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# Frequency of *Equus caballus* papillomavirus in equine aural plaques

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**Abstract.** Despite the reported association between aural plaques and the presence of *Equus caballus* papillomavirus (EcPV), there are few data regarding the distribution of viral types in different geographic regions or possible correlations for different papillomaviruses and lesion characteristics. We detected the presence and frequency of EcPV (1–7) DNA in aural plaque biopsies of horses from different regions of Brazil and identified the patterns of these infections or coinfections and their possible association with lesion severity. A total of 108 aural plaque biopsies from horses in the 5 geopolitical regions of Brazil were examined. We performed PCR to detect EcPV DNA in the biopsies. At least 1 type of EcPV was detected in 97% of the samples. EcPV coinfection was observed in 59% of the samples. Compared to the other viruses, EcPV-4 was found at the highest frequency in coinfection (84%) or individually identified (32%). EcPV-2 and -7 were not detected. No significant association was found between lesion characteristics (type and distribution) and either the viral type detected or the presence of coinfection. EcPV is widely distributed in Brazil, both isolated and in coinfection; the viral type does not appear to influence the clinical characteristics of equine aural plaques.

**Key words:** Dermatology; *Equus caballus* papillomavirus; horses; PCR.

An aural plaque is a variation of cutaneous papillomatosis. The lesion is characterized by well-demarcated, bright, erythematous or depigmented tissue that can be observed in the inner aspect of one or both ears of a horse, and can exist as individual, multiple, or coalescent forms.[14] Affected horses may be asymptomatic or show some degree of ear sensitivity.[18,21] Additionally, horses with aural plaques can lose commercial value and are not allowed to participate in some exhibitions, competitions, and auctions in Brazil.[15]

Despite the previously reported association of aural plaques with the presence of *Equus caballus* papillomavirus (EcPV) 3, 4, 5, and 6,[6,8,9,17,20,21] few data are available regarding the distribution of viral types, the presence of coinfection, and the relationship with clinical presentation, either in different regions of Brazil or worldwide. Furthermore, the range of EcPV types screened is wider than that reported in previous studies.[6,8,9,17,20,21] Such data may be useful for a global perspective on the possible distribution of EcPV variability in horses, particularly considering that Brazil is a large country with 5 different regions, each one similar in size to some countries, and contains different horse breeds, management strategies, and climates. Accordingly, we detected the presence and frequency of EcPV (1–7) DNA in aural plaque biopsies from horses from different regions of Brazil and assessed associations between the presence of EcPV, including coinfection with more than one type, and ear lesion characteristics.

Our study was performed in accordance with the policies of the Institutional Animal Care and Use Committee (120/2013-CEUA) of São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, Botucatu, Brazil. Horses were selected according to the availability of their owners, who approved all procedures performed on their animals. A total of 108 aural plaque biopsies were collected from the ears of naturally infected horses of a median age of 5 y (1–22 y). Aural plaque diagnosis was made by clinical evaluation. Both sexes and various breeds of horses used for work, exhibition, sport, and recreation in the 5 geopolitical regions of Brazil (north, northeast, midwest, southeast, and south) were included in our study. Horses that were overly reactive to manipulation were sedated with xylazine hydrochloride (Sedomin, König do Brasil, Mairinque, São Paulo, Brazil; 1 mg/kg body weight, IV). The aural plaque biopsy from one ear per animal was obtained using a disposable, sterile 6-mm punch, without prior ear disinfection;

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**Table 1.** Frequency (%) of *Equus caballus* papillomavirus (EcPV) in equine aural plaque biopsies in the studied regions throughout Brazil.

| Region | EcPV-positive | EcPV-1 | EcPV-3 | EcPV-4 | EcPV-5 | EcPV-6 |
|---|---|---|---|---|---|---|
| Midwest | 94 (16/17) | 12 (2/17) | 29 (5/17) | 94 (16/17) | ND (0/17) | ND (0/17) |
| North | 100 (22/22) | 59 (13/22) | 100 (22/22) | 96 (21/22) | ND (0/22) | 14 (3/22) |
| Northeast | 100 (22/22) | 82 (18/22) | 41 (9/22) | 91 (20/22) | ND (0/22) | 5 (1/22) |
| South | 90 (18/20) | ND (0/20) | 30 (6/20) | 50 (10/20) | ND (0/20) | 40 (8/20) |
| Southeast | 100 (27/27) | 15 (4/27) | 15 (3/27) | 89 (24/27) | 7 (2/27) | 26 (7/27) |
| Average frequency | 97 (105/108) | 34 (37/108) | 42 (45/108) | 84 (91/108) | 2 (2/108) | 18 (19/108) |

