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Rapid Detection of *Orthopoxvirus* by Semi-Nested PCR Directly From Clinical Specimens: A Useful Alternative for Routine Laboratories

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Orthopoxvirus (OPV) has been associated with worldwide exanthematic outbreaks, which have resulted in serious economic losses as well as impact on public health. Although the current classical and molecular methods are useful for the diagnosis of OPV, they are largely inaccessible to unsophisticated clinical laboratories. The major reason for the inaccessibility is that they require both virus isolation and DNA manipulation. In this report, a rapid, sensitive and low-cost semi-nested PCR method is described for the detection of OPV DNA directly from clinical specimens. A set of primers was designed to amplify the conserved OPV *vgf* gene. The most useful thermal and chemical conditions were selected and minimum non-inhibitory dilutions were determined. More than 100 Brazilian *Vaccinia virus* (VACV) field clinical specimens were tested using this semi-nested PCR in order to confirm its applicability. *Cowpox virus* was also detected by PCR from the ear scabs of scarified Balb/c mice. In addition, the method was highly sensitive for the detection of VACV DNA in murine blood and excreta, which are among the suggested reservoirs of OPV. Together, these data suggest that semi-nested PCR can be used for initial screening for OPV and as a routine diagnostic laboratory method. ***J. Med. Virol. 82:692–699, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: viral diagnosis; vaccinia outbreaks; poxvirus; *Orthopoxvirus*

INTRODUCTION

Orthopoxviruses (OPVs) are large, enveloped, linear double-stranded DNA viruses. They are classified as a genus within the Poxviridae family [Moss, 2007]. Four OPVs have been reported previously to infect humans. The first of these is *Variola virus* (VARV), which is the etiological agent of smallpox, which had been eradicated. The other three are zoonotic species, including *Monkeypox virus* (MPXV), *Cowpox virus* (CPXV), and *Vaccinia virus* (VACV). These species have been associated with outbreaks of infection in Africa, Europe, South America and Asia [Heymann et al., 1998; Haenssle et al., 2006; Trindade et al., 2006; Singh et al., 2007]. The incidence of zoonotic OPV infections has been increasing in recent years [reviewed by Ferreira et al., 2008]. These findings can be explained in part by the suspension of smallpox vaccination in the early 1980s. Cessation of vaccination has also led to the emergence of an unimmunized generation against OPVs [Kulesh et al., 2004].

The diagnosis of OPV infections involves clinical, serological, virological, microscopic and molecular techniques [Kulesh et al., 2004; Lobato et al., 2005; Trindade et al., 2006; Saijo et al., 2008; Vestergaard et al., 2008; Strenger et al., 2009]. Zoonotic OPV has the potential to cause either local or disseminated vesicular-pustular lesions, which are associated with fever, lymphadenopathy, malaise and acute muscle pain [Fenner et al., 1989]. The infection induces a robust humoral immune response. Due to this response, anti-OPV antibodies can be detected by ELISA, immunofluorescence or neutralization tests in sera several days after the initial infection [Pelkonen et al., 2003; Karem

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et al., 2005; Lederman et al., 2008]. The epidermal lesions typically present high titers of virions, and viruses can be isolated from vesicular secretions or by inoculation of permissive cell lines or the chorioallantoic membrane (CAM) of embryonated hen's eggs [Sarkar et al., 1974; Leite et al., 2005; Singh et al., 2006; Kurth et al., 2008]. The viral particles obtained from the lesions are then visualized by electron microscopy. This method is laborious due to required additional specimen manipulation, including fixing, staining and labeling [reviewed by Trindade et al., 2007]. Molecular and immunological techniques, including real-time PCR [Putkuri et al., 2009], nested and semi-nested PCR [Sánchez-Seco et al., 2006; Damaso et al., 2007], PCR-RFLP [Meyer et al., 1997], and Western blot assays [Fedorko et al., 2005] have been applied widely in OPV research. The use of real-time PCR for the detection of VACV directly from lesions without DNA or virus manipulation was described by de Souza Trindade et al. [2008]. However, the other mentioned previously processes require viral isolation and amplification. In addition, some described recently methods have a restricted application because they were designed to detect only specific strains [Damaso et al., 2007; Saijo et al., 2008; Singh et al., 2008] or have become out of date due to novel polymorphisms in new OPV isolates [Meyer et al., 1997; Leite et al., 2007].

