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Marcus Vinicius de Aragão Batista

Uso de novas ferramentas computacionais no estudo da diversidade genética de papilomavírus bovino associado à epidemiologia molecular da papilomatose bovina cutânea

Recife
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Orientador: Prof. Dr. Valdir de Queiroz Balbino
Coorientador: Prof. Dr. Antonio Carlos de Freitas

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Banca Examinadora:

Dr. Valdir de Queiroz Balbino
Universidade Federal de Pernambuco

Dr. Antonio Carlos de Freitas
Universidade Federal de Pernambuco

Dr. Marcos Antônio de Morais Júnior
Universidade Federal de Pernambuco

Dr. Edílson Divino de Araújo
Universidade Federal de Sergipe

Dr. Tiago Alessandro Espinola Ferreira
Universidade Federal Rural de Pernambuco

Recife

2013

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“A ciência avança através de respostas provisórias, conjecturais, em direção a uma série cada vez mais sutil de perguntas que penetram cada vez mais fundo na essência dos fenômenos naturais”.

Louis Pasteur

Resumo

Os papilomavírus (PV) formam um grupo altamente diverso que infectam mamíferos, aves e répteis. Entre esses vírus, o papilomavírus bovino (BPV) tem grande importância veterinária, causando lesões cutâneas e mucosas em gado e outros animais, que podem progredir para câncer. Portanto, estudar a diversidade de BPV que infectam os rebanhos brasileiros se torna relevante. Deste modo, esse estudo objetivou desenvolver uma nova abordagem computacional baseada em entropia para selecionar regiões genômicas filogeneticamente informativas dos PV, assim como avaliar e discutir a diversidade de tipos de BPV que infectam rebanhos da região Nordeste do Brasil. Para isto, a complexidade informacional de cada região genômica foi calculada e aquelas com baixa entropia foram selecionadas para inferência filogenética. Foram coletadas amostras de lesões cutâneas de bovinos do Nordeste do Brasil. O DNA foi extraído, amplificado e os produtos foram sequenciados. Foi possível identificar regiões claramente homólogas que são conservadas entre os diferentes PV. As análises também revelaram a presença de 11 diferentes tipos de BPV nas amostras, assim como prováveis novos tipos e subtipos de BPV. Estes resultados indicam que a entropia pode, com sucesso, selecionar bons marcadores genômicos para inferência filogenética. Além disso, eles adicionam um conhecimento significativo sobre a incidência e diversidade dos BPV que infectam o gado brasileiro. Em conjunto, estes conhecimentos podem ser utilizados para o desenvolvimento de novos sistemas de diagnóstico, mais eficazes no controle da papilomatose bovina.

Palavras-chave: Papilomavírus; Análise filogenética; Entropia; Epidemiologia molecular; Diversidade genética.

Abstract

Papillomaviruses (PV) are a highly diverse group that infects mammals, birds and reptiles. Among these viruses, bovine papillomavirus (BPV) has great veterinary importance, causing skin and mucous lesions in cattle and other animals, which can progress to cancer. Therefore, it is relevant to study the diversity of BPV infecting the Brazilian cattle. In this way, this study aimed to develop a new computational approach based on entropy to select phylogenetically informative genomic regions of PV, as well as evaluate and discuss the diversity of BPV types infecting cattle herds in northeastern Brazil. For this purpose, the informational complexity of each genomic region was calculated and those with low entropy were selected for phylogenetic inference. Samples were collected from skin lesions of cattle from northeastern Brazil. DNA was extracted, amplified and the products were sequenced. It was possible to identify regions that are conserved among different PV, which are probably shared with a common ancestor. The analysis also revealed the presence of 11 different types of BPV in the samples, as well as putative new types and subtypes of BPV. These results indicate that entropy can successfully select good genomic markers for phylogenetic inference. Moreover, they add significant knowledge about the incidence and diversity of BPV infecting Brazilian cattle. Together, this knowledge can be used for the development of new diagnostic systems, more effective in the control of bovine papillomatosis.

Key words: Papillomavirus; Phylogenetic analysis; Entropy; Molecular epidemiology; Genetic diversity.

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Lista de Abreviaturas, Siglas e Símbolos

ATP	Adenosina Trifosfato
BLAST	Ferramenta Básica de Busca por Alinhamento Local
BPV	Papilomavírus Bovino
Da	Daltons
DNA	Ácido Desoxirribonucléico
HPV	Papilomavírus Humano
ICTV	Comitê Internacional de Taxonomia de Vírus
kb	Kilobase
LCR	Região Longa de Controle
MCMC	Monte Carlo via Cadeias de Markov
MDBK	Rim Bovino Madin-Darby
NCBI	Centro Nacional para Informação Biotecnológica
nm	Nanômetro
NNI	Mudança de Vizinho mais Próximo
ORF	Matriz Aberta de Leitura
PCR	Reação em Cadeia da Polimerase
PV	Papilomavírus
RNA	Ácido Ribonucléico
RNAm	Ácido Ribonucléico Mensageiro
SPR	Corte e Enxerto de Subárvore
TAE	Tris Acetato EDTA
VLP	Partícula Semelhante à Vírus
WHO	Organização Mundial de Saúde

Sumário

1. Introdução

Papilomavírus (PV) é um grupo altamente diverso de vírus que é conhecido por infectar vários hospedeiros diferentes no grupo dos amniotas. Eles podem infectar o epitélio escamoso da pele e mucosas, podendo causar várias lesões benignas ou malignas. Existe uma associação causal de tipos de PV com câncer, fazendo com que esses vírus atraiam grande interesse por parte dos pesquisadores. Entretanto, existem PV que estão associados com lesões cutâneas benignas. Além disso, vários tipos de PV já foram isolados de animais aparentemente saudáveis, indicando que eles coexistem com seus hospedeiros por longos períodos de tempo de forma latente.

Nos seres humanos, os PV estão associados ao desenvolvimento de câncer, principalmente o de colo do útero, que é o segundo em número de casos entre as mulheres no mundo. Em animais de interesse econômico, como os bovinos, os PV estão associados ao câncer do trato digestório e bexiga urinária, sendo motivo de grandes perdas econômicas para o produtor. Além da indubitável importância econômica, o papilomavírus bovino (BPV) tem um valor inestimável quando utilizado como modelo *in vivo* para papilomavírus humano (HPV) na investigação da biologia viral, na associação direta entre infecção viral e neoplasia, na relação entre vírus-hospedeiro-ambiente, na resposta imune do hospedeiro ao vírus e no desenvolvimento de vacina anti-papilomavírus.

O conhecimento da biologia dos BPV se torna ainda mais relevante uma vez que o Brasil é o segundo maior produtor mundial de carne bovina, e o terceiro em exportações, sendo extremamente importante que esse produto possua

qualidade satisfatória, aumentando as possibilidades de expansão de mercado interno e externo, além da melhoria da qualidade de vida dos consumidores.

Nem todos os tipos de BPV estão relacionados com o desenvolvimento de câncer. No Brasil, poucos estudos descrevem a detecção molecular de BPV em lesões cutâneas de bovinos. Assim, conhecer a diversidade dos tipos de BPV que infectam bovinos neste país se torna bastante relevante. Além disso, já foi relatado que em HPV, variantes moleculares de um mesmo tipo viral podem determinar um aumento no risco de infecção persistente e/ou de desenvolvimento de câncer cervical. Embora ainda não existam informações sobre a ocorrência deste fato em BPV, é bastante coerente inferir que variantes moleculares destes vírus também possam estar associados com diferentes níveis de patogenicidade ou até de potencial oncogênico.

Do mesmo modo, o entendimento da diversidade genética e evolução dos PV podem ajudar a relacioná-los com propriedades biológicas e patológicas específicas. A análise comparativa das sequências genéticas destes vírus pode tornar possível o desenvolvimento de novos métodos de detecção e genotipagem mais eficazes, desenho de drogas específicas, além do desenvolvimento de formulações vacinais mais eficientes e de amplo espectro.

Apesar da necessidade de se estudar a diversidade genômica dos BPV, ainda existe um custo elevado para se sequenciar o genoma completo de vários isolados em um estudo populacional, fazendo-se necessária a aplicação de métodos e abordagens de Bioinformática que auxiliem estes estudos, visando à diminuição dos custos e esforços. Associado a isso, o conhecimento acerca dos mecanismos evolutivos que conduzem a diversidade genética dos PV ainda é limitado, e entender estes processos é muito importante para o desenvolvimento

futuro de novos métodos de diagnóstico e também de tratamentos mais eficazes. Assim, um método computacional baseado em entropia foi aplicado para a resolução de problemas filogenéticos visando o melhor entendimento das relações evolutivas dos PV.

2. Revisão da Literatura

Os papilomavirus (PV) são um grande grupo de vírus que infecta uma ampla variedade de espécies de vertebrados. Estes vírus estão envolvidos com lesões epiteliais e mucosas de mamíferos, aves e répteis. A importância clínica dos PV tem sido amplamente reconhecida devido ao potencial de induzir neoplasia em seus hospedeiros, embora eles também possam ser detectados em tecidos epiteliais saudáveis (Campo, 2002; Robles-Sikisaka *et al*., 2012).

2.1 Estrutura e genoma dos Papilomavírus

PV apresentam uma estrutura não envelopada, possuindo uma molécula de DNA dupla fita circular que mede aproximadamente oito kilobases (kb). Seu capsídeo é composto por 72 subunidades em forma de pentâmeros (capsômeros) e possui peso molecular de aproximadamente 5,2 x 10^5 daltons (Da). O capsídeo viral exibe uma simetria icosaédrica com diâmetro aproximado de 50-55 nm (Fig. 1), conferindo ao vírus morfologia esférica quando observado por meio de microscopia eletrônica (Shah e Howley, 1996; Garcea e Chen, 2007).

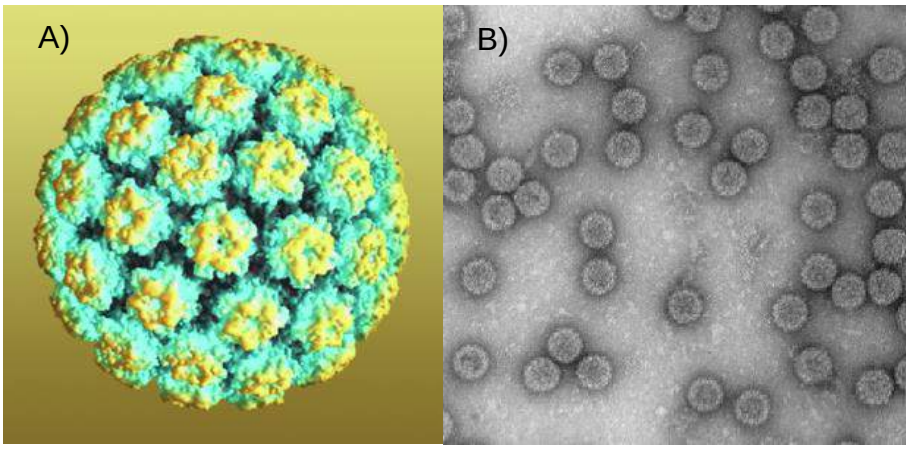

Figura 1: Estrutura dos papilomavírus. A) Representação esquemática da estrutura do capsídeo dos papilomavírus (Fonte: Modis *et al*., 2002). B) Eletromicrografia de papilomavírus bovino (Fonte: Garcea e Chen, 2007).

O genoma dos PV normalmente consiste de oito matrizes abertas de leitura (ORF – *Open Reading Frames*), das quais seis são classificadas como genes de expressão precoce (E – *Early*) e duas como genes de expressão tardia (L – *Late*). Uma região não-codificante conhecida como região longa de controle (LCR – *Long Control Region*) também está presente no genoma dos PV, localizada entre as regiões E e L (Fig. 2). Os genes dos PV estão dispostos em uma única fita do DNA, ocorrendo algumas sobreposições entre vários destes genes. Por exemplo, há sobreposição entre os sítios de início e término de vários genes adjacentes, e a ORF *E4* está quase que completamente contida na ORF *E2* (Hughes e Hughes, 2005).

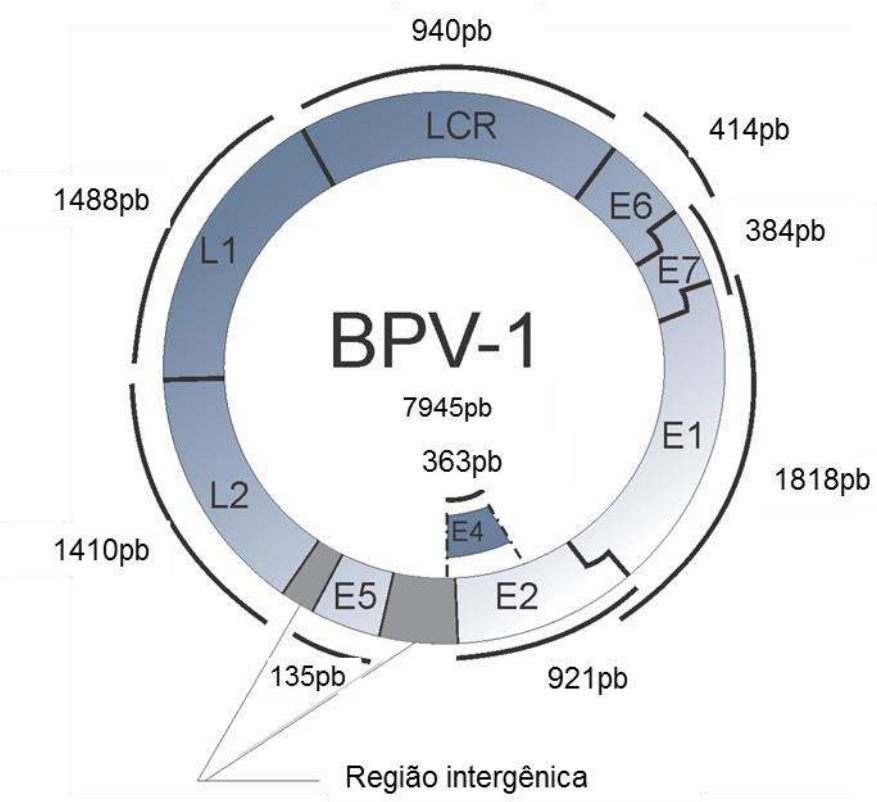

Figura 2: Organização genômica de BPV-1, evidenciando a posição dos genes E e L, assim como a região controladora LCR (Fonte: Freitas *et al*., 2011).

A região E do genoma dos PV codifica proteínas regulatórias (E1, E2, E4, E5, E6 e E7), incluindo aquelas responsáveis pela replicação do DNA viral, e representa aproximadamente 45% de todo o genoma. Enquanto a região L codifica proteínas do capsídeo viral (L1 e L2), compreendendo cerca de 40% do DNA do vírus. A LCR, que apresenta tamanho entre 400 e 1000 pb, contém elementos promotores e reguladores da replicação viral (Fig. 2). Esta região é a mais variável e representa 15% do genoma do vírus, sem capacidade de codificar proteínas, contendo apenas alguns elementos de regulação como os fatores de transcrição celular e origem de replicação (Chang, 1990; Zheng e Baker, 2006).

O segmento LCR contém o promotor p97 junto com sequências potencializadoras e silenciadoras que regulam a replicação do DNA controlando a transcrição das ORFs (Burd, 2003; Zheng e Baker, 2006). Normalmente está dividido em três regiões: 5' terminal, central e 3' terminal. A região central é onde a maior parte dos fatores de transcrição se liga. Por causa disso, é conhecida como região intensificadora da LCR, sendo ela que regula a transcrição dos oncogenes *E6* e *E7* (zur Hausen, 1996). A região 5' terminal da LCR está delimitada pelo códon de terminação do gene *L1* e por um sítio de ligação de *E2*. Furth e Baker (1991) relataram que essa região possui um sítio para o término da transcrição e outro para a poliadenilação do transcrito policistrônico. Enquanto a região 3' terminal compreende um sítio de ligação de *E2* e o códon de iniciação do gene *E6*. Muitas vezes, a transcrição é iniciada a partir do promotor p97, determinando um único transcrito policistrônico iniciado em *E6* (Howley e Lowy, 2001).

Os genes dos PV são normalmente identificados por similaridade com genes já conhecidos. Assim, alguns pseudogenes foram identificados em PV,

confundidos como genes verdadeiros devido à sua similaridade relativamente alta e posição no genoma. Por exemplo, a ORF *E3* foi relatada em alguns poucos PV, mas nunca foi provado que ela codifique alguma proteína. Até pouco tempo acreditava-se que a ORF *E8* fosse outro exemplo deste processo. Entretanto, em BPV-1 e HPV-31, foi mostrado que a ORF *E8* codifica a proteína E8^E2C, fusionada por *splicing*, que funciona como regulador negativo da transcrição e replicação viral (Zheng e Baker, 2006). Além disso, em PV que infectam coelhos, *E8* foi caracterizado como oncogene, com características similares à E5 de BPV-1 e vários HPV (Harry e Wettstein, 1996; Han *et al*., 1998; Zheng e Baker, 2006).

2.2 Proteínas dos Papilomavírus

Dentre todas as proteínas, E1 e E2 são as de maior importância na replicação e transcrição do DNA (Tyring, 2000). O gene *E1* é reconhecido como o maior e mais conservado da região precoce do genoma de diferentes PV. A proteína E1 é uma fosfoproteína de 70 a 80 kDa, com atividade de helicase associada a adenosina-trifosfato (ATP), que atua em conjunto com E2 na origem da replicação viral (Masterson *et al.*, 1998). A proteína E1 se liga em sequências ricas em AT na origem, facilitada pela proteína E2. Após a ligação, um hexâmero é formado, que atua desespiralizando a molécula de DNA (Longworth e Laiminis, 2004). A proteína E2 é uma fosfoproteína de aproximadamente 50 kDa com três domínios funcionais que, além de atuar na replicação, também regula a transcrição das ORFs dos PV. Ela forma dímeros que se ligam em sítios específicos na LCR, podendo ativar ou reprimir a expressão de genes precoces, dependendo de sua concentração (Longworth e Laiminis, 2004).

Segundo Bryan e Brown (2001), a proteína E4 possui uma aparente relação com a liberação do vírus de células infectadas. Entretanto, sua função ainda não está bem estabelecida. Ela é bastante expressa e abundante nas camadas celulares superiores dos tecidos epiteliais. Sua síntese ocorre a partir do *splicing* de um RNAm policistrônico, estando fusionado a cinco aminoácidos da proteína E1. Sugere-se que esta fusão indica a maturação viral, uma vez que essa proteína interage e perturba a rede de citoqueratina, aumentando a fragilidade celular, o que contribui para a liberação dos vírus maduros da célula (Doorbar *et al.*, 1990; Raj *et al.*, 2004).

Os PV codificam três oncoproteínas, conhecidas como E5, E6 e E7. A proteína E5 é pequena e altamente hidrofóbica, possuindo várias funções nas células hospedeiras, através da interação com proteínas citoplasmáticas e do retículo endoplasmático (Longworth e Laiminis, 2004; Krawczyk *et al*., 2011). A depender do tipo de PV, essa proteína pode apresentar maior ou menor atividade oncogênica (McMurray *et al.*, 2001). Por exemplo, nos HPV, ela é pouco oncogênica, mas tem uma função importante aumentando bastante o potencial transformante da proteína E7. Contrariamente, nos BPV, a proteína E5 é a principal responsável pela transformação celular, aparentemente interagindo com receptores de fatores de crescimento. Essa interação modifica a resposta celular que resulta na apoptose, aumentando assim a proliferação das células infectadas. Existe pouca similaridade entre a proteína E5 de HPV e a de BPV, sugerindo que elas agem através de diferentes alvos celulares (Bravo e Alonso, 2004; Longworth e Laiminis, 2004).

Existe um grande interesse em se estudar as oncoproteínas E6 e E7 de HPV, devido ao fato delas interagirem com proteínas que atuam no controle do

ciclo celular, podendo levar à imortalização das células e, consequentemente, ao desenvolvimento de tumores (Finzer *et al.*, 2002). A oncoproteína E6 possui um tamanho de aproximadamente 150 aminoácidos e contém um domínio de ligação de zinco com dois motivos C-x-x-C. Em humanos, ela sozinha pode levar à imortalização de células epiteliais mamárias. Entretanto, uma eficiente imortalização dos queratinócitos humanos requer a expressão tanto de E6 quanto de E7 (Hawley-Nelson *et al.*, 1989; Longworth e Laiminis, 2004).

As primeiras ideias sobre as funções da proteína E6 vêm dos estudos das interações com a proteína celular p53, que é uma supressora de tumor bem caracterizada. Na presença de dano no DNA, a p53 interrompe o ciclo e pode levar a apoptose. Esse mecanismo é utilizado também para evitar que vírus infectando essas células se espalhem (Longworth e Laiminis, 2004). Entretanto, os PV desenvolveram mecanismos que bloqueiam este processo, o que pode levar ao desenvolvimento de neoplasias. Para isso, a oncoproteína E6 se liga à p53 em um complexo com a ubiquitina-ligase chamado E6AP, levando à consequente degradação da p53 (Fig. 3) (Longworth e Laiminis, 2004). Para facilitar o ciclo de infecção do vírus, além da degradação da p53, a proteína E6 também pode interagir com a p300, assim como aumentar a atividade da telomerase (McMurray *et al.*, 2001).

Assim como E6, a oncoproteína E7 também tem participação importante para a patogênese dos PV. Essa proteína é pequena, composta por aproximadamente 100 aminoácidos. Apresenta um motivo conservado (L-x-C-x-E) necessário para a associação da E7 com a proteína supressora de tumor do retinoblastoma (pRB), além de um domínio de ligação de zinco composto por dois

motivos C-x-x-C que funciona como domínio de dimerização (Liu *et al.*, 2006; McLaughlin-Drubin e Münger, 2009).

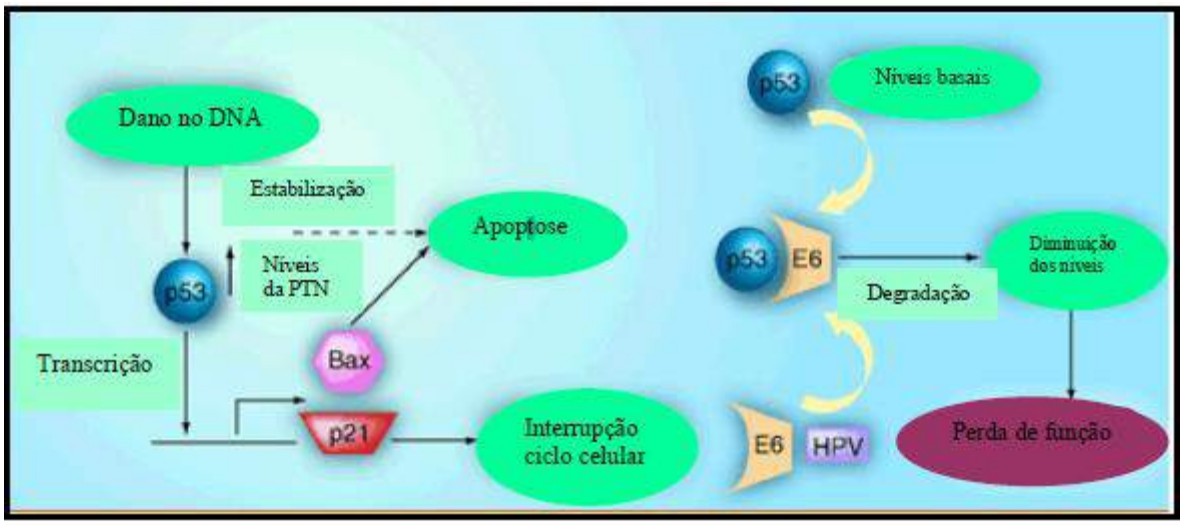

Figura 3: Função da proteína p53 e os efeitos da sua interação com a oncoproteína E6 (modificado de *www.medscape.com*).

Muitas das atividades biológicas da oncoproteína E7, que provavelmente são importantes para o ciclo de infecção viral, ainda são desconhecidas. Entretanto, a habilidade de se ligar e degradar a pRB, que está relacionada com a transformação celular, aparenta ser a sua principal função (Collins *et al.*, 2005). As proteínas da família RB formam complexos com fatores de transcrição como E2F, impedindo a progressão do ciclo celular entre as fases G1 e S. Uma vez associada com a oncoproteína E7, a pRB é sequestrada e degradada através da via de ubiquitinação, resultando na ativação de genes responsáveis pela progressão do ciclo celular, conforme pode ser visto na Figura 4 (Longworth e Laiminis, 2004). Também foi postulado que a proteína E7 pode se associar com inibidores de quinase dependente de ciclina p21 e p27, sendo esta interação, aparentemente, o principal fator de estímulo ao crescimento celular em infecções por HPV (zur Hausen, 2000).

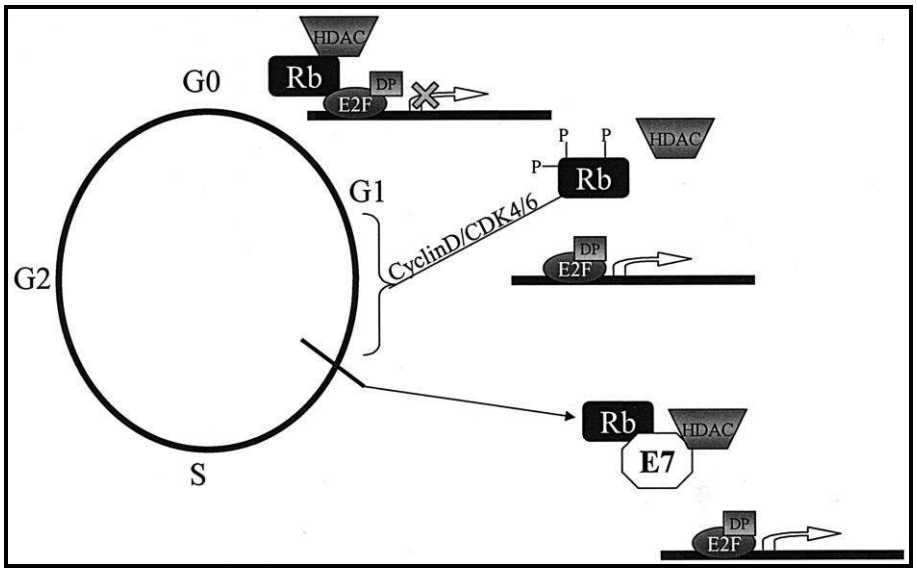

Figura 4: Ilustração esquemática mostrando as atividades de regulação do ciclo celular e os efeitos da interação da proteína E7 com pRB (Fonte: Longworth e Laiminis, 2004).

O capsídeo dos PV é formado por duas proteínas que são codificadas pelo vírus, as proteínas L1 e L2 (Howley e Lowy, 2001). A principal proteína deste capsídeo é a L1, com peso molecular de aproximadamente 55 kDa, cujos pentâmeros têm a propriedade intrínseca de automontagem, formando capsídeos vazios conhecidos como partículas semelhantes a vírus (*virus like particles* ou VLPs) (Bishop *et al.*, 2007). Esta é a proteína mais conservada entre os PV, e ela corresponde a cerca de 80 a 90% do capsídeo viral no vírion (Joyce *et al.*, 1999).

A proteína secundária do capsídeo dos PV é a L2, que participa da montagem e empacotamento do genoma viral, uma vez que ela se liga ao DNA do vírus no momento da maturação. Ela interage com a proteína L1 através de domínios específicos, sendo muito importante para a determinação da morfologia dos PV (Okun *et al.*, 2001; Florin *et al.*, 2002). Estas proteínas estruturais também foram relacionadas com a ligação entre os vírus e os receptores de superfície

celular, importante no desencadeamento da infecção (Day *et al.*, 2008; Sapp e Day, 2009).

2.3 Ciclo de infecção dos Papilomavírus

Os PV replicam seus genomas dentro do núcleo das células infectadas dos hospedeiros e apresentam tropismo específico por queratinócitos. Possivelmente, a infecção por PV ocorre através de microlesões do epitélio que expõem as células das camadas basais (mitoticamente ativas) para a entrada dos vírus (Longworth e Laiminis, 2004). Alguns receptores celulares que se ligam às proteínas L1 e L2 do capsídeo viral são conhecidos, e essa interação faz com que ocorra uma mudança na conformação das proteínas estruturais que resulta na exposição da região amino terminal da proteína L2. Uma série de eventos, ainda não conhecidos completamente, ocorre induzindo a internalização do vírus (Sapp e Day, 2009). Após a entrada do vírus na célula, três formas de infecção podem ocorrer: a infecção latente, a infecção produtiva e a transformação maligna.

Muitos PV parecem utilizar preferencialmente um ciclo de infecção latente, uma vez que vários tipos puderam ser detectados em sítios aleatórios da pele saudável de humanos e outros animais (Antonsson e Hansson, 2002; Antonsson *et al.*, 2003). Na infecção latente, o material genético do vírus permanece dentro do núcleo de forma episssomal, replicando-se juntamente com o DNA da célula hospedeira. Condições de supressão imune em humanos podem levar à ativação de infecções latentes, resultando nas lesões (Fig. 5) (Jablonska e Majewski, 1994; de Villiers, 1998; Forslund *et al.*, 2003).

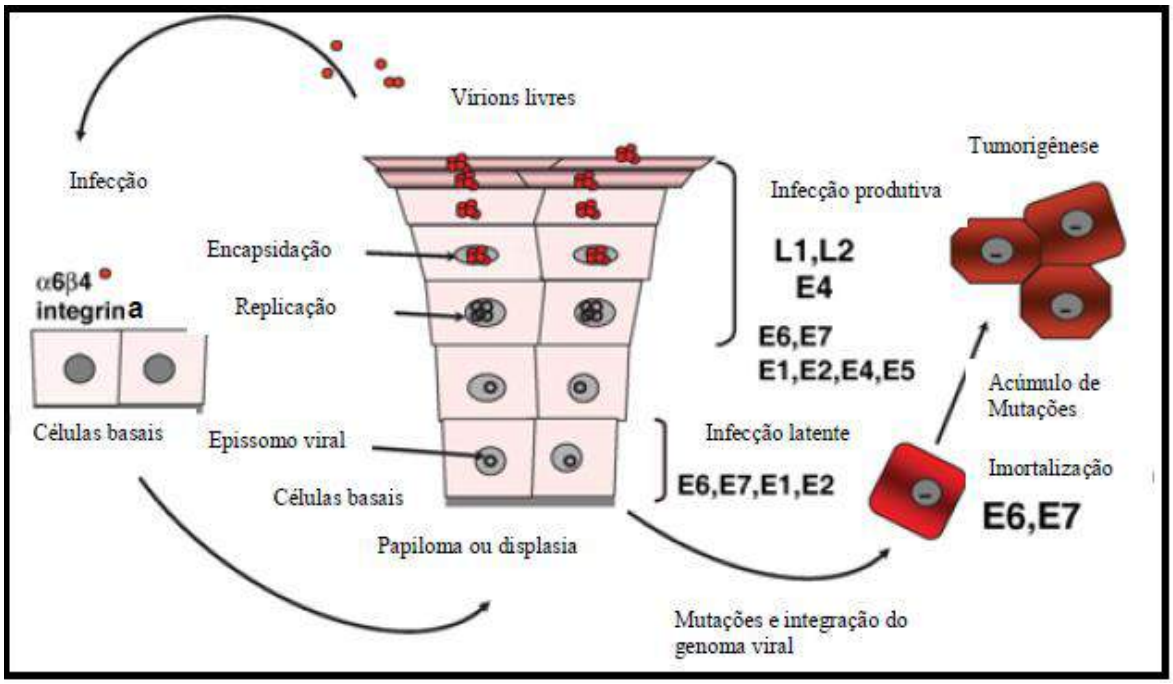

Figura 5: Representação esquemática do ciclo de infecção dos PV (modificado de Narisawa-Saito e Kiyono, 2007).

Na infecção produtiva, o número de cópias do genoma do vírus aumenta de acordo com a diferenciação celular, ou seja, o DNA viral é replicado independentemente do DNA do hospedeiro. As células filhas infectadas migram para as camadas superiores do epitélio, onde se inicia a expressão dos genes tardios, formando partículas virais maduras. Essas partículas são liberadas, infectando novas células hospedeiras (Narisawa-Saito e Kiyono, 2007). Modificações morfológicas no epitélio podem ser observadas durante as infecções produtivas, resultando no desenvolvimento de papilomas e lesões intraepiteliais de baixo grau (Fig. 5) (Longworth e Laiminis, 2004).

Nas células basais de humanos, onde o DNA se encontra na forma episssomal, os níveis de expressão das proteínas E6 e E7 são considerados baixos. Entretanto, se essas infecções continuarem por anos, ou até mesmo por décadas, as células passam a apresentar altos níveis de expressão dessas oncoproteínas devido a mutações ou integração do genoma viral. Esse aumento

nos níveis de expressão dos oncogenes E6 e E7 parece ajudar na imortalização dessas células, tornando-as tumorigênicas (Fig. 5). Este processo é conhecido como transformação maligna (Narisawa-Saito e Kiyono, 2007).

2.4 Classificação dos Papilomavírus

Após décadas de pesquisas e milhares de sequências de isolados de PV terem sido produzidas, foi possível estabelecer um banco de dados que permitiu a proposição de um sistema de classificação que pode ser estável, à medida que novos PV forem encontrados (de Villiers *et al*., 2004).

Apesar dos primeiros tipos de PV terem sido descobertos há mais de 30 anos (Orth *et al*., 1977), ainda existe alguma dificuldade em se encontrar sistemas de cultivo celular apropriados para a propagação desses vírus, o que limita o estabelecimento de uma taxonomia baseada em propriedades biológicas (de Villiers *et al*., 2004). Com o isolamento dos primeiros genomas virais, surgiram algumas classificações baseadas em painéis de restrição, hibridizações por *Southern blot* sob condições não estringentes e hibridizações líquidas (de Villiers *et al*., 2004).

Na década de 1980, os primeiros genomas completos de PV foram disponibilizados (Chen *et al*., 1982; Danos *et al.*, 1982; Schwarz *et al.*, 1983; Seedorf *et al.*, 1985). Atualmente, muitas sequências de genomas completos de PV têm sido depositadas em bancos de dados públicos, estando disponíveis com anotações funcionais, dando respaldo para uma nova classificação baseada em sequências de nucleotídeos.

