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ESTUDO DA BIOLOGIA E DIVERSIDADE DO PAPILOMAVÍRUS BOVINO EM LESÕES CUTÂNEAS E SÍTIOS NÃO EPITELIAIS DE BOVINOS E EQUINOS

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Maria Angélica Ramos da Silva

Estudo da biologia e diversidade do papilomavírus bovino em lesões cutâneas e sítios não epiteliais de bovinos e equinos

Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

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“... amai-vos, eis o primeiro ensinamento; instruí-vos, eis o segundo.”

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Resumo

Os papilomavirus bovino (BPV) são oncovirus com DNA circular dupla fita e usualmente espécie-específico. Embora o BPV cause doenças de importância veterinária, o conhecimento sobre sua diversidade e biologia ainda é limitado. Esse trabalho objetivou estudar a biologia e diversidade da infecção por BPV em lesões cutâneas de bovinos e sítios não epiteliais de bovinos e equinos. No capítulo I, buscou-se avaliar a diversidade de PV em lesões cutâneas. Foram encontrados os BPV 1, 2, 3, 6 e o Papilomavírus associado ao sarcóide felino (FeSarPV) e a presença de co-infecções. No capítulo II, foram avaliados mecanismos etiopatogênicos em lesões papilomatosas induzidas por BPV. Foi observada a expressão da oncoproteína E5 em todos os fibropapilomas analisados e uma superexpressão da conexina 26 nos fibropapilomas, comparado com o tecido normal. No capítulo III, foi verificada a presença do DNA de BPV em espermatozoide e líquido seminal de sêmen comercial de touros e seu efeito na função espermática. O DNA do BPV2 foi encontrado em todas as amostras avaliadas, porém sem causar redução na função espermática. No Capítulo IV, foi avaliada a presença e expressão de BPV em sangue de bovinos sadios e afetados por papilomatose. Os BPV 1 e 2 foram encontrados em animais assintomáticos e com papilomatose assim como sua expressão. No Capítulo V, foi verificada a presença e expressão de BPV em sêmen fresco de touros saudáveis. O DNA de BPV2 foi encontrado em 35% das amostras e a expressão das proteínas E2 e E5 foi verificada em 55% das amostras de sêmen positivas para BPV2. No capítulo VI foi investigada a presença e expressão genes de BPV em células do sangue e sêmen de cavalos saudáveis por PCR e RT-PCR. Os BPV 1 e 2 foram encontrados no sangue de 20% dos cavalos avaliados e no sêmen 35% dos animais. A expressão da oncoproteína E5 foi verificada em 36% das amostras de sangue e em sêmen positivas para o BPV. No Capítulo VII, foram comparados dois sistemas de detecção de BPV por PCR, um com *primers* tipo específico e outro com *primers* consenso em lesões cutâneas e fuidos de bovinos. Os *primers* tipo específico para detecção de BPV mostraram-se mais sensíveis do que os *primers* consenso, além disso, através destes foi verificada a alta prevalência de co-infecções nas amostras estudadas. Porém, os *primers* consenso, amplificaram alta diversidade de BPV, e prováveis novos tipos de BPV nas amostras estudadas. Desta forma, os trabalhos oriundos desta tese, permitiram descrever a diversidade de BPV nas lesões cutâneas de bovinos do Brasil e um de seus prováveis mecanismos de ação, além disso, contribuem para fortalecer a hipótese de que os BPV são capazes de infectar tecidos não-epiteliais, através da verificação da presença e expressão de genes virais em células sanguíneas e do sêmen de bovinos e equinos. Os resultados obtidos nesta tese vêm contribuir para o melhor conhecimento de ferramentas moleculares que podem ser empregadas nos estudos de presença e caracterização do BPV nos diversos tecidos bovinos.

Palavras-chave: BPV, FeSarPV, Conexina 26, Sêmen, Sangue, Expressão, PCR.

Abstract

Bovine papillomavirus (BPV) are double-stranded circular DNA oncovirus and usually species-specific. Although BPV causes diseases of veterinary importance, knowledge about their biology and diversity is still limited. This study investigated the natural history of BPV infection in cattle and cutaneous epithelial sites of cattle and horses. In Chapter I, we sought to evaluate the diversity of PV in cutaneous lesions. We found BPV 1, 2, 3, 6 and papillomavirus associated with feline sarcoid (FeSarPV) and co-infections. In Chapter II, we detected the BPV DNA in sperm and seminal fluid of trade bull semen and its effect on sperm function. The DNA of BPV2 was found in all samples, without causing a reduction in sperm function. In Chapter III, we evaluated etiopathogenic mechanisms of BPV induced lesions. We observed the expression of E5 oncoprotein in all analyzed fibropapillomas and an overexpression of connexin 26 in fibropapillomas compared to normal tissue. In Chapter IV, we evaluated the presence and expression of BPV in blood of healthy and affected cattle by papillomatosis. The BPV 1 and 2 were found in asymptomatic and papillomatosis-affected animals as well as its expression. In Chapter V, we detected the presence and expression of BPV in fresh semen of healthy bulls. BPV2 DNA was found in 35% of samples and the expression of E2 and E5 proteins was found in 55% of BPV2-positive semen. In Chapter VI was investigated the presence and expression of BPV genes in blood and semen cells of healthy horses by PCR and RT-PCR. BPV 1 and 2 were found in 20% of blood horses and in 35% of semen evaluated. Expression of E5 oncoprotein was found in 36% of blood and semen samples positive for BPV. In Chapter VII, we compared two PCR methods for BPV detection in skin lesions and fluids: the use of BPV type-specific and consensus primers. The type-specific primers for detection of BPV were more sensitive than consensus primers and could detect co-infection of BPV in the samples. Consensus primers amplified a high diversity of BPV, and probable new BPV types in the samples studied. Thus, this thesis allowed describing the diversity of BPV in the skin lesions of Brazillian cattle and one of its possible mechanisms of action and also contributes to strengthen the hypothesis that BPV may infect non-epithelial sites, by verifying the presence and expression of viral genes in blood and semen cells of cattle and horses. The results obtained in this thesis contribute to a better understanding of molecular tools that can be employed in studies of presence and characterization of BPV in various bovine tissues.

Key words: BPV, FeSarPV, Connexin 26, Semen, Blood, Expression, PCR

Lista de Ilustrações

SUMÁRIO

1. Introdução

Papilomavírus (PV) é um grupo diversificado de pequenos vírus, não envelopado, de DNA fita dupla circular e epiteliotrópico. Estão classificados na família *Papillomaviridae,* a qual compreende 29 gêneros. Esses vírus são conhecidos como espécie-específicos, porém alguns podem causar infecção cruzada em equídeos e outros animais. O Papilomavirus bovino (BPV) induz infecções de considerável importância veterinária em bovinos e equinos. A papilomatose bovina é uma doença infecto-contagiosa que compromete a produtividade do rebanho. Na região Nordeste, em especial no Estado de Pernambuco (região da Zona da Mata), a ocorrência de papilomatose bovina pode chegar a 30% em alguns rebanhos provocando grandes perdas econômicas para os criadores. O sarcóide equino, também causado pelo BPV, é a neoplasia cutânea mais frequente entre os equídeos, cujo comportamento clínico é localmente agressivo devido à capacidade infiltrativa.

Embora o BPV cause doenças de importância veterinária, o conhecimento sobre sua diversidade ainda é limitado. Além disso, pouco se sabe acerca dos mecanismos etiopatogênicos das lesões induzidas pelo BPV. Esses conhecimentos poderão contribuir para o desenvolvimento de medidas profiláticas e terapêuticas para o controle das papilomaviroses.

Embora os papilomavírus sejam descritos classicamente como epiteliotrópicos, a atividade destes vírus já foi verificada em sítios não epiteliais como sangue e sêmen de bovinos e sangue equinos. Alguns estudos sugerem que o sangue possa atuar como sítio de reserva e propagação para o BPV. Dessa forma,

após infectar o epitélio, sítio de entrada do vírus no corpo do animal, a lesão causada pelo BPV induziria a resposta do sistema imune, atingiria os linfócitos e, destas células, seria levado para os demais sítios do corpo. Essas descobertas podem trazer significantes contribuições para o estudo da transmissão do BPV, porém poucos estudos foram realizados bucando entender os mecanismos de trasmissão de BPV e os principais tipos virais envolvidos na infecção de sítios não-epitelias e a sua transmissão por essas rotas.

2. Revisão da Literatura

2.1 Papilomavírus

Os papilomavírus (PV) formam um grupo altamente diversificado de vírus que infectam os amniotas (Freitas *et al.,* 2011), porém são espécie-específicos e até mesmo em condições experimentais, não infectam outro hospedeiro que não o seu natural (Campo, 2006). Os poucos casos conhecidos de infecção cruzada entre PV envolve o papilomavírus bovino (BPV) e outras espécies animais (Freitas *et al.,* 2011).

Inicialmente, os papilomavírus eram classificados na subfamília *Papilomavirinae*, dentro da família *Papovaviridade* que incluíam os Polyomavírus (Bernard, 2005). Posteriormente, os PV foram re-classificados e formam a grande família Papilomaviridae, que compreende 29 gêneros (*Alphapapillomavirus* a *Dyoiotapapillomavirus)* e mais de 200 tipos virais (de Villiers *et al.,* 2004; Bernard *et al.,* 2010). Embora a maior parte dos tipos virais caracterizados correspondam a PV humanos (150), 64 tipos virais foram identificados em hospedeiros mamíferos não-humanos, três tipos foram isolados de pássaros e outros dois de répteis (Bernard *et al.,* 2010).

Para a identificação de novos tipos de PV tem sido utilizada a sequência de nucleotídeos do gene L1, por ser o mais conservado do genoma. Um PV isolado é reconhecido como novo tipo se o seu genoma completo for clonado e a sequência da ORF (quadro aberto de leitura, de *Open Reading Frame*) L1 divergir em mais de 10% do tipo de PV conhecido mais próximo. Diferenças entre 2% e 10% de

homologia definem um subtipo, e menor que 2% uma variante (de Villiers et al., 2004).

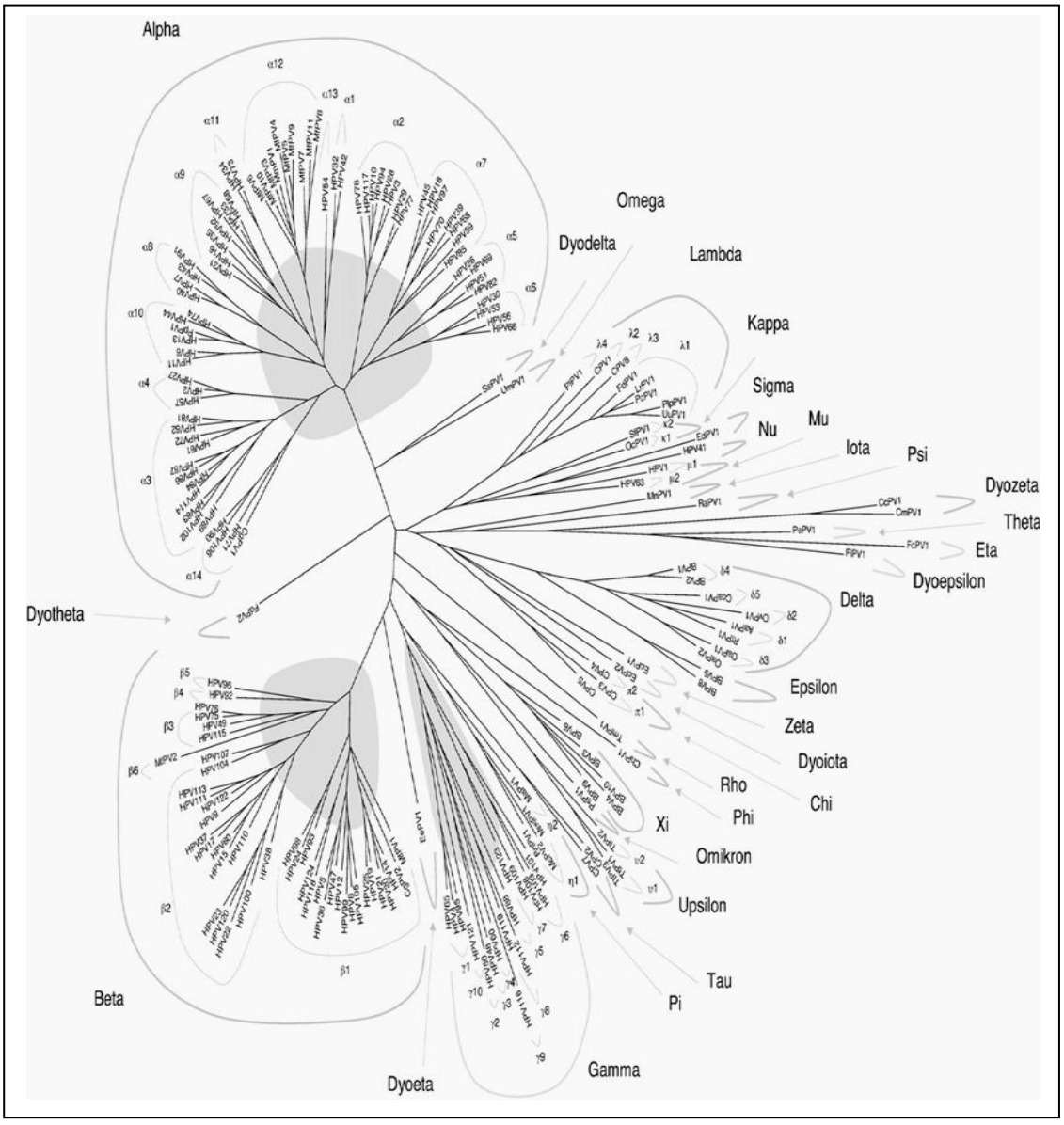

Figura 1 Árvore filogenética da família *Papillomaviridae* (Bernard *et al.,* 2010).

Todos os PV possuem genoma de DNA fita dupla circular, com tamanho aproximado de 8Kb e peso molecular de cerca de $5{,}6x10^2$ Dalton e pode ser dividido, em geral, em três principais regiões: precoce (E, do inglês *early*), tardia (L, de *late*) e longa região de controle (LCR, *long control region*) (Figura 2). As três

regiões são separadas por dois sítios de poliadenilação (pA): o sítio na região precoce (pAE) e outro na região tardia (pAL) (Zheng & Baker, 2006).

A informação genética está distribuída em pelo menos oito ORF no genoma dos papilomavírus. Os genes de expressão precoce (E) codificam proteínas envolvidas na replicação do DNA (E1 e E2), na transcrição (E2 e E4) e no processo de transformação celular (E5, E6 e E7). Os genes de expressão tardia (L) codificam as proteínas L1 e L2 que formam o capsídio viral (Campo, 2006). A proteína L1, a principal proteína do capsídeo, está arranjada em 72 pentâmeros (60 hexaméricos e 12 pentaméricos) e possui capacidade de se auto-arranjar em VLPs, *virus-like particles* (partículas semelhantes ao vírus). A proteína L2 é uma ligante de DNA, necessária para a encapsidação do genoma (Campo, 1995). Diferentemente dos HPV, em BPV a proteína E5 é a principal oncoproteína, seguida por E6 e, com um papel mais modesto na transformação celular, a E7 (Nasir e Campo, 2008).

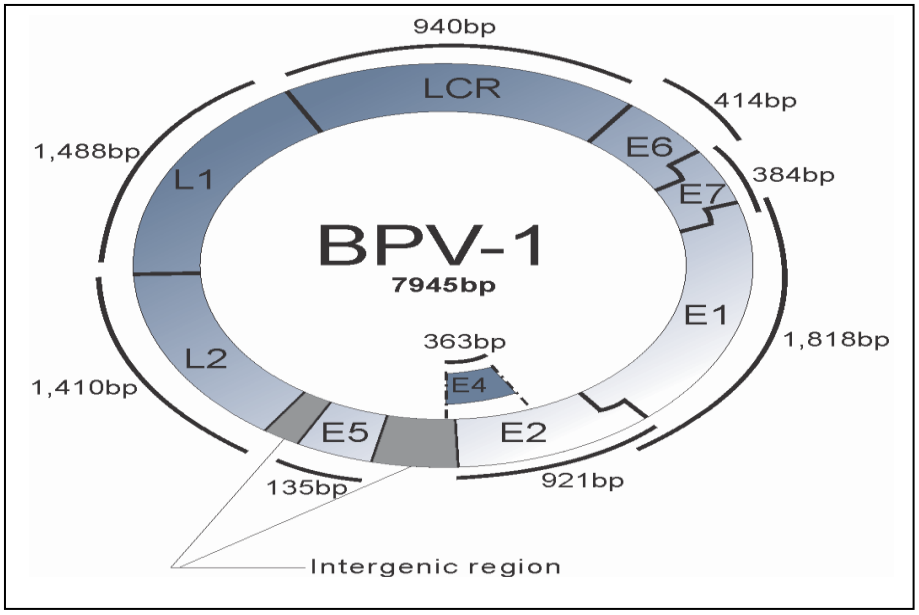

Figura 2: Diagrama da organização do genoma de BPV1. O genoma viral está representado em círculo, demonstrando as posições dos genes virais E6, E7, E1, E2, E4, E5, L2, L1 e o LCR entre os genes L1 e E6 (Freitas et al., 2011).

A região LCR é um segmento de cerca de 850 pb (10% do genoma de HPV), possui função não-codificante, mas contém a origem de replicação. Além disso, contém muitos sítios ligantes de fatores de transcrição que são importantes na regulação da RNA polimerase (Zheng & Baker, 2006). Os vírions possuem estrutura não envelopada icosaédrica, com tamanho aproximado de 55 nm, que contém o DNA complexado com histonas do hospedeiro e condensado em nucleossomos (Chang, 1990; zur Hausen, 1996).

A replicação do vírus ocorre nas células basais do epitélio, estimulando a divisão celular e provocando hiperproliferação, formando as verrugas ou papilomas (Campo, 2006). Em geral, os papilomas regridem espontaneamente sem causar problemas clínicos a seus hospedeiros (Campo, 2002). Esta hiperproliferação ocorre, mais frequentemente, nas células epiteliais da pele ou mucosas, mas alguns tipos de vírus podem infectar fibroblastos.

Os PV induzem tumores benignos que, eventualmente, quando fatores genéticos ou ambientais estão envolvidos, podem resultar em conversão maligna (Campo, 2006).

O ciclo infeccioso dos PV está intimamente ligado à diferenciação epitelial, uma vez que estes vírus se replicam no epitélio escamoso estratificado da pele e mucosas. As células infectadas se dividem e espalham lateralmente (Figura 3). Algumas células migram para as camadas suprabasais e se diferenciam, neste processo genes virais são ativados culminando na formação do capsídio viral (zur Hausen, 2002). A liberação dos vírions é dependente da desintegração celular normal, tipicamente observada na superfície apical do epitélio. A dificuldade de mimetizar a diferenciação do epitélio estratificado em culturas de células tem sido um desafio para o estudo do ciclo de vida desses vírus. Dessa forma, muito do que

se sabe sobre a morfogênese deriva do estudo de versões recombinantes das proteínas estruturais L1 e L2 (Buck et al., 2005).

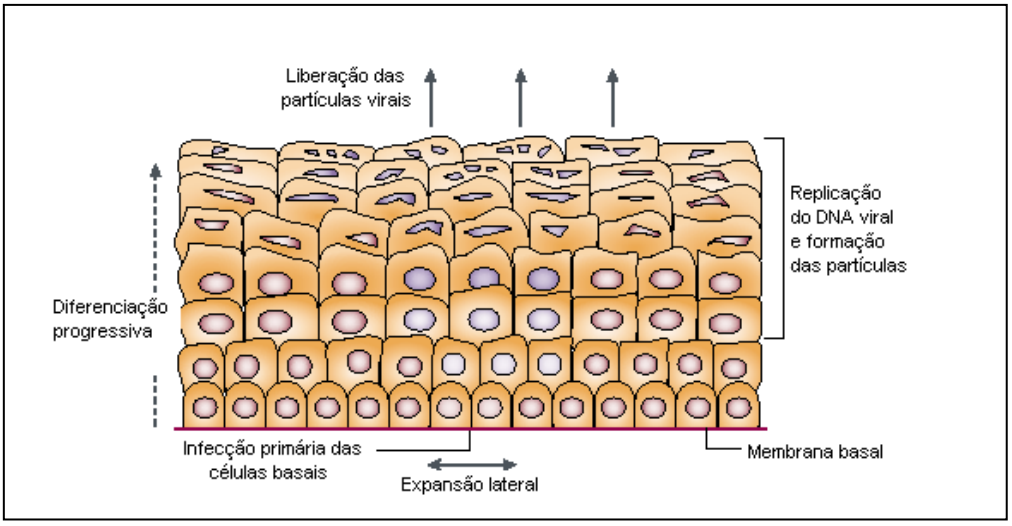

Figura 3: Ciclo infeccioso do Papilomavírus, seguindo a diferenciação do epitélio (zur Hausen, 2002).

Ainda não foi completamente elucidado como fatores carcinogênicos e agentes promotores estão envolvidos em diferentes estágios do desenvolvimento de papilomas e carcinomas, porém foram descobertos dois estágios do mecanismo de carcinogênese, a iniciação e a promoção, que têm componentes independentes (zur Hausen, 1996). A ação oncogênica viral envolve a expressão de genes que codificam proteínas precoces (E6 e E7) dentro da célula hospedeira. Estas oncoproteínas interferem no controle do ciclo celular através de interação com proteínas celulares específicas, tais como a proteína 53 (p53) e a proteína do retinoblastoma (pRB) (Campo, 2003).

A transmissão das papilomaviroses é facilitada pela presença de abrasões na superfície do epitélio. As infecções anogenitais são principalmente transmitidas pelo contato sexual (zur Hausen, 1996). O papilomavírus humano (HPV) é a principal doença transmitida sexualmente (Dunne *et al.,* 2006) e também o principal agente etiológico de neoplasias do epitélio cutâneo e mucoso. Cerca de 40 tipos de HPV estão associados com o câncer (zur Hausen, 2002). Grande parte das infecções é assintomática ou subclínica e mantêm-se indetectável através do tempo. Os PV parecem coexistir com seus hospedeiros por longos períodos de tempo, alguns ocorrem preferencialmente em um ciclo de vida latente, pois grande variedade de tipos de PV foi isolada de pessoas e animais aparentemente saudáveis (Antonsson & Hansson, 2002; Antonsson *et al.,* 2003a; Antonsson *et al.,* 2003b; Ogawa *et al.,* 2004).

Os PV que infectam animais têm tido grande importância na investigação da biologia do vírus, da sua relação com o hospedeiro e sua resposta imune, além de possibilitarem o desenvolvimento de vacinas anti-papilomavírus. A ligação entre infecção por PV e neoplasia e a relação entre o vírus e co-carcinógenos ambientais foram primeiro estabelecidas para PV de animais, particularmente os PV de coelho, (CRPV), bovino (BPV) e oral canino (COPV) (Nasir e Campo, 2008).

2.2 Papilomavírus bovino

Atualmente, existem 12 tipos de BPV (BPV-1 a 12) descritos na literatura (Hatama, 2012). Originalmente, os BPV foram classificados em dois subgrupos, A e B, tomando-se como base a estrutura genômica e a patologia. Os BPV foram amplamente descritos em rebanhos bovinos de todas as regiões do mundo. Alguns estudos demonstraram sua incidência em rebanhos da Europa, América, Ásia, Oceania e África (Singh *et al.,* 2009; Munday *et al.,* 2010; Schimitt *et al.,* 2010; Carvalho *et al.,* 2012; Hatama, 2012).

