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Design, synthesis, and evaluation of peptides derived from L1 protein against bovine papillomavirus-1/2 identified along Mexico's cattle export route

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Abstract

Introduction: Bovine papillomatosis affects animal health and represents one of the greatest economic losses in the livestock sector. New control and prevention methods to protect the livestock industry from this disease are necessary. The aim of this study was to evaluate a candidate peptide for antibody production against bovine papillomavirus (BPV). **Material and Methods:** A total of 64 cattle underwent wart excision among 5,485 cattle distributed over 2 to 4 farms per state and 12 farms in total in the four Mexican states of Tabasco, Chiapas, Veracruz, and Nuevo León. The prevalence of bovine papillomatosis per farm was calculated by wart visualisation. The warts were genotyped by PCR and sequenced, then a phylogenetic tree was built using MEGA X software. A synthetic peptide was designed in the ABCpred, Bepipred 2.0, Bepipred IDBT, Bepitope, LBtope, and MHC II predictor online server software's based on the C-terminal region of the L1 protein. Mice antibody production was induced by subcutaneous immunisation with 50 µg of synthetic peptide and evaluated by indirect ELISA. **Results:** The prevalence of BPV was higher in Tabasco, Chiapas, and Veracruz. Bovine papillomaviruses 1 and 2 were found in all representative samples. A phylogenetic tree showed that Mexican sequences were located in exclusive clades yet were highly related to international ones. The peptide immunisation induced antibody titres of 1:10,000/1:1,000,000 against synthetic peptide and whole wart lysate (WWL), respectively. **Conclusion:** Co-infections of BPV-1 and -2 were found in all four states. Immunisation of BALB/C mice with BPV-1/2–derived synthetic peptide based on the C-terminal region of the major viral capsid protein L1 induced the production of specific antibodies able to recognise BPV-1/2 viral particles from bovine WWL.

Keywords: BPV-1/2, papillomavirus, bovine, wart, PCR.

Introduction

Bovine papillomatosis is characterised by the appearance of cutaneous, mucosal, and genital warts. It is caused by non-enveloped icosahedral bovine papillomavirus (BPV), which possesses an 8-kb circular double-stranded DNA genome. The virus has a 1–3-month incubation period and occurs primarily at an early age, although infection and wart onset can occur at any age (26). It enters through a skin wound and is disseminated by direct or indirect contact, through interaction with the major and minor viral proteins L1 and L2 (17, 28, 29). The risk of developing papillomatosis increases with immunosuppression and declining animal health (13). Bovine papillomavirus has 24 reported genotypes, 1, 2, and 4 being the most prevalent ones in cattle worldwide (1). Types 1 and 2 belong to the *Deltapapillomavirus* genus and cause cutaneous and genital warts, whereas type 4 belongs to the *Xipapillomavirus* genus and promotes

carcinogenesis (26). In Mexico, papillomatosis has been visually identified in the states of Yucatan and Veracruz (21, 25); however, only in Tamaulipas have its cases been genotyped, identifying BPV-1 and BPV-2 (22). In the last 12 years, illegal cattle (4, 6, 9) and semen traffic from South and Central America to Mexico has increased, and farmers from southern Mexico report a coinciding increase in papilloma-like skin infections.

Synthetic peptides used to prevent diseases have become an important strategy in immunisation protocols for disease control. A peptide derived from the N-terminal L2 minor protein of BPV-4 has been evaluated in a bovine model for prophylactic purposes and shown to reduce the number and size of warts (2). In describing how they modelled the human papillomavirus (HPV) capsid, Modis *et al.* (16) mentioned that the L1 major protein C-terminal region is predicted to be highly immunogenic, especially the amino acids at positions 420–429, which expose sufficient space for antibody binding. A murine model that used virus-like particles (VLPs) of BPV-9 induced IgG antibody production (27), and VLPs from BPV-1 immunisation in murine and bovine models showed a significant number of antibodies produced which were capable of identifying the virus (11). One problem with this approach is that VLPs reproduce conformational-type epitopes specific for the genotype, thus producing antibodies that could not always detect cross-reactive epitopes (linear or conformational) (3). The main purpose of this study is to identify circulating genotypes in cattle from the states of Nuevo León, Veracruz, Chiapas, and Tabasco, which are part of a cattle export route in Mexico, to design a synthetic peptide based on the L1 major viral capsid of the genotypes present and to evaluate the antibody production capacity derived from the immunisation of a murine model.

Material and Methods

Collection of samples and prevalence analysis. Representative cutaneous or genital warts were taken from 16 cattle in each of the states of Nuevo León, Veracruz, Tabasco, and Chiapas in convenience sampling. Morphology was used to identify papilloma-like warts (plane or cauliflower). Owner consent was obtained to surgically remove warts from the base, including the skin. Prevalence per farm (of which there were 12) was calculated by counting bovines with cutaneous/genital warts (Table 1) and dividing the total head of cattle on the farm by this number. The samples were transported in a cooler box, and upon reception at the laboratory the hair was removed and the samples were washed with sterile phosphate-buffered saline (PBS) and stored at −80°C until used.

