First report of a variant bovine papillomavirus type 2 (BPV-2) in cattle in the Iberian Peninsula

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Bovine papillomavirus,
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Spain.

Summary
Infections caused by bovine papillomavirus (BPV) have been described worldwide. Some types, like BPV-1 and BPV-2, have been reported in association with skin warts and fibropapillomas in cattle and sarcoids in equids. In this study we have investigated the presence of BPV in cutaneous warts isolated from a steer in Spain. Cutaneous fibropapillomatosis was confirmed by histopathological analysis. Complete genome was amplified by multiple-primed rolling circle and the L1, E5 and E6 genes were sequenced. The isolate was classified as a variant of BPV-2 on the basis of the L1 gene sequences. Genetic variability of L1, E5 and E6 genes was compared with BPV-2 isolates from different hosts in several continents. Some mutations involved non-synonymous substitutions when compared to the prototype strain. One of these non-conservative mutations was located in the jelly roll β-barrel of the EF loop of the capsid protein (encoded by L1). This study presents the first report of a variant of BPV-2 infection in the Iberian Peninsula and contributes to extend the knowledge of the spreading and circulation of BPV.

Primo report di una variante di Papillomavirus bovino tipo-2 (BPV-2) nella Penisola iberica

Parole chiave
BPV-2,
Multiple-primed rolling-circle amplification (RCA),
Papillovirus bovino (BPV),
Spagna.

Riassunto
Le infezioni causate da Papillomavirus bovino (BPV) sono state ampiamente descritte. In particolare, le infezioni da BPV-1 e BVP-2 sono state associate a verruche della pelle e fibropapilloma nei bovini e sarcoidi negli equini. In questo studio è stata analizzata la presenza di BPV in verruche cutanee riscontrate in un bovino della Penisola iberica. La fibropapillomatosi cutanea è stata confermata dall'analisi istopatologica e il genoma completo è stato amplificato con metodica *multiple-primed rolling-circle* (RCA). I geni L1, E5 e E6 sono stati sequenziati. Il ceppo è stato classificato come variante di BPV-2 sulla base della sequenza del gene L1. Quando le sequenze dei geni L1, E5 ed E6 sono state confrontate con quelle omologhe di ceppi di BPV-2 isolati da ospiti diversi nei vari continenti, sono state evidenziate mutazioni dissenso rispetto al ceppo prototipo. Una di queste mutazioni non conservative è stata rilevata nel *jelly roll β-barrel* del loop EF della proteina del capside (codificata da L1). Questo studio, che rappresenta la prima relazione sull'infezione da una nuova variante di BPV-2 nella Penisola iberica, contribuisce ad aumentare le conoscenze sulla diffusione e circolazione di BPV.

The bovine papillomaviruses (BPV) are associated with skin warts in cattle that usually develop on the forehead, neck, upper chest and back (Borzacchiello and Roperto 2008). Bovine papillomaviruses are characterized by high viral diversity and to date 13 types have been recognized, BPV-1 to BPV-13, the latter has been described in 2013 (Lunardi *et al.* 2013). As in all other *Papillomaviridae*, the genome of BPV is circular and has up to 10 genes, which encode 8 early proteins (E1-E8), and 2 late proteins (L1 and L2). In addition, there is a long control region (LCR) between late (L1) and early (E6) genes.

Infections caused in cattle by BPV have been described worldwide, and the genotype BPV-2 is one of the most prevalent. It has been isolated from bovine cutaneous warts (CW) in Brazil (Silva *et al.* 2010, Carvalho *et al.* 2012, da Silva *et al.* 2012), India (Pangty *et al.* 2010), Japan (Hatama *et al.* 2011), Germany (Schmitt *et al.* 2010) and New Zealand (Munday and Knight 2010), and from bovine digital dermatitis in Austria (Brandt *et al.* 2011a). It has also been associated with urinary bladder tumors in Brazil (Wosiacki *et al.* 2005), India (Pathania *et al.* 2012), Italy (Borzacchiello *et al.* 2003, Roperto *et al.* 2013), Romania (Balcos *et al.* 2008) and Azores Archipelago, Portugal (Resendes *et al.* 2011). Recent studies report the detection of BPV-2 in cattle warts, both as simple infections and in co-infections with other BPV types (Schmitt *et al.* 2010, Carvalho *et al.* 2012) or with feline sarcoid-associated PV (da Silva *et al.* 2012). Additionally, productive infection of BPV-2 has been shown in peripheral blood of asymptomatic or papillomatosis-affected cattle (Roperto *et al.* 2011, Silva *et al.* 2013) or reproductive tissues like uterus/ovarium (Yaguiu *et al.* 2006) or placental epithelium (Roperto *et al.* 2012); BPV-2 has been found as well in seminal fluid, milk or urine from infected animals (Lindsey *et al.* 2009, Silva *et al.* 2011).

