The Veterinary Journal 197 (2013) 368–373

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Molecular epidemiology of bovine papillomatosis and the identification of a putative new virus type in Brazilian cattle

Marcus V.A. Batista [a,*], Maria A.R. Silva [a], Nayara E. Pontes [a], Marcio C. Reis [b], Annunziata Corteggio [c], Roberto S. Castro [b], Giuseppe Borzacchiello [c], Valdir Q. Balbino [a], Antonio C. Freitas [a,*]

[a] Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Recife, PE, Brazil
[b] Department of Veterinary Medicine, Rural Federal University of Pernambuco, Pernambuco, Recife, PE, Brazil
[c] Department of Pathology and Animal Health, University of Naples, Federico II, Via Veterinaria, Napoli, Italy

ARTICLE INFO

Article history:
Accepted 20 January 2013

Keywords:
Bovine papillomavirus
Molecular epidemiology
Genetic diversity
Phylogenetic analysis

ABSTRACT

Bovine papillomaviruses (BPVs) are a diverse group of double-stranded DNA viruses, of which 12 viral types have been detected and characterized so far. However, there is still a limited understanding of the diversity of BPV. Several putative new BPVs have been detected and some of these have been recently characterized as new viral types. However, only a very limited amount of information is available on the pathology associated with these novel viral types yet this information could be of significant value in improving our understanding of the biology of BPV. The objective of this study was to examine some of the epidemiological features of cutaneous bovine papillomatosis in Brazilian cattle, in particular to establish the relationship between BPV types isolated from beef and dairy cattle herds and the lesions they cause.

Seventy-two cutaneous lesions were collected from 60 animals. Histopathological, PCR and sequencing assays were conducted to characterize the lesions and detect the BPV types responsible. Phylogenetic analysis was carried out using the maximum likelihood method. BPV types 1–6 and 8–10 were found, as well as a putative new BPV type that belongs to the *Deltapapillomavirus* genus. The tumors were all classified as fibropapillomas. This is believed to be the first record of BPV types 3 and 10 associated with fibropapillomas. These results confirm that there is a wide range of BPV types that infect cattle, and that an understanding of this diversity is necessary for improved methods of therapeutic treatment.

Introduction

Bovine papillomaviruses (BPVs) are a diverse group of double-stranded DNA viruses that are classified in three different genera, namely *Xipapillomavirus*, *Deltapapillomavirus* and *Epsilonpapillomavirus*. So far 12 viral types have been detected and characterized although BPV7 still remains unclassified (de Villiers et al., 2004; Bernard et al., 2010; Zhu et al., 2012). BPVs are responsible for various forms of cutaneous and mucous lesions, which can regress or grow into malignant lesions, especially when combined with environmental co-factors (Jarrett et al., 1978). Some types may cause the development of urinary bladder (BPV1 and BPV2) and upper digestive tract (BPV4) tumors in cattle (Borzacchiello and Roperto, 2008).

Studies have suggested that certain BPV types only affect some particular tissues, and cause specific lesions. Thus, BPV1 has been linked to teat and penile fibropapillomas, BPV2 to cutaneous warts and alimentary fibropapillomas, BPV3 to cutaneous papillomas, BPV4 to pure epithelial papillomas of the upper gastrointestinal tract, BPV5 to rice grain fibropapillomas on the udder, BPV6 to frond papillomas of the teats, BPV8 to cutaneous papillomas, and BPV types 9 and 10 to squamous epithelial papillomas of the udder (Borzacchiello and Roperto, 2008). However, there are reports of the detection of BPV types away from these predilection sites (Bloch et al., 1996; Carvalho et al., 2012).

BPV-related diseases are of considerable economic importance worldwide in both beef and dairy cattle (Borzacchiello and Roperto, 2008). In Brazil, which is the second largest beef producer in the world (USDA, 2011), BPV types 1, 2, 6 and 8 have been identified in skin warts of cattle from the south of the country (Claus et al., 2007; Sá e Silva et al., 2010), while Carvalho et al. (2012) detected 10 different types of BPV along with a putative new BPV11 subtype in the North-East region.

