Bovine papillomavirus DNA in neoplastic and nonneoplastic tissues obtained from horses with and without sarcoids in the western United States

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Objective—To determine the incidence of bovine papillomavirus (BPV) type 1 or 2 in sarcoids and other samples of cutaneous tissues collected from horses in the western United States.

Animals—55 horses with sarcoids and 12 horses without sarcoids.

Procedure—Tissue samples (tumor and normal skin from horses with sarcoids and normal skin, papillomas, and nonsarcoid cutaneous neoplasms from horses without sarcoids) were collected. Tissue samples were analyzed for BPV-1 or -2 DNA, using a polymerase chain reaction (PCR) and restriction fragment length polymorphism. The PCR products from 7 sarcoid-affected horses were sequenced to evaluate percentage homology with expected sequences for BPV-1 or -2.

Results—Most (94/96, 98%) sarcoids contained BPV DNA. Sixty-two percent of the tumors examined had restriction enzyme patterns consistent with BPV-2. Thirty-one of 49 (63%) samples of normal skin obtained from horses with sarcoids contained BPV DNA. All samples subsequently sequenced had 100% homology with the expected sequences for the specific viral type. All tissues from healthy horses, nonsarcoid neoplasms, and papillomas were negative for BPV DNA.

Conclusions and Clinical Relevance—Bovine papillomaviral DNA was detected in essentially all sarcoids examined. There appears to be regional variation in the prevalence of viral types in these tumors. The fact that we detected viral DNA in normal skin samples from horses with sarcoids suggests the possibility of a latent viral phase. Viral latency may be 1 explanation for the high rate of recurrence following surgical excision of sarcoids. (*Am J Vet Res* 2001;62:741–744)

Sarcoids in horses were first described in 1936.[1] They are the most common tumor in horses.[2] Clinical characteristics of sarcoids vary widely from benign verrucous tumors to locally aggressive ulcerative fibroblastic lesions that can spread along nerve sheaths and fascial planes. Tumors commonly recur after surgical excision. Spread to regional lymph nodes is rare, and distant metastasis has not been reported. Histopathologically, sarcoids are fibroblastic tumors with disorganized well-differentiated fibroblasts and pseudoepitheliomatous hyperplasia.[3,4] Histopathologic diagnosis of sarcoids requires evidence of dermal and epidermal abnormalities.

Although the mechanism of transmission is unknown, epizootics of sarcoids in horses support an infectious cause.[5] One of the first reports[6] in which an infectious cause for sarcoids was proposed involved a large herd of donkeys; animals housed in close association with sarcoid-affected donkeys had increased risk for developing tumors, compared with those housed separately from affected donkeys.

Bovine papillomaviruses (BPV) were first postulated to play a role in sarcoid development in horses in the early 1950s when investigators reported that experimental infection of horses with virally induced wart extracts of cattle produced lesions that had many characteristics of naturally developing sarcoids.[7] Bovine papillomaviruses are small DNA viruses and are members of the family Papovaviridae. There are 6 subtypes of BPV, which are divided into 2 groups (A and B). Viruses in subgroup A can transform fibroblasts and epithelial cells, whereas viruses in subgroup B can only transform epithelial cells. In cattle, natural infection with BPV is common and typically results in development of a wart or papillomatous lesion. These papillomatous lesions contain large numbers of viral particles and shed the virus.

Lesions in horses that are experimentally induced by use of wart extracts appear similar to sarcoids[7]; however, there is a lack of epithelial proliferation and an increase in inflammatory infiltrates in many of the experimentally induced lesions, compared with naturally developing sarcoids. Furthermore, all of the experimentally induced tumors regressed without treatment (spontaneous regression rate of 100%). Although wart extracts of cattle are capable of inducing warts in cattle and sarcoid-like tumors in horses, sarcoid extracts are not. It is conceivable that the inflammatory response and high rate of regression in the experimentally induced tumors may reflect differences between natural and experimental infections.