Even though cattle are the natural host for BPV, some genotypes such as BPV-1 and BPV-2 have also been reported to infect other animal species. Bovine papillomaviruses type-2 has been detected in skin tags and in the digestive tract of buffaloes and yaks in India (Pangty *et al.* 2010, Somvanshi *et al.* 2012, Bam *et al.* 2013), urinary bladder tumour in water buffaloes in Turkey (Roperto *et al.* 2013) and in fibropapillomas and sarcoids in zebras, giraffes and sable antelopes in South Africa (van Dyk *et al.* 2009, van Dyk *et al.* 2011). In addition, BPV-2 has also been found associated with equine sarcoid in horses in Austria, Switzerland, Poland, Belgium, UK, Canada, USA and Australia (Bloch *et al.* 1994, Carr *et al.* 2001, Chambers *et al.* 2003a, Bogaert *et al.* 2010, Haralambus *et al.* 2010, Szczerba-Turek *et al.* 2010, Wobeser *et al.* 2010, Brandt *et al.* 2011b) and in donkeys (Reid *et al.* 1994). Furthermore, BPV-2 has been detected in peripheral blood and semen of healthy horses (Silva *et al.* 2012).

Bovine papillomavirus types 1 and 2 can produce different manifestations as skin warts and fibropapillomas (Borzacchiello and Roperto 2008), placenta infections and bladder tumours in cattle (Roperto *et al.* 2012). The benign lesions like warts or fibropapillomas usually regress but they may also occasionally persist, leading to a high risk of evolution into cancer of both epithelial and mesenchymal origin, particularly in the presence of environmental carcinogenic co-factors (Borzacchiello and Roperto 2008).

In this study we analysed skin warts located in the head and neck of a 15-month old yearling steer reared in an extensive grazing farm in the Central Mountain Range of Spain. DNA was extracted from a 0.1 g slice using 500 µL of lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) with homogenization, followed by 3 hours of incubation at 60°C with Proteinase K (500 µg/mL) and phenol: chloroform extraction. DNA was resuspended in 50 µL H_2O and stored at -20°C until use. The DNA was amplified using multiple-primed rolling circle amplification (RCA) technique (Rector *et al.* 2004) with TempliphiTM 100 Amplification (GE Healthcare) following the manufacturer's instructions and using different DNA concentrations (0.02 µg/µL and 1 µg/µL). Multiple primed RCA amplifications were carried out with 0.5 µL of DNA in a final volume of 10 µL. RCA products (4 µL) were digested overnight with restriction enzymes (*Xba*I, *Sma*I, *Kpn*I, *Bam*HI and *Hind*III) (Biotools) and DNA was purified from the bands of electrophoresis of the appropriate size (Speedtools PCR clean-up kit, Biotools). Discrete bands were obtained after *Kpn*I (around 4 kb) and *Bam*HI digestions (around 2 and 6 kb) (Figure 1) while a band migrating at approximately 8 kb, compatible with undigested BPV size, was observed in the 2 remaining digestions.

The two bands from both digestions of around 4 kb and 6 kb (Figure 1, lanes 2 and 1, respectively), were excised from the agarose gel and cloned in the vector pUC19 in a total volume of 10 µL with the T4 DNA ligase (Roche Applied Sciences). One Shot TOP10 competent *Escherichia coli* (Invitrogen, Carlsbad CA, USA) were transformed with the resulting plasmids. The extraction of plasmid DNA from recombinant clones was performed with QIAprep Miniprep Spin kit (Qiagen, Hilden, Germany), and partial sequencing of several clones of each construction was performed in an ABI Prism 3730 automated sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) at the Genomic Unit of the Scientific Park of Madrid-UCM, using M13 forward (-20) primer (5´-GTAAAACGACGGCCAG-3´), M13 reverse primer (5´-CAGGAAACAGCTATGAC-3´) and walking primers (5´-TATAGCTTGCATCCCTCCTTGTTGA-3´; 5´- AACCTTACTATTAGTGTAGCTGCAG-3´; 5´-GCTGAAGATGCTGCTGGAAACA-3´). The clone