Although the methods referred to above are employed in research centers, they are often not useful in unsophisticated routine clinical laboratories. OPV diagnosis in such laboratories, based on detection of anti-OPV antibodies by ELISA, cannot differentiate antibodies associated with acute infection from antibodies resulting from prior vaccination [Jahrling et al., 2007]. This is a concern in OPV diagnosis because patients vaccinated against smallpox patients can be susceptible to other OPV infections [Silva-Fernandes et al., 2009]. Attempting to reduce both, the cost and time for OPV diagnosis, a highly sensitive conventional semi-nested PCR technique was developed to amplify the highly conserved OPV virus growth factor (*vgf*) gene directly from dried scabs and vesicular contents. This study discusses a simple and rapid technique, which has facilitated the diagnosis of more than 100 Brazilian cases of VACV (BV) infection during the last 2 years. In addition, the potential for further use of this method as an epidemiological tool was assessed under laboratory conditions. The method was able to detect OPV DNA directly from rodent specimens, which are the believed to be reservoirs for OPV. The *vgf* semi-nested PCR method does not require viral isolation or DNA manipulation and therefore can be useful for unsophisticated routine clinical diagnostic laboratories as a rapid screening tool for OPV.

MATERIALS AND METHODS

Cells and Viruses

Vero cells were propagated at 37°C in Eagle's-minimum essential medium (MEM; GibcoBRL, Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS), 25 μg/ml fungizone (Amphotericin B, Cristália, Brazil), 500 U/ml penicillin, and 50 μg/ml gentamicin (Schering-Plough, Rio de Janeiro, Brazil). VACV strain *Western Reserve* (VACV-WR) and CPXV strain *Brighton Red* (CPXV-BR), kindly provided by Dr. C. Jungwirth (Universitat Wurzburg, Germany), were grown in Vero cells (ATCC number CCL81) and purified subsequently using a sucrose gradient as described previously [Joklik, 1962]. VACV-WR was then used for the PCR standardization, minimum non-inhibitory dilutions (MNIDs) and sensitivity assays.

Murine Sample Collection

Clinical specimens were collected from *Mus musculus* Balb/c mice to assess the semi-nested PCR of *vgf* sensitivity and MNID. Four male 4-week-old Balb/c mice were held in cages with access to filtered water and autoclaved industrial food. To confirm the absence of OPV, all animals were tested previously by the neutralization assay [Abdalrhman et al., 2006]. To mimic epidermal lesions of OPV, scabs were obtained by scarification in phosphate buffered saline (PBS) of Balb/c mice ears as described previously [Tscharke and Smith, 1999]. Feces and urine were collected with microcentrifuge tubes positioned directly below the anus or penis of the mice. Blood was also collected in microcentrifuge tubes with EDTA after the mice were killed. All samples were then stored at −70°C.

Feces and scabs were homogenized in PBS (0.1 g clinical specimen/0.9 ml PBS). The samples were then centrifuged at 2,000*g* for 3 min, and the supernatants were then collected. Pools of blood, urine and feces supernatants were spiked subsequently with VACV-WR to assess the sensitivity of *vgf* semi-nested PCR and that of MNID.

Collection and Preparation of Human and Bovine BV Clinical Samples

Vesicle contents and dried scabs from cattle udders and the hands of the milking personnel were collected during BV outbreaks (Table II). This collection was accomplished using 1-ml insulin syringes with 0.45 mm × 13 mm needles and cotton swabs or a pair of forceps. Collected samples were transported under cold conditions to the laboratory and stored at −70°C until processing. Vesicular liquid swabs were added to 200 μl of PBS and centrifuged at 2,000*g* for 3 min. Scabs were macerated in PBS (0.1 g scab/0.9 ml PBS) using a homogenizer (Politron, Littau, Switzerland) and clarified by centrifugation at 2,000*g* for 3 min. Two microliters of each supernatant was used in the *vgf* semi-nested PCR. Several expected PCR products were sequenced directly (ET Dynamic Termnator for MegaBACE—GE Healthcare, Fairfield, NJ) and compared with available GenBank sequences using the online BLAST software (http://www.ncbi.nlm.nih.gov/blast). In parallel, two microliters of the same clarified BV field samples were added to 198 μl of PBS and

inoculated in a CAM and a Vero cell monolayer [Leite et al., 2005; Lobato et al., 2005; Trindade et al., 2006] for VACV detection. To avoid any possibility of laboratory cross-contamination, all of the samples were manipulated individually.

