

## Equine sarcoids - Part 3: association with bovine papillomavirus

### *Equine sarcoïden – Deel 3: associatie met boviene papillomavirus*

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#### ABSTRACT

The genetic material of the bovine papillomavirus (BPV) can be detected in virtually all equine sarcoids. Eight different types have been described, all inducing benign proliferation of epithelium in cattle. BPV-1 and -2 are less strictly species-specific and can induce equine sarcoids in horses. Historically, association between BPV and equine sarcoids has been demonstrated using inoculation studies and detection of BPV DNA and BPV gene expression. The BPV genome is composed of 6 early and 2 late genes, with E5 and E6 being the most important transforming genes. Specific BPV-1 variants associated with equine sarcoids have been reported, suggesting circulation of the virus between horses. In horses, a non-productive BPV infection occurs, with only transcription of early genes, responsible for genome maintenance, regulation of cell growth and cell transformation. There is no formation of new infectious virus particles as is the case in the natural host.

#### SAMENVATTING

Het genetisch materiaal van het boviene papillomavirus (BPV) kan worden aangetoond in vrijwel alle sarcoïden. Er worden 8 verschillende BPV-typen beschreven die alle goedaardige epitheliale proliferaties veroorzaken bij runderen, maar enkel BPV-1 en -2 zijn minder strikt speciespecifiek en kunnen bij paarden sarcoïden induceren. De associatie tussen BPV en sarcoïden werd aangetoond door middel van inoculatiestudies en de detectie van BPV-DNA en BPV-genexpressie. Het BPV-genoom is samengesteld uit 6 vroege en 2 late genen, waarbij E5 en E6 de meest belangrijke transformerende genen zijn. Specifieke BPV-1-varianten geassocieerd met sarcoïden worden beschreven, wat er kan op wijzen dat het virus van paard naar paard kan overgedragen worden. Bij paarden vindt een niet-productieve infectie plaats, waarbij er enkel een transcriptie is van vroege genen die verantwoordelijk zijn voor het onderhoud van het genoom, de celgroeiregulatie en de celtransformatie. Er is geen vorming van nieuwe infectieuze viruspartikels zoals bij de natuurlijke gastheer.

#### CLASSIFICATION AND GENOME ORGANIZATION

Bovine papillomaviruses are small non-enveloped DNA viruses (Figure 1) and are members of the family *Papillomaviridae*, a large family of animal and human viruses that normally infect epithelial cells causing hyperproliferative lesions. To date, 8 BPV types (BPV-1-8) have been characterized and classified into 3 genera: the Delta-, Epsilon- and Xi-papillomaviruses (de Villiers *et al.*, 2004). BPV-1 and -2 belong to the Delta-papillomaviruses and induce fibropapillomas (warts). BPV-3, -4 and -6 are members of the Xi-papillomaviruses and infect only the epithelium, inducing true papillomas. BPV-5 and -8 belong to the Epsilon-papillomaviruses and cause both fibropapillomas and epithelial papillomas (Bloch *et al.*, 1994b; Tomita *et al.*, 2007). Phylogenetic analysis based on

