Bovine papillomavirus E2 and E5 gene expression in sperm cells of healthy bulls

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Received: 9 April 2013 / Accepted: 14 November 2013 / Published online: 30 November 2013
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Abstract Papillomaviruses are found in epithelial lesions and are linked to different carcinogenic processes in humans and other animals. Although BPV has been characterized as epitheliotropic, the presence of viral DNA has been detected in other tissues and fluids, such as fresh semen. The aim of this study was to evaluate the presence and expression of BPV in sperm cells of bulls (*Bos taurus*) asymptomatic for papillomatosis. A PCR assay was carried out with specific primers to test BPV2 in 26 semen samples. The presence of BPV transcripts was assessed by RT-PCR to E2 and E5 genes. BPV2 DNA was detected in nine out of 26 samples and the expression of E2 and E5 were detected in five out of nine BPV positive samples. This is the first record of BPV2 expression in bull sperm cells.

Keywords Bovine papillomavirus · Semen · Viral gene expression

Papillomaviruses (PVs) are double-stranded DNA tumour viruses identified in a broad range of animal species that belongs to the amniotes, including human being [1]. So far,

13 genotypes of Bovine papillomavirus (BPV1–13) have been characterized [21] and classified in three different genera: Deltapapillomaviruses (BPV1 and 2), Epsilonpapillomaviruses (BPV5 and 8) and Xipapillomaviruses (BPV3, 4, 6, 9, 10 and 11). BPV7 is classified as an unassigned PV genus [12, 14]. BPVs from Delta-PVs and BPV5 can infect epithelial and subepithelial fibroblasts inducing fibro-epithelial tumours in cattle, known as fibropapillomas while BPVs from another genus can induce true papillomas [22]. Normally, papillomas regress as a result of cell-mediated immune response [17]. However, occasionally papillomas may develop in squamous cell carcinoma as a result of involvement of environmental and genetic co-factors [3, 5]. Recently, the co-infection among BPVs in skin lesions (warts) has been described, but the real significance of this finding need to be clarified [7, 25, 31].

PVs are described as epitheliotropic viruses, although their presence has been detected in different tissues and cells [11, 20, 36]. Few studies have contributed to improved understanding of PV transmission, however, its spread through non-epithelial, however, its spreading of through non-epithelial tissues and fluids has been suggested [6, 11].

In humans, infected sperm are able to penetrate the oocyte, to deliver HPV genome in the oocyte and HPV genes can be actively transcribed by the fertilized oocyte [10]. There are few reports focusing on the study of BPV in reproductive tract and its cells [13, 35] although the BPV-1 were described in the prepuce and penis [16] and BPV-2 were found infecting placenta and actively expressed in equine semen [30, 34]. In an early study, we demonstrate the high incidence of BPV2 in commercial doses of bull semen from national and multinational companies [33]. However, studies are needed to understand the biological significance of BPV presence in semen. The present study aimed to evaluate the presence of BPV in sperm cells of healthy bull, as well as the viral gene expression.

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The semen was collected from 26 healthy bulls (*Bos taurus*) belonging to 20 diverse farms located in several cities in an area of 300 km radius around Recife, Pernambuco State, in the North-East of Brazil. All bulls were asymptomatic for cutaneous papillomatosis. The samples were collected by electro ejaculation and cooled on ice. After that, the samples were centrifuged at $1,200\times g$ for 10 min and the cell pellet obtained was washed twice with PBS (0.9 % saline phosphate buffered solution, pH 7.4) and again centrifuged at $1,200\times g$ for 10 min to separate the sperm cells from seminal plasma and debris. The final pellet obtained was re-suspended in 200 μ L of PBS for subsequent DNA and RNA extraction.

All the samples were submitted to DNA and RNA extraction by Invirsob® Spin Universal RNA Mini Kit, (Invitex, Germany), in accordance with the manufacturer's protocol. The quality of purified DNA was checked by β -globin gene PCR, as previously described [13]. The amplification was carried out in a final volume of 25 μ L containing 100 ng of DNA, $1\times$ Master Mix (Promega, USA) and 0.2 μ M of specific primers. BPV2 DNA was detected by using primers targeting L1 BPV gene, as previously described [37]. Two negative controls were used, a no template control (NTC), and a control with DNA of Madin-Darby bovine kidney (MDBK; ATCC-CCL22) cells. A BPV2 genome cloned into pAT153 plasmid was used as positive control. PCR products were electrophoresed on 2 % agarose gel and the DNA was visualized after being stained with ethidium bromide. A sequencing using corresponding BPV2 primers was conducted using the BigDye Terminator V3.1 Cycle sequencing kit (Applied Biosystems, USA). The DNA sequences were analyzed with Staden Package software (<http://staden.sourceforge.net>) for the quality analysis of chromatogram readings and the formation of the consensus sequences. The identified sequences were analyzed by means of the BLAST tool (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

The RNA samples were digested with RNase-free DNase (Promega, USA) and the first strand of cDNA was synthesized with Oligo (dT) 15 primer (Promega, USA) and ImProm-II TM Reverse Transcriptase (Promega, USA). Two negative controls, one without the reverse transcriptase and another without RNA (NTC) were used during the synthesis of cDNA. The essential meiotic endonuclease -1 transcript was amplified from cDNA with primers designed (EME Fw AACTGA GGCCTGAAGAGACC and EMERev GGACTGGGTATC AGGCAGTT) to verify the presence of cDNA from transcripts in sperm cells. RT-PCRs were carried out by using 0.2 μ M of each primer, 0.5 μ L of cDNA and $1\times$ Master Mix (Promega, USA). The PCR parameters were consisted of 40 cycles of denaturation for 45 s at 94 °C, annealing for 40 s at 52 °C and extension for 40 s at 72 °C.

