COMPARISON OF VIRUS ISOLATION AND VARIOUS POLYMERASE CHAIN REACTION METHODS IN THE DIAGNOSIS OF MUCOCUTANEOUS HERPESVIRUS INFECTION

M.L. NOGUEIRA, J.B. AMORIM, J.G. OLIVEIRA, C.A. BONJARDIM, P.C.P. FERREIRA, E.G. KROON*

Laboratório de Vírus, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais Av. Antonio Carlos, 6627 Belo Horizonte, MG, 31270-901 Brazil

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Summary. – We compared two polymerase chain reaction (PCR) assays (simple and multiplex) and viral isolation to detect herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) in 15 clinical specimens from 13 patients with mucocutaneous herpetic infections. HSV-1 or VZV DNA was detected in 13 specimens by simple PCRs (HSV-1 or VZV PCR) and in 12 specimens by multiplex PCR. On the other hand, viral isolation was positive for 9 specimens only. The PCR protocols used in this study are not only more sensitive and faster than the traditional viral isolation and conventional PCR protocols but also can distinguish rapidly HSV-1 from VZV. We propose the PCRs described here for rapid and precise identification of etiological agents of mucocutaneous herpetic infections.

Key words: HSV-1; VZV; PCR; virus isolation; mucocutaneous herpetic infections; diagnostics

Introduction

Some members of the *Alphaherpesvirus* subfamily are causative agents of human mucocutaneous herpetic infections. They are HSV-1 (human herpesvirus 1), HSV-2 (human herpesvirus 2) and VZV-1. They are characteristic by their ability to cause infection of mucocutaneous tissues and latency in the central nervous system. Clinical manifestations occur usually in mucocutaneous tissues or in the nervous system (Roizman, 1990).

The diagnosis of mucocutaneous herpetic infections is usually based on clinical manifestations. However, in immunocompromised hosts, the disease can be atypical by affecting different sites and inducing resistance to treatment. A differential diagnosis between HSV and VZV infections is necessary as they present different susceptibility to acyclovir. Moreover, a suboptimal therapy with acyclovir may lead to resistant viruses (O'Brien and Campoli-Richards, 1989).

Rubben *et al.* (1997) have showed that initial zoster manifestations often mimic HSV-1 infections and that is why a routine complementary assay should be used to ensure a correct diagnosis of mucocutaneous herpetic infections.

A number of methods, such as Tzank smear, viral isolation and virus DNA amplification by PCR, can be used in complementary diagnostics of herpetic infections (Ashley, 1995). The Tzank smear, a cytopathological procedure, is simple, rapid, effective, and cheap but is dependent on prior training and its correct interpretation is based on the skill of the physician. Under ideal conditions, the detection rate of the Tzank smear is 50% for HSV-1 and 80% for VZV, as reported by Salomom *et al.* (1986). However, its major disadvantage is that it cannot discriminate between HSV-1 and VZV infections.

*Corresponding author. E-mail: kroone@mono.icb.ufmg.br; fax: +5531-4436482.

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HCMV = human cytomegalovirus; HSV-1 = herpes simplex virus 1; HHV-6 = human herpesvirus 6; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; VZV = varicella-zoster virus

Table 1. Clinical data of patients

Patient	Lesion type	Gender	Age (years)	Associated disease	Days[b]
EK	Blister	F	42	ND	1
CAT	Varicella	M	8	ND	6
OF	Facial zoster and keratitis	F	85	ND	5
SC01	Facial zoster and keratitis	F	70	ND	10
SC05	Keratitis and blefaritis	F	7	ND	3
HC01	Whitlow	M	23	AIDS	30
HC02[a]	Whitlow	M	23	AIDS	35
HC03[a]	Whitlow	M	23	AIDS	40
SC02[a]	Keratitis	M	54	ND	5
SC03[a]	Keratitis	F	40	ND	4
HC05	Shingles	F	18	Leukemia	8
CMO01	Keratitis	M	11	ND	1
LV1	Stomatitis	F	21	ND	2
LV2	Blister	M	26	ND	1
GI	Blister	F	24	ND	1

[a]Specimens collected after starting antiviral therapy.
[b]Days of duration of clinical lesions from which swabs were taken.
ND = no associated disease detected.