DNA extraction. The sample was mashed with sterile PBS to obtain a supernatant. The phenol-chloroform technique was used for DNA precipitation. Quantification was performed in a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA) and only DNA with an A260/280 absorption relationship of 1.7–2.0 was used for upstream analysis. The integrity of the DNA was confirmed by 1% agar electrophoresis (70 V for 30 min).

PCR amplification and sequencing. To identify BPV-1, a 301-bp fragment of the L1 gene was targeted with forward 5′-GAGCGCCTGCTAACTATAGGA-3′ and reverse 5′-ATCT- GTTGTTTGGGTGGTGAC-3′ primers. For BPV-2, a 164-bp fragment of the L2 gene was targeted with forward 5′-GTTATACCACCCAAA GAAGACCCT-3′ and reverse 5′-TGGTTGCAACA GCTCTCTTTCTC-3′ primers. A 400-bp amplicon was targeted as an E7 gene fragment to identify BPV-4 with forward 5′-GCTGACCTTCCAGTCTTAAT-3′ and reverse 5′-CAGTTTCAATCTCCTCTTCA-3′ primers. Each primer set was exclusive to each genotype. All primers were synthesised by the Instituto de Biotecnología de la Universidad Autónoma de México (IBT), México City. The chain reaction was performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following parameters: initial denaturation for 3 min at 94°C; denaturation through 35 cycles each of 50 s at 94°C; annealing for 1 min at 60°C; extension for 1 min at 60°C and final extension for 5 min at 72°C. Electrophoresis was performed with tris acetate ethylenediaminetetraacetic acid buffer at 70 V for 30 min with a 100-bp molecular weight marker (Jena Bioscience, Jena, Germany). The PCR products were purified with an Agarose Gel Extraction Kit (cat. no. PP-202S; Jena Bioscience) and sequenced at the IBT by pyrosequencing.

Phylogenetic analysis. Local forward sequences were aligned with their respective reverse sequences (a complementary reverse function was performed) and aligned with the Clustal W algorithm in MEGA X (10). Alignment was performed with sequences reported by other countries and logged in the NCBI database (https://www.ncbi.nlm.nih.gov/) using the same algorithm. Sequence regions at ends that were not aligned, as well as internal gaps, were cut to later construct two phylogenetic trees, one for BPV-1 and the other for BPV-2, of maximum likelihood with the bootstrap algorithm (1,000 repetitions). For BPV-1 L1 fragments, the sequence phylogenetic tree was analysed with the following international sequences: AB626705.1 and LC426023.1 from Japan, MH543316.1 from Egypt, MF384294.1 from Switzerland, and MH197482.1 and HPV-16: S71514 from Turkey. For BPV-2 L2 sequence fragments, we used the following sequences: MH187961.1 and KU674833.1 from Brazil, KM455051.1 and MF045490.1 from China, X01768.1 from the USA and HPV-45 KU049750.1 from the UK.

Synthetic peptide design. An L1 consensus amino acid sequence was obtained in CLC Main Workbench 8 (Qiagen, Hilden, Germany) based on

BPV-1 and BPV-2 using sequences logged in GenBank at the NCBI under accession numbers AYW01245.1, BAL04338.1, BBG58099.1, ATW01203.1, AZB49454.1, AMZ04146.1, AIY55520.1, AYE54073.1, AGR88560.1, ASA69644.1 and BBG58097.1, and others from the NIH PaVE database of papillomavirus sequences (https://pave.niaid.nih.gov). The B-cell linear epitope was predicted using the following online servers: ABCpred (threshold of 0.75; https://webs.iiitd.edu.in/cgibin/abcpred/test1_main.pl), Bepipred 2.0 (threshold of 0.75; http://www.cbs.dtu.dk/services/BepiPred/), Bepipred IDBT (threshold of 0.75; http://tools.iedb.org/bcell/), Bepitope (threshold of 1; http://bepitope.ibs.fr/), BCEpred (threshold of 1.9–2.3; https://webs.iiitd. edu.in/cgibin/bcepred/bcepred.pl) and LBtope (threshold of 0.75; https://webs.iiitd.edu.in/raghava/coronavir/lbtope/ORF10/lbtope.php). For helper T-cell L1 epitope prediction, a consensus amino acid sequence was found from analysis of the following bovine leukocyte antigen (BoLA) alleles: BoLA-D18.4, BoLA-HD6, BoLA-JSP.1, BoLA-T2a, BoLA-T2C, BoLA-AW10, BoLA-D18.4, BoLA-JSP.1, BoLA-3:00101, BoLA-3:00201 and BoLA-3:00102. The H-D-I murine allele was also analysed to ensure compatibility with a murine model for the immunisation experiment on the major histocompatibility complex (MHC) II predictor online server (http://tools.immuneepitope.org/mhcii/). The epitopes predicted were considered when the score indicated them to be "good binders". Finally, a candidate peptide derived from the L1 protein was synthesised by Bio Basic (Markham, ON, Canada). Physical and chemical properties were assessed in the Compute pI/Mw tool (https://web.expasy.org/compute_pi/) program and ProtParam (https://web.expasy.org/protparam/) online server at the Expasy Swiss Bioinformatics Resource Portal. Solubility was verified in CRYSTAL2 (http://biomine.cs.vcu.edu/servers/CRYSTALP2/) and Protein Sol (https://protein-sol.manchester.ac.uk). The candidate peptide's specificity against bovine and BPV sequences was analysed with BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Murine model immunisation. BALB/c mice were immunised at four weeks of age with 50 µg of synthetic peptide diluted 1:1 with water. Freund's complete adjuvant was used in the first dose and Freund's incomplete adjuvant in the two following doses, each with a total volume of 100 µL. Doses were administered every two weeks subcutaneously (n = 3). The positive control group was immunised three times with 100 µL of total antigen extract inactivated with 2M binary ethylenimine every two weeks subcutaneously with the method described by Pangty (20). Retro-orbital puncture was performed weekly under anaesthesia with ketamine and xylazine for serum obtention before and after the first dose. Blood was centrifuged at 1,600 rpm for 30 min to separate serum, and sera were stored at −20°C until use. All procedures were approved by the Comité de Ética de Investigación y Bienestar Animal (CEIBA) institutional ethics committee for research animal welfare of the Autonomous University of Nuevo León under protocol number CEIBA-2018-001. The experiments involving animals were performed following the instructions for care and use of experimental animals and complied with the Mexican federal animal welfare law (NOM-062-ZOO-1999).

