Animal Reproduction Science 129 (2011) 146–151

Contents lists available at SciVerse ScienceDirect

Animal Reproduction Science

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

Detection of bovine papillomavirus type 2 DNA in commercial frozen semen of bulls (*Bos taurus*)

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ARTICLE INFO

Article history:
Received 17 February 2011
Received in revised form 9 November 2011
Accepted 14 November 2011
Available online 25 November 2011

Keywords:
Bulls
Papillomavirus
Semen
PCR

ABSTRACT

Papillomaviruses are found in epithelial lesions and are linked to different carcinogenic processes in humans and other animals. Although bovine papillomavirus (BPV) has been characterized as epitheliotropic, the presence of viral DNA has been detected in other sample types, including fresh semen. The aim of this study was to evaluate the presence of BPV DNA in spermatozoa and seminal plasma samples of commercial frozen semen taken from bulls (*Bos taurus*) and its effects on semen function. PCR assays were conducted with specific primers to detect BPV types 1–6 in 40 semen samples of dairy Gir bulls. The semen quality was assessed by the use of parameters such as motility, vigor, acrosomal integrity and DNA integrity. BPV-2 DNA was detected in all of the sperm cell samples and all the seminal samples; however BPV-1, 3, 4, 5 and 6 could not be detected. The presence of BPV DNA was apparently not a cause of reduced sperm function. This is the first record of BPV-2 DNA the commercial frozen semen taken from dairy Gir cattle by several companies that provide semen. Further studies are needed to assess the viability of the virus and the extent to which it can be spread through semen.

1. Introduction

Papillomavirus (PVs) are double strand DNA viruses that infect mammals, birds and reptiles (Bernard et al., 2010). These viruses are linked to the development of lesions in the epithelium and various carcinogenic processes in humans (Bernard, 2005) and other animals, including cattle (Campo, 2006).

Currently, 11 bovine papillomavirus types (BPV) have been described (Hatama et al., 2008; Bernard et al., 2010), and three of these are involved in carcinogenic processes. Papillomas of the upper gastro-intestinal tract are linked to BPV-4 while lesions in the urinary bladder, are induced by BPV-2 and/or 1, and can lead to cancer in cattle that feed on bracken fern *Pteridium aquilinium* (Campo, 2006).

PVs are described as epitheliotropic (Borzacchiello and Roperto, 2008), although their presence has been detected in different body fluids, tissues and cells (Freitas et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009). Thus, it has been suggested that the virus can spread to non-epithelial tissues through fluids (Freitas et al., 2007).

Although there are several studies that have demonstrated the presence of HPV DNA in human semen (Rintala et al., 2004; Bezold et al., 2007; Didelot-Rousseau et al., 2007; Foresta et al., 2010a,b,c), few studies have recorded the presence of BPV DNA in cattle semen (Carvalho et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009).

The infection of the reproductive tract can be caused by several types of viruses and can have severe consequences, such as: (i) the spread of an infectious agent, (ii) infertility/sterility, (iii) cachexia-induced by a decreased level of testosterone synthesis, (iv) the incorporation of viral genome in germ cells and the risk of vertical transmission,

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doi:10.1016/j.anireprosci.2011.11.005

(v) infection of the egg or embryo, causing abortions or abnormalities in the conceptus development (Dejucq-Rainsford and Jégou, 2004).

In Brazil, the artificial insemination industry for cattle markets about 10 million doses of semen each year. About 43% of the bull semen sold in Brazil is dairy bull semen and 57% is beef bull semen. Multinational companies sell semen from Brazilian bulls and about 42% of the semen is imported from other countries (ASBIA, 2009). Thus, when the widespread use of frozen semen is taken into account, it is clear that contamination with BPV may be a very important factor in the process of artificial insemination (AI) and embryo transfer (ET).

The aim of this study was to evaluate the presence of BPV DNA, by means of the PCR technique, in spermatozoa and seminal plasma samples obtained from the frozen semen of bulls (*Bos taurus*) and to evaluate if there is any relationship between the presence of BPV DNA in semen and its quality.