De acordo com a nomenclatura atual, que leva em consideração as propriedades biológicas e organização do genoma, os BPV epiteliotrópicos (BPV-3, 4, 6, 9, 10, 11 e 12) são definidos como Xi-papilomavírus, os BPV1 e 2 como Delta-papilomavírus e os BPV 5 e 8 são classificados no gênero Epsilon-papilomavírus (de Villers *et al.,* 2004; Zhu *et al.,* 2011; Hatama, 2012). Análises filogenéticas baseadas na ORF L1 classificaram um isolado de BPV do Japão como BPV7, originando um novo gênero na família *Papillomaviridae* (Ogawa *et al.,* 2007) (Figura 4). Além destes tipos bem caracterizados, alguns supostos novos tipos de BPV já foram isolados, baseando-se em técnicas de PCR com primers degenerados que amplificam uma região do gene L1 (Antonsson & Hansson, 2002; Ogawa *et al.,* 2004; Ogawa *et al.,* 2007; Tomita *et al.,* 2007; Maeda *et al.,* 2007; Claus *et al.,* 2008; Carvalho *et al.,* 2012).

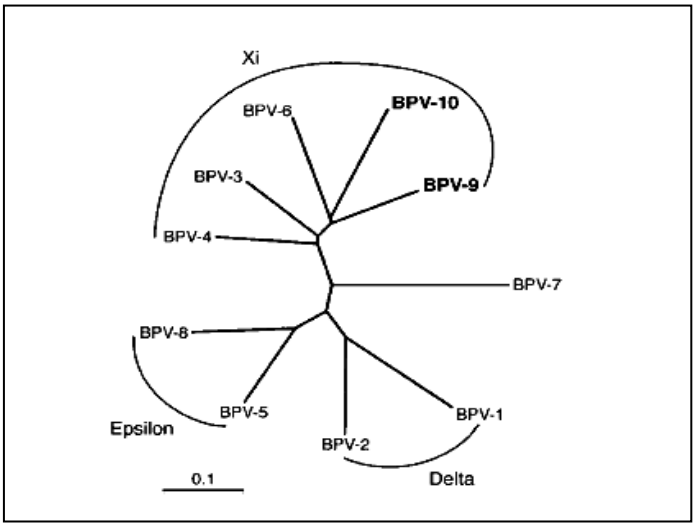

Figura 4 Árvore filogenética com a disposição dos grupos de papilomavírus bovino (Hatama *et al.,* 2008).

A estrutura genômica dos BPV dos gêneros Delta e Epsilon-Papillomavírus é semelhante a outros PV possuindo aproximadamente 8000 nucleotídeos. Os BPV 3, 4 e 6, do gênero Xi-Papillomavírus, possuem cerca de 7300 nucleotídeos e não possuem o gene E6, que codifica uma proteína precoce com função de transformação celular. A região LCR dos BPV 1, 2 e 5 contém 12 sítios ligantes de DNA para E2 (E2BS), um regulador da transcrição viral, enquanto os BPVs 3, 4 e 6 possuem apenas quatro E2BS, com um arranjo muito semelhante aos HPV genitais. Embora os Xi-BPV não possuam a proteína E6, estes vírus conseguem realizar seus ciclos infecciosos com sucesso e até mesmo causar a progressão de papilomas a carcinoma como acontece com o BPV4 e o câncer do trato gastro intestinal (Campo, 2006). A oncoproteína E5, principal oncoproteína entre os BPV, é muito hidrofóbica devido ao seu alto conteúdo de leucina. Geralmente localiza-se em compartimentos endomembranosos celulares onde interage causando inibições das junções Gap intercelulares. Além disso, E5 interage com o receptor celular PDGF e ativa grande número de proteínas quinase responsáveis pelo controle do ciclo celular (Nasir &

Campo, 2008). Outros estudos demonstram que a E5 também interage com o complexo MHC I, permitindo que células infectadas pelo BPV possam se evadir da resposta imune do hospedeiro (Araibi *et al.,* 2004).

Os papilomas que aparecem na superfície da pele geralmente são benignos, e o animal afetado não morre em decorrência dessa infecção. Em muitos casos, os tumores regridem espontaneamente como resultado da reposta imune celular (Knowles *et al.,* 1996). Contudo, os tumores ocasionalmente não regridem e se espalham na superfície da pele. Estas formas de papilomatose são problemáticas e causam prejuízos econômicos aos criadores por impedirem a ordenha em vacas leiteiras, levarem à depreciação do couro do animal e em casos graves levarem à perda de peso com consequente desvalorização da carcaça (no caso do gado de corte) e redução da produção de leite (no caso de gado leiteiro) (Hatama, 2012).

As infecções pelos fibropapilomavírus começam por uma transformação inicial dos fibroblastos sub-epiteliais, seguida por acantose e então papilomatose. As infecções causadas pelos papilomavírus epiteliotrópicos induzem papilomas epiteliais por meio de infecção dos queratinócitos, sem envolvimento dos fibroblastos. Os BPV1 e 2 são os principais agentes de fibropapilomas cutâneos. O BPV1 também pode causar fibropapilomas nas mamas e no pênis e o BPV2, fibropapilomas do trato digestório. O BPV5 causa fibropapilomas em forma de grão de arroz no úbere e os BPV3, 4, 6 e 9 induzem papilomas epiteliais cutâneos, papilomas no epitélio alimentar e papiloma epitelial na teta, respectivamente (Bloch *et al.,* 1994; Campo, 1997) (Figura 5). Embora a natureza patológica dos BPV 7, 8, 10 e 11 não tenha sido ainda completamente determinada, estes têm sido associados com lesões nas tetas, e o BPV12 com lesões na língua (Hatama, 2012).

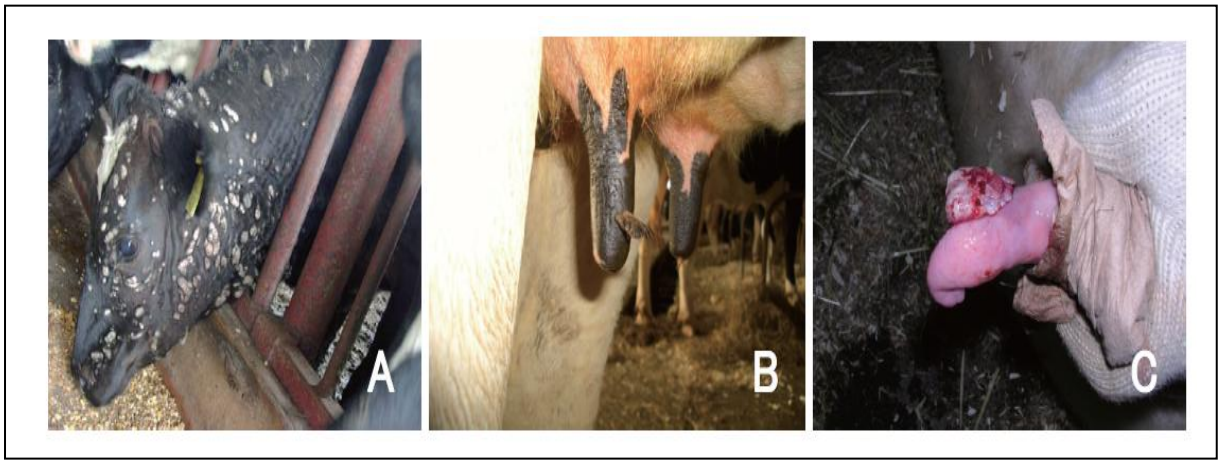

Figura 5: Papilomas cutâneos típicos. A. Fibropapilomas na cabeça e pescoço causados por BPV2. B. Papiloma epiteliais em tetas causados por BPV6. C. Fibropapilomas no pênis causados por BPV1 (Hatama, 2012).

Quando papilomas, ou tumores, são formados na mucosa do trato gastro-intestinal e bexiga, estes algumas vezes podem progredir para carcinomas, principalmente quando associadas a co-fatores ambientais, como o consumo do broto da samambaia *Pteridium aquilinum* (Campo, 2006). Esta é uma planta invasora e cosmopolita de regiões tropicais e temperadas, que infesta campos e contém agentes imunossupressores e mutagênicos. O consumo da samambaia pelo gado pode levar ao desenvolvimento de hematúria enzoótica e câncer de bexiga urinária (Borzachielo *et al.,* 2003; Campo, 2006). Os tumores do trato alimentar são os mais comuns induzidos por BPV. Esses tumores, induzidos por BPV4, quando associados ao consumo de *P. aquillinium*, podem progredir para o carcinoma celular escamoso. Lesões na bexiga urinária induzidas por BPV2 e 1 também podem progredir para o câncer (Figura 6) (Campo, 2002; Borzacchielo, 2007).

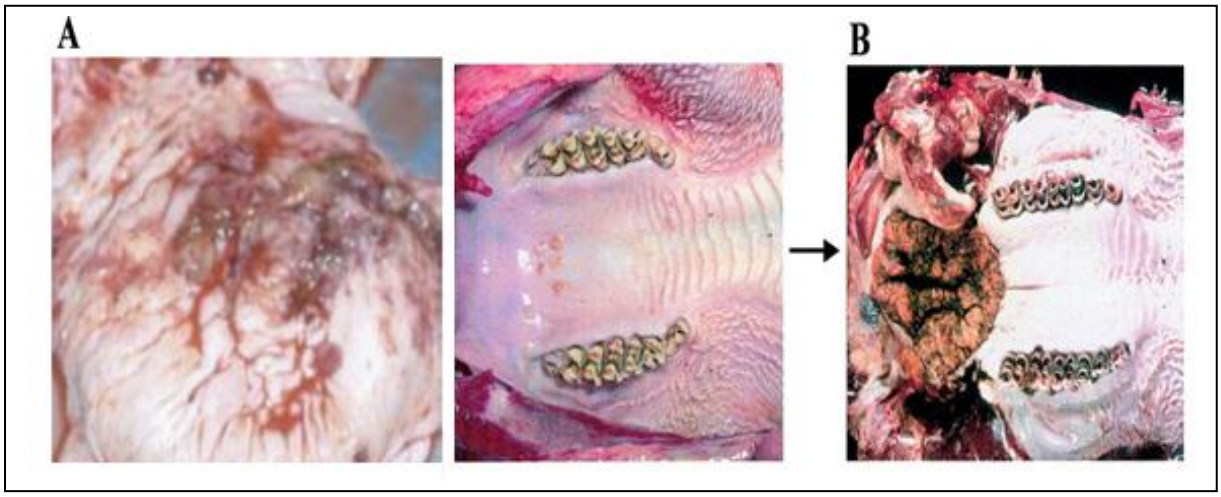

Figura 6: Carcinomas induzidos por BPV. A Câncer de Bexiga; B Progressão de papiloma a câncer do trato gastro-intestinal superior (Borzachiello, 2007; Campo, 2002).

Além de tumores em bovinos, os BPV1 e 2 também estão envolvidos no aparecimento de sarcóide em equídeos. O sarcóide equino é a neoplasia cutânea mais frequente entre os equídeos, cujo comportamento clínico é localmente agressivo devido à capacidade infiltrativa (Plummer, 2005). Este tumor possui seis tipos de apresentação clínica: os tipos oculto ou superficial, verrucoso, fibroblástico, nodular, misto e o maligno (Figura 7) (Scott & Miller 2003), não sofrem metástase, mas tendem a crescer a depender do tratamento (Moriello *et al.,* 2000).

Nos tipos agressivos, podem comprometer a utilização e o bem estar do animal. Vários estudos apontam a detecção de DNA de BPV em quase 100% das amostras de sarcóide equino (Otten *et al.,* 1993; Carr *et al.,* 2001; Martens et al., 2001). Além disso, o mRNA e proteínas de BPV já foram detectados em tecidos de sarcóide (Nasir & Reid, 1999; Carr *et al.,* 2001b; Bogaert *et al.,* 2008). Estudos recentes sugerem que o BPV possa infectar a epiderme de cavalos e causar uma infecção produtiva (Brandt *et al.,* 2008; Bogaert *et al.,* 2010; Brandt *et al.,* 2011).

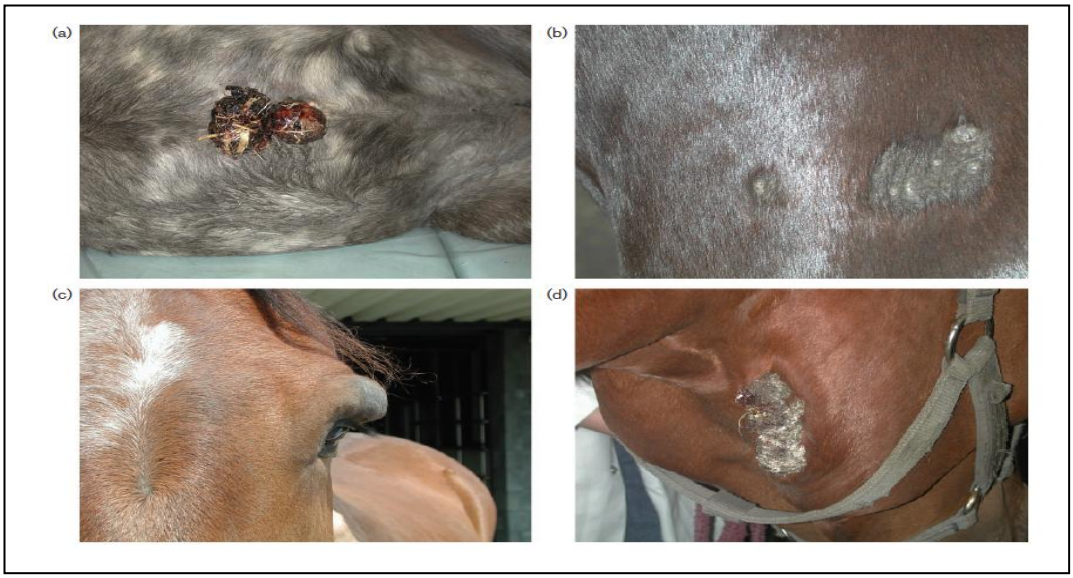

Figura 7: Quatro tipos clínicos de sarcóide. (a) Sarcóide fibroblástico; (b) sarcóide oculto; (c) sarcóide nodular; (d) sarcóide verrucoso (Bogaert *et al.,* 2007) .

Novos achados nos estudos em BPV estão levando diferentes olhares sobre a infecção e patogênese causadas por estes vírus, dentre eles seguem-se a associação entre BPV e tumores linfoepiteliais, vasculares e outros tumores mesenquimais (Borzacchiello *et al.,* 2007) e a associação entre BPV e inflamação (Yuan *et al.,* 2007; Bogaert *et al.,* 2008). A importância da inflamação crônica no processo de oncogênese é alvo de intensa investigação, e a presença e expresão de BPV em sítios de inflamação provê uma importante área de investigação (Borzacchiello *et al.,* 2009).

Atualmente, pouco se sabe como os papilomavírus são transmitidos entre animais. Sabe-se que animais confinados são mais susceptíveis à infecção porque a disseminação do vírus pode ocorrer mais diretamente (de animal para animal) ou indiretamente (por objetos contaminados) (Hama *et al.,* 1988; Nasir & Campo, 2008). Os mecanismos de transmissão de BPV para/ ou entre hospedeiros não específicos não é claro. Estudos recentes demonstrando a formação de capsômeros de BPV na epiderme de equinos apresentando sarcóide podem ajudar a explicar a ocorrência

de sarcóide equino em cavalos que foram mantidos longe de bovinos (Bogaert *et al.,* 2008; 2010a, b; Brandt *et al.,* 2011). Acredita-se que moscas possam ter papel na transferência de BPV de bovino para equino (Nasir & Campo, 2008) e na transmissão de BPV1 entre equídeos (Finlay *et al.,* 2009). Contudo, essa via alternativa de transmissão pode ser menos eficitente (Bravo *et al.,* 2010).

Apesar dos PV serem descritos como estritamente espécie-específico (Campo, 2006), alguns PV, em particular alguns BPV podem infectar grande variedade de hospedeiros. Os diferentes tipos de PV estão distribuídos em uma grande diversidade de vertebrados e assume-se que eles tenham co-evoluido com seus hospedeiros (Bernard, 1994; Antonsson & McMillan, 2006). Todas as espécies de mamíferos hospedam um ou mais tipos de PV (Sundberg *et al.,* 2001). O BPV pode infectar o bovino e além deste, também infecta espécies próximas, tais como búfalos (Silvestre *et al.,* 2009; Pangty *et al.,* 2010) e girafas (van Dyk *et al.,* 2011) causando fibropapiloma e lesões de bexiga (Pathania *et al.,* 2011). Além disso, estes virus podem infectar naturalmente espécies mais distantemente relacionadas, tais como tapir (Kidney & Berrocal, 2008), cavalos (Bogaert *et al.,* 2008), atílopes (van Dyk *et al.,* 2011) e zebras (van Dyk *et al.,* 2009). Uma variante de BPV8 tabém pode causar papilomas em bisão (Literak *et al.,* 2006) (Figura 8).

O papilomavírus associado ao sarcóide felino – *Feline Sarcoid-associated Papillomavirus* (FeSarPV), primariamente identificado em sarcóides felinos, também foi encontrado em fibropapilomas bovinos e dermatites. Tem-se sugerido que o FeSarPV é um PV bovino causando infecção cruzada em felinos, assim como os BPV 1 e 2 causam sarcóides em equinos (Munday & Knight, 2010).

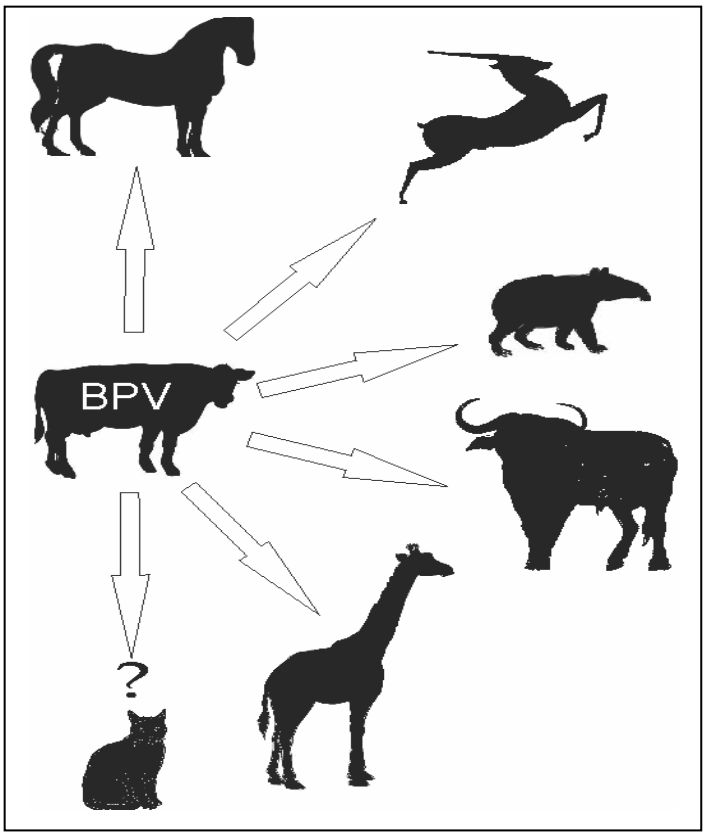

Figura8: Representação esquemática da infecção cruzada causada por BPV. O DNA de BPV foi encontrado em equídeos, antílopes, tapir, búfalos, girafa e, provavelmente, em gatos (Freitas *et al.,* 2012).

A habilidade do BPV1 em infectar hospedeiros relacionados pode ser resultado da domesticação dos bovinos e equinos ou uma aquisição fenotípica dirigida pela transmissão entre espécies do vírus mediada por vetor (Finlay *et al.,* 2009; Gottschling *et al.,* 2011). Assim, mudanças ecológicas podem ter acontecido concomitantemente nos diferentes hospedeiros e então podem ter aumentado a suscetibilidade desses hospedeiros para o BPV, ou simplesmente, o aumento do contato entre os hospedeiros fizeram com que estes se tornassem novos potenciais hospedeiros para o BPV (Gottschling *et al.,* 2011).

2.3 BPV em sítios não epiteliais

Embora o BPV seja descrito com epitélio específico, este foi encontrado em diferentes tecidos e fluidos, tais como sangue periférico, plasma sanguíneo, leite e colostro (Freitas *et al.,* 2003; Wosiacki *et al.,* 2005; Freitas *et al.,* 2007). Além disso, ele foi encontrando em sitios reprodutivos, incluindo oócitos, ovário, útero, células do cúmulus, fluidos uterinos, sêmen e espermatozóide (Carvalho *et al.,* 2003; Yaguiu *et al.,* 2006), assim como placenta e líquido amniótico (Freitas *et al.,* 2007).

Alguns autores sugerem que o sangue possa atuar como sítio de reserva e propagação para o BPV (Freitas *et al.,* 2003; Freitas *et al.,* 2007; Brandt *et al.,* 2008) (Figura 9). Dessa forma, após infectar o epitélio, sítio de entrada do BPV no corpo do animal, a lesão causada pelo vírus induziria uma resposta do sistema imune, atingiria os linfócitos e, destas células, seria levado para os demais sítios do corpo.Yaguiu *et al.* (2008) verificaram a presença de BPV1, 2 e 4 no sangue de vaca e de suas proles. Stocco dos Santos *et al.* (1998) inocularam sangue de vacas infectadas por BPV em vacas saudáveis e verificaram a presença deste vírus após 18 meses no sangue das vacas receptoras e em sua prole.

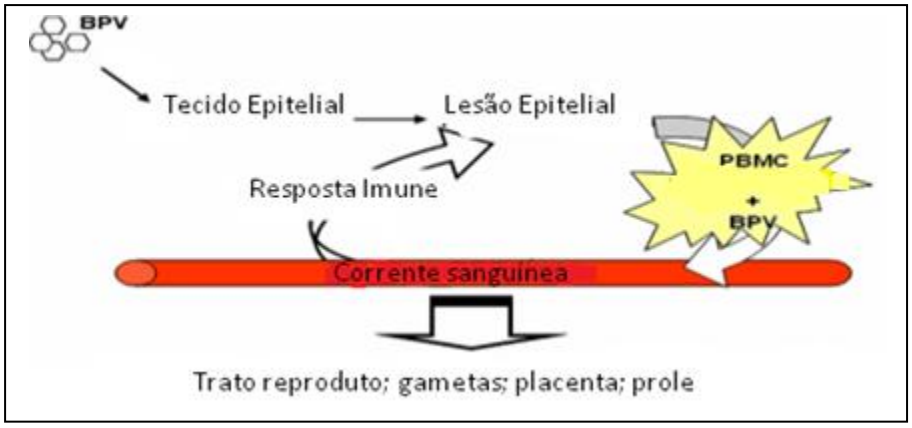

Figura 9: Proposição sobre a forma de transição do BPV entre os tecidos e fluidos bovinos (Freitas *et al.,* 2007).

Em humanos, o DNA e mRNA de diversos PVs foram detectados em sangue de pacientes com câncer cervical (Kay *et al.,* 2005), assim como de doadores saudáveis de sangue (Bodaghi *et al.,* 2005). De acordo com Bravo *et al.,* (2010) as implicações da presença de um único tipo dePV em uma variedade de tecidos no mesmo hospedeiro e em hospedeiros diferentes podem ser muito profundas e indicar maior plasticidade desse tipo viral.

2.4 Papilomavírus bovino e trato reprodutor

Em humanos, a presença do HPV no sistema reprodutor masculino e sêmen, assim como suas possíveis implicações, têm sido amplamente estudados. O DNA do HPV em sêmen tem sido demonstrado na literatura (Olatunbosun *et al.*, 2001; Rintala *et al.*, 2004; Didelot-Rosseau *et al.*, 2007; Giovannelli *et al.*, 2007; Bezold *et al.*, 2007), mesmo em homens assintomáticos para a infecção por HPV (Nielson *et al.,* 2007; Syrjanen, 2010), tanto no plasma seminal quanto em espermatozóides (Syrjanen, 2010; Pakendorf *et al.,* 1998).

Segundo Lai *et al.* (1997), a infecção por HPV pode levar a alteração na motilidade dos espermatozóides, o que pode refletir numa diminuição da capacidade reprodutiva. São comuns na literatura trabalhos com detecção de HPV em sêmen de homens que procuram tratamento para fertilidade (Kyo *et al.,* 1994; Lai *et al.,* 1997; Tanaka *et al.,* 2000; Didelot-Rousseau *et al.,* 2007; Bezold *et al.,* 2007).