Originalmente, os PV faziam parte da família *Papovaviridae*, junto com os poliomavírus. Essa classificação era baseada na similaridade dos capsídeos não

envelopados e do DNA dupla fita circular comum entre eles. Entretanto, eles são agora oficialmente reconhecidos pelo Comitê Internacional de Taxonomia de Vírus (ICTV) como duas famílias distintas, *Papillomaviridae* e *Polyomaviridae*. A separação se deu pela observação de características tais como: 1) diferenças no tamanho do genoma; 2) organizações genômicas notadamente diferentes; 3) baixos níveis de similaridade em sequências de nucleotídeos e aminoácidos e 4) estratégias transcricionais diferentes (de Villiers *et al*., 2004; Bernard, 2006; Wang, 2007).

A ORF *L1*, por ser a mais conservada no genoma dos PV, tem sido utilizada para a identificação de novos tipos virais. Um novo isolado de PV é reconhecido como um novo tipo quando o genoma completo é clonado e o sequenciamento da ORF *L1* demonstrar diferença de identidade de nucleotídeos superior a 10% do tipo conhecido mais próximo. Diferenças de identidade entre 2 e 10% definem um subtipo, e diferenças inferiores a 2% caracterizam uma variante viral (de Villiers *et al.*, 2004; Bernard, 2005).

Os PV são classificados em 29 gêneros, como representado na Figura 6. Diferentes gêneros compartilham menos que 60% de identidade na sequência de nucleotídeos na ORF *L1*. Os gêneros unem tipos filogeneticamente relacionados, mas que são biologicamente diversos (de Villiers *et al.*, 2004; Bernard *et al.*, 2010).

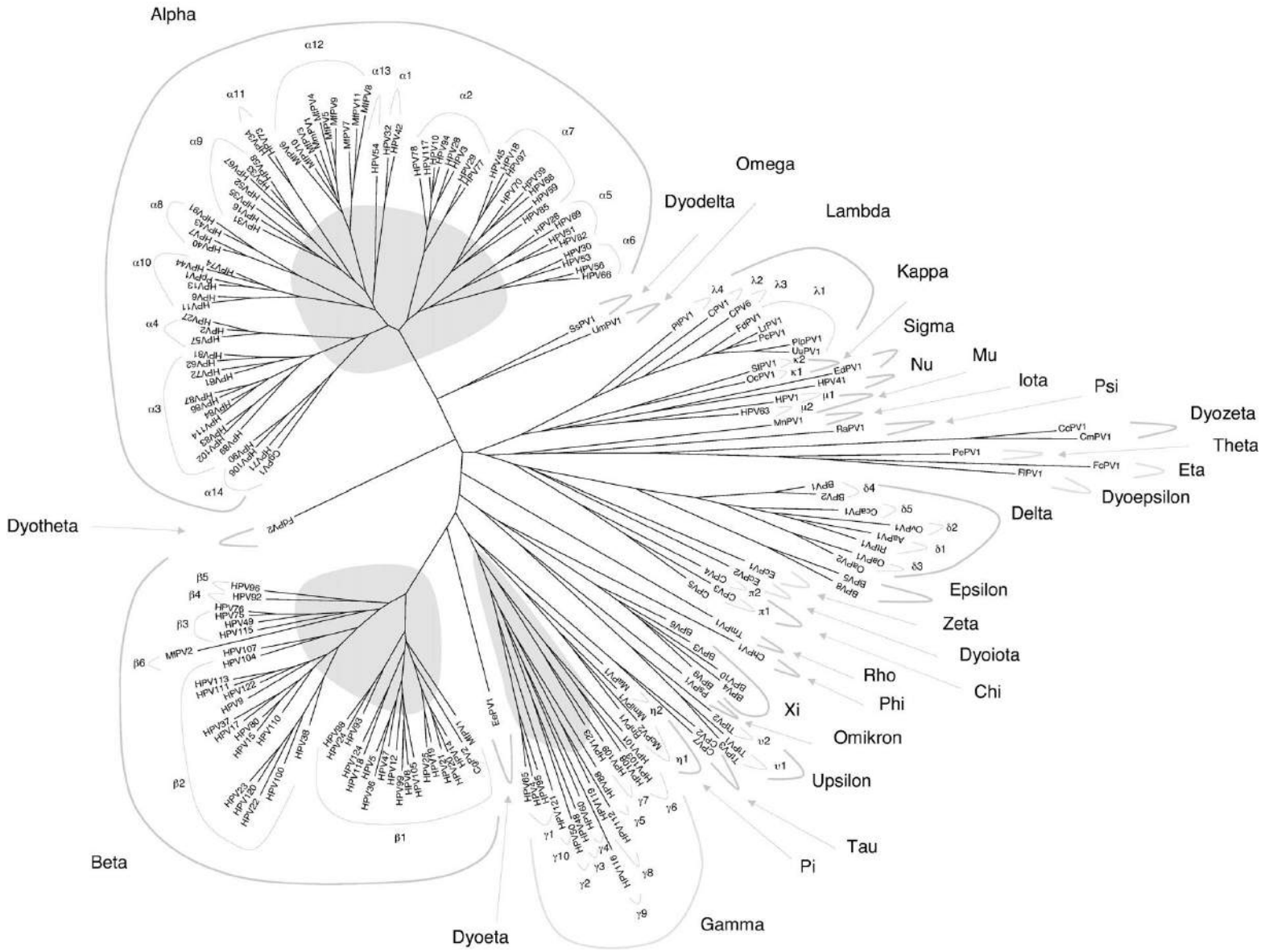

Figura 6: Árvore filogenética inferida a partir da ORF L1 de 189 PV, com ênfase dada aos gêneros (Fonte: Bernard *et al.*, 2010).

2.5 Diversidade e evolução dos Papilomavírus

O conhecimento acerca da diversidade dos PV ainda é limitado, provavelmente subestimado. Enquanto mais que 150 genomas de HPV foram sequenciados, PV de menos de 50 espécies de animais não humanos foram isolados e sequenciados. Entretanto, acredita-se que todos os membros dos Amniotas potencialmente possuam PV específicos (de Villiers *et al*., 2004; Rector *et al*., 2007; Schulz *et al*., 2009). Portanto, mais tipos de PV devem ser sequenciados para aumentar o conhecimento sobre a evolução dos PV. Além disso, a diversidade de subtipos e de variantes pode apresentar um cenário mais detalhado e refinado da diversificação dos PV, aumentando a representatividade de cada tipo de PV (Freitas *et al.*, 2011).

O gene *L1* completo, ou parte dele, normalmente é usado para a detecção e genotipagem dos PV. Assim, inicialmente, as filogenias dos PV eram inferidas a partir da comparação de sequências desse gene (Schulz *et al*., 2009). Este tipo de análise permitia identificar uma série de entidades (gêneros) mais ou menos bem estabelecidas e monofiléticas (de Villiers *et al*., 2004; Gottschling *et al*., 2007a). Entretanto, as árvores radiais publicadas não apresentavam um grupo externo apropriado para polarizar a árvore e nem faziam uma avaliação estatística mais criteriosa dos nós internos, pré-requisitos essenciais para uma análise filogenética mais robusta (Rector *et al*., 2007; Schulz *et al*., 2009).

A atual diversidade dos PV pode ser explicada por múltiplos mecanismos evolutivos (Gottschling *et al.*, 2007b). A divergência vírus-hospedeiro é uma força evolutiva importante, mas ela sozinha não consegue explicar a evolução dos PV e sua diversidade. Assim, mecanismos alternativos como a duplicação viral intra-hospedeiro, recombinação, rearranjo viral ou adaptação viral após mudança de

hospedeiro podem contribuir para explicar a diversificação dos PV (Shah *et al.*, 2010; Gottschiling *et al*., 2011).

2.6 Papilomavírus bovino

Os BPV induzem doenças de considerável importância veterinária, além de possuir um elevado valor como modelo *in vivo* para HPV. Eles infectam o epitélio de vertebrados, podendo causar neoplasias ou persistir assintomaticamente. Os BPV formam um grupo heterogêneo de vírus epiteliotrópicos que reconhecem os bovinos como seu hospedeiro clássico. Treze tipos de BPV foram caracterizados e classificados em três gêneros: *Deltapapillomavirus*, *Epsilonpapillomavirus* e *Xipapillomavirus*. Existe ainda um tipo de BPV (BPV-7) que não está classificado em nenhum gênero (Bernard *et al.*, 2010; Hatama *et al.*, 2011; Zhu *et al.*, 2012; Lunardi *et al.*, 2012). Estes vírus são agentes etiológicos associados com várias formas de lesões cutâneas e mucosas, que podem regredir ou evoluir para um câncer, principalmente quando existe interação com cofatores ambientais como ocorre com animais que se alimentam de brotos de samambaia (Jarret *et al.*, 1978; Borzacchiello e Roperto, 2008).

Os BPV são distribuídos mundialmente tanto em lesões quanto em tecidos normais, em que ocorre a lesão latente. Entretanto, poucos estudos descrevem a detecção molecular de BPV em lesões cutâneas de bovinos no Brasil (Claus *et al.*, 2007, 2008, 2009a, 2009b; Sá e Silva *et al.*, 2010). BPV-1, -2, -6 e -8 foram identificados em lesões cutâneas de gado na região sudeste do país (Claus *et al.*, 2007; Sá e Silva *et al.*, 2010). Além disso, quatro prováveis novos tipos de BPV foram identificados na mesma região (Claus *et al.*, 2008).

A papilomatose bovina está associada com lesões hiperproliferativas, conhecidas como papilomas, em tecidos cutâneos e mucosos (Campo, 2006). Normalmente, ocorre a regressão espontânea das lesões (Jelínek e Tachezy, 2005). Entretanto, a papilomatose bovina possui bastante importância veterinária, uma vez que está associada com câncer e condições de imunossupressão (Campo, 2002).

Até recentemente, acreditava-se que os BPV eram associados com lesões distintas, em sítios anatômicos específicos. BPV-1, -2 e -5 infectam células epiteliais, causando verrugas cutâneas comuns e fibropapilomas (Hama *et al.*, 1988). Por outro lado, os tipos de BPV que são membros do gênero *Xipapillomavirus* induzem lesões epiteliais e cutâneas não-fibroblásticas (Hama *et al.*, 1988). De acordo com Borzacchiello e Roperto (2008), BPV-1 causa fibropapilomas de tetos e pênis, além de tumores na bexiga urinária; BPV-2 está associado com verrugas cutâneas, normalmente na testa, pescoço, tórax e dorso superior, além de fibropapilomas no trato digestório e tumores na bexiga urinária; BPV-3 causa papilomas cutâneos (Freitas *et al.*, 2003); BPV-4 está associado com papilomas epiteliais puros do trato gastrointestinal superior; BPV-5 induz fibropapilomas do tipo grão de arroz no úbere; BPV-6 causa papilomas nos tetos; BPV-8 causa papilomas cutâneos e BPV-9 e -10 estão associados com papilomas escamosos epiteliais no úbere.

Além disso, BPV-4 é um tipo viral relacionado com infecções mucosas e é responsável por causar câncer do trato digestório superior em gado quando associado com brotos de samambaia (*Pteridium aquilinum*). Entretanto, BPV-4 foi detectado em lesões cutâneas (Bloch *et al.*, 1995, Freitas *et al.*, 2007). Enquanto BPV-5 e -6 estão normalmente associados com lesões cutâneas no úbere e tetos,

mas já foram encontrados em lesões da paleta e ao redor dos olhos de alguns animais, sítios não usuais para estes tipos virais (Freitas *et al.*, 2007).

Apesar desta provável associação entre tipo de BPV e o local da lesão, alguns fatores podem levar a interpretações errôneas deste processo, como a coinfecção. Alguns relatos descrevem a ocorrência de coinfecção com diferentes tipos de BPV no mundo (Ogawa *et al.*, 2004; Leishangthem *et al.*, 2008; Pangty *et al.*, 2010; Schmitt *et al.*, 2010). No Brasil, poucos estudos identificaram a presença de mais de um tipo de BPV em uma mesma lesão (Yaguiu *et al.*, 2006, 2008; Claus *et al.*, 2009a; Lindsey *et al.*, 2009). Entretanto, a maioria destes estudos apenas focou em poucos tipos de BPV, principalmente BPV-1 e BPV-2.

Embora os BPV sejam tradicionalmente considerados como um grupo de vírus que exclusivamente infectam células epiteliais, há evidências da presença de DNA, RNA e proteínas de BPV em células mononucleares do sangue periférico (Carvalho *et al.*, 2003; Brandt *et al.*, 2008; Roperto *et al.*, 2008; Yaguiu *et al.*, 2008; Lindsey *et al.*, 2009; Hatama *et al.*, 2011; Roperto *et al.*, 2011), placenta e líquido amniótico (Carvalho *et al.*, 2003; Brandt *et al.*, 2008), gametas (Stocco dos Santos *et al.*, 1998; Silva *et al.*, 2011), urina (Stocco dos Santos *et al.*, 1998), útero (Carvalho *et al.*, 2003; Roperto *et al.*, 2008), ovário (Roperto *et al.*, 2008), sêmen (Stocco dos Santos *et al.*, 1998; Roperto *et al.*, 2008; Schmitt *et al.*, 2010), leite e colostro (Stocco dos Santos *et al.*, 1998; Brandt *et al.*, 2008). Estes achados suportam a hipótese da transmissão vertical de BPV (Freitas *et al.*, 2007; Brandt *et al.*, 2008; Maeda *et al.*, 2007).

Os PV têm sido descritos como sendo espécie-específico (Campo, 2006). Entretanto, alguns PV, principalmente os BPV, infectam vários outros hospedeiros. Os BPV podem infectar, além de bovinos, espécies relacionadas

como bisões (Literak *et al.*, 2006), equinos (Brandt et al., 2011), búfalos (Silvestre *et al.*, 2009; Pangty *et al.*, 2010), girafas (van Dyk *et al.*, 2011), antas (Kidney e Berrocal, 2008), cavalos (Bogaert *et al.*, 2008), antílopes (van Dyk *et al.*, 2011) e zebras (Löhr *et al.*, 2005; van Dyk *et al.*, 2009).

A diversidade genética dos BPV tem sido muito pouco estudada. Entre estes estudos, sete diferentes variantes de E5 de BPV-1 foram detectadas em sarcóide equino, sendo que um polimorfismo alterou um aminoácido de cadeia lateral alifática, sugerindo que ele pode ter implicações para a estrutura da proteína E5. Uma observação importante é que a maioria da variação encontrada está associada com a adaptação aos códons usados em células de mamíferos (Szczerba-Turek *et al.*, 2010).

Cinco diferentes variantes de LCR de BPV-1 e seis diferente variantes de E2 de BPV-1 também foram detectadas na Europa. Três mudanças não sinônimas ocorreram dentro do domínio de transativação de E2. Neste estudo, as variantes estavam infectando cavalos, sugerindo que sarcóides podem estar associados a variantes de BPV-1 que preferencialmente infectam equídeos (Freitas *et al.*, 2011). Em outro estudo, variantes de BPV-1 da Polônia foram divididos em três grupos filogenéticos e um isolado separado, mostrando a grande diversidade genética deste vírus em sarcóide equino (García-Vallvé *et al.*, 2005). Estes achados sugerem que a variabilidade genética encontrada em BPV pode levar a diferentes processos patológicos, assim como podem aumentar a adaptação a outros hospedeiros. Portanto, é muito importante estudar e entender os mecanismos evolutivos que guiam a diversificação dos BPV.

Do ponto de vista filogenético, os BPV são encontrados em pelo menos três linhagens distantemente relacionadas. Estas linhagens não são específicas

para BPV. Vírus que infectam hospedeiros artiodátilos, relacionados com os bovinos, se agrupam com os BPV nestes três grupos. Este padrão de diversificação é um caso de incongruência evolutiva entre a filogenia do hospedeiro e dos PV, indicando que a co-divergência sozinha não pode explicar a diversidade destes vírus (Freitas *et al.*, 2011).

As linhagens que possivelmente originaram os tipos de BPV atuais provavelmente passaram por um processo de divergência prévia antes da diversificação dos hospedeiros. Isto pode explicar a proximidade evolutiva dos BPV à PV que infectam hospedeiros distantemente relacionados (Freitas *et al.*, 2011). Além disso, a transmissão zoonótica é um processo evolutivo importante para os BPV, uma vez que eles foram detectados em diferentes hospedeiros. Portanto, outros mecanismos evolutivos além da coevolução podem ser associados com a diversificação dos BPV. Entretanto, a amostragem ainda é um fator limitante para entendermos estes processos, necessitando-se de mais estudos que avaliem a diversidade genética destes vírus (Freitas *et al.*, 2011).

2.7 Entropia

A quantidade de dados de sequência tem aumentado nos bancos de dados biológicos. Para analisar estes dados, métodos matemáticos e algoritmos computacionais simples, lógicos e consistentes são necessários. Um método, conhecido como entropia, que satisfaz estes requisitos foi criado por Shannon com a introdução da teoria da informação (Shannon, 1948).

Na estatística, Shannon (1948) definiu a entropia de um sistema como a medida de incerteza de sua estrutura. A função de Shannon é baseada no

conceito de que o ganho de informação de um evento é inversamente relacionado à sua probabilidade de ocorrência (Mutihac *et al*., 2001).

A entropia tem sido usada com sucesso para quantificar a conservação de sequências nucleotídicas e protéicas. A conservação de sequência é dada pelo número médio de *bits* necessários para definir um conjunto de sequências alinhadas. Embora esta medida seja útil para o entendimento da estrutura das interações nucleotídicas/protéicas, ela não permite a investigação de sequências individuais (Schneider, 1997).

Para as sequências de DNA, a entropia é calculada para cada posição utilizando a fórmula $H_i = -(\sum^j p_j \, log_2 \, p_j)$, onde H_i corresponde à entropia em cada sítio i; j é igual a 1, 2, 3 ou 4, correspondendo aos nucleotídeos A, C, G ou T, respectivamente; e p_j é a proporção do nucleotídeo j no sítio i. Como as sequências de DNA possuem quatro bases diferentes, a entropia máxima será de dois *bits* por sítio. Para sequências de aminoácidos, uma proteína aleatória deve ter $\log_2 20 = 4{,}32$ *bits* de entropia por sítio.

Análises de entropia de sequências de nucleotídeos e aminoácidos já se mostraram úteis na determinação de padrões de variabilidade genética (Mutihac *et al*., 2001; Valdar, 2002; Krishnamachari *et al*., 2004; Liao *et al*., 2005), sendo uma poderosa ferramenta para determinar locais pouco complexos no genoma, importantes para uma reconstrução filogenética mais precisa.

3. Objetivos

3.1 Geral

Este estudo visou à aplicação de uma nova estratégia computacional baseada em entropia, que pode ser aplicada em estudos de epidemiologia e evolução molecular de tipos, subtipos e variantes de BPV. Além de avaliar a diversidade de tipos de BPV na região Nordeste do Brasil, discutindo aspectos epidemiológicos importantes da papilomatose bovina cutânea, incrementando o conhecimento acerca da biologia destes vírus.

3.2 Específicos

1. Avaliar a diversidade genômica dos diversos tipos de PV conhecidos;
2. Implementar e caracterizar um método computacional baseado em entropia para a seleção de regiões genômicas filogeneticamente mais informativas dos PV;
3. Verificar a eficiência do método diante de regiões genômicas estabelecidas com outros métodos;
4. Aplicar o método baseado em entropia para a seleção de novas regiões informativas utilizadas para a identificação e avaliação da diversidade genética de BPV;
5. Verificar a presença de BPV em lesões cutâneas de bovinos nos estados de Pernambuco e da Bahia;
6. Correlacionar o tipo viral, ou a coinfecção, com a localização da lesão, tipo de tecido e a região geográfica;
7. Caracterizar novos tipos de BPV encontrados nas lesões.

4. Capítulo I

An entropy-based approach for the identification of phylogenetically informative genomic regions of Papillomavirus

Marcus V.A. Batista[a], Tiago A.E. Ferreira[b], Antonio C. Freitas[a], Valdir Q. Balbino[a]

[a] *Department of Genetics, Federal University of Pernambuco, Brazil.*

[b] *Department of Statistics and Informatics, Federal Rural University of Pernambuco, Brazil.*

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An entropy-based approach for the identification of phylogenetically informative genomic regions of Papillomavirus

Marcus V.A. Batista [a], Tiago A.E. Ferreira [b], Antonio C. Freitas [a], Valdir Q. Balbino [a,*]

[a] Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, 50670-901 Recife, Pernambuco, Brazil
[b] Department of Statistics and Informatics, Federal Rural University of Pernambuco, 52171-900 Recife, Pernambuco, Brazil

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ABSTRACT

The papillomaviruses form a highly diverse group that infect mammals, birds and reptiles. We know little about their genetic diversity and therefore the evolutionary mechanisms that drive the diversity of these viruses. Genomic sequences of papillomaviruses are highly divergent and so it is important to develop methods that select the most phylogenetic informative sites. This study aimed at making use of a novel approach based on entropy to select suitable genomic regions from which to infer the phylogeny of papillomavirus. Comparative genomic analyzes were performed to assess the genetic variability of each gene of Papillomaviridae family members. Regions with low entropy were selected to reconstruct papillomavirus phylogenetic trees based on four different methods. This methodology allowed us to identify regions that are conserved among papillomaviruses that infect different hosts. This is important because, despite the huge variation among all papillomaviruses genomes, we were able to find regions that are clearly shared among them, presenting low complexity levels of information from which phylogenetic predictions can be made. This approach allowed us to obtain robust topologies from relatively small datasets. The results indicate that the entropy approach can successfully select regions of the genome that are good markers from which to infer phylogenetic relationships, using less computational time, making the estimation of large phylogenies more accessible.

1. Introduction

Papillomaviruses (PVs) belong to a diverse group of epitheliotropic viruses that are found in a wide variety of mammals, birds and reptiles – the Papillomaviridae. They can infect skin and mucosal squamous epithelium, causing asymptomatic infections and various benign or malignant lesions (Campo, 2002). PVs have a circular double strand DNA molecule measuring approximately 8 kb, surrounded by a non-enveloped capsid composed of 72 pentamers (Shah and Howley, 1996). The genome is often divided into eight ORFs that partially overlap along one strand of the DNA molecule. This area is classified into three distinct regions: an early region (E1–E8), the late region (L1 and L2) and the long control region (LCR) with no coding potential (Baker et al., 1987; Wang, 2007).

Because it is the most conserved gene in the PV genome, the L1 ORF has been used for the identification of new viral types. A PV isolate is recognized as a new viral type when its complete genome is cloned and the sequenced L1 ORF reveals differences greater than 10% compared to the closest known type. Differences of between 2% and 10% define a subtype, while PVs that differ by less than 2% are known as variants (Bernard, 2005; de Villiers et al., 2004). On the other hand, differences exceeding 40% are used as reference for the recognition of new genera, which contain biologically diverse and phylogenetically related types (de Villiers et al., 2004).

Based on these simple principles, the following genera have been recognized: *Alpha-PV*; *Beta-PV*; *Gamma-PV*; *Delta-PV*; *Epsilon-PV*; *Zeta-PV*; *Eta-PV*; *Theta-PV*; *Iota-PV*; *Kappa-PV*; *Lambda-PV*; *Mu-PV*; *Nu-PV*; *Xi-PV*; *Omikron-PV*; and *Pi-PV* (de Villiers et al., 2004). However, new genera, such as *Rho-PV*; *Sigma-PV*; *Tau-PV*; *Upsilon-PV*; *Phi-PV*; *Chi-PV*; *Psi-PV*; *Omega-PV*; *Dyodelta-PV*; *Dyoepsilon-PV*; *Dyozeta-PV*; *Dyoeta-PV*; *Dyotheta-PV* and *Dyoiota-PV* have recently been added (Bernard et al., 2010).

However, we still have little knowledge of PV diversity, and efforts need to be made to increase the sampling of PVs, mostly non-human PVs, so we can better understand their evolution. A number of studies have been made which begin to increase our understanding of the factors that influence the evolution of PVs (Bravo and Alonso, 2007; Chan et al., 1992, 1995; Gottschling et al., 2007a,b, 2011; Shah et al., 2010).

* Corresponding author. Address: Laboratory of Bioinformatics and Evolutionary Biology, Department of Genetics, Federal University of Pernambuco, Av. Professor Moraes Rego s/n, Cidade Universitária, 50670-901 Recife, Pernambuco, Brazil. Tel./fax: +55 81 2126 8512.
E-mail address: vqbalbino@pq.cnpq.br (V.Q. Balbino).

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For example, by using a more rigorous phylogenetic approach, which takes into consideration the choice of an appropriate outgroup, as well as the assessment of confidence values of internal nodes, Gottschling et al. (2007b) recognized that multiple evolutionary mechanisms must guide PV diversification. Another robust study has used the method of importance sampling (Shah et al., 2010), providing evidence for the existence of discrepancies between the divergence patterns of PVs and their hosts, confirming that the complex evolution of these viruses cannot be explained solely by co-speciation events. More recently, Gottschling et al. (2011) compared robust phylogenies of PVs and their hosts using different statistical approaches and suggested that codivergence is an important evolutionary force, but that it alone cannot explain the great diversity of PVs observed. Although these studies provide very reliable data, they needed to use computationally intense methods requiring high-performance processors.

When assessing the phylogenetic relationships between a group of organisms, a good way to decrease the computational time and minimize the bias introduced by genomic regions of uncertain homology is to detect and remove those regions from the multiple sequence alignment in order to find only the most phylogenetic informative regions of the genome (Criscuolo and Gribaldo, 2010). As these regions are usually associated with nucleotide/amino acid conservation, the Shannon entropy (Shannon, 1948) could help to detect them, since it is very suitable for measuring genetic variability and detect binding sites (Johansson and Toh, 2010; Schneider et al., 1986; Schneider and Stephens, 1990). Many approaches have been used to assess character variability and they have proved to be useful for the identification of conserved genomic regions (reviewed by Valdar (2002)). In highly diverse data sets, it is important to analyze conserved genomic regions because they have a higher probability of being associated with functional domains in proteins, improving the search for homologous sites among the genomes. So, the entropy measure seems to be a good estimator to select those regions.

In this context, it is important to understand the exact role of co-evolution between PVs and their hosts, and other evolutionary forces that may influence PV diversification, because then medical questions could be better addressed to improve our knowledge on PV infections and cancer. However, the evaluation of these mechanisms is based on biological sequence analysis, and the sequences of genes/proteins of PVs are divergent. Therefore, we propose a novel approach based on the entropy measure to select conserved genomic sites in order to reconstruct PV phylogeny. This method has proven to reduce computational time spent in the phylogenomic analysis of these viruses, allowing the achievement of robust studies without the need of super processors.

2. Material and methods

2.1. Sequences and local database development

Fifty three complete genome sequences representing the diversity of PV types (six human papillomavirus (HPV) types and 47 non-human PV types) were retrieved from the public database of the National Center for Biotechnology Information (NCBI) (see Supplementary Table S1). Due to the large number of HPVs identified, only one representative of each genus containing HPVs was selected in order to avoid bias in the analysis.

From the complete genomes, the annotations of each gene were retrieved, as well as their nucleotide and amino acid sequences using BLAST (Altschul et al., 1990). Then, the sequences were processed using BioEdit v. 7.0.9 (Hall, 1999) and stored in a local database, along with the intrinsic information of the genomes, such as size and nucleotide composition.

2.2. Sequence alignment and genomic variability

The amino acid sequences of each PVs protein were aligned using ClustalW (Thompson et al., 1994) incorporated into the Molecular Evolutionary Genetics Analysis version 4.0 (MEGA4) software (Tamura et al., 2007). Problematic regions which were difficult to align were realigned with a higher gap opening and extension penalty, and then manually adjusted. The amino acids alignments were back translated into codons to obtain the aligned nucleotide sequences.

For each gene, the following parameters were estimated: total number of aligned sites; number of sites with gaps; conserved sites; variable sites; singleton sites; parsimony informative sites; the total number of mutations; and the overall distance. To achieve this, the programs used were DNA Sequence Polymorphism (DnaSP) version 5.10.00 (Librado and Rozas, 2009) and MEGA4 (Tamura et al., 2007). The transition/transversion ratio (R) was calculated using Tree-Puzzle v. 5.2 (Schmidt et al., 2002).

2.3. Entropy calculation and secondary structure prediction

The sequences were submitted for entropy analysis using Data Analysis in Molecular Biology and Evolution (DAMBE) v. 5.2.31 (Xia and Xie, 2001), in order to evaluate the variability and complexity of each nucleotide site. A window size equal to 100 units was used with the objective of reducing noise. The entropy was calculated for each position using the Shannon entropy formula: $H_i = -(\sum_{j=1}^{4} p_{ij} \log_2 p_{ij})$, where H_i corresponds to the entropy of each site i; j is equal to 1, 2, 3 and 4, corresponding to the A, C, G and T nucleotides, respectively; and p_{ij} is the proportion of the nucleotide j in the site i. All sites with low complexity, defined as those that exhibited entropy values less than or equal to 1.6, were selected to reconstruct the PV phylogeny, which allows sufficient variability to establish the evolutionary relationships of PVs. Some sites were included in order to maintain the codons.

In order to validate the method, we tried to establish a structural relationship between the regions selected by entropy and the secondary structure of PVs' proteins (α-helix, β-sheets and loops regions). For each protein, secondary structure prediction was performed using the JPred 3 server (Cole et al., 2008), which incorporates the Jnet algorithm. This server uses a combination of prediction methods to increase the success rate determining secondary structures. In addition to the increased accuracy, JPred 3 can predict the secondary structure based on a multiple alignment of amino acid sequences, producing a consensus of structures obtained from each sequence.

2.4. Phylogenetic analyses

The selected regions of each gene were submitted to ModelTest v. 3.7 (Posada and Crandall, 1998). ProtTest v. 2.4 (Abascall et al., 2005) was used for proteins. The Akaike Information Criterion (AIC) methodology of model selection (Akaike, 1973) was used to define the substitution model that best fitted the data. When the alignment length was small compared to the number of parameters, we used the corrected AIC (AICc) measure of model fit (Hurvich and Tsai, 1989; Sugiura, 1978; Posada and Buckley, 2004). The model for the concatenated genomic regions was also verified.

We used Tree-Puzzle v. 5.2 (Schmidt et al., 2002) to evaluate the existence of a phylogenetic signal in the dataset. This verification was performed by likelihood mapping, which analyzes groups of four randomly selected sequences called quartets (Strimmer and von Haeseler, 1997). In order to check the phylogenetic signal decay caused by saturation, plots were constructed using DAMBE (Xia and Xie, 2001), representing the behavior of transition and transversion rates as the genetic distance increases between

sequences. Due to the high levels of saturation detected, two datasets were used in the analysis with nucleotides: one including and another excluding the third codon position, for comparison purposes.

Phylogenetic analysis was performed with the concatenated genes/proteins using a 2.67 GHz Core 2 Duo processor with 4 GB of RAM running Windows XP SP3. The genes E4 and E5 were excluded for not being present in at least 80% of the evaluated PV types and for presenting high entropy values. Thus, the data matrix used in the analysis contained the genes/proteins E1, E2, E6, E7, L1 and L2. In parallel, other analyzes were performed with the L1 gene, as well as with the concatenated genes/proteins E1–E2–L1 and E1–E2–L2–L1, in order to assess which set of genes/proteins best reconstructs the evolutionary history of PVs, as well as to confirm the efficacy of the entropy method with a more representative number of viruses.

The reconstruction of the PV phylogeny was performed using Neighbor-Joining (Saitou and Nei, 1987; Studier and Kepler, 1988), Maximum Parsimony, Maximum Likelihood and Bayesian Inference methods. Except for Maximum Parsimony, all methods were calculated using the evolutionary models and parameters that best fitted the dataset. Trees based on Neighbor-Joining and Maximum Parsimony methods were generated using PAUP* v. 4.0b10 (Swofford, 2002), with 1000 nonparametric bootstrap replicates as confidence values for the branches. For Maximum Parsimony, heuristic searches were used with TBR method, and the initial trees were obtained by random addition of taxa with 10 replicates.

Maximum Likelihood analysis was performed using PhyML v. 3.0 (Guindon et al., 2010). NNI branch swapping was used to estimate tree topology by performing heuristic search. A BioNJ tree was used as the initial tree and the taxa were added randomly. The robustness of the branches was assessed using 1000 bootstrap replicates. Bayesian Inference analysis was performed using MrBayes v. 3.1 (Ronquist and Huelsenbeck, 2003), which uses the Markov Chain Monte Carlo (MCMC) simulation technique to approximate the posterior probability of trees. The final topology was estimated with 1,000,000 interactions using 100,000 cycle burn-in and sampled every 100 interactions. Stationarity was assessed by visualization of ln likelihood scores vs. generation plots to determine the point at which likelihood values stabilized.

3. Results

3.1. Genome structure and genetic variability

Based on the annotation available from GenBank, all viral types presented the genes E1, E2, L1 and L2. However, only approximately 75% of PVs contained the E4 gene, 43.4% the E5 gene, and 84.9% the E6 and E7 gene. A few PVs included unusual genes with poorly-defined functions and overlapping sequences.

After the alignment, the E1 gene was the longest with 2,367 bp, whereas E5 gene was the shortest with 509 bp. The analysis showed that the most conserved genes are L1 and E1 with 165 (9.4%) and 146 (6.2%) conserved sites, respectively. E4 and E5 genes were more variable, with no conserved sites. Although relatively extensive, the L2 and E2 genes presented very low number of conserved sites (Table 1).

The variability among the PV genes was quite high, as verified by the presence of sites with gaps and variable sites. In six genes (E2, E4, E5, E6, E7 and L2), the number of gaps was much greater than the number of variable sites. In the L1 gene, the number of variable sites surpassed the number of sites with gaps. The E1 gene presented a number of variable sites close to the number of sites with gaps (1,174 and 1,047, respectively). Among the variable sites, most of them were parsimony informative. The overall distance, an average measure of sequence divergence, was high, demonstrating that PVs have highly diverse sequences (Table 1). This variability pattern was also observed when we analyzed the amino acid sequences (Table 2).

An important parameter for the correct inference of phylogeny concerns the ratio (R) between transition and transversion rates, which allows us to verify the presence of bias in the nucleotide substitutions. Out of the eight genes analyzed, three (E1, E5 and L1) had R values >1.0, showing an excess of transitions. For the other genes (E2, E4, E6, E7 and L2), R values were lower than 1.0, indicating that a greater number of transversions was occurring (Table 1).

