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Reports

In Vivo Experimentation with Simian Herpesviruses: Assessment of Biosafety and Molecular Contamination

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In vivo studies with highly pathogenic viruses prompt concerns regarding the persistence of infectious virus in pathology specimens. Although formalin fixation of tissues may inactivate infectious virus, fixation may also degrade viral nucleic acid and antigens, thereby limiting detection of virus in tissues by polymerase chain reaction (PCR) amplification or immunohistochemistry (IHC). We sought to: 1) assess the rate of inactivation of infectious virus in tissue specimens during formalin fixation, 2) assess IHC recognition of viral antigens and PCR detection of viral DNA after long-term (14 d) formalin fixation, and 3) investigate microtome contamination by DNA carry-over to subsequently sectioned tissues. Infectious baboon herpesvirus HVP2 could be recovered from fresh tissues of infected mice but not those fixed in formalin for ≥24 h. The intensity of IHC staining of viral antigen was unaffected by the duration of formalin fixation. PCR detection of viral DNA was negatively impacted by formalin fixation and/or heat inherent to paraffin processing; however, amplification of very short DNA sequences using real-time PCR was not affected. Lastly, microtome contamination by viral DNA was demonstrated by PCR screening of uninoculated control tissues that were sectioned after sectioning infected tissues. In summary, infectious virus is inactivated after only 24 h of formalin fixation whereas IHC staining remains sensitive in tissues fixed for up to 14 d. Formalin fixation does degrade DNA, but viral DNA can be detected by PCR amplification of very short DNA sequences. In addition, viral DNA can contaminate a microtome knife such that subsequently sectioned uninoculated control tissues exhibit false positive PCR amplification.

Abbreviations: BV, monkey B virus (*Cercopithecine herpesvirus 1*); HVP2, baboon herpesvirus (*Herpesvirus papio 2*); CNS, central nervous system; DMEM, Dulbecco Modified Eagle Medium; IHC, immunohistochemistry; PCR, polymerase chain reaction

A prominent occupational hazard facing researchers and animal handlers that work with macaques is the potential for zoonotic infection by monkey B virus (*Cercopithecine herpesvirus 1*, BV). BV is relatively innocuous in the natural macaque host, but when transmitted to humans, the virus can produce an ascending encephalomyelitis that is often fatal.[18] The zoonotic potential of other closely related simian herpesviruses is virtually unknown. A murine model was developed recently to characterize the pathogenesis of simian herpesvirus infections in an aberrant host.[2,12,13] This model was developed specifically to address the question of cross-species neurovirulence inherent to this group of viruses.

Obviously, there are important biosafety concerns in working with animals that are infected with simian herpesviruses, especially BV. Animals experimentally infected with BV must be housed and handled under animal biosafety level 3 conditions.[11] Because a crucial step in model development includes the basic clinicopathologic characterization of BV (gross and histological examination), it becomes important to know, rather than to assume, that virus has been inactivated in formalin fixatives, for the safety of laboratory personnel handling fixed tissues.

In addition to characterization of virus-induced lesions, it is also often important to localize the course of virus as it spreads from the site of inoculation to the central nervous system (CNS). The temporal location of the virus can be ascertained by viral isolation from strategically harvested tissues, but this process necessitates working with infectious virus, an additional potentially hazardous step from a biosafety standpoint. Alternatively, the virus can be localized by immunohistochemical (IHC) detection of viral antigen or polymerase chain reaction (PCR)-based detection of viral DNA. However, these techniques can be compromised by formalin fixation of tissues. For instance, current recommendations for IHC staining of formalin-fixed tissue indicate that the tissues be removed after 24 h of formalin fixation, to prevent significant antigen masking.[5] Similarly, formalin fixation causes cross-linking and ultimately fragmentation of DNA and RNA and thus has the potential to interfere with PCR amplification of viral nucleic acids.[7] Therefore, it is important to determine the shortest length of time that is necessary for formalin fixation to inactivate infectious virus while limiting negative effects on IHC or PCR.