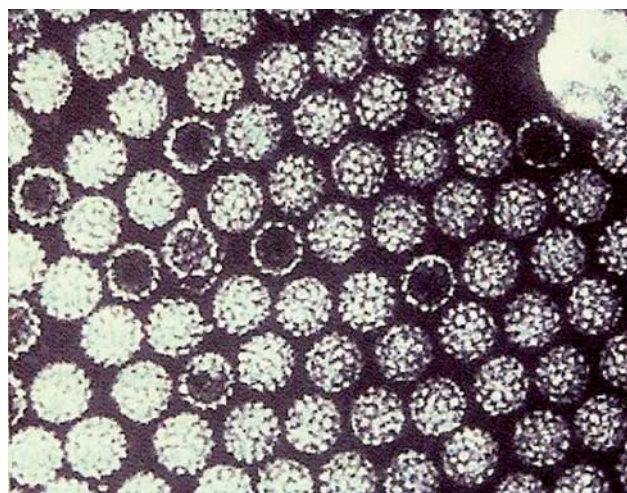


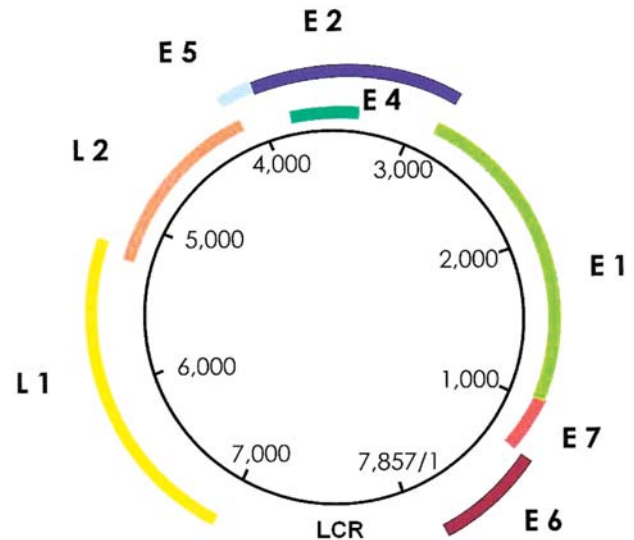
Figure 1. Electron microscopic picture of human papillomavirus particles (Stanley *et al.*, 2006).

the complete L1 ORF suggests that BPV-7 should be classified in a novel genus (Ogawa *et al.*, 2007).

BPV has a genome of 7900 bp of double-stranded DNA and contains 6 early and 2 late genes (Chen *et al.*, 1982; Campo, 1988) (Figure 2). Early genes (E1, E2, E4, E5, E6 and E7) are responsible for replication and transformation, whereas late genes (L1 and L2) encode the structural proteins. The early and late regions are separated by a stretch of non-transcribed DNA, called the long control region (LCR), which contains the transcriptional promoters and enhancers, the origin of DNA replication and binding sites for numerous cellular transcription factors as well as for the virally encoded early gene product E2 (McBride *et al.*, 1991; Phelps *et al.*, 1999; Chambers *et al.*, 2003a).

The most extensively studied BPV gene is E5, which plays a prominent role in sarcoïd development. E5 is a small hydrophobic protein, consisting of 44 amino acids, residing in the membranes of the Golgi apparatus and the endoplasmic reticulum. *In vitro* studies on murine fibroblasts have shown that BPV E5 is one of the most important genes in neoplastic transformation of the cell (Marchetti *et al.*, 2002). As soon as E5 is expressed, a rapid transformation occurs without the help of other proteins. E5 contributes to cellular transformation of fibroblasts in different ways. It has been shown to induce activation of the platelet-derived growth factor  $\beta$  (PDGF- $\beta$ ) receptor by binding to it (Petti and DiMaio, 1992; DiMaio and Mattoon, 2001). Activation of PDGF- $\beta$  receptors of the dermal fibroblasts by E5 results in mitogenesis in nearby epithelial cells (Carr *et al.*, 2001b). This event may explain the typical pseudoepitheliomatous hyperplasia. Furthermore the vacuolar H<sup>+</sup>-ATPase is targeted by BPV E5 (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). This proton pump is essential for the function of cellular compartments that process growth factors. By binding of E5, an alteration in this process occurs. The acidification of the endosomes is blocked and degradation of the receptors for growth factors is inhibited resulting in a prolonged stimulation time, besides an increased receptor recycling (Carr *et al.*, 2001b). This hypothesis is objected by the findings of Ashbey *et al.* (2001) who found no alteration in the function of the vacuolar H<sup>+</sup>-ATPase. The latter authors suppose that the binding to the vacuolar H<sup>+</sup>-ATPase has only a transport function in the cell in order to reach other intracellular targets. The BPV E5 also binds to 16K ductin/subunit c, a component of the gap junctions, responsible for the contact between neighboring cells. Through this binding, the contact is disrupted and the transformed cell can replicate uncontrolled (Campo, 1997). Interaction with 16K leads also to the alkalization of the endosomes and the Golgi apparatus with consequent intracellular retention of MHC class I molecules (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). The absence of MHC class I on the cell surface would help the infected cells to evade host immunosurveillance (Chambers *et al.*, 2003a).

