viruses (7–9). We have standardized the dry/frozen swab method (without VTM) and have shown that swabs without VTM can be applied for detection of EVs (10). The present study was designed to test the applicability of a standardized PCR method that used dry/frozen swabs (without VTM) for collection of clinical samples from 40 volunteer subjects. Our aim is to compare the detection rate of the EV-RNA from the oropharyngeal swabs of volunteers using cotton and synthetic swabs without VTM and a classical synthetic swab with VTM.

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Materials and methods

Forty volunteers (aged 25–60), employees of the Slovak Medical University (SMU), were enrolled in the present study. After getting the Institutional Ethics Committee permission and signing the consent forms, the swabs were taken from participants of the study in the early morning on an empty stomach (to ensure uniformity of collection) using an oropharyngeal swab. Three different types of sterile swabs were included and compared: two swab types without VTM in which one type had a synthetic tip with a synthetic stick applicator (Copan, Brescia, Italy) and the other type had a cotton tip with a wooden stick applicator (Greiner Bio-One GmbH, Frickenhausen, Germany); the third swab type was a classical standard swab with VTM (synthetic tip with synthetic stick applicator, Copan). Samples were tested in parallel. After collection of the sample, the swabs without VTM were transferred into sterile tubes, one per swab. Six swabs from each of the set of three swab-types per person were collected (total of 18 swabs/person). For each given swab type, one pair of swabs/person was processed immediately, the second pair/person was stored in a refrigerator (+4°C), and the third pair/person was frozen at –80°C. Stored swabs were processed 12 days later. Prior to processing, swabs from the VTM were transferred to new empty tubes.

The processing procedures were the same for all swab-types used. To these swabs in tubes, 500 µl of RNase-free water was added and vortexed 3 times for 10 sec. After a 10 min incubation period at ambient temperature, the swabs were squeezed out and discarded. The liquid suspension was frozen at -80°C until use, except for those processed immediately.

Viral RNA was extracted from 100 µl of liquid using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, California, USA) and end step eluted to 50 µl of RNase-free water according to manufacturer´s instructions. The purified RNA was used immediately or stored at –80°C until analysis. The presence of the EV genome was determined by in-house two-step method (combination of PCR with reverse transcriptase and nested PCR) according to de Leeuw et al (11) and further modified in our laboratory (12). Primers directed to the highly conserved sequences in the 5' untranslated region of the EV genome obtained from Microsynth AG (Balgach, Switzerland). PCR reactions were performed in DNA Engine Pletier Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The EV-mRNA was reverse transcribed to cDNA. The cDNA amplification reverse-transcription-polymerase chain reaction (RT-PCR) was carried out in a single tube method by SuperScript III One-Step RT-PCR System with Platinum Taq HiFi Kit (Invitrogen, Carlsbad, California, USA) using primers 41-1 and 41-2 (Tab. 1). Totally, 5 µl of extracted RNA was added to 20 µl of RT-PCR mastermix in each tube. Reaction conditions were set as follows: cDNA synthesis at 45°C for 30 min, pre-denaturation at 94°C for 2 min, 40 cycles of amplification (denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 1 min) and finally extended at 68°C for 5 min and cooling at 4°C.

For the nested PCR reaction, we used the Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, California, USA) and primers 17-1 and 17-2 (Tab. 1). In total, 2.5 µl of RT products were transferred to a 22.5 µl nested PCR mastermix in individual tubes. The amplification conditions were set as follows: pre-denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min followed by cooling at 4°C. Positive and negative controls as well as water blank controls were used in each run while for internal control, beta-actin was used.

The PCR products (477 bp length for RT-PCR and 178 bp length for nested PCR) were separated in 2% agarose gel (in 0.5x TBE) with ethidium bromide (0.01%) and analyzed in horizontal electrophoresis (Bio-Rad Laboratories, Hercules, California, USA). Electrophoresis run at 150 V, 85 mA for 1.5 hours and products were visualized and documented under UV light in a Gel Doc XR+ imaging system (Bio-Rad Laboratories). Processing and storage of the volunteer samples were carried out in laboratories allocated for processing of clinical samples at our National Reference Center (Enterovirus Laboratory, SMU, Bratislava, Slovakia).

Results

The results were evaluated as positive if at least one swab of the pair examined showed PCR positivity. Enteroviral RNA was found in the throat swabs of 6/40 (15%) volunteers (Tab. 2). Three of the six PCR-positive volunteers had clinical symptoms of cold/discomfort during the sampling time and one volunteer later developed symptoms identified as hand-foot-and-mouth disease. The other two PCR-positive volunteers were asymptomatic. Other volunteers did not report any clinical symptoms.