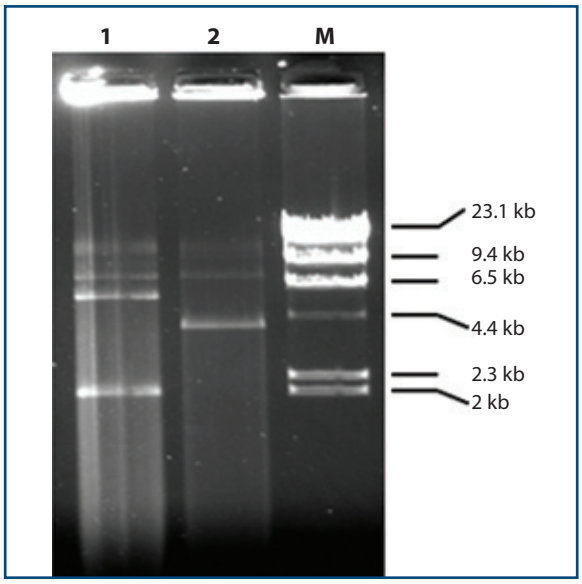

Figure 1. *Digestion profile of the rolling circle amplification (RCA) performed on 10 ng of total DNA extracted from a skin wart.* Each digestion was carried out with 4 µL of RCA product: *Bam*HI (lane 1) and *Kpn*I (lane2). Lane M: λ/*Hind*III ladder (Biotools).

with the *Kpn*I digestion allowed the nucleotide sequencing of the L1 and L2 genes and partially of the LCR region, while the clone with the *Bam*HI digestion allowed the nucleotide sequencing of the complete E5, E6 genes and the partial sequencing of the L1 and L2 genes. Sequences were deposited in GenBank under the Accession Numbers KF171968 (L1), KF293659 (E5), and KF171969 (E6).

Sequences were compared to the GenBank database using the BLAST algorithm. All sequences showed high homology with published BPV-2 sequences. The complete L1 gene had a length of 1494 nt and 497 amino acids (aa). The L1 sequence of the Spanish isolate (KF171968) was compared to other complete and partial sequences of BPV-2 L1 available in GenBank (Table I). The L1 nucleotide sequence from the Spanish isolate showed 14, 11 and 7 nt changes with M20219, X01768 and KC256805, respectively. These changes generate conservative amino acid changes (N323D, S340T, L386V and R484K) and 2 non-conserved changes (L176P, N178M) (Table I). Only the conservative

Table I. *Amino acidic variation among complete and partial L1 sequences from 15 cows (*Bos taurus*), five buffalos (*Bos bubalis*), two yaks (*Bos mutus*) and three unknown hosts (ND, not determined).* GenBank Accesion number KF171968 (the Spanish isolate) corresponds to the present work. Positions are numbered with respect to the first amino acid of the BPV-2 prototype sequence (GenBank accession number M20219). Shaded boxes represent absence of sequence data. White cells represent identity with the prototype. ND, not determined.