Collection and Preparation of Scabs From Balb/c Mice Inoculated With CPXV

To determine the specificity of the PCR for the detection of CPXV, other relevant OPV, the ears of ten 4-week-old Balb/c mice were scarified with 10^6 PFU of CPXV-BR [Tscharke and Smith, 1999]. After 5 days, the scabs were collected with forceps and prepared using the same method described for the BV samples. After preparation, the samples were submitted to semi-nested PCR, CAM, and Vero cell monolayer inoculation, as described for the BV specimens. A control group was scarified with PBS. Experimentation was carried out in accordance with the regulations and guidelines of the Ethical and Animal Use Committee of Universidade Federal de Minas Gerais, Brazil.

Primers and PCR

For the *vgf* semi-nested PCR standardization, an external E primer (5′ACAATGGATATTTACGAC3′) was designed from aligning *vgf* sequences from Brazilian isolates of VACV [Drumond et al., unpublished work] and other OPV sequences that were available (GenBank accession nos. [AY243312.1 (VACV-WR); AY678276.1 (VACV-LISTER); DQ792504.1 (*Horsepox virus*—HSPV); AY484669.1 (*Rabbitpox virus*—RPV); DQ437590.1 (VARV); AF482758.2 (CPXV)]. These sequences were used in association with the VGF-F (5′CGCTGCT-ATGATAATCAGATCATT′) and VGF-R (5′ GATATGGT-TGTGCCATAATTTTTAT 3′) primers. These primers were designed by Fonseca et al. [1998], with both lacking the restriction sites (Fig. 1). Therefore, PCR targeting of *vgf* was carried out by the use of a two-step reaction protocol. Several chemical and thermal conditions were evaluated to determine the optimal conditions. The best conditions were established based on amplicon yield and specificity (corresponding to an expected fragment of 381 bp), described as follows. In the first step, 2 μl of template was added to 18 μl of the PCR reaction mixture containing 0.2 mM primers (VGF-F and E), 10 mM of dNTP set, 2.0 mM $MgCl_2$, and 2 U of Taq DNA polymerase (Promega, Madison, WI). In addition, 10 × buffer that was supplied by the manufacturer was used. Reactions were performed using a DNA Mastercycler Epgradient (Eppendorf, Hamburg, Germany), with the following protocol: incubation at 95°C for 9 min, 30 cycles of denaturation (94°C, 1 min), annealing (45°C, 1 min), extension (72°C, 1 min), and a final extension (72°C, 10 min). The semi-nested PCR step was carried out using 1 μl of undiluted product from the initial PCR as the template. The same chemical and thermal conditions were used, while the internal primers were changed to VGF-F and VGF-R. The PCR products were electrophoresed on 8% PAGE gels and silver stained [Sambrook et al., 1989]. These same conditions were used for the sensitivity assays and for the determination of MNID for each clinical specimen. In order to confirm the specificity of OPV, other exanthematic infectious agents were submitted to PCR, including (i) a *Herpes virus* bovine scab kindly provided by Dr. Z. Lobato (Minas Gerais Federal University, Brazil), (ii) a *Parapoxvirus* caprine scab, kindly provided by Dr. C. Mazur (Fluminense Federal University, Brazil), and (iii) a Brazilian *Sthaphylococcus aureus* strain isolated from a hospital infection, kindly provided by Dr. L. Parucker (Santa Catarina Federal University, Brazil).

Determination of MNIDs and Sensitivity Assays for the Clinical Specimens

PCR MNIDs were determined for murine scabs, feces, urine and blood. Undiluted, 1,000-, 100- and 10-fold specimens diluted in PBS were used for the MNID tests. Each sample was spiked with 10^5 PFU of purified VACV-WR. In the positive control, a clinical specimen was not added. In the negative control, virus was not added.

PCR sensitivity was determined using clinical specimens spiked with decimal serial dilutions ranging from 10^4 to 1 PFU of VACV-WR as templates. The reactions were completed following the MNID selected previously corresponding to each clinical specimen. The sensitivity was defined by the highest viral dilution detected by PCR.

The sensitivity and MNID assays described above were completed with and without the addition of BSA at a concentration of 500 ng per reaction [Abu Al-Soud and Radstrom, 2000].