The BPV E6 protein is localized in membrane and



**Figure 2. Genome organisation of papillomaviruses (Muñoz *et al.*, 2006).**

nuclear fractions and contains 2 highly conserved zinc finger domains typical of DNA-binding transcription activator proteins. However, cell transformation by E6 seems to be independent of its transcription transactivation function (Ned *et al.*, 1997). Human papillomavirus (HPV) E6, the most important transforming protein of HPV, is known to bind to p53 and subsequently stimulate its degradation (Scheffner *et al.*, 1990). However, BPV E6 does not bind to p53 (Rapp *et al.*, 1999). Instead, the transformational ability of BPV E6 is linked to its ability to bind ERC-55/E6BP, a calcium binding protein (Chen *et al.*, 1995) and in part CBP/p300, a transcriptional co-activator (Zimmermann *et al.*, 2000) which interferes with the normal cell functions. BPV E6 also binds the focal adhesion protein paxillin (Tong *et al.*, 1997; Tong and Howley, 1997; Vande Pol *et al.*, 1998) and the  $\gamma$  subunit of the clathrin adaptor complex AP-1 (Tong *et al.*, 1998) leading to disruption of cytoskeleton and vesicular traffic pathways. The cytoskeleton is vital for the maintenance of cellular morphology, motility, division and cell-cell and cell-matrix interactions and the AP-1 complex plays an important role in the control of cell proliferation and differentiation (Chambers *et al.*, 2003a).

The function of the other early BPV genes is less well studied, but it has been established that E1 is responsible for initiation of replication and maintenance of the genome. E2 is, besides its role in replication, an important regulator of transcription. The exact role of E4 is still unknown, but its functions suggest a role in facilitating and supporting viral genome amplification, the regulation of late gene expression, control of virus maturation and mediation of virus release (Roberts, 2006). E7 has a minor role in cell transformation and is a regulator of the BPV copy number (Campo, 1988). This genome organization is somewhat different from the epitheliotropic BPVs (BPV-3, -4 and -6) which lack the E6 gene. Instead

they have an E5 (formerly defined as E8) ORF between E7 and the LCR on the genome (Campo, 1992; Morgan and Campo, 2000). Also in HPV a different organization is seen, where E6 and E7 are the most important transforming genes, and E5 is only active in the early stages of neoplastic transformation (Blair *et al.*, 1998).

Specific BPV-1 variants associated with equine sarcoids have been reported. Most variation in nucleotide sequences of papillomaviruses in general is found in the transforming genes E5, E6 and E7. Chambers *et al.* (2003b) found 7 E5 sequence variants in 34 sarcoids, none of them identical to the prototype sequence found in cattle, which might reflect the specific clinical presentation of equine sarcoids. On the other hand, E1 and E2 have a more conserved sequence, probably due to strict conditions for replication and transcription (Chan *et al.*, 1992; Nasir *et al.*, 2007). LCR variants of sarcoid BPV-1 with a higher activity in equine cells compared to bovine cells have been identified suggesting that these BPV variants have an enhanced function in equine sarcoids (Nasir *et al.*, 2007). These findings suggest that sarcoids are associated with variant BPV-1 genomes that preferentially infect horses and are maintained within equids (Nasir *et al.*, 2007).

#### PAPILLOMAVIRUS INFECTION IN THE NATURAL HOST

Natural infection with papillomaviruses occurs in epithelial cells and induces benign, self-limiting cutaneous and mucosal proliferations, called "warts". During acute virus infection, replication of the virus genome is strictly linked to the state of differentiation of the infected cell. The virus initially infects the basal keratinocytes. The early genes are then expressed in the undifferentiated basal and suprabasal layers. Viral DNA is replicated in the differentiating spinous and granular layers and expression of the late structural proteins is limited to the terminally differentiated cells of the squamous layer, where the new virus particles are encapsidated and released into the environment as the cells shed (Chambers *et al.*, 2003a).