Antibody specificity evaluation. Reactivity was evaluated using the indirect-ELISA method as follows: 96-well plates were sensitised with whole wart lysate (WWL) or synthetic peptide (5 µg/well), both diluted 1:1 in carbonate-bicarbonate buffer with pH of 9.6 and incubated at 4°C overnight. Subsequently, two washes with PBS-Tween 20 0.1% (200 µL/well) were performed before the plates were blocked with 5% skim milk in PBS-Tween 0.1% and incubated at 37°C for 1.5 h. After four washes, sera were added from immunisation days 0, 7, 14 and 35 in a 1:100–1:1,000,000 dilution) and the plates were incubated at 37°C for 1 h. After a further four washes, 75 µL/well of HRP-conjugated anti-mouse antibody was added at 1:3000 dilution and the plates were again incubated at 37°C for 40 min. After a final four washes, 50 µL/well of tetramethylbenzidine substrate was added and the plates were incubated for a final time at 37°C for 20 min. Then 50 µL/well of 1% SDS were added to stop the reaction and the plates were read at 405 nm. The antibody titre capable of detecting the virus was established when the absorbance value exceeded the cut-off value, which was established according to the following formula: Cut-off = blank average + (blank standard deviation ×2).

Statistical analysis. Statistical analysis was conducted in Prism 6 software (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance and Tukey's test were performed ($P < 0.05$).

Results

The southern states showed higher prevalence levels (Tabasco farms having 9.2% to 23.44%, Chiapas 11.6% to 32.8% and Veracruz 10% and 11%) than the northern state of Nuevo León (3.77% to 11.43%) (Table 1). Most representative samples that were subjected to genotyping by PCR presented BPV-1 (96.88% of all samples, 37.50% in this percentage presenting exclusively BPV-1) and a minor part presented BPV-2 (this variant only occurring as a co-infection with BPV-1, comprising the remaining 59.38% in the 96.88%) (Figs 1 and 3). The genotype could not be identified in two samples from Tabasco and Chiapas (Figs 2 and 3) even though they presented the cauliflower morphology characteristic of BPV warts.