RESULTS

vgf Semi-Nested PCR Standardization

A 381 bp DNA fragment corresponding to a region of the *vgf* gene was amplified specifically by PCR under multiple thermal and chemical conditions. The greatest yield was obtained in reactions performed with 30 cycles of amplification in the first step of PCR, followed by the use of this undiluted amplicon (1 μl) as a template in the semi-nested step (Fig. 1). The sequence from the amplified fragment showed complete identity with the VACV *vgf* gene (AY2433121 and others). No specific viral bands were observed in the negative control or in the *Herpes virus*, *Parapoxvirus*, and *S. aureus* reactions.

vgf Semi-Nested PCR MNID and Sensitivity

The MNID corresponds to the lowest dilution of a specific clinical specimen in which PCR is able to detect viral DNA. The four murine clinical specimens examined in reactions without BSA had different inhibition levels when tested using the *vgf* semi-nested PCR. Feces, urine and blood had the same MNID, which was 1/100 (Table I). The reactions were completely inhibited in the original or 10-fold-diluted clinical specimens. With clinical specimens diluted 100 fold a fragment of the predicted size was amplified (381 bp). In addition,

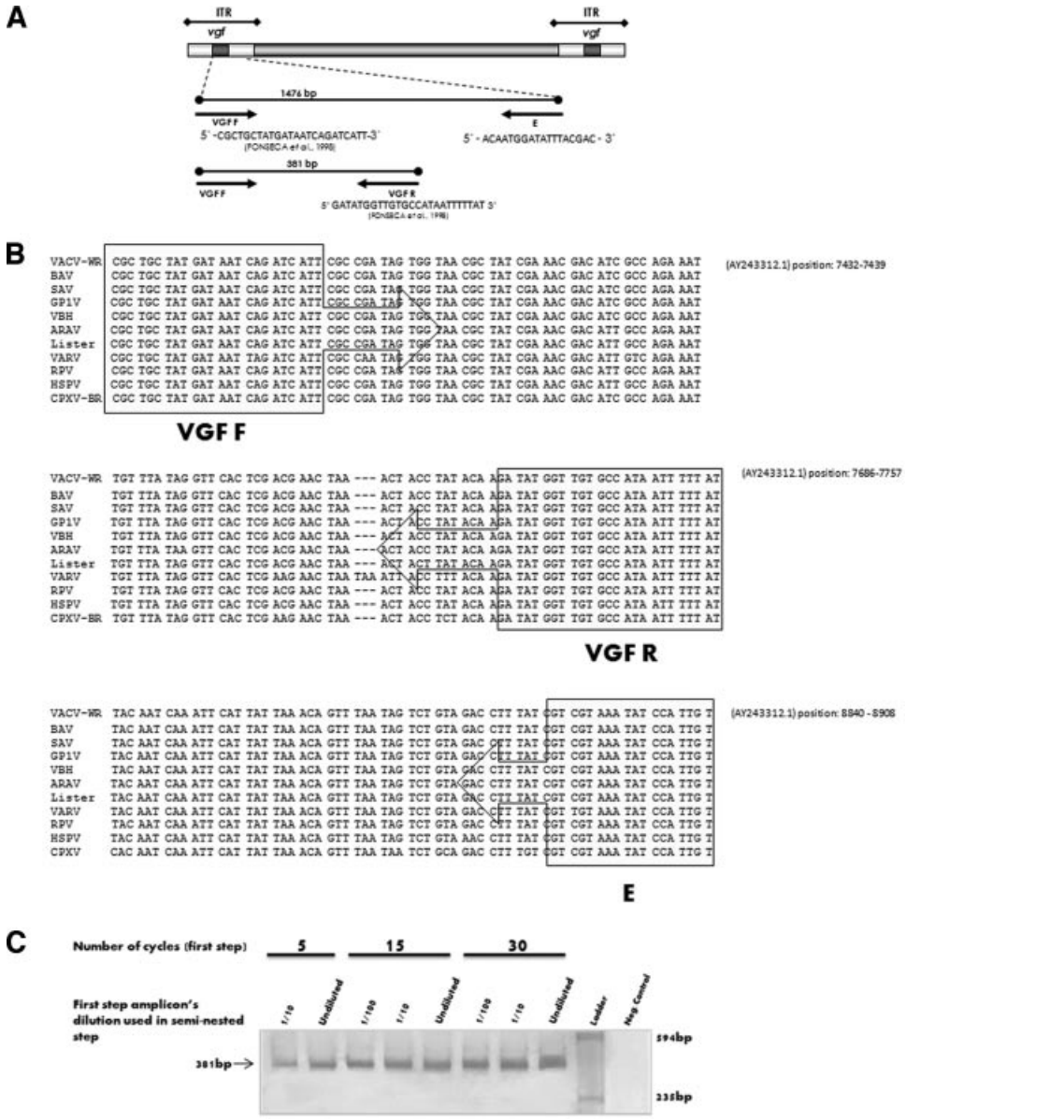

Fig. 1. **A**: Schematic representation of primer locations and sequences in the VACV-WR genome. **B**: VGF-F and VGF-R primers were described by Fonseca et al. [1998]. The E primer was designed based on the alignment of Brazilian VACV strains, *vgf* sequences (Drumond et al., unpublished work), and other OPV *vgf* sequences available. To date there are no available *Buffalopox virus vgf* sequences. **C**: Semi-nested PCR *vgf* standardization: a 381 bp fragment was amplified using 10^5 PFU of VACV-WR as a template. Different numbers of cycles were tested in the first step, and various dilutions of the first-step amplicon were added in the semi-nested step. The PCR products were electrophoresed on 8% PAGE gels and silver stained. ITR: inverted terminal repeats.

TABLE I. Semi-Nested PCR *vgf* MNIDs

Clinical specimen	BSA−	BSA+
Feces	1/100	Undiluted
Urine	1/100	Undiluted
Blood	1/100	1/10
Scab	1/1,000	1/100

Comparison between MNID assays with (BSA+) and without (BSA-) BSA.
MIND, minimum non-inhibitory dilution; BSA, bovine serum albumin. In MIND assays were used 10^5 PFU of VACV-WR/reaction.

the scabs had a higher level of inhibition, with a MNID of 1/1000.

The addition of BSA to *vgf* semi-nested PCR reduced significantly inhibition levels. For feces and urine, the MNID was reduced 100 fold, and VACV DNA could be detected even without dilution. BSA also reduced the inhibition in blood and scabs by 10 fold. In these samples, viral DNA was detected starting at an MNID of 1/10 and 1/100, respectively (Table I).