Table 1
Genomic diversity of Papillomavirus all variables were calculated using MEGA4, DnaSP and TreePuzzle programs.

ORF (size)	Sites with gaps (indels)	Conserved sites	Variable sites			Total no. of mutations	Overall distance	R = Ts/Tv
			Singletons	Parsimony informative	Total			
E1 (2367)	1047 (44.2%)	146 (6.2%)	50	1124	1174 (49.6%)	2951	0.500	1.12 ± 0.02
E2 (1806)	1251 (69.3%)	24 (1.3%)	19	512	531 (29.4%)	1411	0.576	0.77 ± 0.02
E4 (1041)	1023 (98.3%)	0 (0%)	0	18	18 (1.7%)	54	0.696	0.82 ± 0.01
E5 (507)	443 (87.0%)	0 (0%)	0	66	66 (13%)	187	0.671	1.18 ± 0.04
E6 (945)	777 (82.2%)	10 (1.1%)	2	156	158 (16.7%)	440	0.615	0.91 ± 0.04
E7 (945)	852 (90.2%)	5 (0.5%)	5	83	88 (9.3%)	244	0.624	0.82 ± 0.03
L1 (1749)	501 (28.6%)	165 (9.4%)	59	1024	1083 (62%)	2523	0.453	1.21 ± 0.03
L2 (1998)	1644 (82.3%)	6 (0.3%)	6	342	348 (17.4%)	983	0.626	0.76 ± 0.01

Table 2
Protein diversity of Papillomavirus. All variables were calculated using MEGA4 program.

Protein (size)	Sites with gaps (indels)	Conserved sites	Variable sites			Overall Distance
			Singletons	Parsimony informative	Total	
E1 (789)	349 (44.2%)	43 (5.4%)	18	379	397 (50.4%)	0.600
E2 (602)	417 (69.3%)	4 (0.7%)	6	175	181 (30.0%)	0.701
E4 (347)	341 (98.3%)	0 (0%)	0	6	6 (1.7%)	0.881
E5 (169)	149 (88.2%)	0 (0%)	1	19	20 (11.8%)	0.833
E6 (315)	259 (82.2%)	4 (1.3%)	1	51	52 (16.5%)	0.741
E7 (315)	284 (90.2%)	2 (0.6%)	3	26	29 (9.2%)	0.759
L1 (583)	167 (28.6%)	44 (7.6%)	26	346	372 (63.8%)	0.502
L2 (666)	548 (82.3%)	3 (0.4%)	3	112	115 (17.3%)	0.752

Table 3
Low entropy regions selected for phylogenetic analysis.

Gene	Low entropy areas $H \leqslant 1.6$ (interval showing the position in each gene)				Total number of low entropy sites (bp)
	A1	A2	A3	A4	
E1	247–321	804–959	994–1368	1482–2351	1476
E2	30–473	542–628	1519–1617	1669–1752	714
E4	–	–	–	–	–
E5	–	–	–	–	–
E6	221–307	405–503	–	–	186
E7	650–700	–	–	–	51
L1	19–867	987–1610	–	–	1473
L2	151–345	1288–1417	1897–1915	–	363

The C-terminal region was more conserved in the E1 protein, where the helicase domain is located. The high sequence conservation of E2 protein was found in the N-terminal end, related to the transactivation domain and C-terminal end, which exhibits a DNA binding domain. Despite the high genetic variability found in E4 and E5 proteins, it was possible to observe that there were more conserved segments corresponding to functionally important regions. E6 and E7 oncoproteins have zinc-binding domains that are conserved. Interestingly, the LxCxE domain that binds to the retinoblastoma tumor suppressor protein (pRB) was not as highly conserved.

For the structural proteins, it was observed that many conserved sites in several intermolecular interaction domains are responsible for formation and stabilization of the viral capsid. In general, the L1 protein was quite conserved throughout its length, and it was possible to identify several conserved motifs spaced by small variable regions. The L2 protein contained two major conserved domains in the N- and C-terminal regions.

3.2. Entropy analysis and secondary structure association

The analysis identified several regions with low entropy in all evaluated genes, as described in Table 3. The E1 and E2 genes contained four regions with low entropy, while L2 gene presented three regions. L1 and E6 genes had two regions, and E7 gene presented only one region with low entropy. E4 and E5 genes had no regions with entropy values below 1.6. Table 3 and Supplementary Fig. S1 describe the areas with low entropy found in each of the genes and the total size of those regions used in the phylogenetic analysis.

The secondary structure prediction of the E1 protein revealed several α-helix regions, interspersed with β-sheet and loop regions, distributed homogeneously (Supplementary Fig. S2a). The amino-terminal region of E2 protein exhibits a set of α-helix regions followed by a β-sheet, interspersed with loops. Around the central part of the protein there is a large loop region, while in the carbon-terminal portion it was possible to identify five β-sheet regions with α-helix inserted at the end (Supplementary Fig. S2b).

The E4 protein, however, presented itself as a large loop structure, with no α-helix or β-sheet (Supplementary Fig. S2c). On the other hand, E5 oncoprotein exhibited a large α-helix, only with loops at the extremities (Supplementary Fig. S2d). The E6 protein had α-helix, β-sheet and loops regions interspersed throughout the molecule (Supplementary Fig. S2e). The secondary structure prediction of the E7 protein presented, in its central and carbon-terminal region, three β-sheets and one α-helix structure (Supplementary Fig. S2f).

The capsid proteins (L1 and L2) had several β-sheet regions distributed along their entire length. L1 exhibited several α-helices along the protein, especially in the central region, while L2 had only two α-helix regions at the amino-terminus part of the protein. In addition, L1 had several regions of β-sheet at its amino-terminal part. Large loop regions were also present along the L2 protein and the carbon-terminal part of L1 (Supplementary Fig. S2g and h). After prediction, we associated the secondary structure of PV proteins with the previously determined low entropy genomic regions. Most of these informative regions were associated with α-helices and β-sheets structures, interspersed with small loop regions (Fig. 1).

3.3. Phylogenetic analysis of papillomavirus based on low entropy genomic regions

The nucleotide substitution models that best fitted the genes and the evolutionary models for PV proteins are described in Table 4. The Table also includes the models used for the analysis involving the concatenated genes/proteins.

Phylogenetic signal tests were carried out separately for each genomic region, for both amino acids and DNA analyses. In all these analyses, E1, E2, E6, L1 and L2 genomic regions exhibited phylogenetic signals. The saturation test showed that all genes were saturated. Due to a different evolutionary pressure, substitution saturation was usually associated with the codon third position. Analyses with nucleotide sequences (with and without the codon third position) and amino acids were carried out, for comparison purposes. The topologies obtained with all datasets analyzed showed relatively congruent topologies (Supplementary Figs. S3 and S4).

The inferences were made in relation to two datasets, involving the E6–E7–E1–E2–L2–L1 and E1–E2–L2–L1 combinations of genes/proteins (Fig. 2). The objective was to show that, regardless of the choice of genes/proteins used, analysis of the low entropy regions gives rise to a robust phylogeny.

Using the low entropy regions did not generate fully congruent trees. However, high confidence values have been achieved for the majority of the nodes. It was possible to obtain very robust phylogenetic trees, although they had low statistical support for some internal nodes. It was not possible to observe large discrepancies comparing the topologies and the statistical support of the nodes among the trees constructed from the nucleotides, nucleotides without the third codon position and amino acids sequences, although the cladograms based on amino acids sequences showed some branch confidence values that were slightly higher.

In all combinations of genomic regions analyzed, several monophyletic clusters could be clearly distinguished, representing the PV genera. At some level, all genera, mostly *Delta-PV*, *Epsilon-PV*, *Xi-PV*, *Beta-PV*, *Kappa-PV*, *Mu-PV*, *Lambda-PV*, *Nu-PV*, *Alpha-PV*, *Omikron-PV*, *Theta-PV* and *Eta-PV*, were monophyletic and they presented high bootstrap and posterior probability values (Fig. 2).

Some of the clades containing the PV genera corresponded to the clusters of the hosts, such as primates, artiodactyl, lagomorphs, carnivores, cetaceans, birds and reptiles. These clusters were supported under the different methods of tree reconstruction, as well as using different combinations of genes. However, several types of

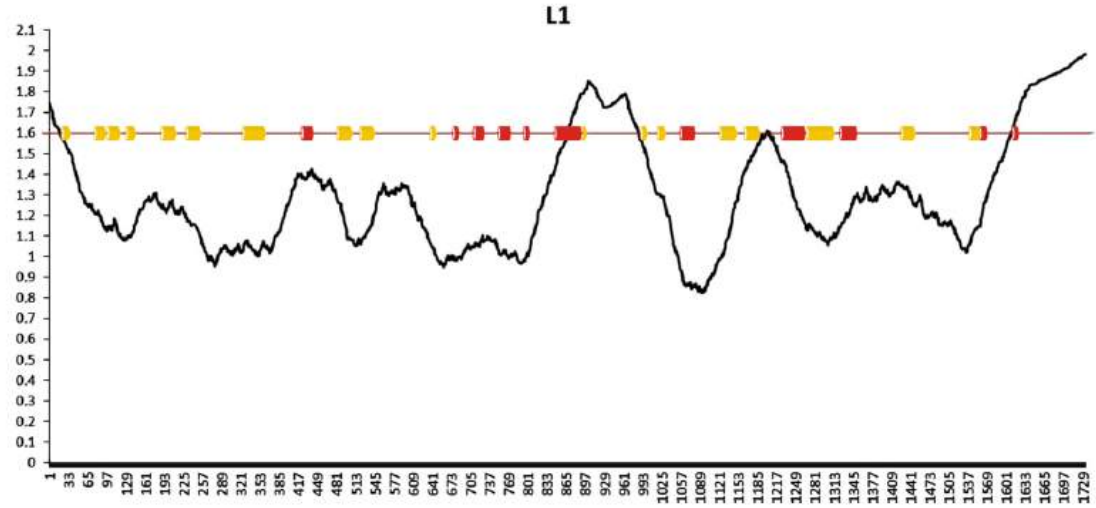

Fig. 1. Entropy per site plot of L1 gene. Regions with entropy values equal to and below 1.6 bits have less information complexity, which indicates the most conserved parts of the genomes. Predicted consensus secondary structure of L1 protein is plotted in the graph. The α-helix regions are in red, the β-sheet ones are in yellow and lines represent loop regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Evolutionary models that most fit the data for the low entropy genomic regions of each gene/protein of Papillomavirus according to ModelTest and ProtTest programs. The last three lines show concatenated genes that formed the combinations used in this analysis.

Genes/proteins	Nucleotide substitution model	Reference	Amino acid substitution model	Reference
E1	GTR + I + G	Tavaré (1986)	RtREV + I + G + F	Dimmic et al. (2002)
E2	GTR + I + G	Tavaré (1986)	RtREV + G + F	Dimmic et al. (2002)
E4	GTR	Tavaré (1986)	VT + G + F	Muller and Vingron (2000)
E5	GTR + G	Tavaré (1986)	FLU + F	Dang et al. (2010)
E6	GTR + G	Tavaré (1986)	WAG + I + G + F	Whelan and Goldman (2001)
E7	TVMef + G	Posada (2003)	LG + I + G + F	Le and Gascuel (2008)
L1	GTR + I + G	Tavaré (1986)	WAG + I + G	Whelan and Goldman (2001)
L2	TVM + I + G	Posada (2003)	WAG + I + G + F	Whelan and Goldman (2001)
E1–E2–L2–L1	GTR + I + G	Tavaré (1986)	LG + I + G + F	Le and Gascuel (2008)
E6–E1–E2–L2–L1	GTR + I + G	Tavaré (1986)	LG + I + G + F	Le and Gascuel (2008)
E6–E7–E1–E2–L2–L1	GTR + I + G	Tavaré (1986)	RtREV + I + G + F	Dimmic et al. (2002)

PV did not cluster together according to the phylogeny of their hosts. For example, PVs that infect primates did not form a monophyletic group in all analyses. MfPV1, which infects a non-human primate, clustered together with HPV24; a polyphyly was observed in the group of PVs that infect cattle; PVs that infect canines also showed themselves to be polyphyletic; OcPV1 and SfPV1, which infect rabbits, clustered together with HPV1; UmPV1 which infects one Carnivora member and SsPV1 that infects one artiodactyl clustered together with the PVs that infect primates (Fig. 2).

Despite the relative robustness of the tree, taking into account the highly variable dataset, some nodes showed well-supported phylogenetic contradictions when taking into consideration all four different methods used to reconstruct the phylogenetic trees. In addition, some viruses randomly clustered together when using different partitions, for instance EcPV1, TmPV1, MnPV1, RaPV1 and BPV7 (Fig. 2).

Phylogenetic analyses based on genomic regions with low entropy were more quickly computed than the analyses with the original datasets because of the smaller dataset size (Supplementary Table S2). The entropy method selected regions with sizes 15–52% smaller, which represented computational times approximately 30–70% faster, depending on the dataset used.

4. Discussion

Some attempts to obtain a complete overview of the evolution of PVs have been made (Bravo and Alonso, 2007; Chan et al., 1992, 1995; Gottschling et al., 2007a,b, 2011; Shah et al., 2010). However, this is the first time that an entropy-based method has been used to identify phylogenetically informative genomic regions in order to reconstruct the PV phylogeny. In this study, we used the complete genomes of PVs that infect a wide variety of hosts. However, we did not take into consideration the intratypic variation of PVs (variants and subtypes), and so we do not know how representative the GenBank reference isolates are.

The PV genes are highly variable, both in terms of nucleotide and amino acid sequences. However, it is interesting to observe that there is a relative conservation regarding to certain protein domains. Therefore, PVs appear to maintain constant structurally and functionally important regions, whereas other regions are more flexible in terms of evolution. This is consistent with previous knowledge that the protein regions that evolve more slowly are usually associated with molecular recognition functions (Worth et al., 2009). For example, the L2 protein contains two conserved domains at the N- and C-termini for which functional evidence

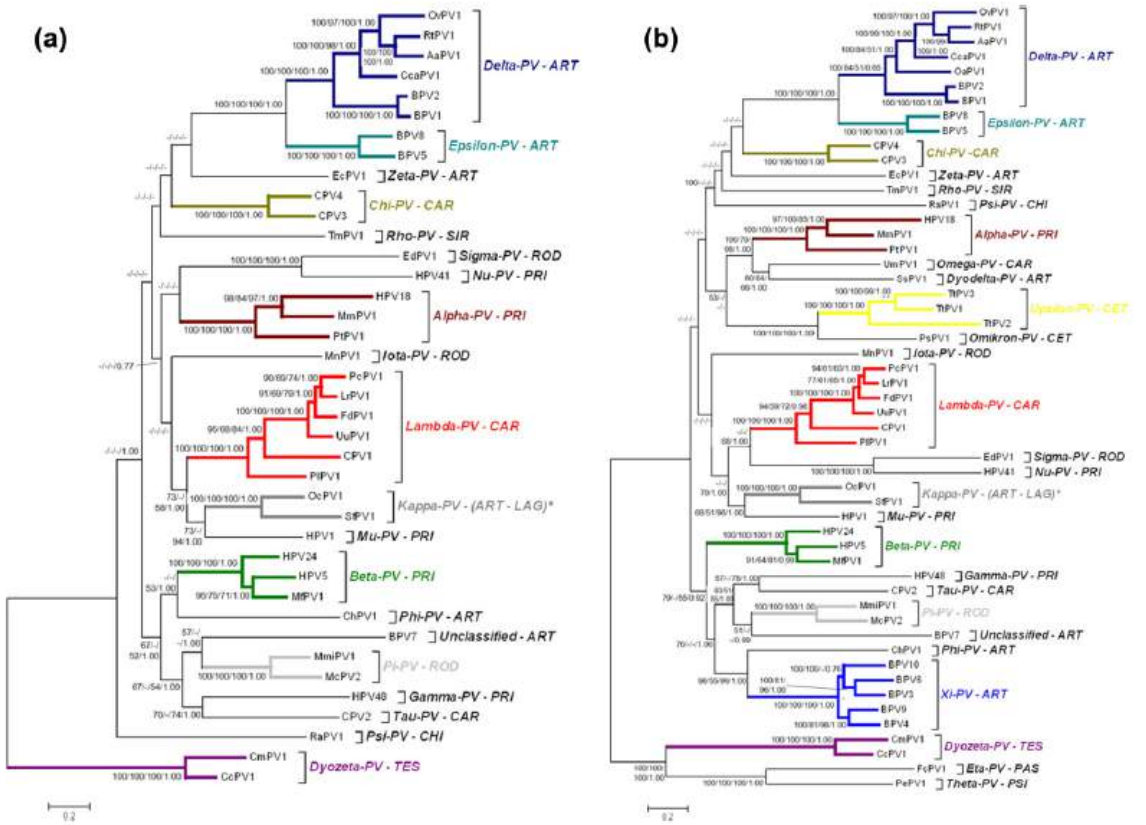

Fig. 2. Maximum Likelihood phylogenetic trees of representative PVs based on amino acid sequences, using low entropy regions. (a) Analysis carried out with E6–E7–E1–E2–L2–L1 protein combination. (b) Analysis carried out with E1–E2–L2–L1 protein combination. Branch support values are represented (left to right: NJ/MP/ML/Bayesian probabilities). Topological incongruences and bootstrap values under 50% are not represented. Papillomavirus genera (Greek letters) are represented along with host order they infect: ART – Artiodactyla; PRI – Primates; CAR – Carnivora; CET – Cetacea; SIR – Sirenia; CHI – Chiroptera; ROD – Rodentia; LAG – Lagomorpha; TES – Testudinata; PAS – Passeriformes; PSI – Psitaciformes. Asterisks indicate the only Papillomavirus genus (Kappa-PV) that contains viruses infecting two different host orders (OcPV1 infects Artiodactyla and SfPV1 infects Lagomorpha).

has been reported (Bossis et al., 2005; Bousarghin et al., 2003; Finnen et al., 2003; Florin et al., 2006; Kämper et al., 2006; Richards et al., 2006; Roden et al., 2000).

However, we have also noted in this study that the L × C × E domain of E7 protein was not well conserved, despite its association with cancer development, according to the report of Caldeira et al. (2000). They have observed that the E7 protein of some HPVs does not exhibit this domain and the virus can still induce cell proliferation. Consequently, although PV genes have accumulated many mutations over evolutionary time, apparently function has been maintained. This idea is important because selecting functionally important genomic regions to infer the phylogeny of this group becomes very relevant due to the huge genetic variability found in these viruses.

The behavior of transitions and transversions is as important as knowing the gene sequence variation within a group of organisms. Estimating the possible bias of these rates is very important for understanding the evolution of genomes, as well as the correct reconstruction of their phylogeny (Yang and Yoder, 1999). In this analysis, we found values near one (between 0.7 and 1.2) which is expected given the high level of sequence divergence.

As a consequence of PVs genomic variability, we are suggesting a new approach to phylogenetic reconstruction using low entropy genomic regions. Regions in a DNA sequence that are widely divergent could show different evolutionary histories, disturbing the overall phylogenic reconstruction from the group of organisms. So, it is important to find and delete these regions when we deal with a highly variable dataset such as the one analyzed here. The entropy method applied to nucleotide and amino acid sequences has proved to be useful in determining patterns of genetic variability (Caffrey et al., 2004; Krishnamachari et al., 2004; Liao et al., 2005; Mutihac et al., 2001; Pilpel and Lancet, 1999; Schneider et al., 1986; Valdar, 2002; Zou and Saven, 2000). So, it is a powerful tool for determining less complex sites in the genome, which is important for a more accurate phylogenetic reconstruction. Therefore, entropy is a numerical measure that indicates regions that are most probably homologous.

Entropy based methods have been shown to be more accurate than other trimming approaches. In comparison to other methods, Criscuolo and Gribaldo (2010) showed that their entropy method gave best confidence values for the monophyly of Unikonts, as well as providing a more accurate prediction for the monophyly of Archaeplastida and Unikonts, and the phylogenetic distinction between jacobids and Archaeplastida.

Importantly, this is the first time that entropy has been used to select PV genome regions for phylogenetic reconstruction. This

approach has gained importance in recent years, resulting in the development of software which selects regions in a multiple sequence alignment that are suitable for phylogenetic inference and then computing a score related to the entropy value (Criscuolo and Gribaldo, 2010). In the case of PVs, despite the great genetic variation found, it was possible to observe low informational complexity genomic regions, which are related to the conserved sites of each gene. Logically, less variable genes have more low entropy regions. These regions have a higher probability of being related to important functional domains in the proteins of these viruses. For example, according to Kim et al. (2003), regions related to transcription factor binding sites have a low probability of mutations.

Therefore, the high level of genetic variability exhibited by PVs can be explained by the presence of functionally and structurally important conserved regions, which maintains protein stability and indicates a common ancestry. Thus it is important to associate low informational complexity regions with the secondary structure of the proteins. However, many regions selected by entropy are related to loop regions. Despite the existence of a potential flexibility in loops, increasing tolerance to variation in those regions, some conservation can be found depending on whether catalytically active residues are present in these structures or whether they adopt important positions for protein fold (Camps et al., 2007). Moreover, α-helix and β-sheet regions may influence the adjacent loop regions, like a shield effect, decreasing the number of amino acids changes in these places due to evolutionary pressure known as background selection, as observed in Fusconaia, Pleurobema, Lemiox and Ptychobranchus (Chapman et al., 2008). So we can infer that the low entropy regions are probably structurally important, and therefore useful for the construction of PV phylogeny.

Accordingly, the entropy was used in order to improve phylogenetic signal to reconstruct the evolutionary relationships of a group of viruses, not only based on information theory, but also associated with biological concepts. Moreover, with the intention of further validate the method, three different genomic combinations were analyzed, using four, five or six concatenated genes, and we obtained congruent results for all three datasets, which shows the efficiency of the method independently of the genomic region analyzed.

Using the entropy measure approach, we were able to obtain topologies and branch lengths similar to the ones obtained by Gottschling et al. (2007b), corroborating the idea that diversification of PVs are due to multiple evolutionary mechanisms as suggested by Gottschling et al. (2007b). Congruence between virus and host phylogeny is required to confirm that co-evolution by itself guides PV diversification (Chan et al., 1992; Rector et al., 2007). García-Vallvé et al. (2005) proposed that PVs co-evolve with their hosts, even though some genes show different evolutionary histories, indicating that some of these genes were acquired later in the evolution of PVs. Although those ideas are quite plausible in first instance, our results suggest that there is disagreement between the phylogeny of PVs and their hosts, and that co-evolution by itself cannot explain the diversification of PVs, consistent with the findings of Gottschling et al. (2007b), Shah et al. (2010) and Gottschling et al. (2011).

A major problem found in the elucidation of PV phylogeny is the limited availability of sequenced PV types, subtypes and variants. While many HPV types are known, there is an insufficient sampling of non-human PVs. This small representation may explain the low bootstrap values found in the internal nodes. Although the phylogeny obtained in this study resembles the one found by Gottschling et al. (2007b), which used another character trimming method (Castresana, 2000), we were able to obtain it using a fewer regions of the genome, which exhibited low entropy. This means that we got similar results using less data, resulting in lower computational cost.

Another advantage of the method is that the congruence obtained between topologies based on three gene combinations used in our study indicated that it is possible to reconstruct PV phylogeny by using four, five or six concatenated genes, since the most informative regions of these genes are used. Besides the decrease in computational cost, this process is relevant as it shows that there is no need to sequence the entire genome of new PV types for them to be included in phylogenomic studies. Therefore, entropy can be used to determine hot spots of information in the genomes for inclusion in phylogenetic studies.

Even though some studies have shown the effectiveness of other methods which eliminate regions that disrupt the dataset phylogenetic signal (e.g. Castresana, 2000; Criscuolo and Gribaldo, 2010; Talavera and Castresana, 2007), it is important to develop and apply new methods and approaches that increase this efficiency to make the estimation of large phylogenies more accessible.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.09.013.

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5. Capítulo II

Detection of bovine papillomavirus types, co-infection and a putative new BPV11 subtype in cattle

C.C.R. Carvalho, **M.V.A. Batista**, M.A.R. Silva, V.Q. Balbino, A.C. Freitas

Department of Genetics, Federal University of Pernambuco, Brazil.

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Transboundary and Emerging Diseases

ORIGINAL ARTICLE

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

C. C. R. Carvalho[1], M. V. A. Batista[1,2], M. A. R. Silva[1], V. Q. Balbino[2] and A. C. Freitas[1]

[1] Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Recife, PE, Brazil

[2] Laboratory of Bioinformatics and Evolutionary Biology, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Recife, PE, Brazil

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

Correspondence:A.
C. de Freitas. Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Federal University of Pernambuco, Av. Professor Moraes Rego s/n, Cidade Universitaria, 50670-420, Recife, Pernambuco, Brazil.
Tel.: +55 81 2126 8520;
Fax: +55 081 2126 8522;
E-mail: acf_ufpe@yahoo.com.br

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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.

Introduction

Brazil has the second largest cattle population in the world (about 200 million cattle) and is currently the largest beef producer and the sixth largest producer of milk in the world (with around 21 million dairy cows) (FAO, 2006; IBGE, 2008). The benign and malignant tumours caused by the infection with bovine papillomavirus (BPVs) are emerging diseases important for beef and dairy cattle in the world (Borzacchiello and Roperto, 2008).

Papillomaviruses are a family of viruses that infect a wide variety of animals, including bovine. Bovine papillomavirus infect basal epithelial cells leading to the formation of tumours known as papillomas or warts. The lesions are usually benign and tend to regress, however, with the interaction of environmental co-factors they can turn into malignant tumours (Campo and Jarret, 1994). Eleven BPV types (BPV1–11) have been sequenced and characterized (Bernard et al., 2010; Hatama et al., 2011). BPVs are classified in three different genera: *Deltapapillomavirus* (BPV1 and BPV2), *Xipapillomavirus* (BPV3, BPV4, BPV6, BPV9, BPV10 and BPV11) and *Epsilonpapillomavirus* (BPV5 and BPV8). Another viral type, BPV7, is still unclassified (Ogawa et al., 2007).

Although BPVs are described as solely infecting keratinocytes and fibroblasts (Campo and Jarret, 1994; Borzacchiello and Roperto, 2008), there is evidence for the presence and expression of BPV in other body fluids and tissues (Carvalho et al., 2003; Freitas et al., 2003, 2007; Roperto et al., 2011). These findings corroborate the hypothesis of vertical transmission of BPV (Stocco dos Santos et al., 1998; Freitas et al., 2003, 2007).

Recent studies describe the detection of potentially new BPV types using PCR and sequencing techniques (Tomita et al., 2007; Claus et al., 2008; Hatama et al., 2008). According to de Villiers et al. (2004), different BPV types share a genetic identity of <90%, viral subtypes display a

sequence identity between 90% and 98%, whereas variant strains of the same viral type have sequence identity of more than 98%. Degenerate primers have been used for HPV and BPV genotyping (Antonsson and Hansson, 2002; Ogawa et al., 2004; Claus et al., 2008). Nevertheless, the sensitivity of these primers could be compromised because of the sequence variability between papillomaviruses from these two hosts (Schmitt et al., 2010).

A few studies report the occurrence of co-infection with different types of BPV worldwide. In India and Germany, different BPV types were identified in the same lesion (Pangty et al., 2010; Schmitt et al., 2010). In Brazil, the presence of BPV types 1, 2, 4 and 6 in the same lesion was also reported (Yaguiu et al., 2006; Claus et al., 2007, 2009b; Lindsey et al., 2009).

An active surveillance program to identify the incidence and distribution of these viruses is paramount for preventive and therapeutic strategies, as well as towards the establishment of reliable diagnostic methods. Therefore, the aim of the present study was to screen bovine cutaneous lesions for the presence of known and potentially novel BPV types.

Materials and Methods

Cattle selected for this study were from two dairy farms with high incidence of cutaneous papillomatosis, located in Pernambuco and Bahia states, Northeastern Brazil. Seventy-two cutaneous lesions samples from 66 animals were collected via excision from different sites on the skin of the animals. Biopsy samples were wrapped in foil, immersed in liquid nitrogen, transported on ice to the laboratory and stored at −80°C.

DNA extraction

Genomic DNA were extracted from tissue samples (30 mg) collected from each lesion by using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to protocols of manufacturer. DNA samples were quantified using Nanovue (GE, Fairfield, CT, USA). The quality of the purified DNA was checked by β-globin gene PCR, as described by Freitas et al. (2003).

Detection of viral DNA

For detection of viral DNA, 100 ng of DNA was amplified by PCR in a final volume of 25 μl, 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 degenerate primers FAP59 and FAP64 under conditions described by Ogawa et al. (2004), with the annealing temperature set at 50°C. Subsequently, DNA isolates were subjected to PCR using BPV type-specific primers and respective annealing temperatures as outlined in Supporting Information Table S1, according to the amplification protocol described by Stocco dos Santos et al. (1998). PCR reactions were carried out in a MJ Research PTC 200 thermocycler.

Amplicons obtained by FAP59/64 PCR and by specific primers were sequenced to identify/confirm the viral type. All amplification products were visualized by 2% TAE agarose gel electrophoresis and subsequent ethidium bromide staining. Controls included in each PCR involved BPV1 to six viral genome cloned into PAT 153 plasmid and sequenced as positive control. Primers for BPV7 to BPV10 were analysed *in silico*, and the positive samples were sequenced to confirm the result. Bovine DNA extracted from Madin-Darby Bovine Kidney (MDBK) cells as negative control. Given that BPV3 primers recognized both the L1 gene from BPV3 and BPV6, sequencing was performed to separate the two types.

Sequencing and genotyping

Samples that tested positive for the presence of putative (i.e. supposed) new viral types were once again amplified by PCR using a High Fidelity DNA polymerase (GE) and the degenerated primers indicated above. Following PCR amplification and purification using DNA Purification System (Promega), PCR products were cloned into the pGEM-T vector (Promega) and transformed in competent DH5α bacteria. Bacterial clones were randomly selected for confirmations. At least two different positive clones were sequenced, with an ABI 3100 Applied Biosystems DNA sequencer and Sanger BigDye terminator v 3.1 method with cycle sequencing kit, twice in both directions to identify new putative BPV types.

Sequencing quality and contig assembly were carried out using Pregap4 and Gap4 programs (Staden, 1996). Only sequences with a Phred value above 30 were considered for the contig assembly. Local sequence alignments were carried out with BLAST (Altschul et al., 1990). Multiple sequence alignment of published and potentially new BPV types was carried out by ClustalW (Thompson et al., 1994). The extremities of the multiple sequence alignment were deleted. The newly identified sequences BPV/UFPE01 and BPV/UFPE02 were submitted at GenBank [GU201950, GU201951].

Phylogenetic analysis was carried out using the Maximum Likelihood method with TIM3 + G as nucleotide substitution model in PAUP* v. 4.0b10 (Swofford, 2002). The tree topology was estimated with heuristic search. An initial BIONJ tree was used, and the taxa were randomly added. To determine the statistical support of the

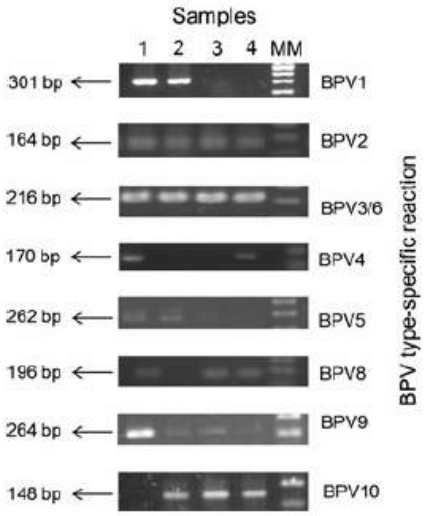

Fig. 1. Electrophoresis gels with the bovine papillomavirus type-specific PCR results. Lines 1–4 represent the samples selected randomly, that are not necessarily the same for each reaction. A molecular marker (MM) of 100 bp was used.

obtained branches, 1000 non-parametric bootstrap replicates were used. Accession number of published sequences mentioned herein is given in Supporting Information Table S2.

Results

Viral distribution

All BPV types included in this study were detected in the samples, except for BPV7 (Figs 1 and 2). BPV2 and BPV3 were the most frequently found viral types. DNA of three recently described BPV types, BPV8, BPV9 and BPV10, was also detected. The co-infection was observed with up to four different BPV types (Fig. 3 and Table 1). A total of 64/72 (89%) skin samples were co-infected with at least two BPV types.

New BPV11 subtype

Sequence analyses indicated the presence of two isolates (BPV/UFPE01 and BPV/UFPE02) of a putative new BPV11 subtype. The identity between the BPV/UFPE01 isolate and BPV11 was 97%, and between the BPV/UFPE02 isolate and BPV11 was 96%, indicating that they are BPV11 subtypes. Between the two new isolates, variations were detected only in two nucleotide sites, producing 99% identity.

Phylogenetic analysis

The final sequence alignment including the sequence of the novel BPV11 subtype isolates display 491 nucleotide, with 115 (approximately 23%) conserved and 373 (approximately 76%) variable sites. Of the variable sites, 348 were parsimony informative and 25 sites were singletons. Three sites were present in only one sequence and they were not computed.

The results shown in Fig. 4 confirmed that both isolates belong to the same viral type, with 86% of confidence based on bootstrap. The phylogenetic analysis showed that these isolates clustered together with the recently described BPV11, confirming that they were new subtypes of this viral type. These two novel isolates are

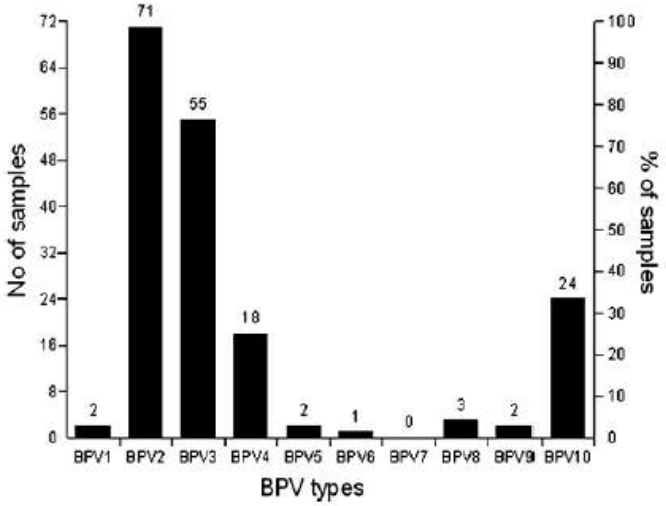

Fig. 2. Distribution of bovine papillomavirus types detected in 72 skin lesions.