Although 12 BPV genomes have been identified it is likely that there are far more – especially when it is taken into account that more than 150 human papillomavirus (HPV) genomes are known.

* Corresponding authors. Tel.: +55 81 2126 8520.
E-mail addresses: mvabatista@hotmail.com (M.V.A. Batista), acf_ufpe@yahoo.com.br (A.C. Freitas).

1090-0233/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tvjl.2013.01.019

PCR assays conducted with degenerate primers followed by sequencing have identified about 31 putative new BPV types (Forslund et al., 1999; Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007; Claus et al., 2008). Some of these putative types were recently characterized as new BPV types after their complete genomes had been sequenced (Ogawa et al., 2007; Tomita et al., 2007; Hatama et al., 2008, 2011; Zhu et al., 2012). Very limited data are available on the pathology associated with these novel viral types but such information could provide a valuable means of improving our understanding of the biology of bovine papillomavirus, and allow progress to be made in the treatment and diagnosis of BPV-related diseases. The purpose of the present study was to characterize the distribution of BPV types that can cause cutaneous lesions in cattle herds in the North-East of Brazil.

Materials and methods

Study population

The cattle selected for the study came from beef and dairy farms in North-East Brazil where cutaneous papillomatosis occurs. A total of 60 animals with cutaneous papillomatosis were identified and samples obtained. Multiple samples were obtained from animals with several skin lesions to assess co-infection, resulting in the collection of 72 cutaneous lesions. These were obtained from different anatomical parts of the animal, such as the head, neck, and udder.

Histopathology

Histological diagnosis was carried out following the guidelines laid down by the World Health Organization (WHO) for the histological classification of epithelial and melanocytic tumors of the skin of domestic animals (Goldschmidt et al., 1998).

DNA extraction

Genomic DNA was extracted from tissue samples from each lesion by using the DNeasy Blood and Tissue kit (Qiagen), in compliance with the manufacturer's protocols. The extracted DNA was quantified using Nanovue (GE). The DNA quality was checked by bovine β-globin gene PCR, as described by Freitas et al. (2003).

Detection of viral DNA and genotyping

Viral DNA was amplified by conducting PCR assays using a Master Mix kit (Promega) following the manufacturer's instructions. The reactions were carried out in a two-stage process. First, all of the DNA samples were screened for the presence of BPV DNA using the degenerate primers FAP59/64 under the conditions described by Ogawa et al. (2004) and the modifications described by Carvalho et al. (2012). Second, the DNA samples were subjected to PCR using BPV type-specific primers, in accordance with the amplification protocol described by Stocco dos Santos et al. (1998) and the modifications described by Carvalho et al. (2012). All of the amplification products were visualized by 2% Tris–acetate–EDTA (TAE) agarose gel electrophoresis and subsequent ethidium bromide staining. The positive and negative controls were as described by Carvalho et al. (2012). Amplicons were obtained by FAP59/64 PCR and by specific primers which were sequenced to identify/confirm the viral type.

Identification of a putative new BPV type

The identification of viral types with FAP59/64 degenerate primers requires confirmation by sequencing. If the obtained L1 gene sequence shows a divergence of more than 10% from the closest known type, it is considered a novel type (de Villiers et al., 2004). Samples that tested positive for the presence of a putative new BPV type, were again amplified by PCR, using a high fidelity DNA polymerase (GE) and the degenerated primers referred to above for confirmation. The PCR products were cloned into the pGEM-T vector (Promega) and transformed into competent DH5α bacteria. Bacterial clones were randomly selected for confirmation. At least two different positive clones were sequenced twice, in both directions, by means of an ABI 3100 DNA sequencer (Applied Biosystems).

