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Technical note

Histopathology and PCR detection of bovine fibropapillomatosis in cattle in San Luis Potosí, Mexico

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Abstract:

Bovine papillomavirus (BPV) occurs worldwide and has myriad signs, including cutaneous papillomas, fibromas and fibropapillomas. Histology and PCR were used to identify the presence of BPV in tissue samples collected from cattle manifesting skin lesions suggestive of papillomas, fibromas and fibropapillomas in production units in the state of San Luis Potosí, Mexico. Eleven skin biopsies were taken from animals between 5 and 18 months' age in stabled, semi-stabled and pastured beef and dairy production systems. Lesions were suggestive of papillomas, fibropapillomas and squamous cell carcinomas. Samples were evaluated by histopathology. Detection of BPV was also done using DNA extracted from the samples and analyzed by PCR with the FAP59/FAP64 and MY09/MY11 oligonucleotide pairs. The lesions were classified into fibromas (45.45 %) and fibropapillomas (54.54 %). Lesion type distribution exhibited no patterns by anatomical location, animal age, production system or end purpose. Most (72.72 %, n= 8) of the samples were positive for BPV by PCR; 45.45 % (n= 5) with the FAP pair and 54.54 % (n= 6) with the MY pair. This is the first study

identifying the presence of BPV in San Luis Potosí. The results will be useful in establishing detection and control measures to improve production system health measures and end product quality.

Key words: Bovine papillomavirus, Bovine fibropapillomatosis, Histopathology, PCR, San Luis Potosí.

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Bovine papillomavirus (BPV) causes bovine fibropapillomatosis, an infectious, species-specific disease found worldwide. It mainly affects young cattle and is associated with various predisposing factors such as immunosuppression conditions, animal age, nutritional status, parasitosis, improper management, stress and immunosuppressive drugs, among others[1,2]. Papillomaviruses are a family of small, non-enveloped oncogenic viruses that infect birds, mammals and fish. The Papillomaviridae family comprises 29 genera that encompass 189 viral types of which 120 have been isolated from humans, 64 from mammals, 3 from birds, and 2 from reptiles[2,3].

Ten BPV viral types capable of causing infection at different anatomical sites in bovines have been characterized to date. Lesion characteristics respond to viral type. For example, BPV-1 is known to produce papillomas and fibropapillomas in the penile region, while BPV-2 manifests as papillomas and fibropapillomas on the skin and in the digestive tract. Bovine papillomavirus types 3 and 8 cause skin tags, and BVP-4 has been associated with the appearance of papillomas in the gastrointestinal tract. Fibropapillomas on the udder can be caused by BVP-5 while papillomas on the udder are associated with BVP-6, -9 and -10[3-7].

Fibromas, papillomas or fibropapillomas are benign proliferative neoplasms. They can be exophytic or endophytic, solitary or multiple, partially delimited, plaque-like and papillary. Their appearance can vary from that of a grain of rice to the texture of cauliflower, and their texture can be dry or firm. They can become necrotic and detach, and may exhibit secondary bacterial contamination[8,9].

Lesions caused by BVP can regress spontaneously or remain for six to eighteen months. Depending on their location, multiple lesions can lead to loss of body condition. Clinical signs of BVP vary by location on the body; for example, if located in the interdigital space, they can cause pain, leading to lameness or prostration. Rarely do BVP lead to clinical manifestations in the gastrointestinal tract although they can cause anorexia or bloat. When

infecting the mammary gland BVP can make milking difficult or complicate with secondary infections and generate mastitis. Lesions in the vagina or on the penis can interfere with intercourse, may bleed or become infected and can interfere with reproduction[10-12].