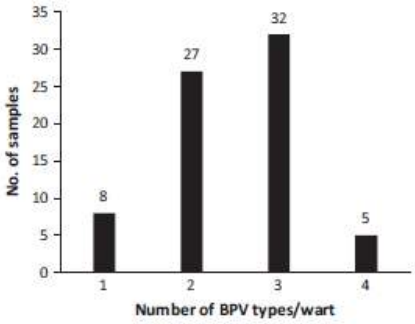

Fig. 3. Number of samples infected associated with the number of bovine papillomavirus types detected.

Table 1. Percentage of samples infected with one or more bovine papillomavirus (BPV) types

Viral types present	Absolute frequency	Relative frequency (%)
BPV types 1, 2 and 3	2	2.8
BPV types 2 and 10	3	4.2
BPV types 2 and 3	21	29.1
BPV types 2, 3 and 10	13	18.1
BPV types 2, 3 and 4	10	13.8
BPV types 2, 3, 4 and 10	4	5.6
BPV types 2, 3 and 5	2	2.8
BPV types 2, 3 and 8	1	1.4
BPV types 2, 3 and 9	1	1.4
BPV types 2, 3, 9 and 10	1	1.4
BPV types 2 and 4	3	4.2
BPV types 2, 4 and 8	1	1.4
BPV types 2, 6 and 10	1	1.4
BPV types 2, 8 and 10	1	1.4
BPV type 10	1	1.4
BPV type 2	7	9.6
Total	72	100

also closely related to BPV4, and to the strains BPV/BR-UEL2 and BPV/BR-UEL3 described by Claus et al. (2008). The isolates described in this study were clustered together in the genus *Xipapillomavirus*, along with BPV3, BPV4, BPV6, BPV9, BPV10 and 11 (Fig. 4).

The majority of the branches were statistically well supported with 50% of confidence. Although some statistical inconsistencies in the clade representative of the genus *Xipapillomavirus* were noticed, the branch that corresponds to the putative new BPV11 subtype was well supported with 68% of confidence (Fig. 4).

Discussion

In this study, the presence of different types of BPVs in skin lesions of cattle affected by papillomatosis was evaluated. This is the first study using specific primers to detect 10 different BPV types. This strategy led the identification of co-infection in cattle. Besides, two putative new BPV11 subtypes were also detected, showing the diversity of this virus.

BPV1 is a viral type often found in skin lesions (Freitas et al., 2003, 2007). In the present study, this viral type was detected in lesions located in the back and dewlap of two animals. Interestingly, there was a high incidence of BPV2 and BPV3, described as characteristic of skin lesions (Campo and Jarret, 1994). So far, BPV3 is rarely reported in the world, because the majority of studies about BPV infection focus on BPV1 and BPV2. In this study, BPV3 was found in the majority of lesions. BPV4 is a viral type related to mucosal infections and it is responsible for upper digestive tract cancer in cattle. Here, BPV4 was detected in skin lesions (Supporting Information Figure S1). Although unusual, its presence in cutaneous epithelium was earlier reported (Bloch et al., 1996). Nevertheless, the role of BPV4 in skin lesion remains to be elucidated. BPV5 and BPV6 are usually associated with skin lesions in teats and udder. In this study, BPV5 and BPV6 were detected in lesions of the shoulder clod and around the eye of the animal, unusual sites for these viral types. BPV7 was not detected in this study. However, recently described BPV8, BPV9 and BPV10 were detected suggesting that BPV7 was not present in the samples. BPV8, BPV9 and BPV10 were described in cutaneous lesions in Japan (Tomita et al., 2007; Hatama et al., 2008) and after found in Brazil – BPV8 (Claus et al., 2009a) and India – BPV10 (Rai et al., 2011). BPV9 and BPV10 have not been detected in the American continent yet. So far, the carcinogenic potential of these three recently described viral types remains to be determined, and further studies on the pathobiological significance of these viruses are needed.

The occurrence of co-infections was observed in several examined lesions (Table 1 and Fig. 2). Earlier co-infection has been detected in cutaneous lesions with different BPV types in Brazil (Yaguiu et al., 2006; Claus et al., 2007, 2009b; Lindsey et al., 2009). Other studies demonstrated the presence of co-infection with BPV (Leishangthem et al., 2008; Pangty et al., 2010; Schmitt et al., 2010). This is the first study showing co-infections of BPV types 1–10 by using specific primers. At least two viral types were verified in the majority of lesions. There is no information in literature in relation to the consequences of co-infection in the animals. The constant presence of diverse lesions over the body could be a consequence of co-infection. Possibly, co-infection could prevent the regression of the lesions because of suppression of immunological mechanisms.

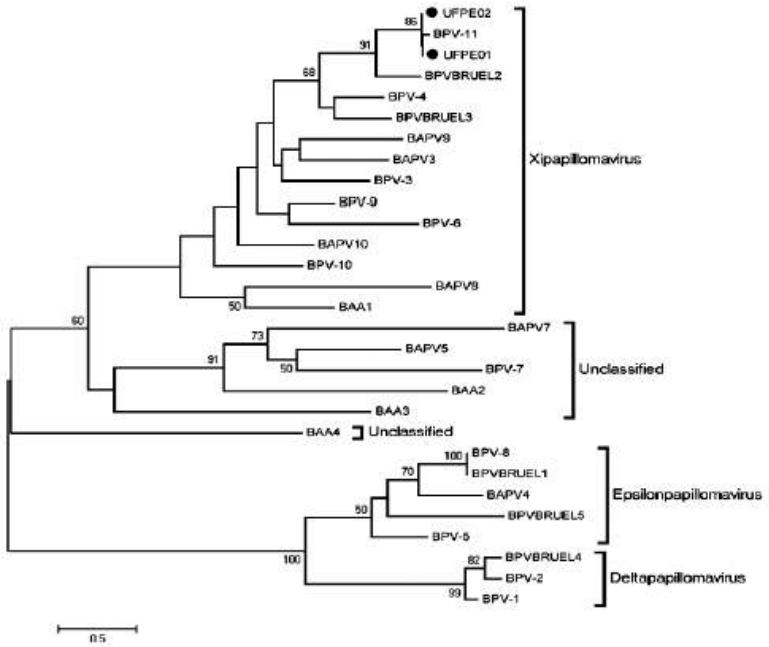

Fig. 4. Maximum Likelihood tree of bovine papillomavirus, comprising the 11 characterized types and 18 putative new types, based on partial sequences of L1 ORF. Four groups of viruses are distinguished, which form the previously described genera (*Xipapillomavirus*, *Deltapapillomavirus*, *Epsilonpapillomavirus* and Unclassified). Numbers in the nodes are bootstrap support values of the branches determined by 1000 replicates, and the values below 50% are not shown. Circles represent isolates bovine papillomavirus/UFPE01 and BPV/UFPE02 identified in this study.

The use of PCR and sequencing techniques led to the identification of two putative novel BPV11 subtypes (isolates BPV/UFPE01 and BPV/UFPE02). The BPV11-like isolates were detected in lesions from three different animals. Lesion morphologies are shown in Supporting Information Figure S2. Two of the animals showed lesions on the back and around the eyes, respectively. However, at this point, it is not possible to ascertain whether such lesion morphology is characteristic of BPV11, because the animal was co-infected with BPV2 and BPV11. Studies related to genetic diversity and evolution of BPVs are sparse. Despite the recognition of over a hundred types of HPV, only eleven BPV types have been described reflecting the scarcity of studies in this area. As far as we are aware, only few studies have been conducted with the detection of putative new BPV types in Brazil (Claus et al., 2008; Silva et al., 2010). These data suggest that other BPV types may also be present and known types may be more widely distributed than originally thought. The genetic diversity of BPV could explain the complex biology of this virus. Although still unknown, the identification of novel subtypes and variants of BPV could indicate different pathological aspects. Additional studies aimed at detecting and characterizing potential new viral types and their variants are needed.

This is the first study evaluating the BPV diversity in Brazilian cattle, assessing the presence of eleven different BPV types. Ten of 11 BPV types were identified, and the presence of a novel BPV11 subtype has been reported for the first time, indicating that BPV11-like viruses also occur in South America. In addition, the detection of viral types in unusual sites was also reported. Thus, our data suggest that certain viral types are not restricted to specific lesions as previously thought. Further molecular epidemiological investigations on the incidence and diversity of BPV infection in cattle will assist in establishing a more accurate view of the distribution of this virus. This could help the development of accurate and specific diagnostics, prophylactics and therapeutic requirements.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative lesion of BPV4 infection on the back of one animal.

Figure S2. (A) Representative lesion of the putative new BPV11 subtype infection. (B) The same lesion extracted and in detail.

Table S1. Primers used in BPV genotyping.

Table S2. Genbank accession number and size of the sequences used in this analysis.

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6. Capítulo III

Entropy-based approach for selecting informative regions in the L1 gene of bovine papillomavirus for phylogenetic inference and primer design

M.V.A. Batista, A.C. Freitas, V.Q. Balbino

Departamento de Genética, Universidade Federal de Pernambuco, Brasil.

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M.V.A. Batista, A.C. Freitas and V.Q. Balbino

Departamento de Genética, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife, PE, Brasil

Corresponding author: M.V.A. Batista
E-mail: mvabatista@hotmail.com

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ABSTRACT. Bovine papillomaviruses (BPVs) cause many benign and malignant lesions in cattle and other animals. Twelve BPV types have been identified so far, and several putative novel BPV types have been detected based on the analysis of L1 gene fragments, generated by FAP59/64 and MY11/09 primers. Phylogenetic trees are important in studies that describe novel BPV types. However, topological mistakes could be a problem in such studies. Therefore, we made use of entropy to find phylogenetic informative regions in the BPV L1 gene sequences from all 12 BPVs. Six data sets were created and phylogenetically compared to each other using neighbor-joining and maximum likelihood methods of phylogenetic tree reconstruction. We found two major regions in the L1 gene, using an entropy-based approach, which selects regions with low information complexity. More robust phylogenetic trees were obtained with these regions, when compared to the ones obtained with FAP59/64 and MY11/09 primers. More robust phylogenetic trees are important to accurately position novel BPV types, subtypes and variants. We conclude that an entropy-based approach is

a good methodology for selecting regions of the L1 gene of BPVs that could be used to design more specific and sensitive degenerate primers, for the development of improved diagnostic methods.

Key words: Bovine papillomavirus; L1 gene; Phylogenetic analysis; Entropy

INTRODUCTION

Papillomaviruses (PVs) form a diverse group of non-enveloped viruses with a circular double-stranded DNA that infects a wide variety of hosts. The genome is approximately 8 kb in size and contains around eight genes. The L1 gene encodes the major capsid protein and has been used to classify PVs into genera, species, types, subtypes, and variants (de Villiers et al., 2004; Bernard et al., 2010). Among PVs, bovine papillomaviruses (BPVs) have a major role in veterinary medicine. They cause benign and malignant lesions in cattle and are associated with equine, zebra, and buffalo lesions (Lörh et al., 2005; Silvestre et al., 2009; van Dyk et al., 2009; Bogaert et al., 2010; Somvanshi, 2011).

To date, 12 BPV types have been identified and classified into three genera (Deltapapillomavirus, Epsilonpapillomavirus, and Xipapillomavirus); one of them remains unassigned (Campo, 2006; Ogawa et al., 2007; Tomita et al., 2007; Hatama et al., 2008, 2011; Zhu et al., 2012). Furthermore, several putative novel BPV types and subtypes have been detected based on analyses of L1 gene fragments of approximately 450 bp (Antonsson and Hansson, 2002; Ogawa et al., 2004; Claus et al., 2008; Carvalho et al., 2012). These fragments have been generated using FAP59/64 or MY11/09 primers designed to detect human PVs (Manos et al., 1989; Forslund et al., 1999). The DNA sequence variability between human PVs and BPVs is considerable; thus, the sensitivity of these primers could be compromised.

Every study describing the detection or characterization of novel BPVs uses a phylogenetic tree to classify isolates into genera and prove statistically that they are novel types. However, the observed trees are not as robust as they should be, which is of central concern because topological bias could be inserted in the analysis, causing possible interpretation/classification errors.

A successful entropy-based approach has recently been described for the selection of phylogenetic informative genomic regions in PVs (Batista et al., 2011). The aim of this study was to make use of entropy to find new regions in the L1 gene of BPVs that are more suitable for phylogenetic inferences.

MATERIAL AND METHODS

The analysis was carried out using L1 gene sequences of the 12 BPVs characterized thus far. Sequences were retrieved from GenBank database and aligned using the Muscle algorithm incorporated in Molecular Evolutionary Genetics Analysis version 5 (Tamura et al., 2011). The GenBank accession Nos. are BPV1 (X02346), BPV2 (M20219), BPV3 (NC_004197), BPV4 (X05817), BPV5 (NC_004195), BPV6 (AJ620208), BPV7 (DQ217793), BPV8 (NC_009752), BPV9 (NC_010192), BPV10 (NC_010193), BPV11 (AB543507), and BPV12 (JF834523).

A total of six data sets were created for comparison. First, the complete L1 gene was used. Second, the phylogenetically most informative regions were selected using an entropy-

based approach described by Batista et al. (2011). A cutoff value of 1.0 was used, and every nucleotide site with an average entropy value under this cutoff was selected (total entropy). The third data set was the region defined by FAP59/64 primers. Fourth, the region defined by MY11/09 primers was used. The fifth data set was a 768-bp region obtained using the entropy-based approach (entropy region 1). The sixth data set was a 540-bp region also obtained using the entropy-based approach (entropy region 2).

For the phylogenetic analysis, the jModelTest 0.1.1 software (Posada, 2008) was used to select the model that best fit each data set. The models were selected under the Bayesian Information Criterion. The nucleotide substitution model selected for the complete L1 gene, total entropy, entropy region 1, and FAP59/64 region sequence alignments was GTR+G. For the MY11/09 region and entropy region 2, the substitution models were TPM3uf+G and TPM3uf+I+G, respectively.

The neighbor-joining method was used to reconstruct BPV trees in Molecular Evolutionary Genetics Analysis version 5 (Tamura et al., 2011). Maximum likelihood trees were created for each data set using the best fitting nucleotide substitution model in PhyML 3.0 (Guindon et al., 2010). Five substitution rate categories were used. The tree topology search was carried out with an algorithm developed with the best of the nearest-neighbor interchange and subtree pruning and regrafting methods. The quartet measures of Component 2.0 (Page, 1989) were used to compare the obtained topologies. The complete L1 gene tree was used as the template, and all other trees were compared to it. In addition, the confidence values of the nodes were compared.

RESULTS

The entropy-based approach uncovered five regions with low entropy values ($H \leq 1.0$), which are the phylogenetically most informative regions (Figure 1). Two regions with low entropy were selected to assess their phylogenetic potential. Compared with the FAP59/64 region, entropy region 1 was 278 bp longer. In addition, entropy region 2 was 70 bp longer than the MY11/09 region.

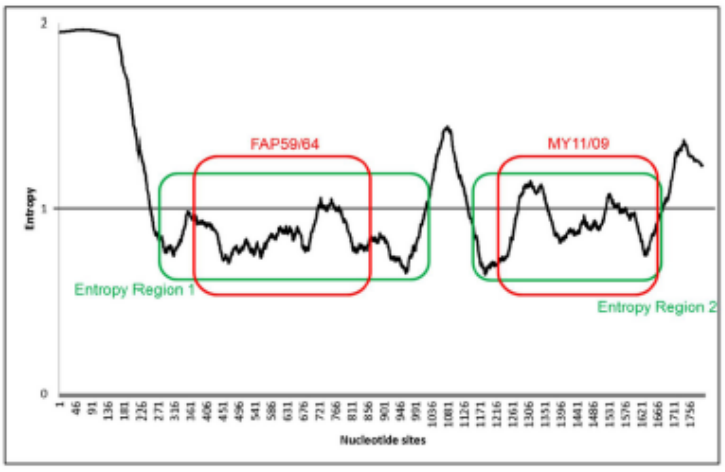

Figure 1. Entropy per site plot of the bovine papillomavirus L1 gene. Regions with entropy values equal to and below 1.0 bits were selected to the phylogenetic inference. Green boxes indicate two entropy regions analyzed in this study. Red boxes indicate regions comprised by FAP59/64 and MY11/09 primers.

Phylogenetic analysis using both methods for reconstructing trees showed that the trees using the complete L1 gene were more robust than the others (Figures 2 and 3). In general, clusters corresponding to genera could be identified but not without some changes inside these groups. However, the maximum likelihood tree of the complete L1 gene was more consistent, with higher confidence values. Although the trees constructed using the total entropy region were slightly less robust than those constructed with the complete L1 gene, their topology was very similar (see Figures 2 and 3). The quartet method showed that the smallest values of topological distance from the complete L1 gene for neighbor-joining and maximum likelihood trees were 0.032 and 0.051, respectively (Table 1).

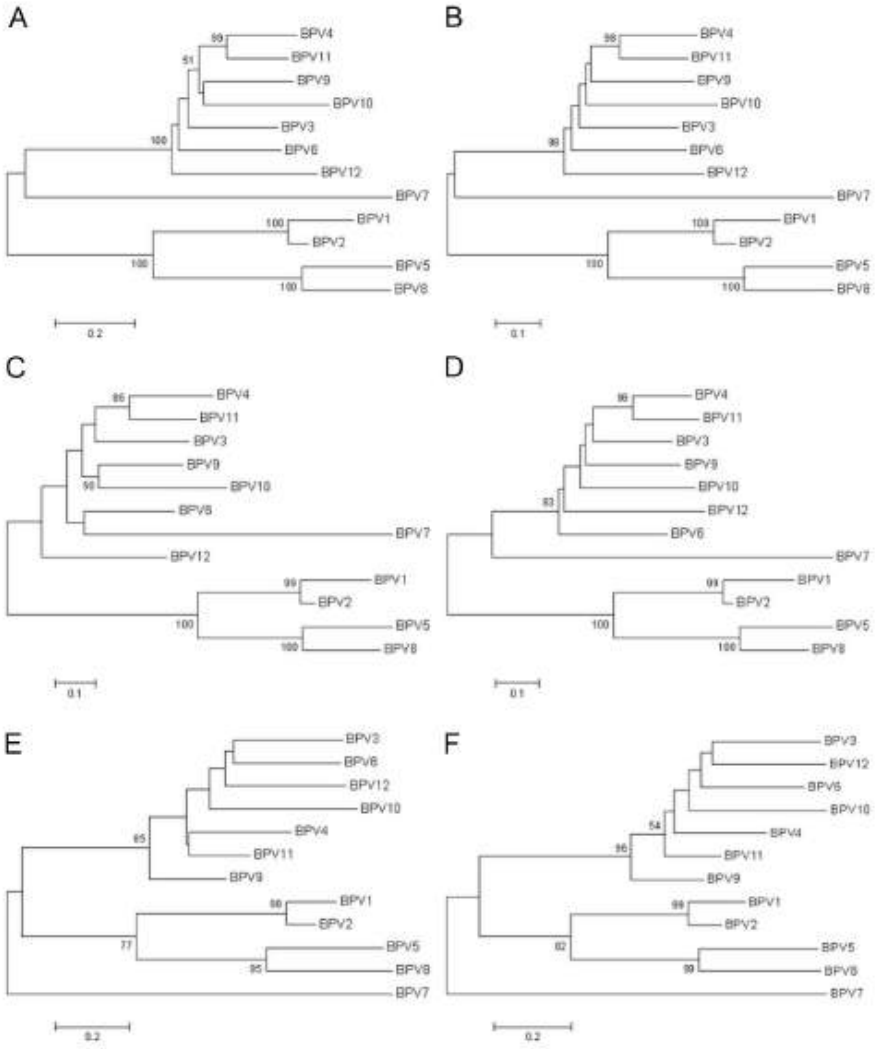

Figure 2. Phylogenetic trees of 12 bovine papillomaviruses inferred by the neighbor-joining method. Different data sets were used: **A.** the complete L1 gene; **B.** total entropy; **C.** FAP59/64 region; **D.** entropy region 1; **E.** MY11/09 region; **F.** entropy region 2. Bootstrap values under 50% are not shown. Tree branch length is in scale.

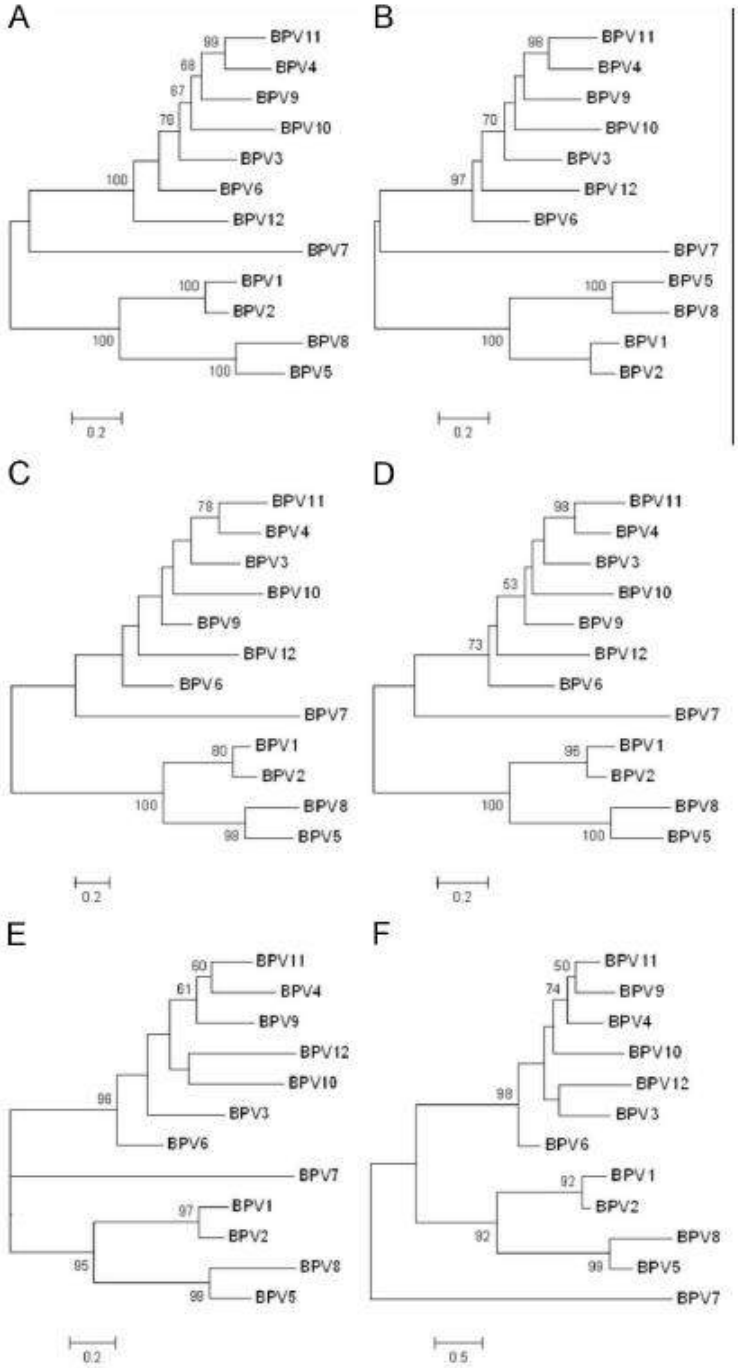

Figure 3. Phylogenetic trees of 12 bovine papillomaviruses inferred by the maximum likelihood method. Different data sets were used: **A.** the complete L1 gene; **B.** total entropy; **C.** FAP59/64 region; **D.** entropy region 1; **E.** MY11/09 region; **F.** entropy region 2. Bootstrap values under 50% are not shown. Tree branch length is in scale.

Table 1. Topological comparisons among the obtained trees.

Comparison		SD-NJ	SD-ML	s-NJ	d-NJ	s-ML	d-ML
Entropy	Complete L1	0.032	0.051	479	16	470	25
Entropy region 1	Complete L1	0.149	0.154	421	74	419	76
	Entropy	0.125	0.103	433	62	444	51
Entropy region 2	Complete L1	0.372	0.117	311	184	437	58
	Entropy	0.360	0.067	317	178	462	33
	Entropy region 1	0.354	0.170	320	175	411	84
FAP	Complete L1	0.156	0.154	418	77	419	76
	Entropy	0.188	0.103	402	93	444	51
	Entropy region 1	0.172	0.000	410	85	495	0
	Entropy region 2	0.455	0.170	270	225	411	84
MY	Complete L1	0.313	0.141	340	155	425	70
	Entropy	0.301	0.091	346	149	450	45
	Entropy region 1	0.315	0.184	339	156	404	91
	Entropy region 2	0.067	0.109	462	33	441	54
	FAP	0.400	0.184	297	198	404	91

Dissimilarity values: SD = symmetric difference; NJ = neighbor-joining tree; ML = maximum likelihood tree; s = resolved and same; d = resolved and different.

Comparison of the neighbor-joining trees from the FAP59/64 region and entropy region 1 showed that entropy region 1 presented a more robust topology, which was confirmed with the quartet method (see Figure 2 and Table 1). Neighbor-joining trees from the MY11/09 region and entropy region 2 were also compared and were similar (see Figure 2). This result was confirmed with the quartet measure that showed a topology distance of 0.067 (see Table 1). However, the tree for entropy region 2 showed bootstrap values slightly higher than those of the MY11/09 region.

We observed that the FAP59/64 region and entropy region 1 presented the same topology when the maximum likelihood trees were analyzed (see Figure 3). The confirmation was obtained with the quartet measure that showed no topology distance between them (see Table 1). However, the tree for entropy region 1 had higher confidence values, indicating a more robust phylogenetic tree. The tree for entropy region 2 had a topology more similar to that of the complete L1 gene than to that of the MY11/09 region (see Figure 3). The quartet measure showed that entropy region 2 had a topology distance value of 0.117 to the complete L1 gene tree, whereas the MY11/09 region had a value of 0.141 (see Table 1).

DISCUSSION

In this study we assessed and proposed novel regions in the L1 gene of BPVs to make phylogenetic inferences based on partial sequences. An entropy-based approach was used to select those regions, and they were identified as phylogenetically informative based on a previous study (Batista et al., 2011). The analysis supports the idea that new L1 gene regions should be taken into account in studies that aim to detect novel BPV types using degenerate primers.

Phylogenetic analysis based on those regions showed that they are more informative than the regions determined using FAP59/64 and MY11/09 primers. These primers are widely used in studies that describe the detection or characterization of novel BPV types and subtypes (Antonsson and Hanson, 2002; Ogawa et al., 2004; Claus et al., 2008; Hatama et al., 2008; Carvalho et al., 2012). Because those studies use a phylogenetic tree to classify isolates into a genus and statistically prove that they are indeed novel types, the use of phylogenetically more

informative regions in the L1 gene of BPVs is critical for increased accuracy.

The two regions assessed in this study (entropy regions 1 and 2) comprised the regions of the primers FAP59/64 and MY11/09. However, the results showed that the increase in length of these regions, as suggested by the entropy approach, improved the phylogenetic signal. In addition, the final size of the two entropy regions is appropriate for any polymerase chain reaction and sequencing reactions, which makes them suitable in a BPV detection system.

Robust phylogenetic trees were obtained from the low entropy regions of the BPV L1 gene. These trees had very small topological distances to the complete L1 gene trees. This result showed that the approach proposed by Batista et al. (2011) is suitable for the selection of phylogenetically informative regions of the BPV L1 gene. Although the complete L1 gene trees were more robust than the entropy-based ones, we obtained very similar topologies with fewer data using this approach. Another interesting discovery was that the trees obtained using the maximum likelihood method was more robust than those constructed with the neighbor-joining method. Even though probabilistic methods are known to be more accurate than distance-based methods, many BPV detection or characterization studies still use the neighbor-joining method to reconstruct BPV phylogeny (Antonsson and Hanson, 2002; Ogawa et al., 2004, 2007; Tomita et al., 2007; Claus et al., 2008; Hatama et al., 2008, 2011; Lunardi et al., 2010; Zhu et al., 2012).

The fact that entropy region 1 was 278 bp longer than the FAP59/64 region and entropy region 2 was 70 bp longer than the MY11/09 region explains the improved topologies obtained. This result is in accordance with the idea that increasing sequence length is a good way to improve the support, resolution, and accuracy of phylogenetic inference, as suggested by Wortley et al. (2005). Entropy region 1 had 174 more parsimony informative sites than FAP59/64 does, and entropy region 2 had 37 more parsimony informative sites than MY11/09 has. These results show that the entropy approach not only extended the regions but also increased the information that was available for phylogenetic inference. However, the contribution of those sites to the phylogenetic trees was more evident in the neighbor-joining trees.

The entropy-based approach used in this study selected phylogenetically more informative regions in the L1 gene of BPVs. Two entropy regions were analyzed and compared to regions of established degenerate primers. In general, the two entropy regions were associated with more robust phylogenetic trees, which are important for positioning novel BPV types, subtypes, and variants accurately. This issue is central to BPV detection and characterization studies. The results of this study point to a solid methodology for the selection of regions in the L1 gene of BPVs, which could be applied to the design of novel degenerate primers with greater specificity and sensitivity for detecting those viruses. This finding is significant for the development of improved diagnostic methods and, consequently, the establishment of a surveillance program to identify the incidence and distribution of BPVs.

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7. Capítulo IV

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

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

[a] *Department of Genetics, Federal University of Pernambuco, Brazil.*

[b] *Department of Veterinary Medicine, Rural Federal University of Pernambuco, Brazil.*

[c] *Department of Pathology and Animal Health, University of Naples Federico II, Italy.*

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

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

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

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ABSTRACT

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

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

Introduction

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

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

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

Although 12 BPV genomes have been identified it is likely that there are far more – especially when it is taken into account that more than 150 human papillomavirus (HPV) genomes are known. PCR assays conducted with degenerate primers followed by

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

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

Materials and methods

Study population

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

Histopathology

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

DNA extraction

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

Detection of viral DNA and genotyping

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

Identification of a putative new BPV type

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

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

Phylogenetic analysis

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

Results

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

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

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

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

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

The phylogenetic tree confirmed that the BPV/UFPE04BR isolate belongs to a new viral type, with 99% of confidence based on bootstrap (Fig. 3). This isolate is related to the BPV1, BPV2 and BPV/BRUEL4 isolates described by Claus et al. (2008), which suggests that it belongs to the *Deltapapillomavirus* genus. The majority of

Table 1
Distribution of bovine papillomavirus (BPV) types found in cutaneous lesions in cattle from Brazil. Geographical, animal and lesion information is available for some samples. Missing information is indicated as NA.