Bovine papillomavirus-like DNA has been detect-

Received Apr 10, 2000.
Accepted Jun 15, 2000.
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Supported by the Center for Equine Health, University of California, Davis, Calif.
Presented in part at the 17th Annual Forum, American College of Veterinary Internal Medicine, Chicago, Ill, May 1999.

ed in most sarcoid tumors surveyed by use of a **polymerase chain reaction (PCR)** and Southern hybridization techniques.[8-10] The vast majority of tumors evaluated in those studies contained BPV-1-type DNA, with the minority containing BPV-2-type DNA. Bovine papillomavirus-1 and -2 are members of group A and infect fibroblasts and epithelial cells. Those studies used tumors collected from horses in specific geographic regions, including the eastern United States, Germany, and Switzerland. The objective of the study reported here was to determine the relative frequency of BPV-1 and -2 DNA in tissues from horses in the western United States with and without sarcoids.

Material and Methods

Sample collection—Tissue specimens were obtained from horses examined at the University of California-Davis Veterinary Medical Teaching Hospital. We also used tissue specimens sent to us by referring veterinarians. We obtained 1 or more samples of sarcoid tissue from horses with sarcoids. Whenever possible, 2 samples of normal skin were collected from horses with sarcoids: 1 from an area located 3 to 4 cm from the tumor (**near normal skin [NNSK]**) and another from a site a minimum of 20 cm from any tumors (**distant normal skin [DNSK]**). We obtained samples from 96 sarcoids and 49 samples of normal skin from 55 horses with sarcoids for evaluation. In addition, we obtained samples from other sources, including 12 horses with nonsarcoid tumors. These included 7 samples of normal skin (6 horses), 8 warts (5 horses and 3 cattle), 2 melanomas (2 horses), 1 squamous cell carcinoma (1 horse), and 1 sample of lymphoma tissue (1 horse) for evaluation for BPV DNA.

An additional experiment was designed to assess the possibility that the surface of samples of normal skin was contaminated with viral DNA shed from tumor beds and could cause false-positive results. Swab specimens of skin were collected from 4 sites on 1 sarcoid-affected horse. A sterile swab was moistened with sterile saline (0.9% NaCl) solution and vigorously rubbed on the skin surface immediately prior to biopsy of the same site. Swab specimens were placed in a sterile container and processed for DNA extraction.

Tissue processing and DNA extraction—All tissue and swab specimens were immediately fixed in 10% formaldehyde and routinely processed (serially dehydrated in alcohol solutions and embedded in paraffin). Extraction of DNA was performed. Sections (50 µm) were cut from each tissue and placed on noncoated slides.[a] The microtome blade was cleaned with a RNase solution[b] between samples to prevent cross contamination. In addition, a 5-µm section was cut from each tissue and stained with H&E. The area (tumor tissue or normal skin) was examined on the H&E slide, and the corresponding area was excised from the 50-µm section and placed into a sterile 1.5-ml tube. Tissues were deparaffinized by addition of 1 ml of xylene and gentle rocking for 20 minutes. Xylene was decanted, and the process was repeated. After removal of paraffin, xylene was removed, using 2 serial washes with 100% ethanol. A vacuum desiccator was used to dry tissue samples prior to DNA extraction. Tissues were mechanically disrupted after addition of 100 µl of a digestion buffer (50 m*M* tris, 1 m*M* EDTA, 0.5% Tween 20) containing 2 µl of Proteinase K (10 µg/µl) and incubated overnight at 65 C. The following day, all samples were heated to 95 C to inactivate enzymes and centrifuged (14,000 × g) to pellet remaining debris. Quantity of DNA was determined by spectrophotometric absorbance on a spectrophotometer set at 260 nm.