On a microscopic level papillomas consist of papillary projections of squamous epithelium, supported by fibrovascular stroma. These epithelial projections exhibit marked hyperplasia and hyperkeratosis, as well as ortho- and parakeratosis. In some papillomas, keratinocytes, mainly those of the stratum spinosum, have abundant clear cytoplasm or a perinuclear halo and pyknotic nuclei, which are called koilocytes (cells with cytopathic changes). Conditions present in certain regressing papillomas include reduction of epidermal hyperplasia, increased fibroblast proliferation, collagen deposits, and lymphocyte infiltration. Fibropapillomas have two components: a lining epithelium which alternates with fibrous tissue arranged in short interlocking bundles, and reactive fibroblasts. The lining epithelium does not exhibit cytopathological changes, but does have marked hyperplasia and plexiform acanthosis[1,13,14]. In large lesions, the epithelium may erode and come to resemble fibroids, in which proliferation of fibroblasts with dense collagen deposits has been observed[15].

Different strategies have been developed for using PCR to detect BPV in fibromas and fibropapillomas. The FAP59/FAP64 oligonucleotide pair, designed based on analysis of conserved regions of the human papillomavirus (HPV) L1 gene, has proven effective to this end. In addition to being useful in detecting a wide spectrum of HPV types in skin tumors and healthy skin, it has also been applied in detection of cutaneous papillomaviruses in various species, including BPV types 1-12. Similarly, the MY09/MY11 pair, originally designed to detect mucosa- and genital-associated HPV types, have been shown capable of amplifying regions of the L1 gene in BPV types 1, 3, 5 and 6[16,17].

In Mexico, data on molecular detection and identification of BPV infection have only been reported for cattle in the state of Tamaulipas[18]. No epidemiological data on the incidence, prevalence, or the BPV viral types most frequently involved in the development of papillomas or fibropapillomas in cattle have been reported from other regions of the country. The present study objective was to identify the presence of different BPV types in tissue samples from skin lesions with a histopathological diagnosis of papillomas and/or fibropapillomas collected from cattle from two regions in the state of San Luis Potosí, Mexico.

Incisional and excisional biopsies of skin exhibiting lesions suggestive of fibromas, papillomas, and/or fibropapillomas were collected from eleven animals in two regions of San Luis Potosí (Table 1, Figure 1).

Table 1: Descriptive information on sampled cattle

ID	Breed	Age (months)	Sex	Production system	Location
1	Swiss-Zebu	18	Female	Stabled	Tamuín
2	Swiss-Zebu	5	Male	Pastured	Éban
3	Lidia	6	Male	Stabled	Villa de Reyes
4	Swiss Cross	6	Male	Semi-stabled	Villa de Zaragoza
5	Holstein	15	Female	Stabled	Soledad de Graciano Sánchez
6	Holstein	10	Female	Stabled	Soledad de Graciano Sánchez
7	Holstein	10	Female	Stabled	Soledad de Graciano Sánchez
8	Holstein	12	Female	Stabled	Soledad de Graciano Sánchez
9	Holstein	13	Female	Stabled	Soledad de Graciano Sánchez
10	Zebu Cross	7	Male	Pastured	Tamuín
11	Swiss Cross	8	Male	Semi-stabled	Tamasopo

Figure 1: Sample site locations (red dots) in San Luis Potosí; at sites where more than one sample was collected the number is indicated

Each tissue sample was divided into two portions. One was placed in 15 ml Falcon tubes containing 10% formalin and evaluated by histopathology. The other was placed in a phosphate buffer solution (PBS) at pH 7.2 and stored in a cryogenic container until later PCR analysis. All samples were transported to the Immunology and Virology Laboratory of the Autonomous University of San Luis Potosí (Universidad Autónoma de San Luis Potosí - UASLP) for processing.

The sample portions intended for histopathological evaluation were fixed in 10% formalin, embedded in paraffin and processed by routine histological techniques. Thin sections (3 to 5 µm) were cut and stained with hematoxylin and eosin (H&E).

Extraction of DNA from the collected samples was done by processing 25 mg tissue with the DNeasy Blood & Tissue reagent set (Qiagen, Valencia, California, USA) following manufacturer instructions. The extracted DNA was stored at -80 °C until use. Its purity and quantify were verified with a Nano-200 drop spectrophotometer (Allsheng, Beijing, China). Integrity of the DNA was verified with 2% TAE-agarose gel electrophoresis.

