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Short communication

Molecular and histological characterization of bovine papillomavirus in North West Italy

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ABSTRACT

Bovine papillomaviruses (BPVs) are group of worldwide-spread DNA virus that infect primarily cattle determining diseases of considerable economic relevance. Recently, research on BPVs, received a great impulse owing to the development of specific biomolecular analysis, mostly based on L1 gene sequencing, that resulted in the identification of new viral types. This work is aimed at the identification and molecular and histopathological characterization of BPVs circulating in North West Italy, one of the main national cattle breeding areas.

In this study, 71 bioptic specimens were submitted both to histological examination and to PCR and sequencing analysis. Histopathology revealed various lesion types; however, no connections were demonstrated between involved viral types and histopathological findings. BPV DNA was demonstrated in all the analyzed samples and several viral types were detected. Particularly, molecular investigations revealed a broad diffusion of highly pathogenic BPV1 and 2 *Deltapapillomavirus* and presence of BPV3 and 9 *Xipapillomavirus.* Two cases of co-infection were also demonstrated. Phylogenetic analysis revealed presence of different clusters and therefore a noteworthy genetic variety among the analyzed viral types.

This study provides information on the main BPVs types in North West Italy and our results demonstrate the complexity of viral epidemiology which is characterized by circulation of multiple viral types even inside single herds. Knowledge of the prevalence and of the variety of BPVs is a milestone for the development of appropriate prophylactic and therapeutic measures.

1. Introduction

BPVs are a group of double-stranded DNA viruses, belonging to the *Papillomaviridae* family. BPVs infect primarily cattle determining diseases of considerable economic relevance as they cause various forms of cutaneous and mucosal lesions ranging from papillomatosis to malignant tumors at the urinary bladder and digestive tract level (Batista et al., 2013). Recently, developement of specific biomolecular analysis, mostly based on L1 gene sequencing (Bernard et al., 2010) resulted in identification and characterization of novel viral types. Particularly, improvement of diagnostic procedures resulted in new classification approaches and determined the evolution of the former viral classification, (Jarrett et al., 1984) dividing viral types in subgroups A (BPV type 1, 2 and 5) and B (BPV type 3, 4 and 6) in reason of their genetic and biological features, in a new taxonomy based predominantly on L1 nucleotide sequence similarities (Araldi et al., 2014). Currently, 13 BPVs have been characterized and classified in four genera: *Xipapillomavirus*, *Deltapapillomavirus, Epsilonpapillomavirus* and *Dyoxi-papillomavirus. Xi-papillomavirus* genus (BPV type 3, 4, 6 – previously included in subgroup B – 9, 10, 11 and 12) infects keratinocytes inducing epithelial papillomas while *Delta-papillomavirus* genus (BPV1, 2 – previously included in subgroup A – and 13) determines fibropapillomas of paragenital areas, skin, alimentary tract and urinary bladder. Finally, BPV5 – previously included in subgroup A – and 8, belong to the *Epsilon-papillomavirus* genus and cause papillomas and fibropapillomas mostly at the udders and teats level (Lunardi et al., 2013a) while BPV7 has recently been assigned to *Dyoxi-papillomavirus* genus although classification has not yet been approved by ICTV (Rector and Van Ranst, 2013). Even though BPVs infection normally does not cause cattle death, it dramatically reduces productive performances since it determines a slow animal growth, loss of weight and, as long as teats and udders are involved, a decrease in milk production (Bocaneti et al., 2014). Disease economic relevance along with BPV epidemiology complexity inspired this study aimed at identification and characterization of

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BPV types circulating in North West Italy, one of the major national cattle breeding areas, in order to evaluate appropriate control and therapeutic measures.

2. Material and methods

2.1. Sampling

The characterization study was conducted on 71 bioptic specimens of papilloma-like lesions collected from 70 cattle and 1 donkey and conferred for the production of an autogenous vaccine by vet practitioners to the Istituto Zooprofilattico Sperimentale laboratories from 2011 to 2014. Tissues were both fixed in 10% neutral buffered formalin for histological examination and frozen at −80 °C for biomolecular analysis.

