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Research Article

Research of Bovine Papillomaviruses Types 1 and 2 in the Genital Tract of Cows

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ABSTRACT

Papillomaviruses are oncogenic viruses, causing papillomas and fibropapillomas. Bovine papillomavirus-1 (BPV-1) DNA has been found in cow uterine flushings, and BPV-2 in cow ovarian and uterine tissues, uterine flushing, oocytes, and cumulus cells. So far, has been proven BVP presence in the uterus of cattle through molecular techniques, but the pathogen association with uterine lesions is yet not clear. The presence of BPV-1 and 2 was investigated in the genital tract of 80 cows. Eighty dairy cows between three and five years of age were slaughtered and sampled for laboratory analysis. Uterine cervix cytology samples for Papanicolaou testing were collected with a swab smeared in glass slides and fixated with spray. Reproductive organ fragments (Ovaries, uterine tubes, uterine horns, uterine body, and cervix) were collected for histopathology and fixed in 10% buffered formalin. Tissue fragments were collected in a sterile/stereo universal collecting cup, and whole blood was collected in a vacuum tube with EDTA, both frozen at -20°C for further nested-PCR. Different techniques were used: macroscopy, histopathology (hematoxylin and eosin stain -HE), cytologic atypia of the uterine cervix (Papanicolaou), nested-PCR for the L1 gene of BPV viral capsid using the primers FAP59/FAP 64, and Delta Epsilon F/Delta Epsilon R. Both metritis and endometritis were macroscopically found in 5.0% of cows. Cytology showed cell atypia in 25.0% of cows: karyomegaly, binucleation, and multinucleation, while histopathology confirmed nonspecific endometritis in 25.0% of cows. BPV was not detected by nested-PCR.

Keywords: Cattle; Reproductive tract; Cytology; Histopathology; Nested-PCR

INTRODUCTION

PapillomaViruses (PV) are small oncogenic viruses, causing papillomas and fibropapillomas in the epithelium and mucosa of different animal species [1].

In cattle, the different BVP types are mostly associated with tissues such as skin warts (BVP-1 and BVP-2), teat and udder papillomas (BVP-1, BVP-5, and BVP-6), penis papilloma (BVP-1), and bladder cancer (BVP-1 and BVP-2) [2]. Given the intense papillomatosis spread in herds, investigation of different transmission routes and their respective mechanisms has required special attention (Table 1).

Tissues	BVP Types
Skin warts	BVP-1 and BVP-2
Teat and udder papillomas	BVP-1, BVP-5, and BVP-6
Penis papilloma	BVP-1
bladder cancer	BVP-1 and BVP-2
ovarian and uterine	BPV-2

Table 1: The different BVP types are mostly associated with tissues.

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BPV-2 has been found in ovarian and uterine tissues, uterine flushing, and cow oocytes and cumulus cells; therefore, viral infection can develop out of epithelial tissue. These findings point to BPV transmission by embryo transfer and *in vitro* fertilization procedures [3,4].

It was detected BVP-2 DNA in commercial bovine frozen semen [5]. Thus, BPV can be inserted into the uterus of cows by artificial insemination, as the sanitary control of BPV is not mandatory in batches of industrialized bovine semen.

BPV can be transmitted by animal-to-animal contact or by contact with contaminated surfaces or other fomites such as mechanical milking, ropes, as well as drinking and feeding troughs [6]. Tissue lesions or microcracks contribute to infection by BPV since they lead to exposure of heparin sulfate peptidoglycans in the cell cytoplasm, in which they become a binding site for the L1 protein, causing viral endocytosis [7].

In cattle, oncotic cytology and histopathology are little used in studies of precancerous lesions. So far, what has been proven is BVP presence in the uterus of cattle through molecular techniques, but the pathogen association with uterine lesions is yet not clear.

The aim was to study the presence of BPV-1 and 2 in the genital tract of cows and to associate it with the presence of macroscopic and microscopic lesions.

MATERIALS AND METHODS

This study was approved by the Animal Experimentation Ethics Committee of the Biological Institute (CETEA-IB) on October 29, 2015, registered under protocol number 145/15. It meets the Ethical Principles in Animal Experimentation adopted by the Brazilian Society of Science in Laboratory Animals (SBCAL/COBEA), by the Brazilian Guideline for Care and Use of Animals for Scientific and Didactic Purposes (DBCA).

Eighty dairy cows between three and five years of age were slaughtered and sampled for laboratory analysis. All animals came from inspected slaughterhouses in the Vale do Paraiba region, which is one of the major milk-producing areas in São Paulo State, Brazil. After slaughter the reproductive tracts of cows were removed and submitted to macroscopic analysis, visualizing conformation, color, and lesions of ovaries, uterine tubes, uterine horns, uterine body, cervix, vagina, and vulva.