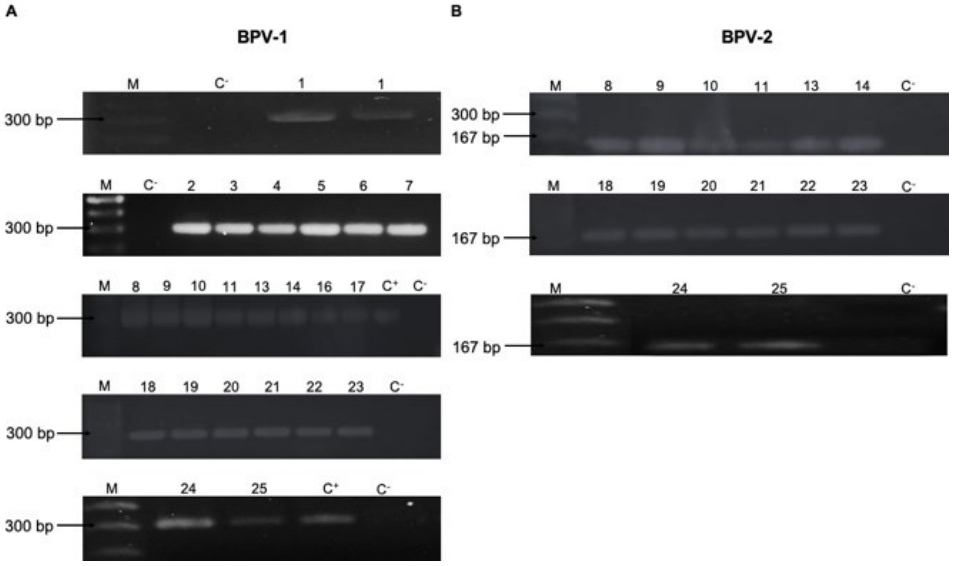

Fig. 1. Bovine papillomavirus BPV-1 and BPV-2 shown in representative samples which were positive for viral genetic material when electrophoresed in 1.5% agarose gel
M – 100 base pair molecular weight marker; C^+ – BPV-1 positive sample from Nuevo León; C^- – DNA-free reaction mix; A – BPV-1 presence in cutaneous/genital warts: lanes 1–11, 13–14 and 16–25; B – BPV-2 presence in cutaneous/genital warts: lanes 8–11, 13–14 and 18–25 (Nuevo León, lanes 1–5 and 24–25; Veracruz, 18–23; Chiapas, 6–7 and 14–17; and Tabasco, 8–13)

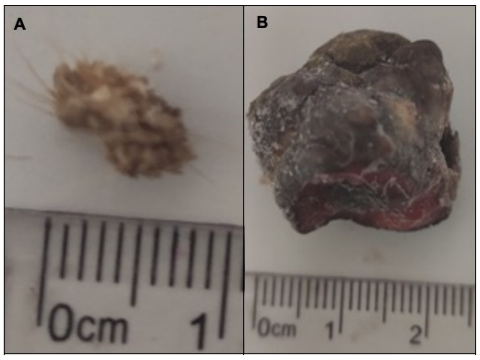

Fig. 2. Samples negative for bovine papillomavirus (BPV)-1/2/4 and therefore with genotype unknown
A – sample from Emiliano Zapata, Tabasco; B – sample from Libertad, Chiapas. Samples had cauliflower morphology suggesting that they be considered BPV positive

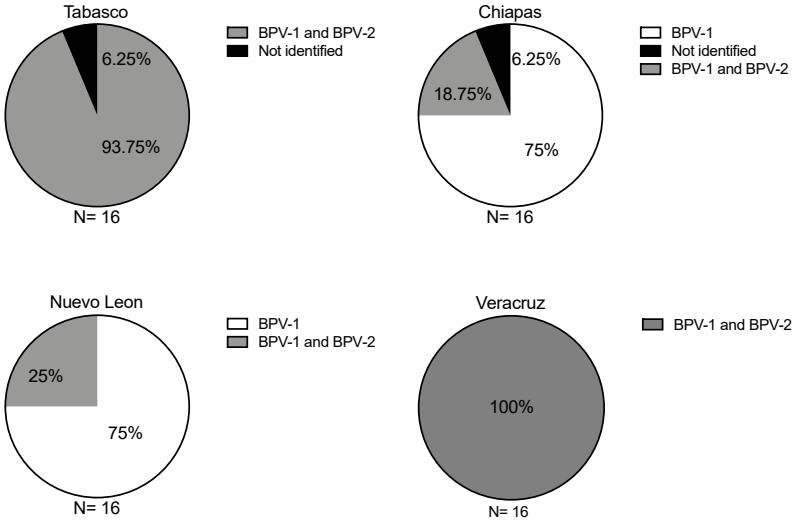

Fig. 3. Bovine papillomavirus (BPV)-1 and BPV-2 genotype distribution per state from representative samples. BPV-1 was found as a unique genotype or as a co-infection with BPV-2, but BPV-2 was found only as a co-infection

Table 1. Bovine papillomatosis prevalence in Nuevo León, Chiapas, Veracruz and Tabasco, Mexico

State:	Papillomatosis	n
Nuevo León		
Farm 1 (Linares)	3.77%	450
Farm 2 (Linares)	18%	250
Farm 3 (Cadereyta)	11.43%	700
Chiapas		
Farm 4 (Palenque)	12.83%	1200
Farm 5 (Libertad)	11.6%	500
Farm 6 (Salto de Agua)	32.8%	125
Farm 7 (Agua Fria)	20%	50
Veracruz		
Farm 8 (Temapache)	10.67%	150
Farm 9 (Tihuatlán)	10%	160
Tabasco		
Farm 10 (Emiliano Zapata)	9.2%	1500
Farm 11 (Balancan)	16.25%	80
Farm 12 (Tenosique)	23.44%	320