Polymerase chain reaction—The PCR initially was used to assess equine genomic DNA in all samples except the wart sections obtained from cattle. For the PCR, 10 µl of DNA was added to 90 µl of reaction mix containing 20 pmol of each primer, 2 units of DNA polymerase,[c] 100 µmol each of dATP, dCTP, dTTP, and dGTP, and 0.5 to 1.25 m*M* $MgCl_2$, 0.1% Triton X-100, 50 m*M* KCl, and 10 m*M* tris-HCl. Amplification was carried out for 40 cycles (1 minute at 95 C, 1 minute at 55 C, and 2 minutes at 72 C) followed by a final 10-minute extension at 72 C. Amplification of a segment of the N-*ras* gene, using primers reported elsewhere,[11] was used to confirm equine genomic DNA.

The same PCR protocol then was used to detect BPV DNA. Primers that have been used to amplify a region of the E5 gene in BPV-1 and -2 were used to amplify fragments (244 and 248 **base pairs [bp]**, respectively).[8] A positive-control sample consisting of wart DNA from cattle as well as negative-control samples of deionized water and wart DNA from horses were included in every amplification. All amplification products were electrophoresed on a 3% agarose gel and stained with ethidium bromide. A 123-bp λ ladder was included on all gels to estimate fragment length. The PCR amplification products from 7 tumors and skin samples were sequenced to determine percentage homology with the expected viral sequence.

Restriction fragment length analysis—Restriction fragment length polymorphism (RFLP) was used to determine the subtype of BPV in tissue specimens. The PCR products were incubated with restriction endonucleases Hinf-I[d] and Bst-XI.[e] Briefly, enzymes were incubated with PCR products for 1 hour (2.6 U of Hinf-I/µg of DNA at 37 C or 30 U of BST-XI/µg of DNA at 45 C) in incubation buffer (5 m*M* tris-HCl, 10 m*M* NaCl, 1 m*M* $MgCl_2$, 0.1 m*M* dithioerythritol, pH 7.5). After incubation, samples were electrophoresed on a 3% polyacrylamide gel and stained with ethidium bromide. A 123-bp λ ladder was included on all gels to determine fragment length. The restriction endonuclease BST-X1 cleaved the 244-bp amplified fragment from BPV-1 into 2 fragments (130 and 114 bp, respectively) but did not cleave the amplified fragment from BPV-2. The restriction endonuclease Hinf-1 cleaved the 248-bp fragment amplified from BPV-2 into 2 fragments (129 and 119 bp, respectively) but did not cleave the amplified fragment from BPV-1.

Sequencing of PCR products—The PCR product (90 µl) was purified, using a commercially available kit.[f] Ten microliters of the resulting concentrated product was sequenced in both directions with 3 µmol of each of the original PCR primers, using an automated sequencing system. Briefly, samples were processed with a commercially available sequencing reaction kit.[g] Data were analyzed, using sequencing software.[h]

Results

We evaluated 161 equine tissue specimens. The N-*ras* primers successfully amplified a fragment of the expected size from all equine tissues. The majority (94/96, 98%) of specimens of sarcoids collected from 55 horses contained BPV DNA. All sarcoid-affected horses had at least 1 sarcoid with positive results, except for 1 horse from which only a single sample was obtained, and the result for that specimen was negative. All 4 tissue samples from nonsarcoid cutaneous neoplasms were negative for BPV DNA, including a sample from a squamous cell carcinoma removed from a horse that also had 2 sarcoids in the same vicinity as the carcinoma. Other BPV-negative tumors included 2 melanomas and a cutaneous lymphoma. All of the 5 papillomas and 7 samples of normal skin from horses without sarcoids were negative for BPV DNA.

We evaluated 49 samples of normal skin from 23 sarcoid-affected horses. Of these, 31 (63%) contained BPV DNA. Location of the skin biopsy was recorded for 46 samples (30 NNSK and 16 DNSK). Of the 31 samples with positive results, 22 were NNSK, and 8 were DNSK; location was not recorded for the other sample with positive results. We collected DNSK and NNSK samples from 11 horses. Of this subset, both samples yielded positive results in 4 horses, only the NNSK sample had positive results in 3 horses, only the DNSK sample had positive results in 1 horse, and both samples had negative results in 3 horses.

