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First report of bovine papillomavirus genotypes in cutaneous lesions by polymerase chain reaction (PCR) and direct sequencing in Panama

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Bovine papillomavirus (BPV) causes benign tumours in the mucosal and cutaneous epithelium and is characterized by the presence of warts. The present study includes the molecular identification of BPV strains in samples of warts using degenerate polymerase chain reaction (PCR) primers FAP59/64. Skin biopsies were taken from crossbred cattle from two experimental farms of Instituto de Investigación Agropecuaria de Panamá (IDIAP) during the period from July 2016 to February 2018. Fourteen samples were positive by PCR amplification and sequenced at the laboratory of the Institute of Legal Medicine and Forensic Sciences (IMELCF). The analysis of the sequences allowed the identification of strains related to seven viral types; this was the first time that this type of study was carried out in Panama. The present study showed that PCR amplification with the primers FAP59/64, which partially amplify the L1 gene, followed by direct sequencing was useful for genotyping BPV. This study possibly identified local strains of BPV2 and BBA2; however, it is necessary to carry out more studies to establish the diversity and distribution of this virus in the country. The results in this study are important for the development of prophylactic and therapeutic measures that contribute to reducing the economic losses associated with BPV in Panama.

Key words: Livestock, biotechnology, molecular genetics, virology, animal health.

INTRODUCTION

Papillomavirus has been identified as the causative agent of benign and malignant neoplasms that infect epithelial tissue in humans and a wide variety of animals (Campo, 2003; Rector and Van Ranst, 2013; García-Pérez et al., 2014). It can present as a cutaneous papilloma, benign fibroplasia, urinary bladder tumour or oesophageal cancer, causing significant economic losses (Vázquez et al., 2012; Carvalho et al., 2013). In cattle, bovine papillomavirus (BPV) induces exophytic lesions (papillomas, warts) and flat lesions (flat warts, cervical intraepithelial neoplasia) in cutaneous and mucosal epithelia (de Villiers et al., 2004; Dyne et al., 2018).

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Lesions in general are benign and usually revert without causing any serious clinical problem in the host; however, they can persist without being eliminated from the animal. Persistent lesions can be debilitating and can become a focal point for malignant transformation to squamous cell carcinoma, particularly in the presence of environmental or genetic cofactors (Campo, 2003).

Although infections caused by BPV in cattle do not cause much damage, they produce great economic losses due to their impact on aesthetics and the quality of cattle in livestock shows and hinder the commercialization of products derived from animals infected with BPV, such as leather for the production of footwear and other clothing (Catroxo et al., 2013; Araldi et al., 2014). However, superinfections in lesions and milking difficulties when papillomas appear on the udders can cause considerable health and management complications, and finally, some genotypes are associated with the development of carcinogenic lesions (Campo et al., 1992; Borzacchiello et al., 2003).

BPV is usually transmitted by direct contact with infected animals and is introduced into the skin by cutaneous lesions. It is a disease of economic importance because it causes negative effects in cattle judging and sale because bovine papillomatosis causes a loss in body condition, particularly when there is secondary bacterial infection (Salib and Farghali, 2011). Warts present on the udder also interfere with the milking process (Radostitis et al., 2007). This disease affects animals older than 2 years; however, bovine animals of all ages can develop these lesions (Kumar et al., 2013). Immunosuppressive factors play a role in the progression of bovine papillomatosis, as mentioned by Radostitis et al. (2007), including internal and external parasites. Likewise, a high incidence of bovine enzootic leukosis virus has been observed in conjunction with BPV because of a probable co-infection, causing chromosomal aberrations in peripheral blood lymphocytes (Yaguiu et al., 2008)