In cattle, mainly young animals are affected; later on they acquire immunity so that older animals rarely develop warts (Nicholls and Stanley, 2000). Initially transformation of subepithelial fibroblasts is seen, followed by epithelial acanthosis and the typical papilloma formation. BPV-1 mainly affects the genital region (teat and penile papillomas and the surrounding skin), while BPV-2 can infect the entire skin surface as well as the epithelial layers of the gastro-intestinal tract (Jarrett, 1984; Campo, 1987; Campo, 1997). In Europe, warts in cattle are commonly observed on the abdomen, limbs and dorsum, whereas in the USA mainly the head, neck and shoulders are affected (Phelps *et al.*, 1999). Most animals will spontaneously cure after several weeks to months as a result of a cell-mediated immune response. Reinfection is prevented by neutralizing antibodies against capsid proteins. This immunity is type specific, so infection with other BPV

types is still possible (Nicholls and Stanley, 2000).

In a small number of cases, no regression or even malignant transformation to squamous cell carcinoma is seen. Initiation of malignant transformation is linked to the deregulated expression of the early virus genes, which results in an uncontrolled proliferation and loss of differentiation of the infected cells (Campo, 1997). The presence of BPV-2 DNA in urinary bladder cancer in cattle is known, both naturally as well as experimentally induced (Campo and Jarrett, 1986; Borzacchiello *et al.*, 2003; Yuan *et al.*, 2006).

#### HISTORY OF ASSOCIATION BETWEEN BPV AND EQUINE SARCOIDS

Epidemiological data suggest an etiological role of an infectious agent in the development of equine sarcoids (Jackson, 1936; Voss, 1969; Ragland, 1970). Many similarities can be observed between equine sarcoids and papillomatosis in other species: fast growth, tendency for multiplicity and spreading over the body by contact between different parts of the body (Jackson, 1936). Epizootics have been described in closed herds of horses and zebras, pointing to an infectious agent (Ragland *et al.*, 1966; Nel *et al.*, 2006). Inoculation studies conducted in the early fifties suggested a role of BPV in sarcoid development (Olson and Cook 1951). Nevertheless, transmission studies from sarcoids to cattle were not successful (Ragland *et al.*, 1970). Other viruses, such as the equine cutaneous papillomavirus (Gorman, 1985; Obanion *et al.*, 1986) and a retrovirus (England *et al.*, 1973; Cheevers *et al.*, 1982), were supposed to be involved, but later on enough evidence was collected to assign an etiological role in equine sarcoid development only to BPV-1 and -2 (Lancaster *et al.*, 1977; Angelos *et al.*, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Bloch *et al.*, 1994a; Reid *et al.*, 1994b; Nasir *et al.*, 1997; Nasir and Reid, 1999; Carr *et al.*, 2001b).

#### Inoculation studies

The earliest suggestion that sarcoids may have an infectious origin was made in the early fifties. Horses were experimentally infected with cell-free extracts from cattle papillomas resulting in tumors grossly and histologically mimicking equine sarcoids (Olson and Cook, 1951). Later studies have confirmed these findings (Ragland and Spencer, 1968; Voss, 1969; Ragland and Spencer, 1969; Ragland *et al.*, 1970; Koller and Olson, 1972; Lancaster *et al.*, 1977; Makady *et al.*, 1990). Yet many differences were also observed between the induced tumors and naturally arising sarcoids. Histologically, only the dermal layers were changed without involvement of the epidermis and picked fence formation was not observed. Moreover, all induced tumors regressed spontaneously within 4 to 12 months and neutralizing antibodies against BPV were formed resulting in resistance against re-infection. In natural infection, spontaneous regression exists but is less common. Moreover, no



neutralizing antibodies against BPV are formed and horses remain sensitive to re-infection (Ragland and Spencer, 1969; Lancaster *et al.*, 1977; Brostrom *et al.*, 1979). Furthermore, attempts to identify BPV particles in natural cases of equine sarcoids by electron microscopy or using anti-BPV antibodies have been unsuccessful (Barthold and Olson, 1978; Sundberg *et al.*, 1984). One reason for the observed differences might be that with experimental inoculation numerous viral particles as well as bovine antigens are inserted in the organism inducing a strong immune reaction. In natural infection, possibly only one or a few virus particles infect the cell causing latent or non-productive infection (Carr *et al.*, 2001a). Moreover, the horse is not the natural host for BPV and cannot support the vegetative portion of the viral life cycle (Gorman, 1985; Sousa *et al.*, 1990). In natural sarcoids BPV DNA is kept in a non-integrated episomal state in the nucleus, only the early genes are transcribed and only a few copies of the genome are produced (Goodrich *et al.*, 1998). This might explain why inoculation of sensitive calves with equine sarcoid material does not induce papillomas and why these animals remain sensitive for BPV infection (Ragland *et al.*, 1970).