The purpose of this study was threefold: 1) to determine the minimum time necessary for formalin inactivation of infectious herpesvirus in whole-mouse carcasses, 2) to determine whether the time necessary to formalin-inactivate herpesviruses negatively affects either IHC detection of viral antigen or PCR detection of viral genes, and 3) to evaluate whether PCR screening of paraffin-embedded tissue sections for viral DNA

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can be spuriously affected by contamination of the microtome blade and consequent carry-over of viral DNA to subsequently sectioned tissues.

Materials and Methods

Animals. All animal experiments with simian herpesviruses were approved by the Institutional Animal Care and Use Committee of Oklahoma State University. Young adult (weight, 12 to 14 g) female, specific-pathogen-free BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in cages with isolator filter cage tops and maintained at a uniform temperature (21 to 22 °C) and humidity (50% ± 10%) and on a 12:12-h dark:light cycle. Mice received rodent chow (Purina 5001, St Louis, MO) and water ad libitum.

Virus. We used a neurovirulent strain (X313) of *Herpesvirus papio 2* (HVP2), an α-herpesvirus of baboons, in these studies. HVP2 is very closely related genetically and antigenically to BV, and the clinicopathologic disease produced by neurovirulent HVP2 in mice is nearly identical to that produced by BV infection.[3,12,13] Despite these similarities, HVP2 is a biosafety level 2 pathogen and therefore ideal for performing these safety assessment studies. The X313 strain originally was isolated from a yellow baboon (*Papio cynocephalus cynocephalus*) and has been described.[3,9] The virus was grown and quantitated in Vero cells as previously described.[4]

Experimental design. Mice were inoculated intramuscularly in the left hindlimb with 10^5 plaque-forming units of HVP2 strain X313 as previously described.[12] This dose is 10 times the dose lethal to 50% of the population and guarantees virus-induced disease in 100% of animals. Control mice were similarly inoculated with sterile phosphate-buffered saline. As previously reported, HVP2-infected mice developed characteristic herpetic skin lesions over the site of inoculation that were accompanied by CNS disease.[12,14] All mice were euthanized at 8 d postinfection. Immediately after euthanasia, fresh tissue samples were collected (see next paragraph) from 5 infected mice and 5 uninfected controls. The pleural and peritoneal cavities of the remaining infected and control mice were opened by making a ventral midline incision, and the carcasses were submerged in neutral buffered formalin. Thereafter, 5 mice each from the HVP2-infected and control groups were removed from formalin after 1, 2, 3, 7, and 14 d of formalin fixation.

Subsequent processing of samples was performed in an identical manner, regardless of whether samples were fresh (time 0) or from tissue fixed in formalin for 1, 2, 3, 7, or 14 d. A sample (0.5 cm × 0.5 cm × 0.2 cm) of skin from the site of inoculation was collected with forceps and scissors. The vertebral column was isolated, and the entire spinal cord was exposed by performing a dorsal laminectomy with fine-point scissors. The lumbar spinal cord segment was isolated and removed with forceps and scissors. The skin and lumbar spinal cord samples were collected and pooled from 3 of the 5 mice for routine tissue processing and paraffin embedding. The skin and spinal cord samples from the remaining 2 mice were pooled and homogenized for virus isolation and DNA extraction for PCR. Lastly, virus isolation was performed on homogenates obtained from fresh skin and lumbar spinal cord tissue that was routinely processed and paraffin-embedded.

Virus isolation. Tissues were homogenized in 0.5 ml of Dulbecco Modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum, penicillin–streptomycin, and amphotericin B.[4] Aliquots (0.25 ml) of the homogenates were plated onto confluent monolayers of Vero cells in 24-well plates. Plates were incubated for 1 h at 37 °C, an additional 0.25 ml DMEM added to each well, and incubated overnight at 37 °C. The medium–homogenate then was removed, cell monolayers washed with DMEM, and fresh medium added. Cultures were maintained at 37 °C and observed microscopically for viral cytopathic effect daily for 1 wk.