Numbers in parentheses are no. of positive samples/total no. of samples. ND = not detected.

the samples were frozen in liquid nitrogen and stored at −80°C until DNA extraction. After the biopsy, topical insect repellents were applied to the biopsied site. In addition, recently published data (clinical characteristics of the lesion) on the same group of horses[11] were analyzed in conjunction with the results of EcPV detection obtained in our study.

DNA extraction was performed on 20 mg of sample (macerated in liquid nitrogen; GenElute mammalian genomic DNA miniprep kit, Sigma-Aldrich, St. Louis, MO) in accordance with the manufacturer's instructions. The DNA purity and concentration were determined by spectrophotometry (NanoDrop 2000, Thermo Scientific, Wilmington, DE), based on an A260/280 ratio of 1.8. Polymerase chain reaction (PCR) for EcPV-3, -4, -5, and -6 was performed as described previously.[20] For EcPV-1, -2, and -7, gradients were performed to establish the optimal annealing temperature for each primer pair. The primers used were described previously.[12,20] Each reaction consisted of a total volume of 25 μL and contained 2.5 μL of DNA template, 12.5 μL of GoTaq master mix (Promega, Madison, WI), 300 nM each forward and reverse primer, and 8.5 μL of ultrapure DNase- and RNase-free water. PCR sensitivity was determined using 10-fold dilutions of positive samples. To verify the presence of cellular DNA, PCR using equine *β-actin* gene primers[12] was also performed for all EcPV-negative samples. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

The PCR products were purified (GenElute PCR clean-up kit, Sigma-Aldrich), according to the manufacturer's instructions, and sequenced (3500 ABI genetic analyzer, Life Technologies, Carlsbad, CA). The resulting DNA sequences were analyzed with MEGA 6.[16] Sequences with >80% coverage and identity and an E-value close to zero were considered matches.

Using clinical data obtained by our group for horses affected with aural plaques,[11] the ear distribution of aural plaques (I: only 1 quadrant affected; II: 2 quadrants affected; or III: ≥3 quadrants affected) and the type of lesion (A: ears with 1–5 punctate lesions, B: ears with >5 lesions, or C: ears with coalescing aural plaque lesions) were evaluated. Associations were performed using this information and the presence of EcPV type and coinfection.

Descriptive statistics were applied to determine the median age of the horses studied (PROC MEANS, SAS v.9.4, SAS Institute, Cary, NC). A frequency distribution was determined for categorical data (viral type, coinfection, regions, and sex; PROC FREQ, SAS Institute). Using 2016 clinical data,[11] chi-squared and Fisher exact tests (PROC FREQ) were performed to determine associations linking the type of EcPV detected and the presence of coinfection to the type and distribution of the lesion. Additionally, Fisher exact test was employed to test for significant differences in viral frequency between pairwise combinations of regions (PROC FREQ).

Of the studied horses, 58% (63 of 108) were female and 42% (45 of 108) male; the median age was 5y (range: 1–22 y). The biopsies were taken from 108 of the 164 ears of the same group of horses clinically reviewed in our recently published data.[11]

Of the biopsies analyzed, 97% (105 of 108) were positive for at least one viral type (Table 1). Coinfection was observed in 59% (62 of 105) of the samples (Table 2). No significant association was found when assessing lesion characteristics (type and distribution), EcPV type detected, or coinfection presence (Table 2). The *β-actin* gene was amplified in all EcPV-negative samples ($n = 3$), confirming the presence of cellular DNA in these samples. PCR for EcPV-1, -3, -4, -5, and -6 was able to detect DNA at dilutions up to $10^{-2}$, $10^{-6}$, $10^{-8}$, $10^{-2}$, and $10^{-4}$, respectively.