Overall, BSA increased the sensitivity of PCR in the range of 100–1000 fold. Without BSA, the PCR sensitivity for feces and blood was 10^2 PFU, while for the

urine and scabs it was 10^3 PFU. A total of 500 ng of BSA per reaction increased the detection limit of the PCR to 1 PFU of VACV-WR in feces, urine, blood and scabs (Fig. 2). The PCR MNID and sensitivity assays presented a unique and specific amplified band of approximately 381 bp. No specific viral bands were observed in any of the negative controls.

vgf Semi-Nested PCR Applicability Tests: Field BV and Murine CPXV Clinical Specimens

To test the applicability of the semi-nested *vgf* PCR, human and bovine dried scabs and vesicle contents collected during outbreak of bovine vaccinia (BV) were submitted to both PCR and to the classical VACV diagnostic methods of Vero cell inoculation and CAM infection.

A total of 108 BV samples were examined in this study. In general, CAM inoculation was more sensitive than inoculation of Vero cells. Typical VACV white pocks were observed in 77.7% of the inoculated eggs, while 57.4% of cell monolayers developed cytopathic effects (Table II). In addition, 12 samples were toxic for Vero cells and CAM, even after a 10-fold dilution (data not shown). The *vgf* semi-nested PCR generated a 381 bp fragment in 108 (100%) of the BV field specimens. In addition, the expected *vgf* fragment was also amplified from infected mice scabs. These scab induced the formation of cytopathic effects in Vero cells and the development of hemorrhagic red pocks in the CAM. Once the applicability of PCR was established, the specificity was confirmed by sequencing several representative samples. All sequences had a high identity with OPV species. Table II summarizes the results of OPV detection in clinical samples.

DISCUSSION

Zoonotic OPV infections have been associated with worldwide exanthematic outbreaks, serious economic losses and considerable impact on public health [Heymann et al., 1998; Haenssle et al., 2006; Trindade et al., 2006; Singh et al., 2007]. To identify the etiological agent of these outbreaks, both classic and molecular methods have been applied. These methods include viral isolation, DNA detection, DNA restriction, serology and microscopy [Kulesh et al., 2004; Lobato et al., 2005; Trindade et al., 2006; Saijo et al., 2008; Vestergaard et al., 2008]. Although applicable in research centers, these methods are inaccessible to routine clinical diagnostic laboratories.