Histology and IHC. Skin samples overlying the site of inoculation and samples of the spinal cord (entire lumbar segment) were collected from the fresh or fixed carcasses, routinely processed, paraffin-embedded, and sectioned at 5 µm. Sections were either stained with haematoxylin and eosin to confirm virus-induced lesions or were mounted on positively charged slides for IHC. IHC identification of viral antigens was performed as previously described[12] by using polyclonal hyperimmune rabbit anti-serum. IHC staining for the intermediate filament protein vimentin was performed on an automated stainer using Confirm anti-vimentin clone 3B4 (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's recommended protocol.

DNA extraction and PCR. DNA was extracted from fresh, formalin-fixed, and paraffin-embedded tissues by using a commercial kit (DNeasy Tissue Kit, Qiagen, Valencia, CA) according to the manufacturer's instructions and quantitated by spectrophotometric analysis. All samples were analyzed using a standard 'hot-start' PCR assay for detection of HVP2 DNA.[14] PCR reactions (total volume, 50 µl) contained 1× PCR buffer, 25 pmol each of primers KT1 and KT10[15], 25 mM $MgCl_2$, 5% DMSO, 1 M betaine (Sigma Chemical, St Louis, MO), 0.2 mmol dNTPs, 25 U *Taq* polymerase (Applied Biosystems, Foster City, CA), and template DNA (4% of total DNA collected from the tissue extracts). Reactions underwent initial denaturation at 96 °C for 3 min, followed by 40 cycles of 96 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min. After a final 7 min at 72 °C, 5 µl of each reaction was loaded onto a 0.6% agarose gel to visualize the expected 1.17-kb DNA product.

Quantitative real-time PCR[15] was used to confirm results of standard PCR analysis. HVP2-specific primers and probe were designed based on the aligned sequences of the UL41 gene from 8 HVP2 isolates by using the PrimerExpress software (version 2.0, Applied Biosystems). The sequence of the forward primer was 5′ TGC GCC AAC CTC TAC CA 3′, and that for the reverse primer was 5′ TGT CGG TCG TGT GGA CGT 3′. A TaqMan MGB (minor groove binder) probe (5′ CCA ACA CCG TCG CG 3′) was labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with a non-fluorescing quencher. All primers, probes, and reagents for real-time PCR were purchased from Applied Biosystems.

To relate the amount of virus to the number of cells present in the sample as a means of normalizing for the cell density of different tissue types as well as for varying sample mass, we used TaqMan Pre-designed Assay for 18S rRNA (Applied Biosystems) as directed by the manufacturer. PCR reactions were performed in 96-well plates and contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 50 µM each HVP2 primer, 250 nM HVP2 probe, and (4% of total DNA collected from the tissue extracts) in a final volume of 25 µl. PCR amplification and detection was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) by using the following conditions: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 2-step cycles of 95 °C for 15 s and 60 °C for 1 min. All PCR reactions were done in triplicate.

Assessment of microtome contamination. We cut and pooled 5 sections (thickness, 5 µm) from a tissue block previously shown by PCR to be strongly positive for virus. This tissue block was removed from the microtome and replaced with a block contain-

Table 1. Effect of formalin fixation on virus isolation, immunohistochemical recognition of viral antigens, and PCR detection of viral genes

	Formalin fixation (d)											
	0		1		2		3		7		14	
	CNS	Skin	CNS	Skin	CNS	Skin	CNS	Skin	CNS	Skin	CNS	Skin
Histologic lesions	+	+	+	+	+	+	+	+	+	+	+	+
Immunohisto-chemistry	+	+	+	+	+	+	+	+	+	+	+	+
Virus isolation	+	+	–	–	–	–	–	–	–	–	–	–
PCR[a] of tissue homogenates	+	+	+	+	+	–	+	–	–	–	–	–
PCR of paraffin- embedded tissue	+	+	–	ND	–	ND	–	ND	–	ND	–	ND
Real-time PCR[b] of paraffin- embedded tissue	+	ND	+	ND	+	ND	+	ND	+	ND	+	ND