Samples	State	Herd	Animal	Age category	Gender	Lesion morphology	Lesion location	BPV types
1	Pernambuco	Dairy	1	Adult	Female	Cauliflower	NA	2, 3 and 4
2	Pernambuco	Dairy	2	Adult	Female	Cauliflower	NA	2, 3 and 10
3	Pernambuco	Dairy	3	Adult	Female	Cauliflower	NA	2 and 3
4	Pernambuco	Dairy	4	Adult	Female	Cauliflower	NA	2 and 6
5	Pernambuco	Dairy	5	Adult	Female	Cauliflower	NA	2 and 4
6	Pernambuco	Dairy	6	Adult	Female	Cauliflower	NA	2 and 3
7	Pernambuco	Dairy	7	Adult	Female	Cauliflower	NA	2 and 3
8	Pernambuco	Dairy	8	Adult	Female	Cauliflower	NA	2 and 10
9	Pernambuco	Dairy	9	Adult	Female	Cauliflower	NA	2 and 3
10	Pernambuco	Dairy	10	Adult	Female	Cauliflower	NA	2 and 3
11	Pernambuco	Dairy	11	Adult	Female	Cauliflower	NA	2 and 3
12	Pernambuco	Dairy	12	Adult	Female	Cauliflower	NA	2 and 3
13	Pernambuco	Dairy	13	Adult	Female	Cauliflower	NA	2, 3 and 4
14	Pernambuco	Dairy	14	Adult	Female	Cauliflower	NA	10
15	Pernambuco	Dairy	15	Adult	Female	Cauliflower	NA	2, 8 and 10
16	Pernambuco	Dairy	16	Adult	Female	Cauliflower	NA	2, 3 and 10
17	Pernambuco	Dairy	17	Adult	Female	Cauliflower	Back	2 and 3
18	Pernambuco	Dairy	17	Adult	Female	Cauliflower	Udder	2, 3 and 9
19	Pernambuco	Dairy	18	Adult	Female	Cauliflower	Shoulder	2, 3 and 10
20	Pernambuco	Dairy	18	Adult	Female	Cauliflower	Right eye	2, 3 and 10
21	Pernambuco	Dairy	18	Adult	Female	Cauliflower	Udder	2 and 3
22	Pernambuco	Dairy	19	Adult	Female	Cauliflower	Shoulder	2, 3 and 5
23	Pernambuco	Dairy	19	Adult	Female	Cauliflower	Back	2, 3 and 5
24	Pernambuco	Dairy	20	Adult	Female	Cauliflower	Neck	2, 3, 4 and 10
25	Pernambuco	Dairy	20	Adult	Female	Cauliflower	Muzzle	2, 3, 4 and 10
26	Pernambuco	Dairy	21	Adult	Female	Cauliflower	Dewlap	2, 3, 4 and 10
27	Pernambuco	Dairy	21	Adult	Female	Cauliflower	Back	2, 3, 9 and 10
28	Pernambuco	Dairy	21	Adult	Female	Cauliflower	Hind hooves	2, 3 and 4
29	Pernambuco	Dairy	22	Adult	Female	Cauliflower	Ear	2 and 3
30	Pernambuco	Dairy	22	Adult	Female	Cauliflower	Neck	2 and 3
31	Pernambuco	Dairy	23	Adult	Female	Cauliflower	Scapula	2, 3 and 4
32	Pernambuco	Dairy	24	Adult	Female	Cauliflower	Udder	2 and 3
33	Pernambuco	Dairy	24	Adult	Female	Cauliflower	Back	2, 3 and 4
34	Pernambuco	Dairy	25	Adult	Female	Cauliflower	Neck	2, 3 and 4
35	Pernambuco	Dairy	25	Adult	Female	Cauliflower	Back	2 and 3
36	Pernambuco	Dairy	26	Adult	Female	Cauliflower	Neck	2 and 3
37	Bahia	Beef	27	Adult	Female	Cauliflower	Udder	2, 3 and 10
38	Bahia	Beef	27	Adult	Female	Cauliflower	Back	2 and 10
39	Bahia	Beef	28	Adult	Female	Cauliflower	Back	2, 3 and 8
40	Bahia	Beef	29	NA	NA	NA	NA	2, 3 and 10
41	Bahia	Beef	30	NA	NA	NA	NA	2 and 3
42	Bahia	Beef	31	Young	Male	Flat	Head	2, 3 and 4
43	Bahia	Beef	31	Young	Male	Flat	Back	2, 3 and 4
44	Bahia	Beef	32	Young	Male	Flat	Back	2, 3 and 4
45	Bahia	Beef	33	Young	Male	Flat	Head	2, 3, 4 and 10
46	Bahia	Beef	34	Young	Male	Peduncle	Dewlap	2, 3 and 4
47	Bahia	Beef	35	Adult	Female	Cauliflower	Dewlap	2, 3 and 10
48	Bahia	Beef	36	Adult	Female	Cauliflower	Dewlap	1, 2 and 3
49	Bahia	Beef	37	Adult	Female	Cauliflower	Head	2, 3 and 10
50	Bahia	Beef	38	Adult	Female	Cauliflower	Back	1, 2 and 3
51	Bahia	Beef	39	Adult	Female	Flat	Dewlap	2 and 3
52	Bahia	Beef	40	Adult	Female	Cauliflower	Back	2, 3 and 10
53	Bahia	Beef	41	Adult	Female	Cauliflower	Back	2 and 10
54	Bahia	Beef	42	Young	Male	Cauliflower	Dewlap	2, 3 and 10
55	Bahia	Beef	43	Adult	Female	Cauliflower	Back	2, 3 and 10
56	Bahia	Beef	44	Adult	Female	Peduncle	Back	2 and 3
57	Bahia	Beef	45	Adult	Female	Peduncle	Dewlap	2, 3 and 10
58	Bahia	Beef	46	Young	Male	Cauliflower	Head	2, 3 and 10
59	Bahia	Beef	47	Young	Male	Flat	Back	2 and 3
60	Bahia	Beef	48	Young	Male	Flat	Dewlap	2, 3 and 10
61	Bahia	Beef	49	Young	Male	Peduncle	Head	2
62	Bahia	Beef	50	Adult	Female	Peduncle	Head	2
63	Bahia	Beef	51	Young	Male	Atypical	Dewlap	2 and 3
64	Bahia	Beef	52	Young	Male	Atypical	Head	2 and 3
65	Bahia	Beef	53	Young	Male	Peduncle	Dewlap	2
66	Bahia	Beef	54	NA	NA	NA	NA	2
67	Bahia	Beef	55	Young	Male	NA	Back	2
68	Bahia	Beef	56	Young	Male	NA	Back	2 and 4
69	Bahia	Beef	57	Young	Male	NA	Dewlap	2, 4 and 8
70	Bahia	Beef	58	Young	Male	NA	NA	2
71	Bahia	Beef	59	Adult	Male	NA	Dewlap	2 and 4
72	Bahia	Beef	60	NA	NA	NA	NA	2

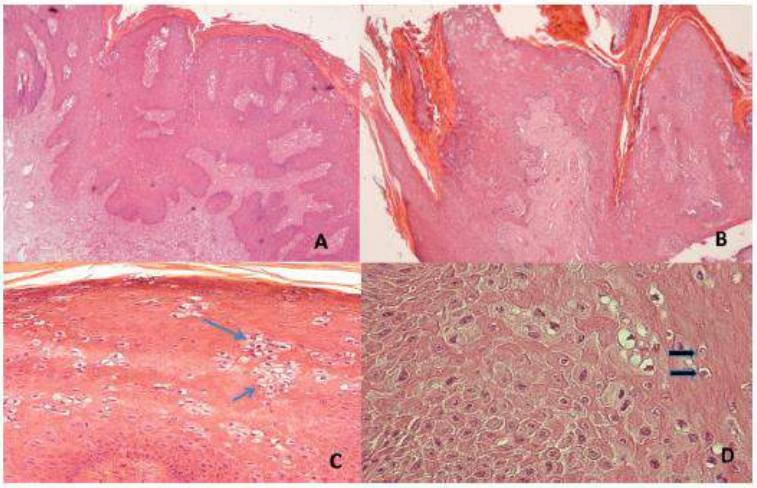

Fig. 1. Histological analysis of bovine skin fibropapilloma. Figures (A and B) illustrate epidermal hyperplasia and fibrovascular stroma with the stratum corneum exhibiting hyperkeratosis. In figures (C and D) koilocytes are indicated by arrows.

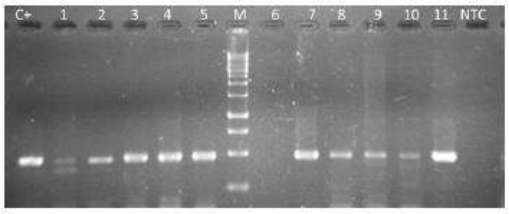

Fig. 2. Amplification of BPV DNA in skin lesions. A representative electrophoresis gel of PCR products using FAP59/64 primers is shown. Lanes 1–11, samples; C+, positive control; NTC, no template control; M, 1Kb bp molecular weight marker.

the branches were statistically well-supported with 50% confidence. In addition, the clade that corresponded to the *Deltapapillomavirus* genus was very well supported (Fig. 3).

Discussion

In this study, we investigated the relationship between BPV types isolated from beef and dairy cattle herds and the lesions they cause. A large number of cases of bovine papillomatosis were assessed with emphasis being laid on many different viral types. In addition, a putative new BPV type was detected, which demonstrates the great diversity of BPV.

The distribution of the BPV types did not follow any geographical pattern. It is very possible that BPV is widespread in Brazil as suggested by Claus et al. (2008). Although we only found BPV1 in Bahia State, BPV1 has been previously identified in Pernambuco State (Diniz et al., 2009; da Silva et al., 2012). In addition, there is evidence that BPV types may be widespread in all Brazilian regions, regardless of the herd, gender, and age. Although cattle of any age can be affected, calves and yearlings were shown to have a higher incidence of bovine papillomatosis (Hama et al., 1988; Freitas et al., 2011). It is likely that specific factors such as nutrition, tick infestation, age, and immune disorders are contributory factors in the dissemination of BPV.

The relationship between BPV types and lesion morphology is poorly understood. In this study, no link between BPV types and lesion morphology was observed. Histologically, all the BPV lesions were attributed to fibropapillomas. BPV types 1 and 2 were previously related to cutaneous fibropapillomas. However, BPV types 3 and 10 were linked to cutaneous papillomas and squamous epithelial papillomas, respectively (Borzacchiello and Roperto, 2008). As far as we are aware, this is the first time that BPV types 3 and 10 have been linked to fibropapillomas.

Co-infection was found in the majority of the lesions (Table 1). The presence of co-infection could be caused by a decrease in host resistance, which may include immunosuppression, as suggested by Carvalho et al. (2012). However, further studies are necessary to verify this. In addition, some BPV types were detected in different lesions of the same animal. As these viral types were found in other animals from the same farm, the animals could be reinfected several times through animal to animal contact. Another explanation is that these BPV types could be carried to other sites of the animal through blood cells, where they were previously found (Diniz et al., 2009; Roperto et al., 2011).

In this study, BPVs 8, 9 and 10 were found in several different anatomical sites. BPV8 was found on the back and dewlap. BPV8 had previously been linked to cutaneous papillomas and the healthy skin of teats, and the back (Tomita et al., 2007; Borzacchiello and Roperto, 2008; Claus et al., 2009). This is the first time that BPV8 has been found in the dewlap. In addition, in this study, BPV9 was detected on the udder and back of the animal and BPV10 on the shoulder, around the right eye, neck, muzzle, dewlap, back, udder and head. Previously, BPV9 and BPV10 were attributed to squamous epithelial papillomas of the udder and teats (Borzacchiello and Roperto, 2008; Hatama et al., 2008). There is no evidence of the detection of BPV9 and BPV10 on the back of the animal. Moreover, BPV10 was, for the first time, found on the shoulder, around the right eye, neck, muzzle, dewlap and head. All of these results show that recently described BPV types 8, 9 and 10 are not restricted to any anatomical site in cattle. Apparently, the body site where the animal is exposed to BPV infection is more significant determinant than the genotype of the virus. Of course, this is a preliminary investigation, and further studies are required to investigate this phenomenon.

Knowledge of the genetic diversity and evolution of BPVs is still in its early stages. Only 12 BPV types have been described so far, despite the great diversity found in HPV. This fact reflects the lack of studies into the question of BPV diversity. In this study, we

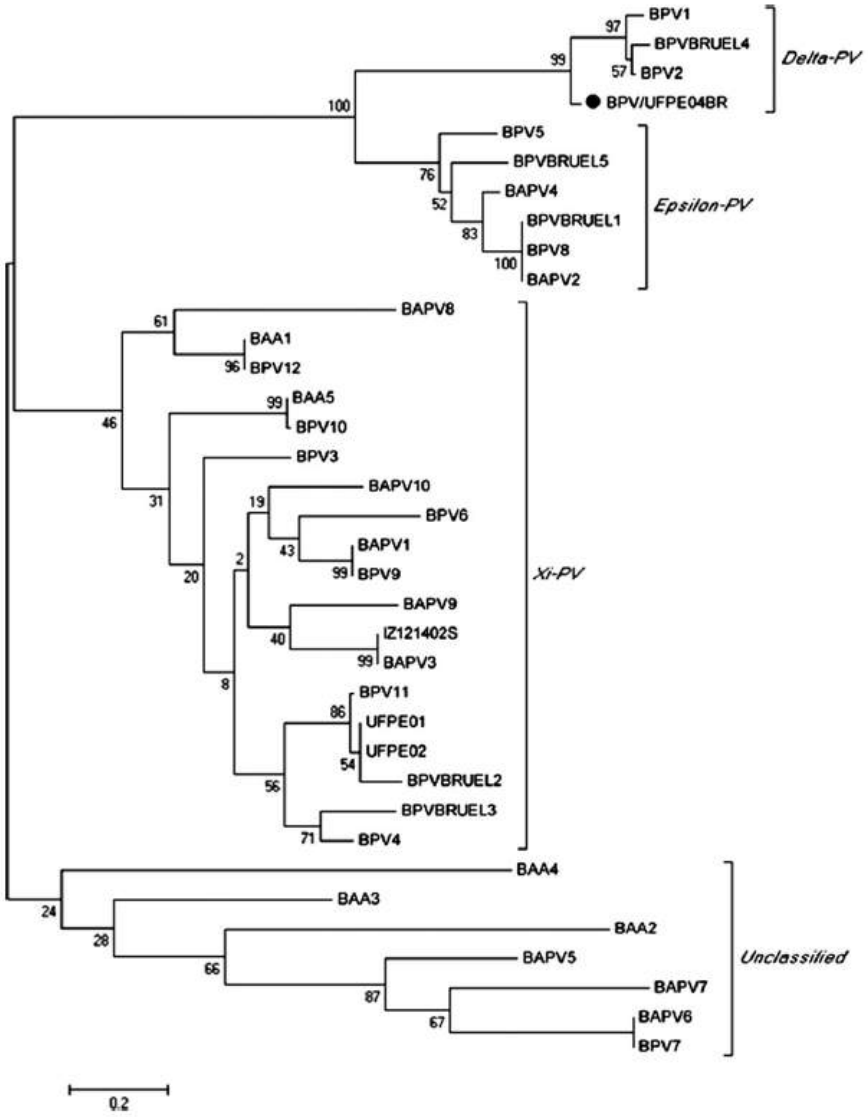

Fig. 3. Maximum Likelihood tree of bovine papillomavirus, which comprises 12 BPV types and 25 putative new BPV types, based on partial sequences of L1 ORF. Three groups of viruses are distinguished, which forms the previously described genera (*Xipapillomavirus*, *Deltapapillomavirus* and *Epsilonpapillomavirus*). Unclassified isolates are also presented. Numbers in the nodes are bootstrap support values of the branches determined by 1000 replicates, and the values below 50% are not shown. The black circle represents the isolate BPV/UFPE04BR identified in this study.

describe a putative new BPV type that belongs to the *Deltapapillomavirus* genus. Our results suggest that there is a great diversity of BPV types that infect cattle, and the understanding of this diversity is necessary for improved therapeutic treatment.

Conclusions

This study evaluated the diversity of BPV types in Brazilian cattle, and carried out an assessment of the relationship of the epidemiological information about them. It is possible that BPV types are widespread throughout the country, with no distinction between beef and dairy cattle herds. Ten BPV types were detected, and the presence of an unreported novel BPV type has also been described. In addition, we report the detection of recently described viral types (BPVs 8–10) in unusual sites. Diverse re-infection events or dissemination through blood could be the cause of BPV detection in different lesions on the same animal, and future studies should examine this evidence, which will be essential in understanding the natural history of BPV infections. Our data suggest that these BPV types are not restricted to any particular anatomical site. Further molecular epidemiological investigations on the incidence and diversity of BPV infection in cattle are now required to allow an accurate diagnosis to be carried out and the development of appropriate vaccines.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Uncited references

Bernard et al. (2010), Campo et al. (1994), Freitas et al. (2012), and Rai et al. (2011).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2013.01.019.

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8. Discussão geral

Este estudo focou na aplicação de um novo método de bioinformática que pode ser aplicado em estudos de epidemiologia e evolução molecular de papilomavírus (PV). Associado a isto, este estudo também objetivou verificar a ocorrência dos diferentes tipos de papilomavírus bovino (BPV) que infectam rebanhos na região Nordeste do Brasil.

Algumas tentativas de se obter uma visão completa da evolução dos PV foram feitas (Chan *et al.*, 1992, 1995; Bravo e Alonso, 2007; Gottschling *et al.*, 2007a, 2007b, 2011; Shah *et al.*, 2010). Entretanto, esta é a primeira vez que um método baseado em entropia foi utilizado para identificar regiões genômicas filogeneticamente mais informativas para a reconstrução das relações filogenéticas dos PV. Mais especificamente, este método pode ser aplicado para a seleção de regiões do gene *L1* de BPV importantes para inferência filogenética baseada em sequências parciais. Com isto, novas regiões deste gene podem ser propostas para ser utilizadas em estudos que visem detectar novos tipos de BPV através do uso de *primers* degenerados.

Além disso, neste estudo, a presença de diferentes tipos de BPV em lesões cutâneas de bovinos afetados por papilomatose foi avaliada. Alguns aspectos epidemiológicos importantes também foram discutidos, como padrões geográficos de distribuição, localização e morfologia das lesões. Este é o primeiro estudo a utilizar uma combinação de *primers* específicos e degenerados para detectar 10 diferentes tipos de BPV. Esta estratégia levou à identificação de coinfecção no gado, além da descoberta de novos subtipos e tipos de BPV.

Ao se comparar as sequências dos genes entre os vários PV existentes, é possível observar uma grande variabilidade. Entretanto, na análise de sequências dos PV é possível notar que existem regiões conservadas que estão relacionadas com certos domínios proteicos. Consequentemente, embora os genes dos PV tenham acumulado muitas mutações ao longo do tempo evolutivo, a função das proteínas se mantém. Assim, o método baseado em entropia desenvolvido neste estudo se torna relevante por selecionar regiões genômicas que são importantes estrutural e funcionalmente para inferir a filogenia deste grupo de vírus bastante diverso.

Outro ponto extremamente importante quando se lida com sequências bastante variáveis é a remoção de partes dos dados que perturbam a reconstrução da filogenia de um grupo de táxons. A quantidade de mutações pode ser tão grande, que os caracteres são semelhantes devido a eventos homoplásicos. Os métodos baseados em entropia de remoção destas regiões se mostraram ser mais acurados que outros métodos (Criscuolo e Gribaldo, 2010).

Com a abordagem de entropia foi possível obter topologias e comprimentos de ramos similares aos obtidos por Gottschling *et al.* (2007b), corroborando com a ideia de que a diversificação dos PV ocorre devido à múltiplos mecanismos evolutivos. Nossos resultados sugerem que existem discrepâncias entre a filogenia dos PV e a dos seus hospedeiros, e assim, a coevolução sozinha não poderia explicar a diversificação dos PV, o que é consistente com os achados de Gottschling *et al.* (2007b), Shah *et al.* (2010) e Gottschling *et al.* (2011).

Embora as árvores filogenéticas obtidas neste estudo se assemelhe com a encontrada por Gottschling *et al.* (2007b), que utilizou outro método de edição

de caracteres (Castresana, 2000), dois pontos favorecem a utilização do método aqui desenvolvido: primeiro, fomos capazes de obter uma topologia semelhante utilizando bem menos regiões do genoma dos PV, resultando em um menor custo computacional; segundo, houve congruência quando comparados filogeneticamente diferentes combinações de genes, ou seja, foi possível reconstruir as relações filogenéticas dos PV independente da combinação de genes empregada, desde que as regiões mais informativas deles sejam utilizadas.

Este método baseado em entropia foi utilizado para encontrar regiões informativas no gene *L1* de BPV. As análises filogenéticas mostraram que estas regiões possui mais sinal filogenético que as regiões delimitadas pelos *primers* FAP59/64 e MY11/09, que são amplamente utilizados em estudos que descrevem a detecção ou caracterização de novos tipos e subtipos de BPV (Antonsson e Hanson, 2002; Ogawa *et al.*, 2004; Claus *et al.*, 2008; Hatama *et al.*, 2008; Carvalho *et al.*, 2012). Como estes estudos utilizam árvores filogenéticas para classificar os isolados em um gênero e provar estatisticamente que são realmente novos tipos, o uso das regiões mais informativas do gene L1 de BPV é crítico para uma maior acurácia das inferências.

As regiões selecionadas pela entropia incluem as regiões delimitadas pelos *primers* FAP59/64 e MY11/09. Entretanto, o aumento no tamanho destas regiões, como sugerido pela abordagem da entropia, melhorou o sinal filogenético, fazendo com que menos erros topológicos ocorram. Além disso, o tamanho final destas regiões selecionadas é apropriado para qualquer reação de PCR e sequenciamento, tornando-as adequadas para um sistema de detecção de BPV.

O fato das regiões selecionadas pelo método baseado em entropia serem maiores que as delimitadas pelos *primers* FAP59/64 e MY11/09 explica as topologias melhoradas, o que está de acordo com a ideia que aumentar o tamanho da sequência é uma boa maneira de melhorar o suporte, a resolução e a acurácia da inferência filogenética, como sugerido por Wortley *et al.* (2005). Esta nova região aumentou bastante o número de sítios informativos para parcimônia, mostrando que a abordagem baseada em entropia não apenas ampliou as regiões, mas aumentou a informação filogenética associada.

Por outro lado, a avaliação da diversidade genética dos BPV que infectam rebanhos no Nordeste do Brasil se inicia com a detecção dos tipos virais nas lesões. Como dados epidemiológicos acerca da papilomatose bovina são bastante escassos, é importante conhecer mais sobre a biologia da infecção dos BPV. Neste contexto, este estudo revelou alguns aspectos novos relacionados com o ciclo de infecção dos BPV, como dados de ocorrência de tipos pouco encontrados no mundo e a detecção de tipos de BPV em sítios incomuns dos animais.

Neste estudo foi possível identificar uma alta ocorrência de BPV tipos 2 e 3. Até então, BPV-3 era raramente relatado no mundo, uma vez que a maioria dos estudos focava em BPV-1 e BPV-2. BPV-4 é um tipo viral tradicionalmente relacionado com infecções mucosas, sendo responsável por causa câncer no trato digestório superior em bovinos. Aqui, BPV-4 foi detectado em lesões cutâneas. Embora não usual, sua presença no epitélio cutâneo tinha sido relatada (Bloch *et al.*, 1996). Entretanto, o papel do BPV-4 em lesões cutâneas ainda está para ser elucidado. BPV tipo 5 e 6 estão normalmente associados com lesões cutâneas de tetos e úbere. Mas neste estudo foi possível detectá-los em lesões

da paleta e ao redor dos olhos do animal, sítios não usuais para estes tipos virais. Os BPV tipo 8, 9 e 10 foram descritos em lesões cutâneas no Japão (Tomita *et al.*, 2007; Hatama *et al.*, 2008). Os BPV tipo 9 e 10 foram detectados pela primeira vez no continente americano neste estudo. Aparentemente, o sítio do corpo em que o animal é exposto à infecção por BPV é mais determinante que o tipo do vírus. Apesar de estes achados mostrarem uma nova perspectiva sobre a infecção dos BPV, novos estudos sobre a significância patobiológica destes tipos virais são necessários.

A ocorrência de coinfecções também foi um resultado importante. Na grande maioria das lesões foram detectados mais de um tipo viral. A coinfecção por diferentes tipos de BPV tinha sido relatada (Yaguiu *et al.*, 2006; Claus *et al.*, 2007, 2009b; Leishangthem *et al.*, 2008; Lindsey *et al.*, 2009; Pangty *et al.*, 2010; Schmitt *et al.*, 2010). Entretanto, este é o primeiro estudo que avaliou coinfecções por BPV tipos 1-10 utilizando *primers* específicos. Ainda não são conhecidas as consequências desta coinfecção nos animais. Entretanto, a presença constante de múltiplas lesões no corpo dos animais pode ser uma consequência da coinfecção.

Através dos nossos resultados foi possível observar que a distribuição dos tipos de BPV não seguiu nenhum padrão geográfico no Brasil. É possível que os tipos de BPV estejam completamente espalhados em todo o país, como sugerido por Claus *et al.* (2008). Os tipos de BPV parecem estar disseminados entre todas as regiões do Brasil, independente do tipo de rebanho (de corte ou leiteiro). Aparentemente, também não há associação entre os tipos de BPV e o gênero do animal, uma vez que os vírus foram detectados tanto em machos como em fêmeas. Além disso, não pudemos estabelecer qualquer relação entre os tipos

de BPV e a idade dos bovinos. Acreditava-se que embora animais de todas as idades pudessem ser afetados, bezerros e novilhos apresentavam maior incidência de papilomatose bovina (Hama *et al.*, 1988; Freitas *et al.*, 2012). É provável que fatores específicos como nutrição, infestação por carrapatos, idade e o estado imunológico do animal possam contribuir em conjunto para esta incidência.

A associação entre os tipos de BPV e a morfologia das lesões é pouco compreendida. Neste estudo não foi possível observar associação entre os tipos de BPV e a morfologia da lesão. Do ponto de vista histológico, encontramos associação entre BPV tipo 1, 2, 3 e 10 com fibropapilomas. Os BPV tipo 1 e 2 haviam sido relacionados com fibropapilomas (Borzacchiello e Roperto, 2008). Entretanto, esta é a primeira vez que foi observado a associação entre os BPV tipo 3 e 10 com fibropapilomas. Mas novos estudos são necessários para a elucidação do verdadeiro papel destes tipos virais na patogenia dos BPV.

Alguns tipos de BPV foram detectados em diferentes lesões de um mesmo animal. Como estes tipos virais também foram encontrados em outros animais da mesma fazenda, pode ter ocorrido vários eventos de reinfecção através de contato animal à animal. Outra explicação seria que estes tipos de BPV poderiam ser carreados para outros locais do animal através das células sanguíneas, uma vez que BPV foi identificado nestas células (Diniz *et al.*, 2009; Roperto *et al.*, 2011).

O conhecimento acerca da diversidade genética dos BPV ainda é escasso. Apenas 13 tipos de BPV foram descritos até o momento, apesar da grande diversidade encontrada nos HPV. Este fato reflete o pequeno número de estudos que visam ampliar o conhecimento acerca da diversidade dos BPV.

Entretanto, neste estudo, nós detectamos um provável novo tipo de BPV, que pertence ao gênero *Deltapapillomavirus*, e um provável novo subtipo de BPV-11. Estes resultados sugerem que há uma grande diversidade de tipos de BPV no Brasil, e a compreensão desta diversidade pode ajudar no desenvolvimento de novos métodos terapêuticos.

De uma maneira geral, este estudo contribui de forma significativa para o aumento do conhecimento relacionado com a diversidade genética dos PV, principalmente dos BPV. O método baseado em entropia aqui desenvolvido se mostrou uma excelente alternativa para estudos relacionados com a evolução dos PV. Além disso, este método foi aplicado com sucesso em sequências do gene *L1* de BPV, muito utilizado em estudos que visam à identificação de tipos, subtipos e variantes virais. Os resultados apontam para uma metodologia sólida que pode ser utilizada para selecionar regiões informativas, indicando *primers* para a amplificação e o sequenciamento destas regiões. Este achado é importante para o desenvolvimento de métodos diagnósticos mais sensíveis e específicos, o que permitiria o estabelecimento de um programa de monitoramento mais eficiente da papilomatose bovina.

Associado a isto, conseguimos avaliar de maneira bastante ampla a ocorrência de BPV no Nordeste do Brasil. Identificamos 10 de 11 tipos de BPV testados em lesões localizadas em locais incomuns nos animais. Além destes, prováveis novos tipos foram encontrados, sugerindo uma diversidade ainda maior de BPV presente no país. Esta grande diversidade genética encontrada provavelmente está associada com a complexa biologia destes vírus. Embora esta relação ainda esteja desconhecida, a identificação de novos tipos de BPV pode indicar diferentes aspectos patológicos que podem acometer os animais.

Assim, novas investigações epidemiológicas e evolutivas que visem aumentar o conhecimento acerca da diversidade genética dos BPV auxiliarão no estabelecimento de uma visão mais acurada da distribuição destes vírus, ajudando no desenvolvimento de métodos para o controle da doença.

9. Conclusões gerais

1. Embora os papilomavírus (PV) apresentem grande diversidade genômica, regiões conservadas que são importantes para a estrutura e função de suas proteínas podem ser encontradas a partir de uma medida de entropia, caracterizando a ancestralidade comum.
2. Uma abordagem baseada em entropia foi desenvolvida para selecionar as regiões filogeneticamente mais informativas do genoma dos PV. Esta metodologia foi aplicada com sucesso na reconstrução filogenética deste grupo de vírus, em que foi possível observar que múltiplos mecanismos evolutivos estão associados com a evolução dos PV.
3. O método baseado em entropia se mostrou bastante eficiente, uma vez que foi possível obter árvores filogenéticas robustas utilizando um número menor de dados, implicando em menor custo computacional. Além disso, independente do conjunto de genes a ser utilizado, se as regiões de baixa entropia forem utilizadas, é possível obter árvores filogenéticas robustas. Assim, esta abordagem se torna bastante relevante em estudos de genômica populacional de PV, diminuindo a necessidade de se sequenciar o genoma completo dos vírus.
4. Esta abordagem foi aplicada na análise de sequências do gene *L1* de papilomavírus bovino (BPV). Com ela, foi possível encontrar duas regiões filogeneticamente mais informativas do que as regiões delimitadas pelos *primers* FAP59/64 e MY11/09. Estes resultados são importantes por posicionar de forma mais acurada os tipos, subtipos e variantes de BPV, requisito central em estudos de detecção e caracterização de novos

isolados. Associado a isto, esta metodologia pode ser aplicada para o desenho de novos *primers* degenerados com maior especificidade e sensibilidade para detectar estes vírus, o que é relevante para o desenvolvimento de novos sistemas de diagnóstico, e consequentemente, para o estabelecimento do monitoramento de BPV.

5. Este foi o primeiro estudo a avaliar a diversidade de BPV em rebanhos brasileiros avaliando tantos tipos virais ao mesmo tempo. Vários tipos de BPV foram detectados, além de um provável novo tipo de BPV e um provável novo subtipo de BPV-11.

6. Nossos dados sugerem que os tipos de BPV não estão restritos a nenhum sítio do corpo dos bovinos, sendo o local da exposição ao vírus e o transporte através das células do sangue eventos importantes na transmissão do vírus e consequente desenvolvimento das lesões. Além disso, nenhum padrão de distribuição geográfica foi encontrado, mostrando que estes vírus podem estar disseminados em todo o país de forma uniforme.

7. Estas informações, em conjunto, contribuem para o aumento do conhecimento da biologia e da diversidade dos BPV no Brasil, podendo ser úteis na avaliação de rotas de entrada destes vírus na região, assim como no desenvolvimento de métodos diagnóstico e terapêuticos mais acurados. Entretanto, novas investigações epidemiológicas e evolutivas ainda precisam ser realizadas sobre a incidência e diversidade das infecções por BPV em bovinos, levando em consideração não só os tipos e subtipos, mas também as variantes de BPV.

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11. Anexos

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New variants of E6 and E7 oncogenes of human papillomavirus type 31 identified in Northeastern Brazil

Bárbara S. Chagas [a], Marcus V.A. Batista [a], Vilma Guimarães [b], Valdir Q. Balbino [a], Sergio Crovella [a,c,d], Antonio C. Freitas [a,c,*]

[a] Department of Genetics, Federal University of Pernambuco, Recife, PE, Brazil
[b] Institute of Integral Medicine Prof. Fernando Figueira, Recife, PE, Brazil
[c] Laboratory of Immunopathology Keizo Azami, Federal University of Pernambuco, Recife, PE, Brazil
[d] Genetic Service IRCCS Burlo Garofolo,Trieste, Italy

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ABSTRACT

Objective. We sought to characterize E6 and E7 oncogenes genetic variability of HPV-31 isolated from cervical scraping samples of Northeastern Brazilian women.

Methods. E6 and E7 were amplified with specific *primers*, cloned and sequenced. The sequences obtained were aligned with the GenBank reference sequences with the aim of evaluating the possible genetic variants.

Results. We identified several genetic variants in E6 and E7 sequences from HPV-31 positive women. Three nucleotide changes in E6 were described for the first time in this study. Some nucleotide changes were non-synonymous substitutions.

Conclusion. The knowledge of region/country HPV specific genetic variations is relevant to understand the epidemiology and the development of effective vaccines.

Introduction

Infection with human papillomavirus (HPV) represents the most important risk factor in the genesis of cervical cancer [1]. Currently more than 120 types of HPV have been identified and about 40 types affect the genital tract through sexual contact [2].

The criterion for classification of HPVs into types, subtypes and variants is based on the analysis of the highly conserved L1 gene nucleotide sequence coding for the major capsid protein [3].

The differences found in the nucleotide sequences between variants of one HPV type can result in amino acid changes leading to distinct oncogenic potentials between different variants. Variations in LCR region nucleotide sequence, containing sequences important for regulation of viral replication and early genes transcription, could result in loss of binding capacity of a specific transcription factor, as well as changing its recognition site and the affinity of a specific transcription factor [4]. When considering L1 region, one or more amino acid changes could lead to a conformational change in the capsid protein, interfering with the conformation of viral neutralization relevant epitopes [5]. Moreover the importance attributed to variations in the E6 gene is directly related to the occurrence of variations in the critical regions for p53 protein degradation and in the regions involved in host immune recognition [6].

A previous study reported distinct HPV incidences in different Brazilian regions, evidencing the major frequency of HPV-31 in women from Pernambuco, Northeast region of Brazil [7,16]. Thus, this study aimed to assess the genetic variability of the HPV-31 E6 and E7 genomic regions in cervical scraping samples from Northeastern Brazilian women. These genetic variations were associated with predicted T and B-cell epitopes, and the phylogenetic relationships of these variants were determined.

Methods

The samples used in this study were obtained by cervical scraping from a total of six patients aged between 29 and 67 years old (s.d. 15.579), two are European Caucasian-derived and four are African-derived, with different lesions and life styles, as seen in Table 1. The patients were attended at the Department of Gynecology of "Instituto de Medicina Integral Prof. Fernando Figueira (IMIP)" in Pernambuco state, Northeastern Brazil.

All six HPV-31 samples were tested for E6 and E7 genetic variability. Samples were PCR amplified using specific primers for E6 (450 bp) and E7 (297 bp) genomic regions (Supplementary Table S1). PCR products were cloned into vector pGEM-T (Promega) and sequenced using the kit ABI PRISM BigDye™ Terminator Cycle Sequencing v 3.1.

The sequences obtained were checked for quality and assembled using the Staden package [8]. Sequence identity was determined

* Corresponding author at: Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil. Fax: +55 81 21268512.
E-mail address: antonio.freitas@pq.cnpq.br (A.C. Freitas).

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Table 1
Characteristics of the participants: HPV-31 variant status, lesions, age, ethnicity and life style are reported.

Patient	HPV-31 Variant	Lesion	Age (years)	Residence	Ethnicity	No. of sexual partners	No. of pregnancies	Contraceptive usage	Smoke
1	HPV31/UFPE-01	CINIII	29	Urban area	AD	1	1	Yes	Never
2	HPV31/UFPE-02	CINI	34	Urban area	AD	1	1	No	Never
3	HPV31/UFPE-03	None	37	Urban area	ECD	3	None	No	Never
4	HPV31/UFPE-04	None	67	Urban area	ECD	1	2	No	Never
5	HPV31/UFPE-05	None	44	Urban area	AD	3	4	Not always	Not anymore
6	HPV31/UFPE-06	Cervical cancer	62	Urban area	AD	2	3	No	Not anymore

CINIII = Cervical intraepithelial neoplasia type III.
CINI = Cervical intraepithelial neoplasia type I.
ECD = European Caucasian-derived.
AD = African-derived.

using BLAST [9]. Multiple sequence alignment was performed using ClustalW [10].

T-cell epitope prediction was performed using ProPred-1 [11] and ProPred [12] servers for the prediction of MHC class I and class II binding regions, respectively. B-cell epitope prediction was performed using Bcepred server [13] with the default parameters.

The phylogenetic trees were constructed using PAUP* software version 4.0b10 [14] with the Neighbor Joining method, using GTR model for the E6 gene and K80 + I + G for the E7 gene, estimated from Bayesian Information Criterion (BIC) by jModelTest v.0.1.1 program [15].

GenBank accession numbers of the new variants were as follows: GU595167 (HPV31/UFPE-01 E6), GU595168 (HPV31/UFPE-01 E7) and HQ149098 (HPV31/UFPE-04 E7).