The quality of the sequencing and the contig assembly were assessed using Pregap4 and Gap4 programs (Staden, 1996). Only sequences with a Phred value above 30 were considered for the contig assembly. Local sequence alignments were carried out to determine the sequence identity with BLAST (Altschul et al., 1990). A multiple sequence alignment was carried out by Muscle (Edgar, 2004) and ClustalW (Thompson et al., 1994) algorithms, incorporated into MEGA5 software (Tamura et al., 2011). The identity of the nucleotide and amino acid sequences was determined by means of BioEdit v. 7.1.3 software (Hall, 1999).

Phylogenetic analysis

A phylogenetic analysis was carried out with amino acid sequences of BPV types and putative novel types, using the maximum likelihood method with LG + G as amino acid substitution model in PhyML 3.0 (Guindon et al., 2010). The tree topology was estimated by employing the best of the nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) methods. An initial BIONJ tree was used, and the taxa were randomly added. 1000 non-parametric bootstrap replicates were employed to determine the statistical support of the obtained branches. The sequences used in this study are described in the Supplementary Table S1 (Appendix A).

Results

Data on the cattle population used in this study and the lesions sampled are summarized in Table 1. In total, 72 samples were collected from two states of North-East Brazil, namely, Pernambuco ($n = 36$) and Bahia ($n = 36$). The lesion morphologies detected included cauliflower, flat, and peduncle, as well as some atypical morphological shapes. Lesions were collected from the back, udder, shoulder, eye, neck, muzzle, dewlap, ear, scapula, hind hooves and head (Table 1). The samples were subjected to histopathological analysis to characterize the lesions, all of which proved to be fibropapillomas.

BPV1 was found in lesions on the dewlap and back; BPV2 and 3 were found in almost all of the lesions; BPV4 was found in lesions on the scapula, hind hooves, head, neck, back, and dewlap); BPV5 and 6 were detected on the shoulder and around the eyes. In this study, BPV8 was found on the back, and in the dewlap; BPV9 was detected on the udder and back; and BPV10 was found on the shoulder, around the right eye, neck, muzzle, dewlap, back, udder and head (Table 1). A curious finding was that some BPV types were detected in multiple anatomical parts of the same animal, e.g. animals 19, 20, 22 and 31 (Table 1). Another interesting point was that co-infection was present in a large number of the samples. However, we could not observe a pattern linking BPV type to a particular geographical location, herd, age category, or sex (Table 1).

The relationship between BPV types and lesion morphology is poorly understood. Although we know that BPV types are linked to the morphology of the lesion, in practice it was difficult to establish the nature of this link, owing to the huge number of co-infections (Table 1). Histologically, the tumors consisted of dermal fibrovascular stroma with low to moderate numbers of fibroblasts. The overlying cutaneous epithelium was hyperplastic with characteristic rete pegs at the periphery of the papillae. The tumors were all classified as fibropapillomas (Fig. 1).

A putative new BPV type was identified in the samples by the use of FAP59/64 primers (Fig. 2), and the isolate was termed BPV/UFPE04BR (GenBank accession number JQ897975). The identity between the BPV/UFPE04BR sequence and BPV2 L1 sequence was 74.1%. This suggested that the BPV/UFPE04BR isolate was a new BPV type. This isolate was collected from a cow, in a beef herd with a semi-intensive management system. The animal had lesions of low intensity, most of which were flat. The lesions collected for molecular analysis were of flat morphology.

The BPV/UFPE04BR sequence is 399 bp in length. However, the final sequence alignment, including the sequence of the novel BPV (type) isolate, had 498 nucleotides, with 115 (approximately 23%) conserved and 368 (approximately 74%) variable sites. Of the variable sites, 339 were parsimony informative and 29 were singletons. Fifteen sites were found in only one sequence and these were not computed.

The phylogenetic tree confirmed that the BPV/UFPE04BR isolate belongs to a new viral type, with 99% of confidence based on boot-