BPV is a small, non-enveloped virus whose genome consists of a double-stranded DNA molecule (dsDNA), approximately 8 kb, within a T = 7 icosahedral capsid (da Silva et al., 2016); the dsDNA encodes early functional and structural proteins and late proteins, which are expressed in different phases of the viral cycle (Carvalho et al., 2013). The gene encoding the L1 protein is the most conserved within the genome and has been used by many research groups to identify new viral variants (Silva et al., 2013). To classify the types of BPV, the same gene coding for the L1 protein is used; a segment of the gene is amplified and, through sequencing and use of bioinformatic tools, is compared to reconstruct evolutionary relationships, expressed graphically as phylogenetic trees (Chan et al., 1995). Under this classification, the group of BPVs belongs to the C and D supergroup, ungulate fibropapillomas and fibropapillomas that cause true papillomas, respectively (Chan et al., 1995; Antonsson and Hanson, 2002). BPV belongs to the Papillomaviridae family, and several genera and species have been classified in cattle. Of the genus *Deltapapillomavirus* (considered high risk), viral types BPV1, BPV2, BPV13 and BPV14 belonging to species *Deltapapillomavirus 4* have been identified; of the genus *Epsilonpapillomavirus,* viral types BPV5 and BPV8 belonging to species *Epsilonpapillomavirus 1* have been identified; and of the genus *Xipapillomavirus,* viral types BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV15 and BPV23 belonging to species *Xipapillomavirus 1* have been identified and viral type BPV12 belonging to species *Xipapillomavirus 2* has been identified. There are species within this group, such as BPV17, BPV23 and BPV UFPE05BR, that have not been classified. The genus *Dyoxipapillomavirus* has 2 species; however, they have not been classified: BPV7 and BPV22. *Dyokappapapillomavirus,* BPV16, BPV18, and BPV22 are also without classification within this genus.

BPV1 and BPV2 have also been identified from equine sarcoids in horses as a result of transmission between species (Ataseven et al., 2016; Daudt et al., 2018).

Commonly, genetic characterization is performed by polymerase chain reaction (PCR) using degenerate primers (FAP59/FAP64) that amplify the L1 region of the virus, followed by sequencing of the products (Forslund et al., 1999).

In Panama, there are no diagnostic methodologies or information on the genotypes of circulating BPV, and due to the increasing rate of BPV in the world mainly affecting cattle, it is important to have techniques to identify and propose programmes for the prevention, control and eradication of the virus by identifying the circulating genotypes. The use of PCR using degenerate primers, followed by sequencing, has allowed the identification of several types of papillomavirus in humans and animals (Forslund et al., 1999; Antonsson and Hansson, 2002; Carvahlo et al., 2012).

The aim of this study was to develop a methodology for the diagnosis of bovine papillomavirus by PCR and direct sequencing and to identify genotypes present in cutaneous lesions compatible with the presence of the virus during the onset of an outbreak of this disease on 2 farms in 2 regions of the Republic of Panama.

MATERIALS AND METHODS

A total of 14 (S1 to S14) skin biopsy samples were taken from the neck, abdomen and back of animals with warts from the Carlos Manuel Ortega Experimental Station of the Agricultural Research Institute of Panama (Instituto de Investigación Agropecuaria de Panamá - IDIAP), located in Gualaca, province of Chiriqui, and from animals from the Experimental Station El Ejido, province of Los Santos, from July 2016 to February 2018. All samples were obtained from *Bos taurus* × *Bos indicus* crossbreeds. Each sample collected was immediately stored at -21°C until processing in the laboratory.

DNA extraction was performed using the commercial Quick Extract solution (Epicenter, USA) following the manufacturer's instructions, with some modifications. DNA concentration was