Results

Within the six samples of HPV-31 analyzed, we observed four isolates (HPV31/UFPE-01 and HPV31/UFPE-02; HPV31/UFPE-04 and HPV31/UFPE-06) that were identical. The other samples (HPV-31/UFPE-03 and HPV31/UFPE-05) showed E6 and E7 regions identical with respect to the HPV-31 reference sequence (J04353). When analyzing HPV-31 E6 genomic region, we identified five specific mutations (Table 2).

The comparative analysis of E7 gene revealed four distinct variations (Table 2). Nucleotide substitutions resulting in premature stop codons or frameshift changes were not detected.

Phylogenetic analysis based on E6 and E7 nucleotide sequences showed that the six samples from Northeastern Brazil did not group together. HPV31/UFPE-01 and HPV31/UFPE-02 isolates are exactly alike and different from the reference sequence. HPV31/UFPE-03 and HPV31/UFPE-05 isolates are identical and equal to the reference sequence. Isolates HPV31/UFPE-04 and HPV31/UFPE-06 presented nucleotide substitutions only in the E7 gene. There were no correlations between the isolates and patients ethnicity (Table 1). Fig. 1 shows the trees of these isolates and others from different geographical regions. All trees were rooted with HPV-16, the viral type closer to HPV-31.

Then we performed B-cell and T-cell epitopes prediction in order to evaluate the impact of mutations on the immune function of the E6 and E7 transforming proteins. Fig. 2 reports the consensus of T-cell and B-cell epitopes obtained for E6 and E7 proteins. As shown we observed amino acid changes in sites belonging to B-cell and/or T-cell epitopes.

Table 2
Genetic variability of HPV-31 samples E6 and E7 regions. The nucleotides conserved with respect to the reference sequence (J04353) are marked with an asterisk (*). In bold, nucleotide variations not described in the literature. (–) No change of amino acid. Reference: prototype sequence of HPV-31. T: threonine, A: alanine, K: lysine, R: arginine, V: valine, H: histidine, Y: tyrosine, E: glutamate.

Isolates	Positions of E6 nucleotide substituitions					Positions of E7 nucleotide substituitions			
	141	190	213	368	413	67	111	136	184
Reference	T	A	A	A	C	C	C	G	A
HPV31/UFPE-01	**C**	**G**	T	**G**	T	**T**	**T**	**A**	**G**
HPV31/UFPE-02	**C**	**G**	T	**G**	T	**T**	**T**	**A**	**G**
HPV31/UFPE-03	*	*	*	*	*	*	*	*	*
HPV31/UFPE-04	*	*	*	*	*	*	*	*	**G**
HPV31/UFPE-05	*	*	*	*	*	*	*	*	*
HPV31/UFPE-06	*	*	*	*	*	*	*	*	**G**
Position of altered codons	47	64	71	123	138	23	37	46	62
Reference	–	T	–	K	A	H	–	E	K
Altered amino acid	–	A	–	R	V	Y	–	Y	E

Discussion

When considering the association HPV–cervical cancer, HPV-16 is the most frequent worldwide, with the exception of Indonesia where HPV-18 is the most common [16]. Although in Brazil, a country with continental dimensions, HPV-16 is also the most frequent, the second most important type differs according to the Brazilian regions. Baldez da Silva et al. 2009 [16] showed that HPV types incidence in metropolitan region of Recife (Northeastern Brazil) were: HPV-16 (78.67%), HPV-31 (15.49%), HPV-18 (2.82%) and HPV-33 (2.82%).

We have analyzed the genetic variability of HPV-31 E6 and E7 genomic regions, in cervical samples from six Northeastern Brazilian women. We identified mutations in E6 and E7 sequences of HPV-31, and many of these variations were observed in sites belonging to B-cell and/or T-cell epitopes.

HPV E6 and E7 proteins are important for several viral properties such as replication and transcription of viral DNA, interaction with the cytoskeleton network, immortalization and transformation [17]. E6 and E7 interact with a wide range of cellular proteins although they are better known for their ability to bind to and inactivate p53 and pRb, respectively [18–20].

The nucleotide changes detected at positions 213 and 413 of HPV-31 E6 gene were previously described [21,22], but changes at positions 141, 190 and 368 are described for the first time in this study. In Brazil, until now, only one study analyzed genetic variability in HPV-31 isolates from the Midwest region [22]; in our study we were able to find HPV-31 variants different from those ones. Moreover, we report for the first time four nucleotide changes in HPV-31 E7, at positions 67, 111, 136 and 184. E6 and E7 genes presented a peculiar substitution pattern, indicating that these variants may be circulating in the population of Northeast Brazil.

Although there is little information regarding the biological consequences of HPV genetic variations in the process of cervical carcinogenesis, it is reasonable to hypothesize that they may have relevance to different aspects of the carcinogen development process. When considering HPV-31, even less information is available. However, genetic variability studies of E6 and E7 genes of HPV-16 suggested that each viral variant was associated with risk of cervical neoplasia [23–29].

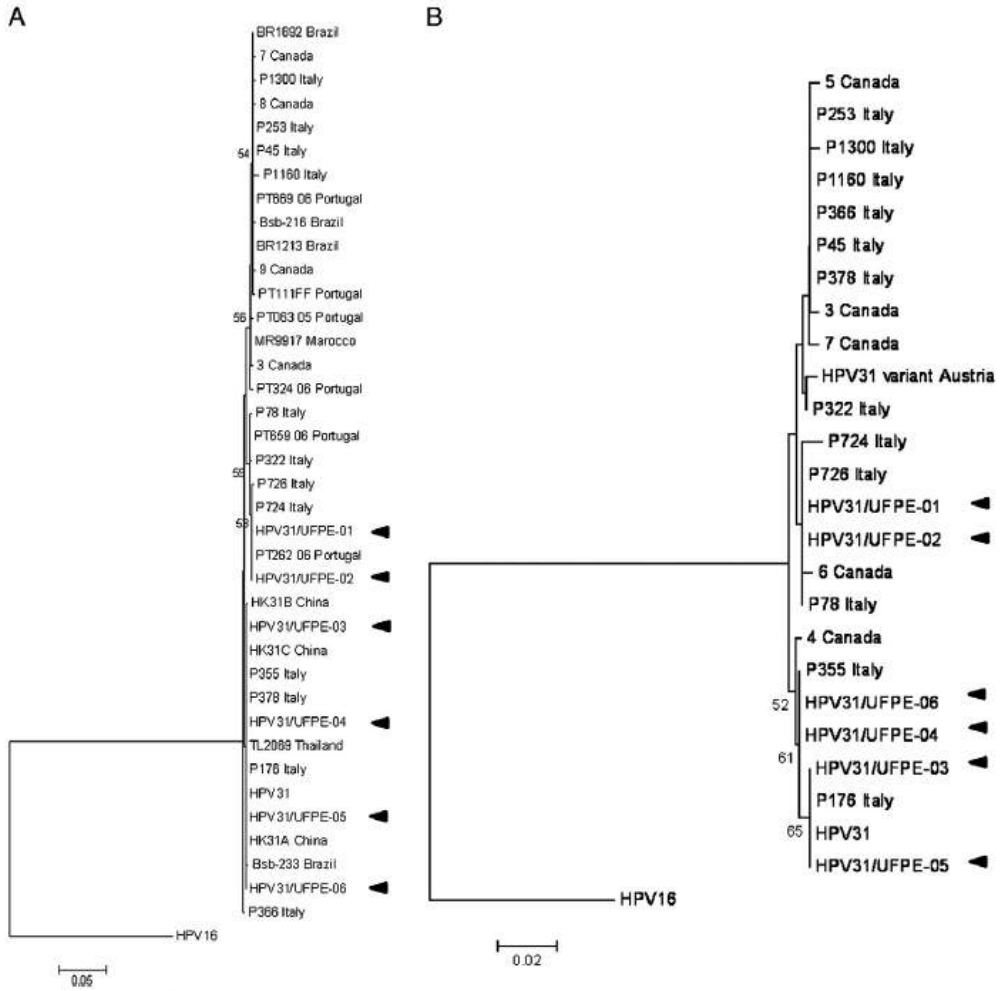

Fig. 1. Phylogenetic trees based on the Neighbor Joining method of representing the relationships between variants of HPV-31. A) Tree based on gene E6. B) Tree based on gene E7. Bootstrap values (1000 replicates) was significant (above 50%) are represented in the branches. Arrows indicate the isolates found and characterized in this study.

Another possible parameter involved with HPV-31 E6 and E7 variants and oncogenecity is the cellular immune response. Many of E6 and E7 genetic variations observed were located in B-cell and/or T-cell epitope sites. It is known that genetic polymorphism of both HPV and HLA influence the presentation of viral peptides to T-cells [20,30].

Although HPV-31 is the third most worldwide distributed viral type [31], studies involving HPV-31 E6 and E7 variants and cellular immune response are scarce in the literature. We described nucleotide substitutions that alter various T-cells peptide binding epitopes, as shown in Supplementary Table S3 and S4. These changes may influence the immune recognition of HPV infected cell.

When looking at modified amino acids caused by nucleotide changes in E6 and E7 genomic regions, we observed some changes in the polarity, hydropathic potential and amino acid side chain, respectively. These changes are related to alterations in the conformational structure of proteins, with possible functional effect. These mutations could also interfere with viral gene expression and host immunological functions. We did not observe variations in the conserved p53 and pRb binding domains responsible for cellular immortalization and transformation and involved in oncogenetic processes.

From the phylogenetic point of view, the currently known HPV types show great diversity. However, among these viral types there are subtypes and variants that can acquire biological advantages through fixed mutations in their genome. We identified two isolates with non-synonymous nucleotide substitutions never reported before. According to Calleja-Macias et al. 2005 [21], even small variations could result in small adaptive improvements, possibly altering the composition of a HPV population.

Our data suggest that HPV-31 E6 and E7 variants did not evolve following the patterns of co-evolution with human ethnic groups as occurs with HPV-16 and HPV-18 variants [5]. The same inconsistence was observed in a previous study with HPV-31 LCR [22]. We also observed that the phylogenetic behavior of the isolates is independent from ethnicity (Table 1 and Fig. 1). However, North East Brazil is characterized by strong ethnical admixture, possibly explaining isolates distribution in different clades of the phylogenetic trees. These genes could also present a different evolutionary rate in

Fig. 2 Predicted T-cell and B-cell epitopes. A) Epitopes within the E6 protein of HPV31/UFPE variants. B) Epitopes within the E7 protein of HPV31/UFPE variants. In blue, a consensus of the predicted T-cell epitopes for all alleles. In green, a consensus of the predicted B-cell epitopes for the combined physical–chemical properties. Black arrows indicate mutations sites presented in HPV31/UFPE-01 and HPV31/UFPE-02 isolates. Red arrow indicates mutation site presented in HPV31/UFPE-01, HPV31/UFPE-02, HPV31/UFPE-04 and HPV31/UFPE-06 isolates. Isolates HPV31/UFPE-03 and HPV31/UFPE-05 are identical to the reference sequence.

comparison to other HPV-31 ones. Another explanation would be that these HPV-31 variants might have existed before the spread of humans and the clusters specific for the ethnic groups arose subsequently, as suggested by Calleja-Macias et al. 2005 [21].

Knowing HPV genetic variations in a country of continental proportions like Brazil is relevant to the understanding of epidemiology, development of more effective drugs and vaccines, as well as improvement of diagnostic methods. Changes in amino acid sequences between variants from different geographical regions evidence the possibility of functional divergence between them, suggesting that the variants detected in Northeastern Brazil may have different adaptive fitness. Our findings describe new Northeastern HPV variants and contribute to better understand the genomic diversity of viruses that occur in Brazil.

Conflict of interest statement
The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ygyno.2011.07.008.

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Review

Recent insights into Bovine Papillomavirus

Freitas, A. C.*, Silva M. A. R., Jesus, A. L. S., Mariz, F. C., Cordeiro, M. N., Albuquerque, B. M. F. and Batista, M. V. A

Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Recife, PE, Brazil.

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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.

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

*Corresponding author. E-mail: acf_ufpe@yahoo.com.br.

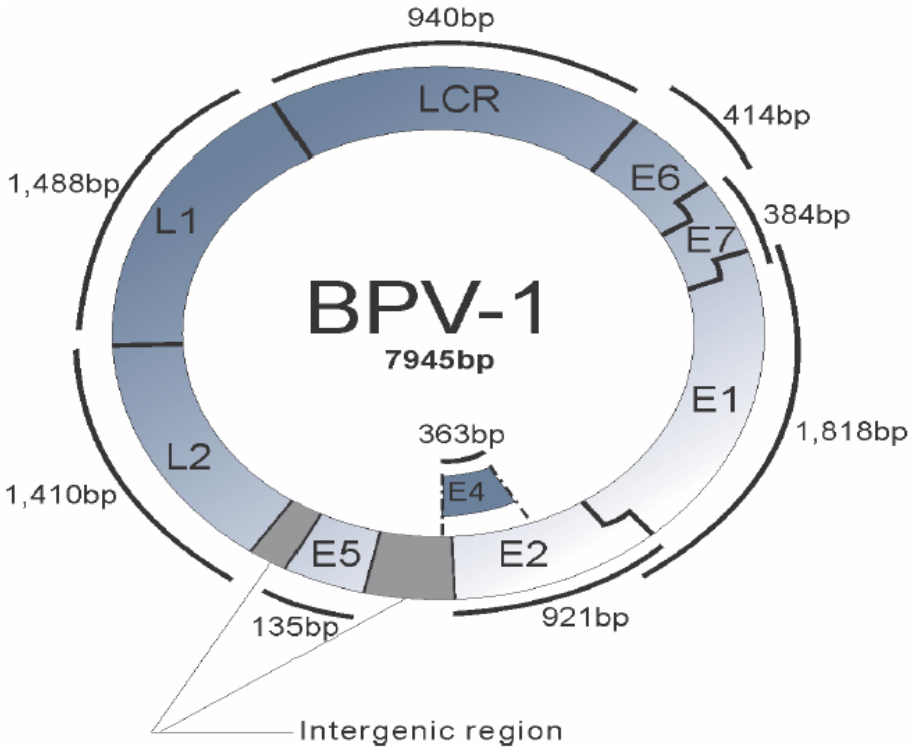

Figure 1. Genomic organization of BPV-1 showing the genomic positions of viral genes E6, E7, E1, E2, E4, E5, L2, L1, and the long control region (LCR) between L1 and E6 genes; as well as the intergenic regions between E2 and E5, and between E5 and L2.

according to the viral life cycle into the host cell. The E1 protein has helicase activity and plays its role on the viral replication (Lambert, 1991). The E2 gene product is responsible for recognition and ligation to the replication origin and, furthermore, it has mitotic chromosome binding activity in order to ensure equal distribution of viral episomes among daughter-cells (Baxter et al., 2005). The E4 gene, completely overlapping E2 gene but in a different reading frame, produces a small protein profusely found in keratinocytes cytoplasm during productive replication (Anderson et al., 1997). Three early proteins are necessary for BPV-mediated carcinogenic process, so, called oncoproteins: E5, E6 and E7 (Nasir and Campo, 2008). E5 is a membrane-associated hydrophobic protein, which plays a role on disrupting cellular growth control. BPV E6 protein is known to have a multitude of binding partners and activities on the virus life cycle. In *Xipapillomavirus*, E6 gene is replaced by an E5-like gene, which was initially defined as E8.

Nevertheless, the comprehension that most (although not all) of the functions of this protein are shared with BPV-1 E5, prompted its redefinition as E5 (Campo, 2006). The E7 protein appears to cooperate with E5 and E6 for cellular transformation, whose production with the two others oncoproteins increases the transformation efficiency (Bohl et al., 2001).

The viral capsid structure is constituted by two proteins (Modis et al., 2002; Xu et al., 2006) encoded by the late genes in viral genomes. The L1 gene is useful for classification and construction of phylogenetic trees (Bernard, 2005). The capsid is formed by 360 copies of L1 protein, organized as 72 capsomers (pentameric assembled), and 12 copies of L2 protein. Although being present in less number, L2 minor capsid protein is necessary for viral morphogenesis (Modis et al., 2002).

BPV DIVERSITY

The present PV diversity can be explained by multiple evolutionary mechanisms (Gottschling et al., 2007). Virus host-divergence is an important evolutionary force,

however this force solely cannot explain the evolution of PVs and their diversity, thus alternative mechanisms such as within-host virus duplication, recombination, viral sorting, or viral adaptation after a host switch, may therefore contribute considerably to explain the PV diversification (Shah et al., 2010; Gottschiling et al, 2011).

Although it is not very clear how these evolutionary mechanisms act on PV diversity, several robust methods have been used. Gottschling et al. (2007) used a rigorous phylogenetic approach, which took into consideration the choice of appropriate outgroups, as well as the assessment of confidence values of internal nodes. A robust study has used the method of importance sampling to Bayesian divergence time estimation, which indicates prior divergence of at least 6 PVs lineages associated with an ancestral mammalian host (Shah et al., 2010). Gottschiling et al, (2011) used different statistical approaches to assess topological and branch-length congruence, evidencing the importance of alternative mechanisms other then codivergence. Another statistical approach based on entropy was used to assess the evolution of PVs, showing that hot spots in the genome could be used as markers in order to infer PV phylogeny (Batista et al., 2011). These robust phylogenetic analyses provide the basis for contemporary classification of PVs, which is very important for any medical and veterinary researches.

The understanding of PV diversity is limited, probably underestimated. As there are more than 150 sequenced HPV genomes, less than 50 non-human papillomavirus species have been isolated and sequenced. So, more new PV types should be sequenced to increase our knowledge about PV evolution. The diversity of subtypes and variants could show a more detailed and refined scenario of PV diversification, increasing insights into the representativeness of each PV type. When it comes to BPV, 12 types are currently well described and about 14 new putative types were isolated (Antonsson and Hansson, 2002; Ogawa et al., 2004; Campo, 2006; Ogawa et al., 2007; Tomita et al., 2007; Claus et al., 2008; Hatama et al., 2008; 2011; Zhu et al., 2011).

In phylogenetic analysis, BPVs are found in at least three distantly related lineages. First, BPV-1, BPV-2, BPV-5 and BPV-8 form a paraphyletic group with OvPV-1 and OvPV-2, which infects a close related host. Other PVs that infect Artiodactyla are also close relatives of those BPVs. However, this group is clustered together with equine and canine PVs. Second, BPV-3, BPV-4, BPV-6, BPV-9, BPV-10, BPV-11 and BPV-12 are grouped together with caprine PV (ChPV-1). This group is related with a large cluster that involves human, canine and rodent PVs. BPV-7, an unclassified PV, has an uncertain phylogenetic position, which makes it difficult to infer its relatedness to other PVs.

Therefore, all this diversity found in PVs that infect one host (*Bos taurus*) is a case of evolutionary incongruence between host and PV phylogeny, indicating that co-divergence alone cannot explain the PV diversity (Gottschling et al., 2007, 2011; Shah et al., 2010).

Some conflicting phylogenetic positions of types within *Xipapillomavirus*, which includes some BPV types, have been shown when analyzing early or late genes phylogenies (García-Vallvé et al., 2005; Köhler et al., 2011). In general, the topological inconsistency between early and late genes phylogenies have been explained with ancient recombination events (Gottschling et al., 2007; Shah et al., 2010). This also could be the explanation for the contradicting positions of BPVs within *Xipapillomavirus*. For BPVs, at least three lineages seem to originate the currently known types. These lineages probably passed through a prior divergence process preceding the host divergence. This could also explain the proximity of BPVs to PVs that infect distantly related hosts. In addition, zoonotic transmission of PVs is rare event but it occurs in BPVs as they were found in zebras, horses and buffaloes (Silvestre et al., 2009; van Dyk et al., 2009; Bogaert et al., 2010a). Other evolutionary mechanisms could be associated with BPV diversification, however sampling is still a limiting factor.

BPV DETECTION AND DISTRIBUTION

BPV DNA is detected by a variety of polymerase chain reaction (PCR)-based techniques. These PCRs are based frequently on the detection of one or two BPV types using degenerated or type-specific primers. Genotyping is performed either by real-time detection (Rai et al., 2011) or by sequence analysis (Brandt et al., 2008) or restriction fragment length polymorphism (RFLP) analyses (Carr et al., 2001) of the generated PCR fragments. Consensus primers capable of identifying potentially more than two BPV types have also been described (Ogawa et al., 2004). Besides, PCR assays, designed originally for the detection of human papillomaviruses have been used to genotype different BPV types (Antonsson and Hansson, 2002; Ogawa et al., 2004). PCR assays using degenerate primers that amplify partial fragments of the L1 gene, followed by sequencing, have suggested the existence of numerous yet uncharacterized BPV types in cattle herds from diverse geographical regions. Using the primers FAP59/FAP64 and MY09/MY11, 12 putative new BPV types were detected in teat skin warts and healthy teat skin of cattle from Japan and Sweden (Antonsson and Hansson, 2002; Ogawa et al., 2004).

Bovine papillomavirus has been widely found in cattle worldwide. Cases have been reported in the incidence of BPV in cattle in Europe, America, Asia and Oceania. BPV-1, -6, -8 and -10 were found in bovine warts from a German cowshed (Schmitt et al., 2010). In Japan, heifers were found to have benign teat tumors causing by BPV-6 (Maeda et al., 2007). In another work, Ogawa et al. (2004)

detected BPV-1, -3, -5 and -6 in papilloma specimens. Bovine cutaneous warts were reported from India and identified as BPV types 1 and 2 (Singh et al., 2009; Pangty et al., 2010) and recently Rai et al. (2011) identified BPV-10 in teat warts from cattle at a dairy farm in India. Cattle from Brazil have also been investigated for the presence of BPV. It was identified BPV-1, -2, -6 and -8 in skin warts of cattle from southern Brazil (Claus et al., 2007; Sá e Silva et al., 2010). Results from our group, in northeastern Brazil, also revealed the presence of ten different types of BPV in the samples, with the exception of BPV-7 (Carvalho et al., in press).

As considered before, BPV is also associated with cancer in cattle. BPV-4 infection and associated tumors of upper GI tract have been found in Brazil, the Nasampolai Valley of Kenya, Western Highlands of Scotland and in southern Italy (Jarrett et al., 1978; Borzacchiello et al., 2003). Field cases of urinary bladder cancer in cattle associated with BPV-1 and -2 infections were reported in continental Europe, Azores Islands, some regions of Kenya, Brazil, New Zealand, India and China (Borzacchiello and Roperto, 2008).

A similar investigation revealed notable diversity among BPV types detected in papillomas of four cattle herds in southern Brazil. The study identified four putative new BPV types designated as BPV/BR-UEL2 to BPV/BR-UEL5 (Claus et al., 2008). Phylogenetic analysis using complete L1 ORF sequences revealed that the one of the isolates was similar with BPV-4 (78%), which suggested its classification in the genus *Xipapillomavirus* (Lunardi et al., 2010).

In a work of our group it was also detect possible new types and variants in samples from herd in northeastern Brazil, in which sequence analyses indicated the presence of two isolates (BPV/UFPE01 and BPV/UFPE02) of a putative new BPV-11 subtype (unpublished data). These two novel isolates are also closely related to BPV-4, and to the strains BPV/BR-UEL2 and BPV/BR-UEL3 described by Claus et al. (2008). Currently, the group continues the analysis of new BPV DNA sequences from cutaneous warts with very promising results for the identification of new types of BPV in Brazilian cattle.

BOVINE PAPILLOMATOSIS

Bovine papillomatosis is an infectious disease worldwide distributed among herds. The BPV is responsible for this contagious illness, whose remarkable clinical sign is the hiperproliferative lesions, known as papillomas, on cutaneous tissue and mucosa (Campo, 2006). Despite being primarily considered epitheliotropic, BPV DNA has already been isolated from peripheral blood mononuclear cells, milk, urine, seminal fluid and sperm cells of animals infected with BPV-1, BPV-2 and BPV-4 (Carvalho et al., 2003; Yaguiu et al., 2006, 2008; Roperto et al., 2008, Lindsey et al., 2009).

Similarly to others papillomatosis, it is usually observed the spontaneous regression of lesions, defined as benign proliferative neoplasm (Jelínek and Tachezy, 2005). Many times its importance is not verified by many owners and veterinarians badly clarified. Much more than an esthetic issue, bovine papillomatosis has recently grown in importance due its association with cancer and immunosuppression conditions (Campo, 2002). This infection, according to the viral type and environmental co-factors, causes distressing symptoms in cattle, as cutaneous fribropapillomas (BPV-1, and -2), cancer of the upper gastrointestinal tract (BPV-4), papillomatosis of teats and udder (BPV-1, -5, -6, -9 and 10) and penis (BPV-1) and cancer of the urinary bladder (BPV-1 and -2) and cutaneous papillomas (BPV-8) (Borzacchiello and Roperto, 2008) (Figure 2). Even the benign progression demands attention, once hyperplasic lesions may depreciate the pelt in affected animals; when located in the udder, it may lead to secondary infections and lactation problems. In fact, Campo (2006) related several economic consequences, as cows with teat papillomas cannot be milked, young calves cannot suckle, and often the peduncolated papillomas snap off, the sites become infected and mastitis may ensue with distortion of the milk canals. Animals can also develop extensive papillomas in the upper gastrointestinal tract and, consequently, present difficulty to feed and breathe, resulting in a debilitated animal that may come to death (Campo, 1997).

At the moment, there is no vaccine or effective treatment for the control of papillomatosis. There are few BPV treatments available with levels of success varying between 15-50%. However, it was evident the economic unavailability to repeat the treatment in animals that did not recover after the first therapeutic intervention (Silva et al., 2004).

BPV AND CO-INFECTION

Some reports describe the occurrence of co-infection with different types of BPV worldwide. In Japan, Ogawa et al. (2004) verified the presence of BPV in up to four BPV types and putative new BPV types in the same papilloma in the Japanese herd. In Brazil, the simultaneous presence of BPV-1 and -2 was detected in the same lesion (Yaguiu et al., 2006, 2008; Lindsey et al., 2009). Also, it was found five different combination of multiple BPV infection in cattle (Claus et al., 2009). Co-infection with BPV-1 and -2 was described in India (Leishangthem et al., 2008; Pangty et al., 2010) and co-infection with BPV-1 and -11 was assessed using a multiplex BPV genotyping assay in bovines in Germany (Schmitt et al., 2010) and in Brazil using specific BPV primers (Carvalho et al., in press).

Co-infection of FeSarPV, a new putative PV type

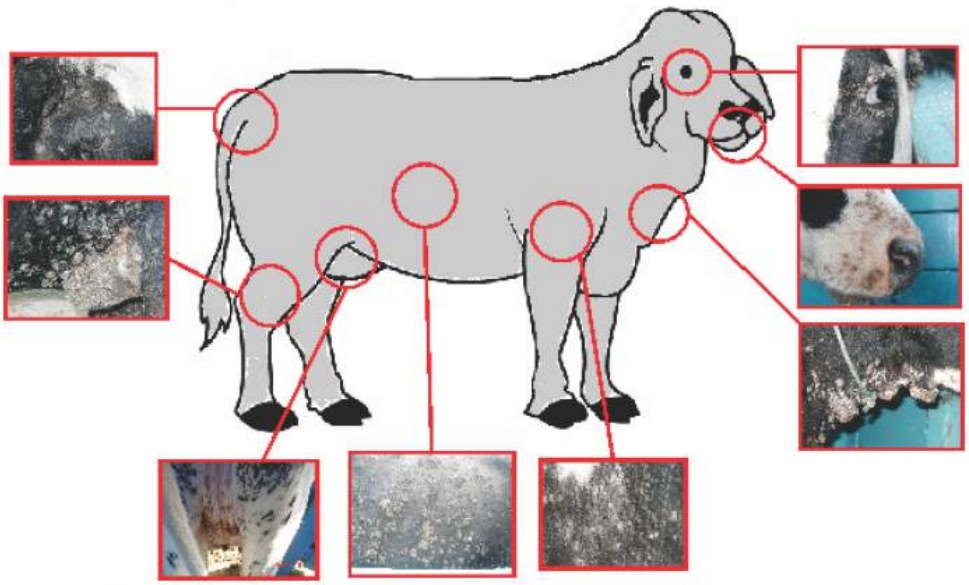

Figure 2. Schematic view of bovine papillomatosis in different sites of the cow.

related to Delta-PVs, and BPV-2 was described in New Zealand (Munday and Knight, 2010) and Brazil (Silva et al., unpublished data). In healthy cattle, the papillomas normally regress, but in cattle which have been fed on bracken fern (*Pteridium aquilinum*), there is a good correlation between persistent papillomatosis and cancer (Campo, 1997). However, the persistence of skin warts has been seen in a large number of animals (Claus et al., 2009). As several animals are constantly affected with warts in diverse body parts, it could be consequence of co-infection, which could lead to fall of immune response and prevent the regression of the lesions.

During several years, most of the first six well characterized BPVs have been described as causative agents of specific lesions in distinct body sites of bovines: BPV-1 has caused teat frond and penile fibropapillomas; BPV-2 has been described as the agent of common warts and esophageal fibropapillomas; BPV-3 and -8 in the epithelial papillomas of the skin; BPV-4 has been described as the agent of papillomas of the alimentary canal, showing specificity to the mucous epithelium; BPV-5 has caused rice grain fibropapillomas of the udder, and BPV-6 has been isolated from teat frond papillomas, BPV-9 and -10 has been associated to epithelial squamous papillomas of the udder (Campo, 1997; Borzacchiello and Roperto, 2008). However, in the late years, a diversity of multiple bovine papillomaviral infection has been described in bovine and other mammals (Ogawa et al., 2004; Bogaert et al., 2008; Claus et al., 2009; van Dyk et al., 2011) suggesting that certain viral types are not restricted as previously thought. Claus et al. (2009) observed the occurrence of several BPV types in a specific anatomical region; the detection of the same viral type in distinct body sites and determining papillomas with diverse gross aspects; and lesions with similar morphological characteristics caused by distinct papillomavirus.

Besides cutaneous warts lesions in cattle, the presence of more than one putative new BPV type was also observed in the normal skin (Ogawa et al., 2004). The simultaneous presence of BPV-1 and -2 was demonstrated in others bovine tissue such as blood and reproductive cells (Yaguiu et al., 2006, 2008; Diniz et al., 2009; Lindsey et al., 2009). BPV-1 and -2 was found co-infecting giraffe (van Dyk et al., 2011), zebra (van Dyk et al., 2009) and horse (Bogaert et al., 2008).

According to Schmitt et al. (2010), the occurrence of diverse co-infection by BPV in a single sample suggests that natural competition of different BPV types may not occur on the skin.

However, it is not clear if all BPV types founded in the lesion are transcriptionally active. Detection of viruses in apparently latency may be a result of evasion from the immune system (Schmitt et al., 2010). Nonetheless, the distribution of BPV types appeared to resemble the situation known from skin HPV types, where co-infections of more than 10 genotypes are detected frequently at very low copy numbers (Antonsson et al., 2000). In preliminary data obtained by our research group, we found very low copy number of BPV in cutaneous lesion

co-infected by several viral types (unpublished data).

TRANSMISSION OF BPV

Currently, little is known about how the disease is transmitted between animals. About this important question, it is known that confined populations are more vulnerable because virus dissemination may occur by direct (animal to animal) or indirect (contaminated objects) contact (Hama et al., 1988; Nasir and Campo, 2008). Besides the established skin–skin pathway, another via like arthropod vector and vertical trans-mission has been suggested (Freitas et al., 2003; Finlay et al., 2009). However these alternatives via of transmission might be less efficient (Bravo et al., 2010).

The increasing interest of studying BPV in the blood revealed this tissue as a source of spreading to BPV through non-epithelial tissues and fluids (Stocco dos Santos et al., 1998; Freitas et al., 2007). This hypothesis may be corroborated by the detection of BPV in different tissues and cells, including reproductive sites as oocytes, ovary, uterus, cumulus cells, and uterine lavage (Freitas et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009). The vertical transmission of BPV has been suggested (Stocco dos Santos et al., 1998; Freitas et al., 2003; Yaguiu et al., 2008). Also for humans, it has been shown that HPV- infected women can transmit the infection to the fetus by transplacental mechanisms (Rombaldi et al., 2008).

The mechanism behind the transmission of BPV to/between no specific hosts is not clear. Recent findings of BPV in epidermis and formation of L1 capsomers of equine sarcoid and active-BPV in normal skin of equine (Bogaert et al., 2008; 2010a, b; Brandt et al., 2011) could help explaining the occurrence of equine sarcoid in animals kept far away from any bovine virus source, especially when living in close contact with other affected equids (Brandt et al., 2011). It is believed that flies can be a vector for BPV and transmit the virus between bovine and horses (Nasir and Campo, 2008; Finlay et al., 2009). However, there is no further information about this virus–vector– host system. The zoonotic potential and the medical implications for the corresponding transmission route need to be explored (Bravo et al., 2010). Alternatively, BPV infection may be transmitted via stable management practices, or passed into existing wounds from contaminated pasture. Considerably more research is necessary to investigate all of these possibilities (Chambers et al., 2003). The mechanism of transmission of BPV in a cattle herd and to other mammals should be most studied since BPVs are disseminated infecting and co-infecting these animals due to its plasticity.

BPV AND CROSS-SPECIES INFECTION

Although PVs have been described as specie-specific (Campo, 2006) some PVs infect a variety of hosts. PVs appear to be widespread and have been found in a large number of vertebrate species and are assumed to have co-evolved with their hosts (Bernard, 1994; Antonsson and McMillan, 2006). Strict host specificity of PVs might act as a barrier that prevents close physical contact between different viruses, but a series of PVs infect a variety of phylogenetically distant hosts (Bravo et al., 2010). Virtually all mammalian species are hosts for one or more papillomaviruses (Sundberg et al., 2001). BPV can infect cattle but also infect close relatives of cattle such as buffalo (Silvestre et al., 2009; Pangty et al., 2010) and giraffe (van Dyk et al., 2011) causing fibropapillomas and bladder lesions (Pathania et al., 2011). Moreover, these viruses naturally infect more distantly related species, such as tapirs (Kidney and Berrocal, 2008), horses (Bogaert et al., 2008), sable antelope (van Dyk et al., 2011), and zebras living either in zoos (Löhr et al., 2005) or in the wild (van Dyk et al., 2009) causing sarcoids, and fibrosarcomas when inoculated into rodents (Robl and Oslon, 1968). Also, a variant of BPV-8 can induce papillomas in Bison (Literak et al., 2006) (Figure 3).