A detecção do DNA do HPV no interior do espermatozóide e infecção *in vitro* pelo HPV foram demonstradas (Foresta *et al.,* 2010; 2011). Além disso, espermatozóides humanos transfectados com DNA de HPV são capazes de penetrar ovócitos de hamster e expressar seus genes nos ovócitos penetrados. A

atividade transcricional do HPV16 foi confirmada *in vivo* em espermatozóides (Lai *et al.,* 1997). Como a adsorção de DNA exógeno por parte das células espermáticas é inibida por partículas no sêmen (Spadafora, 2007) a presença de DNA de HPV dentro do espermatozóide e sua transcrição podem significar que o HPV seja capaz de infectar as linhagens espermáticas precursoras do indivíduo. A infecção de células espermáticas por HPV e a interação dos genomas viral e do hospedeiro podem levar a distribuição do genoma de HPV para o embrião.

A presença de BPV em gametas e sítios do trato reprodutor de bovinos pode representar risco potencial de transmissão vertical e pode trazer implicações para a fertilidade (Freitas *et al.,* 2007; Silva *et al.,* 2012).

O primeiro estudo buscando avaliar a relação do BPV e o trato reprodutivo em bovinos verificou a presença de material genético de BPV1 e 2 no trato reprodutivo e gametas de fêmeas bovinas (Carvalho *et al.,* 2003). Posteriormente, Yaguiu *et al.,* (2006) verificaram a presença de BPV1 e 4 no trato reprodutivo de fêmeas e do BPV2 em sêmen de bovinos. Lindsey *et al.* (2009) verificaram a presença de BPV2 em amostras de sêmen congelado e fresco de animais que sofriam de papilomatose crônica.

O vírus pode ter acesso ao sêmen através de linfócitos infectados, considerando que a detecção de BPV em células polimorfonuclares do sangue periférico foi relatada (Roperto *et al.,* 2011). Além disso, o sêmen pode ser contaminado através de infecções do trato reprodutor, traumas no epitélio ou inflamações nas glândulas acessórias (próstata, vesícula seminal ou bulbouretral) (Bielanski, 2007).

Os primeiros trabalhos sugerindo a transmissão vertical de BPV envolveram a detecção do vírus em sangue de vacas e suas proles (Stocco dos Santos *et al.,*

1998; Freitas *et al.,* 2003; Yaguiu *et al.,* 2008), em sítios do trato reprodutor de vacas como ovócito, ovário, útero, células do cumulus e de lavagem uterina (Carvalho *et al.,* 2003). Em paralelo, a detecção de BPV em anexos embrionários como a placenta e no líquido amniótico também foi relatada (Freitas *et al.,* 2007). O linfócito foi então hipotetizado como veículo responsável por carrear o BPV pela corrente sanguínea e disseminá-lo do epitélio para o trato reprodutor (Freitas *et al.,* 2007). A detecção do material genético do BPV em sangue e plasma (Freitas *et al.,* 2007; Diniz *et al.,* 2009), assim como os achados de expressão de oncoproteínas e de proteínas estruturais do vírus em linfócitos corroboraram essa hipótese (Roperto *et al.,* 2008; 2011).

Recentes descobertas de Roperto *et al.* (2012) talvez representem um divisor de águas na investigação da transmissão vertical do papilomavírus. No referido trabalho, os autores demonstraram não somente a produção da proteína E2, importante para a replicação do DNA viral, como a formação de partículas virais de BPV-2 a partir das células infectadas do epitélio uterino e coriônico da placenta de bovinos naturalmente infectados. Adicionalmente, a oncoproteína E5 foi caracterizada formando um complexo com o receptor do fator de crescimento ß derivado de plaquetas - *platelet-derived growth factor ß receptor* (PDGFßR) nas células do trofoblasto e isso poderia estar associado a uma organogênese anormal e comprometimento da gestação.

3. Objetivos

3.1 Geral

Esse trabalho objetiva estudar a biologia e diversidade da infecção pelo BPV em lesões cutâneas de bovinos e sítios não epiteliais de bovinos e equinos.

3.2 Específicos

1. Avaliar a diversidade de PV em lesões cutâneas de bovinos;
2. Avaliar mecanismos etiopatogênicos em lesões papilomatosas induzidas por BPV, através da análise da expressão da oncoproteína E5, juntamente com a análise da expressão da conexina 26 bovina;
3. Avaliar a presença do DNA de BPV em espermatozóides e líquido seminal de sêmen comercial de touros e seu efeito na função espermática;
4. Verificar a presença e expressão de BPV em sangue de bovinos sadios e afetados por papilomatose bovina;
5. Verificar a presença e expressão do BPV em sêmen fresco de touros saudáveis;
6. Investigar a presença e expressão de BPV em células do sangue e sêmen de cavalos saudáveis;
7. Comparar dois sistemas de detecção de BPV por PCR, PCR com primers tipo específico e PCR com primers consenso, em lesões cutâneas e fluidos de bovinos.

4. Capítulo I

Co-infection of Bovine papillomavirus and Feline-associated papillomavirus in bovine cutaneous warts

Artigo publicado no periódico Transboundary and Emerging Disease

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Abstract

The diversity of papillomavirus (PV) found in bovine cutaneous warts from Brazilian cattle were evaluated using the PCR technique with the utilization of consensus primers MY09/11 and by PCR using BPV type-specific primers followed by sequencing. Eleven cutaneous warts from 6 cattle herds were selected. Six warts were positive for the presence of PV. It was found the presence of Bovine Papillomavirus (BPV) types 1, 2, 3, 6 and feline sarcoid-associated PV (FeSarPV) in cutaneous wart lesions as well as the presence of co-infections. To the best of our knowledge this is the first time that FeSarPV is described co-infecting a cutaneous wart in Brazil. The present study confirms the previous finding of FeSarPV infecting cattle. These results show the necessity of more studies to investigate the diversity of PV in cattle, its diversity and the possibility of co-infection in cattle and other animals.

Keywords: BPV, FeSarPV, cattle

Introduction

Papillomaviruses (PVs) are a diverse group of double-strand DNA viruses, identified in more than 20 species of mammals, birds and reptiles. PVs are classified in the Papillomaviridae family, which contains 18 different genera (Bernard et al, 2010). These viruses are known as species-specific and in experimental conditions do not infect another host than their natural one (Campo, 2006).

BPV1 and BPV2 are classified in the genus *Deltapapillomavirus*. So far, 12 types of BPV (BPV1-12) have been described (Zhu et al, 2011). These viruses are associated with different histopathological lesions and three types are involved with the development of carcinogenic process, especially when associated with environmental co-factors and genetic factors (Campo, 2006). Several studies have showed that BPV1 and BPV2 may produce cross-infection in horses, donkeys, mules (Chambers et al, 2003), zebras (van Dyk et al, 2009) and Buffaloes (Pangty et al, 2010).

Feline sarcoids have been found in North America, UK, Sweden, Australia and New Zealand (Schulman et al, 2001; Munday et al, 2010). Among these tumors, it was detected a new putatitve papillomavirus type named feline sarcoid-associated papillomavirus (FeSarPV) and evidences showed that FeSarPV may cause a non-productive infection in cats and it uses the feline as a non-permissive host (Teifke et al, 2003; Munday et al, 2010a). FeSarPV DNA was found in sarcoids of cats from North America and New Zeland (Munday et al, 2010a). Recently, FeSarPV DNA was verified in bovine fibropapillomas and dermatitis (Munday et al, 2010b).

In Brazil, there are a few investigations based on the definition of BPV types infecting and co-infecting cutaneous warts. The knowledge of the circulating PV types

in the field is important to conduct strategies of control and prevention of papillomaviruses in cattle and other animals. In this study the presence of BPV co-infection associated with FeSarPV was assessed.

Cutaneous wart samples were collected from diverse body sites from 11 adult cattle. Breeds of bovine were Nelore or Girolando and the samples were collected from both male and female sexes. These animals were originated from beef and milk cattle herd with semi-intensive and extensive management located in Bahia State, Northeastern Region of Brazil. Segments of warts were removed by parallel incision in the skin surface using a disposable sterile scalpel and kept in sterile tube containing 10% formaline. After fixation the pieces were washed, processed for embedding in paraffin, cut to 7 mm of thickness, stained with hematoxylin-eosin (Michalany, 1980), and observed under an optical microscope.

All collected samples were submitted to DNA extraction by “Qiagen DNeasy® Blood and Tissue kit” (Qiagen, Germany), in accordance with the manufacturer protocol. Each DNA sample was screened for the presence of bovine β-globin DNA by Polymerase Chain Reaction (PCR) amplification using the primers: Fw: 5’-AACCTCTTTGTTCACAACCAG-3’ and Rev: 5’- CAGATGCTTAACCCACTGAGC-3’. Primer set amplifies a 430bp product and provides an indication of a good DNA quality. PCR was carried out in accordance with protocol described by Freitas et al, (2003). The amplification was performed in a volume of 25µL containing 100ng of DNA, 1X Master Mix (Promega, USA) and 0.2µM of specific primers.

The virus presence was verified using the consensus primers MY09/11 previously described by Manos et al, (1989). PCR was performed using 0.2 µM of each primer, 100ng of DNA and 1X Master Mix (Promega, USA). The cycling conditions were described by Ogawa et al, (2004). As negative control, we used a no

template control and BPV2 genome cloned in PAT153 plasmid as positive control. The positive samples were subjected to PCR using BPV1-6 type-specific primers and respective annealing temperatures as showed in Table 1, according to the amplification protocol by Yaguiu et al, (2008).

The PCR products were electrophoresed on 2% agarose gel with TAE buffer and DNA was visualized by staining with ethidium bromide. Subsequently, a direct sequencing was performed using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA). Sequencing quality and contig assembly were carried out using Pregap4 and Gap4 programs (Staden, 1996). Only sequences with Phred value above 30 were considered for the contig assembly (GenBank accession numbers: JQ071444-JQ071449). Local sequence alignments were carried out with BLAST (Altschul et al, 1990).

Multiple sequence alignment of the isolates, BPVs, FdPVs and the other members of *Deltapapillomavirus* genus was done by Muscle algorithm incorporated in MEGA version 5 (Tamura et al, 2011). The GenBank accession number of the sequences used is showed in the Supplementary Table S1. The phylogenetic analysis was carried out using Neighbor-Joining method with JTT + Γ model of amino acid substitution in MEGA version 5 (Tamura et al, 2011). Statistical confidence of nodes was assessed with 1000 replicates of bootstrap. Bovine and feline PV sequences were analyzed along with the isolates of this study.

The primers for β-globin gene amplified a fragment of 450bp in all DNA samples and as expected, no template control tested negative. Primers MY09/11 amplified PV DNA in 6 of the 11 cutaneous warts samples (55%). The sequenced amplicons showed 98-99% of similarity to BPV1 in two fibropapilloma samples and 97-99% to BPV2 in two samples. Also, it was observed similarity of 99% to BPV6 in

one sample and 100% of similarity to FeSarPV in one cutaneous wart sample. The minor variation within the sequences (about 97 up 99%) was attributed to errors during sequencing rather than the presence of multiple PV variants. The isolate similar to FeSarPV was collected from a cow, in a beef herd with extensive management. The animal presented low intensity of lesions, with flat lesions mostly. The PCRs with specific primers showed the presence of BPV2 in five samples (83%), BPV3 in five samples (83%), BPV1 in two samples (33%) and BPV6 in one sample (17%). Three samples (50%) showed co-infection to three BPV types, including the FeSarPV positive sample. Two samples (29%) showed co-infection to two BPV types (BPV2 and 3), and one sample showed infection to only BPV6.

Table 1. Animal characterization, PV identification and histopathological analysis of cutaneous warts from cattle

Isolate	Herd Farm	PV Type[a] (Similarity) (%)	BPV type (specific detection)	Histopathology
1VU	Milk herd	BPV6 (99)	6	True Papilloma
11V	Beef herd	BPV2 (97)	2,3	Fibropapilloma
18V	Milk herd	BPV2 (99)	1,2,3	Fibropapilloma
21V	Milk herd	BPV1 (98)	2,3	Fibropapilloma
20V	Milk herd	BPV1 (99)	1,2,3	Fibropapilloma
29V	Beef herd	FeSarPV (100)	2,3	Fibropapilloma

BPV, Bovine Papillomavirus, PV, papillomavirus.
[a]Obtained by sequencing of MY 9/11 PCR amplicon.

The phylogenetic analysis confirmed the BPV types detected with good statistical confidence (Figure 1). The isolate 29V (identified as FeSarPV) was clustered together with the *Deltapapillomavirus* members, possibly it belongs to this genus. The tree showed the close relatedness between FeSarPV and Artiodactyl PVs (e.g. OaPV1, OaPV2, BPV1 and BPV2), instead of feline papillomaviruses

FdPV1 and FdPV2. Almost all these clusters were well supported by the bootstrap analysis. The tree showed some genetic variability between the isolates and the reference sequences, as indicated by the identity analysis.

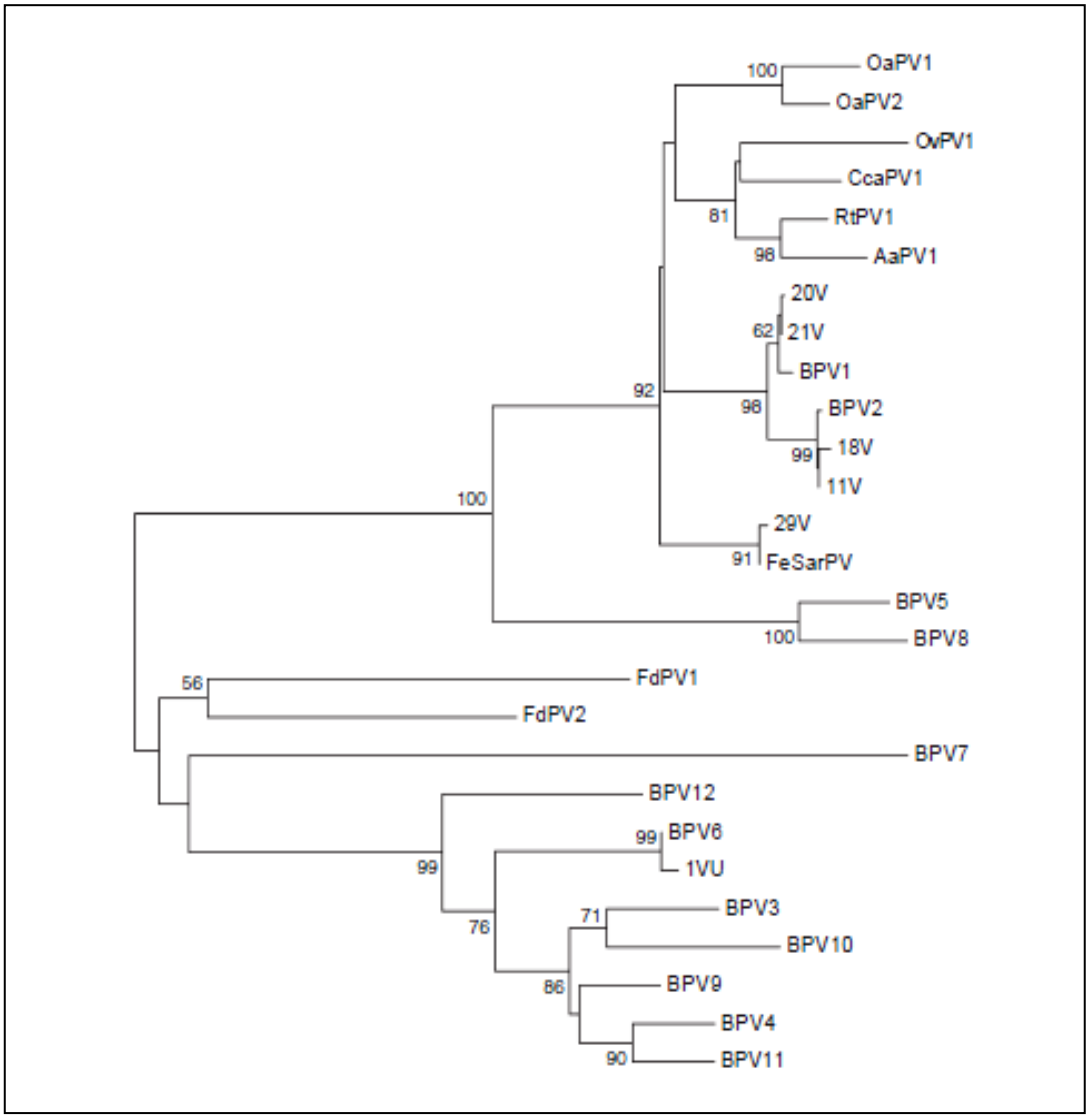

Figura 1 Phylogenetic tree based on L1 fragment amino acid sequences comprising the isolates of this study, bovine and feline papillomaviruses, along with *Deltapapillomavirus* members. Numbers in the nodes represent bootstrap confidence values above 50%. Branch lengths are in scale.

In this study, the PV DNA was detected in 6 of 11 cutaneous wart samples using PCR primer set MY09/11. PCR is regarded as a highly sensible technique and it is commonly used for PV detection, but consensus primers with degenerated bases

may decrease its sensibility (Gravitt et al, 2000). However, other investigations used consensus primers to BPV detection and the sensibility level ranged from 53 to 100% (Ogawa et al, 2004; Munday et al, 2010b; Silva et al, 2010). Although the primers MY09/11 had been made to detect HPV in human mucous epithelial tissue (Manos et al., 1989), these primers have been used for detection of a wide range of HPVs (Gravitt et al, 2000), BPVs (Ogawa et al, 2004; Silva et al, 2010) and FeSarPV (Munday et al, 2010b).

BPV1, 2, 4, 6 and 8 were detected in bovine wart from herd of different Brazilian regions (Freitas et al, 2003; Claus et al, 2009). In this study, BPV1, 2, 3, 6 and FeSarPV DNA were detected in cutaneous warts, as well as the co-infection between BPV and FeSarPV. The co-infection between BPV1 is in accordance with previous studies (Yaguiu et al, 2008; Diniz et al, 2009; Pangty et al, 2010). However this is the first relate of BPV detection associated with FeSarPV in American continent. Munday et al, (2010b) also found co-infection of FeSarPV and BPV2 in cuntaneous wart samples in New Zealand. The persistence of skin warts has been seen in a large number of animals in the farms studied. According to our findings, this observation could be explained by the occurrence of multiple infections in the cutaneous wart skin lesions. FeSarPV was found in a lesion with two different types of BPV, thus we can suggest that BPV types apparently do not inhibit FeSarPV presence in the lesion.

In this study, FeSarPV was detected in bovine cutaneous warts using consensus primers. Initially, this virus was found in feline sarcoid, but this virus may have a ruminant as natural host as suggested by Munday et al, (2010b). Previously, it was detected FeSarPV in feline sarcoids from North America and New Zealand (Munday et al, 2010a) and for the first time it was described in fibropapillomas and

dermatitis of bovine from New Zealand using specific primers set (Munday et al, 2010b).

The phylogenetic analysis showed that FeSarPV was clustered in the *Deltapapillomavirus* genus, together with OaPV1, OaPV2, BPV1, BPV2, and others artiodactyl PVs, corroborating with the findings of Teifke et al, (2003) and Munday et al, (2010a). FeSarPV had high identity compare to BPV1 and BPV2, demonstrating one possible evolutionary relationship among them. Besides, as BPV have been described in other animals such as horses, donkeys, mules, zebras, buffaloes and bison (Chambers et al, 2003; Literak et al, 2008; van Dyk et al, 2009; Pangty et al, 2010), our results indicate that FeSarPV could be a bovine papillomavirus that infects another host (feline), through an inter-host transmission process and corroborate with Munday et al, (2010b) where the authors verified the FeSarPV presence in bovine fibropapillomas. Therefore, the present results can contribute to confirm that the detection of FeSarPV in cattle was not incidental and it needs further investigations. Even though the L1 fragments analyzed were small, the statistical support of the FeSarPV and artiodactyl PVs cluster was high indicating that this hypothesis could be raised. Efforts are being made to sequence the complete genome of FeSarPV.

Although we have detected co-infection between BPV and FeSarPV, more studies are necessary for understanding the importance of this relation. Feline sarcoid is a rare tumor, mostly found in cats from rural area or exposed to cattle (Sculman et al, 2001). It is believed that flies could transfer BPV from cattle to horses or among horses (Finlay et al, 2009). According to Munday et al, (2010b) cat injuries caused by hunting or fights could exposure parts of its body to virus. Some of these parts are related to sarcoid development and thus hunting and fights are pointed as a possible way of virus entry into dermis.

Further studies are required to verify the prevalence of FeSarPV and other types of BPV in cattle. A better knowledge of PV types affecting bovines as well as cross-infections caused by PV could have some implications in the future for development of strategies in prevention and control of animal papillomaviruses.

Acknowledgement

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5. Capítulo II

Expression of connexin 26 in bovine cutaneous fibropapillomas

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Abstract

Bovine papillomaviruses (BPVs) can infect epithelial cells and fibroblasts, inducing fibropapillomas in cattle. Gap junctions are communication channels between cells composed of proteins known as connexins (cxs). Cx expression is altered during skin tumour development in humans. The present study evaluated the expression of the major BPV oncoprotein E5 and cx26 in bovine cutaneous fibropapillomas. Both tumoral and normal samples were positive for BPV (100%). The fibropapillomas stained positive for E5, whereas the normal skin was negative. Cx26 was faintly expressed in the normal skin epithelium. Eighteen out of twenty (90%) fibropapillomas stained positive for cx26. Cytoplasmic and juxtanuclear immunoreactivity for cx26 were found in fibropapillomas. Western blot analysis confirmed the higher expression of cx26 in fibropapillomas compared to normal skin samples.

Keywords: Bovine papillomavirus; Cutaneous fibropapillomas; Connexin; E5

Introduction

Bovine Papillomaviruses (BPVs) are double-stranded oncogenic DNA viruses and usually species-specific (Bernard et al., 2010). However, BPVs also cross-infect other species, including buffaloes, bisons and equids (Literak et al., 2006; Nasir and Campo, 2008; Silvestre et al., 2009). Twelve genotypes of BPV (BPV-1 to BPV-12) have been characterised and classified in three different genera: Deltapapillomaviruses (BPV-1 and BPV-2), Epsilonpapillomaviruses (BPV-5 and BPV-8) and Xipapillomaviruses (BPV-3, -4, -6, -9,-10, -11 and -12) and an as yet unassigned PV genus (BPV-7) (Bernard et al., 2010; Hatama et al., 2011; Zhu et al., 2011). BPVs from Deltapapillomaviruses and BPV-5 can infect epithelial and dermal fibroblasts, inducing fibroepithelial tumours in cattle known as fibropapillomas (Nasir and Campo, 2008).

Normally, fibropapillomas regress as a result of a cell-mediated immune response (Knowles et al., 1996). However, occasionally fibropapillomas may progress to squamous cell carcinomas (SCCs) (Campo, 2006;Borzacchiello and Roperto, 2008).

E5, the major BPV oncoprotein, is expressed in the basal epidermal layer and infected fibroblasts (Anderson et al., 1997; McCance, 2002) and is localised on the membranes of the endoplasmic reticulum and Golgi apparatus of host cells (Pennie et al., 1993). The highest expression of the protein is observed in the basal layer and in the differentiating keratinocytes of spinous or granular layers of bovine cutaneous papillomas (skin warts) (Burnett et al., 1992; Bohl et al., 2001), as well as in bovine urinary bladder cancers (Borzacchiello, 2003).

E5 induces cell transformation by binding to the platelet-derived growth factor (PDGF) β receptor and also perturbs other cellular functions such as alkalinisation of the Golgi apparatus through direct 62 inhibition of V-ATPase activity (DiMaio and Mattoon, 2001; Borzacchiello et al., 2006).