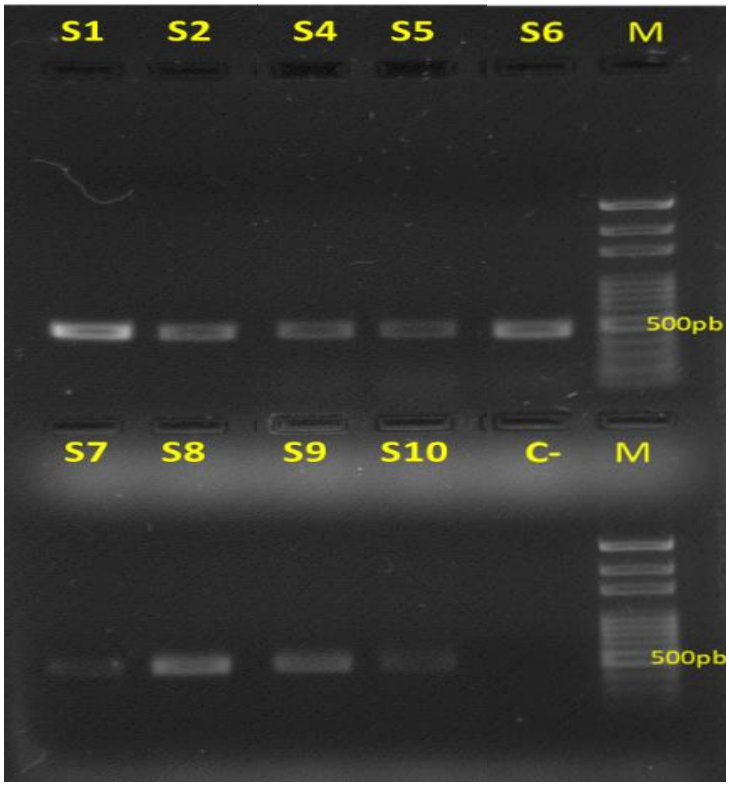

Figure 1. PCR products (478 bp) for the L1 gene of bovine papillomavirus in wart samples using primers FAP59/64. Samples S1, S2, S4 and S5 to S10 represent positive samples; line C represents the negative control, and M represents molecular weight markers from 100 to 1300 base pairs.

measured via absorbance at 260 nm using a spectrophotometer, and DNA quality was verified using a 1% agarose gel. PCR was performed in the region of the FAP gene that encodes the viral protein L1. The protocol used was proposed by Carvalho et al., (2013). The reaction was performed using a final volume of 25 µl, which included 1 to 5 ng of DNA, 0.5 µM each primer, FAP59 (5´-TAACWGTIGGICAYCCWTATT-3´) and FAP64 (5´-CCWATATCWVHCATITCICCATC-3´), 0.2 mM each DNTP, 1X PCR buffer, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase. Amplification consisted of an initial denaturation of 5 min at 95°C, followed by 35 cycles of 60 s at 95°C, 60 seconds at 52°C and 60 s at 72°C, with a final extension of 5 min at 72°C. The amplification products were analysed by electrophoresis in a 1% agarose gel. The presence of a band of 470 base pairs (bp) indicated that the virus was present.

PCR products were purified using the MinElute Gel Extraction Kit (Qiagen). Cycle sequencing was performed directly on the purified PCR products using BigDye Terminator chemistry version 3.1 (Applied Biosystems). All samples were sequenced in both directions using Sanger sequence technology (Sanger et al., 1975) in an ABI 3500 DNA analyser from Applied Biosystems at the Biomolecular Analysis Laboratory of the Institute of Legal Medicine and Forensic Sciences (IMELCF). The quality of the generated sequences was verified using MEGA 7 (Molecular Evolutionary Genetics Analysis version 7 (Kumar et al., 2016). Sequence alignment was performed using Clustal Omega (Goujon et al., 2010) applying the predetermined parameters. Homology analysis of the sequences was performed using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). The comparison of the nucleotide sequences was performed with MEGA 7, and for the construction of the phylogenetic tree, maximum parsimony with 2000 bootstraps was used. Depending on the similarities observed after the analysis, sequences of references were included to determine viral genus relations.