FeSarPV, primarily identified feline sarcoid, was verified in bovine fibropapillomas and dermatitis by Munday and Knight, (2010). It has been suggested that FeSarPV is a bovine PV causing a non-productive cross-infection in felines as well as BPV-1 and BPV-2 causes sarcoids in equids. Recently, it was found L1 capsomers in epidermis of equine with sarcoid suggesting a productive infection by BPV (Brandt et al., 2011). A newly proposed BPV type BRUEL- 4 (Claus et al., 2008) was identified in a sarcoid tumor of a horse, revealing a new viral type associated with equine sarcoid (Sá e Silva et al., 2010).

The ability of BPV-1 to infect related hosts can be a result of human domestication of cattle and horses or a phenotypic acquisition driven by vector-mediated interspecies transmission (Finlay et al., 2009; Gottschling et al., 2011). Thus, ecological changes happened concomitantly in the different hosts may have increased their susceptibility to BPV cross-infection and/or have simply increased the frequency of physical contact between them to grant BPV improved access to a potential new host (Gottschling et al., 2011).

CONCLUSION

Bovine papillomavirus is a group of viruses extensively studied in the last years. BPV has always been considered as an excellent experimental model to investigate HPV infection and carcinogenesis. It is also useful to understand the oncogenic potential of the virus, the relationship between virus and co-factors, and the development of anti-viral vaccines. In this review, we broach new insights into the mechanisms of BPV co-infection, cross-species infection and transmission.

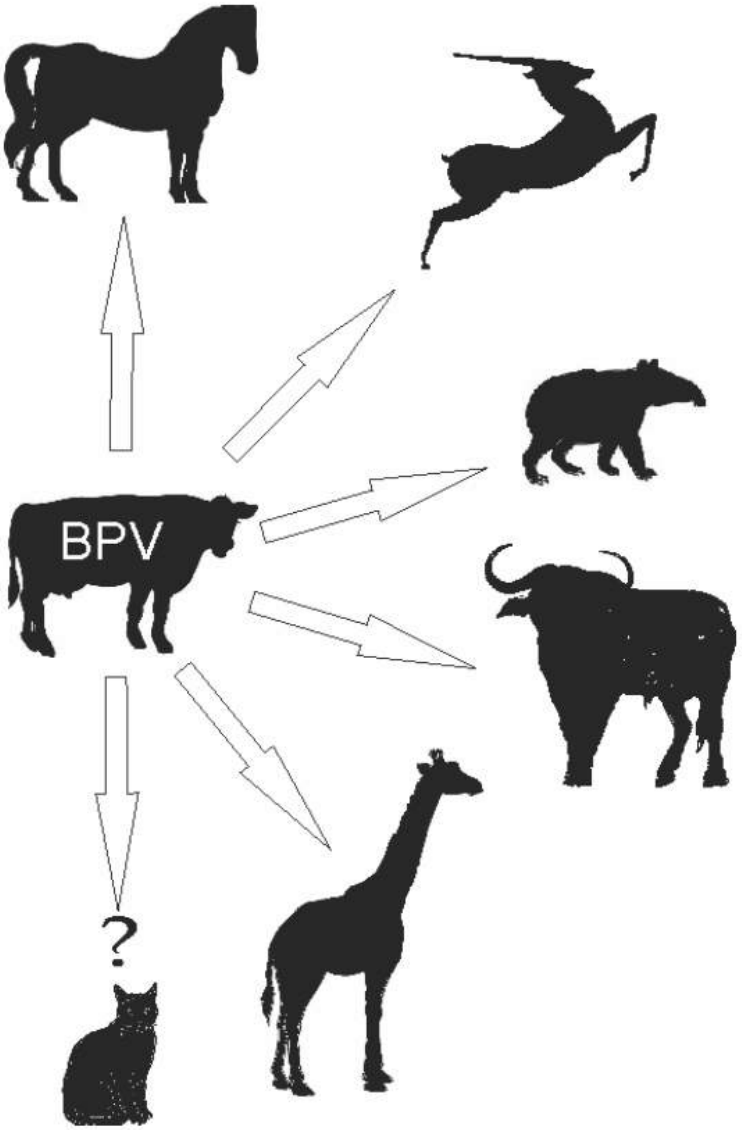

Figure 3. Schematic presentation of cross-infection caused by BPV. BPV DNA was found in close and distant related hosts: horse, sable antelope, tapir, buffalo, giraffe and possible cat.

New aspects involving the mechanisms of BPV transmission and cross-species infection have broken some paradigms about these viruses. The BPV status as an epitheliotropic and species-specific viruses can no longer be seen that way. The heterologous BPV infection has been consistently documented by several research groups worldwide, as well as the evidence of the presence of the virus in non-epithelial tissues.

The co-infection by multiple BPV has also generated interesting discussions. The occurrence of several BPV types in a specific anatomical region suggests that both multiple papillomavirus infections and high viral diversity can be frequent in cattle. The identification of multiple BPV infections may contribute to the understanding of the

epidemiological, clinical, and immunological features of cutaneous papillomatosis in cattle. Particularly for the immunological approach, this multiple infection brings important implications when is considered immunization strategies to eradicate papillomatosis, since the introduction of vaccines against a single BPV type may contribute to the spread of other genotypes able to cause skin lesions with similar morphological characteristics in cattle.

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SHORT COMMUNICATION

Co-infection of Bovine Papillomavirus and Feline-Associated Papillomavirus in bovine cutaneous warts

M. A. R. da Silva[1], C. C. R. Carvalho[1], L. C. A. Coutinho[2], M. C. Reis[2], M. V. de Aragão Batista[1], R. S de Castro[2], F. B. R. dos Anjos[3] and A. C. de Freitas[1]

[1] Department of Genetics, Federal University of Pernambuco, Pernambuco, Recife, Brazil
[2] Department of Veterinary Medicine, Rural Federal University of Pernambuco, Pernambuco, Recife, Brazil
[3] Department of Pathology, Federal University of Pernambuco, Pernambuco, Recife, Brazil

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Correspondence:
A. C. de Freitas, Department of Genetics of the Federal University of Pernambuco, Av. Prof. Moraes Rêgo, 1235, Cidade Universitária, Recife, Pernambuco 50740521,Brazil.
Tel.: 0055 (081) 2126 8569;
Fax: 0055 (081) 2126 8522;
E-mail: antonio.freitas@pq.cnpq.br

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Summary

The diversity of papillomavirus (PV) found in bovine cutaneous warts from Brazilian cattle was evaluated using the PCR technique with the utilization of consensus primers MY09/11 and by PCR using Bovine Papillomavirus (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. The presence of BPV types 1, 2, 3, 6 and feline sarcoid-associated PV (FeSarPV) in cutaneous wart lesions, as well as the presence of co-infections, was found. 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.

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).

Bovine Papillomavirus1 (BPV1) and Bovine Papillomavirus2 (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 shown 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 tumours, a new putatitve papillomavirus type named feline sarcoid-associated papillomavirus (FeSarPV) was detected and evidence 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., 2010). FeSarPV DNA was found in sarcoids of cats from North America and New Zeland (Munday et al., 2010). Recently, FeSarPV DNA was verified in bovine fibropapillomas and dermatitis (Munday and Knight, 2010).

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 haematoxylin-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, Hilden, Germany), in accordance with the manufacturer's 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 25 μl containing 100 ng of DNA, 1X Master Mix (Promega, Fitchburg, WI, USA) and 0.2 μM 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 shown in Table 1, according to the amplification protocol by Yaguiu et al. (2008).

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 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, Foster City, CA, 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 performed by Muscle algorithm incorporated in MEGA version 5 (Tamura et al., 2011). The GenBank accession number of the sequences used is shown in the Supplementary Table S1. The phylogenetic analysis was carried out using Neighbour-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 analysed along with the isolates of this study.

The primers for β-globin gene amplified a fragment of 450 bp 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, similarity of 99% to BPV6 in one sample and 100% similarity to FeSarPV in one cutaneous wart sample were observed. 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.

The phylogenetic analysis confirmed the BPV types detected with good statistical confidence (Fig. 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

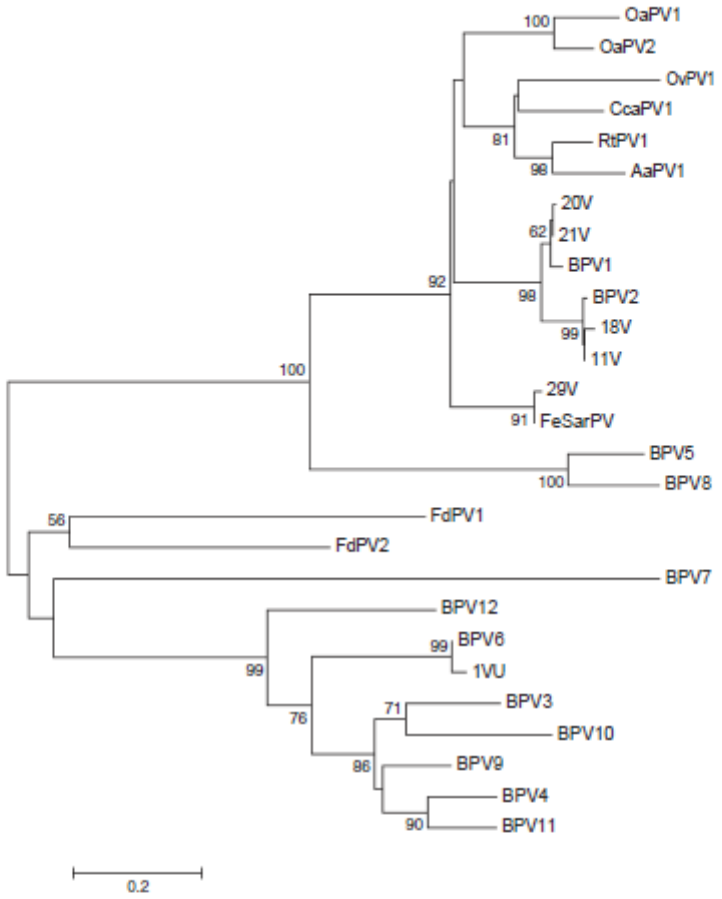

Fig. 1. Phylogenetic tree based on L1 fragment amino acid sequences comprising the isolates of this study, bovine and feline papillomaviruses, along with *Deltapapillomavirus* members. Numbers in the nodes represent bootstrap confidence values above 50%. Branch lengths are in scale.

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.

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 and Knight, 2010; 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 and Knight, 2010).

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 study of BPV detection associated with FeSarPV in American continent. Munday and Knight (2010) 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 and Knight (2010). Previously, FeSarPV in feline sarcoids from North America and New Zealand (Munday et al., 2010) was detected, and for the first time, it was described in fibropapillomas and dermatitis of bovine from New Zealand using specific primers set (Munday and Knight, 2010).

The phylogenetic analysis showed that FeSarPV was clustered in the *Deltapapillomavirus* genus, together with OaPV1, OaPV2, BPV1, BPV2, and other artiodactyl PVs, corroborating with the findings of Teifke et al. (2003) and Munday et al. (2010). FeSarPV had high identity compared with 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., 2006; 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 and Knight (2010) 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 analysed were small, the statistical support of the FeSarPV and artiodactyl PVs cluster was high, indicating that the hypothesis that FeSarPV could really be a BPV variant that infects a different host is valid. 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 relationship. Feline sarcoid is a rare tumour, mostly found in cats from rural area or exposed to cattle (Schulman 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 and Knight (2010), 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.

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Supporting Information

Additional supporting information may be found in the online version of this article. A formal description of the model structure is presented.

Table S1. List of PVs, their respective hosts and GenBank accession number.

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Novel E6 and E7 oncogenes variants of human papillomavirus type 31 in Brazilian women with abnormal cervical cytology

Bárbara Simas Chagas [a,*], Marcus Vinicius de Aragão Batista [b], Sergio Crovella [c,d], Ana Pavla Almeida Diniz Gurgel [a], Jacinto Costa Silva Neto [e], Ivi Gonçalves Soares Santos Serra [f], Carolina Maria Medeiros do Amaral [a], Valdir Queiroz Balbino [b], Maria Tereza Cartaxo Muniz [g], Antonio Carlos de Freitas [a,*]

[a] Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Federal University of Pernambuco, Pernambuco, Brazil
[b] Laboratory of Bioinformatics and Evolutionary Biology, Department of Genetics, Federal University of Pernambuco, Pernambuco, Brazil
[c] Laboratory of Variability and Human Genetics, Department of Genetics, Federal University of Pernambuco, Pernambuco, Brazil
[d] Genetic Service IRCCS Burlo Garofolo, Trieste, Italy
[e] Molecular and Cytological Research Laboratory, Department of Histology, Federal University of Pernambuco, Pernambuco, Brazil
[f] Gynaecological Unit, Center of Integrated Medicine in Sergipe, CEMISE, Sergipe, Brazil
[g] Laboratory of Molecular Biology of Center of Pediatric Oncohematologia, University of Pernambuco, Pernambuco, Brazil

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ABSTRACT

HPV-31 has been widely described as an important oncogenic type, showing high incidence in worldwide and especially in Northeastern Brazil. We sought to identify the presence of specific mutations in HPV-31 E6 and E7 oncogenes in women with abnormal cervical smear. We enrolled 150 gynecological patients from Sergipe State, Northeastern Brazil. HPV screening was carried out by polymerase chain reaction (MY09/11). E6 and E7 oncogenes were amplified with specific primers and sequenced. The sequences obtained were aligned with the GenBank reference sequences in order to search for genetic variants. We identified genetic variants in E6 and E7 sequences from HPV-31. Two new nucleotide changes in E6 and E7 were described for the first time in this study. A novel mutation in E6 resulted in amino acid change in a site belonging to T-cell epitope with MHC II binding activity. There was no significant difference in the distribution of HPV-31 E6 and E7 variants when compared to all selected clinical/epidemiological characteristics. HPV-31 isolates have been clustered into three main groups called lineages A, B and C. We describe new HPV-31 variants in Brazil, contributing to better understand the genomic diversity of these viruses.

1. Introduction

Cervical cancer is the second most common form of neoplasia in women worldwide. Epidemiologic evidences indicate that infection with high- risk human papillomavirus (HPV) is the principal cause of invasive cervical cancer (ICC) and cervical intraepithelial neoplasia (CIN) (Schiffman et al., 1993; Walboomers et al., 1999; Bosch et al., 2002). To date, more than 200 papillomavirus types have been characterized and sixty of these are detected in cervical epithelium (de Villiers et al., 2004; Bernard et al., 2010).

HPV belongs to the *Papillomaviridae* virus family. HPV-31 is classified into the *Alphapapillomavirus* genus, phylogenetically clustered in Alpha-9 species groups (de Villiers et al., 2004; Bernard et al., 2010; Schiffman et al., 2010) and it has been described as an important oncogenic type (Clifford et al., 2006). A new HPV type is defined by the presence of less than 90% of identity compared to established prototypes in L1 gene sequence, associated with the cloning and sequencing of its complete genome (de Villiers et al., 2004; Bernard et al., 2010). In addition, the term subtype is used to identify HPV genomes with L1 nucleotide sequences that differ between 2–10% from the closest type, and the variants differ less than 2% in nucleotide sequence of L1, and 5% in LCR (de Villiers et al., 2004; Prado et al., 2005).

There is a proposed hypothesis about the differences in pathogenicity existing among variants of a genotype (Xi et al., 2007). Studies have demonstrated that HPV-16 variants differ in their association with cervical cancer (Xi et al., 1995, 2007; Zehbe et al., 1998, 2001; Berumen et al., 2001) and viral persistence (Londesborough et al., 1996; Ferenczy and Franco, 2002; Sichero et al., 2007; Lee et al., 2008). Non-synonymous mutations in the E6 oncogene could alter the biological or immunogenic properties of the

* Corresponding authors. Address: Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil. Fax: +55 81 21268512.
E-mail addresses: babisimas@gmail.com (B.S. Chagas), antonio.freitas@pq.cnpq.br (A.C. de Freitas).

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encoded protein (Ellis et al., 1995; Zehbe et al., 2003). However, clinical relevance of HPV-31 variants has been rarely described (Schiffman et al., 2010; Cento et al., 2011; Chagas et al., 2011). Based on whole genome analysis, Chen et al. (2011) classified HPV-31 variants into three different phylogenetic entities called A, B and C lineages. This classification is important to the understanding of the variant-related pathogenesis of cervical lesion (Xi et al., 2012).

Thus, in this study, the presence of specific mutations in E6 and E7 oncogenes of HPV-31 was investigated in women with low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL). The association between the grade of cervical lesion and the predicted T- and B-cell epitopes with these polymorphisms was verified. In addition, the phylogenetic relationships among the HPV-31 isolates were analyzed in order to understand their distribution and possible geographical and ethnicity patterns.

2. Material and methods

2.1. Study groups

We enrolled 150 women aged between 18 and 55 years, with a mean age of 36.2 years (s.d., 11.1) at the Gynecology Unit in "Center for Integral Attention to Women's Health (CAISM)" in Sergipe State, Northeastern Brazil. The study included women with abnormal cervical cytology divided in low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). We collected cervical cells by scraping in PBS solution (pH 7.4); cervical-cells were stored at −80 °C. DNA was extracted using DNeasy Blood Tissue Kit (Qiagen), following the manufacturer protocol.

We obtained approval of the Ethical Committee (CEP/CCS/UFPE N° 491/11) and informed consent from all women enrolled for the study.

2.2. HPV screening

The obtained DNA was submitted to amplification by PCR of human *MDM2* gene in order to assess the quality of DNA, in order to avoid possible false negative results (Ma et al., 2006). HPV screening was carried out by polymerase chain reaction (PCR), using degenerate primers MY09/11 (Manos et al., 1989). HPV typing was performed by PCR using specific primers for HPV-31 (Karlsen et al., 1996). E6 and E7 genomic regions from all DNA samples were amplified using specific primers and Taq DNA polymerase High Fidelity (Chagas et al., 2011).

2.3. Sequencing of E6 and E7 ORFs

From each sample, one independent PCR product was generated and sequenced (both strands). Only sequence variations detected in both strands were evaluated. Sequencing of E6 and E7 was performed using ABI PRISM BigDye® Terminator Cycle Sequencing V.3.1 kit (Applied Biosystems) and with the same reverse and forward primers used in the amplification reaction.

2.4. Data analysis

The obtained sequences were checked for quality and assembled using the Staden package (Staden, 1996). HPV-31 E6 and E7 sequences were compared to sequences from GenBank using BLAST (Altschul et al., 1990). Multiple sequence alignment was performed using ClustalW (Thompson et al., 1994).

HPV-31 variants were associated with various characteristics of the study subjects. Statistical analysis was done using Fisher's exact test, when the frequencies were smaller than five. The association was considered significant when $p < 0.05$.

T-cell epitope prediction was performed using ProPred-1 (Singh and Raghava, 2003) and ProPred (Singh and Raghava, 2001) servers for the prediction of MHC classes I and II biding regions, respectively. B-cell epitope prediction was performed using Bcepred server (Saha and Raghava, 2004) with the default parameters.

Maximum likelihood (ML) phylogenetic trees were inferred for concatenated and separated E6 and E7 genomic regions using PhyML v. 3.0 (Guindon et al., 2010). The evolutionary models TPM3uf + I and K80 + I + G were estimated by jModelTest V. 0.1.1 (Posada, 2008) for E6 and E7 analyses, respectively. The best of NNI and SPR methods of tree searching was used to estimate tree topology. The robustness of the branches was assessed using 1000 bootstrap replicates.

3. Results

A total of 150 cervical samples were screened with L1 consensus primers (MY09/11): 90 (60.0%) were positive for HPV, and further genotyping with type specific primers revealed that 22 (14.7%) samples were positive for HPV-31, 47 (31.3%) were positive for HPV-16 and 5 (3.3%) were positive for HPV-33. Considering the 90 samples in which it was possible to identify HPV sequences, 16 samples displaying more than one HPV type were detected: the results indicated that HPV-16 + HPV-31 was the most frequent (12, 75%), followed by HPV-16 + HPV-33 (3, 18.8%) and HPV-31 + HPV-33 (1, 6.3%).

35 HPV-31 positive samples (22 single HPV-31, 12 HPV-16 + HPV-31 and 1 HPV-31 + HPV-33) were randomly selected to evaluate the genetic variability. Among these, it was observed that 19 patients (54.3%) presented high-grade squamous intraepithelial lesion (HSIL) and 16 patients (45.7%) presented low-grade squamous intraepithelial lesion (LSIL).

3.1. Genetic variability of E6 oncogene

Nucleotide sequences of the complete E6 oncogene from all patients with cervical lesion (High/Low grade) were compared with the HPV-31 reference sequence (J04353). Sixteen patients (55.2%) showed nine different types of E6 oncogene mutations, of which five were missense mutations at codons 60, 64, 123, 138 and 144 for amino acids tyrosine, alanine, arginine, valine and glycine, respectively (Table 1). The remaining four nucleotide variations led to silent mutations (Table 1). The sites presenting the most frequent mutations were 320 (51.7%, 15 of 29), 520 (48.3%, 14 of 29), 428 (41.4%, 12 of 29), 285 (37.9%, 11 of 29) and 404 (37.9%, 11 of 29). Other mutations were A297G (6.9%, 2 of 29), A475G (6.9%, 2 of 29), T248C (3.4%, 1 of 29), and C537G (3.4%, 1 of 29).

A novel mutation (C537G) in E6 was found and resulted in an amino acid substitution R144G (Table 1). GenBank accession number of this new variant is: JX185395. We performed T-cell and B-cell epitopes prediction in order to evaluate the impact of this new mutation on the immune function of the E6 transforming protein. We identified an amino acid change in a site belonging to T-cell epitope with MHC II binding activity (E6 140-148 WRRPG-TETQ). The same amino acid change was also observed in a site belonging to B-cell epitope.

Nucleotide substitutions resulting in premature stop codons or frameshift changes were nor detected.

Table 1
Nucleotide and amino acid variability of HPV-31 E6 and E7 regions. The nucleotides conserved with respect to the reference sequence (J04353) are marked with a dot (.). In bold, nucleotide variations not described in the literature. Reference: prototype sequence of HPV-31. H: histidine, T: threonine, Y: tyrosine, A: alanine, K: lysine, R: arginine, V: valine, G: glycine, E: glutamate. WT: Wild type.

	Nucleotide position at E6[a]									Nucleotide position at E7[a]						
WT nucleotide	248	285	297	320	404	428	475	520	537	580	592	626	670	695	743	Variant lineage
	T	C	A	A	G	A	A	C	C	G	T	C	C	G	A	
HPV31/UFPE-08	NE									.	C	.	.	.	G	A
HPV31/UFPE-11	.	T	.	T	A	G	.	T	.	NE						C
HPV31/UFPE-13	.	T	.	T	A	G	.	T	.	A	.	.	T	A	G	C
HPV31/UFPE-14	.	T	.	T	A	G	.	T	.	A	.	.	T	A	G	C
HPV31/UFPE-16	.	T	.	T	A	G	.	T	.	A	.	.	T	A	G	C
HPV31/UFPE-17	.	T	.	T	A	G	.	T	.	NE						C
HPV31/UFPE-18	.	T	.	T	A	G	.	T	.	A	.	.	T	A	G	C
HPV31/UFPE-21	.	.	G	T	.	.	G	T	.	.	.	T	T	A	G	B
HPV31/UFPE-07	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A
HPV31/UFPE-25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
HPV31/UFPE-31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-35	.	.	.	.	.	.	.	.	.	NE						A
HPV31/UFPE-36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
HPV31/UFPE-37	NE									A	.	.	T	A	G	C
HPV31/UFPE-09	.	T	.	T	A	G	.	T	.	A	.	.	T	A	G	C
HPV31/UFPE-10	.	T	.	T	A	G	.	T	.	NE						C
HPV31/UFPE-12	.	T	.	T	A	G	.	T	.	.	.	T	T	A	G	C
HPV31/UFPE-15	.	T	.	T	A	G	.	T	.	.	.	.	.	.	G	C
HPV31/UFPE-19	.	T	.	T	A	G	.	T	.	A	.	.	T	A	G	C
HPV31/UFPE-20	.	.	.	T	.	.	.	.	.	NE						A
HPV31/UFPE-22	C	.	G	T	.	.	G	T	.	.	.	T	T	A	G	B
HPV31/UFPE-23	.	.	.	T	.	.	.	T	.	.	.	.	.	.	G	C
HPV31/UFPE-24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
HPV31/UFPE-27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
HPV31/UFPE-38	NE									.	.	.	.	.	G	A
HPV31/UFPE-41	NE									.	.	.	.	.	.	A
HPV31/UFPE-42	NE									.	.	.	.	.	.	A
HPV31/UFPE-43	NE									.	.	.	.	.	.	A
WT amino acid		H	T				K	A	R			H		E	K	
Amino acid position		60	64				123	138	144			23		46	62	
Amino acid change		Y	A				R	V	G			Y		Y	E	

NE: Isolates not evaluated to E6 or E7.

3.2. Genetic variability of E7 oncogene

The comparative analysis of E7 transforming gene with the HPV-31 reference sequence (J04353) revealed that twenty-three isolates (76.7%) presented six variable sites. Three mutations were missense at codons 23, 46 and 62 for amino acids tyrosine, tyrosine and glutamic acid, respectively (Table 1). The remaining three mutations were silent ones (Table 1). The sites that presented the most frequent mutations were 743 (76.7%, 23 of 30), 670 (33.3%, 10 of 30), and 695 (33.3%, 10 of 30). Other mutations were G580A (23.3%, 7 of 30), C626T (10%, 3 of 30) and T592C (3.3%, 1 of 30). One novel mutation (T592C) in E7 was found, although it did not result in amino acid change (Table 1). GenBank accession number of this new variant is: JX185396. Nucleotide substitutions resulting in premature stop codons or frameshift changes were not detected.

3.3. Phylogenetic analysis

The evolutionary relationships among several isolates of HPV-31 were assessed using E6 and E7 nucleotide sequences. The phylogenetic tree generated from the concatenated E6 and E7 sequences clustered the isolates of HPV-31 into three main groups called lineages A, B and C (Fig. 1). Those lineages were classified according to the topology and nucleotide sequence differences from >1% to <10%. Despite the isolate HPV31/UFPE-23 was different from lineage C, its average genetic distance to the isolate HPV31/UFPE-15 was only 0.3 ± 0.2%, so it was classified as belonging to the lineage C. The HPV-31 isolates from this study did not cluster together in one clade. As depicted in Fig. 1, HPV-31 variants did not follow the any patterns of patients' ethnicity or a geographical distribution.

3.4. Distribution of HPV-31 variants lineages according to clinical/epidemiological characteristics

In all 35 HPV-31 positive samples (with and without co-infection), A, B and C lineages were detected in 20 (57.1%), 2 (5.7%) and 13 (37.1%), respectively. However, only 22 samples positive for HPV-31 (without co-infection) were used to analyze the distribution of variants lineages by clinical and epidemiological characteristics. In this case, A and C lineages were detected in 13 (59.1%) and 9 (40.9%) women respectively, while B lineage was absent. There was no significant difference in the distribution of HPV-31 E6 and E7 variants when compared to all selected clinical/epidemiological characteristics (Table 2).

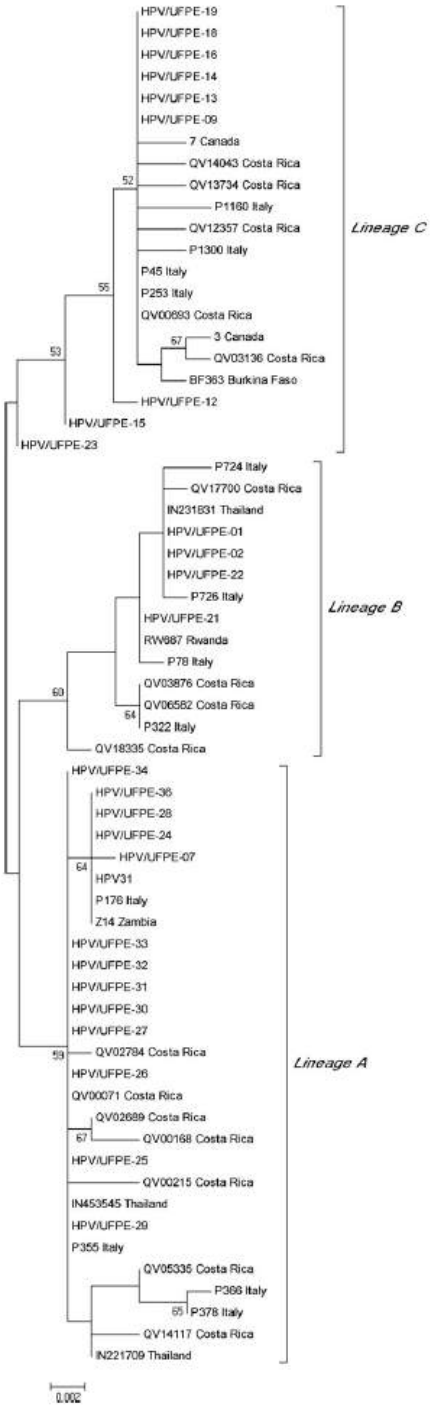

Fig. 1. Maximum likelihood phylogenetic tree of HPV-31 variants based on concatenated E6 and E7 genes. Three clusters were identified as lineages A, B and C. Only bootstrap values above 50% are represented in the branches.

Table 2
Distribution of HPV-31 variants with study subjects demographics and clinical characteristics.

Variable	A variants no. (%)	B variants no. (%)	C variants no. (%)	*p*-value
Total no.	13	0	9	
Age				
18–30	7 (53.8)	0	4 (44.4)	1
⩾ 31	6 (46.2)	0	5 (55.6)	
Racial group				
Caucasian	2 (15.4)	0	1 (11.1)	1
African	3 (23.1)	0	3 (33.3)	
Pardo	8 (61.5)	0	5 (55.6)	
Current use of hormonal contraceptives				
No	5 (38.5)	0	4 (44.4)	1
Yes	8 (61.5)	0	5 (55.6)	
Current smoking				
No	12 (92.3)	0	8 (88.9)	1
Yes	1 (7.7)	0	1 (11.1)	
Cytology				
LSIL[a]	5 (38.5)	0	6 (66.7)	0.38
HSIL[b]	8 (61.5)	0	3 (33.7)	
No. of sexual partners				
1–4	11 (84.6)	0	8 (88.9)	1
⩾ 5	2 (15.4)	0	1 (11.1)	

[a] LSIL: Low-grade squamous intraepithelial lesion.
[b] HSIL: High-grade squamous intraepithelial lesion.

4. Discussion

The study of HPV genetic variability may contribute to better understanding the mechanisms of transformation and progression of cervical cancer. In fact, it has been suggested that variants of the same HPV type are biologically distinct and may have differential pathogenic risks (Sichero et al., 2007). HPV-16 is the most detected viral type in cervical cancer worldwide, followed by the type-18. However, the incidence of HPV-31 is very high in Northeastern Brazil and Southern Europe (Baldez da Silva et al., 2009, 2012; Cento et al., 2011). So, in this study we have analyzed the genetic variability of HPV-31 E6 and E7 genomic regions, in cervical samples from Northeastern Brazilian women.

Nucleotide changes previously described in E6 oncogene of HPV-31 were observed at positions 248, 297, 320, 475, 520 (Chagas et al., 2011), 285, 404 (Calleja-Macias et al., 2005) and 428 (Garbuglia et al., 2007). The nucleotide change at position 537 is described for the first time in this study. When considering HPV-31, E7 oncogene, we observed changes previously described at positions 626, 670, 695, 743 (Chagas et al., 2011) and 580 (Chen et al., 2011). Moreover, in E7 of HPV-31, we reported a novel nucleotide change at position 592. E6 and E7 oncogenes showed a peculiar substitution pattern, suggesting that these variants may be circulating with higher frequency in the population from Northeast Brazil. In those genes, the non-synonymous mutations C285T, A297G, A475G, C520T, C537G, C626T, G695A, A743G, leads to change of polarity, hydropathic potential and amino acid side chain, potentially influencing the correct folding of the oncoprotein (Chagas et al., 2011).

Nucleotide variations in E6 and E7 oncogenes play a critical role in the development of cervical carcinogenesis due to their ability to inactivate p53 and pRb proteins, respectively (Kast et al., 1994). Epidemiological studies have shown association between HPV-16 genetic variability and cervical carcinogenesis. The amino acid changes of HPV-16 E6 D25E and L83V are associated with the elevated risk of development of cervical carcinomas (Zehbe et al., 1998, 2011; Matsumoto et al., 2000; Kammer et al., 2002; Lee et al., 2008; Cai et al., 2010; Freitas et al., 2012).

According to Sichero et al. (2012), the association of E-350G variant (E6, HPV-16) and cancer risk may be explained by its ability to evade the host immune system and/or by the increased oncogenic potential due to biological properties of viral proteins. Functional studies demonstrated that the E-350G variant has an increased Tp53 degradation (Asadurian et al., 2007), immortalization and transformation abilities (Richard et al., 2012), degradation of BAX and binding to E6BP (Lichtig et al., 2006). The published reports about the clinical relevance of HPV-31 variants are limited. In this study one genetic variation observed in E6 (C537G) was located in B-cell and T-cell epitopes site and this change may influence the immune recognition of cell infected by HPV/evasion of the natural immune response (Chagas et al., 2011). In HPV-16, the common E6 variant L83V was suggested as associated with the HLA class I alleles B*44, B*51 and B*57 with approximately four to five fold increased risk for cancer (Zehbe et al., 2003). In another study, a HPV-16 E6 variant (R10G) was demonstrated to alter a B*07 binding epitope which may influence immune recognition by cytotoxic T lymphocytes (Ellis et al., 1995).

The obtained phylogenetic trees clustered the HPV-31 isolates into three distinct lineages as defined by Chen et al. (2011). Although the tree is well supported, two isolates from Costa Rica were clustered in different lineage, supporting the idea that the classification should be carried out using the whole genome. However, the phylogenetic tree obtained on the basis of our findings shows the evolutionary relationships of the HPV-31 oncogenes, which could shed light into the evolution of carcinogenesis process among HPV-31 lineages.

In general, E6 and E7 phylogenetic trees did not show any geographic and ethnic patterns among HPV-31 isolates, in accordance with other studies (Chagas et al., 2011; Chen et al., 2011). Although the evolution of HPV-16 and HPV-18 variants is associated with the diversification of human ethnic groups (Schiffman et al., 2006), other HPVs did not show this evolutionary characteristic.