E5 also binds to 16 k ductin/subunit c of vacuolar ATPase, which is a major component of gap junctional intercellular communication (GJIC). E5 binds to ductin and causes loss of GJIC in bovine cell culture (Faccini et al., 1996). Gap junctions are channels that mediate cell-to-cell communication and tissue integrity, and they have been proposed to play a role in regulating cell proliferation and in the maintenance of tissue homeostasis (Richard, 2000). GJIC has also been implicated in coordinating the activity of dividing cells (Green, 1988). The subunit proteins of GJIC are connexins, members of a multigene family consisting of about 20 different members (Kumar and Gilula, 1996). Alterations regarding the expression level or type of cx in a given cell type correlate with carcinogenesis (Rouan et al., 2001). Cx26 is the main gap junction protein in skin tissue, although its expression is found also in other epithelial tissues. Many studies describe altered cx26 expression in different human malignancies (Jamieson et al., 1998; Gee et al., 2003). In particular, cx26 expression is altered in skin tumours, being overexpressed in fibropapillomas (Sawey et al., 1996; Mesnil et al., 2005). Decreased expression of cx26 has been reported recently in canine mammary tumours (Gotoh et al., 2006).

In an ongoing study of the expression of cxs in BPV- induced tumours, we demonstrated that expression of cx43 is reduced in a subset of BPV induced bovine urinary bladder cancers (Corteggio et al., 2011). The aim of the present study is to evaluate the expression of cx26 in BPV-induced bovine cutaneous fibropapillomas.

Materials and methods

Tissue samples

Twenty cutaneous fibropapillomas (T1-T20) were collected from various body parts of twenty adult cows bred in farms located in Bahia and Pernambuco State (Brazil), the province of Caserta (Italy) and Moldova region (Romania).Three normal skin samples (N1-N3) from healthy cows were also included in this study. Segments of tissues were removed by parallel incision in the skin surface with the aid of a disposable sterile scalpel. The samples were divided into two parts: one was fixed in 10% neutral buffered formalin for histological examination and the other was used for molecular analysis (samples T12-T20 were not available for Western blot analysis).

The fixed tissues were embedded in paraffin wax by routine methods. Paraffin sections (4 μm thick) were stained with haematoxylin and eosin (HE) for histopathological assessment according to the World Health Organization Histological classification of epithelial and melanocytic tumours of the skin (Goldschmidt et al., 1998).

PCR analysis

DNA was extracted using the Qiagen DNeasy Blood and Tissue kit. DNA from samples T12-T20 was recovered from paraffin sections as described previously (Borzacchiello et al., 2007). The quality of the purified DNA was checked by PCR for the β-globin gene using the primers: forward: 5'-AACCTCTTTGTTCACAACCAG-3' and reverse: 5'- CAGATGCTTAACCCACTGAGC-3'. PCR products were amplified in a final volume of 25 μL containing 100 ng DNA, 25 units/ml of Taq DNA polymerase, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 200 μM dTTP, 1,5 mM MgCl2 (Master Mix, Promega) and 0.2 μM each oligonucleotide primer. The PCR conditions were

the following: denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 112 50 s, annealing at 67 °C for 1 min and extension at 72 °C for 1 min, with a final cycle at 72 °C for 5 min. The primers amplified a 450 base pairs (bp) product as expected (Freitas et al., 2003).

PCR for detection of BPV was performed using specific primers for BPV-1 and BPV-2. Each sample (10 µl) was amplified in 50 µl reaction mixture containing 3 mM MgCl2, 1 U Platinum *Taq* (Invitrogen), 25 pmol each primer and 200 µM dNTPs. The reaction was carried out in a Cycler (Bio-RadLaboratories) using forward (5′-CACTGCCATTTGTTTTTTTC -3′) and reverse (5′-GGAGCACTCAAAATGATCCC-3′) primers that amplify a DNA fragmente of the E5 open reading frame (ORF) of BPV-2 (153 bp, from nt 3842 to 3995) (Roperto et al., 2008). PCR conditions were as follows: denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 1 min, with a final extension for 5 min at 72 °C. For BPV-1 DNA detection thefollowing primers were used: forward: 5'- GGAGCGCCTGCTAACTATAGG -3'and reverse 5'-ATCTGTTGTTTGGGTGGTGAC-3', which yielded an amplicon of 301 bp of L1 gene (from nt 5721 to 6021).The reaction was performed as follows : denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing for 40 s at 68 °C, and extension at 72 °C for 1 min (Carvalho et al., 2012). PCR products were separated by electrophoresis in 2% agarose gels with tris acetate-EDTA buffer (TAE: 40 mM Tris; 1m MNa2 EDTA; 20 mM acetic acid) and the DNA was visualised under ultraviolet light after staining with ethidium bromide. The amplicons were purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega) and directly sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Immunofluorescence and confocal microscopy

For immunofluorescence labelling, sections were dewaxed and rehydrated. Antigen enhancement was performed by pretreating with microwave heating (twice for 5 min each at 525 W). The sections were blocked with donkey antiserum for 30 min. The sheep anti-E5 primary antibody (kindly provided by Prof. M.S. Campo, University of Glasgow, Scotland) was applied overnight at 4°C in ahumidified chamber at 1:50 dilution in phosphate buffered saline (PBS; pH 7.4, 0.1 M). The slides were washed three times with PBS, then incubated with Alexa Fluor 488 donkey anti-sheep (A-11015, Molecular Probes) for 1 h at room temperature (RT) in a humidified chamber at 1:100 dilution. Finally, after washing with PBS, the slides were mounted in PBS/glycerol (1:1) (Borzacchiello et al., 2007). A confocal laser scanning microscope LSM-510 (Zeiss) was used for scanning and photography. The negative control was obtained by omitting the primary antiserum and replacement of the primary antibody with a non-relevant antibody of the same IgG subtype produced in the same animal species, but of irrelevant specificity.

For two colour immunofluorescence staining of E5 with cx26, the sheep anti-E5 primary antibody was used and detected by Alexa Fluor 488 donkey anti-sheep as above, the primary rabbit antibody specific for cx26 (0-24: sc-130729, Santa Cruz Biotechnology) was applied overnight at room temperature at 1:20 dilution in a humidified chamber and detected with TRITC-conjugated donkey anti-rabbit IgG antibody diluted 1:50 (Jackson Immuno Research Laboratories Inc., West Grove, OK, USA). After final washing, the slides were mounted in aqueous medium. For scanning and photography, Alexa Fluor 488 was irradiated at 488 nm and detected via a 505-560 nm band pass filter. TRITC was irradiated at 543 nm and detected with a 560 nm band pass filter. Two-channel frame by frame multi-tracking was used

for detection to avoid “cross-talk” signals. The different frames were scanned separately, with appropriate installation of the optical path for excitation a 161 nd emission for each scan according to the manufacturer’s instructions.

Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis were performed on tumour samples (T1-T6) and on three normal skin samples (N1-N3). The samples were snap frozen in liquid nitrogen and homogenised in ice-cold lysis buffer (50 mM Tris pH7.5;150 mM NaCl; 1mM EDTA; 0.25% deoxycholic acid, 1% Triton X-100) with 20 mM sodium pyrophosphate, 0.1 mg/m Laprotinin, 2 mM phenylmethylsulphony fluoride (PMSF), 10 mM sodium orthovanadate (Na2VO3) and 50 mM sodium fluoride (NaF). Protein concentrations were determined by use of a protein assay kit (Bio-Rad Laboratories).

Equal amounts of lysate samples were boiled and loaded on bis/acrylamide gels, separated by electrophoresis and proteins were blotted from the gel onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in tris buffered saline (TBS: 12.5 mM Tris HCl pH 7.4; 125 mM NaCl) at room temperature, washed with TBS-0.1% Tween and incubated with a 1:200 dilution of anti cx26 antibody (0- 24: sc-130729, Santa Cruz Biotechnology). After appropriate washing steps, peroxidase conjugated anti-rabbit IgG (sc-2004, Santa Cruz Biotechnology) was applied for 1 h at a 1:1,000 dilution. The blots were stripped and reprobed against mouse anti-actin antibody (CP01, Calbiochem) at 1:5,000 dilution to confirm equal loading of proteins in each lane.

Protein expression levels were quantitatively estimated by densitometry using a Gel Doc scanner (BioRad) equipped with a densitometric workstation. The protein concentrations were normalised to the actin level and expressed as the densitometric ratio.

Statistical analysis

Statistical analysis was performed using independent sample *t*-test and$P<0.05$ was considered to indicate a significant difference. The statistical analysis was performed using SPSS 13 software.

Immunohistochemistry

For immunohistochemistry (IHC), sections were dewaxed and rehydrated. Antigen enhancement was performed by heating in a microwave twice for 5 min each at 525 W. A primary rabbit antibody specific for cx26 (0-24: sc-130729, Santa Cruz Biotechnology) diluted 1:50 in PBS, was applied overnight at room temperature. Sections from the tumour samples analyzed by Western blot were also incubated with a monoclonal mouse anti-PCNA (Proliferation Cell Nuclear Antigen; M0879, clone PC10, Dako) diluted 1:300 and applied overnight at 4° C. After washing three times with PBS, the slides were incubated for 30 min with biotinylated secondary antibody (labelled streptavidin biotin, LSAB Kit, Dako). Sections were washed three times with PBS and then incubated with streptavidin conjugated to horseradish peroxidase (LSAB Kit, Dako). Colour development was obtained as previously reported (Borzacchiello et al., 2007). Sections were counterstained with Mayer's haematoxylin. A human hepatocellular carcinoma sample was used as a positive

control for cx26. Negative controls included omission of the primary antiserum and replacement of the primary antibody with a non-relevant antibody of the same IgG-subtype produced in the same animal species. Proliferation index (PI) was derived from the PCNA labeled sections by counting 500 cells over 10 fields (examined with the 40 X objective).

Results

Gross and histopathological examination

The cutaneous warts consisted of multiple cauliflower-like lesions. The outgrowths were sessile. On HE stained sections, the tumours were seen to consist of epidermal hyperplasia with acanthosis and orthokeratotic hyperkeratosis. Formation of keratohyaline granules and cytoplasmic vacuolisation was recorded in some cells. Many koilocytes were also seen, consistent with papillomavirus infection. Moreover, some samples showed an inflammatory response, with congestion of vessels and cellular infiltration. Histologically, the tumours were classified as cutaneous fibropapillomas.

PCR for bovine papillomavirus DNA

DNA of PCRquality was recovered from all the examined samples. The primers for the β-globin gene amplified a fragment of 450 bp in all samples. A fragment of the expected size (153 bp) for BPV-2 was amplified in all the analysed samples (100%) and a fragment of the expected size (300 bp) for BPV-1 was amplified in 12 out of twenty fibropapillomas (60%).

The normal skin samples were BPV-1 positive. Amplicons were sequenced, confirming the presence of BPV-1 and BPV-2 DNA in the samples.

Immunofluorescence for BPV E5 expression

Expression of the BPV-1/2 E5 oncoprotein was detected by indirect immunofluorescence in all fibropapillomas (Table 1). No staining of the normal skin was observed. All fibropapillomas (100%) stained positive for E5 (Fig.1).The immunoreactivity was found in basal and in more differentiated fibropapilloma layers. Differences in the intensity and patterns of E5 expression among the samples were observed. E5 was mostly recorded intracytoplasmically, although in some samples 236 the neoplastic cells displayed a very characteristic juxtanuclear and/or membranous staining pattern. Results are summarised in Table 1.

Table 1

Expression of bovine papillomavirus E5 viral oncoprotein and connexin 26 in bovine skin fibropapillomas.

Samples	E5 expression	Connexin 26 expression
T1	Y	+++
T2	Y	+
T3	Y	+
T4	Y	++
T5	Y	+
T6	Y	+++
T7	Y	+
T8	Y	0
T9	Y	++
T10	Y	+++
T11	Y	+
T12	Y	+++
T13	Y	++
T14	Y	0
T15	Y	+++
T16	Y	++
T17	Y	++
T18	Y	++
T19	Y	++
T20	Y	+++
N1	N	+
N2	N	+
N3	N	+

Immunoreactivity scoring was determined in a 'blind' study by two observers (GB and MARS). The intensity of immunolabelling for each specimen was scored on a four-tiered scale as follows: 0, Absent or very weak signal; +, Weak signal; ++, Moderate signal; +++, Strong signal. Y, Yes; N, Negative.

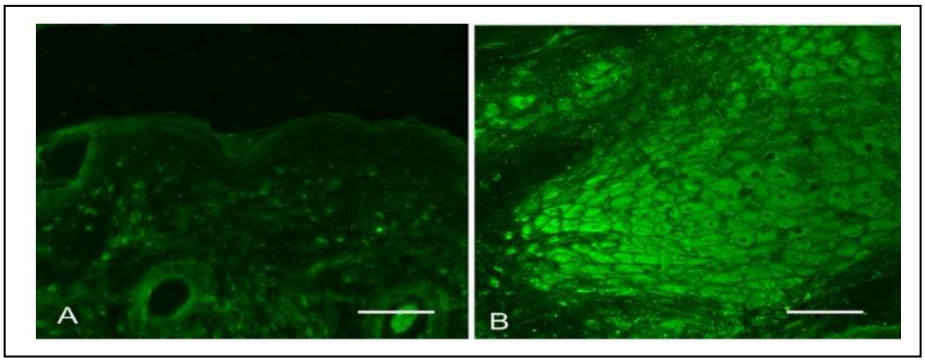

Fig. 1. Expression of bovine papillomavirus (BPV) E5 oncoprotein in normal skin and fibropapillomas. (A) The normal epithelial cells from skin do not express E5. Bar = 1.5 mm. (B.) E5 oncoprotein is detected in the cytoplasm of neoplastic cells. Bar = 1.5 mm.

Western blot analysis for detection of cx26 expression

Cx26 was detected both in normal skin and in tumour samples by Western blotting with an anti-cx26 antibody. In all samples, the antibody recognised a band of the right molecular weight. Noticeable difference in cx26 expression levels between normal skin and fibropapillomas was revealed by densitometric analysis, indicating that the cx26 is overexpressed in tumours when compared to normal skin (Fig.2). Experiments were repeated three times, yielding the same results.

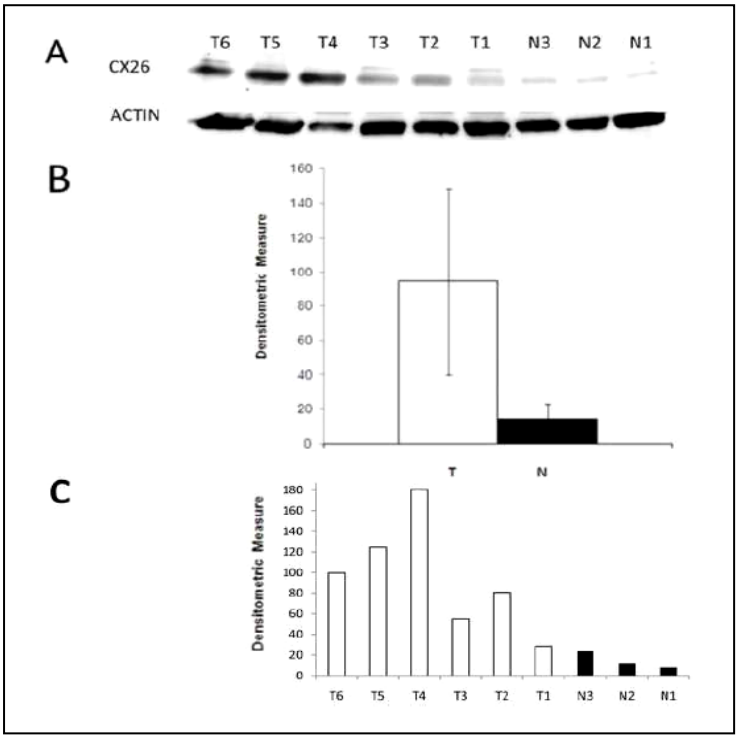

Fig. 2. Western blot analysis of cx26 in bovine normal skin and fibropapillomas. (A) Increased amount of cx26 was evident in tumour samples compared to normal skin (upper blot).The blot was stripped and reprobed with anti-actin antibody (lower blot) to confirm equal loading of proteins in each lane. (B) Mean densitometric values and standard deviations. A significant difference between samples was recorded (*t* test; $P<0.05$). (C) Individual densitometric measures of cx26 expression for each sample. All tumour samples show higher cx26 expression level when compared to normal samples.

Immunohistochemistry for detection of cx26 expression

The immunohistochemical labelling for cx26 of the normal and neoplastic lesions is summarised in Table 1. In all the three normal skin samples, cx26 was faintly expressed in the upper layers of normal epithelial cells (Fig.3). Hair follicles and eccrine sweat glands displayed a predominant membranous staining pattern. Eighteen out of twenty (90%) fibropapillomas stained for cx26. Cx26 was expressed in the basal and spinous layers of fourteen samples (77%), whereas in the remaining four (22%) the immunoreactivity was detected only in the cells of the basal layers. Neoplastic cells from fibropapillomas displayed a predominately cytoplasmic immunoreactivity whose intensity ranged from moderate to strong (Fig.4A). Most cells

from five fibropapillomas (27%) showed a juxtanuclear staining pattern (Fig.4B). A human hepatocellular carcinoma sample used as positive control showed cytoplasmic staining (Fig.4C). Cutaneous fibropapillomas incubated with an isotype matched irrelevant control antibody did not stain (negative control) (Fig.4D).

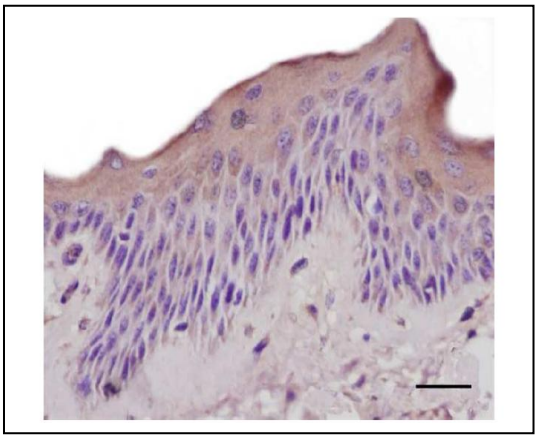

Fig. 3. Cx26 expression in normal bovine cutaneous epithelium. Cells of the superficial layers display faint cytoplasmic immunolabelling. Detection of cx26 antigen by the streptavidin biotin immunoperoxidase method, counterstained with Mayer's haematoxylin. Bar = 1.5 mm.

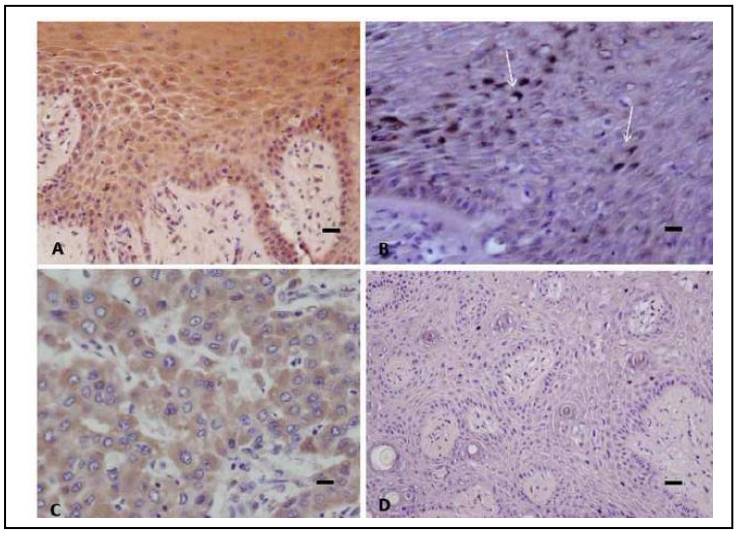

Fig. 4. Cx26 expression in bovine cutaneous fibropapillomas. (A) Cx26 is strongly expressed in the cytoplasm of the neoplastic cells of basal and spinous layers. Bar = 0.5 mm. (B) Juxtanuclear expression of cx26 is shown (white arrows). Detection of cx26 antigen by the streptavidin-biotin immunoperoxidase method, counterstained with Mayer's haematoxylin. Bar = 0.5 mm. (C) Neoplastic hepatocytes express cx26 in the cytoplasm. Bar = 0.5 mm. (D) Cutaneous fibropapilloma incubated with an isotype matched irrelevant control antibody with no reactivity. Bar = 1 mm.

PCNA immunoreactivity was detected in all the analyzed tumour samples. The percentage of labeled nuclei ranged from 85.5 to 95.5, with a mean P.I. of 92.96%. No correlation between higher cx26 expression levels and P.I. was found among the analysed fibropapillomas, suggesting that cx26 upregulation does not affect proliferative capacity of neoplastic cells.

Double immunofluorescence for colocalization of E5 and cx26

Samples T4, T6 and T12 were double labelled in order to investigate a possible colocalization between E5 and cx26 in cutaneous fibropapillomas. All the analysed samples showed cytoplasmic colocalization of E5 and cx26 as judged by the yellow fluorescence of the merge images (Fig. 5).

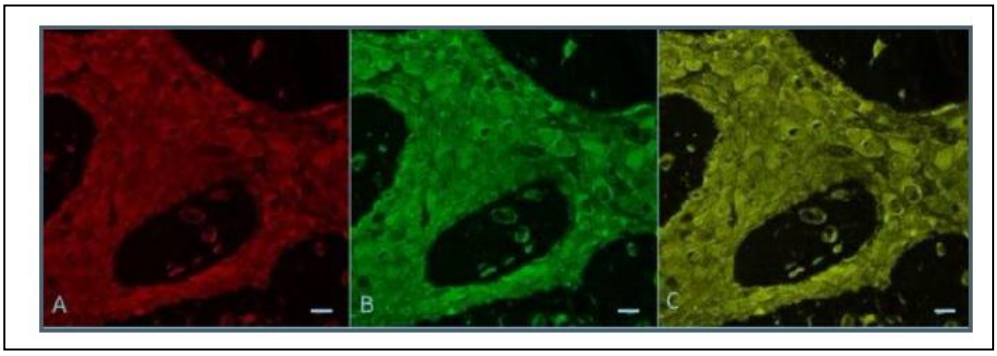

Fig.5. Colocalization of E5 and cx26 in cutaneous fibropapillomas. (A) Cx26 is expressed in the cytoplasm of the neoplastic cells (red TRITC), (B) E5 is expressed in the cytoplasm of the neoplastic cells expressing cx26 (green Alexa fluor 488), (C) Colocalization (yellow) of cx26 and E5 in neoplastic cells. Bar = 1 mm.

Discussion

BPV-2 was detected in all 20 bovine cutaneous fibropapillomas by PCR, with twelve samples showing co-infection with BPV-1. This is in accordance with previous studies, where the simultaneous presence of BPV-1 and -2 has been demonstrated in other bovine tissues (Oelze et al., 1995; Kumar et al., 1996; McCance, 2002; Diniz

et al., 2009). Our findings also demonstrate that BPV-1 and BPV-2 can be readily detected in bovine cutaneous fibropapillomas from Eastern (Romania) and Southern (Italy) Europe.

BPV is recognised as the aetiological agent of bovine cutaneous fibropapillomas (Borzacchiello and Roperto, 2008; Nasir and Campo, 2008). The transforming activity of the virus is mainly due to the expression of its major oncoprotein, E5 (Venuti et al., 2011). E5 oncoprotein is predominantly localised in the Golgi apparatus and endomembranes. This viral oncoprotein is able to bind to the 16 k ductin/subunit c of vacuolar ATPase in vitro and causes loss of gap junctional intercellular communication in cultured bovine primary fibroblasts (Goldstein et al., 1991; Faccini et al., 1996; Schapiro et al., 2000). We have demonstrated that the major BPV oncoprotein E5 is expressed only in tumours but not in the normal skin, further strengthening the concept of E5 being causally involved in the development of cutaneous fibropapillomas. E5 was localised in both the basal and more differentiated layers of fibropapillomas, suggesting that this oncoprotein is expressed during the early and late stages of virally induced carcinogenesis.