The PCR analysis was run using two oligonucleotide pairs: FAP-59/FAP-64 (Macrogen, Seoul, South Korea) (FAP-59: 5'-TAACWGTIGGICAYCCWTATT-3'; FAP-64: 5'-CCWATATCWVHCATITCICCATC-3'), and MY-09/MY-11 (IDT, San Diego, California, USA) (MY-09: 5'-CGTCCAAAAGGAAACTGAGC-3'; MY-11: 5'-GCACAGGGACATAACAATGG-3'). Both oligonucleotide pairs detect the open reading frame of the gene for the majority protein of the L1 capsid, which is highly conserved in all types of bovine and human PV.

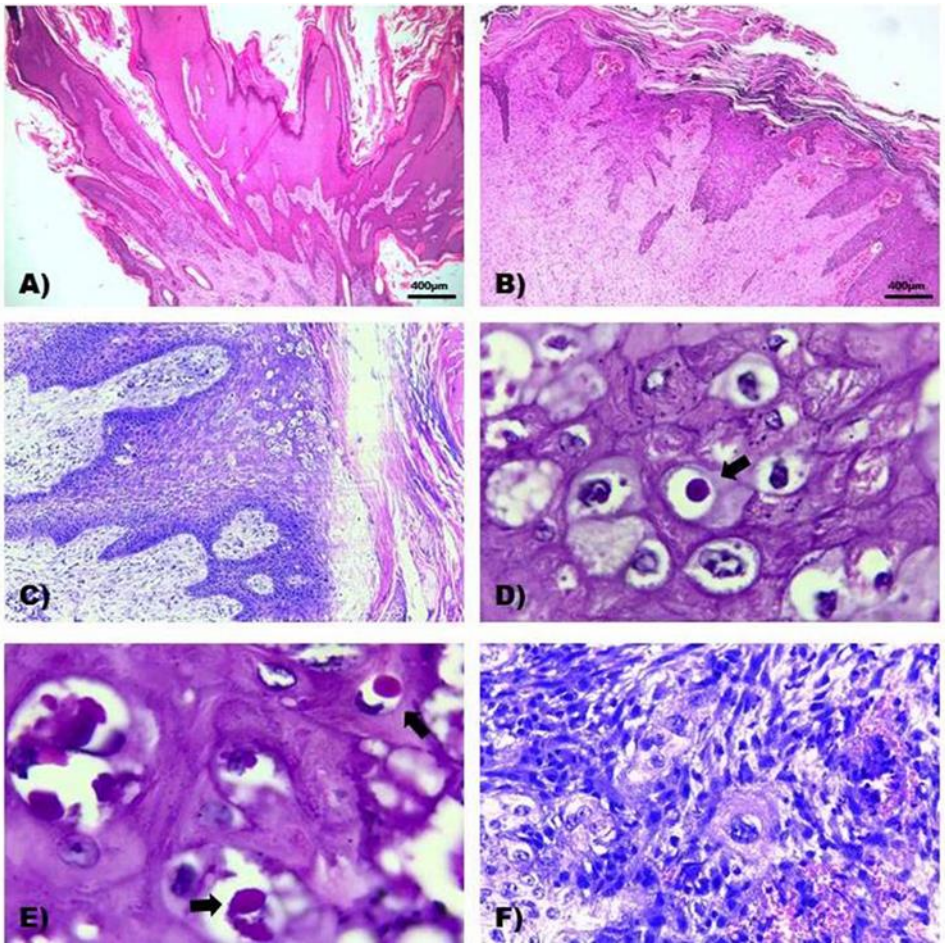
The PCR mixtures (50 µl) were prepared with the Invitrogen PCR Reagent Set (Invitrogen, Massachusetts, USA). These mixtures contained 1 × buffer, 200 µM DNTp, 2mM $MgCl_2$, 20 pM oligonucleotides, 5 UI Taq polymerase and 10 ng DNA. Analyses were run in a Multigene Optimax Thermal Cycler (LabNET, California, USA). For the MY09/MY11 pair, the PCR program was 10 min initial denaturation at 94 °C; 35 cycles of 90 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C; and a final extension of 5 min at 72 °C. For the FAP59/FAP64 pair, the program was 10 min initial denaturation at 94 °C; 45 cycles of 90 sec at 94 °C, 90 sec at 50 °C and 90 sec at 72 °C; and a final extension of 5 min at 72 °C.