2. Materials and methods

2.1. Semen samples

Frozen semen samples ($n = 40$) taken from dairy Gir bulls (*Bos taurus*) were obtained from four companies in Brazil, three of which are multinational companies. The samples were thawed in a water bath at 37 °C for 30 s and centrifuged at 1200 × *g* for 10 min to separate the sperm cells from the seminal plasma and diluents. The supernatant (200 μL) was used as a seminal plasma sample. The cell pellet obtained after centrifugation, was washed twice with PBS (0.9% saline phosphate buffered solution, pH 7.4) and again centrifuged at 1200 × *g* for 10 min. The final pellet obtained was re-suspended in 200 μL of PBS for subsequent DNA extraction.

2.2. DNA extraction

All the semen samples and grasshopper tissues (Tropidacris collaris) were submitted to DNA extraction by Qiagen DNeasy® Blood and Tissue kit (Qiagen, Germany), in accordance with the manufacturer's protocol. The DNA obtained from grasshoper was used as a control for cross-contamination with BPV, since the BPV is not naturally found in this organism. The quality of the purified DNA from semen was checked by β-globin gene PCR, as described by Freitas et al. (2003). In the case of the DNA from grasshopper, it was evaluated by PCR for 5S DNA with universal primers (kindly provided by Dr. M.J.L. Lopes, Laboratory of Animal Genetics – UFPE). The amplification was conducted in a final volume of 25 μL containing about 50 ng of DNA, 1X Master Mix (Promega, USA) and 0.2 μM of specific primers.

2.3. Viral DNA detection by PCR

The presence of the virus was determined by using specific primers to detect BPV-1 to 6 (Table 1). The set of primers target the L1 (BPV-1,-2,-3,-5 and -6) and E7 (BPV-4) genes. Standardization reactions were conducted and BPV-1 to 6 viral genome cloned into PAT 153 plasmid was used as positive control (Fig. 1). All the clones were used for each primer set to ensure their primer specificity. All the primers were specific to each viral type, except the BPV-3 primers that amplified the BPV-3 and 6 clones. PCR was conducted by using 0.2 μM of each primer, about 100 ng of DNA and 1X Master Mix (Promega, USA).

PCR was conducted using cycling parameters described by Stocco dos Santos et al. (1998) and consisted of an initial denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation for 40 s at 94 °C, annealing for 40 s at 68 °C with primers for BPV-1, 55 °C with primers for BPV-2, 60 °C with primers for BPV-3, 4 and 5, and 6 and extension for 1 min at 72 °C. As negative control, a no template control (NTC) i.e. water, was used, together with DNA of Madin-Darby bovine kidney (MDBK; ATCC-CCL22) cells. To confirm the presence of BPV-2 DNA in semen samples, a PCR was conducted with two different set of primers, one set targeting the E2 gene and another targeting the E5 gene (Roperto et al., 2008). The PCR cycle consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 52 °C for 40 s, and extension at 72 °C for 40 s. PCR products were electrophoresed on 2% agarose gel with TAE buffer at a constant voltage (100 V) for approximately 35 min. DNA was visualized under UV light after staining the gel with ethidium bromide. L1 and E5 BPV-2 amplicons were purified with Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA). The amplicons of E2 BPV-2 were cloned into the pGEMR-TEasy vector (Promega pGEM–T Easy Vector System, Promega, USA), and transformed into competent JM 109 *E. coli* cells (Promega, USA). The DNA recombinant clones were isolated with PureYeld™ Plasmid Miniprep System (Promega, USA). Subsequently, a direct sequencing using corresponding BPV-2 primers was conducted using the BigDye Terminator V3.1 Cycle sequencing kit (Applied Biossystems, USA).

The DNA sequences were analyzed with Staden Package software (http://staden.sourceforge.net) for the quality analysis of chromatogram readings and the generation of the consensus sequences. The identified sequences were analyzed by means of the BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.4. Evaluation of vigor and sperm motility

An aliquot of semen, previously heated to 37 °C, was analyzed subjectively under optical microscope (Olympus, Japan) for estimated progressive sperm motility (0–100%) and vigor (0–5) (Souza et al., 2006).

2.5. Evaluation of sperm acrosome and chromosome status

To assess the acrosomal integrity of the sperm, slides containing 10 μL of semen were prepared, stored at 4 °C, protected from light and analyzed with the aid of FITC-conjugated Peanut agglutinins – FITC-PNA technique as described by Roth et al. (1998). Assays for evaluation of the DNA integrity of the sperm were carried out by using acridine orange dye in accordance with the recommendations of Evenson et al. (2002).