Braz J Vet Pathol, 2019, 12(1), 1 – 4
DOI: 10.24070/bjvp.1983-0246.v12i1p1-4

BJVP
Brazilian Journal of Veterinary Pathology
ISSN 1983-0246

Short communication

Equine papillomavirus detection in aural plaques by qPCR

Luiza S Zakia[1], Mariana Herman[1], Roberta M. Basso[1], Juliana M. Hernadez[2], Joao P. Araujo Jr.[3], Alexandre S. Borges[1], Jose P. Oliveira-Filho[*1]

[1]Department of Veterinary Clinical Sciences, School of Veterinary Medicine and Animal Science, São Paulo State University (Unesp), Botucatu/Sao Paulo/Brazil.
[2]Research Group in Veterinary Medicine (GIVET), School of Veterinary Medicine, Unilasallista-Corporación Universitaria Lasallista, Caldas-Antioquia-Colombia.
[3]Biosciences Institute, Botucatu, São Paulo State University (Unesp), Botucatu/Sao Paulo/Brazil.
***Corresponding author**: E-mail address: jose.oliveira-filho@unesp.br

Submitted May, 23rd 2018, Accepted November, 11th 2018

Abstract

Papillomatosis occupy a prominent position both in human and veterinary medicine, since it is a viral skin disease with potential to develop malignancy. *Equus caballus papillomavirus* (EcPV) are associated with several diseases in horses, i.e. classical papillomatosis associated with EcPV 1; squamous cell carcinoma associated with EcPV 2; tumors in mucous membranes on the genital area (EcPV 2 and EcPV 7); aural plaque associated with EcPV 1, 3, 4, 5 and 6; and equine sarcoid, associated with bovine papillomavirus (BPV 1 and 2). The aural plaque is characterized by small papules (1-2 cm), hypochromic and keratinized on the internal surface of the pinnae and can evolve and coalesce into larger lesions. To obtain a specific diagnostic test, both sensitive and fast to identify these viruses, a quantitative real time PCR (qPCR) was standardized for EcPV 3, 4, 5 and 6. Applying the qPCR technique in the 103 equine aural plaque samples resulted in 90.29% of at least one viral type prevalence, which was distributed as following: EcPV3, 36.89%; EcPV4, 82.52%; EcPV5, 0.97%; and EcPV6, 10.68%. This study represents an evolution in the area related to aural plaque and equine papillomatosis and raises new questions for future research.

Key words: horse, EcPV, real-time PCR, aural plaque.

Four *Equus caballus papillomavirus* (EcPV; i.e., 3, 4, 5 and 6); are frequently reported in auricular papilomatosis (1, 2, 11, 12), also known as aural plaque (6, 9). This is a dermatologic disease of the internal auricular pavilion that can be found unilaterally or bilaterally (8). It has benign character, without spontaneous regression, and can cause sensitivity on the ears (8, 11). It has verrucous aspect and can appear isolated, measuring 1 to 2 cm or in multiple lesions, coalescing and occupying the whole internal pavilion (6). The diagnosis can be made by histology, where the lesions are characterized by thickness of the epidermal layer associated to hyperkeratosis and hypomelanosis (8) and/or by conventional polymerase chain reaction (PCR) (12).

Currently, other three EcPV (i.e. 1, 2 and 7) have also been associated with dermatology disorders in horses (3, 7, 9). EcPV 1 belongs to Zeta-papillomavirus genera, whose biological properties are to create cutaneous lesions in horses (9, 10). Although the EcPV 1 DNA has been detected in aural plaque samples (2, 12), it is mainly associated with classic equine papillomatosis (7, 9), which is characterized by the appearance of keratinized lesions, normally on the muzzle and around the lips. Generally, occurs in young animals, more frequently younger than one year, with remission of the lesions in about three months (6). EcPV2 belongs to the Dyoiota genera and is correlated to genital papillomatosis and squamous cell carcinoma. This type of virus has been isolated from penile squamous cell carcinoma, intraepithelial penile neoplasia and penile papillomas. It was also detected on a latent form

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DOI: 10.24070/bjvp.1983-0246.v12i1p1-4

in the genital mucosa of healthy male horses. The EcPV 7, that belongs to the Dyorho genera, has also been identified in penile masses (9).