ND, not done.
[a]Positive detection determined by visual demonstration of a PCR product after agar gel electrophoresis, ethidium bromide staining, and transillumination.
[b]Samples that gave a slope of ≤ –2.8 between triplicate average values for 10-fold dilution Ct values were considered positive (–3.3 indicates 100% efficiency).[15] Samples not meeting this criterion were negative. The 18S rRNA gene was used as a cellular standard to normalize for tissue mass. No-template control wells were negative.

ing rat tissue obtained from an unrelated study (negative for herpesvirus by PCR), and 5 sections were cut and pooled; the microtome blade was not cleaned or replaced between blocks or sections. The blocks were alternately sectioned 4 additional times, with pooling of the 5 samples from each sectioning. DNA was extracted from each of the pooled samples as described earlier and tested in both standard and real-time PCR assays.

Results

Virus isolation. Skin from the site of inoculation and lumbar spinal cord (CNS) samples were either not fixed (fresh) or fixed in formalin for increasing lengths of time and tested for the presence of infectious virus. Infectious virus could be recovered only from fresh, unfixed tissues collected immediately after euthanasia (Table 1). Virus could not be isolated from tissue homogenates obtained from carcasses submerged in formalin, regardless of the length of time of formalin fixation (24 h to 14 d). In addition, infectious virus could not be isolated from fresh tissues that were run through a tissue processor (for paraffin embedding). Vero cell cultures inoculated with homogenates of fixed tissues did not exhibit any attenuation in growth or morphology that would suggest a deleterious effect from exposure to the formalin-fixed tissue homogenates.

Histology and IHC. Histologic examination of tissue sections stained with hematoxylin and eosin revealed virus-induced lesions at the site of inoculation and within the CNS.[12,14] Viral antigen was detected readily by IHC of tissues collected from carcasses fixed in formalin for 14 d (Figure 1 A–C). Subjectively, there was a slight decrease in the intensity of staining at 14 d as compared with tissues fixed for ≤7 d; but this decrease was not sufficient to affect interpretation of positive staining or the identification and distribution of virus-infected neurons. In contrast, formalin fixation compromised vimentin staining (Figure 2 A–C). Decreased staining intensity was noticed first in tissues fixed for 7 d. By 14 d of formalin fixation, there was a marked drop in vimentin staining intensity, which was accompanied by an increase in nonspecific background staining (Figure 2C).

PCR detection of viral DNA in tissue homogenates and paraffin-embedded tissue sections. We used a standard PCR assay that amplifies a fairly large product (1.1 kb) to determine the effect of formalin fixation on the integrity of viral DNA in samples. This PCR assay detected viral DNA in homogenates of fresh skin and in skin samples that were fixed in formalin for 24 h (Table 1). Viral DNA could also be detected in fresh CNS samples and from homogenates of CNS tissue that were formalin-fixed for as long as 3 d. The standard PCR assay failed to identify viral DNA in any sample fixed for more than 3 d. Viral DNA could be detected by PCR in fresh tissue that was routinely processed and paraffin-embedded (without formalin fixation) but not in any sample that was formalin-fixed and paraffin-embedded (Table 1).

Because fragmentation of DNA is expected to occur during formalin fixation,[7] we performed real-time PCR as a potentially more sensitive PCR method to detect virus in formalin-fixed, paraffin-embedded tissue samples. The real-time PCR assay we used amplifies a very small sequence (52 bp, compared with the 1.1-kb sequence for the standard PCR) that is well below the 300- to 400-bp fragments reported to result from formalin fixation.[7] All paraffin-embedded tissue samples were positive for viral DNA by real-time PCR (Table 1).

Microtome contamination. In previous studies, we encountered on several occasions positive PCR results when testing tissues that were not expected to be positive. In light of sequence analysis of the PCR products and records of the tissue sections that had been cut, these aberrant results were likely due to carry-over contamination from previously sectioned tissue samples. To test this theory, tissue blocks that were known to be PCR-positive (by both standard and real-time PCR) for viral DNA were sectioned alternately with tissue blocks from an unrelated study that were confirmed to be negative for viral DNA. DNA was extracted from the sections and tested by both standard and real-time PCR assays for viral sequences. All samples obtained from paraffin-embedded blocks containing CNS tissue from HVP2-infected mice were positive for virus by both standard (Figure 3) and real-time (data not shown) PCR assays. Of the 5 negative control samples sectioned immediately after sectioning the positive control block, 1 was virus-positive by both PCR methods, indicating carry-over from the previously sectioned positive sample.