The presence of BPV transcripts were evaluated for two different BPV genes, E5 and E2. The two set of primers

were described [28, 33]. PCRs were carried out according conditions described [33]. The purified amplicon from PCR were cloned into the pGEMR-TEasy vector (Promega pGEM-T Easy Vector System, Promega, USA), sequenced and analyzed as described above.

Following β -globin PCR with scored positive for all DNA isolates for semen, we assessed for the presence of BPV2 DNA. The primers for BPV2 amplified a fragment of the expected size (160 bp) in nine out of 26 (35 %) semen samples (Fig. 1). The amplicons sequenced from PCR products revealed a similarity with BPV2 ranging from 97 to 99 % at the nucleotide level.

The cDNA samples were assessed for the transcripts of essential meiotic endonuclease-1 bovine gene followed by the assessment of E5 and E2 BPV2 transcripts in BPV2 positive samples. Five out of nine samples (55 %) were positive for the presence of BPV2 transcripts. Three samples were positive for E2 and E5 BPV2 expression, and two samples had only E2 or E5 expression (Fig. 2a, b). The two negative controls produced during cDNA synthesis scored negative. The results are summarized in the Table 1. The sequenced clones of BPV2 E2 and E5 amplicons confirmed the transcripts presence in the semen.

This is the first study, to the best of our knowledge, which describes the expression of BPV2 in sperm cells of bulls. Our results confirm and better define our previously obtained data on the detection of BPV2 in commercial semen [33]. Also, these results corroborate with our previous study where BPV was demonstrated being expressed in equine semen [34].

Our findings correspond with the previous results [6, 20, 36] who also detected BPV2 DNA in bull semen and inside the spermatozoa. However, this study is more comprehensive than the previous one because it was possible to investigate bull semen from a large number of farms, 20 in a total, and this study was developed in an extensive area of 300 km radius. In the case of humans, several studies have found the presence of HPV in semen [2, 8, 9, 19, 26].

We demonstrated the presence of E2 and E5-BPV2 transcripts in sperm cells of bulls. Previous studies showed the expression of E5 oncogene in other non-epithelial tissue, such as blood cells [4, 28] and semen [34]. Moreover, early and late proteins were found in white blood cells [29]. PV-containing blood cells were suggested to be responsible for spreading the infectious agent to numerous organs, including semen [11, 29]. This hypothesis may be corroborated by the detection of BPV in different tissues and cells, including reproductive sites as oocytes, the ovary, the uterus, cumulus cells, uterine lavage [6, 13, 20, 36]. It is reasonable to suppose that the presence of active BPV in semen of asymptomatic animals could facilitate the dissemination of BPV through this route.

The presence of BPV DNA and RNA in semen could also be explained by reproductive tract infections.

Fig. 1 Presence of BPV2 in bull semen detected by BPV2 L1 gene PCR. M: molecular marker, C+: positive control, C-: negative control, lanes 1–26 samples

Fig. 2 BPV2 gene expression in bull semen. 1 RT-PCR for E2 BPV2 mRNA. M: molecular marker; C+: positive control; C-: no template control, lanes 1–9 samples. 2 RT-PCR for E5 BPV2 mRNA. M: molecular marker, C+: positive control, C-: negative control, lanes 1–9 samples

Table 1 DNA presence of BPV2 and expression of E2 and E5 in bovine semen

Samples	BPV2 detection	BPV2 expression	E2 expression	E5 expression	E2 and E5 expression
1	+	+	+	+	+
2	+	–	+	–	–
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	–	–	–
6	+	–	–	–	–
7	+	+	–	+	–
8	+	–	–	–	–
9	+	–	–	–	–

+ stands for positive and
– stands for negative

Fibropapillomas were detected in both the prepuce and penis. Furthermore, it can spread along the perineum and even up toward the back. The fibropapillomas can cause loss of reproductive function and may lead animals to slaughter [16]. In humans, HPV DNA and RNA have been found not only in the penile shaft, glans and urethra but also in the ductus deferens, epididymis and testis [15, 23, 24, 32]. Moreover, there are studies showing the presence of BPV and HPV in the sperm cell by in situ hybridization [20]. Therefore, sperm may be a site of infection by BPV.

The HPV RNA presence was found in human seminal plasma and sperm cells [18]. However, in bovine there is no study about the RNA presence in sperm cells. These results seem to suggest that BPV as well as HPV could infect sperm cells, and express certain genes.

Previous reports suggest the vertical transmission of some PVs. In humans, sperm transfected with HPV genes are able to penetrate in oocytes, to deliver HPV genome, and HPV genes can be actively transcribed by the penetrate

oocyte [10]. Also, the transplacental transmission of PVs appears to take place in humans and cows [13, 27, 30].

This study adds a new dimension to what we know about BPV transmission mechanisms and pathogenesis. The better understanding of the implications of active BPV-containing semen can contribute to improve strategies to avoid BPV dissemination in the cattle herd and its implications in the reproduction.

Acknowledgments We would like to express our gratitude to the Brazilian Federal Agencies (CNPq and CAPES) for their financial support and Dr Érica Moraes for her technical support.

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