The PCR products were analyzed by electrophoresis of 5 µl of product on 2% TAE-agarose gel at 80 V for 80 min. The gel was then impregnated with ethidium bromide (Sigma-Aldrich, Missouri, USA) and an image taken with a Gel Doc EZ System photodocumenter (Bio-Rad, Hercules, California, USA).

All eleven skin biopsies exhibited common histological characteristics such as irregular hyperplasia and marked hyperkeratosis of the epidermis, erosions, ulcers and serocellular crusts. Some also had papillary projections supported by fibrovascular stroma alternating with dense collagen and ballooning degeneration of the epithelium, with inclusion bodies. Proliferation of mature fibrous connective tissue was observed in the dermis, interspersed with reactive fibroblasts, dense collagen, newly formed lymphatic vessels, and multiple aggregates of lymphocytes, plasma cells and macrophages. In one tissue section, scattered atypical epithelial cells were observed which exhibited loss of the nucleus cytoplasm

relationship, abundant intensely eosinophilic cytoplasm, a large nucleus with notches, chromatin displaced to the periphery and one to three nucleoli evident (Figure 2).

Figure 2: Histological lesions representative of the fibromas, papillomas and fibropapillomas identified in the bovine skin samples



A) Fibropapilloma: Epidermis exhibits irregular hyperplasia and marked diffuse hyperkeratosis, as well as formation of papillary projections supported by fibrovascular stroma. H&E, 4X; B) Fibroma: Epidermis exhibits irregular hyperplasia and marked diffuse hyperkeratosis, dermis exhibits proliferation of fibrous connective tissue interspersed with congested blood vessels and aggregates of lymphocytes and plasma cells. H&E, 4X; C) Fibroma: Epidermis exhibits irregular hyperplasia and marked hyperkeratosis, and ballooning degeneration of keratinocytes present in different strata, dermis exhibits proliferation of reactive fibroblasts and aggregates of lymphocytes and plasma cells. H&E, 10X; D and E) Fibroma: keratinocytes exhibit ballooning degeneration and presence of amorphous amphophilic structures compatible with inclusion bodies (Arrow). H&E, 100X; F) Fibroma: some epithelial cells show marked anisokaryosis with loss of the nucleus cytoplasm relationship, abundant intensely eosinophilic cytoplasm, a large nucleus with notches, chromatin displaced to the periphery, and 1 to 3 evident nucleoli. H&E, 40X.

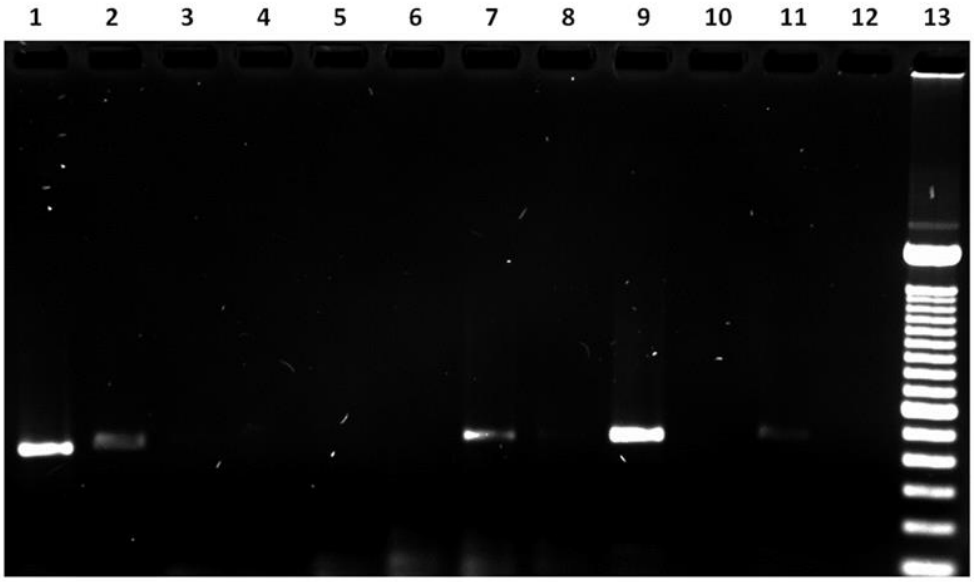
The lesions observed histologically were suggestive of a benign viral-type neoplastic process compatible with fibromas and fibropapillomas. This conclusion is based on characteristics such as irregular hyperplasia and epidermal hyperkeratosis; presence of papillary projections of the epidermis; proliferation of mature fibrous connective tissue interspersed with reactive fibroblasts, dense collagen, and aggregates of lymphocytes, plasma cells and macrophages. Koilocytes and intranuclear amphophilic inclusion bodies were also observed (Table 2).