Virus isolation is usually the "gold-standard" method of complementary diagnostics of herpetic infections (Thomas *et al.*, 1994; Slomka *et al.*, 1998). However, its results are highly variable, depending on the cell line used, and it also requires special technical procedures, which are often not available in clinical laboratories. These disadvantages make another approach to identify the virus a real need (Ashely, 1995).

PCR has been used for the diagnosis of several diseases, especially infectious ones. The method is simple, rapid, sensitive, and specific, presenting several advantages when compared with conventional methods (Cewley, 1995). Several reports have described the use of PCR in the diagnostics of viral diseases, especially herpesvirus infections. In the case of mucocutaneous herpetic infections, the use of PCR is as efficient as or even more than the virus isolation or Tzank smear. Several authors have used specimens from swabs, vesicles, crusts, archival tissues and virological cultures with a step with DNA extraction with organic reagents in their protocols (Kido *et al.*, 1991; Thomas *et al.*, 1994, Nahass *et al.*, 1992, 1995).

Recently (Nogueira *et al.*, 1998a), we have described a rapid PCR protocol for the diagnosis of mucocutaneous herpetic infections in which the DNA extraction step with organic reagents was omitted . This simplified approach presents some advantages, such as rapidness, elimination of DNA contamination of specimens due to multiple handling, and no requirement of organic reagents that could inhibit the assay.

In this study, we used this simplified protocol to compare "simple" and "multiplex" PCR procedures with the virus isolation in the diagnosis of HSV-1 or VZV in mucocutaneous herpetic infections.

Materials and Methods

Cells. Vero cells were obtained from ATCC and MRC-5 cells (human pulmonary diploid fibroblasts) were from the Banco de Células, Fundação Ezequiel Dias (FUNED), Belo Horizonte. These cells were cultured at 37°C in Eagle's Minimum Essential Medium (MEM, Sigma) supplemented with 5% or 10% of fetal calf serum (FUNED) in a 5% CO_2 atmosphere.

Specimens. We examined 15 clinical specimens from 13 patients with mucocutaneous herpetic infections. The specimens were collected at the onset of the antiviral therapy unless other wise stated. The patient lesions were cleaned with sterile saline and sterile swab cotton was rubbed onto the base of the ulcers. The swabs were then immersed in 1 ml of MEM containing 1% fetal bovine serum, penicillin, amikacin, and amphotericin B. The specimens were transported on ice and stored at -70°C until analysis. The clinical data of the patients are summarized in Table 1.

Virus isolation was performed according to Nahass *et al.* (1992). Briefly, 100 µl of the clinical specimens was inoculated into a MRC-5 or Vero cell culture in a 25 cm^3 flask , the virus was left to adsorb for 1 hr and then the medium was replaced. The culture was monitored daily until cytophatic effect (CPE) appeared or at most for 14 days. An aliquot (10 µl) from cultures with CPE was collected, diluted and subjected to PCR.

HSV-1 PCR was performed according to Nogueira et al. (1998a). Briefly, a 10-fold dilution of the clinical specimen was heated at 100°C for 10 mins and an 8 µl aliqout was used in PCR. The reaction mixture (20 µl) contained 2 mmol/l $MgCl_2$, 1 mmol/l dNTPs, 1% glycerol, 1 U of Taq polymerase (Promega), a PCR buffer, and 10 pmoles of each primer (HSV1TK-3', 5'-TCAGTTAGCCTCCCCCATC-3', nt 46672–46690, and HSV1TK-5', 5'-ATGGCTTCGTACCCCTGCC-3', nt 47802–47784). These primers amplified only the HSV-1 DNA but not the HSV-2, VZV, human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6) DNAs (Nogueira *et al.*, 1998a; Nogueira and Kroon, unpublished data). PCR amplification was carried out as follows: 35 cycles of 1 min at 94°C, 55°C and 72°C, and 1 cycle of 15 mins at 72°C. The expected amplified DNA had 1130 bp and corresponded to the TK gene of HSV-1. It was subjected to electrophoresis in 1% agarose gel and staining with ethidium bromide.