Numerous studies have characterized the prevalence of papillomavirus in humans, but few have assessed the frequency of domestic animal papillomaviruses, especially equine papillomaviruses. Of these, studies to determine the seroprevalence or genoprevalence of EcPV-2 have been conducted in horses,[3] and other studies also have detected EcPV at different horse anatomical sites, such as genital plaques (EcPV-4),[8] aural plaques (EcPV-5 and -6),[8] penile masses (EcPV-7),[8] and penile and preputial squamous cell carcinoma (EcPV-2 and -3).[19] In 3 previous studies conducted in Brazil, the presence of papillomavirus was detected in 61% (28 of 45),[6] 52% (11 of 21),[20] and 100% (11 of 11)[21] of the equine aural plaque samples analyzed. However, in the first study,[6] the authors assessed only EcPV-3 and -4, and all 3 studies involved samples only from São Paulo State.[6,20,21]

EcPV-1 was previously identified in lesions of horses with facial papillomatosis[4] and in a single aural plaque sample.[20] In our study, this viral type was detected in 34% (37 of 108) of EcPV-positive biopsies; however, it was always detected in coinfections with other equine papillomavirus types. Although EcPV-1 was found to be the third most prevalent virus, it is difficult to determine whether it participated in the development

**Table 2.** Frequency (%) of the distribution and type of aural plaque lesions according to *Equus caballus* papillomavirus (EcPV) detection.

| EcPV infection type(s) | Frequency | Quadrant affected | | | Type of lesion | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | A | B | C |
| 3 | 2 (2/105) | 50 (1/2) | — | 50 (1/2) | 100 (2/2) | — | — |
| 4 | 32 (34/105) | 38 (13/34) | 18 (6/34) | 44 (15/34) | 41 (14/34) | 21 (7/34) | 38 (13/34) |
| 6 | 7 (7/105) | 43 (3/7) | 29 (2/7) | 29 (2/7) | 57 (4/7) | 14 (1/7) | 29 (2/7) |
| 1 and 3 | 1 (1/105) | — | — | 100 (1/1) | — | 100 (1/1) | — |
| 1 and 4 | 12 (13/105) | 23 (3/13) | 54 (7/13) | 23 (3/13) | 62 (8/13) | 15 (2/13) | 23 (3/13) |
| 3 and 4 | 16 (17/105) | 35 (6/17) | 24 (4/17) | 41 (7/17) | 53 (9/17) | 6 (1/17) | 41 (7/17) |
| 3 and 6 | 2 (2/105) | 50 (1/2) | 50 (1/2) | — | 50 (1/2) | — | 50 (1/2) |
| 4 and 5 | 1 (1/105) | — | — | 100 (1/1) | — | — | 100 (1/1) |
| 4 and 6 | 3 (3/105) | — | — | 100 (3/3) | — | 33 (1/3) | 67 (2/3) |
| 1, 3, and 4 | 16 (17/105) | 41 (7/17) | 41 (7/17) | 18 (3/17) | 65 (11/17) | 24 (4/17) | 12 (2/17) |
| 1, 3, and 6 | 2 (2/105) | 50 (1/2) | — | 50 (1/2) | 50 (1/2) | — | 50 (1/2) |
| 1, 4, and 5 | 1 (1/105) | — | — | 100 (1/1) | — | — | 100 (1/1) |
| 1, 4, and 6 | 1 (1/105) | — | — | 100 (1/1) | — | — | 100 (1/1) |
| 3, 4, and 6 | 2 (2/105) | 100 (2/2) | — | — | 100 (2/2) | — | — |
| 1, 3, 4, and 6 | 2 (2/105) | 50 (1/2) | 100 (1/2) | — | 100 (2/2) | — | — |

Quadrant affected: I = only 1 quadrant affected; II = 2 quadrants affected; III = 3 or more quadrants affected. Type of lesion: A = ears with 1–5 punctate lesions; B = ears with >5 lesions; C = ears with coalescing aural plaque lesions. Numbers in parentheses are no. of positive samples/total no. of samples. Dash (—) indicates not observed.