Table 2: Production type, histology and PCR results

ID	Production type	Histological Diagnosis	PCR FAP	PCR MY
1	Beef	Fibroma	-	+
2	Beef	Fibropapilloma	-	-
3	Exhibition	Fibropapilloma	-	+
4	Beef	Fibroma	+	+
5	Dairy	Fibropapilloma	+	+
6	Dairy	Fibropapilloma	+	+
7	Dairy	Fibroma	-	-
8	Dairy	Fibroma	-	-
9	Dairy	Fibropapilloma	-	-
10	Beef	Fibroma	+	-
11	Beef	Fibropapilloma	+	-
Total			5	6

In the FAP59/FAP64 PCR analysis an amplicon was observed between 480 and 538 bp. In 45.45 % (n= 5) of the samples, this corresponds to the PCR amplification product of the BPV L1 gene. No PCR product was observed in 27.27 % (n= 3) of the samples. Overall, 72 % of the samples analyzed with this oligonucleotide pair were positive for DNA sequences corresponding to the BPV L1 gene (Figure 3, Table 2).

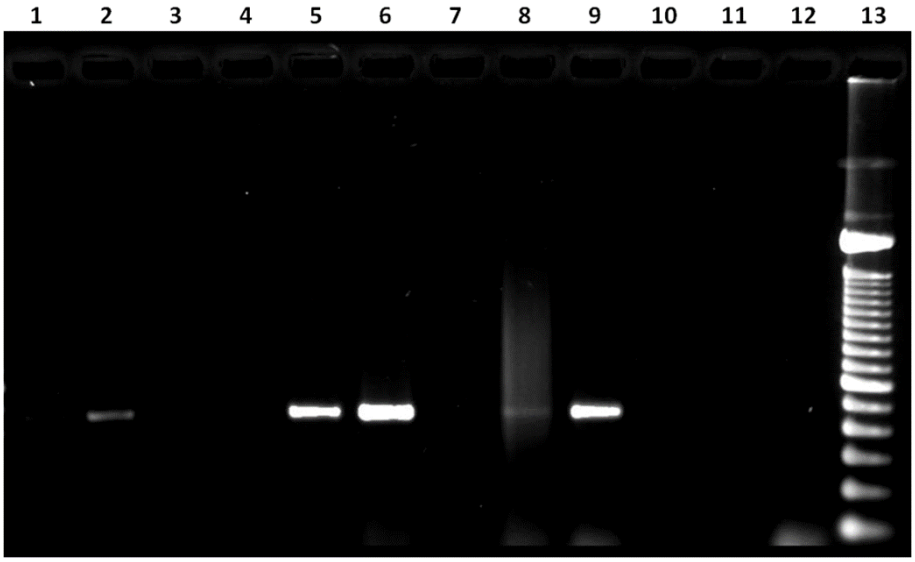
Figure 3: FAP59/FAP64 PCR results



Lane 1: sample 5, 485 bp amplicon; Lane 2: sample 6, 511 bp amplicon; Lane 7: sample 10, 521 bp amplicon; Lane 9: sample 11, 515 bp amplicon; Lane 11: sample 4, 513 bp amplicon; Lane 12: negative control (no DNA); Lane 13: 100 bp ladder. Lanes 3, 4, 5, 6, 8 and 10: no PCR product observed.