Discussion

The potential for zoonotic infection with BV is a prominent occupational hazard for researchers and animals handlers that work with rhesus macaques. Similar risks exist for researchers working in the laboratory with BV or BV-infected small animals. Although potential exposure can be minimized by appropriate personal protective equipment, brief handling of animals only at the time of euthanasia, use of blunt-ended forceps and scissors

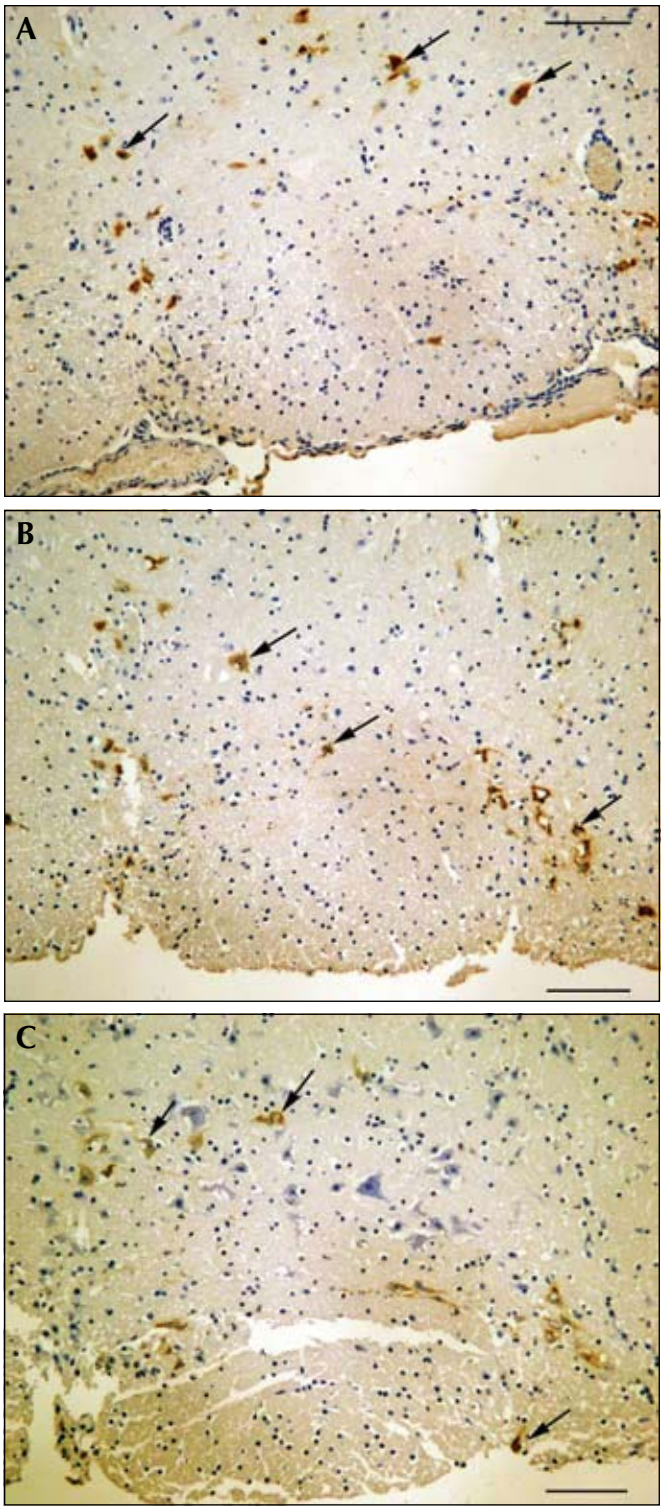

Figure 1. Brainstems from BALB/c mice inoculated with 10^5 PFU of HVP2 strain X313. Tissues were fixed in formalin for (A) 24 h, (B) 3 d, or (C) 14 d. After formalin fixation, tissues were processed routinely, paraffin-embedded, and sectioned; sections underwent immunohistochemical staining for viral antigens and counterstained with Mayer's hematoxylin. Viral antigen can be seen within neurons (arrows) in all sections. A mild loss of staining intensity is apparent when tissues fixed for (C) 14 d are compared with tissues fixed for (A) 24 h or (B) 3 d. Bar, 100 µm.