Aural plaque lesions can cause loss of economic value of the animals affected and the horses may even be prevented from participating in events and auctions in Brazil (8). Likewise, some animals present sensitivity of the ears (9, 12) generating problems with handling these animals. Therefore, the aim of this study was to standardize a diagnostic test for the EcPV 3, 4, 5 and 6, previously reported as mainly associated to equine aural plaque, using quantitative real time PCR (qPCR) methodology.

Samples were collected from 103 adult equines, with no breed, sex or coat color predilection and all presenting macroscopic lesions of aural plaque. The age of the animals varied between one and 22 years, (mean 6.5 years). A total of 60 females and 43 males distributed in 39 farms were used in this study. These farms were distributed in 21 cities, in nine states of the five regions of Brazil. All the procedures were approved by the Ethic Committee of the University (120/2013).

The biopsy procedure was performed with the horses sedated with intravenous detomidine hydrochloride (0.01 mg/Kg BW). The biopsies, one ear per animal, were performed with a 6-mm punch of the major lesion of the ear, without prior ear disinfection. A fly repellant was applied locally later. The tissue samples were snap frozen in liquid nitrogen and stored at –80ºC until DNA extraction.

Samples (20 mg) were macerated in liquid nitrogen for DNA extraction using the GenElute Mammalian Genomic DNA Miniprep (Sigma®) kit, following the manufacture's instructions. Then the eluted samples were spectrophotometry evaluated for quality and quantity (Nanodrop 2000); and stored at -20ºC until PCR procedure.

The specific primers and probes from a DNA fragment of L1 region of each viral type (EcPV 3, 4, 5 and 6) were designed using the PrimerExpress® (Applied Biosystems) program and tested in silico online at Blast® (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 1). The qPCR reaction was prepared with a final volume of 20 µL, being 10 µL of qPCR master mix (Go Taq® Probe qPCR Master Mix, Promega®), 1 µL of primer forward, 1 µL of primer reverse, 0.5 µL of the probe, 5.5 µL of nuclease free water and 2 µL of DNA sample. The qPCR was performed in a 7500 Fast Real Time PCR System thermocycler (Life Technologies), using an activation period of two minutes at 95ºC, followed by 40 cycles at 95ºC for 15s and 60ºC for 60s for EcPV 3 and 4 and 40 cycles at 95ºC for 15s and 62ºC for 60s for EcPV 5 and 6. In addition, the qPCRs specificities were also proven by direct Sanger sequencing of PCR products, as previously described (11), and the sequences obtained from each qPCR were identical to the EcPV (3, 4, 5 and 6) sequences deposited in the Genbank™.

Table 1. Primers and probes of the four viral types tested in this study.

Virus	Primer Forward 5'-3' (11)	Primer Reverse 5'-3' (11)	Probe 5'-3'
EcPV 3	TTGCGCCAGGTTTCCACATCTA	TTGTTCTGGCCTTGTGCACGTT	ACACCCAGTGGCTCTTTGGTTAGT
EcPV 4	ACAATGGTGTTTGCTGGCACGA	ACCGTGCAAAGCTGCAGAATGA	GGTGTCCAAAGATATGCGGCCTGTA
EcPV 5	GGCTCCGTAGACATTCCTAAAG	CTGTTACACCCACGCCTAAT	GGCATGGAAGTAGGACGAGGTCA
EcPV 6	CTACCAGAGGAACGAGCTTTAC	TAGCTCCCAATCCTCCAAGATA	AGGCATGTGGAGGAGTTTGAGCTT

The relative sensibility of the qPCR was analyzed using the seriated dilution curve (10X) with 11 points for each viral type, and a known positive sample in conventional PCR. All the samples with a cycle threshold (Ct) above the established one in each dilution curve were considered positive. For the EcPV 3, an amplification curve was obtained until the sixth point of dilution (10-6) (slope, -3.49; efficiency, 93.2%), making it possible to determine a Ct of 0.45 that was used to standardize the test and establish the Ct of each sample being considered positive for the EcPV 3 until 35. For the EcPV 4, an amplification curve was obtained until the eighth point of dilution (10-8) (slope, -3.49; efficiency, 93.3%), making it possible to determine a Ct of 0.2 that was used to standardize the test and establish the Ct of each sample being considered positive for the EcPV 4 until 38. For the EcPV 5, an amplification curve was obtained until the ninth point of dilution (10-9) (slope, -3.6; efficiency, 90.0%), making it possible to determine a Ct of 0.035 that was used to standardize the test and establish the Ct of each sample being considered positive for the EcPV 5 until 36. For the EcPV 6, an amplification curve was obtained until the sixth point of dilution (10-6) (slope, -3.58; efficiency, 90.2%), making it possible to determine a Ct of 0.2 that was used to standardize the test and establish the Ct of each sample being considered positive for the EcPV 6 until 38.