Sixty-five percent of the normal skin samples obtained from sarcoid-affected horses were positive when tested for BPV DNA. These results differ from reports[8-10] in which normal skin and other tissues from sarcoid-affected horses were consistently negative when tested for BPV DNA. To rule out the possibility that sections were contaminated during processing, all normal skin samples were sectioned, extracted, and amplified again during a separate experiment; however, results were consistent with the initial findings. In addition, to rule out the possibility that DNA shed from the surface of a sarcoid may have contaminated biopsy specimens of normal skin, swab and biopsy specimens were collected from 4 locations on a sarcoid-affected horse. Specimens were collected sequentially from areas that were progressively closer to the tumor bed. These swabs specimens were collected immediately prior to biopsy of the same site. In all 4 sites, the biopsy specimens were positive when tested for BPV DNA, and the swab specimens were negative when tested for BPV DNA.

Of the 54 horses with BPV-positive sarcoids, 53 were analyzed by use of RFLP to determine the specific viral type. Twenty (38%) had sarcoids that contained only BPV-1 DNA, 29 (55%) had sarcoids that contained only BPV-2 DNA, and 4 (7%) had sarcoids that contained both types of BPV DNA. Sequence analysis of the PCR product from 7 tumors revealed 100% homology with the expected subtype.

Discussion

Ninety-four of 96 (98%) sarcoids from horses in the western United States contained BPV DNA. These results are consistent with those for sarcoids from other geographic regions in which 88 to 100% of sarcoids contained BPV DNA.[8-10]

Most investigators have found a preponderance of BPV-1 DNA, with BPV-2 DNA found in only 8 to 27% of sarcoids examined.[9,10,12] In the study reported here, 62% of sarcoids had BPV-2 DNA. A small number of horses appeared to have both viral subtypes in the lesions, which is consistent with other reports. This geographic variation also has been reported in a study[12] in which investigators examined sarcoid tissue from horses in the midwestern United States. Whether the type of BPV found is of importance with respect to the type of sarcoid or its clinical progression remains unclear at this time. We were unable to obtain historical information regarding many of the sarcoids evaluated in our study. It would be interesting to prospectively evaluate the clinical characteristics of a population of sarcoids to determine whether viral type correlates with tumor progression, treatment success, or other clinical variables.

For a negative-control sample, specimens of papillomas from horses were incubated with the BPV E5 primers. In all cases, amplification products of appropriate size were not observed, supporting the conclusion that equine papillomavirus was not the agent amplified from these sarcoids.

All of the amplification products that were subsequently sequenced had 100% homology with the expected BPV subtype, as determined on the basis of RFLP results, further supporting the conclusion that BPV and not another variant was in the tissues tested. Both viral subtypes are members of the group-A BPV, are capable of infecting fibroblasts and epithelial cells, and can induce fibropapillomas in cattle. In situ hybridization for BPV DNA of tissue sections from sarcoids revealed viral DNA in the dermal layer within the fibroblasts of the tumor and not within the epithelial cells.[12,13] Although sarcoids of horses contain epithelial and fibroblastic changes, they are classified as fibroblastic tumors. Epithelial changes seen in sarcoids likely are a result of growth-promoting factors expressed on the neoplastic fibroblasts that stimulate proliferation of surrounding epithelial cells.

In contrast to the findings of others, BPV DNA was detected in 65% of the samples of normal skin obtained from sarcoid-affected horses. In all cases, biopsy specimens were interpreted as normal skin without evidence of neoplasia or an increase in the proliferative fraction of cells. It is possible that during sectioning of the tissues a small piece of neoplastic tissue that projected into the biopsy specimen of normal skin may have been missed; however, this is unlikely, because even the NNSK samples were collected at a location a minimum of 3 to 4 cm from the tumor margin. Projections of neoplastic tissues would not explain the number of samples of normal skin with positive results in the DNSK.