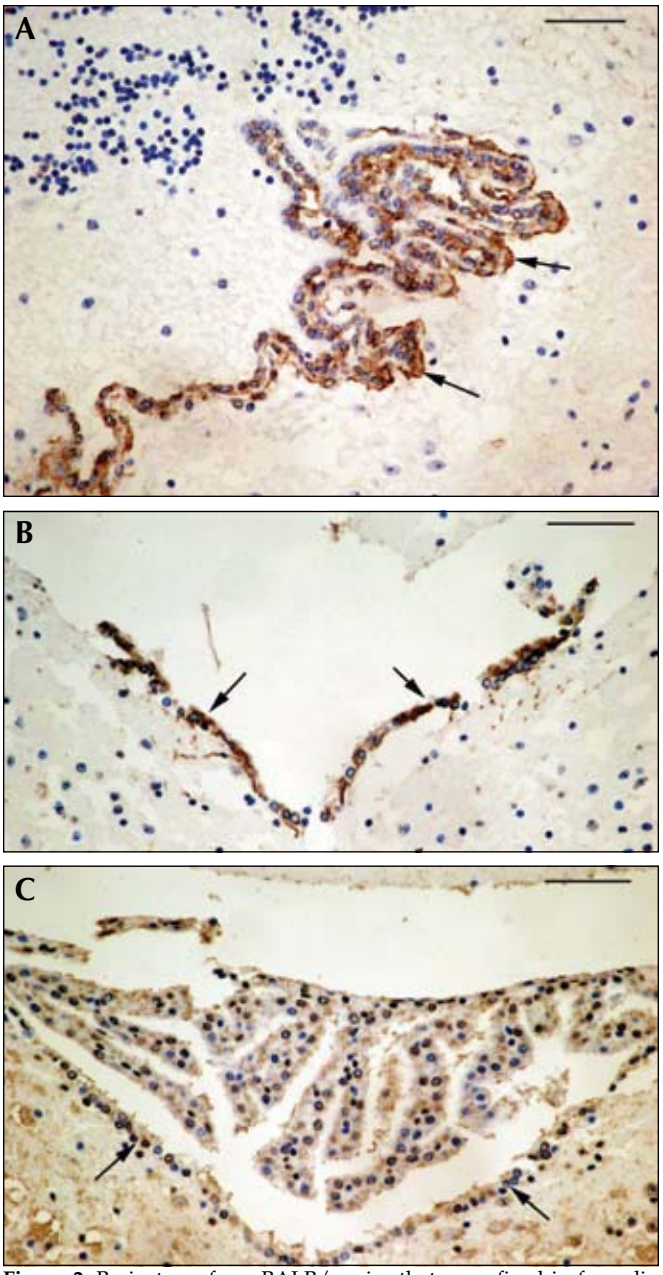

Figure 2. Brainstems from BALB/c mice that were fixed in formalin for (A) 24 h, (B) 3 d, or (C) 14 d. After formalin fixation, tissues were processed routinely, paraffin-embedded, and sectioned. Sections then underwent immunohistochemical staining for the intermediate filament vimentin and were counterstained with Mayer's hematoxylin. The ependymal cells (arrows) are intensely positive in sections obtained from tissues fixed for (A) 24 h or (B) 3 d. Beginning at 7 d of fixation, and most noticeably, at (C) 14 d of fixation, staining intensity was diminished, inconsistent, and accompanied by nonspecific background staining. Bar, 40 µm (A, B); 50 µm (C).

for dissection, and fixation of carcasses in formalin, potential risks for exposure still remain. This study was undertaken to assess the risks associated with performing routine histopathologic processing of herpesvirus-infected tissues and to identify conditions that would maximally reduce exposure risk while minimizing effects on experimental results.