In total, 90.3% (93/103) of the samples were positive for at least one viral type. For the EcPV 3, 4, 5 and 6, 38/103 (36.89%), 85/103 (82.52%), 1/103 (0.97%) and 11/103 (10.68%) of the samples were positive, respectively. Types 3 and 4 were detected simultaneously in 35/103 (33.98%), types 3 and 6 in 3/103 (2.91%), types 4 and 5 in 1/103 (0.97%), types 4 and 6 in 4/103 (3.88%) and types 3, 4 and 6 in 2/103 (1.94%). To evaluate the reproducibility of the obtained results for the incidence of the EcPVs, the SAS 9.4 program was used, in which was obtained a trust interval of 0.273-0.465 for EcPV 3,

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0.7295-0.8863 for EcPV 4, 0.0002-0.0524 for EcPV 5 and 0.0540-0.1814 for EcPV 6.

Another study performed by the same group five years ago, evaluated the prevalence of EcPV 3 and 4 in 45 aural plaque samples, and 62.3% were positive, 8.89% for EcPV 3 and 37.78% for EcPV 4. When the results are compared, it is possible to see the increased incidence of both viral types. The percentage of positive samples in this study is higher than the previously one, possibly because of the inclusion of the detection of the EcPV 5 and 6. However, of the 103 animals tested in our study, 9.7% (10/103) were negative for the four viral types tested. Other authors (10) reported the growth number of papillomavirus in humans and animals and it has been described this especially in equine papillomatosis (3). The EcPVs tested in the present study were the types 3, 4, 5 and 6 since these are the most prevalent virus associated with aural plaque lesions (1, 2, 11, 12). EcPV 1 has been detected in aural plaque samples previously (2, 12); however, it was described in only one prevalence study and was never detected without another papillomavirus type, only in co-infections with one or more papillomavirus type, suggesting it does not necessarily participate in the development of the lesions (2).

As described previously in the literature, these viruses are partially resistant to the environment conditions and can be found in its latent form in healthy tissue. However, other authors have failed to detect the EcPV in samples from the internal pavilion from healthy animals (1).

According to the statistical analysis, all four viral types analyzed in this study showed a short confidence interval; therefore, a good precision and reproducibility. In order to test the relative sensitivity of the test, the results of these 103 samples tested with qPCR were compared with samples tested for conventional PCR (2) in another study performed by our group with the same samples. For the EcPV 4, four samples were positive only for the conventional PCR and two only for qPCR. Comparing both tests, the sensitivity of the qPCR was 97.70%, the specificity and positive predicted value were 100% and negative predicted value was 89.47%; the accuracy was 98.01%. For the EcPV 3 ten were positive only at the PCR and five at the qPCR; so, when both tests were compared, the sensitivity, specificity, positive predicted value and negative predicted value were 88.37%, 100%, 100% and 92.42%, respectively; and the accuracy was 95.19%. The only sample in each EcPV 5 was detected in conventional PCR, qPCR was also capable of detecting it. For the EcPV 6, nine were positive in PCR and only one in the qPCR; therefore, the sensitivity of the qPCR was 57.89%, the specificity and positive predicted value were 100% and negative predicted value was 91.40%; the accuracy was 92.31%. This analysis showed that conventional PCR tends to be more sensitive; however, qPCR was capable of detecting a few samples that tested negative for conventional PCR and still reached high sensitivity/specificity values. The present study failed to detect EcPV in 9.71% of the samples, while the other study (2) failed in only 3%. Although the previous study (2) also performed PCR for EcPV1, it most likely did not interfere with the relative sensitivity of qPCR when comparing both tests because this virus was not isolated alone in a sample, only being detected in coinfections.

Therefore, it is possible to conclude that both techniques are crucial to the complete diagnosis of the etiology of equine aural plaque and despite the relatively high economical costs of qPCR, it is extremely important in order to map the geographic location of each virus and its prevalence.

Acknowledgements

The authors acknowledge the support of the Fundação de Amparo a Pesquisa do Estado de São Paulo (Fapesp, 2013/15995-8), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 117049/2014-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

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