n – total sampled bovines per farm of all ages

Genotypes were confirmed by sequencing, selecting those with an A260:280 absorbance ratio of 1.8 indicating high purity when extracted. Alignment of sequences in BLASTn revealed high homology between each obtained genotype sequence and that of the corresponding genotype recorded in the NCBI database (Tables 2 and 3); BPV-1 sequences showed higher homology (99%) with their corresponding sequences than BPV-2 sequences (91–97%) (Tables 2 and 3) against the reference sequences with accession number KC595244.2 and MH187961.1, for that the virus genotype 2 sequences were still identifiable and conserved, which made the genotypes identified by PCR certain to be the correct ones. Phylogenetic analysis located Mexican sequences in exclusive clades (Figs 4 and 5), although the BPV-1 phylogenetic tree separated local sequences into three clades instead of one monophyletic group, as was observed in the BPV-2 phylogenetic tree (Fig. 5). In the analysis of the partial sequences of the L1 protein, it was observed that all the clades started from the same node from which the HPV-16 sequence separated from the bovine sequences, showing a common ancestor located at the root, and a closer relationship between the BPV-1 sequences than these sequences had to the reference one, which was another confirmation of the identity of the local sequences obtained. All the BPV-1 sequences were equally related as they had the same distance from the common ancestor (three nodes), but it was observed that the sequences from countries other than Mexico were in an exclusive clade, which shows that they are more related. Local sequences, on the other hand, were found in three clades mixing the different states, two of these at the same distance from the common ancestor, and a clade composed of two sequences from the state of Nuevo León was found slightly further away from this ancestor. Regarding the sequences derived from the L2 minor viral capsid protein, the Veracruz local sequence (Veracruz RS20) was in an exclusive clade along with the common ancestor, whereas the Tabasco sequence (Tabasco RS8) was found in another exclusive clade at greater distance from the common ancestor; both local sequences were separated from international ones (Fig. 5). It was demonstrated that the sequences obtained from local states were conserved but still highly related to sequences from around the world.

Chiapas was the state with the lowest percentage of co-infections at 18.75%, followed by Nuevo León at 25%, Tabasco at 93.75%, and Veracruz at 100% (Figs 3 and 6). It is important to mention that Veracruz is one of the largest cattle collection centres, and larger than Tabasco and Chiapas. Meanwhile Nuevo León is the final feedlot destination for exportation or national consumption as it is in the north of the country (Fig. 6). It is remarkable that, as cattle move north, the co-infection ratio decreases. Nuevo León acts as a bottleneck by virtue of its position in selecting cattle, whereas Veracruz concentrates the incoming genotypes from South American countries and the concentration facilitates virus transmission.

Table 2. Nucleotide sequence identity analysis of bovine papillomavirus 1 L1 fragments isolated from samples positive for the virus

Sequence	Expect (E) value	Identity % (alignment with KC595244.2)	Gaps
Chiapas (RS7)	$7e^{-129}$	99%	2/265
Chiapas (RS6)	$4e^{-118}$	99%	0/242
Nuevo León (RS4)	$2e^{-129}$	99%	3/267
Nuevo León (RS2)	$2e^{-101}$	99%	0/209
Nuevo León (RS5)	$3e^{-131}$	99%	2/266
Veracruz (RS20)	$1e^{-125}$	99%	1/257
Tabasco (RS8)	$1e^{-125}$	99%	1/257
Nuevo León (RS3)	$2e^{-126}$	99%	2/261
Nuevo León (RS1)	$6e^{-128}$	99%	4/267

Table 3. Nucleotide sequence identity analysis of bovine papillomavirus 2 L2 fragments isolated from samples positive for the virus

Sequence	Expect (E) value	Identity % (alignment with MH187961.1)	Gaps
Veracruz (RS20)	$7e^{-25}$	97%	0/71
Tabasco (RS8)	$6e^{-45}$	91%	3/132

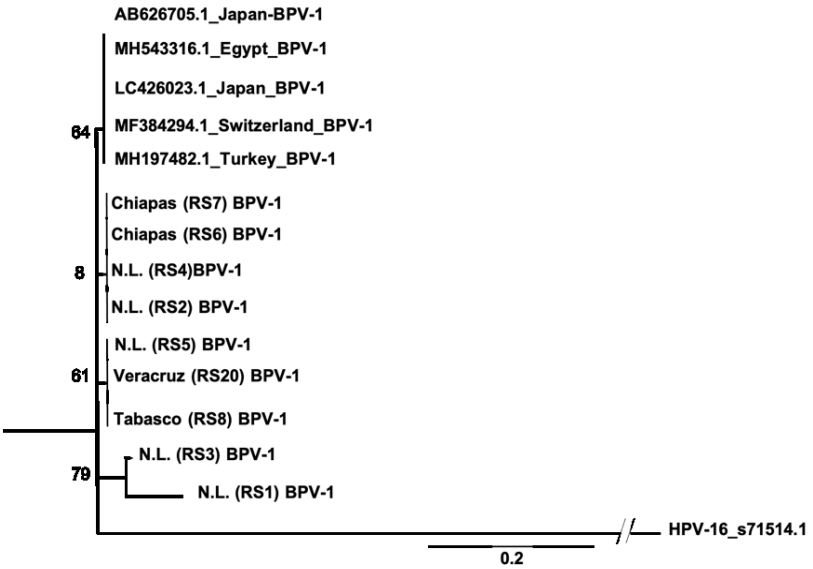

Fig. 4. Bovine papillomavirus (BPV)-1 phylogenetic tree of Mexican isolates (partial L1 sequence). Sequences were compared with sequences from different nationalities recorded in the NCBI database, of which the accession numbers are indicated. The comparison used a neighbour-joining method in MEGA 10.1. RS – representative sample