Uterine cervix cytology samples for Pap testing were collected with a swab smeared in glass slides and fixated with spray (Carbovax™ Polyethylene Glycol 1450 Flake, Dow, Midland, MI, USA). The uterine cervix smear was strained using the Papanicolaou method, modified to show morphology, maturity degree, and cellular metabolic activity. Baths of ethyl alcohol, hematoxylin, Orange G, EA- 36, and xylol were used for diaphanization, and finally, the slides were sealed with coverslips and resin [8, 9].

Reproductive organ fragments (Ovaries, uterine tubes, uterine horns, uterine body, and cervix) were collected for histopathology and fixed in 10% buffered formalin for up to 24 hours. Tissue samples underwent elution in ethyl alcohol, diaphanization with xylol, impregnation in liquid paraffin, and embedding in paraffin [10]. The material was cut in a microtome (3 μm thick), laid out in a water bath (60°C), and mounted on a glass slide treated with albumin to facilitate adhesion of histological cut to the slide. Afterward, the slide was kept in an oven at 60°C until deparaffinization and then stained with hematoxylin and eosin. The slide was sealed with a coverslip and resin.

Tissues for the histopathological and cytological studies were processed in the Laboratory of Pathologic Anatomy, and the nested-PCR at the Laboratory of Bovine Viruses of the Biological Institute.

Tissue fragments were collected in a sterile/stereo universal collecting cup, and whole blood was collected in a vacuum tube with EDTA, both frozen at -20°C for further nested-PCR. DNA was extracted using the Cador Pathogen kit (Quiagen, Germantown, MD, USA), which is based on cell membrane lysis, and was performed in an automated extraction system (Qiacube HT, Quiagen, Germantown, MD, USA). After thawing, reproductive tract fragments were macerated and added with 500 μL of 0.9% buffered saline solution (pH 7.2). This material was transferred to an extraction rack, and the DNA extraction kit protocol was followed using a Qiacube HT system (Quiagen, Germantown, MD, USA). After this procedure, the material was stored at -20°C.

The negative BPV PCR control was Nuclease-Free Water, and the positive PCR control was BPV-1 from a bovine skin papilloma. DNA segment was amplified using primers for the gene encoding the BPV-1 viral capsid protein L1: FAP59, 5'-TAACWGTIGGICAYCCWTATT-3' and FAP64, 5'-CCWATATCWVHCATITCICCATC-3' with 478bp; and primers for the gene encoding the BPV-1 and BPV-2 viral capsid protein L1: Delta Epsilon F, 5' CCAGAYTAYYTMAAAATGGC-3' and Delta Epsilon R, 5'-ATAAMKGCTAGCTTATATTC-3' with 430 bp [11-13].

FAP59 and FAP64 genes were amplified by one cycle of 5 min at 94°C, followed by 45 cycles of 1 min at 94, 49.3, and 72°C, and of 5 min at 72°C. The reaction mixture consisted of 3.75 μL nuclease-free water with 1.075 μL of 10 pmol FAP59 and 10 pmol FAP64 and 12.5 μL PRC master mix (Promega, Madison, WI, USA), and a total volume of 20 μL was used for 5 μL DNA reactions.

BVP Delta Epsilon gene was amplified using the PCR master mix (Promega, Madison, WI, USA), consisting of 8.5 μL nuclease-free water, 1.0 μL of each Delta Epsilon F and R primer set (10 pmol/μL), 12.5 μL PCR master mix (Promega, Madison, WI, USA), and 2.0 μL DNA template for a total of 25 μL reaction. The amplification cycle comprised denaturation cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, followed by a final extension at 72°C for 10 min.

The amplified products were analyzed by electrophoresis (Bio-Rad, Hercules, CA, USA) (100V/60 min) in 1.5% agarose gel in Tris borate buffer, EDTA pH 8.0, and visualized in red gel

(1:150). The gel image under UV light was recorded in a photo-documentator (Gen-DocTM - Bio-Rad, Hercules, CA, USA) coupled to a computer.

RESULTS AND DISCUSSION

The present study used cows slaughtered in inspected facilities located in the Vale do Paraba region, São Paulo State, Brazil. Eighty uteri were collected from cows aged between three and five years, which had been discarded from breeding. Clinical examination showed that cows were in good general physical conditions, with only one animal having skin papillomas. The animals' clinical history and gynecological examination could not be obtained from the farms.