Cxs form gap junctions, which are cellular structures that transport ions and small molecules between adjacent cells and contribute to cellular homeostasis. The disruption of homeostasis often results in tissue dysfunction giving rise to cancer (Mesnil and Yamasaki, 1993). Alteration of cxs expression has been reported in multiple human tumour types (Sawey et al., 1996; Jamieson et al., 1998; Gee et al., 2003; Mesnil et al., 2005).

Only a few studies have reported cxs expression in neoplasms in veterinary species (Gotoh et al., 2006; Corteggio et al., 2011). In our study, cx26 was faintly expressed in normal epithelium, whereas its expression was upregulated in

fibropapillomas. We also found cx 26 colocalized with E5 oncoprotein in the same neoplastic cells, suggesting a possible correlation between E5 expression and cx26 dysregulation.

Our results are in agreement with several previous studies performed on virally and chemically induced skin tumours in humans and mice (Salomon et al., 1988; Kamibayashi et al., 1995; Sawey et al., 1996; Lucke et al., 1999).

Upregulation of cx26 in hyperplastic conditions and its loss in invasive cancer is a possible function of this connexin acting as a tumour suppressor protein (Mesnil et al., 1997). Besides alterations of cx26 expression, altered intracellular localisation of this protein was also observed. Cx26 is naturally expressed by several organs and tissues (Pennie et al., 1993; Mesnil et al., 2005) but the protein is mostly found abnormally localised in the cytoplasm of neoplastic cells (Krutovskikh et al., 1991; Kamibayashi et al., 1995; Knowles et al., 1996; Jamieson et al., 1998; Gee et al., 2003; Corteggio et al., 2011).

In some fibropapillomas, cx26 was expressed in a juxtanuclear position, indicating possible localisation in the Golgi apparatus. Concentration of cx26 in the Golgi apparatus was reported by Hernadez-Blazquez et al., (2001) in a mouse skin fibropapilloma cell line under conditions that block the traffic through the Golgi apparatus to the plasma membrane. E5 promotes the alkalinisation of the Golgi apparatus, altering the intracellular trafficking (Schapiro et al., 2000). Therefore, it is reasonable to assume that E5 could contribute to inhibition of the traffic of cx26, causing its accumulation in the Golgi apparatus and cytoplasm.

Cells expressing E5 exhibit alterations in GJIC (Oelze et al., 1995; Faccini et al., 1996) and it is possible that the dysregulation of cx26 plays a role in the GJIC

alteration. However, the mechanism behind the loss of GJIC is probably multifactorial (Aasen et al., 2003) and further studies are needed to better understand the possible contribution of cx26 to this process.

There were no differences in Cx26 staining patterns between tumours with BPV-2 only and tumours co-infected with BPV-1 and BPV-2. Dysregulation of cx26 does not appear to be dependent on the presence of the two different viral genotypes. However, since cx26 expression is upregulated in BPV-2 induced bovine urinary bladder tumours (personal observations), it is possible that cx26 dysregulation is a specific effect of BPVs transforming activity.

Further studies are required to identify the role of cx26 dysregulation in BPVs induced tumours.

Conclusions

Expression of cx26 is increased in BPV E5 positive bovine fibropapillomas, suggesting a pathogenic role for cx26in naturally occurring bovine fibropapillomas. As an extension of this work, different cxs and other proteins composing the GIJC are under investigation by this research group to further evaluate their possible involvement in this type of tumour.

Acknowledgements

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6. Capítulo III

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

Artigo publicado no periódico Animal Reproduction Science

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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 carried out 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.

Keywords: Bovine Papillomavirus, semen, PCR.

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 & 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 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, 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. Material and methods

2.1. Semen samples

Forty frozen semen samples taken from dairy Gir bulls (*Bos taurus*) were obtained from 4 companies in Brazil, three of which are multinational companies. The samples were thawed in a water bath at 37°C for 30 seconds 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 carried out 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 carried out by using 0.2 µM of each primer, about 100 ng of DNA and 1X Master Mix (Promega, USA).

PCR was carried out 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 40s 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 carried out 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 95ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 45 s, annealing at 52ºC for 40 s, and extension at 72ºC for 40 s. PCR products were electrophoresed on 2% agarose gel with TAE buffer at a constant voltage (100 V) for approximately 35 minutes. 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 carried out 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).

Table 1
Primers for detection of: bovine β-globin gene, ribosomal 5S gene and bovine papillomavirus.

Primer	Sequence	Region	Fragment size
bovine β-globin	F: 5′-AAC CTC TTT GTT CAC AAC CAG 3′ R – 5′ CAG ATG CTT AAC CCA CTG AGG 3′	Bovine β-globin gene	450 bp
Universal 5SRibosomal	F – 5′ACC GAC CAT ACC ACG GTG AA 3′ R – AAG CGG TCC CCC ATC TAA GT 3′	Ribosomal 5S gene	100 bp
BPV-1	F – 5′ GGA GCG CCT GCT AAC TAT AGG 3′ R – 5′ ATC TGT TGT TTG GGT GGT GAC 3′	L1 gene (5721–6021nt GeneBank Accession X02346)	300 bp
L1BPV-2	F – 5′ GTT ATA CCA CCC AAA GAA GAC CCT 3′ R – 5′ CTG GTT GCA ACA GCT CTC TTT CTC 3′	L1gene (6888–7051nt GeneBank Accession M20219)	163 bp
BPV-3/6	F – 5′ CAG TCA ATT GCA ACT AGA TGC C 3′ R – 5′ GGC TGC TAC TTT CAA AAG TGA 3′	L1 gene (6620–6836nt GeneBank Accession AF486184)	216 pb
BPV-4	F – 5′ GCT GAC CTT CCA GTC TTA AT 3′ R – 5′ CAG TTT CAA TCT CCT CTT CA 3′	E7 gene (642–812nt GeneBank Accession X05817)	170 bp
BPV-5	F – 5′ GGC ATG TAG AGG AAT ATA AGC 3′ R-5′ TTC TCT GAG ATC AAT ATT CC 3′	L1 gene (6646–6908nt GeneBank Accession AF457465)	262 bp
E5BPV-2	F – 5′ CAC TGC CAT TTG TTT TTT TC 3′ R – 5′ GGA GCA CTC AAA ATG ATC CC 3′	E5 gene (3842–3995nt Accession M20219.1)	153 pb
E2BPV-2	F – 5′ GGC ACA GAT CTT GAT CAC CTT 3′ R – 5′TCC AGG AGG TAG TGG GAC AT 3′	E2 gene (3726–3801nt Accession M20219.1)	76 pb

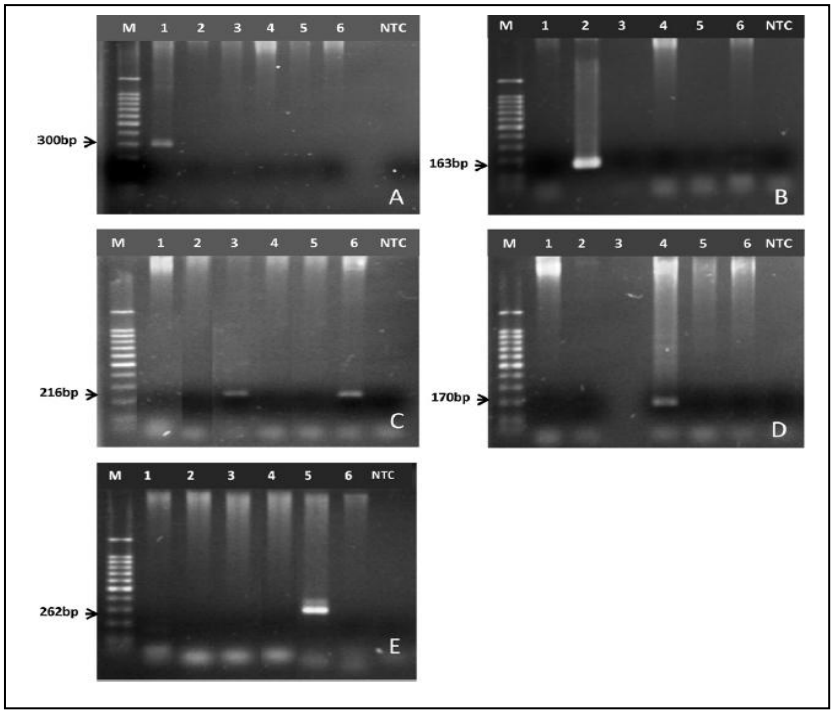

Fig. 1. Evaluation of specificity of BPV -1 to 6 primers. A–BPV-1 primer set; B–BPV-2 primer set; C–BPV-3 & 6 primer set; D–BPV-4 primer set; E–BPV-5 primer set. Fig 1 A, B, C, D, E: Lane M – molecular weight; Lane – 1 BPV-1 clone, Lane 2 – BPV-2 clone; Lane 3 – BPV-3 clone; Lane 4 – BPV-4 clone; Lane 5 – BPV-5 clone; Lane 6 – BPV-6 clone; NTC: no template control.

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).

3. Results

The primers for the β-globin gene amplified a fragment of 450bp in all sperm cell (n=40/40) DNA and in 25% (n=10/40) of DNA from seminal plasma with diluents. Only positive samples were used for PCR detection of BPV to avoid misleading results. Confirming the absence of cross-contamination and the reliability of the method employed, all controls (the NTC, virus-free DNA from MDBK cells and grasshopper DNA) tested negative.

In this study, BPV-2 DNA for L1, E2 and E5 genes were detected in all of the DNA semen samples (Fig. 2). However, BPV-1, 3, 4, 5 or 6 DNA was not detected. Five amplicons for L1, E2 and E5 gene PCR were sequenced and analyzed to confirm the BPV-2 results. They shared a 97 to 100% identity with BPV-2 compared with NCBI bank.

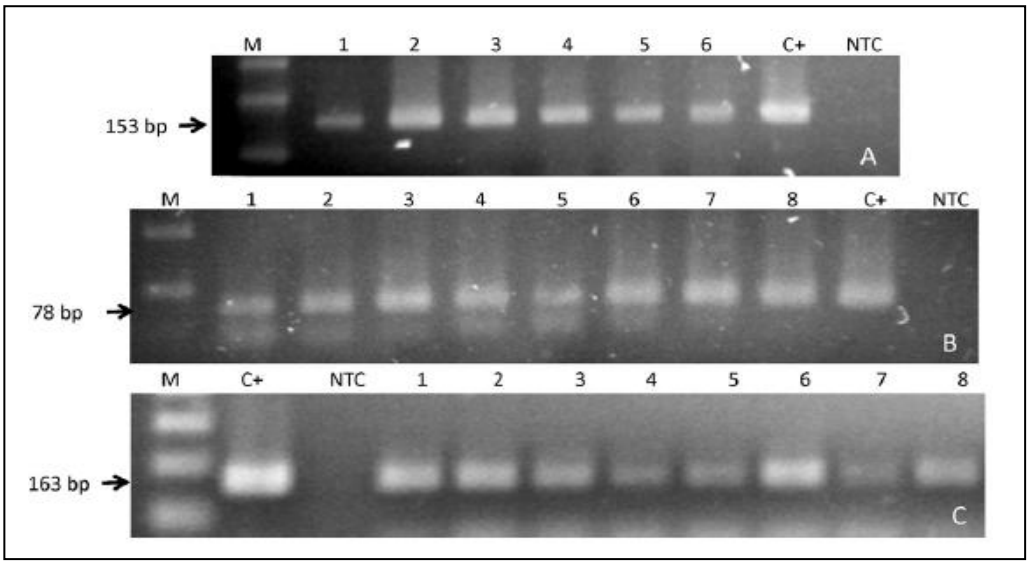

Fig. 2. Detection of BPV-2 in sperm cell. A. PCR for E5 BPV-2 gene. Lanes 1–6 samples; C+: positive control; NTC: no template control; M: 100 pb molecular weight marker. B. PCR for E2 BPV-2 gene. Lanes 1–8 samples; C+: positive control; NTC: no template control; M: 100 pb molecular weight marker. C. PCR for L1 BPV-2 gene. Lanes 1–8 samples; C+: positive control; NTC: no template control; M: 100 pb molecular weight marker.

The mean and standard deviation of quality parameters analyzed for all frozen semen samples are: sperm motility 60.0 ± 16.8%, vigor 3 ± 0.4, acrosomal integrity 68.5 ± 20.8% and DNA integrity of the spermatic cell 97 ± 3.8%.

4. Discussion

Infections caused by BPV are common in Brazil and the BPV types which have been found are BPV-1, -2, -4, -6 and -8 (Carvalho et al., 2003, Freitas et al., 2003; Wosiacki et al., 2005, 2006, Yaguiu et al, 2006, Claus et al., 2007, 2009; Diniz et al., 2009; Silva et al., 2010). In this study, BPV DNA was detected in sperm cells and seminal fluids of bulls from 4 dairy bull semen companies that provide semen to all states in Brazil. Many farmers buy semen from these companies, and there is a significant prevalence of BPV infection in dairy cattle in the country. BPV-2 DNA were detected in all the analyzed samples, although, BPV-1, 3, 4, 5 and 6 DNA were not

detected. The results of this study agree with the findings of Yaguiu et al. (2006) and Lindsey et al. (2009) who also detected BPV-2 DNA in all the tested frozen semen. However, they were unable to detect BPV-1 and 4. BPV-2 has been detected in several states in Brazil (Yaguiu et al., 2006; 2008; Claus et al., 2008; 2009; Diniz et al., 2009; Silva et al., 2010). It is hoped that these results can help understand the wide distribution of this virus type since the vertical transmission of BPV has been suggested (Freitas et al, 2003; Yaguiu et al, 2008). However, the role played by sperm cells contaminated with BPV DNA in the transmission of the virus is still unknown. In the case of humans, it has been shown the transplacental transmission of HPV and the transmission of HPV DNA to blastocyst through sperm cells (Chan et al., 1996; Kadze et al., 2002; Rombaldi et al., 2008). Moreover, it has been demonstrated the inhibition of two-cell embryo development after exposition to HPV DNA (Hennenberg et al, 2006). We suppose that infection with BPV DNA in semen could be associated with spontaneous bovine abortions, with no aetiological diagnosis, that is frequent in cattle (Campero et al, 2003).

The ability of BPV to infect non-epithelial tissues was discussed by Bravo et al., (2010). Freitas et al. (2003; 2007) and Lindsey et al. (2009) detected BPV-1, -2 and -4 in various body fluids such as blood, plasma, milk and colostrums. Other researchers have detected BPV-1, 2 and 4 DNA in cells and tissues of the reproductive tract, including oocytes, ovaries, uterus, cumulus cells, uterine lavage (Carvalho et al., 2003; Yaguiu et al., 2006), placenta and amniotic fluid (Freitas et al., 2007; Yaguiu et al, 2008). BPV-2 L1, E5 and E2 proteins were observed in lymphocyte, which and suggests that these cells may be carriers of BPV-2 in the bloodstream (Roperto et al., 2011). Infections of the reproductive tract and blood cells have been identified as sources of semen contamination by micro-organisms

(Bielanski, 2007). Thus, the presence of BPV-2 DNA in the semen samples could be due to the presence of BPV-2 in other sites, such as blood and reproductive tract in bovine.

Sperm cells are able to maintain adsorbed exogenous DNA even after the washing process that removes the inhibitory molecules (Brackett et al., 1971; Spadafora, 2007). Thus, if there is viral DNA in seminal plasma, this molecule can enter a sperm cell, as shown by Yaguiu et al., (2006) and Lindsey et al. (2009) who demonstrated the presence of BPV-2 DNA inside the spermatozoa, by means of in situ hybridization. This study confirmed the presence of BPV DNA in all the sperm cell samples as well as all the seminal plasma samples, which suggests that seminal plasma may act as a source of contamination to sperm cells. The presence of free BPV DNA in semen should be a cause of concern, as in the case of humans where HPV DNA fragments from E6 oncogene can cause a fragmentation of the sperm DNA (Connelly et al., 2001; Lee et al., 2002). The presence of E6 BPV DNA was not evaluated, but the main oncogene in BPV, E5 was found in every sample. Moreover as seen in the investigation of Roperto et al., (2011) where the BPV was shown to produce a productive infection in white blood cells, it is possible that this virus could also infect sperm cells. The high prevalence of the BPV DNA may also suggest a possibility of latent or asymptomatic infection by the virus. More studies are needed to evaluate these possibilities.

Although there is no information on whether or not BPV are infectious after freezing, this study has revealed a significant presence of viral DNA in cryopreserved semen samples from various national distributors in Brazil. Foresta et al. (2010a) also found a significant presence of HPV in the human sperm taken from testicular cancer patients and suggested that screening for HPV should be considered. There is no

test to detect BPV in breeding bulls or in the commercialized semen, but the screening for BPV should also be considered.

In this study, it has been shown that the presence of BPV DNA in frozen semen does not affect the quality of sperm. According to the Brazilian College of Animal Reproduction, BCAR (1998), the values obtained for vigor, motility, acrosomal integrity and DNA integrity are those that can be expected for a fertile bull. In the case of humans, Rintala et al. (2004) did not observe any changes in motility, vigor and concentration of spermatozoa resulting from the presence of high risk HPV; however Foresta et al. (2010b) observed reduced sperm motility that was linked to the presence of HPV. This is the first study that has analyzed these parameters in BPV- affected-semen, but it was not possible to make a comparison with a control group due to the high prevalence of BPV DNA in the samples. If BPV affects the quality of a sperm cell, this may have a bearing on the selection of semen from virus-free bulls, since the World Organization for Animal Health (OIE) does not require notification of the papilomaviruses despite the economic losses they cause to the farmers.

This is the first study undertaken to detect BPV DNA in bull semen from a wide range of national and multinational companies trading bull semen. The extent of the prevalence of BPV DNA-affected semen shows the need for further studies to understand the implications of the presence of BPV in semen and its impact on the process of artificial insemination (AI) and embryo transfer (ET).

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7. Capítulo IV

Presence and expression of bovine papillomavirus in blood of healthy and papillomatosis-affected cattle

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Running Title: BPV expression in cattle blood

Abstract

Papillomavirus (PV) are double-stranded DNA viruses that can cause benignant and malignant tumours in the amniotes. There are twelve types of Bovine papillomavirus (BPV-1 to 12) and their presences have been found in reproductive tissues and body fluids. This study aimed to evaluate the presence and expression of BPV in blood of healthy and papillomatosis-affected bovine using PCR and RT-PCR. BPV1/2 were detected in eight out of 12 (66%) blood samples of asymptomatic bovine and in eight out of nine (88%) in blood of papillomatosis-affected bovine. Six out of eight (75%) asymptomatic samples positive for BPV presence also showed expression for BPV. Four out of six (67%) samples were positive for E2 expression while three out of six (50%) samples were positive for E5 expression. Five out of eight (63%) symptomatic samples positive for BPV also showed the BPV expression. Five out of five were positive for E2 expression while one out of five (20%) were positive for E5 expression.. Two out of six (33%) blood samples of asymptomatic bovine and one out of five (20%) symptomatic blood samples scored positive for both, E2 and E5 expression. This is the first study showing the expression of BPV genes in blood of asymptomatic and papillomatosis-affected animals. This study reveals the importance of better understanding the role of BPV in non-epithelial sites, and its consequences to healthy and epidemiology of BPV in bovine.

Keywords: BPV, blood, viral gene expression, cattle.

1. Introduction

Papillomaviruses are double-stranded DNA tumour viruses identified in several animals, including humans (Bernard et al., 2010). Their open reading frames are divided into early (E) and late (L) regions. The early region encodes non-structural proteins E1 to E7. E1 and E2 control the replication and transcription of the genome, and the known oncoproteins are E5, E6 and E7. The late region encodes structural proteins L1 and L2, which form the capsid (Campo, 2006). Bovine papillomavirus can infect basal epithelial cells and subepithelial fibroblasts leading to the formation of tumors known as papillomas or warts (Nasir and Campo, 2008). The lesions are usually benign and tend to regress as a result of cell-mediate immune response (Knowles et al., 1996). However with the action of environmental co-factors they can turn into malignant tumors (Campo, 2006; Borzacchiello and Roperto, 2008). Some recent studies describe the co-infection among BPVs in skin tumours (Pangty et al., 2010; Schimitt et al., 2010; Carvalho et al., 2011) and the cross-specie infection caused by BPV (Bogaert et al., 2008, Silvestre et al., 2009, van Dyk et al., 2011, Freitas et al., 2011).

PVs are described as epitheliotropic, although their presence have been detected in different tissues and cells (Freitas et al., 2003; Yaguiu et al., 2006, Lindsey et al., 2009, Silva et al., 2011). Recently, it has been shown that human and bovine PV DNA is detectable in peripheral blood mononuclear cells (PBMCs) (Freitas et al., 2003; Bodaghi et al., 2005; Roperto et al., 2008; Roperto et al., 2011), plasma and serum (Widschwendter et al., 2003; Freitas et al., 2007). The expression of structural and nostructural genes as well as the respective proteins have been **demonstrated**

in bovine lymphocytes with bladder cancer (Roperto et al., 2008; Roperto et al., 2011).

Few studies contributes to improve the understanding of PV transmission, however, it has been hypothesized that the lymphocyte can harbor the virus in bloodstream (Stocco dos Santos et al., 1998; Freitas et al., 2003; Diniz et al., 2009) and spread through non-epithelial tissues and fluids (Freitas et al., 2007). Recent data showing that the lymphocytes may be PV carriers and the blood can be a potential new route of PV transmission seem to support this hypothesis (Bodaghi et al., 2005; Roperto et al., 2008, 2011). The presence of PV in blood of asymptomatic individuals has been described in the literature, but the significance of this finding still remains to be fully clarified (Stocco dos Santos et al., 1998; Wosiacki et al., 2005; Lindsey et al., 2009).

Although previous studies have shown the viral expression in blood of BPV-infected bovine that suffer bladder cancer (Roperto et al., 2008, 2011), it is not clear if the BPV transcription occurs in animals with only benign tumours as cutaneous papillomas. This study aimed to evaluate the presence and expression of BPV in blood of healthy and papillomatosis-affected bovine.

2. Materials and methods

2.1 Animals

Twenty-one bovines were selected to this study. The animals belong to six different farms located in Bahia State, in the North-East of Brazil. All the farms had papillomatosis-affected and healthy animals. Some animals of each farm symptomatic and asymptomatic for papillomatosis were selected.

2.2. Blood samples

The blood of the animals (05 mL) was collected on EDTA-containing tubes and 200 µL of total blood were used for DNA and RNA extraction.

2.3. DNA and RNA extraction and cDNA synthesis

All the samples were submitted to DNA and RNA extraction by Invirsob® Spin Universal RNA Mini Kit, (Invitek, Germany), in accordance with the manufacturer´s protocol. The quality of the purified DNA was checked by β-globin gene PCR, as described by Freitas et al. (2003). The amplification was carried out in a final volume of 25µL containing 100ng of DNA, 1X Master Mix (Promega, USA) and 0,2 µM of specific primers.

Subsequently, 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 (no template control) were used during the synthesis of cDNA. β-actin transcript was amplified from cDNA with primers previously described by Robinson et al, (2007) to verify the cDNA quality. PCR was carried out by using 0.2 µM of each primer, 05µL ofcDNA and 1X Master Mix (Promega, USA). The PCR parameters were consisted of 40 cycles of denaturation for 45s at 94°C, annealing for 40s at 52°C and extension for 40s at 72°C.

2.3. BPV detection by PCR

The presence of BPV1/2 DNA was assessed with primers targeting E5 and L2 BPV genes previously described by Roperto et al., (2008). Two negative controls were used, a no template control (NTC), and a control with DNA of Madin-Darby bovine

kidney (MDBK; ATCC-CCL22) cells. BPV1 and 2 genomes cloned into pAT153 plasmid were used as positive control. PCR products were electrophoresed on 2% agarose gel with TAE buffer and run at a constant voltage (100 V) for approximately 35 minutes. DNA was visualized under UV light after being stained with ethidium bromide.