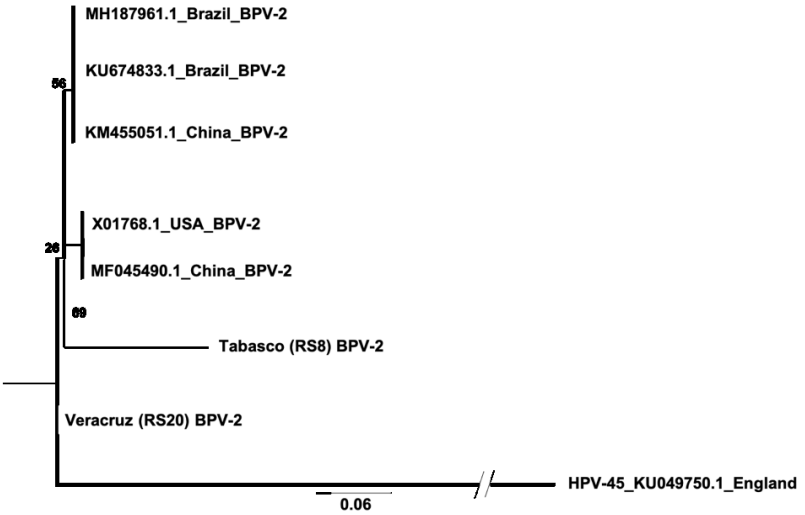

Fig. 5. Bovine papillomavirus (BPV)-2 phylogenetic tree of Mexican isolates (partial L2 sequence). Sequences were compared with sequences from different nationalities recorded in the NCBI database, of which the accession numbers are indicated. The comparison used a neighbour-joining method in MEGA 10.1. RS – representative sample

Once the circulating genotypes (Fig. 6) were identified, and the sequences analysed transpired to be highly conserved locally and internationally, a candidate peptide was selected for immunisation in the mouse model with the purpose of antibody production. The selected peptide was derived from BPV-1 and BPV-2 genotypes. Peptide selection was based on the matches between the outputs of online servers of the C-terminal region of the L1 protein amino acid sequence, considering the higher scores (Table 4 and Fig. 7). The sequence measures 14 nucleotides to fit the B-cell epitope length. Figure 9B shows a yellow region indicating antigenicity and green non-antigenic regions within the peptide; most of the C-terminal region was shown to be antigenic. Because the immunisation experiment was to be performed in a mouse model, affinity with this was verified with B cell epitope prediction in Bepipred IDBT software. The *in silico* result indicated compatibility with the H-D-I allele (data not shown), and a possibility of the peptide being an MHC II epitope in the MHC II predictor online server, it being forecast to be a "good binder" (data not shown). In addition, the chemical and physical properties analysed demonstrated a 4.68 isoelectric point, no hydrophobicity, and a mass weight of 1948.09 Da. Because it is considered a hapten,

administration with an adjuvant is recommended, and thus the immunisations were conducted with Freund's adjuvant (complete in the first dose and incomplete in the last two doses).

To corroborate that the candidate peptide was specific for both BPV-1 and BPV-2, the peptide amino acid sequence was analysed in BLASTp. The result was 100% homology with the L1 sequence of BPV-1 and BPV-2 (Fig. 8), demonstrating a dual identity. Therefore, this candidate peptide was selected for chemical synthesis, and immunisation with the synthetic peptide (high purity, data not shown) was performed in a murine model.

Immunisation with synthetic peptide induced the production of specific antibodies, which possessed reactivity against the synthetic peptide (Fig. 9B) up to a 1:1,000,000 titre on days 7, 14, and 35 ($P < 0.001$). This contrasted with positive control immunisation of WWL inactivated against WWL (Fig. 9A), antibodies deriving from which reacted up to a 1:10,000 titre on the same days ($P < 0.001$). Also, a highly significant difference ($P < 0.001$) could be observed between titres in Figs. 9A and 9B, last on day 14, and between all dilutions on day 35 (Fig. 9B, $P < 0.005$).