The aim of this study was to design a simple, sensitive, rapid, inexpensive and direct diagnostic conventional PCR test that can be applied in routine laboratories. The laboratories would include facilities where modern real-time and other current research laboratory methods are not yet available. An OPV diagnostic test undertaken directly on clinical specimens seemed to be a rational option, since viral isolation and DNA extraction from dried scabs and vesicle content are not possible in many laboratories. However, several compounds in clinical specimens could be associated with PCR inhibitory activity, including phenolic substances and carbohydrates [Abu Al-Soud and Radstrom, 2000]. In an attempt to optimize the method, some reactions were completed with the addition of BSA. A mechanism of action for BSA has not been established, but some consider that it can bind directly to PCR inhibitors [Abu Al-Soud and Radstrom, 2000; Malorny and Hoorfar, 2005]. In this study, BSA reduced significantly the action of PCR inhibitors, thereby increasing the sensitivity of the reaction and allowing the use of clinical specimens with small dilutions (Table I). The high sensitivity of the *vgf* PCR method is likely explained by (i) the use of a semi-nested strategy, (ii) the existence of two copies of *vgf* gene in some VACV genomes [reviewed by Ferreira et al., 2008], and (iii) the high resolution and reproducibility of the polyacrylamide gel electrophoresis-silver staining method [Sambrook et al., 1989].

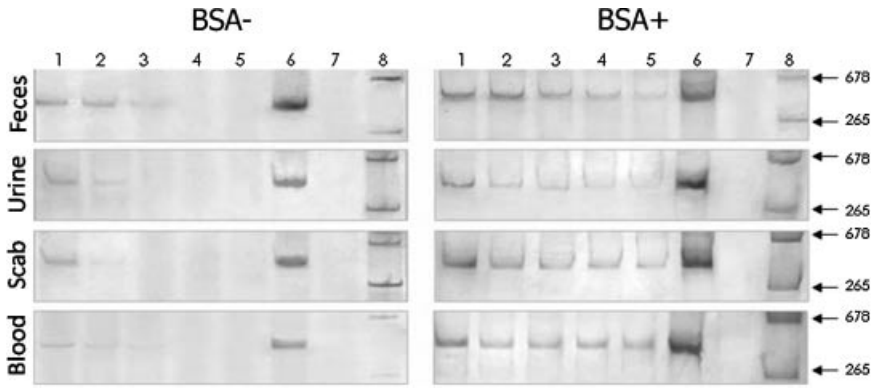

Fig. 2. Semi-nested PCR *vgf* sensitivity assays. Clinical specimens collected from uninfected Balb/c mice were processed and spiked with VACV-WR serial decimal dilutions (10^4–1 PFU). The samples were submitted to semi-nested PCR for the *vgf* gene amplification (381 bp). **Lane 1**: 10^4 PFU, **lane 2**: 10^3 PFU, **lane 3**: 10^2 PFU, **lane 4**: 10^1 PFU, **lane 5**: 1 PFU, **lane 6**: positive control (purified VACV-WR), **lane 7**: negative control, and **lane 8**: molecular marker. The tests were done in the presence of 500 ng BSA (**right**) or in its absence (**left**). The PCR product was electrophoresed in PAGE 8% and silver stained. BSA: bovine serum albumin.

TABLE II. Detection of VACV in Field Samples by *vgf* Semi-Nested PCR, Vero Cells, and CAM Inoculation