#### Detection of BPV DNA

Since the seventies, several studies have demonstrated the presence of BPV DNA in equine sarcoids with molecular techniques. Measurements of DNA-DNA reassociation kinetics on fresh or frozen material yielded 80-86 % of sarcoids positive for BPV DNA (Lancaster *et al.*, 1977). Southern blot hybridization on fresh or frozen material was positive in 86-100 % of the sarcoids (Trenfield *et al.*, 1985; Angelos *et al.*, 1991; Reid *et al.*, 1994b). PCR on paraffin embedded material demonstrated BPV DNA in 74-91 % of all cases (Teifke *et al.*, 1994; Bloch *et al.*, 1994a) and PCR on fresh or frozen samples resulted in a 100 % detection rate (Otten *et al.*, 1993; Martens *et al.*, 2001).

An explanation for the fact that BPV DNA can sometimes not be demonstrated in 100 % of the examined sarcoid samples might be the insensitivity of the technique used e.g. when only a low copy number of viral genomes per cell is present (Angelos *et al.*, 1991) or after unsuited or too long formalin fixation (Teifke *et al.*, 1994; Bloch *et al.*, 1994a).

#### Detection of BPV gene expression

Nasir and Reid (1999) demonstrated that in 95 % of the sarcoids where BPV DNA can be detected with PCR, expression of viral genes is also present. BPV E2 and E5 mRNA were detected in respectively 90 % and 80 % of the sarcoids. This was the first evidence of direct involvement of BPV in the pathogenesis of equine sarcoids. In additional studies, BPV E2, E5, E6 and E7 were found to be expressed in up to 100 % of the sarcoids (Chambers *et al.*, 2003b; Nixon *et al.*, 2005; Bogaert *et al.*, 2007). Carr *et al.* (2001b) could

demonstrate the E5 protein in all examined sarcoids. Collectively, these studies clearly provide evidence that the viral genes are expressed and that the presence of BPV DNA in equine sarcoids is not coincidental.

#### PATHOGENESIS OF BPV INFECTION OF THE HORSE

Although most papillomaviruses are extremely species- and tissue-specific, it has now been established that a minority of them can also infect other hosts resulting in a different pathological outcome. BPV infection of the horse is one of the most extensively studied species-crossing infections. Both BPV-1 and BPV-2 infections occur, independent of each other, but no correlation exists between BPV type and clinical presentation of the equine sarcoid (Reid *et al.*, 1994b). Regional differences in frequency of both types are observed: in Europe, 74 - 93 % of all examined samples of sarcoids are infected with BPV-1, compared to only 7 - 26 % BPV-2 (Angelos *et al.*, 1991; Otten *et al.*, 1993; Teifke *et al.*, 1994; Martens *et al.*, 2000; Martens *et al.*, 2001; Bogaert *et al.*, 2007). In the USA however, both types are seen in equal proportions (Teifke *et al.*, 1994; Carr *et al.*, 2001a). In a single tumor only one BPV type has been found up till now, but in case of multiple tumors on the same horse, both BPV types can be demonstrated (Otten *et al.*, 1993; Martens *et al.*, 2001; Carr *et al.*, 2001a). These findings point towards lack of immunity to superinfection (Otten *et al.*, 1993). In Australia, a BPV type has been found in a small number of sarcoids different from type 1 and type 2. Maybe this is a third and until now unidentified type (Trenfield *et al.*, 1985).