RESULTS AND DISCUSSION

Of the total samples examined, 100% were positive for BPV. The presence of the 470 bp fragment established as indicative of infection caused by the virus is shown in Figure 1. This fragment size is similar to that reported by Carvalho et al. (2013), who studied the virus in a herd of Holstein cattle affected by chronic cutaneous papillomatosis. However, Araldi et al., (2014), using the same set of primers, reported a 478 bp fragment in cutaneous papillomas in samples of Angus Red cattle in Sao Paulo, Brazil. Similarly, Claus et al. (2009), in a study conducted in beef cattle in Paraná, Brazil, reported a 480 bp amplicon size using the same sets of primers as in the present study (FAP59/FAP64). However, all amplicons, after subsequent sequencing, were confirmed

Table 1. Comparison of cutaneous lesions, viral types, percentage of similarity, E value of probability and type of accession by region, farm and animal evaluated at 2 locations in the Republic of Panama.

Location	DNA sample	Lesion type	Viral type	Similarity (%)	E	GenBank Accession No.
El Ejido	S1	large cauliflower	BPV2/B160620	98.7	0.0	LC426022.1
El Ejido	S2	small cauliflower	BPV2/B160620	67.6	2e-41	LC426022.1
Gualaca	S3	bulging round	BPV2/B160620	93.9	3e-100	LC426022.1
Gualaca	S4	horny round	BPV2/B160620	98.6	0.0	LC426022.1
Gualaca	S5	bulging round	BPV2/B160620	98.3	0.0	LC426022.1
Gualaca	S6	bulging round	BPV25/14RS13/BR	98.8	0.0	MG252779.1
Gualaca	S7	bulging round	BPV25/14RS13/BR	97.6	0.0	MG252779.1
Gualaca	S8	flat round	BPV2/B160620	98.6	0.0	LC426022.1
Gualaca	S9	leafy horny	BPV UFPE05BR/BPV11	91.2	2e-165	JQ897976.1
Gualaca	S10	bulging round	BPV2/B160620	95.8	0.0	LC426022.1
Gualaca	S11	cauliflower	BPV/ISO 04	99.7	0.0	MF384288.1
Gualaca	S12	cauliflower	BPV6	99.5	0.0	AB845589.1
Gualaca	S13	flat	BAA2	79.1	1e-82	AF485376.1
Gualaca	S14	scaly	BPV2/B160620	98.7	0.0	LC426022.1

as positive for BPV. The variability in the size of base pairs was previously described by Carvalho et al., (2013), who reported sizes ranging between 469 and 484 bp in different viral strains. This fact highlights the importance of using sequencing, in addition to using specific segments of the viral fragment that we wish to amplify because it allows comparative studies of the different genotypes found in an outbreak where the presumptive diagnosis is BPV.

Seven of the sequenced samples, S1, S3, S4, S5, S8, S10 and S14, showed similarities (93.9% to 98.7%) and eigenvalues (E; 0.0 to 3e-100) indicative of the BPV2 strain of the species *Deltapapillomavirus 4* (GenBank Accession No LC426022.1; submitted September 25, 2018, by Nanako Yamashita University of Tokyo, Division of Infection Control and Disease Prevention, Department of Veterinary Medical Science, Graduate School of Agricultural and Life sciences; 1-1-1 Yayoi, Bunkyo-ku, Tokyo, Tokyo 1138657, Japan). The similarity of BPV2 in sample S2 was the lowest among the samples (67.41, E = 2e-41).

Two samples, S6 and S7, showed a similarity of 98.8 and 97.6% (E = 0.0), respectively, with papilloma type 25 (BPV25) isolate 14RS13/BR (unclassified *Xipapillomavirus*; GenBank Accession No MG252779.1; submitted October 23, 2017, by Daudt, C., Da Silva, F.R.C., Cibulski, S.P., Junqueira, D.M. and Canal C.W. Laboratorio de Virología, Universidade Federal do Rio Grande do Sul, Bento Goncalves, 9090, Porto Alegre RS 91540-000, Brazil).