L1 (major capsid protein)				Residues																	
Accession No.	Host	Origin	Length (aa)	176	178	323	340	386	442	444	445	447	450	454	457	464	465	466	467	468	484
M20219	*Bos taurus*	ND	498	L	N	N	S	L	K	W	S	D	E	L	D	R	F	L	A	Q	R
KF171968	*Bos taurus*	Spain	498	P	M	D	T	V													
X01768	*Bos taurus*	ND	498	I		D		V	R												
KC256805	ND	China	498	I		D		V	R												
GQ369512	*Bos taurus*	India	54																		
GQ369513	*Bos taurus*	India	54																		
GQ369514	*Bos taurus*	India	54																		
HE600126	*Bos taurus*	India	54																		
HQ144251	*Bos taurus*	India	55									E					Y	I			
HQ144252	*Bos taurus*	India	55										D	F			Y	R			
HQ144253	*Bos taurus*	India	55							A	R						I	K			
HQ144254	*Bos taurus*	India	55							S											
HQ144255	*Bos taurus*	India	54																		
HQ166712	*Bos taurus*	India	67					V													
JQ071445	*Bos taurus*	Brazil	142					V													
JQ071446	*Bos taurus*	Brazil	142			D		V	R												
GQ369510	*Bubalus bubalis*	India	54																		
GQ369511	*Bubalus bubalis*	India	54																		
HE600123	*Bubalus bubalis*	India	54																		
HE600124	*Bubalus bubalis*	India	54																		
HE600125	*Bubalus bubalis*	India	54																		
HE603639	*Bos mutus*	India	54												H	K			P	H	
HE603640	*Bos mutus*	India	54																P	H	
EF151531	ND	India	42																		
EF151532	ND	India	42																		

mutations N323D and L386V were coincident in all 3 isolates (Table I). The 2 non synonymous substitutions are located in the EF loop inside the jelly roll β-barrel (Wolf *et al.* 2010). Some isolates from cattle from Brazil and India showed one or both N323D and L386V mutations mentioned above and others at new positions. Residues 465 to 469, which correspond to a short α-helix in the C-terminal part of the L1 protein, are particularly interesting, as conservative (F465Y, L466I) and non-conservative changes (F465I, L466R, L466K, A467P or Q468H) have been detected in some Indian isolates. However, these mutations were not observed in the Spanish isolate (Table I).

The analysis of the E5 ORF from the Spanish isolate showed 100% homology with the corresponding gene in the prototype sequence (M20219). However, some differences were observed with BPV-2 E5 protein sequences from zebras and horses from South Africa (Table II).

The E6 sequence showed several amino acidic changes with the prototype sequence M20219: S5T, P23L, V45L and N135K; no other BPV-2 E6 genes from GenBank were available for the analysis at the time

Table II. *Amino acidic variation among complete and partial E5 sequences from one cow (*Bos taurus*), 30 horses (*Equus caballus*), and three zebras (*Equus zebra*).* GenBank Accesion number KF171968 (the Spanish isolate) corresponds to the present work. Positions are numbered with respect to the first amino acid of the BPV-2 prototype sequence (GenBank accession number M20219). Shaded cells represent absence of sequence data. White cells represent identity with the prototype. ND, not determined.

E5 (major transforming protein)				Residues				
Accession No.	**Host**	**Origin**	**Length (aa)**	**6**	**9**	**24**	**40**	**41**
M20219	*Bos taurus*	ND	45	F	F	L	T	G
KF171968	*Bos taurus*	Spain	45					
AF102551	*Equus caballus*	ND	26					
AY232264	*Equus caballus*	Switzerland	45					
FJ865503	*Equus caballus*	Austria	45					
FJ865504	*Equus caballus*	Austria	45					
FJ895874	*Equus caballus*	Canada	41					
FJ895875	*Equus caballus*	Canada	41					
FJ895876	*Equus caballus*	Canada	41					
FJ895877	*Equus caballus*	Canada	41					
HQ541333	*Equus caballus*	South Africa	45			M	S	N
HQ541334	*Equus caballus*	South Africa	45	S		M	S	N
HQ541335	*Equus caballus*	South Africa	45		S	M	S	N
HQ541336	*Equus caballus*	South Africa	45		L	M	S	N
HQ541337	*Equus caballus*	South Africa	45			M	S	N
HQ541338	*Equus caballus*	South Africa	45			M	S	N
HQ541339	*Equus caballus*	South Africa	45			M	S	N
HQ541340	*Equus caballus*	South Africa	45			M	S	N
HQ541341	*Equus caballus*	South Africa	45			M	S	N
HQ541342	*Equus caballus*	South Africa	45			M	S	N
HQ541343	*Equus caballus*	South Africa	45			M	S	N
HQ541344	*Equus caballus*	South Africa	45			M	S	N
HQ541345	*Equus caballus*	South Africa	45			M	S	N
HQ541346	*Equus caballus*	South Africa	45			M	S	N
HQ541347	*Equus caballus*	South Africa	45			M	S	N
HQ541348	*Equus caballus*	South Africa	45			I	S	N
HQ541349	*Equus caballus*	South Africa	45			I	S	N
HQ541350	*Equus caballus*	South Africa	45			I	S	N
HQ541351	*Equus caballus*	South Africa	45			M	S	N
HQ541352	*Equus caballus*	South Africa	45					
HQ541353	*Equus caballus*	South Africa	45					
HQ541354	*Equus caballus*	South Africa	45			P		
FJ648526	*Equus zebra*	South Africa	45					
FJ648527	*Equus zebra*	South Africa	45					
FJ648528	*Equus zebra*	South Africa	45					