Macroscopic diagnosis of the reproductive tract revealed that 72 cows (90.0%) had no pathological changes. Four cows (5.0%) showed macroscopic lesions suggestive of endometritis (inflammation restricted to the endometrium), characterized by edema, endometrial hyperplasia, fluid accumulation, hemorrhage, adherent fibrin fragments, and necrotic debris. Four animals (5.0%) showed evidence of metritis, which is the inflammation of all layers of the uterine wall and presents generalized edema, dark serous, chocolate discharge of foul odor, petechiae in the mucosa, and fine fibrin bundles adhered with necrotic debris. Papillomatous lesions were not observed in the macroscopic analysis of cows' uterus, vagina, and vulva.

Of the 80 cytological smears analyzed for uterine cervix cytology, cell atypia, such as karyomegaly, binucleation, and multinucleation, was found in 20 cows (25%) and accompanied by neutrophils. Neutrophils are commonly present in cytology samples of cows with endometritis [14]. In human beings, the Bethesda cervical-vaginal cytologic classification system considers that cell atypia of indeterminate significance, characterized by karyomegaly, binucleation, and multinucleation, highlights pathology to be investigated [15].

Sixty cows (75.0%) had no histopathological changes, 20 (25.0%) had nonspecific endometritis accompanied by cell atypia (karyomegaly, irregular nuclear membranes, prominent nucleoli, coarse and disorganized chromatin, cell pleomorphism), eosinophils, neutrophils, hyperplasia, and ulcerations. Between 10% to 20% of cows with repeated estrus without any apparent cause have endometritis [16].

In the present study, all cow samples (whole blood, ovary, uterine tubes, uterine horns, uterine body, and uterine cervix) were negative for nested-PCR of the two sets of L1 gene primers for BPV (Delta Epsilon F/Delta Epsilon R and FAP 59/FAP64).

Degenerate primers FAP59/FAP64 were initially designed for HPV detection, but have been applied in studies on BVP diversity in cattle. Delta Epsilon F/Delta Epsilon R degenerate primers have been used in BVP-1 and BVP-2 studies, present in bovine skin and mucosa. It was observed that the Delta Epsilon F/Delta Epsilon R primer set was more efficient than the FAP59/FAP64 primer set in detecting BVP-2.

In Japan, they have identified 10 more alleged BVP types, thus, just as HPV, BPV types can also be widely diverse. Therefore, this small number of known virus types appears to be a methodological failure in determining those present in infections, rather than the lack of antigenic and molecular diversity in BVP. In Brazil, regardless of the level of technification of livestock farming, BVP infection can be found in beef cattle herds, and mainly in dairy herds, in practically the entire country. Despite the high infection rates, the determination of the type of virus circulating in herds is still quite sporadic [17,18]. All Polymerase Chain Reaction (PCR) techniques have different positive rates when used on different clinical samples. The standardization of the nested-PCR assay validated the positive control in a bovine cutaneous papilloma, genotyped as BVP-1. DNA extraction from tissue and whole blood samples used an automated nucleic acid extractor, reducing manipulator errors. Primers from the conserved and non-conserved regions of the L1 gene were used to increase the nested-PCR sensitivity to BVP.

As BPV was not detected by nested-PCR in the genital tract, other risk factors may be associated with the identified lesions. This is because culled animals had been disposed of and were young and of reproductive age. In this sense, infertility may have been the cause of discarding.

In general, the uterine region is contaminated during parturition and can lead to postpartum infections. Early detection of abnormalities allows veterinarians to rapidly select a proper treatment while considering factors that may compromise the return of normal uterine function and ovarian activity. Cell inflammation and atypia were in the uterus and most of infectious origin and usually caused by inadequate reproductive management practices, mainly mating, obstetric assistance, and follow-up after calving.

During calving, the uterus is contaminated with environmental bacteria, which are eliminated by normal uterine involution. The uterine environment is compromised by changes in local defense mechanisms and consequent persistence of pathogenic bacteria, establishing different uterine infection cases [19,20].

Occurrence of retained placenta, dystocia, twin births, miscarriages, and early-term gestation are among the main risk factors associated with uterine infection establishment [21-23]. The establishment, severity, and persistence of different types of infection are related to uterine environment conditions, genetic factors, and innate/acquired immunity [24]. Expression of clinical signs depends on the interaction between immune response, and quantity and pathogenicity of microbial agents [25].

In cattle farming, cows must calve once a year, and those which do not are culled from the herd (slaughtered) so that farm costs and benefits can be balanced. From a livestock point of view, such a goal is achieved through environmental hygiene, rational production, and reproductive management.

CONCLUSION

Bovine papillomavirus was not detected by the molecular tests used, which corroborates the absence of macroscopic and microscopic papillomatous lesions in the genital tracts of the studied cows.

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