2.4Determination of BPV expression

The presence of BPV transcripts were evaluated for two different BPV genes, E2 and E5. The two set of primers used for the reactions were described by Roperto et al., (2008) and Silva et al., (2011). PCRs were carried out according conditions described by Silva et al., (2011). To confirm presence of BPV transcripts, the purified amplicon from PCR was cloned into the pGEM-TEasy vector (PromegapGEM–T Easy Vector System, Promega, USA), and transformed into competent JM 109 E. coli cells (Promega, USA). The DNA of recombinant clones were isolated with PureYeldTM Plasmid Miniprep System (Promega, USA), and a direct sequencing was conducted by using an ABI PRISM® 3100 Genetic Analyzer (Applied Biossystems, USA) with the primers for BPV1/2. 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).

3. Results

3.1 Presence of BPV

It was assessed for the presence of BPV1/2 DNA in all the blood samples that scored positive for the β-globin PCR.The NTC and virus-free DNA from MDBK cells tested negative, thus confirming the absence of cross-contamination and the reliability of the method employed. The blood samples from asymptomatic animals scored positive for eight out of 12 (66%) isolates and for symptomatic individuals eight out of nine (88%) amplified a positive signal (Fig.1).

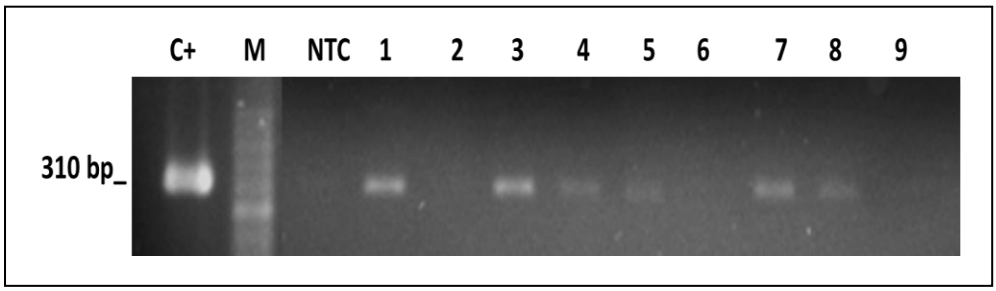

Fig. 1. Representation of BPV1/2 detection in blood.PCR for E5L2 BPV2 gene. C+: positive control; M: 50 bp molecular weight marker; NTC: no template control;Lanes 1 – 9 samples.

3.2 Expression of BPV

The cDNA samples were assessed for the transcripts of β-actin gene. The samples score positive were evaluated for the presence of viral transcripts for the E2 and E5 BPV genes. In blood samples, nine out of 11 (56%) samples positives for BPV had the E2 transcripts amplified, while four out 11 samples (36%) were positive for E5 expression. Three samples (27%) were positive for E2 and E5 expression simultaneously. In asymptomatic blood group, four out of six samples expressing BPV genes (67%) were positive for E2 expression, while 3 out of six (50%) score positive for E5 expression. The blood of papillomatosis-affected animals score positive for E2 expression in five out of five (100%) samples expressing BPV and one out of five (20%) isolates expressed the E5 gene. These results are summarized in

the table 1 and showed in the figures 2 and 3 and were confirmed by direct sequencing of the E2 and E5 gene obtained by RT-PCR.

Table 1 Viral expression of E2 and E5 BPV genes in bovine blood positive for BPV detection.

Samples	*BPV2 expression*	*E2 expression*	*E5 expression*	*E2 and E5 expression*
Asymptomatic	6/8 (75%)	4/6 (67%)	3/6 (50%)	2/6 (33%)
Symptomatic	5/8 (63%)	5/5 (100%)	1/5 (20%)	1/5(20%)
Total	11/16 (69%)	9/11 (82%)	4/11 (36%)	3/11 (27%)

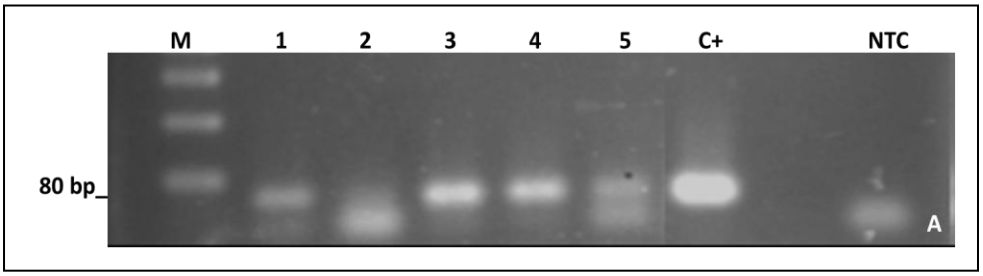

Fig. 2.Representative figure of BPV expression in blood.A RT-PCR for E2 BPV gene. Lanes 1 – 5 samples; C+ positive control; NTC no template control; M: 100bp molecular weight marker.

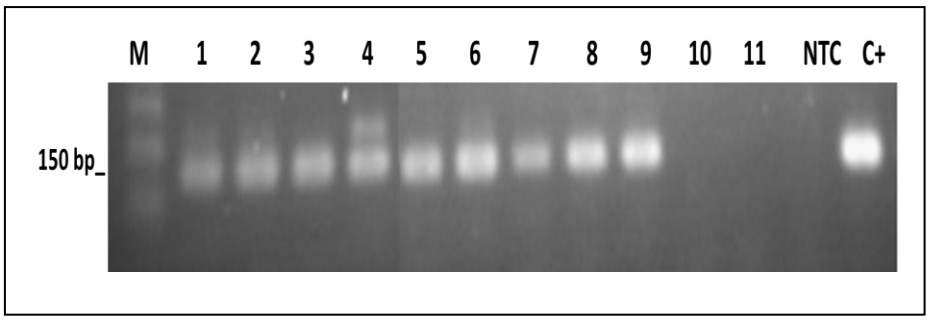

Fig. 3.Representative figure of E5 BPV expression in blood. RT-PCR for E5 BPV gene.M: 100 pb molecular weight marker. Lanes 1 –11 samples; NTC no template control; C+ positive control.

4. Discussion

This is the first study, to the best of our knowledge, describing the expression of BPV2 in blood of healthy and papillomatosis-affected bovine. We found a significant level of blood samples positive for expression of BPV. E2 and E5-BPV2 transcripts were found in blood of healthy and papillomatosis-affected bovine. A previous study also have showed the expression of E5 oncogene in blood cells of bladder cancer-affected animal (Roperto et al., 2008). Moreover, early and late proteins were found in white blood cells (Roperto et al., 2011). However, this is the first study showing the BPV expression in healthy and papillomatosis-affected bovine. In human, HPV-specific RNA is known to occur in blood cells of papillomavirus infected cancer patients (Pao et al., 1991).

Some samples showed the virus presence, but with no virus expression. In these samples the blood is probably acting as a site of virus latency. However, in other samples the virus is active in blood. As active and inactive virus is found in papillomatosis-affected bovine and in asymptomatic bovine, the activation of the virus in blood is independent of the productive infection in epithelial tissue. It is suggested that environmental and genetic factors could contribute to activation of BPV in blood as observed for PV in epithelial tissues (Haussen, 2002; Campo, 2006). Active PV-containing blood cells were suggested to be responsible for spreading the infectious agent to numerous organs (Freitas et al., 2003; Freitas et al., 2007; Roperto et al., 2011). The detection of BPV in different tissues and cells, including reproductive sites as oocytes, the ovary, the uterus, cumulus cells and uterine lavage could corroborate this idea (Freitas et al., 2003; Yaguiu et al., 2006, Lindsey et al., 2009). We suppose that active BPV in blood cells of bovine could facilitate the virus dissemination to non-epithelial sites of asymptomatic and symptomatic bovine.

The presence of BPV in blood of newborn calves has been detected (Stocco dos Santos et al., 1998; Freitas et al., 2003; Yaguiu et al., 2008) suggesting the vertical transmission of BPV. In humans, it has been shown that HPV-infected women can transmit the infection to the fetus by transplacental mechanisms (Rombaldi et al., 2008). More studies are needed to understand the possible importance of active BPV-containing blood to vertical transmission and the role of this mechanism in the asymptomatic and papillomatosis-affected animals to the dissemination of BPV in the herd.

The blood also could be a possible source for horizontal BPV transmission. Stocco dos Santos et al., (1998) experimentally demonstrated evidences that the peripheral blood of animals affected by papillomatosis can act as a vehicle for the transmission of the BPV to healthy cattle. It is believed that flies can be a vector for BPV and transmit the virus between bovine and horses (Nasir e Campo, 2008; Finaly et al., 2009). Up to this moment, information about this system virus-vector-host is inexistent; however, we should not exclude this possibility. The presence of active BPV in blood cells of asymptomatic individuals could represent a source of horizontal transmission in farm negative for papillomatosis.

This study shows the importance of better understanding the role of BPV in non-epithelial tissues, and its consequences to healthy and epidemiology of BPV in bovine.

Acknowledgements

We would like to thank the Brazilian Institutes CNPq for providing financial support to the development of this research and CAPES for providing the scholarships for postgraduate students and the Aggeu Magalhães Research Center (FIOCRUZ, PE) for the sequencing of samples in the present study.

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8. Capítulo V

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

Artigo submetido para publicação no periódico Research in Veterinary Science

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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 sample types, including 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 detect 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. Further studies are needed to assess the implications of active BPV-containing semen to improve strategies avoiding BPV dissemination in the cattle herd and its implications on the reproduction.

Keywords: Bovine Papillomavirus, semen, viral gene expression.

1. Introduction

Papillomaviruses (PVs) are double-stranded DNA tumour viruses identified in a broad range of animal species belonging to the amniotes, including humans (Bernard et al., 2010). So far, 12 genotypes of Bovine papillomavirus (BPV12) have been characterized 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 (Freitas et al., 2011; Hatama et al., 2011; Zhu et al., 2011). 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 (Nasir and Campo, 2008). Normally, papillomas regress as a result of cell-mediate immune response (Knowles et al., 1996). However, occasionally papillomas may develop in squamous cell carcinoma (SCC) as result of involvement of environmental and genetic co-factors (Campo, 2006; Borzacchiello and Roperto, 2008). Recently, it has been described the co-infection among BPVs in skin lesions (warts), but the real significance of this finding needs to be clarified (Pangty et al., 2010; Schimitt et al., 2010; Carvalho et al., 2011)

PVs are described as epitheliotropic, although their presence has been detected in different tissues and cells (Yaguiu et al., 2006; Freitas et al., 2007; Lindsey et al., 2009). Few studies contributes to improve the understanding of PV transmission, however, it has been suggested that the virus can spread through non-epithelial tissues and fluids (Freitas et al., 2007; Freitas et al., 2011).

It has been shown the presence of human and bovine PV DNA in semen and spermatozoa, but the significance of this finding still remains to be fully clarified

(Carvalho et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009; Foresta et al., 2010a; 2011). In humans, the 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 (Foresta et al., 2011).

There are few reports focusing on the study of BPV in reproductive tract and its cells (Stocco do Santos et al., 1998; Freitas et al., 2003) although the BPV-1 were described in the prepuce and penis (Jarret, 1985) and BPV-2 were found infecting placenta (Roperto et al., 2012). In an early study, we demonstrate the high incidence of BPV2 in commercial doses of bull semen from national and multinational companies (Silva et al., 2011). However, studies are needed to understand the biological significance of BPV presence in semen. The present study aimed at evaluating the presence of BPV in sperm cells of healthy bull, as well as a possible gene activity.

2. Materials and methods

2.1. Semen samples

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 electroejaculation and cooled on ice. After, the samples were centrifugated at 1200 g for 10 min and 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 to

separate the sperm cells from seminal plasma and debris. The final pellet obtained was re-suspended in 200 µL of PBS for subsequent DNA and RNA extraction.

2.2 DNA and RNA extraction and cDNA synthesis

All the samples were submitted to DNA and RNA extraction by Invirsob® Spin Universal RNA Mini Kit, (Invitek, Germany), in accordance with the manufacturer´s protocol. Subsequently, 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 (no template control) were used during the synthesis of cDNA. The essential meiotic endonuclease -1 transcript was amplified from cDNA with primers designed (EME Fw AACTGAGGCCTGAAGAGACC and EMERev GGACTGGGTATCAGGCAGTT) to verify the presence of cDNA from transcripts in sperm cells. PCRs were carried out by using 0.2 µM of each primer, 05µL of cDNA and 1X Master Mix (Promega, USA). The PCR parameters were consisted of 40 cycles of denaturation for 45s at 94°C, annealing for 40s at 52°C and extension for 40s at 72°C.

The quality of purified DNA was checked by β-globin gene PCR, as described by Freitas et al., (2003). The amplification was carried out in a final volume of 25µL containing 100ng of DNA, 1X Master Mix (Promega, USA) and 0,2 µM of specific primers.

2.3 BPV detection by PCR

It was detected the presence of BPV2 DNA with primers targeting L1 BPV gene previously described by Yaguiu et al., (2008). 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 with TAE buffer and run at a constant voltage (100 V) for approximately 35 minutes. DNA was visualized after being stained with ethidium bromide under UV light. A sequencing using corresponding BPV2 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 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).

2.4 BPV expression by RT-PCR

The presence of BPV transcripts were evaluated for two different BPV genes, E5 and E2. The two set of primers were described by Roperto et al., (2008) and Silva et al., (2011). PCRs were carried out according conditions described by Silva et al., (2011). The purified amplicon from PCR 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) to confirm presence of BPV transcripts. The recombinant DNA clones were isolated with PureYeld^{TM} Plasmid Miniprep System (Promega, USA). Subsequently, a direct sequencing was conducted and analyzed as described above.

3. Results

3.1 Presence of BPV in semen

Following β-globin PCR with scored positive for all DNA isolates for semen, we assessed for the presence of BPV2 DNA. As expected, the NTC tested negative and this was also the case with the virus-free DNA from MDBK cells, thus confirming the absence of cross-contamination and the reliability of the method employed. 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%.

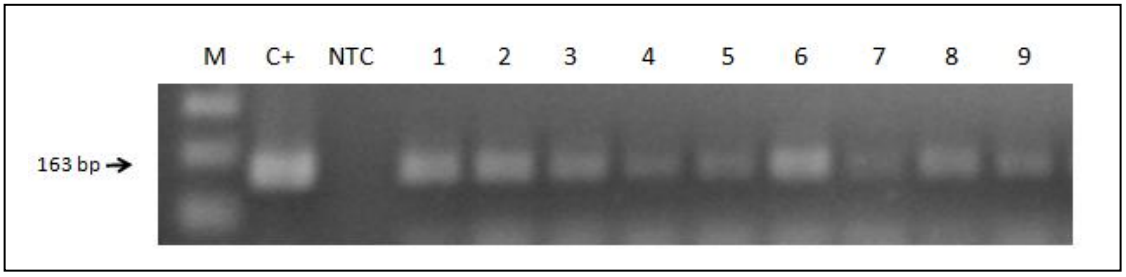

Fig. 1. Presence of BPV-2 in bull semen. PCR for L1 BPV-2 gene. M: 100 bp molecular marker; C+ positive control; NTC no template control; lanes 1 – 9 samples.

3.2 Expression of BPV in semen

First, it was assessed for the transcripts of essential meiotic endonuclease-1 bovine gene in the cDNA samples. Subsequently, it was found the presence of viral transcripts for the E5 and E2 BPV2 genes in positive samples for BPV2. 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 and 2b). The two negative controls produced during cDNA synthesis scored negative. The results for presence and expression of BPV2 in

semen are summarized in the table 1. The sequenced clones of BPV2 E2 and E5 amplicons confirmed the transcripts presence in the semen.

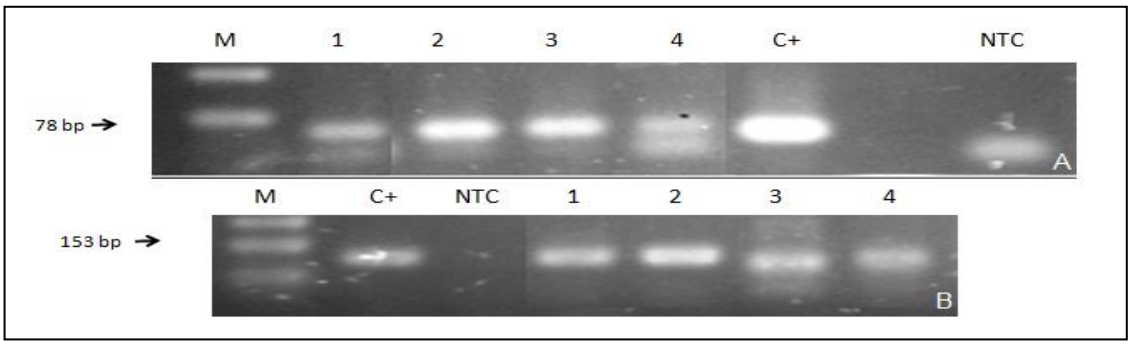

Fig. 2. Expression of BPV2 in semen. A. RT-PCR for E2 BPV2 gene. M: 100 bp molecular marker; lanes 1 – 4 samples; C+: positive control; NTC: no template control.

4. Discussion

This is the first study, to the best of our knowledge, describing the expression of BPV2 in bovine sperm cells. Our results confirm and better define our previously obtained data on the detection of BPV2 in commercial semen (Silva et al., 2011), and also have important implications regarding PV pathogenesis and possibly in the transmission.

Our findings correspond with the previous results of Carvalho et al., (2003), Yaguiu et al. (2006) and Lindsey et al. (2009) who also detected BPV2 DNA in bovine 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 (Lee et al., 2002, Rintala et al., 2004, Bezold et al, 2007, Foresta et al., 2010a,b,c).

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 (Brandt *et al*., 2008; Roperto *et al*., 2008). Moreover, early and late proteins were found in white blood cells (Roperto et al., 2011). PV-containing blood cells were suggested to be responsible for spreading the infectious agent to numerous organs, including semen (Freitas et al., 2007; Roperto et al., 2011). 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 (Carvalho et al., 2003; Freitas et al., 2003; Yaguiu et al., 2006, Lindsey et al., 2009). We believe that the presence of active BPV in semen of asymptomatic animals could facilitate the dissemination of BPV through this route.

An alternative explanation for BPV DNA and RNA in semen is that it might, at least in part, originate from infections in reproductive tract. Fibropapillomas were described occurring both in the prepuce and penis and 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 (Jarret, 1985). 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 (Nielson et al, 2009a,b; Shigehara et al., 2010; Hernadez et al., 2008). Moreover, there are studies showing the presence of BPV and HPV in the sperm cell by in-situ hybridization (Lindsey et al., 2009). So, sperm may be a site of infection by BPV.

The HPV RNA presence was found in human seminal plasma and sperm cells (Lai et al., 1996). 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.

Some reports suggest the vertical transmission of PV. 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 (Foresta et al., 2011). Also, the transplacental transmission of PV appears to take place in humans and cows (Rombaldi et al., 2008; Freitas et al., 2003 and Roperto et al., 2012).

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.

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9. Capítulo VI

Presence and gene expression of bovine papillomavirus in peripheral blood and semen of healthy horses

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Short title: BPV in blood and semen of horses

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Abstract

Papillomavirus (PV) are double-stranded DNA viruses that can cause benignant and malignant tumours in mammals. Twelve genotypes of bovine papillomavirus (BPV1 to 12) have been identified so far. The presence of BPV1 and 2 have been found in body fluids of cattle and horse. This study aimed to investigate the presence of BPV DNA and expression of viral genes in blood and semen cells of healthy horses by PCR and RT-PCR. BPV-1/2 were detected in 14 out of 70 (20%) blood samples and in 11 out of 31 (35%) semen samples. Five out of 14 (36%) blood samples tested positive for E5 expression while no blood sample was positive for L1 expression. Four out of 11 (36%) semen cells samples scored positive for E5 expression while no expression of L1 gene could be detected. This is the first study showing BPV1 gene expression in blood and semen of healthy horses. Our data address to the need for a better understanding of the presence of BPV in non-epithelial tissues of horses and their role in vertical and horizontal transmission of these viruses.

Keywords: BPV, expression, semen, blood, equine.

1. Introduction

The papillomaviruses are a group of double-stranded DNA viruses, which have been identified in a broad range of animal species (Freitas et al, 2011). They are classified in the *Papillomaviridae* family which comprises 29 different genera (Bernard et al, 2010). These viruses are species-specific, and even in experimental conditions, do not infect other host than its natural one (Campo, 2006). However, the bovine papillomavirus types 1 and 2 (BPV1 and BPV2) can cause cross-species infection in horses, donkeys, mules (Chambers et al, 2003; Campo, 2006) and zebras (van Dyk et al, 2009) being detected in different cutaneous lesions: neoplastic (sarcoid) inflammatory (Chambers et al, 2003; Yuan et al, 2007) as well as in healthy skin (Bogaert et al, 2008). Recently, the putative new BPV type "BR-UEL-4", which was firstly described in cattle, has been detected also in equine sarcoid (Silva et al, 2010).

Although papillomaviruses are described as epithelial-specific (Campo, 2006), in bovines BPV has already been found in different tissues and fluids, such as peripheral blood, blood plasma, milk and colostrum (Stocco dos Santos et al, 1998; Freitas et al, 2003; Roperto et al, 2008; Diniz et al, 2009; Roperto et al, 2011). Additionally, the expression of BPV oncoproteins has been demonstrated in lymphocytes of cows suffering from urinary bladder cancer (Roperto et al, 2011). In equids, BPV has been described in blood of sarcoid-affected and healthy animals (Brandt et al, 2008; van Dyk et al, 2009).

The presence of BPV DNA in bovine semen and reproductive tract has already been demonstrated (Yaguiu et al, 2006; Lindsey et al, 2009; Roperto et al,

2012), but without any apparent change in the parameters of vigor, motility, acrosome reaction and DNA integrity (Silva et al, 2011).

In order to better understand the biological significance of the BPV presence in the non-epithelial tissues of horses, we investigated the presence of BPV DNA as well as the expression of E5 and L1 proteins in blood and semen cells of healthy horses.

2. Material and Methods

2.1 Blood samples

Blood samples were collected from 70 horses from two countries - Italy (30 samples) and Brazil (40 samples). 3mL of blood was collected by jugular venipuncture using EDTA-containing tubes and 200 µL of total blood were used for DNA and RNA extraction. The horses were asymptomatic for the presence of equine sarcoid.

2.2 Semen samples

Semen samples were collected from Italy (10 samples) and Brazil (21 samples). A mare in behavioral estrus was used for sexual stimulation and each stallion was mounted on a breeding phantom for semen collection. Immediately before collection, the penis was cleaned with water, including hygienizing the urethral fossa in order to remove the smegma adhered to the penile and preputial surface, as described by Tischner and Kosiniak (1992). Semen was collected using an artificial vagina (BiotechBotucatu®, Sao Paulo, Brazil) recovered internally with latex mucosa containing water at 40-42°C and coated with a plastic mucosa coupled to a collection tube containing a filter for retention of dirt and cellular debris (Amann et al, 1983). After collection, the semen was analyzed for macroscopic (volume, appearance,

color) and microscopic (total motility, progressive motility, vigor, sperm concentration) parameter settings using phase contrast microscopy (Nikon Eclipse E-400®) (Sieme, 2009). The collected semen was diluted with commercial diluent based on skim milk and glucose (Botu Semen, BiotechBotucatu®) in a proportion 1:1 (semen: diluent), placed into 15ml tube and centrifuged at 400g for 5 minutes (CenterBio centrifuge, Sao Paulo, Brazil). The supernatant (consisting of seminal plasma, diluent, cell debris and dead spermatozoids) was discarded and the precipitated cells were resuspended with the same diluent at a ratio of 1:2 (semen:diluent) in order to avoid damage to the transported samples, due to the presence of seminal plasma (Troedsson et al, 2005). Before starting the extraction of DNA and RNA, semen samples were centrifuged twice at 1200g for 10 minutes to remove the seminal diluent and the cell pellet was washed with PBS (0.9% saline phosphate buffered solution, pH 7.4). The final pellet was resuspended in 200µL of PBS.