of the lesions or whether it was simply present whereas another type of EcPV was the actual cause of the lesions. Conversely, EcPV-2 and -7 were not detected. EcPV-5 was detected in only 2% of the samples (2 of 108), always with another type of EcPV; this very low rate of detection suggests that EcPV-5 did not cause the lesion in these horses. In this sense, the prevalence rates reported herein and in previous studies[8,21] show that both EcPV-2 and -7 are not likely to be etiologic agents of equine aural plaques. EcPV-4 was found at the highest frequency (84%) followed by EcPV-3 (42%), and EcPV-6 was detected in 18% of the samples (Table 1). EcPV-3, -4, and -6 were individually identified at frequencies of 2% (2 of 105), 32% (34 of 105), and 7% (7 of 105), respectively. The most commonly found coinfections were EcPV-3 + -4 and both with EcPV-1. EcPV-4 was also detected at a significantly higher rate compared to other viruses in all lesion types ($p < 0.0001$), with a higher distribution ($p < 0.0001$). This finding suggests that this virus may be the most pathogenic EcPV associated with equine aural plaques,[6,20] although further investigation is required because there is no consensus as to whether the presence of the virus plays a direct role in development of the lesion. Other techniques such as in situ hybridization are necessary to better elucidate this condition. EcPV-3 and -6 were also individually detected, albeit in a smaller number of horses, which may suggest pathogenic capacity to establish infection. Again, additional studies are needed to address this hypothesis.

To date, coinfection with different types of bovine papillomavirus (BPV) is mainly reported for domestic animals. Of 72 samples, coinfection was detected in 89% of BPV infections in Brazil.[2] Additionally, coinfection with EcPV-2 and BPV-1 was found in a pony with squamous cell carcinoma.[7] Similar to the findings of these studies, a rate of EcPV coinfection of 59% (62 of 105) was observed in our study; in another study by our group using fewer samples, the rate of coinfection was 65% (11 of 17).[20] The impact of coinfection on the development of papillomavirus lesions in humans has not been fully determined.[5,10]

Host coinfections have been proposed to exist in mutualism or in competition,[10] with the latter being reflected by a low frequency of coinfection, in contrast to a high frequency in the former.[10] Following this logic, the high frequency of coinfection found in our study may reflect synergism of the viruses. Nevertheless, in such a cross-sectional study, it is difficult to determine whether viruses initiate the infection together or whether they are acquired separately.[10] Moreover, with regard to humans, both women[10] and men[13] infected with one type of papillomavirus are at a higher risk of infection with a second type. This could be the case for horses infected with EcPV-4, the most frequently observed EcPV type, either individually or in coinfection. However, some studies show that coinfections occur randomly, with or without a phylogenetic relationship between viruses.[1] Nonetheless, phylogenetic relationships between different types of papillomavirus found in the same lesion have been described.[5] It is also important to emphasize that EcPV-4 and -5 are from the same genus, *Dyoiotapapillomavirus*, and that EcPV-3 and -6 belong to the genus *Dyorhopapillomavirus*.[8] Furthermore, as found in our study, the presence of coinfection did not influence the clinical characteristics (type and distribution of the lesion) of equine aural plaques.

We included the 5 geopolitical regions of Brazil, and 97% of the aural plaque samples assessed were positive for at least

one viral type (EcPV-1, -3, -4, -5, and -6), demonstrating the wide distribution of the virus throughout the country. All analyzed samples from the southeast, northeast, and north regions were positive for EcPV. Although EcPV-3 and -4 were detected in all regions of Brazil, EcPV-3 was detected significantly more frequently in the north and EcPV-4 significantly less frequently in the south. In addition, compared to EcPV-3, EcPV-4 detection was significantly more frequent in the midwest, northeast, and southeast ($p = 0.0002$, $p = 0.0011$, and $p < 0.0001$, respectively). EcPV-5 was only detected in the southeast. EcPV-1 detection was significantly higher in the north + northeast ($p < 0.0001$) compared with midwest + southeast, and EcPV-6 detection was significantly higher in the south + southeast compared with north + northeast ($p = 0.0095$). Furthermore, only the southeast region contained all viral types (EcPV-1, -3, -4, -5, and -6), a finding that likely reflects this region's active and intensive horse market as well as intensive or semi-intensive management practices that have largely been adopted in this region, as in other countries worldwide.

The distribution of EcPV in Brazil may be influenced by many factors, such as weather, management, and the immunologic condition of the horses, although further studies are required to explain the mechanism of EcPV dissemination. Furthermore, using clinical data on the same horses studied herein, we recently showed a significant association between the characteristics of aural plaque lesions (type and distribution previously explained) and management factors, including intensive and semi-intensive management systems, as well as ear grooming.[11] Nevertheless, in the present study, no significant associations were observed between the type and distribution of lesions and the type of EcPV detected or the presence of EcPV with coinfection. Other studies comparing the clinical characteristics of aural plaques and their association with viral type were not found in the literature.

## Declaration of conflicting interests

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