Immediate processing of the samples was the most effective method for all the compared types of swabs (Tab. 2). In one volunteer (#4), stored samples were EV-RNA-negative (at +4°C and –80°C). Although, when the samples were immediately processed, EV-RNA was detectable in only one swab of two paired samples. We therefore conclude that the concentration of the virus in the initial sample was low. In stored samples in another volunteer (#1), only the frozen samples with cotton tips without VTM and classic synthetic swabs with VTM were EV-RNA-positive. The comparison of storage of the swabs specifically without VTM (synthetic and cotton) showed that synthetic swabs that were stored in the refrigerator showed rela-

Table 1. Primers used for RT- and nested PCR detection of EV

Primers		Sequence 5´– 3´	Location at base pairs (bp)
41-1*	forward	CAAGCACTTCTGTTTCCCCGG	165–185
42-2*	reverse	CACCGGATGGCCAATCCA	625–642
17-1**	forward	GCTAGAATTCCAGTCCTCCGGCCCCTGAATG	433–462
17-2**	reverse	AACAATGGATCCATTGTCACCATAAGCAGCCA	580–611
β1***	forward	ATCATGTTTGAGACCTCCAA	424–443
β2***	reverse	CATCTCTTGCTCGAAGTCCA	723–742

* Primers for RT-PCR reaction; ** primers for nested PCR reaction; *** primers for beta-actin

tively better results as compared to the other swabs (but differences were minimal), while cotton swabs showed better results after deep freezing. These results confirmed our unpublished pilot volunteer trial at an international meeting where 16.22% of all 37 participants from different countries showed presence of EV-RNA when samples were processed immediately.

Table 2. Presence of enteroviral RNA in the PCR-positive volunteers after different storage times

	Swabs processed immediately			Swabs processed after 12 days					
				Stored at –80°C			Stored at +4°C		
Volunteer*	CO	SY	CL	CO	SY	CL	CO	SY	CL
1	++*	++	++	++	—	++	—	—	—
2	++	++	++	++	++	++	–+	++	++
3	++	++	++	++	++	++	—	+–	++
4	+–	–+	+–	—	—	—	—	—	—
5	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	+–	–+	+–

*Six volunteers positive for EV-RNA; VTM = virus transport medium; CO = cotton swab without VTM; SY = synthetic swab without VTM; CL = classic synthetic swab with VTM; the swabs were investigated in parallel; "++" = EV-RNA detected in both swabs; "—" = EV-RNA not detected in any of the swabs; "+–" or "–+" = EV-RNA detected only in one of the two swabs

Discussion

Based on our previous standardization procedures (10), we suggest that the positivity after long-term storage depends on high concentration of the virus, which indicates that a high viral load in the volunteer/patient swab at the time of sampling is necessary to ensure successful long-term storage of positive swabs. Instructions from the World Health Organization and other data (13–15) do not recommend the use of swabs with cotton tips and wooden applicator, as they may contain substances that inactivate some viruses and inhibit PCR testing. Our results demonstrate that the patient samples should be either processed immediately or frozen if transport to a diagnostic laboratory is delayed. The samples may be stored for a few days (1–6 days, unpublished data) in the refrigerator at +4°C and then transported on dry ice. If EV infection is suspected, a throat swab is preferable to a buccal swab because in a small pilot study (unpublished data), we detected the presence of EV-RNA in throat swabs using cotton swabs without VTM in 7/10 volunteers (some showed mild fever and cold), while saliva positivity was determined in only two participants who showed signs of viral infection (unpublished data).

Our in-house method, which is a nested PCR reaction, is a highly specific and sensitive method for the detection of EVs (15). The limitation is that the primers cover the highly conserved region in the 5′ untranslated region of EV-RNA, thus detect most of EV species except for EV-D68, EV-A71 and parechoviruses.

Transporting a dry swab to the laboratory for virus diagnosis using molecular methods is a suitable and robust alternative to traditional sample types. This study was carried out to confirm the actual feasibility and applicability of the dry swab method (without VTM) in humans for the diagnosis of EV infection. The limitation of this study lies in a relatively small number of volunteers and in the fact that we have checked for enterovirus positivity by PCR without the tissue culture virus identification or further sequencing to identify the EVs. However, our subjective was to prove the usability and suitability of dry tampons in the molecular diagnosis of EVs. Because the prevalence of EVs in humans is relatively high and infections caused by them often go unnoticed (4–5), we expected to detect a certain percentage of EVs in the oropharynx of adults. Another limitation stems from the fact that our aim was to study and compare the use of the different types of swabs and VTM and the detection of viral RNA, we did not perform quantitative analysis. The selection of volunteers was random, with no evidence of increased incidence of EV infections at the time of sampling.

The outcome of this study demonstrates the feasibility of storage of the patient swabs without VTM in a standard freezer or refrigerator for use of enterovirus diagnostics. When placing patient samples in the refrigerator, we recommend using synthetic swabs, specifically for a period no longer than six days, as after this interval the amount of RNA in the sample begins to decrease (unpublished data). In patient-care facilities, this method has a potential to reduce both the storage space needed and the stress stemming from the necessity of rapid transport to the diagnostic laboratory. In addition, the use of non-VTM swabs could reduce the costs involved due to simplicity of collection, storage and mass transport of samples to the diagnostic laboratory. Swabs without VTM would be useful, especially during a pandemic when supplies of VTM can be scarce.

Conclusions

We conclude that the cotton or synthetic swabs without VTM are both efficient, and could be utilized in screening, surveillance, and routine sample collection for the diagnosis of EV infections via molecular technique. Immediate treatment or freezing of dry throat swabs is recommended, but it is possible to use an alternative method of short-term storage at + 4°C and use of dry ice during transport to the diagnostic laboratory.

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