2.2. Histopathology

Formalin fixed tissue samples were processed by standard paraffin wax techniques. Samples were then cut in 4 ± 2 m sections and stained by hematoxylin and eosin method (HE). Slides were evaluated microscopically at increasing magnifications (10×, 20×, 40×). The histopathologic findings were classified as described by Ginn et al., (2007):

- Squamous papilloma

Hyperplastic epidermis supported by thin dermal stalks with dilated capillaries. Epidermal hyperplasia predominates and it is particularly due to marked expansion of stratum spinosum cells. Cells of the spinosum and/or granulosum stratum show ballooning degeneration of cytoplasm and eccentric pyknotic nuclei (koilocytes), and keratohyalin granules. Stratum corneum exhibits variable degrees of ortho to parakeratotic hyperkeratosis;

- Fibropapilloma

Acanthosis, hyperkeratosis and downgrowth of rete ridges with prevalence of dermal proliferation. The proliferating fibroblasts are arranged in whorls and fascicles;

- Lesions of intermediate type

Marked epidermal hyperplasia associated to evident fibroblasts proliferation.

2.3. PCR and sequencing

DNA extraction from frozen tissues was performed by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden) according to instructions of manufacturer. Two different PCR analysis both targeted to L1 gene were performed as described by Maeda et al. (2007) using two specific primers sets (subAup/subAdw and subBup/ subBdw; illustrated in Table 1) able to detect Delta- and *Epsilon-Papillomavirus,* and *Xi-Papillomavirus*, respectively (Hatama et al., 2011). Briefly, a PCR mixture final volume (50 mL) containing total DNA (1 mL), primers (0,25 mM each), dNTPS (200 mM), MgCl2 (2,5 mM), buffer 10X (5 mL) and Taq DNA polimerase (1,25 units, Qiagen) was submitted to the amplification protocol illustrated in Table 2. Viral DNA was detected by electrophoresis on 2% agarose gel containing Gel Red (Nucleic Acid Gel Stain 10.000x) and visualized by GelDoc (BioRad, Hercules). DNA extracted from tissues belonging to clinically healthy slaughtered cattle that had previously resulted negative both to histological and to PCR analysis was included as negative control.

Table 1
Subgroup A and subgroup B PCR primers.

Subgroup A	Forward – subAup	5′-CCAGAYTAYYTMAAAATGGC-3′
	Reverse – subAdw	5′-ATAAMKGCTAGCTTATATTC-3′
Subgroup B	Forward – SubBup	5′-TWYAATAGGCCCTTTTGGAT-3′
	Reverse – subBdw	5′-TTMCGCCTACGCTTTGGCGC-3′

Table 2
PCR amplification protocol.

Initial denaturation	94 °C; 5′
Denaturation	94 °C; 45″
Annealing	55 °C; 30”
Extension	72 °C; 60”
Final extension	72 °C; 60”
Number of cycles	35

A sample size of 21 BPV PCR-positive specimens, selected in reason of the significance of the clinical case and representativeness of their geographical distribution, were sequenced to identify viral genome type. Furthermore, sequencing analysis were conducted on BPV amplicons obtained from the donkey included in the study and from all the tissues presenting subgroups A and B viruses co-infection.

For sequencing analysis, PCR products were purified using the NucleoSpin Gel and PCR clean-up (Macherey-Nagel, Duren, Germany) and sequencing was performed with forward and reverse primers using Cycle Sequencing Kit (Big Dye Terminator, version 1.1, Applied Biosystems, Austin, USA) along with a 310 DNA Analyzer (Applied Biosystems, Austin, USA). The forward and reverse complementary sequences were aligned using BioEdit software (version 7.0.9.0; Hall, 1999) and the results obtained analyzed by BLAST search (http://blast.ncbi.nlm.nih.gov/) on GenBank database.

2.4. Phylogenetic analysis

Phylogenetic analysis of sequences retrieved in this study was carried out using bovine papillomavirus sequences obtained using two sets of primers (subAup/subAdw, subBup/subBdw) and reference strains for four viral types: BPV1 (accession no. JX678969), BPV2 (accession no. AB823006) BPV3 (accession no. AF486184) and BPV9 (accession no. AB331650). Sequences of the partial L1 region were aligned using BioEdit. In order to illustrate evolutionary relationships among the analyzed sequences, phylogenetic and molecular analysis were conducted by Neighbour Joining method using MEGA software version 6 (Tamura et al., 2013).

3. Results

3.1. Histopathology

All the analyzed specimens were identified as papilloma. In detail, 39 (54,93%) samples were classified as fibropapilloma, 30 (42,25%) as squamous papilloma and 2 (2,82%) samples revealed lesions of intermediate type (Fig. 1). As far as the relationship between BPV types and lesion morphology is concerned, animals infected by subgroup A viruses showed the complete spectrum of lesions described by Ginn et al. (2007). Particularly, pathological lesions caused by BPV1 were recognized as fibropapilloma (58.82%) or squamous papilloma (41.18%), while all BPV-2 positive lesions were depicted as fibropapilloma. The two samples revealing a co-infection of BPV1/BPV3 and BPV1/BPV9 were both classified as fibropapilloma.