of the study. The 2 non-conserved changes (P23L and N135K) are located in several arms between beta-laminar and alpha helix structures.

A slice of tissue was submitted for histopathological analysis at the Department of Pathology of the Veterinary Clinical Hospital of the Veterinary Faculty at the UCM (Madrid, Spain). The formalin-fixed sample was embedded in paraffin by routine methods and sections were stained with haematoxylin and eosin (HE) and evaluated by a certified pathologist. Histopathological analysis confirmed the molecular results. Alterations compatible with papillomavirus infection, as hyperplasia of epidermis, hyperkeratosis or acanthosis, were observed (Figure 2).

To the best of our knowledge, this is the first report of BPV-2 presence in cattle in the Iberian Peninsula. According to the sequencing results, the present isolate could correspond to a new variant of BPV-2, as there were 14 nt substitutions of the 1494 nt that encode L1 when compared to the M20219 prototype strain. This rate of substitutions would define a variant (de Villiers *et al.* 2004). Contrariwise to human papillomaviruses (HPV), where variants have been largely studied, no variants had been described for BPV-2. Some isolates of BPV-2 have shown homology in the L1 gene less than a 100% when compared to the prototype, which would have been enough for considering them as variants (Silva *et al.* 2010, Silva *et al.* 2011). However, besides the possibility of sequencing errors, they have not been classified as such because of the incomplete sequence of the L1 gene.

The genetic analysis of L1 gene of the Spanish isolate and 24 others of BPV-2 showed a total of 48 nucleotide variations corresponding most of them to silent mutations. Five non-synonymous substitutions have been identified in at least 2 isolates (residues 176, 465, 466, 467 and 468). The non-conservative mutation N178M was only seen in the Spanish isolate. Secondary structure is unlikely altered by the mutation L176P or N178M because these residues are located in the EF loop in the jelly roll β-barrel at the N-terminal region. This region is also coincident with a non-conserved region described in HPV (Bishop *et al.* 2007). However, residues 465 to 468 are located in a ten-amino acid conical hollow around the pentamerous axis (positions 460 to 469) that shapes a short α-helix (α5). This motif is highly conserved in most BPV types and coincides with descriptions of HPV types (Bishop *et al.* 2007), and emphasizes the need of conserving the structure of the short helix (residues 460-469) followed by a strand (residues 478-484), which increases the contact edge between capsid subunits.

Additionally to L1 gene analysis, the comparison of the E5 and E6 sequence of the Spanish isolate to other published sequences might contribute to the better knowledge of these proteins. The E5 is the smallest oncoprotein described; it has different biological activities and is essential for efficient cellular transformation (Corteggio *et al.* 2013). The E5 protein interacts with the PDGF receptor in both epithelial and vascular tumours of the urinary bladder, suggesting a possible role of the virus also in mesenchymal carcinogenesis. The E5 of the Spanish variant from this work is unchanged