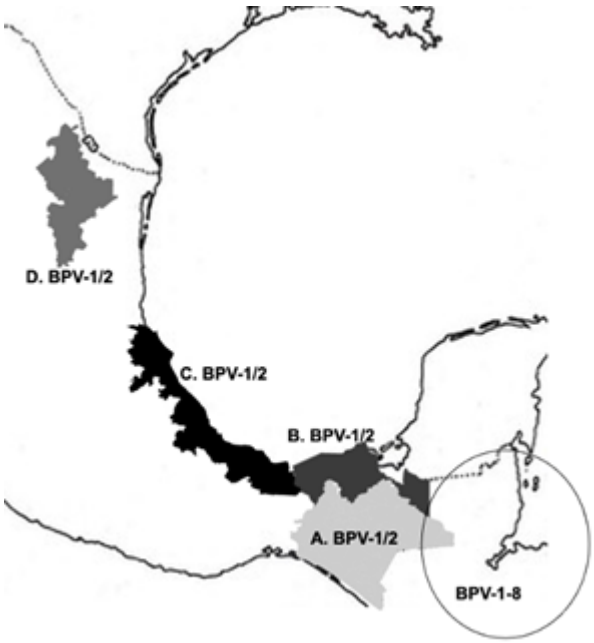

Fig. 6. Geographical bovine papillomavirus (BPV) genotype localisation in Mexico. Tabasco, Chiapas, and Veracruz states are considered breeder states and cattle collection centres for Central America. Nuevo León is a final feedlot destination state for national consumption or international exportation. The intensity of grey indicates the percentage of co-infection
A – Chiapas; B – Tabasco; C – Veracruz; D – Nuevo León

Table 4. Amino acid matches between epitope predictions in different software

Online server	L1 epitope	Position	Threshold	Score
Bepitope	SPATKCASNVIPAK	421	0.75	Yes
ABCpred	SILEDTYRYESPATK	410	0.75	0.87
Bepipred	PSVLQNWEIGVQPPTSSILEDTYRYIESPATKCASNVIPAKEDPYAGFKF	394	0.75	Yes
LBtope	VQPPTSSILEDTYRYIES	404	0.75	85.2
BCEpred	DTYRYIESPATKCASNV	414	1.9	Yes

Underlined letters represent matches

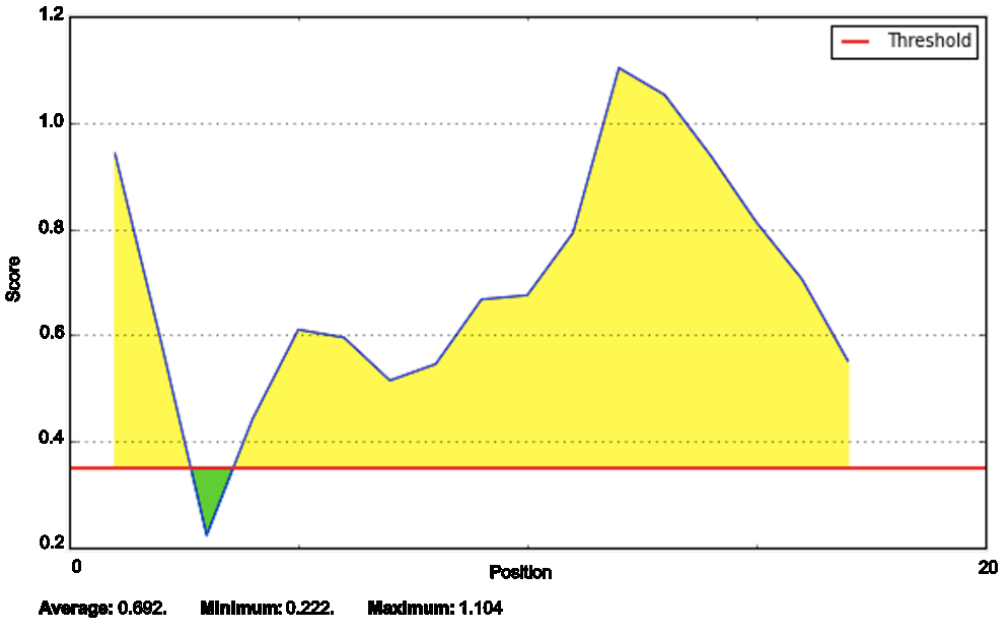

Fig. 7. Candidate peptide prediction by online server. The amino acid sequence of the selected peptide possesses antigenicity. Prediction with default values was performed in Bepipred Linear Epitope Predictor

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
L1 protein [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	APP14105.1
RecName: Full=Major capsid protein L1 [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	P06458.2
major capsid protein [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	BBQ04448.1
L1 protein [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	497	ATX74956.1
L1 protein [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	AIY55520.1
L1 [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	AMZ04146.1
major capsid protein [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	AHY28934.1
L1 protein [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	AGG55387.1
L1 [Bos taurus papillomavirus 2]	Bos taurus papilloma...	61.7	61.7	100%	1e-09	100.00%	497	ASA69644.1
L1 protein [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	495	ATW01070.1
L1 protein [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	495	ATW01105.1
L1 protein [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	495	ATX74933.1
L1 protein [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	495	ARO77073.1
L1 protein [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	495	ASN74466.1
L1 [Deltapapillomavirus 4]	Deltapapillomavirus 4	61.7	61.7	100%	1e-09	100.00%	495	AYW01245.1

Fig. 8. Specificity of the candidate peptide for BPV-1. BLASTp showed it to be specific to BPV-1 and BPV-2 with 100% homology