In the MY09/MY11 analysis, a band was present between 470 and 490 bp in samples 1, 2, 5, 6, 8 and 9 (54.5% of all samples). Again, this corresponds to the PCR amplification product of the BPV L1 gene (Figure 4).

Figure 4: MY09/11 PCR results



Lane 1: sample 5, 472 bp amplicon; Lane 2: sample 6, 479 bp amplicon; Lane 5: sample 9, 492 bp amplicon; Lane 6: sample 1, 488 bp amplicon; Lane 8: sample 3, 483 bp amplicon; Lane 9: sample 4, 486 bp amplicon; Lane 12: negative control (no DNA); Lane 13: 100 bp ladder. Lanes 3, 4, 7, 10 and 11: no PCR product observed.

Most of the examined lesions were indicative of fibroids. However, some papillomas and fibropapillomas can exhibit regression or morphological changes during their evolution which make them resemble fibroids; their presence depend on animal chronicity and immune status[13,15]. Given the timing of the sampling, it is therefore highly probable that the incidence of fibroids reported here constitutes an overestimate since lesion etiology and evolution can follow a common pattern.

The analyzed samples were collected from skin from different anatomical regions and no pattern of lesion restriction to a specific body region was observed. These results coincide with a previous study reporting that skin lesions associated with BPV infection can be generalized and that appearance site may correlate to BPV virus type[4].

The lesions sampled here were only cutaneous, suggesting that the viral types involved were most probably BPV-2, BPV-3, BPV-6, BPV-8, BPV-9 or BPV-10. This possibility requires confirmation through PCR product sequencing.

It seems this is the first study addressing molecular level detection of BPV in cattle in the state of San Luis Potosí. The present results will help to identify areas of opportunity in which greater detection and control is needed to improve animal health and the quality of livestock products.

The most frequent lesions observed in the epidermis during the histological analysis were irregular hyperplasia, marked diffuse hyperkeratosis and ballooning degeneration of keratinocytes, while in the dermis they were proliferation of reactive fibroblasts, fibrous connective tissue and aggregates of lymphocytes and plasma cells. These characteristics are widely reported as lesions suggesting papillomavirus infection[19,20].

Previous studies in Japan using the FAP-59/FAP-64 pair reported a 100 % BPV prevalence in skin lesions[21], while a study in Iran found a 12.5 % prevalence in Holstein cattle[22]. Overall infection frequency for any BPV type was 72 % in the present study. This level is only slightly lower than the 86 % BPV positive sample frequency reported in Brazil[23], and just above the 2 to 70 % positive sample frequency reported in the state of Tamaulipas, Mexico[18]. In 27.27 % of the present samples the histological lesions were highly suggestive of viral infection, but PCR results were negative for BPV. In these cases a possible association of BPV with these lesions cannot be ruled out due to the genomic integration process which occurs in the natural history of BPV infection[4,24].

Papillomavirus infections have been described worldwide but genotype regional prevalence varies[1,25,26]. In the present study, samples were collected from cattle in the central and Huasteca regions of San Luis Potosí. Climate in the former is dry arid while in the latter it is

subtropical. The present sample is too small to make conclusions about prevalence in these two regions, but ranchers in the Huasteca region report a higher incidence of lesions suggestive of BPV infection. This would agree with a previous report of a probable association between cutaneous papillomatosis frequency and tropical rainy climates[27].

This is the first description of BPV in cattle from the state of San Luis Potosí, Mexico. Frequency in the evaluated samples was high, but similar to that found in a region bordering the state. In the present study there were no apparent patterns based on animal age or breed, or production system type. The BPV present in San Luis Potosí has not yet been characterized to the viral type level. This is a vital next step since it will allow characterization of specific distribution patterns (clusters) and consequent development of biological strategies aimed at viral types.

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Conflicts of interest

The authors declare no conflict of interest in terms of the creation, edition and publication of this manuscript.

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