The number of copies of BPV DNA per diploid equine sarcoid cell varies between 0.1 and 500 (Lancaster *et al.*, 1977; Amtmann *et al.*, 1980; Otten *et al.*, 1993; Goodrich *et al.*, 1998; Yuan *et al.*, 2006). They are present in a non-integrated circular episomal state in the cell (Amtmann *et al.*, 1980). In situ hybridization studies have only shown BPV DNA in the nuclei of fibroblasts, especially at the dermo-epidermal junction and not in the epidermis (Teifke *et al.*, 1994). This is in contrast with BPV infection in cattle, where the virus is mainly located in the epidermal layers. Only in the first stages of infection in cattle, BPV DNA is found in the fibroblasts (Phelps *et al.*, 1999). Infection of BPV in a non-permissive host results in a non-productive cycle. There is only transcription of early genes, responsible for genome maintenance, regulation of cell growth and cell transformation (Gorman, 1985; Sousa *et al.*, 1990). A productive infection only occurs in bovine skin in well-differentiated keratinocytes: only here considerable replication and formation of complete virus particles takes place (Campo, 1997; Carr *et al.*, 2001b). It has been shown that intra-type sequence variation occurs within papillomavirus types, which can affect the cellular location and function of viral proteins, including E5, which in turn can affect the pathogenesis and transforming effects of the virus (Reid *et al.*, 1994a; Giannoudis and Herrington, 2001;

Chambers *et al.*, 2003b).

When an equine fibroblast gets infected by BPV-1 or -2, a number of cellular changes can happen resulting in cell transformation and uncontrolled growth. First of all, the MHC involved in immune regulation plays a major role. Other cellular proteins are also involved, such as p53, c-myc and in Arabian Horses DNA-PKcs, and probably many more.

The tumor suppressor gene p53 is coding for a nuclear phosphoprotein which will activate, in case of genetic damage, the transcription of genes inhibiting the cell cycle progression, resulting in DNA repair (Nasir *et al.*, 1999). P53 inhibits expression of the basic fibroblast growth factor and inhibits angiogenesis by up-regulation of thrombospondin-1 (Bucher *et al.*, 1996). These events result in conservation of the genetic stability of the cell (Nasir *et al.*, 1999). In human cancers it is observed that somatic mutation of p53 is the most commonly observed genetic alteration (Levine *et al.*, 1994). In horses and donkeys with equine sarcoids this is not observed, although a point mutation cannot be excluded (Bucher *et al.*, 1996; Nasir *et al.*, 1999). Other explanations for p53 inactivation are complex formation with viral or cellular proteins (Nasir *et al.*, 1999). HPV E6 is known to bind to p53 and subsequently stimulate its degradation (Scheffner *et al.*, 1990). However, BPV E6 does not (Rapp *et al.*, 1999). Yet it is supposed that in one way or another function loss of p53 takes place resulting in uncontrolled proliferation of fibroblasts and angiogenesis (Bucher *et al.*, 1996). Another hypothesis is cytoplasmic sequestration of p53, leading to inability to exert its function in the nucleus (Nasir *et al.*, 1999). Immunohistochemical staining has demonstrated that 9-44 % of the sarcoids show perinuclear staining for p53 (Martens *et al.*, 2000; Nixon *et al.*, 2005; Bogaert *et al.*, 2007). These data indicate that nuclear exclusion seems to be a possible explanation for the deficient p53 mechanism.

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## OPROEP

### Onderzoek naar equine sarcoïd

Op de Vakgroep Heelkunde en Anesthesie van de Huisdieren van de Faculteit Diergeneeskunde te Merelbeke loopt momenteel een onderzoek naar de transmissie van het bovine papillomavirus. Hiervoor zijn we op zoek naar proefpaarden met equine sarcoïden, evenals naar paarden die vroeger reeds behandeld werden voor sarcoïden maar nu sarcoïd-vrij zijn. Deze paarden zullen gebruikt worden om de exacte manier van transmissie te ontrafelen, uitgaande van viraal materiaal afkomstig van zowel runderen als van paarden. Opgelet: deze paarden zullen worden overgekocht door de vakgroep en kunnen dus niet in het bezit blijven van de eigenaar! Indien u in uw cliënteel dergelijke dieren hebt die anders toch moeten geëuthanaseerd of geslacht worden kunt u ons steeds contacteren voor overname van deze dieren.

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