Sample S9 presented a similarity of 91.2% (E = 1e-156) with the Brazil isolate BPV/UFPE05BR (genus unclassified; GenBank Accession No. JQ897976.1 Submitted April 03, 2012, Genética, Universidad Federal de Pernambuco, Av. Moraes Rego S / N, Recife, Pernambuco 50732970, Brazil) and strain BPV11 of the genus *Xipapillomavirus* (species *Xipapillomavirus 1*; GenBank Accession No. AB543507.1; submitted January 25, 2010; Contact: Shinichi Hatama National Institute of Animal Health, Hokkaido Research Station; 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-0045, Japan) (Hatama et al., 2011; Silva et al., 2013).

Sample S11 presented a similarity of 99.7% (E = 0.0) with isolate 04 as well as UK *Deltapapillomavirus* type 4 (GenBank Accession No MF384288.1; Submitted June 26, 2017, and published by Koch et al. (2017)). Genomic comparison of bovine papillomavirus 1 isolates from bovine, equine and asinine lesional tissue samples; Virus Res. 244, 6-12. Sample S12 presented a similarity of 99.5% (E = 0.0) with BPV6 of the genus *Xipapillomavirus 1* (GenBank accession N° AB845589.1; Submitted August 12, 2013; Contact: Shinichi Hatama National Institute of Animal Health, Hokkaido Research Station; 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-0045, Japan, unpublished data).

Sample S13 showed a similarity of 79.1% (E = 1e-82) with the BAA2 isolate of the capsid protein (GenBank accession N° AF485376.1; Submitted February 20, 2002, and published by Antonsson and Hansson (2002)). Healthy skin of many animal species harbours papillomaviruses which are closely related to their human counterparts (Table 1).

A total of 57% of the samples analysed showed similarity with viral type BPV2, which is associated with tumours in the urinary tract (Borzacchiello et al., 2007; Balcos et al., 2008; Resendes et al., 2011). This viral type has the greatest geographic distribution and has been reported in countries such as Germany, Brazil, New Zealand, Japan, India, Italy, Turkey and Korea and isolated from cutaneous papillomas, pulmonary fibromatosis, bladder, semen, blood, milk and urine (Daudt et al., 2018). These samples related to BPV2