VZV PCR was performed similarly to HSV-1 PCR, just different primers were used (VZVTK-3', 5'-AGGAAGTGTTGT CCTGAACGGC-3', nt 65830–65807, and VZVTK-5', 5'-ATGTCAACGGATAAAACCGATGT-3', nt 64806–64829). These primers amplified only the VZV DNA but not the HSV-1, HSV-2, HCMV and HHV-6 DNAs (Nogueira *et al.*, 1998a; Nogueira and Kroon, unpublished data). PCR amplification was carried out as follows: 35 cycles of 1 min at 94°C, 57°C and 72°C, and 1 cycle of 15 mins at 72°C. The expected amplified DNA had 1025 bp and corresponded to the TK gene of VZV. It was visualized in the same way as described above.

Multiplex PCR was performed in the same way as the simple PCRs described above except all the 4 primers were used in the

same reaction. The amplification cycles were the same as those in the HSV-1 PCR. The PCR products were electrophoresed in 1.5% agarose gel. PCR products of 1130 bp and 1025 bp were obtained for HSV-1 and VZV, respectively.

Results

We analyzed 15 specimens from 13 patients with mucocutaneous herpetic infections (Table 1).

We were able to isolate the viruses from 9 of the 15 specimens. From 2 of the positive specimens (specimens CAT and SC05), we isolated the virus in both MRC-5 and Vero cells. We isolated HSV-1 or VZV from 6 and 5 specimens in MRC-5 and Vero cells, respectively. After detection of CPE, the cultures were harvested, centrifuged and the supernatants were submitted to PCR in order to identify the viruses. We were able to isolate and identify HSV-1 from 6 and VZV from 3 specimens (Table 2).

HSV-1 DNA was amplified by simple PCR directly from swabs in 9 cases. These swabs originated from patients with blister, keratitis, blepharitis, and whitlow. VZV DNA was amplified by simple PCR directly from swabs in 4 cases. These swabs were collected from patients with shingles, facial zoster and varicella. All these swabs were tested by both HSV-1 and VZV PCR. Two swabs (SC02 and SC03) were negative by either PCR and were obtained from patients who had used topical treatment.

By employing the multiplex PCR, we were able to detect HSV-1 or VZV in 12 of 15 swabs (Table 2).

On the other hand, we were not able to detect HSV-1 or VZV by the multiplex PCR in swabs SC02 and SC03, which were negative also by the simple PCRs. Swab GI, which was positive by virus isolation and HSV-1 PCR, was negative by repeated multiplex PCR.

Table 2. Comparison of different assays used for detection of herpesviruses in mucocutaneous herpetic infections

Specimen (patient)	Cell	CPE	HSV-1 PCR[a]	VZV PCR[a]	Multiplex PCR[a]
EK	Vero	+	+	–	+
CAT	Vero/MRC-5	+/+	–	+	+
OF	Vero	+	–	+	+
SC01	MRC-5	–	–	+	+
SC05	Vero/MRC-5	+/+	+	–	+
HC01	MRC-5	+	+	–	+
HC02	MRC-5	–	+	–	+
HC03	MRC-5	–	+	–	+
SC02	MRC-5	–	–	–	–
SC03	MRC-5	–	–	–	–
HC05	MRC-5	+	–	+	+
CMO01	MRC-5	–	+	–	+
LV1	Vero	+	+	–	+
LV2	MRC-5	+	+	–	+
GI	MRC-5	+	+	–	–

(+), (–) = positive, negative.
[a]Performed directly on swabs.