2.3 DNA and RNA extraction and cDNA synthesis

The extraction for both DNA and RNA of blood and semen samples was performed using the Invirsob® Spin Universal RNA Mini Kit (Invitek, Germany) following the conditions specified by the manufacturer. In another step, all RNA samples were treated with RNase-free DNase enzyme (Promega, USA) before the cDNA synthesis. For the cDNA synthesis, it was used the Improm-II® Reverse Transcription System (Promega, USA) following the manufacturer's recommendations. Two negative controls were used, one without the addition of reverse transcriptase enzyme, and the other without the addition of RNA (no template control).

2.4 Detection of BPV DNA

The samples were analyzed for the presence of BPV1 and 2 L1 gene using the following primers (L1BPV1aFw 5' GGC TGA GGA CGC TGC TGG TA 3' and Rev 5' TCT CCG AGC CCC CTC TGG TC 3'; L1BPV2a Fw 5' GTT ATA CCA CCC AAA GAA GAC CCT 3' and Rev 5' CTG GTT GCA ACA GCT CTC TTT CTC 3'). The PCRs were carried out using Master Mix (Promega, USA), 0.2µM of each primer and about 100 ng of DNA. The PCR cycle consisted of a denaturation at 94ºC for 40 seconds, annealing at 60ºC for 40 seconds and extension at 72ºC for 40 seconds for L1BPV1 primers. The cycle conditions for L1BPV2 are described in Silva et al (2011). The genomes of BPV1 and BPV2 cloned into pAT153 plasmid were used as positive control. The negative control consisted of DNA extracted from E DERM (normal equine cells) (ATCC CL57 USA), along with a control without DNA (no template control – NTC). The PCR products were analyzed by electrophoresis on 2% agarose gel with 1X TAE buffer (Tris-Acetate-EDTA) stained with ethidium bromide solution (0.5 µg/µL) and visualized under UV light transilluminator.

2.5 Detection of BPV mRNA

Primers for verification of BPV1 and BPV2 L1 and E5 genes expression were designed using Primer Blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The expression of E5 BPV1/2 was assayed with primers E5B1/2F/R (Fw 5' ATT CTC GAG RCC ATG CCR AAT CTA TGG TTT C 3' and Rev 5' AAG GTA CCT TAA AAG GGC AGA CCT GTA C 3') that amplifies a fragment size of 135 bp. The PCRs were carried out using Master Mix (Promega, USA), 0.2µM of each primer and about 100 ng of DNA and the cycle conditions consisted of a denaturation at 94ºC for 1 min., annealing at 50ºC for 1 min. and extension at 72ºC for 1 min. The primers to detect the expression of L1 BPV1 (Fw 5' AAT CAG ATC TAC CTC TTG ACA TTC 3' and Rev 5' CAC AGA GCA TAG CTC TAA TAT AAA 3') and L1 BPV2 (Fw 5' ACT

ACC TCC TGG AAT GAA CAT TTC C 3' and Rev 5' AAA TTT GCC AGT ATC ATA CTC TGA C 3') amplified a fragment size of 499. The PCRs were carried out using Master Mix (Promega, USA), 0.2µM of each primer and about 100 ng of DNA and the cycle conditions consisted of a denaturation at 94ºC for 1 min., annealing at 60ºC for 1 min. and extension at 72ºC for 1 min for both primers.

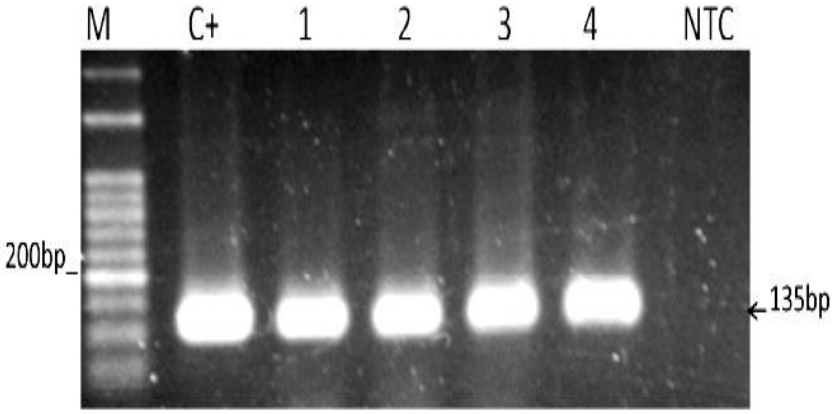
3. Results

As expected, the primers for detection of BPV1 and 2 did not amplified any fragments in the sample of DNA isolated from EDERM cells as well as in the NTC. Twelve out of 70 animals analyzed (17%) were positive for BPV1 DNA (Table 1). 2 out of 70 blood samples (3%) were positive for BPV2 DNA presence. None of the animals showed simultaneous presence of BPV1 and 2 in the blood samples.

Table 1: Presence and expression of BPV DNA and E5 and L1 genes in blood and semen of healthy horses

Samples	*BPV1 DNA presence*	*BPV2 DNA presence*	*E5 gene expression*	*L1 gene expression*
Blood	12/70 (17%)	2/70 (3%)	5/14 (36%)	0/70 (0%)
Semen	11/31 (35%)	0/31 (0%)	4/11 (36%)	0/31 (0%)

Only the 14 samples positive for BPV1 or 2 DNA were tested for viral gene expression. E5 was found to be expressed in 5 out of 14 (36%) blood samples (Fig. 1). All the samples with positive expression of E5, tested positive only for the BPV1. No blood samples were positive for BPV L1 gene expression.



Expression of BPV1 in semen and blood cells. RT-PCR for E5 BPV1 gene. M: 50 bp molecular marker; lanes 1 and 2 represent blood samples; lanes 3 and 4 represent semen samples; C+: positive control; NTC: no template control.

In the semen cells samples, BPV1 DNA was present in 11 out of 31 samples (35%) whereas BPV2 DNA was not found. 4 out of 11 (36%) samples positive for BPV1 showed E5 gene expression (Fig. 1), and as in the blood samples, no semen cells samples were positive for BPV L1 gene expression (Table 1).

The semen from all animals had normal characteristics of volume, appearance and color. The parameters of total motility, progressive motility, vigor and sperm concentration were also the expected for a fertile horse. All animals have proven fertility and ages between 6 and 12 years.

4. Discussion

Recently, the number of studies to detect the presence and expression of BPV in blood or other tissues of horses is increasing (Brandt et al, 2008; van Dyk et al, 2009; Hartl et al, 2011). This study confirms the presence of BPV1, and more rarely BPV2, in the blood of healthy horses. For the first time, we have detected BPV1 E5

gene expression in the blood and semen from healthy horses of different countries (Italy and Brazil), demonstrating the wide spread of this virus.

Brandt et al (2008) showed the presence of BPV in mononuclear cells from sarcoid-affected horses, but they failed to identify the presence of BPV in the blood of healthy animals. Our results are in accordance with those obtained by Van Dyk et al (2009), who demonstrated the presence of BPV1 and 2 in the blood of sarcoid-affected and healthy zebras, with BPV1 being the most frequent. Accordingly, we have also found BPV1 more frequently than 2, confirming that this genotype is mostly found in horses.

The presence and transient expression of BPV1 E5 has been demonstrated in blood cells from experimentally infected foals (Hartl et al, 2012). The presence of BPV in the blood after sarcoid development, and a BPV latent infection in healthy skin, as reported by Bogaert et al (2008) could help explaining how BPV reached the blood.

In previous reports BPVs 1, 2 and 4 were found in reproductive tract tissues of cattle, including oocyte, ovary, uterus, cumulus cells and uterine lavage (Carvalho et al, 2003; Yaguiu et al, 2006), placenta and amniotic fluid (Freitas et al, 2007; Yaguiu et al, 2008; Roperto et al, 2012). However, in horses, this is the first evidence of BPV infection in the reproductive tract cells. The assessed parameters of total motility, progressive motility, vigor and sperm concentration showed no change that would implicate in reduced fertility, as also observed in the case of BPV presence in semen samples from cattle (Silva et al, 2011).

The BPV gene expression in semen and blood cells suggests that BPV can also infect these cells, although the lack of L1 expression indicates a non productive infection. Our results are in accordance with Lindsey et al (2009) demonstrated the

presence of BPV DNA into the spermatozoa of cattle by in situ hybridization assays. Taken together, these and our results about BPV expression in semen cells could suggest that these cells may be a new site for BPV infection in its hosts.

Freitas et al (2003) found the presence of BPV1 in the blood of newborn calf and Yaguiu et al (2008) found BPV1, 2 and 4 in the blood of cows and their progeny, suggesting that the blood and the semen of horses may also represent a risk for vertical transmission. Further studies are needed to clarify this intriguing issue.

Some authors suggest that the blood can act as a reservoir of BPV (Freitas et al, 2003; 2007; Brandt et al, 2008). As we found the presence of BPV in the blood of healthy animals living in farm without history of sarcoid, the presence of these animals can be a source of BPV. The blood could be also a vehicle of horizontal transmission of BPV: it is believed that flies have a role in the transference of BPV between cattle and horses (Nasir and Campo, 2008; Finlay et al, 2009), although this route of transmission may be less efficient than the path already well established among mucosa (Bravo et al, 2010). However, data about this virus-vector-host system are lacking and further investigations are needed.

5. Conclusion

Our data indicate that BPV may infect blood and semen cells of healthy horses, even if this infection is asymptomatic. Our results address to the need for a better understanding of the presence of BPV in non-epithelial tissues of horses and their role in vertical and horizontal transmission of this virus.

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10. Capítulo VII

Comparison of two PCR systems for detection of bovine papillomavirus

Artigo a ser submetido para publicação

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Abstract

Bovine papillomavirus (BPV) is a diverse group of double stranded DNA oncogenic viruses, which have been detected in epithelial lesions and body fluids. Most studies of BPV infection rely on a single method for DNA detection; however the use of any single method or technique may underestimate the true prevalence of this virus. Since the knowledge of BPV diversity and prevalence is very important for development of treatment and diagnostic methods, this study aimed to compare two PCR systems for detection of BPV in skin lesions and fluids: the use of BPV type-specific and consensus primers. Seventy two cutaneous lesions, 57 blood samples and 59 semen samples were collected. PCR with the consensus primers FAP and BPV type-specific primers to BPVs 2, 3, 4, 5, 8, 9 and 10, along with sequencing assays, were used in order to detect the BPV types. Phylogenetic analysis was carried out with maximum likelihood method. Both FAP and BPV type-specific primer sets could amplify BPV types DNA in skin lesions, blood and semen samples. However, BPV type-specific primers were more sensitive than consensus primers and could detect co-infection of BPV in the samples. Consensus primers amplified five BPV types and they were more suitable to detect putative new BPV types. So, it is important to consider both PCR primer systems in order to perform any BPV-associated epidemiological studies, which is necessary to identify co-infection, the presence of novel viruses, and avoid false-negative results.

1. Introduction

The papillomaviruses (PVs) are a group of double-stranded DNA viruses, which have been identified in a broad range of animal species (Freitas et al, 2011). They are classified in the *Papillomaviridae* family which comprises 29 different genera (Bernard et al, 2010). There are more than 150 human papillomavirus (HPV) identified while only 12 Bovine papillomavirus (BPV) types have been described.

BPV can induce papillomas and fibropapillomas in the skin (Nasir and Campo, 2008) and mucous lesions, which can regress or evolve to malignant lesions, mainly when in association with environmental co-factors (Jarret et al., 1977). Some BPV types are involved in urinary bladder (BPV1 and BPV2) and upper digestive tract (BPV4) malignant tumours in cattle (Borzacchiello and Roperto, 2008).

BPV has been widely detected in non-epithelial sites such as gametes and fluids in recent years (Freitas et al., 2003; Roperto et al., 2008; Diniz et al., 2009; Lindsey et al., 2009; Roperto et al, 2011; Silva et al., 2011), and blood has been hypothesized as a carrier for BPV to diverse body parts (Freitas et al., 2007; Roperto et al., 2011). Moreover, blood has been described as a site of productive infection for BPV (Roperto et al., 2008; Roperto et al., 2011).

PVs have been detected and characterized by PCR with consensus primers (Manos et al., 1989; Forslund et al., 1999; Ogawa et al., 2004, 2007). Two sets of primers (FAP59/FAP64 and MY09/MY11), originally designed from two conserved regions of L1 gene of HPV, are widely used for PVs identification in human and a wide range of animals (Manos et al., 1989; Forslund et al., 1999; Antonsson and Hansson, 2002; Ogawa et al., 2004; Schulz et al., 2009). In bovines, about 31 putative new BPV types were detected using these primers (Forslund et al., 1999;

Antonsson and Hansson, 2002; Ogawa et al., 2004; Maeda et al., 2007; Claus et al., 2008; Carvalho et al., 2012). Beyond the use of consensus primers, diverse BPV type-specific primers have been designed for the purpose of specific BPV types identification (Gaukroger et al. 1991; Bloch et al., 1997; Wosiacki et al., 2005; Brandt et al., 2008; Silva et al, 2011; Carvalho et al., 2012).

Some studies in HPV indicate that any single method or technique for the detection may underestimate the true prevalence of this virus (Karlsen et al., 1996; Smiths et al., 1995). In cattle, most studies of BPV infection rely on a single method. Since the knowledge of BPV diversity and prevalence is very important for development of treatment and diagnostic methods, as well as for the understanding of BPV evolution and epidemiology, we have compared two PCR systems for detection of BPV in skin lesions and fluids from Brazilian cattle, the use of BPV type-specific and consensus primers. This report highlights the robustness and weakness of each method and reveals the necessity of using both of them.

2. Material and methods

Skin lesions

A total of 72 skin lesions were collected from beef and dairy cattle of farms in Northeastern Brazil. The cutaneous lesions were collected via excision from different sites on the skin of the animals.

Blood samples

Blood samples were collected from 57 cattle from beef and dairy farms with high incidence of cutaneous papillomatosis in Northeastern Brazil. Three mL of blood

was collected by jugular venipuncture using EDTA-containing tubes and 200 µL of total blood were used for DNA extraction.

Semen samples

Fifty-nine frozen semen samples taken from dairy bulls were obtained from four companies in Brazil. The samples were thawed in a water bath at 37°C for 30 seconds and centrifuged at 1,200 g for 10 min to separate the sperm cells from the seminal plasma and diluents. The obtained cell pellet was washed twice with PBS (0.9% saline phosphate buffered solution, pH 7.4) and again centrifuged at 1,200 g for 10 min. Finally, the pellet was re-suspended in 200 µL of PBS for subsequent DNA extraction.

DNA extraction

Genomic DNA were extracted from each sample by using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to manufacturer's protocols. Extracted DNA was quantified using Nanovue (GE, Fairfield, CT, USA). 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 PCR assays using Master Mix Promega kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Reactions were performed in a two-step process. First, all DNA samples were screened for the presence of BPV DNA using the consensus primers FAP59/64 under the conditions described by Ogawa et al. (2004) and the modifications described by Carvalho et al. (2012). Second, DNA samples were amplified using BPV type-specific primers (BPV2- 5 and 8-10 for skin lesions and BPV2-5 for fluids), according to the amplification protocol described by Stocco dos Santos et al. (1998)

and the modifications described by Carvalho et al. (2012). All amplification products were visualized by 2% TAE agarose gel electrophoresis and subsequent ethidium bromide staining. Positive and negative controls are described by Carvalho et al. (2012). Amplicons obtained by FAP59/64 PCR and by specific primers were sequenced to identify/confirm the viral type, respectively.

Identification of a putative new BPV type

The sample that tested positive for the presence of a putative new BPV type was once again amplified by PCR using a High Fidelity DNA polymerase (GE) and the degenerated primers indicated above for confirmation. PCR products were cloned into pGEM-T vector (Promega) followed by the transformation of competent DH5α bacteria. Bacterial clones were randomly selected for confirmation. At least two different positive clones were sequenced twice, in both directions, with an ABI 3100 Applied Biosystems DNA sequencer and Sanger BigDye terminator v 3.1 cycle sequencing kit.

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

Phylogenetic analysis

Phylogenetic analysis was carried out with amino acid sequences of BPV types and putative novel types isolated from Northeastern Brazil, using the Maximum Likelihood method with WAG + G as amino acid substitution model in PhyML 3.0 (Guindon et al., 2010). The tree topology was estimated using the best solution among Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) methods. An initial BIONJ tree was used, and the taxa were randomly added. In order to determine the statistical support of the obtained branches, 1,000 non-parametric bootstrap replicates were used.

3. Results

The total number of each BPV type amplified by the two PCR systems is summarized in the table 1. The number of positive samples with BPV type-specific primers ranged between 95 to 100% while the number of positive samples with consensus primers ranged from 5 to 54%. The consensus primers FAP59/64 amplified a bigger number of skin lesion samples than fluid samples (blood and semen). Also the BPV type-specific primers had a level of positivity similar in the two types of samples (100% for skin lesion and 95% for semen and blood).

Table 1. Number of samples positive for BPV DNA

Samples	FAP59/64	BPV type-specific
Skin lesions	39/72 (54%)	72/72 (100%)
Blood	3/60 (5%)	57/60 (95%)
Semen	3/40 (7%)	59/59 (100%)

In skin lesions, using the BPV type-specific primers, 89% of the samples presented co-infection. However, we fail to detect co-infection using the consensus primers. Furthermore, the BPV types 4 and 9 were not detected using the consensus primers, while the prevalence of these BPV types using specific primers were 25% (BPV4) and 3% (BPV9). The results of BPV prevalence are shown in the Figure 1. High discrepancies in BPV prevalence were found when comparing the results of BPV type-specific primers and consensus primers. BPV2 showed a prevalence of 99% with BPV type-specific primers while only 16% of the samples were positive for BPV2 using consensus primers. The prevalence of BPV3 was 78% with type-specific primers and only 8% with consensus primers. Also BPV10 showed a high discrepancy of prevalence when comparing the two PCR systems – it showed 33% of prevalence with type-specific primers while 12% of the samples were positive for BPV with consensus primers. In semen, using the BPV type-specific primers, 66% of the samples showed co-infecton while 49% of blood samples presented co-infection. As demonstrated to skin samples, the co-infection was not observed using the consensus primers. BPV2 and 3 had the highest prevalence in all kind os samples (skin, semen and blood).

The consensus primers showed a high diversity of BPVs types and putative new types. Fourteen diverse BPVs types and subtypes were found in the samples (BPV1-6, BPV8-10, BAPV3, BAPV10, BRUEL3-5) while 07 out of 07 possible BPV types tested with BPV type-specific primers were found. 50% of the samples amplified with consensus primers showed a putative new BPV type previously described. Furthermore, the use of consensus primers let us to detect some putative new BPV types. Nucleotide identity analysis showed that one isolate

(BPV/UFPE03BR) is an unreported putative BPV type. Another isolate (BPV/UFPE05BR) found in this study is a new BPV11 subtype (Figure 2). The identity between BPV/UFPE03BR sequence and BPV6 L1 sequence was 71.5%. This suggests that BPV/UFPE03BR isolate is a novel BPV type. The identity between BPV/UFPE05BR sequence and BPV11 L1 sequence was 98%, which indicates that it is a new BPV11 subtype. These two nucleotide sequences were deposited at GenBank with the following accession numbers: JQ897974 and JQ897976.

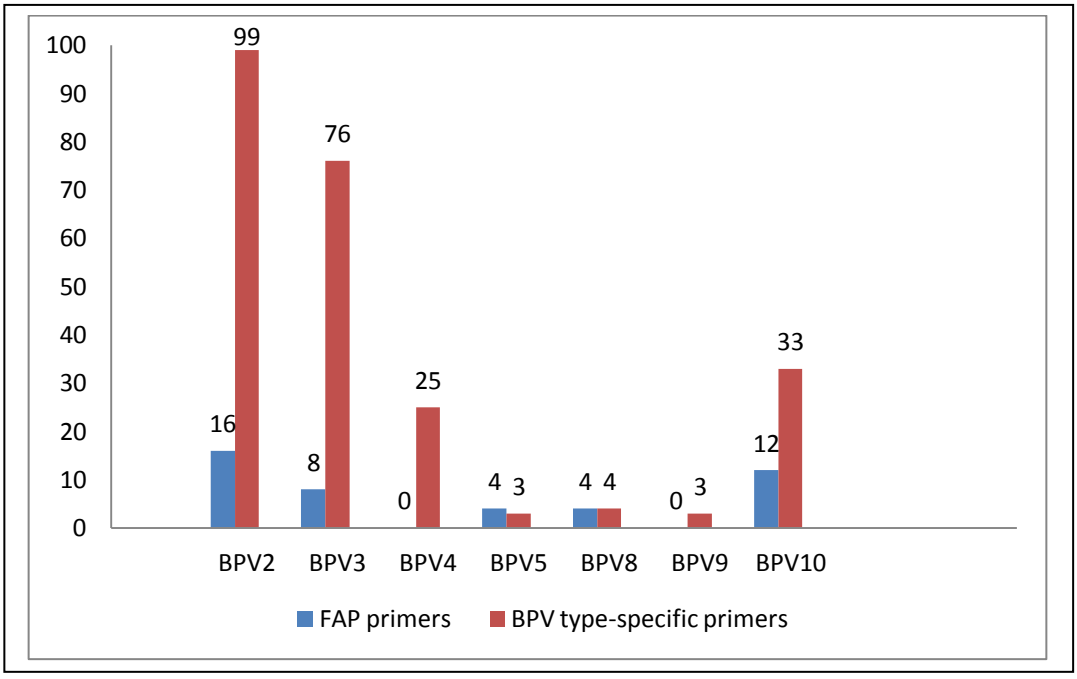

Figure 1. BPV types amplified by two PCR systems

The phylogenetic tree confirmed that the isolate BPV/UFPE03BR belongs to a new viral type, with 59% of confidence based on bootstrap (Figure 2). This isolate is close related to all *Xipapillomavirus* genus members, which indicates that it belongs to this

genus. The isolate BPV/UFPE05BR was clearly associated with BPV11, which confirms that it is a new subtype. The majority of the branches were statistically well supported with at least 50% of confidence. In addition, the clade that corresponded to *Xipapillomavirus* genus was strongly supported (Figure 2).

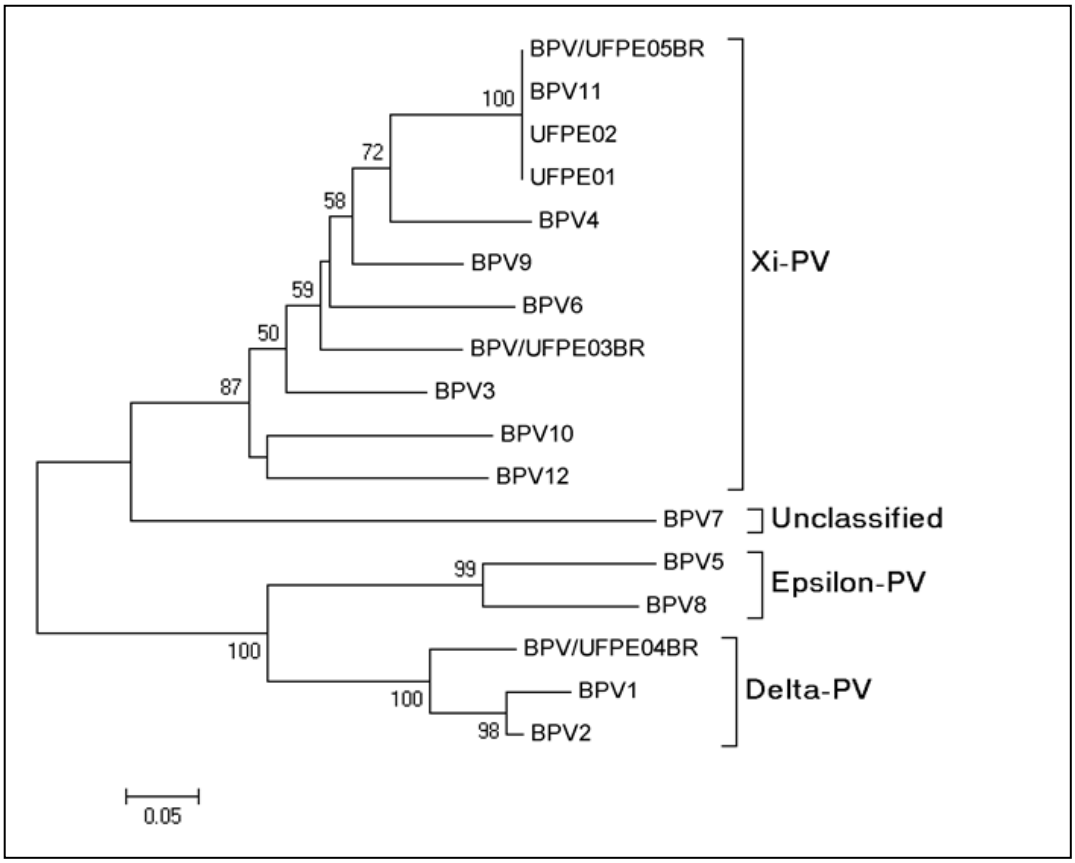

Figure 2: Maximum Likelihood tree of bovine papillomaviruses, which comprises 12 BPV types and some isolates from Brazil, based on partial sequences of L1 ORF. Three groups of viruses are distinguished, which forms the previously described genera (*Xipapillomavirus*, *Deltapapillomavirus* and *Epsilonpapillomavirus*). Unclassified isolates are also presented. Numbers in the nodes are bootstrap support values of the branches determined by 1,000 replicates, and the values below 50% are not shown.