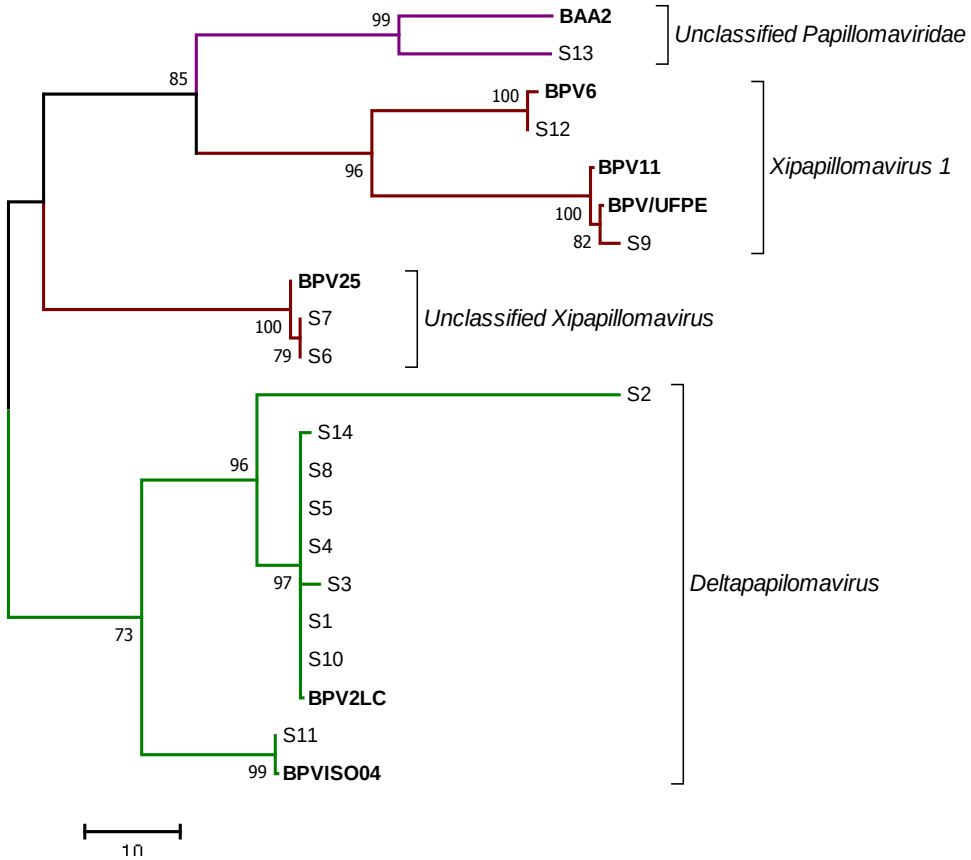

Figure 2. Molecular phylogenetic analysis (MEGA 7) using the maximum parsimony method for sequences of bovine papillomavirus. The percentage of replicated trees in which the taxa were grouped in the bootstrap test (2000 replicates) is shown next to the branches (Felsenstein, 1985).

showed 5 types of lesions: fibropapillomas in the form of large and small cauliflower-like growths observed in El Ejido and flat, bulging and scaly flat forms observed in Gualaca. The samples related to type BPV11 and BPV UFPE05BR presented leafy horny-type lesions, whose presence was reported in South America (Brazil) by Carvalho et al. (2012) and da Silva et al. (2015) and in Japan by Hatama et al. (2011). For isolates with similarity to the BPV25 type (S6 and S7), representing 14.3% of the samples, round bulging lesions were observed. Regarding this viral type, no report has been published since being entered into GenBank in October 2017; therefore, this is the first published article that refers to this viral type. Samples S11 and S12 presented the cauliflower-type form; however, they are related to 2 distinct viral types, *Deltapapillomavirus* and *Xipapillomavirus,* as shown in Figure 2. Sample S12, related to BPV6, showed cauliflower-type lesions, which was also reported by Carvalho et al. (2012) in Brazil and Savini et al. (2016) in Italy and is related to skin, teat and udder lesions (Claus et al., 2009).

Sample S13 was described as flat and was identified as related to BAA2 (Table 1). This particular viral type is related to skin papillomas in humans and was reported by Antonsson and Hansson (2002) in Sweden. This type of BAA2 was not located in any of the supergroups mentioned previously by Chan et al., (1995); therefore, they postulated that this type should be part of another putative supergroup.

The amplification of the fragments by PCR and direct sequencing allowed for the analysis of phylogenetic relationships, as shown in Figure 2, from which several groupings were formed. Most branches were statistically well supported with a minimum of 79% confidence to 100% confidence.

Sample S13 was observed in the upper region of the tree, grouped with the BAA2 isolate of the major protein of the L1 gene capsid, unclassified papillomavirus (bootstrap 99%). Next, sample S12 and viral type BPV6 and sample S9 and types BPV UFPE05BR and BPV11 were observed within the same branch (bootstrap 100%), both related to genus *Xipapillomavirus 1*. However, as it is a single case, it is necessary to perform more isolations; however, at the time of this report, there were

no more animals with papillomatous lesions observed. The subsequent branch included samples S6 (bootstrap 79%) and S7 (bootstrap 100%) and isolate 14RS13/BR of the viral type BPV25, within the genus *Xipapillomavirus* (unclassified). Next, samples related to *Deltapapillomavirus* were observed; within the branch, 3 sub-clusters were observed: sample S2 (bootstrap 96%) and a separate group of samples S14, S8, S5, S4, S3, S1 and S10 with BPV2LC (bootstrap 97%) and sample S11 related to type BPVISO04 (bootstrap 99%).

Conclusion

Before this study, there was no information regarding the subtypes of BPV present in Panama. The molecular characterization described in this report will establish a guide for subsequent studies with a greater number of samples.

The results of this research are important because they contribute to the development of prophylactic and therapeutic measures that minimize economic losses associated with the presence of papillomavirus in cattle in Panama.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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