Although formalin fixation efficiently inactivates infectious virus, a brief period of formalin fixation (24 h) is desirable because formalin can mask antigens crucial for IHC as well as interfere with PCR detection of viral DNA.[5] However, when whole carcasses are fixed, the question arises regarding how much time is sufficient for formalin to penetrate tissues and inactivate infectious virus. Of most concern are CNS tissues, as

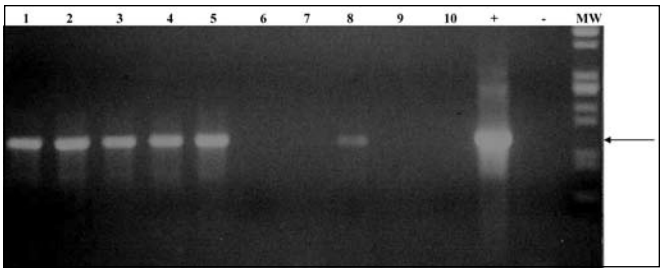

Figure 3. Agar gel electrophoresis of PCR products obtained by amplification of DNA extracted from paraffin-embedded tissue sections. Lanes 1 to 5 are results for tissue sections obtained from the CNS collected from HVP2-infected mice. Lanes 6 to 10 represent visceral tissues from rats obtained from an unrelated study and are known to be negative for simian herpesviruses. The lane numbers correspond to the microtome sectioning sequence (1-6-2-7-3-8-4-9-5-10) of virally infected tissue immediately followed by sectioning of the negative control tissue without cleaning or changing the microtome blade. A PCR product is easily visible in lane 8, indicating contamination of the known negative sample, most likely from carry-over from the previously sectioned positive sample 3. Gradient purified DNA from HVP2 strain OU1-76 was used as a positive template (+) control and water was loaded as a negative (–) template control. The molecular weight ladder (MW) was a combination of HindIII and EcoRI restriction fragments of lambda phage DNA (Promega, Madison, WI).

these are relatively protected within the bony vault of the spinal column and skull and are laden with infectious virus during peak CNS disease. Therefore, one purpose of this study was to assess the length of time for formalin fixation of tissues that would inactivate infectious virus but that would not interfere with subsequent analyses, such as PCR and IHC, that are important in pathogenesis studies.

Given the supposition that formalin penetration and fixation would be delayed in the anatomically confined CNS tissues, we expected that infectious virus would be recovered from CNS tissue after formalin fixation of the whole carcass for only 24 h. However, formalin fixation for as little as 24 h reduced infectious virus to levels below the limit of detection by virus isolation. Similarly, infectious virus could not be detected in fresh tissue that was run through a histological tissue processor for paraffin perfusion of tissue. This finding indicates that the heat or chemicals inherent to tissue processing techniques efficiently inactivate herpesviruses. Heat and formalin inactivation of herpesviruses is not novel and has been used for years for the preparation of inactivated herpesvirus vaccines.[6,10,16,17]

In addition to the biosafety concerns, tissues for histological analysis must sometimes remain in formalin for much longer than 24 h as other experimental procedures, such as RNA isolation or assembly of in vitro bioassays, take priority because they must be performed immediately. Formalin-fixed tissues are also prone to delayed processing during large, labor-intensive animal studies, particularly temporal studies with closely spaced timepoint collections. Formalin cross-links basic amino acids and can attenuate epitopes recognized by the labeling antibody.[5] Therefore, it is important to know how long tissues can remain in formalin before IHC procedures are negatively affected. We found a slight decrease in the staining intensity of viral antigen in tissues that underwent prolonged fixation (7 or 14 d); however, this effect would not have hampered or attenuated evaluation of the distribution of viral antigen within the sections. Vimentin is an intermediate filament protein that is susceptible to antigen masking by formalin fixation.[8] Therefore, vimentin immunoreactivity is often used to control for the integrity of antigens within tissues destined for IHC staining. Vimentin staining was compromised by formalin-induced antigen masking (as expected), but the IHC staining for viral antigens was not similarly affected. These results suggest that at least some of the viral epitopes recognized by the anti-viral *polyclonal* antibody are more resistant to antigen masking by formalin fixation and paraffin processing than is the epitope recognized by the anti-vimentin *monoclonal* antibody. Therefore, the effect of the length of time of formalin fixation on antigen recognition by antibodies is an issue that needs to be assessed individually for each antibody used. Although current recommendations are to remove tissue for immunostaining after 24 h of formalin fixation, the current study indicates that tissue can remain in formalin for 3 d, and possibly as long as 7 d, without negatively affecting even vimentin detection. In addition, a recent study showed that vimentin immunoreactivity in formalin-fixed tissues can outlast the antigen of interest,[1] further confirming the need to individually assess the antibodies used rather than assuming that vimentin immunoreactivity correlates with the immunoreactivity of all antigens in formalin-fixed tissues.