County/state/year	No. of specimens	Source	Designation	Specimen[a]	snPCR *vgf*[b]	Vero[b]	CAM[b]	References
Guarani/MG/2005	2	B	GP1V, GP2V	Scab	2	1	2	Trindade et al. [2006]
Serro/MG/2005	11	B/H	SV	Scab and vesicle	11	7	7	Trindade et al. [2007]
Passatempo/MG/2003	1	B	PSTV	Scab	1	1	1	Leite et al. [2005]
Mariana/MG/2005	13	B/H	MARV	Scab and vesicle	13	7	7	This work
Linhares/ES/2008	4	B/H	LINV	Scab and vesicle	5	4	4	This work
Resplendor/MG/2005	5	B/H	RPLV	Scab and vesicle	5	4	4	This work
Jequeri/MG/2005	8	B/H	JQRV	Scab and vesicle	8	5	5	This work
Paraguassu/MG/2008	8	B	PRGV	Scab	8	3	3	This work
Argirita/MG/2008	4	H	ARGV	Vesicle	4	2	2	This work
Cantagalo/RJ/2001	3	B/H	CTGV-2	Scab and vesicle	3	2	3	
S. Francisco do Itabapoana/RJ/2002	17	B/H	SFIV	Scab and vesicle	17	13	16	
Porciúncula/RJ/2001	2	B	PRNV	Scab	2	1	2	
São Fidéis/RJ/2001	4	B/H	SFDV	Scab and vesicle	4	1	4	
Itaperuna/RJ/2001	2	B/H	IPRV	Scab and vesicle	2	1	2	
Resende/RJ/2002	1	B	RESV	Scab	1	1	1	
Cardoso Moreira/RJ/2002	3	B/H	CMRV	Scab and vesicle	3	1	3	
Cambuci/RJ/2005	1	B	CAMV	Scab	1	0	1	S
Miracema/RJ/2006	4	B/H	MIRV	Scab and vesicle	4	1	4	
Cordeiro/RJ/2001	1	B	CORV	Scab	1	0	1	
Varre-sai/RJ/2006	6	B/H	VRV	Scab and vesicle	6	6	6	
Sto. Atn. de Pádua/RJ/2000	1	H	SAPV	Vesicle	1	0	1	
S. José de Ubá/RJ/2001	1	H	SJUV	Vesicle	1	1	1	
Campos/RJ/2001	2	B/H	CAPV	Scab and vesicle	2	0	1	
Três Rios/RJ/2002	1	H	TRV	Vesicle	1	0	1	
Natividade/RJ/2006	1	B	NATV	Scab	1	1	1	
Sta. Ma. Madalena/RJ/2001	1	B	SMMV	Scab	1	0	1	
Total	108				108 (100%)	62 (57.4%)	84 (77.7%)	

B, *Bos taurus*; H, *Homo sapiens*.
[a]Vesicular liquid presented the same MNID of scab (data not shown).
[b]Number of positive samples in semi-nested PCR *vgf*, Vero cells or CAM.

Although the primary goal of the study was the application of PCR to the diagnosis of OPV outbreak, the method was also shown to be useful for the detection of DNA in murine blood and excreta. Some studies have described the shedding of OPVs, including CPXV [Maiboroda, 1982], VARV [Sarkar et al., 1973], and ectromelia virus [Gledhill, 1962], in host excrements or secretions, including feces, urine and conjunctive liquid. In addition, a recent study showed shedding of VACV in infected Balb/c mice excrements and the potential role of murine feces in viral circulation among rodents was considered [Ferreira et al., 2008]. The intrinsic resistance of OPV particles [Essbauer et al., 2007] and the great variety of chemical micro-niches present in clinical samples such as feces and urine, make it likely that some viral genomic units remain conserved, even for some time after the excretion. This has been observed with other viruses [Bouillant and Hanson, 1965]. Along these lines, if the murine excreta shedding patterns observed in the laboratory are similar to those in wild host reservoirs, *vgf* semi-nested PCR could be useful as an epidemiological tool for monitoring VACV or other zoonotic OPV outbreak areas. Although speculative, this question must be considered because VACV outbreaks are reported increasingly, and the natural reservoir is still unknown.

The screening of BV field clinical samples confirmed the high sensitivity of the method, which is a requirement for epidemiological screening tools. Among the assays examined, PCR presented the greatest sensitivity. All field clinical specimens tested demonstrated amplification of the expected *vgf* fragment gene, indicating infection by VACV. VACV is the only confirmed OPV species that circulates currently in Brazil. In view of the fact that the primer annealing regions are conserved among available sequences in the GenBank database (Fig. 1), other OPV species could also be detected by *vgf* semi-nested PCR. This type of application was demonstrated using CPXV collected from infected murine scabs. The direct detection of OPV from clinical specimens has an important biosafety advantage. This is particularly true for highly virulent OPVs, such as CPXV and MPXV, because isolation procedures for OPV often increase the viral load, which could lead to exposure to a large inoculum.

CONCLUSION

The *vgf* semi-nested PCR method was useful for rapid diagnosis of OPV in bovine, murine and human clinical specimens. This method can be applied for initial OPV screening as a routine laboratory diagnostic method. Other significant aspects of OPV biology can be explored with the use of additional molecular and biological approaches.

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