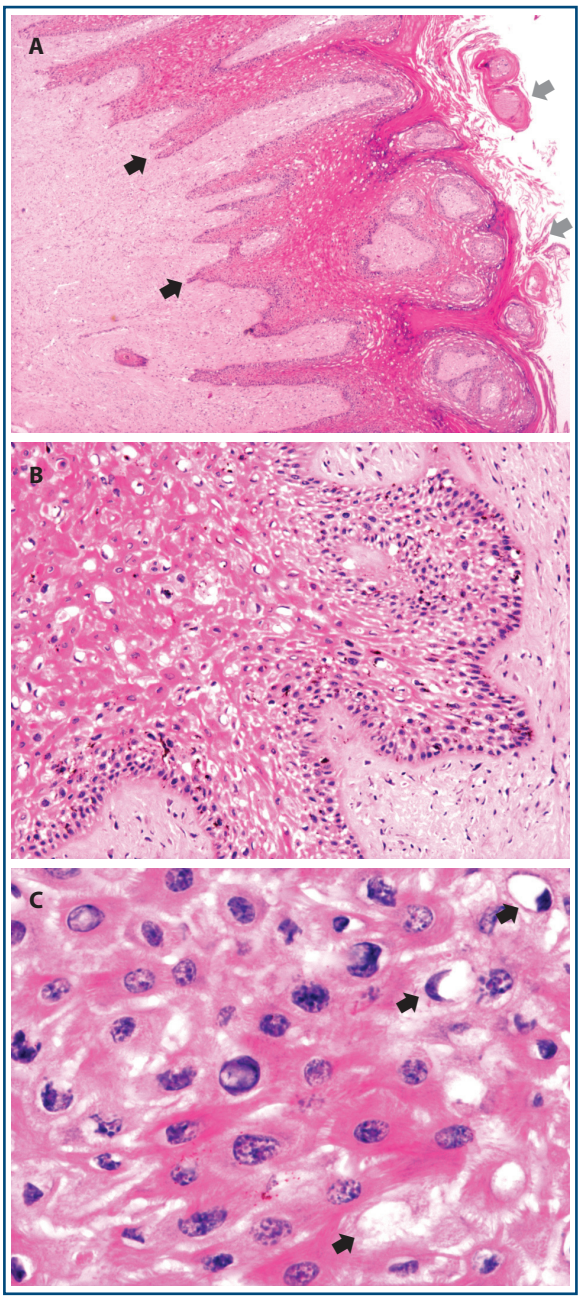

Figure 2. *Histopathological section of a bovine skin wart stained with HE (haematoxylin-eosin).* (**A**) Hyperplasia and acanthosis of epidermis with papillary projections into the dermis (indicated by black arrows). Hyperkeratosis and growth of keratin tubular formations (indicated by grey arrows). (**B** and **C**) Nuclear vacuolization in dermal stratum spinosum, with presence of empty nuclei (indicated by arrows in C). (Magnification: A, x2; B, x10; C, x40).

compared to the prototype sequence (M20219) and BPV-2 sequences from *Equus caballus* in Austria (Haralambus *et al.* 2010) and Canada (Wobeser *et al.* 2010). The inclusion of additional 33 BPV-2 E5 protein sequences of horses (GenBank accession numbers HQ541333 to HQ541354) and zebras (accession numbers FJ648526 to FJ648528) (van Dyk *et al.* 2009) from South Africa in the comparison revealed 2 amino acidic positions frequently altered (L24M and G41N) in the horse sequences from South Africa isolates. These changes are also present in BPV-1 isolates detected in equine sarcoid in Switzerland (Chambers *et al.* 2003b) and in European elk papillomavirus and deer papillomavirus (Horwitz *et al.* 1988). Thus, they might be involved in host range or lesion development.

Mutations were also observed in the E6 gene of the Spanish isolate when compared to prototype M20219, the only sequence of BPV-2 E6 gene available in GenBank. Nevertheless, 1 of the 2 non-conserved changes, K135N, has been described in other BPV types, such as BPV-13 in Brazil (Lunardi *et al.* 2013), BPV-1 associated with hoof canker in Austria (Brandt *et al.* 2011b) and *Bos grunniens* BgPV type 1 in China (Zhu *et al.* 2013), so they must not affect host range or produce a big impact on virulence.

Since BPV was first characterized, it has been found in many countries and hosts, although the worldwide distribution of different types is poorly known. Even though fibropapillomas have been described in cattle in Spain, no BPV had been published up till now. We report for the first time the identification of BPV-2 in the Iberian Peninsula, although type 2 has been described in neighbouring countries and regions including Italy, Germany, Romania and Azores Archipelago (Balcos *et al.* 2008, Schmitt *et al.* 2010, Resendes *et al.* 2011, Roperto *et al.* 2012), it has also been associated with skin warts or bladder tumours in cattle. As this isolate could be considered a variant, its description can contribute to the knowledge of dispersion and circulation of BPV, similar to HPV where several intragenotypic variants with different geographical and ethnic distributions have been identified. This would help to design protocols to protect cattle or avoid infections in other animals.

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