4. Discussion

This study compares two PCR methods for BPV detection and characterization. The knowledge about BPV diversity and epidemiology is very

important to the development of prevention strategies and the understanding of the evolution of this group of viruses. Thus, the employment of an accurate PCR strategy is necessary to obtain reliable data to increase the knowledge about BPV biology.

The consensus primers FAP59/64 had low level of sensitivity when compared to BPV type-specific primers. In a previous study employing consensus and type-specific primers for HPV detection found that the sensitivity of type-specific primers was higher than the consensus primers (Qu et al., 2000). Besides, FAP primers has been used to amplify BPV DNA (Antonsson and Hansson, 2002; Ogawa et al., 2004; Claus et al., 2007 and Carvalho et al., 2012). However, these primers were designed to amplify a HPV L1 region (Forslund et al., 1999). Thus, the mismatches among the consensus primers sequences and BPV L1 sequences could contribute to decrease the sensitivity of these set of primers. Qu et al., (2000) affirmed that the efficiency of consensus primers may be related to the number, position and stability of the mismatch. Furthermore, these authors verified differences in type-specific amplification efficiency attributed to degeneracy synthesis in the consensus primers. There are three mismatches between FAP primers and BPV2, and there are six, four and five mismatches between the FAP primers and BPVs 3, 4 and 10, respectively. This high number of mismatches could help to explain the low sensitivity of FAP primers when compared to BPV type-specific primers.

Nevertheless, consensus primers enable the amplification of a high number of BPV types, including uncharacterized types. In this study, one putative new BPV type and one novel BPV11 subtype were found by using this PCR system. Previous studies using consensus primers detected 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; Carvalho et al., 2012). There may be an underestimation

of the extent of the spread of BPV. Only 12 BPV types have been described so far, despite the great diversity found in HPV. In this context, the use of consensus primers is very important to increase the knowledge about BPV diversity.

In this study, we described a putative new BPV type that belongs to *Xipapillomavirus* genus and a novel BPV11 subtype. These results suggest that there is a great diversity of BPV types that infect cattle, and the understanding of this diversity is necessary for therapeutic development and to improve diagnostic methods. Recent studies describe novel BPV types, demonstrating this diversity (Claus et al., 2008; Hatama et al., 2008; Hatama et al., 2011; Carvalho et al., 2012; Zhu et al., 2012).

The presence of putative novel BPVs in Brazilian cattle suggests that other uncharacterized BPV types could be infecting cattle worldwide. The use of consensus primers is very important in the investigation of novel PV types, which shows the necessity of this kind of approach. Additional studies aimed at detecting and characterizing novel PV types and their variants are needed in order to better understand their biology and their association with different pathological aspects.

In conclusion, both FAP and BPV type-specific primer sets amplified a wide range of BPV types in skin lesions, blood and semen samples. However, BPV type-specific primers were more sensitive than consensus primers and it could detect co-infection of different BPV types in the samples. On the other hand, the consensus primers are very suitable to detect novel BPV types and subtypes, which is also important. Therefore, the choice of the PCR primer system plays an important role on the BPV epidemiological investigations. For a more complete study, both systems are complementary.

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11. Discussão geral

11.1 BPV em lesões cutâneas de bovinos

Neste estudo, através do emprego da técnica de PCR utilizando os primers consenso e primers específicos para BPV, foi detectada a co-infecção de BPV e o FeSarPV em lesões cutâneas de bovinos. Nossos achados estão de acordo com Munday & Knight (2010) que também encontraram estes dois tipos virais em uma mesma lesão de bovinos. A ocorrência de múltiplas infecções nas lesões cutâneas de bovinos pode ter uma associação com a longa persistência dessas lesões observada nos animais estudados.

Tem-se proposto que o FeSarPV, inicialmente isolado em felinos, pode ter um ruminante como seu hospedeiro natural (Munday & Knight 2010). As análises filogenéticas realizadas agruparam o FeSarPV no gênero *Deltapapillomavirus,* corroborando essa ideia.

Embora este estudo tenha mostrado a co-infecção de FeSarPV e BPV em uma mesma lesão, mais estudos são necessários para entender essa relação. Aparentemente esse dois virus não estão inibindo a presença um do outro na mesma lesão. Estudos posteriores são necessários para se determinar a prevalência de FeSarPV e outros BPV no rebanho bovino, pois o melhor conhecimento dos PV que afetam bovinos, assim como infecções cruzadas causadas por PV possuem implicações importantes para o desenvolvimento de estratégias de prevenção e controle das papilomaviroses.

11.2 Alterações na expressão de conexina 26 em fibropapilomas

Lesões co-infectadas por BPV também foram avaliadas quanto à expressão da oncoproteína E5 de BPV1 e 2 e a expressão de uma proteína que compõe as junções gap intercelulares, a conexina 26. Foi observada uma desregulação na expressão da conexina 26 nas lesões cutâneas que expressavam a oncoproteína E5. Alterações na expressão de conexinas têm sido demonstrada em múltiplos tipos de tumor (Sawey *et al.,* 1996; Jamieson *et al.,* 1998; Gee *et al.,* 2003; Mesnil *et al.,* 2005). Porém, apenas poucos estudos reportam a expressão de conexinas em neoplasias de espécies veterinárias (Gotoh *et al.,* 2006; Corteggio *et al.,* 2011).

Em alguns fibropapilomas, a conexina 26 foi expressa em uma posição perinuclear, indicando uma possível localização no aparato de Golgi. Como E5 promove a alcalinização do aparato de Golgi (Schapiro *et al.,* 2000), alterando o tráfego intracellular, pode-se assumir que E5 poderia contribuir para a inibição do tráfego de Cx 26, causando seu acúmulo no citoplasma e aparato de Golgi.

O padrão de expressão da conexina 26 parece não ser dependente da presença de 1 ou 2 tipos de BPV na mesma lesão, e é possível que sua desregulação seja um efeito específico da atividade transformante do BPV, porém mais estudos são necessários para identificar a função da desregulação da conexina 26 em tumores induzidos por BPV.

11.3 BPV em sítios não epiteliais

Os papilomavírus, embora descritos como vírus epiteliotrópicos, têm sido desmonstrados em sangue e outro sítios não epiteliais (Freitas *et al.,* 2007; Lindsey *et al.,* 2009). Neste estudo, foi verificada a presença de BPV em espermatozóide e fluido seminal de doses de sêmen comercial de touros; sangue e sêmen de bovinos e equinos. Além disso, a atividade viral destes virus também foi verificada em células do sêmen e sangue de bovinos e equinos.

Embora nenhuma alteração na função espermática tenha sido verificada nas amostras de sêmen comercial contaminada por BPV, a alta frequência de BPV nestas doses pode representar um risco à transmissão do BPV, visto que, a transmissão vertical de BPV tem sido sugerida (Freitas *et al.,* 2007; Roperto *et al.,* 2012).

Atividade gênica do BPV no sêmen sugere que o espermatozóide seja um novo sítio para infecção por BPV. A presença de transcritos de HPV em espermatozóide foi previamente demonstrada na literatura (Lai *et al.,* 1996). Os resultados encontrados neste estudo sugerem que o BPV, assim como o HPV, pode infectar a células espermáticas e expressar alguns de seus genes.

Estudos prévios demonstraram a presença de BPV em sangue de bovinos (Stocco dos Santos *et al.,* 1998; Diniz *et al.,* 2009) e equinos (Brandt *et al.,* 2008), além de sua expressão em sangue de bovinos que apresentavam câncer de bexiga (Roperto *et al.,* 2011) e de equinos através de infecção experimental (Hartl *et al.,* 2011).

Células sanguíneas contendo o genoma de BPV ativo podem ser responsáveis por disseminar o BPV para numerosos órgãos (Freitas *et al.,* 2003; Freitas *et al.,* 2007; Roperto *et al.,* 2011). A detecção de BPV em diferentes tecidos e

células, tais como ovário, ovócitos, útero podem corroborar essa hipótese (Freitas *et al.,* 2003; Yaguiu *et al.,* 2006, Lindsey *et al.,* 2009).

A presença de BPV em sangue apoia a possibilidade de transmissão vertical desses vírus. Freitas *et al,* (2003) verificaram a presença de BPV1 em sangue de um bezerro recém-nascido. Yaguiu *et al.,* (2008) verificaram a presença de BPV1, 2 e 4 no sangue de vacas em uma propriedade e de sua prole recém-nascida. Stocco dos Santos *et al.,* (1998) inocularam sangue de vacas contaminadas por BPV em vacas saudáveis e verificaram a presença deste vírus após 18 meses no sangue das vacas receptoras e em sua prole.

O sangue pode ser também um veículo de transmissão horizontal de BPV entre bovinos e equinos. Acredita-se que moscas possam ter um papel na transferência de BPVs de gado para cavalo e entre cavalos (Nasir & Campo, 2008; Finlay *et al.,* 2009). Da mesma forma, em nossa opinião, o carrapato também poderia ser um agente disseminador do BPV através do sangue. William *et al.* (1992) observaram que todos os animais que apresentavam papilomatose também eram concomitantemente infestados por carrapato. Mbuthia *et al.* (2003) relataram que após a implantação de controle de carrapato alterou a incidência de várias doenças de pele, inclusive papilomatose. Até o momento, maiores informações sobre esse sistema vírus-vetor-hospedeiro são inexistentes, porém não devem ser excluídas, embora essa via de transmissão seja menos eficiente do que a via já bem estabelecida entre mucosas (Bravo *et al.,* 2010).

11.4 Comparação de dois sistemas para detecção de BPV

O conhecimento sobre a diversidade e epidemiologia dos BPV é muito importante para o desenvolvimento de estratégias de prevenção e o entendimento da evolução desses vírus. Assim, o emprego da melhor estratégia de PCR é necessária para se obter dados confiáveis e aumentar o conhecimento sobre a biologia dos BPV.

Os primers FAP, utilizados anteriormente para detecção de DNA de BPV (Antonsson & Hansson, 2002; Ogawa *et al.,* 2004; Claus *et al.,* 2007; Carvalho *et al.,* 2012) demonstraram menos sensibilidade do que os primers específicos, assim como observado por Qu *et al.,* (2000) em seu estudo com primers consenso e tipo específico para HPV.

Por outro lado, os *primers* consenso se mostraram mais adequados para estudos de detecção e caracterização de novos tipos de BPV. Neste estudo, nós verificamos a presença de um provável novo tipo de BPV, assim como estudos anteriores utilizando *primers* consenso também verificaram (Forslund *et al.,* 1999; Antonsson & Hansson, 2002; Ogawa *et al.,* 2004; Maeda *et al.,* 2007; Claus *et al.,* 2008; Carvalho *et al.,* 2012).

Dessa forma, a escolha dos mais adequado sistema de PCR para amplificação do DNA de BPV é muito importante em investigações epidemiológicas de BPV e, para um estudo mais completo, ambos os métodos são complementares.

12. Conclusões gerais

Este estudo descreve a diversidade de BPV nas lesões cutâneas de bovinos no Brasil, através da descrição da presença de diversos tipos de BPV e do FeSarPV infectando e co-infectando lesões cutâneas de bovinos; Verificamos alterações na expressão da proteína conexina 26 em lesões induzidas por BPV, revelando que este possa ser um dos prováveis mecanismos de atuação do BPV em tumores cutâneos bovinos; Além disso, este estudo constatou a alta prevalência de BPV2 em sêmen congelado comercial de touros, porém não foram observadas alterações nos parâmetros de fertilidade no sêmen desses animais.

Este trabalho contribui para fortalecer a hipótese de que os BPV são capazes de infectar tecidos não-epiteliais, através da verificação da presença e expressão de genes virais em células sanguíneas e do sêmen de bovinos e equinos.

Este estudo verificou que os *primers* tipo-específico são mais sensíveis para detecção de BPV do que os *primers* consenso, porém estes são mais adequados para se detectar novos tipos de BPV. Para um estudo mais completo, ambos os tipo de *primers* são importantes, pois eles são complementares. Desta forma, este estudo também contribui para o melhor conhecimento de ferramentas moleculares que podem ser empregadas nos estudos de presença e caracterização do BPV nos diversos tecidos bovinos.

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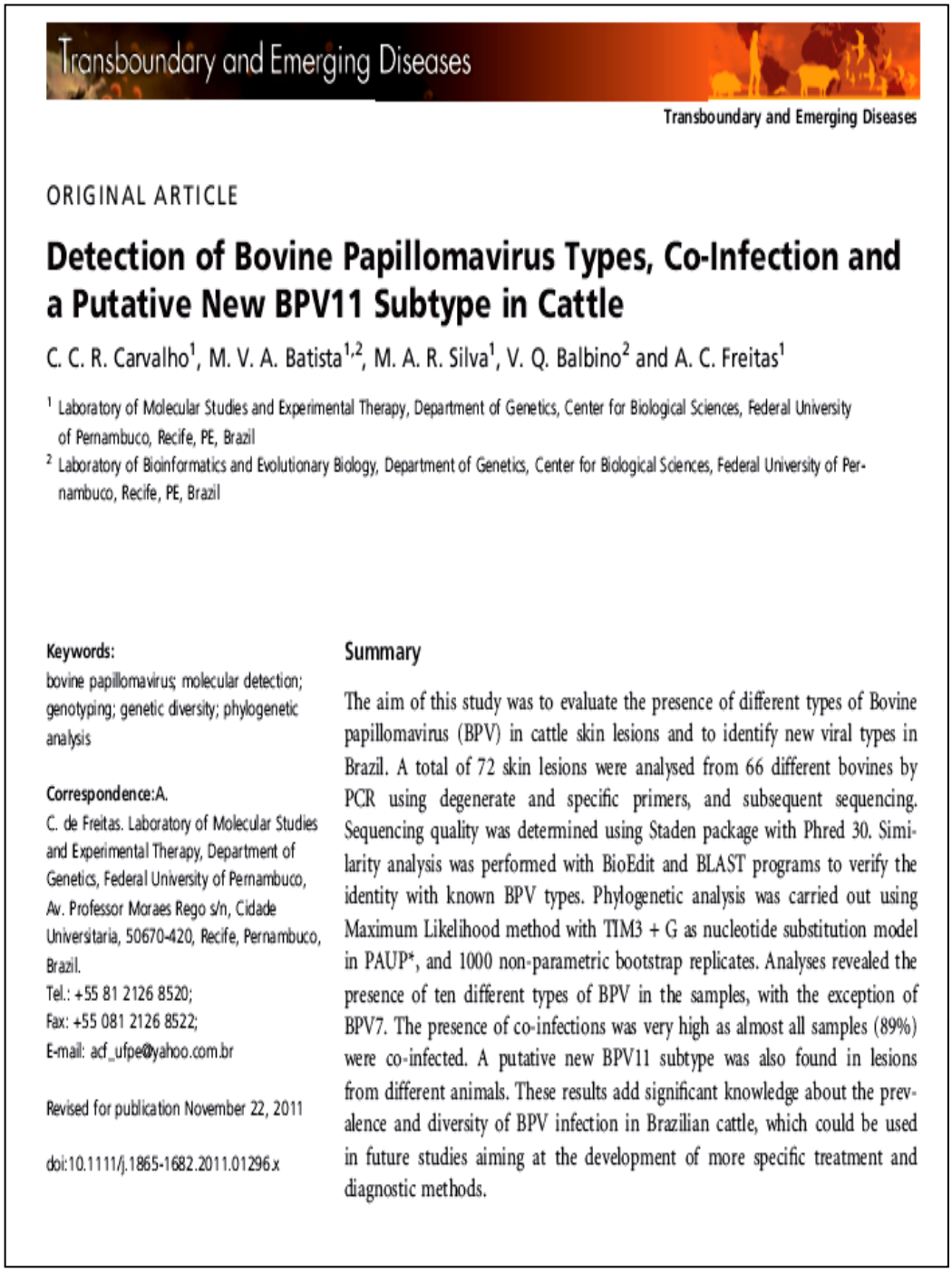
14. Anexos

Participação em artigos científicos durante o doutorado relacionados ao tema da tese:

14.1 Detection of Bovine papillomavirus types, Co-infection and a putative new BPV11 Subtype in cattle



Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Detection of Bovine Papillomavirus Types, Co-Infection and a Putative New BPV11 Subtype in Cattle

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Keywords:
bovine papillomavirus; molecular detection; genotyping; genetic diversity; phylogenetic analysis

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Summary

The aim of this study was to evaluate the presence of different types of Bovine papillomavirus (BPV) in cattle skin lesions and to identify new viral types in Brazil. A total of 72 skin lesions were analysed from 66 different bovines by PCR using degenerate and specific primers, and subsequent sequencing. Sequencing quality was determined using Staden package with Phred 30. Similarity analysis was performed with BioEdit and BLAST programs to verify the identity with known BPV types. Phylogenetic analysis was carried out using Maximum Likelihood method with TIM3 + G as nucleotide substitution model in PAUP*, and 1000 non-parametric bootstrap replicates. Analyses revealed the presence of ten different types of BPV in the samples, with the exception of BPV7. The presence of co-infections was very high as almost all samples (89%) were co-infected. A putative new BPV11 subtype was also found in lesions from different animals. These results add significant knowledge about the prevalence and diversity of BPV infection in Brazilian cattle, which could be used in future studies aiming at the development of more specific treatment and diagnostic methods.

14.2 Recent insights into bovine papillomavirus

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Review

Recent insights into Bovine Papillomavirus

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Bovine papillomaviruses (BPV) are double-stranded DNA viruses that infect the cutaneous and mucosal epithelia inducing hyperplasic lesions in cattle. BPV is the etiologic agent of the papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder. The benign and malignant tumors caused by BPV are emergent diseases important for beef and dairy cattle in the world. Although BPV associated tumors have veterinary and agricultural relevance, they have also been studied as a relevant model of human papillomavirus (HPV). Recent studies in BPV biology have shown a great diversity of BPV types and new putative BPV types infecting and co-infecting the herd in several parts of the world. This review will briefly summarize the genomes and structure of BPV and the bovine papillomatosis; will describe in greater detail the genotypic diversity, BPV cross-species infection, relevant aspects of BPV and co-infection and its possible routes of transmission. These new approaches about BPV may be very useful to understand the oncogenic potential of the virus, the relationship between virus and co-factors, and the development of anti-viral vaccines.

Key words: Bovine papillomavirus, co-infection, virus transmission, BPV diversity.

INTRODUCTION

Papillomaviruses (PVs) are a diverse group of small, nonenveloped, circular double-stranded DNA viruses that occur in a broad range of animal species belonging to the amniotes, including humans (Antonsson and Hansson, 2002). Bovine papillomavirus induces diseases of considerable veterinary importance in farm animals, but has also an enormous value as an in vivo model for HPV. They infect the epithelia of vertebrates, where they can cause neoplasias or persist asymptomatically. After being assorted in the old family Papovaviridae, PVs were re-designated as a distinct family, Papillomaviridae (van Regenmortel et al., 2000). BPVs are a heterogeneous group of epitheliotropic viruses that recognize bovines as its classical host. Twelve BPV types have been characterized and classified into three genera: *Deltapapillomavirus* (BPV-1 and -2), *Epsilonpapillomavirus* (BPV-5 and -8) and *Xipapillomavirus* (BPV-3, -4, -6, -9, -10, -11 and -12), and an as yet unassigned PV genus (BPV-7) (Bernard et al., 2010; Hatama et al., 2011; Zhu et al., 2011). The bovine papillomatosis is an important disease leading to economic depreciation of animals, deterioration of the appearance and of the animal leather. The lesions may progress to cancer due to the synergistic action of genetic or environmental co-factors (Borzacchiello and Roperto, 2008; Leal et al., 2003). Recent insights into BPV biology open new fields of discussion about co-infection, cross-species infection, and transmission of these viruses.

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GENOMES STRUCTURE OF BOVINE PAPILLOMAVIRUS

BPV genomes comprise nearly 8 Kb, which includes a long control region (LCR), early (E) and Late (L) genes (Figure 1). The LCR (about 500-1000 nucleotides) contains transcriptional regulatory sequences and the replication origin (Munger and Howley, 2002).

There are six early genes, all of them expressed

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2008 Doutorado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
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2006 - 2008 Mestrado em Genética.
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Universidade Federal da Paraíba, UFPB, Joao Pessoa, Brasil
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Artigos completos publicados em periódicos

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11. LIRA, R. C., SILVA, Maria Angélica Ramos da, CHAGAS, B. S., PONTES, N. E., STOCCO DOS

SANTOS, R. C.., BECAK, W., SANTOS, J. F., FREITAS, A. C.
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12. CARVALHO, C. C. R, SILVA, Maria Angélica Ramos da, Monteiro, V. L. C;, COELHO, M. C. O. C., BECAK, W., STOCCO DOS SANTOS, R. C.., FREITAS, A. C.
Avaliação da presença do papilomavírus bovino tipo 2 afetados por papilomatose cutânea mas sem hematúria enzoótica In: Congresso Brasileiro de Genética
CBG. , 2007.

Trabalhos publicados em anais de eventos (resumo expandido)

1. SILVA, Maria Angélica Ramos da, CARVALHO, C. C. R, JESUS, A. L. S., RIEGER, T. T., SANTOS, J. F., BECAK, W., STOCCO DOS SANTOS, R. C.., FREITAS, A. C.
Bovine Papillomavirus identification: new primers for improving PCR procedures In: Congresso Brasileiro de Microbiologia, 2007, Brasília.
Anais do Congresso Brasileiro de Microbiologia. , 2007.

Apresentação de trabalho e palestra

1. Silva, M. A. R.
Presença e expressão de Papilomavírus bovino em sêmen e sangue de bovinos saudáveis e afetados por papilomatose, 2011. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais : Brasil/Português; Cidade: Recife; Evento: I Jornada de Pós-Graduação em Genética; Inst.promotora/financiadora: UFPE

2. CARVALHO, C. C. R, da Silva, M. A. R., BATISTA, M. V. A., BALBINO, V. Q., de Freitas, A. C.
Evaluation of the presence of low frequent Bovine Papillomavirus types (8,9 and 10) in Pernambuco state by PCR and sequencing assays, 2009. (Congresso,Apresentação de Trabalho)
Referências adicionais : Brasil/Português; Evento: XX Encontro Nacional de Virologia

3. Silva, M. A. R., JESUS, A. L. S., Carvalho, C. C. R., RIEGER, T. T., SANTOS, F. L., SANTOS, J. F., Freitas, A.C.
Padronização da detecção e tipificação de Papilomavírus bovino através de PCR, 2007. (Congresso,Apresentação de Trabalho)
Referências adicionais : Brasil/Português; Evento: Congresso Brasileiro de Microbiologia

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Artigos aceitos para publicação..... 2
Livros publicados..... 1
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Apresentações de trabalhos (Conferência ou palestra)..... 1
Apresentações de trabalhos (Congresso)..... 5
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Participações em eventos (encontro)..... 8