Discussion

We analyzed 15 specimens from 13 patients with mucocutaneous herpetic infections and we detected viral DNA in 13 of them. We were unable to detect viral DNA by PCR or to detect the virus by isolation in 2 specimens collected after the beginning of topical therapy (SC02 and SC03). In two specimens collected after oral therapy (HC02 and HC03,) we were able to detect viral DNA by PCR even though the virus isolation was negative. These data suggest that specimens for PCR can be collected even after the beginning of therapy, although collection before therapy is more adequate for virus isolation and PCR.

Our approach, based on PCR, has some advantages as compared with conventional PCR protocols, since we have not used organic reagents for DNA extraction that could inhibit the PCR reaction. Our protocol is also faster and cheaper, because there is no need to extract DNA. Another advantage is that with reduced handling the chances of sample contamination are minimum.

Several authors have reported the use of PCR for diagnosis of mucocutaneous herpetic infections. They have used specimens of different kind, such as swabs, crusts, archival samples and virological cultures, and have employed different approaches to amplify the viral DNA. There have been many reports in the literature (Kido *et al.*, 1991; Nahass *et al.*, 1992, 1995) about the use of protocols employing DNA isolation with organic reagents. However, such DNA samples may inhibit PCR amplification.

Virus isolation is still used as the "gold standard" procedure for the diagnosis of mucocutaneous herpetic infections (Thomas *et al.*, 1994). However, results from our and other laboratories (Kido *et al.*, 1991; Nahass *et al.*, 1992, 1995; Thomas *et al.*, 1994; Baron *et al.*, 1996; Risbud *et al.*, 1999) strongly suggest that PCR should be used as an alternative "gold standard" in the diagnostics of mucocutaneous herpetic infections as suggested for herpes simplex encephalitis (Lakeman and Whitley, 1995; Tebas *et al.*, 1998).

The swab is probably the best source of DNA for PCR assay of mucocutaneous herpetic infections (Kimura *et al.*, 1990; Nahass *et al.*, 1995). In our approach, we used a rapid protocol in which we collected a swab from the patient lesions followed by a boiling step. This rapid procedure is suitable for isolation of DNA for PCR assays.

Several reports have showed that PCR could be more sensitive and specific than virus isolation or antigen

detection for the diagnostics of mucocutaneous herpetic infections (Nahass *et al.*, 1995; Safrin *et al.*, 1997; Slomka *et al.*, 1998). The future goal is to obtain higher sensitivity and rapidness for PCR.

The use of HSV-1/VZV general primers or multiplex PCR could provide a faster assay. Some authors have described the use of a multiplex PCR to identify more than one virus or organism in the same reaction (Baron *et al.*, 1996; Cassinotti *et al.*, 1996; Stockton *et al.*, 1998; Nascimento *et al.*, 1998; Risbud *et al.*, 1999; Read *et al.*, 1999).

The use of general primers and subsequent restriction fragment length polymorphism (RFLP) analysis or enzyme-linked immunosorbent assay (ELISA) has been described (Scott *et al.*, 1997; Markoulatos *et al.*, 1997) and the detection of mRNAs is also under evaluation (Mori *et al.*, 1998). Although these procedures are both sensitive and specific they are time-consuming and expensive.

Our approach employing the multiplex PCR is based on a conserved TK gene. The amplicons showed different sizes that enable differential visualization in agarose gel. Moreover, this procedure provides a rapid and specific assay, which permits the prescription of a drug treatment 6 hrs after collecting specimens from a patient.

Besides, the TK gene is the most common target for mutation in acyclovir-resistant strains of HSV-1 and VZV (Field and Goldthorpe 1992; Boivin *et al.*, 1994) and the amplicom analysis (e.g., sequencing) could be used to detect acyclovir-resistant strains.

In conclusion, this study presents two PCR protocols that are more sensitive and faster than the conventional virus isolation. We propose that this approach could be used as rapid and specific complementary assay to identify precisely the etiologic agent of a suspected mucocutaneous herpetic infection.

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