As expected, formalin fixation interfered with the ability to detect viral DNA in tissues by using standard PCR assay. This assay detected viral DNA in skin from the site of inoculation and in CNS tissues fixed for only 24 h. However, after 24 h, the ability to detect viral DNA in the skin was lost, and viral DNA could not be detected in CNS tissues after 3 d of formalin fixation. The ability to detect viral DNA in CNS tissues after 3 d of formalin fixation as compared to only 1 d of fixation of the skin is consistent with the original hypothesis that the affects of formalin fixation of CNS tissues is delayed in whole-carcass fixation. Viral DNA was detected by standard PCR from fresh tissue sections that were processed for paraffin embedding (without formalin fixation); however, viral DNA could not be detected by the standard PCR technique in sections obtained from formalin-fixed, paraffin-embedded tissues.

The development of real-time PCR technology coupled with contemporary methods of laser-assisted microdissection has allowed quantitation of gene expression with precise tissue localization. Furthermore, the typically small size of real-time PCR amplicons (50 to 150 bp) is well below the expected fragmentation size (300 to 400 bp) of DNA that occurs secondary to formalin fixation.[7] Consistent with this theory, viral DNA was detected by real-time PCR in all formalin-fixed, paraffin-embedded tissue samples, even tissues fixed for as long as 14 d. The limitation of the real-time PCR assay used in these studies was extraction of quantitatively and qualitatively suitable DNA from paraffin-embedded sections. However, with DNA extraction optimized, real-time PCR offers an excellent mechanism to quantitate virus with precise anatomic localization provided by histologic sectioning.

There has been anecdotal evidence that microtome blades can become contaminated with nucleic acids during tissue sectioning and that this contamination can carry-over to subsequently sectioned tissues. The results of this study confirmed this suspicion. We found that viral DNA was detectable by both standard and real-time PCR assays in known-negative tissues that were sectioned immediately after sectioning of virus-positive tissue. This finding underscores the importance of sectioning order and of changing microtome blades when using PCR techniques to screen paraffin-embedded tissues for viral DNA.

In summary, this study determines that tissues infected with herpesviruses can be safely handled after 24 h of formalin fixation of the whole carcass. Viral antigen detection was possible in tissues fixed in formalin for as long as 14 d with the polyclonal antisera used in these studies; however, long-term fixation might

induce antigen masking in other antibody–antigen reactions. Because vimentin immunostaining was unaffected in tissues held in formalin for as long as 3 d, we recommend 3 d of formalin fixation to ensure maximal inactivation of virus while retaining antigen integrity for IHC staining and achieving excellent fixation for tissue morphology. Inherent to any model system, there is a remote possibility that differences exist between the baboon virus and monkey B virus in regard to fixation time for inactivation of infectious virus in tissues. Furthermore, the detection of infectious virus in this study is limited to the sensitivity of virus isolation techniques. Therefore, the recommendations here are intended as guidelines and do not preclude the use of good laboratory practices, including attention to biosafety and personnel protection. Lastly, it is advisable to either clean or change microtome blades if using PCR to detect viral DNA in tissue sections, because cross-contamination can occur between serially sectioned samples.

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