In this study, we observed a great diversity among HPV-31 variants isolated in patients from Northeast Brazil. Strains belonging to the three HPV-31 lineages have been detected. The association among HPV-31 variant lineages and cervical lesions is still topic for discussion. In this study, no association between variants lineages and cervical lesions was found, similarly to the findings of Cento et al. (2011). Despite this lack of association, HPV oncogenes are suggested be more flexible in order to adapt the viruses to different biological niches (Chen et al., 2011). So, further studies are necessary in order to clarify the mechanisms that are influenced by these polymorphisms. However, we are aware of the limitations of our study, due to the low number (22) of HPV-31 positive samples analyzed.

In conclusion, our findings describe two new HPV-31 variants in Brazil, contributing to better understand the genomic diversity of these viruses. In addition, the characterization of HPV oncogenes variants in cervical disease may improve the knowledge of the molecular mechanisms underlying disease progression and transformation.

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Competing Interest

The authors B.S.C, M.V.A.B., S.C., A.P.A.D.G., J.C.S.N., I.G.S.S.S., C.M.M.A., V.Q.B., M.T.C.M. and A.C.F. have no conflict of interest to declare.

Ethical approval

The work has been approved by the Human Research – Center of the Biological Sciences - CCB/UFPE Ethics Committee (CEP/CCS/UFPE N° 491/11) and informed consent from all women in the study were obtained.

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Capítulo de livro publicado

Chapter

GENETIC DIVERSITY OF HUMAN AND ANIMAL PAPILLOMAVIRUSES

Antonio Carlos de Freitas,[a] Ana Pavla Almeida Diniz Gurgel,[a] Bárbara Simas Chagas,[a] Valdir de Queiroz Balbino,[b] and Marcus Vinicius Aragão Batista[b]*

[a]Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Pernambuco, Brazil

[b]Laboratory of Bioinformatics and Evolutionary Biology, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Pernambuco, Brazil

ABSTRACT

The papillomaviruses form a highly diverse group that infects mammals, birds and reptiles. There is a huge veterinary and public health importance associated with these viruses. Because of this, human and bovine papillomaviruses are the most studied viruses of this group. Infection with human papillomavirus (HPV) represents the most important risk factor in the genesis of cervical cancer. Bovine papillomavirus (BPV) infects basal epithelial cells leading to the formation of benign and malignant tumors, when in synergism with action of environmental co-factors. Brazil is a country with continental dimensions; therefore the understanding of the genetic variability and the distribution of these viruses is essential for an active surveillance program and the design of more efficient vaccines and diagnostic methods. In what concerns the association of HPV and cervical cancer, HPV-16 is the most frequent type worldwide, with the exception of Indonesia where the HPV-18 happens to be the most common type. Although the HPV-16 is the most frequent, the second most important type differs between different regions in the world. So, this difference in the incidence of HPV genotypes needs to be elucidated, along with the genetic variability of these isolates that could lead to distinct oncogenic potential among different variants and subtypes. Oncogenic variants of HPV-31 were found and some of the mutations were observed in regions involved in host immune recognition. Changes in amino acid sequences between variants from different geographical regions evidence the possibility of functional divergence between them, suggesting that the variants detected may have different adaptive fitness. On the other hand, twelve BPV types are known so far, but only three of them are associated with cancer. There is a great diversity of BPV types among cattle and high co-infection was found. The presence of infection with multiple viral types could lead to suppression of the immunological system making the clinical state of the animals worse. So, further molecular epidemiological investigations on the incidence and diversity of papillomavirus infection will assist in establishing a more accurate view of the distribution of these viruses. This could help the development of accurate and specific diagnostics, prophylactics and therapeutic requirements.

12. Curriculum vitae (Lattes)

Marcus Vinicius de Aragão Batista
Curriculum Vitae

Março/2013

Marcus Vinicius de Aragão Batista
Curriculum Vitae

Dados pessoais

Nome Marcus Vinicius de Aragão Batista
Filiação José Airto Batista e Maria Aleide de Aragão Batista
Nascimento 22/09/1983 - Aracaju/SE - Brasil

Endereço profissional Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Departamento de Genética
Av. Prof. Moraes Rego, 1235, Departamento de Genética, Laboratório de Bioinformática e Biologia Evolutiva
Cidade Universitária - Recife
50670-901, PE - Brasil
Telefone: 81 97124882

Endereço eletrônico
E-mail para contato : genetics_marcus@hotmail.com
e-mail alternativo : genetics.marcus@gmail.com

Formação acadêmica/titulação

2010 Doutorado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Estudo da diversidade genômica e dinâmica evolutiva de Papilomavírus Bovino associado à epidemiologia molecular das papilomatoses bovinas cutâneas
Orientador: Valdir de Queiroz Balbino
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico
Palavras-chave: Papilomavírus, Genômica, Biologia Molecular, Bioinformática, Evolução Viral, Filogenia Molecular, Modelagem Molecular
Áreas do conhecimento : Genômica Viral e Bioinformática

2008 - 2010 Mestrado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Genômica Comparativa e Reconstrução Filogenética de Papilomavírus, Ano de obtenção: 2010
Orientador: Valdir de Queiroz Balbino
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico
Palavras-chave: Papilomavírus, Bioinformática, Genômica, Filogenia Molecular
Áreas do conhecimento : Genética Molecular e de Microorganismos,Bioinformática

2002 - 2007 Graduação em Ciências Biológicas Licenciatura.
Universidade Federal de Sergipe, UFS, Sao Cristovao, Brasil
Título: Abordagem da Virologia nos livros didáticos de Biologia do Ensino Médio na cidade de Aracaju-SE-Brasil
Orientador: Alexandre Luna Cândido
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

Formação complementar

2012 - 2012 Curso de curta duração em Avaliação de Impacto Ambiental.
Associação Brasileira de Educação a Distância, ABED, Sao Paulo, Brasil

2012 - 2012 Phylogeny, Biogeography and Spatial Modeling.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

2011 - 2011 Curso de curta duração em Phylogeny and Evolution of Viruses.
Sociedade Brasileira de Virologia, SBV, Rio De Janeiro, Brasil

2010 - 2010 Curso de curta duração em Gestão Ambiental Desenvol. Sust. Hist. Quest. Amb..
Fundação Getúlio Vargas, FGV, Rio De Janeiro, Brasil

Atuação profissional

1. Universidade Federal de Pernambuco - UFPE

Vínculo institucional

2010 - 2010 Vínculo: Aluno de Doutorado , Enquadramento funcional: Bolsista Mobilidade Estudantil , Carga horária: 40, Regime: Integral

2010 - Atual Vínculo: Aluno de Doutorado , Enquadramento funcional: Pesquisador Colaborador , Carga horária: 40, Regime: Integral

2008 - 2010 Vínculo: Estudante de Mestrado , Enquadramento funcional: Pesquisador Colaborador , Carga horária: 40, Regime: Integral

Atividades

08/2012 - 08/2012 Pós-graduação, Douorado em Biotecnologia
Disciplinas ministradas:
Bioinformática Aplicada à Saúde (Programa de Pós-Graduação em Biotecnologia (PPGB/RENORBIO), Ponto Focal Piauí)

04/2011 - 04/2011 Pós-graduação, Douorado em Biotecnologia
Disciplinas ministradas:
Atuei como Professor Auxiliar da disciplina Bioinformática Aplicada à Saúde, do Programa de Pós-Graduação em Biotecnologia (PPGB/RENORBIO), Ponto Focal Piauí.

03/2008 - Atual Pesquisa e Desenvolvimento, Centro de Ciências Biológicas, Departamento de Genética
Linhas de pesquisa:
Bioinformática e Biologia Molecular Aplicados à Genômica e Evolução Viral

03/2008 - 02/2011 Graduação, Ciências Biológicas
Disciplinas ministradas:
Atuei como Professor Assistente Voluntário das seguintes disciplinas de graduação: , Bioinformática Aplicada à Genética (GN321 - 60 horas - Anual) , Genética de Populações (GN238 - 45 horas - Semestral)

Linhas de pesquisa

1. Bioinformática e Biologia Molecular Aplicados à Genômica e Evolução Viral

Objetivos:Utilizar ferramentas moleculares e computacionais visando entender os processos genéticos e evolutivos da diversificação de vírus de importância médica e veterinária.
Palavras-chave: Bioinformática, Biologia Molecular, Papilomavírus

Áreas de atuação

1. Bioinformática
2. Genética de Populações e Evolução
3. Genômica e Filogenia Molecular
4. Genética Molecular e de Microorganismos
5. Virologia

Projetos

Projetos de pesquisa

2010 - 2012 Uso de ferramentas de Biologia Molecular no estudo dos padrões de transmissão vetorial da leishmaniose visceral canina em uma área de transmissão ativa no Sertão do Estado de Pernambuco, Brasil
Situação: Concluído Natureza: Projetos de pesquisa
Alunos envolvidos: Graduação (3); Mestrado acadêmico (5); Doutorado (4);
Integrantes: Marcus Vinicius de Aragão Batista Valdir de Queiroz Balbino (Responsável)
Financiador(es): Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco-FACEPE

2010 - 2012 Vacina de DNA contra papilomaviroses: uso do papilomavirus bovino como modelo experimental para o desenvolvimento de uma extratégia vacinal contra o câncer de colo de útero em humanos baseada nos genes E5 e L2
Situação: Concluído Natureza: Projetos de pesquisa
Alunos envolvidos: Graduação (3); Mestrado acadêmico (3); Doutorado (4);
Integrantes: Marcus Vinicius de Aragão Batista Antônio Carlos de Freitas (Responsável)
Financiador(es): Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco-FACEPE

2010 - Atual Estudo da diversidade genômica e dinâmica evolutiva de Papilomavírus Bovino associado à epidemiologia molecular das papilomatoses bovinas cutâneas
Descrição: Projeto de tese de Doutorado.
Situação: Em andamento Natureza: Projetos de pesquisa
Alunos envolvidos: Doutorado (1);
Integrantes: Marcus Vinicius de Aragão Batista (Responsável); Valdir de Queiroz Balbino; Antônio Carlos de Freitas
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq

2008 - 2010 Genômica Comparativa e Reconstrução Filogenética de Papilomavírus
Descrição: Projeto de Dissertação de Mestrado
Situação: Concluído Natureza: Projetos de pesquisa
Alunos envolvidos: Mestrado acadêmico (1);
Integrantes: Marcus Vinicius de Aragão Batista Valdir de Queiroz Balbino (Responsável); Antônio Carlos de Freitas
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq

Idiomas

Inglês Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Espanhol Compreende Razoavelmente , Fala Razoavelmente , Escreve Razoavelmente , Lê Razoavelmente

Português Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Prêmios e títulos

2011 Prêmio Hélio Gelli Pereira (Categoria Pós-Graduação), Sociedade Brasileira de Virologia

2008 Menção honrosa pela participação no Prêmio Pós-Graduação, Sociedade Brasileira de Genética

Produção

Produção bibliográfica

Artigos completos publicados em periódicos

1. **BATISTA, M.V.A.**, FREITAS, A. C., BALBINO, V. Q.
Entropy-based approach for selecting informative regions in the L1 gene of bovine papillomavirus for phylogenetic inference and primer design. Genetics and Molecular Research. , v.12, p.400 - 407, 2013.
Referências adicionais : Inglês. Meio de divulgação: Meio digital

2. da Silva, M. A. R., **de Aragão Batista, M. V.**, dos Anjos, F. B. R., de Castro, R. S, Reis, M. C., de Freitas, A. C., CARVALHO, C. C. R., Coutinho, L. C. A.
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3. CARVALHO, C. C. R., **Batista, M. V. A.**, SILVA, M. A. R., BALBINO, V. Q., FREITAS, A. C.
Detection of Bovine Papillomavirus Types, Co-Infection and a Putative New BPV11 Subtype in Cattle. Transboundary and Emerging Diseases (Print). , v.9, p.no - no, 2012.
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4. **Batista, Marcus V.A.**, Ferreira, Tiago A.E., Freitas, Antonio C., Balbino, Valdir Q.
An entropy-based approach for the identification of phylogenetically informative genomic regions of Papillomavirus. Infection, Genetics and Evolution (Print). , v.11, p.2026 - 2033, 2011.
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5. Chagas, Bárbara S., **Batista, Marcus V.A.**, Guimarães, Vilma, Balbino, Valdir Q., Crovella, Sergio, Freitas, Antonio C.
New variants of E6 and E7 oncogenes of human papillomavirus type 31 identified in Northeastern Brazil. Gynecologic Oncology (Print). , v.123, p.284 - 288, 2011.
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6. Freitas AC,, SILVA, M. A. R., JESUS, A.L.S., MARIZ, F. C., CORDEIRO, M. N., DE ALBUQUERQUE, B. M. F., **Batista, M. V. A.**
Recent insights into Bovine Papillomavirus. AFR J MICROBIOL RES. , v.5, p.6004 - 6012, 2011.
Referências adicionais : Inglês. Meio de divulgação: Meio digital

7. BATISTA, M.V.A., CUNHA, M.M.S., CÂNDIDO, A.L.
Análise do tema Virologia em livros didáticos de Biologia do ensino médio. Ensaio: Pesquisa em Educação em Ciências (Impresso). , v.12, p.145 - 158, 2010.
Palavras-chave: Livro Didático, Virologia, Ensino Médio
Áreas do conhecimento : Ensino de Ciências
Referências adicionais : Português. Meio de divulgação: Meio digital. Home page: [http://www.portal.fae.ufmg.br/seer/index.php/ensaio/article/view/263/331]

8. BATISTA, M.V.A., SANTOS, M.I.S., MENEZES, L.C.S., CARNEIRO, M.R.P., CÂNDIDO, A.L.
Enterobacteriaceae e Pseudomonas sp. multiresistentes isoladas de efluentes urbanos em Aracaju, Sergipe. Biologia Geral e Experimental. , v.7, p.15 - 18, 2007.
Referências adicionais : Português. Meio de divulgação: Vários. Home page: [http://www.biologiageralexperimental.bio.br/revistas/revista%202007.1/saúde_pública.pdf]

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1. **BATISTA, M.V.A.**, da Silva, M. A. R., PONTES, N. E., Reis, M. C., CORTEGGIO, A., Castro, R. S., BORZACCHIELLO, G., BALBINO, V. Q., FREITAS, A. C.
Molecular epidemiology of bovine papillomatosis and the identification of a putative new virus type in Brazilian cattle. The Veterinary Journal (London, England. 1997). , 2013.
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2. **BATISTA, M.V.A.**, CHAGAS, B. S., CROVELLA, S., GURGEL, A.P.A.D., SILVA NETO, J. C., SERRA, I. G. S. S., AMARAL, C.M.M., BALBINO, V. Q., MUNIZ, M. T. C., FREITAS, A. C.
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Referências adicionais : Inglês.

Capítulos de livros publicados

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Genetic diversity of human and animal papillomaviruses In: Genetic Diversity: New Research.1 ed.Hauppauge : Nova Publishers, 2012, p. 1-34.
Referências adicionais : Estados Unidos/Inglês. Meio de divulgação: Vários, ISBN: 9781620816332, Home page: https://www.novapublishers.com/catalog/product_info.php?products_id=33439

Trabalhos publicados em anais de eventos (resumo)

1. GOMES JR, P. P., ARAUJO, A. L., FREITAS, M. T. S., **BATISTA, M.V.A.**, BALBINO, V. Q.
An efficient method of DNA extraction of single egg of Aedes aegypti In: XVIII International Congress for Tropical Medicine and Malaria, 2012, Rio de Janeiro.
Annals of XVIII International Congress for Tropical Medicine and Malaria. , 2012.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

2. **BATISTA, M.V.A.**
Desenvolvimento e aplicação de novos métodos computacionais aplicados à diversidade genética e evolução de papilomavírus In: II Jornada de Pós-Graduação em Genética, 2012, Recife.
Anais da II Jornada de Pós-Graduação em Genética. , 2012.
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso

3. FREITAS, M. T. S., **BATISTA, M.V.A.**, LUCENA, R. M., ARAUJO, M. B., TAVARES, V. B., MORAIS JÚNIOR, M.A., NAKAZAWA, Y. M., LEAL-BALBINO, T. C., BALBINO, V. Q.
Identification of bacterial diversity from digestive tract of Triatoma brasiliensis using 16S rRNA gene In: XVIII International Congress for Tropical Medicine and Malaria, 2012, Rio de Janeiro.
Annals of XVIII International Congress for Tropical Medicine and Malaria. , 2012.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

4. ARAUJO, A. L., GOMES JR, P. P., ARAGAO, N. C., SILVA, L. G., CARDOSO, M.V., **BATISTA, M.V.A.**, BALBINO, V. Q.
The use of ovitraps in monitoring of Aedes aegypti populations in Serra Talhada city, Pernambuco, Brazil In: XVIII International Congress for Tropical Medicine and Malaria, 2012, Rio de Janeiro.
Annals of XVIII International Congress for Tropical Medicine and Malaria. , 2012.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

5. **BATISTA, M.V.A.**, FREITAS, A. C., BALBINO, V. Q.
An entropy-based approach for designing degenerate PCR primers suitable for detecting Bovine Papillomavirus In: 7th International Conference of th Brazilian Association for Bioinformatics and Computational Biology - X-Meeting, 2011, Florianópolis.

X-Meeting 2011 Abstract Book. , 2011.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

6. **BATISTA, M.V.A.**, FREITAS, A. C., BALBINO, V. Q.
Codon usage patterns and the evolution of Papillomavirus In: 7th International Conference of th Brazilian Association for Bioinformatics and Computational Biology - X-Meeting, 2011, Florianópolis.
X-Meeting 2011 Abstract Book. , 2011.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

7. **BATISTA, M.V.A.**, FREITAS, A. C., BALBINO, V. Q.
Comparative phylogenetic analysis of Bovine Papillomavirus based on L1 gene: accuracy of different gene regions In: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul, 2011, Atibaia.
Annals of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. , 2011. v.16. p.104 - 105
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

8. DE ALBUQUERQUE, B. M. F., CRUZ, H.L.A., COIMBRA, E.C., AMARAL, C.M.M., **BATISTA, M.V.A.**, SILVA, M. A. R., JESUS, A.L.S., BALBINO, V. Q., FREITAS, A. C.
Development of a real time PCR typification/quantification assay for Bovine Papillomavirus In: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul, 2011, Atibaia.
Annals of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. , 2011. v.16. p.263 - 264
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

9. **BATISTA, M.V.A.**, FREITAS, A. C., BALBINO, V. Q.
Diversidade genética de Papillomavírus Bovino: epidemiologia molecular e uma nova abordagem baseada em entropia In: I Jornada de Pós-Graduação em Genética, 2011, Recife.
Anais da I Jornada de Pós-Graduação em Genética. , 2011.
Referências adicionais : Brasil/Português. Meio de divulgação: Vários

10. SANTOS, M.A.O., **BATISTA, M.V.A.**, BALBINO, V. Q.
Methods for selection of phylogenetic informative regions in mitochondrial genes In: 7th International Conference of th Brazilian Association for Bioinformatics and Computational Biology - X-Meeting, 2011, Florianópolis.
X-Meeting 2011 Abstract Book. , 2011.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

11. CARVALHO, C. C. R., **BATISTA, M.V.A.**, BALBINO, V. Q., FREITAS, A. C.
Molecular studies of Bovine Papillomavirus: Molecular diversity, co-infection and detection of a putative new BPV11 subtype In: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul, 2011, Atibaia.
Annals of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. , 2011. v.16. p.265 - 265
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

12. **BATISTA, M.V.A.**, FREITAS, A. C., BALBINO, V. Q.
The efficiency of a new entropy-based trimming approach to reconstruct Papillomavirus phylogeny based on L1 protein In: 7th International Conference of th Brazilian Association for Bioinformatics and Computational Biology - X-Meeting, 2011, Florianópolis.
X-Meeting 2011 Abstract Book. , 2011.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

13. CARVALHO, C. C. R., BATISTA, M.V.A., REIS, M.C., BALBINO, V. Q., CASTRO, R.B., FREITAS, A. C.
Detection of Bovine Papillomavirus type 11 and a putative new type of BPV in cattle in Northeast region of Brazil In: XXI National Meeting of Virology - V Virology Meeting of Mercosul, 2010, Gramado.
Virus Reviews and Research. Rio de Janeiro: SBV, 2010. v.15. p.214 - 215
Palavras-chave: BPV11, Detection, New type, Papillomavirus
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

14. CHAGAS, B. S., GURGEL, A.P.A.D., BATISTA, M.V.A., CROVELLA, S., FREITAS, A. C.
Evaluation of changes on E6 and E7 oncogenes of Human Papillomavirus type 31 of patients with cervical lesion from Northeast region of Brazil In: XXI National Meeting of Virology - V Virology Meeting of Mercosul, 2010, Gramado.
Virus Reviews and Research. Rio de Janeiro: SBV, 2010. v.15. p.216 - 216
Palavras-chave: Aminoacid, Cervical cancer, E6, E7, Human Papillomavirus
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

15. BATISTA, M.V.A., FREITAS, A. C., BALBINO, V. Q.
Evidence of virus-host adaptation based on genome size in Papillomavirus In: XXI National Meeting of Virology - V Virology Meeting of Mercosul, 2010, Gramado.
Virus Reviews and Research. Rio de Janeiro: SBV, 2010. v.15. p.236 - 237
Palavras-chave: Papillomavirus, Evolution, Genomics, Bioinformatics
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

16. CHAGAS, B. S., BATISTA, M.V.A., CROVELLA, S., FREITAS, A. C.
New variants of E6 and E7 oncogenes of Human Papillomavirus type 31 identified in Northeastern Brazil and their relationship with predicted T-cell epitopes In: XXI National Meeting of Virology - V Virology Meeting of Mercosul, 2010, Gramado.
Virus Reviews and Research. Rio de Janeiro: SBV, 2010. v.15. p.215 - 216
Palavras-chave: Cervical cancer, Epitopes, E6, E7, Human Papillomavirus
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

17. BATISTA, M.V.A., FERREIRA, TAE, FREITAS, A. C., BALBINO, V. Q.
The use of entropy for selecting phylogenetic informative genomic regions and the evolution of Papillomavirus In: XXI National Meeting of Virology - V Virology Meeting of Mercosul, 2010, Gramado.
Virus Reviews and Research. Rio de Janeiro: SBV, 2010. v.15. p.236 - 236
Palavras-chave: Papillomavirus, Phylogeny, Entropy, Genomics, Bioinformatics
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

Trabalhos publicados em anais de eventos (resumo expandido)

1. **BATISTA, M.V.A.**, FERREIRA, TAE, FREITAS, A. C., BALBINO, V. Q.
Genomic diversity and phylogenetic reconstruction of Papillomavirus using a new approach based on low entropy genomic regions In: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul, 2011, Atibaia.
Annals of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. , 2011. v.16. p.42 - 42
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários
Trabalho apresentado de forma oral, como concorrente ao prêmio "Hélio Gelli Pereira"

2. CHAGAS, B. S., **BATISTA, M.V.A.**, GUIMARÃES, V., BALBINO, V. Q., CROVELLA, S., FREITAS, A. C.
New variants of E6 and E7 oncogenes of human papillomavirus type 31 identified in Northeastern Brazil In: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul, 2011, Atibaia.
Annals of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. , 2011. v.16. p.41 - 41
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários
Trabalho apresentado de forma oral, como concorrente ao prêmio "Hélio Gelli Pereira"

3. SILVA, G.Z.G., MEDEIROS, S., GOMES, R., SANTOS, M.A.O., **BATISTA, M.V.A.**, CUNHA, W.L., GOMES, L., LIMA, T.L.D., FERREIRA, TAE, BALBINO, V. Q.
SandFly Database: development of an integrated platform of biological and molecular data of sandflies (Diptera: Psychodidae) of medical and veterinary importance In: IV Workshop de Genética e Biologia Molecular de Insetos Vetores de Doenças Tropicais, 2010, Recife.
Anais do IV Workshop de Genética e Biologia Molecular de Insetos Vetores de Doenças Tropicais. , 2010. p.120 - 122
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

Apresentação de trabalho e palestra

1. **BATISTA, M.V.A.**
A Bioinformática e a Evolução de Papilomavírus, 2011. (**Conferência ou palestra**, Apresentação de Trabalho)
Referências adicionais : Brasil/Português. Meio de divulgação: Vários
Palestra.; Local: Universidade Federal de Sergipe; Cidade: São Cristóvão; Evento: V Simpósio Sergipano de Microbiologia e I Encontro de Controle de Qualidade Microbiológica; Inst.promotora/financiadora: Universidade Federal de Sergipe

2. **BATISTA, M.V.A.**
Comparative phylogenetic analysis of Bovine Papillomavirus based on L1 gene: accuracy of different gene regions, 2011. (Comunicação, Apresentação de Trabalho)
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários; Local: Tauá Hotel & Convention Center; Cidade: Atibaia; Evento: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul; Inst.promotora/financiadora: Sociedade Brasileira de Virologia

3. **BATISTA, M.V.A.**
Genomic diversity and phylogenetic reconstruction of Papillomavirus using a new approach based on low entropy genomic regions, 2011. (Comunicação, Apresentação de Trabalho)
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários
Apresentação oral.; Local: Tauá Hotel & Convention Center; Cidade: Atibaia; Evento: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul; Inst.promotora/financiadora: Sociedade Brasileira de Virologia

4. **BATISTA, M.V.A.**
Molecular studies of Bovine Papillomavirus: Molecular diversity, co-infection and detection of a putative new BPV11 subtype, 2011. (Comunicação, Apresentação de Trabalho)
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários
Apresentação em painel.; Local: Tauá Hotel & Convention Center; Cidade: Atibaia; Evento: XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul; Inst.promotora/financiadora: Sociedade Brasileira de Virologia

5. **BATISTA, M.V.A.**
Evidence of virus-host adaptation based on genome size in Papillomavirus, 2010. (Comunicação, Apresentação de Trabalho)
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários
Apresentação em painel.; Local: FAURGS; Cidade: Gramado; Evento: XXI National Meeting of Virology - V Virology Meeting of Mercosul; Inst.promotora/financiadora: Sociedade Brasileira de Virologia

6. **BATISTA, M.V.A.**
The use of entropy for selecting phylogenetic informative genomic regions and the evolution of Papillomavirus, 2010. (Comunicação, Apresentação de Trabalho)
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários
Apresentação Oral.; Local: FAURGS; Cidade: Gramado; Evento: XXI National Meeting of Virology - V Virology Meeting of Mercosul; Inst.promotora/financiadora: Sociedade Brasileira de Virologia

Produção técnica
Demais produções técnicas

1. **BATISTA, M.V.A.**
Bioinformática aplicada à Microbiologia, 2012. (Outro, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 4 horas. Meio de divulgação: Vários
Minicurso ministrado no VI Simpósio Sergipano de Microbiologia e II Encontro de Controle de Qualidade Microbiológica.

2. **BATISTA, M.V.A.**
III Curso de Bioinformática: Análise de Dados Moleculares (Turma 1), 2012. (Outro, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 75 horas. Meio de divulgação: Meio digital

3. **BATISTA, M.V.A.**
III Curso de Bioinformática: Análise de Dados Moleculares (Turma 2), 2012. (Outro, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 75 horas. Meio de divulgação: Meio digital

4. **BATISTA, M.V.A.**
IV Curso de Bioinformática: Análise de Dados Moleculares, 2012. (Extensão, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 75 horas. Meio de divulgação: Meio digital

5. **BATISTA, M.V.A.**
I Curso de Bioinformática: Ferramentas e Aplicações (Turma 1), 2011. (Outro, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 45 horas. Meio de divulgação: Vários

6. **BATISTA, M.V.A.**
I Curso de Bioinformática: Ferramentas e Aplicações (Turma 2), 2011. (Outro, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 45 horas. Meio de divulgação: Vários

7. **BATISTA, M.V.A.**
II Curso de Bioinformática: Análise de Dados Moleculares, 2011. (Outro, Curso de curta duração ministrado)
Referências adicionais : Brasil/Português. 75 horas. Meio de divulgação: Vários

Orientações e Supervisões

Orientações e supervisões

Orientações e supervisões em andamento

Iniciação científica

1. Isabela Hazin Antunes de Souza. **Variabilidade genética de papilomavírus bovino isolados no estado de Pernambuco**. 2012. Iniciação científica (Ciências Biológicas - Bacharelado) - Universidade Federal de Pernambuco
Referências adicionais : Brasil/Português.
Co-orientador.

Eventos

Eventos
Participação em eventos

1. Apresentação de Poster / Painel no(a) **II Jornada de Pós-Graduação em Genética**, 2012. (Outra)
Desenvolvimento e aplicação de novos métodos computacionais aplicados à diversidade genética e evolução de papilomavírus.

2. **I Workshop em Filogenia Molecular**, 2012. (Oficina)
.

3. **São Paulo School of Advanced Science on e-Science for Bioenergy Research**, 2012. (Outra)
.

4. Apresentação de Poster / Painel no(a) **I Jornada de Pós-Graduação em Genética**, 2011. (Outra)
Diversidade genética de Papillomavírus Bovino: epidemiologia molecular e uma nova abordagem

baseada em entropia.

5. Apresentação Oral no(a) **XXII Encontro Nacional de Virologia e VI Encontro de Virologia do Mercosul**, 2011. (Congresso)
Genomic diversity and phylogenetic reconstruction of Papillomavirus using a new approach based on low entropy genomic regions.

6. Apresentação de Poster / Painel no(a) **7th International Conference of th Brazilian Association for Bioinformatics and Computational Biology - X-Meeting**, 2011. (Congresso)
The efficiency of a new entropy-based trimming approach to reconstruct Papillomavirus phylogeny based on L1 protein.

7. Apresentação Oral no(a) **XXI National Meeting of Virology - V Virology Meeting of Mercosul**, 2010. (Congresso)
The use of entropy for selecting phylogenetic informative genomic regions and the evolution of Papillomavirus.

8. **1st Brazilian-German Meeting of Plant Systems Biology and Bioenergy**, 2010. (Encontro)
.

9. **IV Simpósio Virtual em EaD**, 2010. (Simpósio)
.

Organização de evento

1. **BATISTA, M.V.A.**
III Curso de Bioinformática: Análise de Dados Moleculares (Turma 1), 2012. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Meio digital
Curso com carga horária global de 75 horas.

2. **BATISTA, M.V.A.**
III Curso de Bioinformática: Análise de Dados Moleculares (Turma 2), 2012. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Meio digital
Curso com carga horária global de 75 horas.

3. **BATISTA, M.V.A.**
IV Curso de Bioinformática: Análise de Dados Moleculares, 2012. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Meio digital

4. **BATISTA, M.V.A.**
I Curso de Bioinformática: Ferramentas e Aplicações (Turma 1), 2011. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Vários
Curso com carga horária global de 45 horas.

5. **BATISTA, M.V.A.**
I Curso de Bioinformática: Ferramentas e Aplicações (Turma 2), 2011. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Vários
Curso com carga horária global de 45 horas.

6. **BATISTA, M.V.A.**
I Jornada de Pós-Graduação em Genética, 2011. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Vários

7. **BATISTA, M.V.A.**
II Curso de Bioinformática: Análise de Dados Moleculares, 2011. (Outro, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Vários

Curso com carga horária global de 75 horas.

Bancas

Bancas
Participação em banca de trabalhos de conclusão

Graduação

1. **BATISTA, M.V.A.**, SILVA, W. M., CARDOSO, M.V.
Participação em banca de Moisés Thiago de Souza Freitas. **Diferenciação genética entre duas populações simpátricas de Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae) do município de Sobral (CE) Brasil**, 2011
(Ciências Biológicas - Bacharelado) Universidade Federal de Pernambuco
Referências adicionais : Brasil/Português.

Citações

Web of Science Total de citações: 1; Total de trabalhos : 3; Data : 01/06/2012; Fator H: 1;
Nome(s) do autor utilizado(s) na consulta para obter o total de citações:
Batista M* V*

SCOPUS Total de citações: 1; Total de trabalhos : 4; Data : 01/06/2012
Nome(s) do autor utilizado(s) na consulta para obter o total de citações:
Batista M.V.

Totais de produção

Produção bibliográfica

Artigos completos publicados em periódico	8
Artigos aceitos para publicação	2
Capítulos de livros publicados	1
Trabalhos publicados em anais de eventos	49
Apresentações de trabalhos (Comunicação)	7
Apresentações de trabalhos (Conferência ou palestra)	2

Produção técnica

Trabalhos técnicos (relatório técnico)	1
Trabalhos técnicos (outra)	3
Curso de curta duração ministrado (extensão)	1
Curso de curta duração ministrado (outro)	10
Relatório de pesquisa	3

Orientações

Orientação concluída (trabalho de conclusão de curso de graduação)	1
Orientação em andamento (iniciação científica)	1

Eventos

Participações em eventos (congresso)	9
Participações em eventos (simpósio)	6
Participações em eventos (oficina)	2
Participações em eventos (encontro)	11
Participações em eventos (outra)	10
Organização de evento (outro)	13
Participação em banca de trabalhos de conclusão (graduação)	1

Outras informações relevantes

Fui aprovado em Concurso Público no cargo de Professor de 3º Grau de Biologia Molecular e Bioinformática, do CENTRO DE BIOTECNOLOGIA da Universidade Federal da Paraíba, CAMPUS I, aberto pelo Edital nº 65 de 04/09/2012, publicado no DOU nº 175 de 10/09/2012, págs.62 a 65, seção 3.

Fui aprovado em Concurso Público no cargo de Professor Substituto no Eixo Profissional Ciências da Natureza, Matemática e suas Tecnologia (Biologia) do Instituto Federal de Educação, Ciência e Tecnologia de Pernambuco pelo Edital Nº 58/2011-GR.

Fui aprovado em Concurso Público no cargo de Professor Temporário na Matéria Genética da Universidade Federal Rural de Pernambuco pelo Edital Nº 05/2011.

Fui aprovado em Concurso Público no cargo de Professor Substituto - Área Ciências e Biologia do Colégio de Aplicação da Universidade Federal de Pernambuco pelo Edital Nº16, com resultado divulgado no Diário Oficial da União - Seção 3 Nº 51, Pg. 53-54, 16 de março de 2011.

Fui aprovado em Concurso Público no cargo de Professor Substituto - Disciplina Botânica da Universidade Federal Rural de Pernambuco pelo Edital Nº 12/10.