To attempt to control for contamination, all samples of normal skin were reexamined in a separate experiment that excluded tissues from sarcoids. In all cases, the results were repeatable. The type of BPV DNA found in the normal skin was always the same as the type found in the sarcoid tissues from the same horse. Although there was a higher percentage (75%) of skin samples with positive results in the NNSK group, a substantial number (50%) of samples had positive results in the DNSK.

An additional experiment was performed in an effort to rule out the possibility that viral DNA shed from the surface of a sarcoid may have contaminated the surrounding normal skin, creating a false-positive result. Swab specimens collected from the site of a skin biopsy immediately prior to obtaining a biopsy specimen were negative when tested for viral DNA, whereas the biopsy specimens had positive results. If viral DNA was shed and contaminated the skin surface, we would have expected to amplify viral DNA from the swab specimens as well as the subsequent biopsy specimens. Given that BPV DNA is localized to dermal fibroblasts, shedding of infected cells from the superficial epithelium would be unlikely.

Although quantitative PCR was not performed, there appeared to be less viral DNA amplified from normal skin than from sarcoid tissues. The importance of this finding is unknown, but perhaps the virus maintains itself as a latent low-copy number plasmid in normal skin. If this is the case, then viral infection alone is not sufficient for induction of a sarcoid. Some other factor such as cell injury via trauma or another initiator of transformation may be required for development of an overt sarcoid. A multiple-step progression also may explain the high incidence of transformation of sarcoids to more malignant phenotypes after inadequate treatment or excision. Detection of BPV DNA in normal skin could suggest that viral infection is an incidental finding and not important for the progression of sarcoids toward malignancy in horses. However, none of the skin samples from clinically normal horses or horses with other types of skin tumors contained BPV DNA. In humans, papillomavirus infection of cervical epithelial cells is a risk factor for development of cervical neoplasia.[14] Viral infection of cervical epithelia increases the risk for but does not directly result in neoplastic transformation. Infection with human papillomavirus is considered 1 of the initiating factors in a multiple-step progression toward neoplastic transformation and development of cervical cancer in women. Similarly, it is conceivable that BPV may infect the skin of horses and remain in a latent phase within the fibroblasts of the dermis until some other factor triggers transcriptional activation. This may explain the high incidence of tumor recurrence after surgically complete excision, because surgical trauma may induce proliferation and expression of latent virus, resulting in regrowth of the tumor. To support this hypothesis, we recently reported expression of a viral transforming gene in sarcoids of horses but not in DNA-positive samples of normal skin.[15] Triggering factors remain to be elucidated. Increased risk for sarcoids at sites of previous injury suggests that stimulation of cellular proliferation may be involved in viral activation. Alternatively, a genetic predisposition could result in an insufficient immune response against virally infected tumor cells, a loss of cell-cycle regulation, or an increase in cellular proliferation, all of which could be important in a multiple-step progression toward development of a sarcoid.

The fact that we found BPV DNA in nearly all sarcoids in these horses is consistent with other reports, and viral infection and transcription of transforming genes is likely important in this neoplastic transformation. Geographic variation appears to exist regarding the incidence of specific types of BPV. Detection of viral DNA in normal skin from sarcoid-affected horses raises the question of viral latency. Additional factors are likely to be required for viral activation or neoplastic transformation.

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[a]Superfrost/Plus, Fisher Scientific, Pittsburgh, Pa.
[b]RNase Away, Molecular Biologic Products, San Diego, Calif.
[c]*Taq* DNA polymerase, Promega, Madison, Wis.
[d]Hinf-I, Boehringer Mannheim, Indianapolis, Ind.
[e]Bst-XI, Boehringer Mannheim, Indianapolis, Ind.
[f]QIAquick PCR purification kit, QIAGEN, Valencia, Calif.
[g]ABI PRISM dye terminator cycle sequencing ready reaction kit with Ampli *Taq* DNA polymerase FS, Perkin-Elmer/ABI, Foster City, Calif.
[h]ABI PRISM A sequencing 2.1.1 software, Perkin-Elmer/ABI, Foster City, Calif.

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