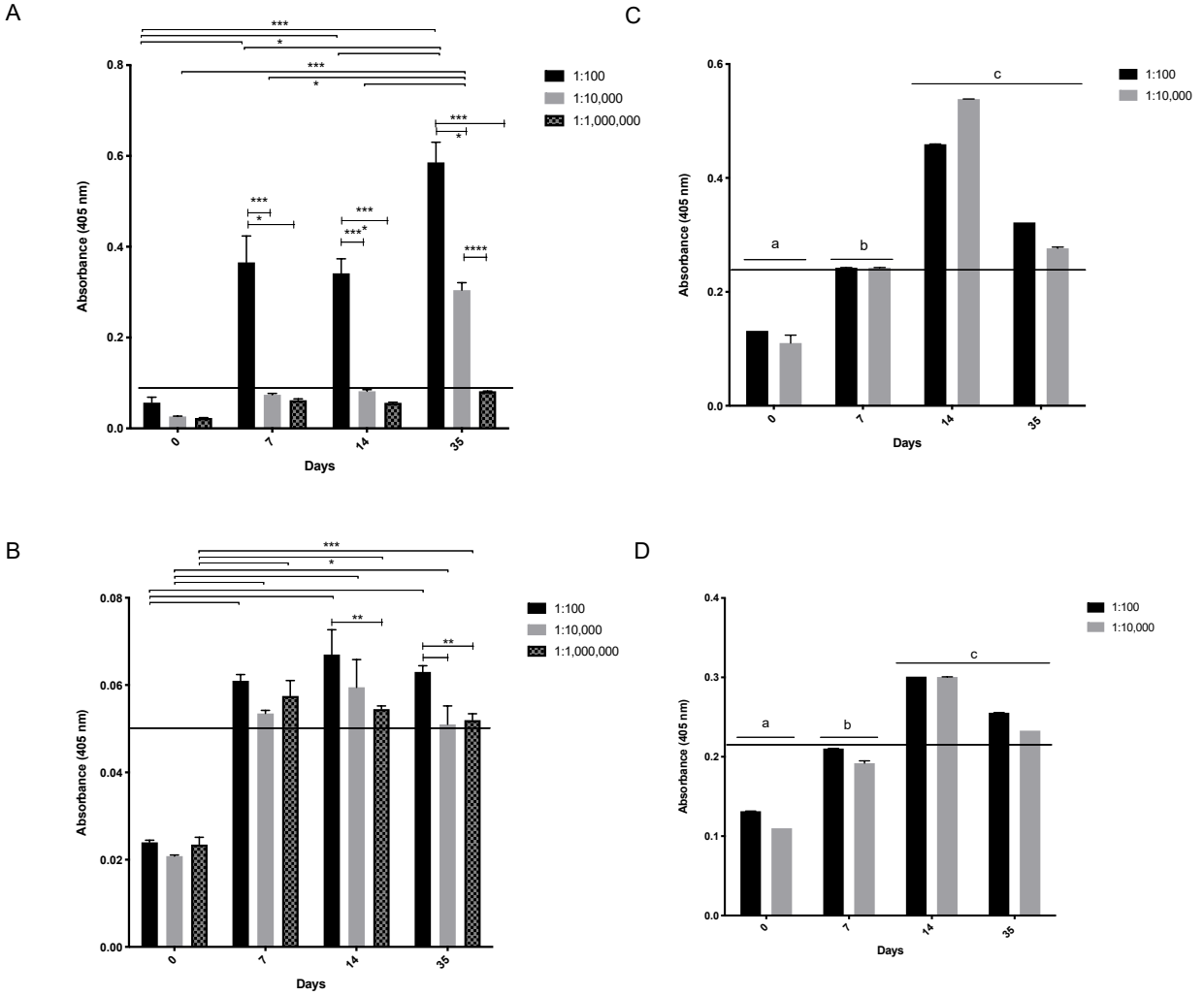

Fig. 9. Sera reactivity against WWL/synthetic peptide and antibody specificity against BPV
A – Anti-wart antibody reactivity against WWL at 1:100 dilution on day 7 and 1:10,000 on days 14 and 35; B – Anti-peptide serum reactivity against synthetic peptide at a titre of 1:1,000,000 on days 7, 14 and 30. Cut-off points (horizontal solid line) were calculated at 0.087 and 0.053, respectively. **** – extremely significant difference (Tukey test $P < 0.0001$); *** – highly significant difference (Tukey test $P < 0.0002$) (n = 2); ** – significant difference (Tukey test $P < 0.0021$); * - significant difference (Tukey test $P < 0.0332$) (n = 2); C – Anti-wart serum reactivity against peptide at titres of 1:10,000–1:1,000,000 on day 14 and at a titre of 1:10,000 on day 35; D – Anti-peptide serum reactivity against WWL at a titre of 1:10,000 on days 7, 14 and 35, and at a titre of 1:1,000,000 on days 14 and 35. Letters a, b, and c represent significant difference based on the cut point (horizontal line) of 0.219 in both C and D (Tukey's test $P < 0.05$) (n = 2)

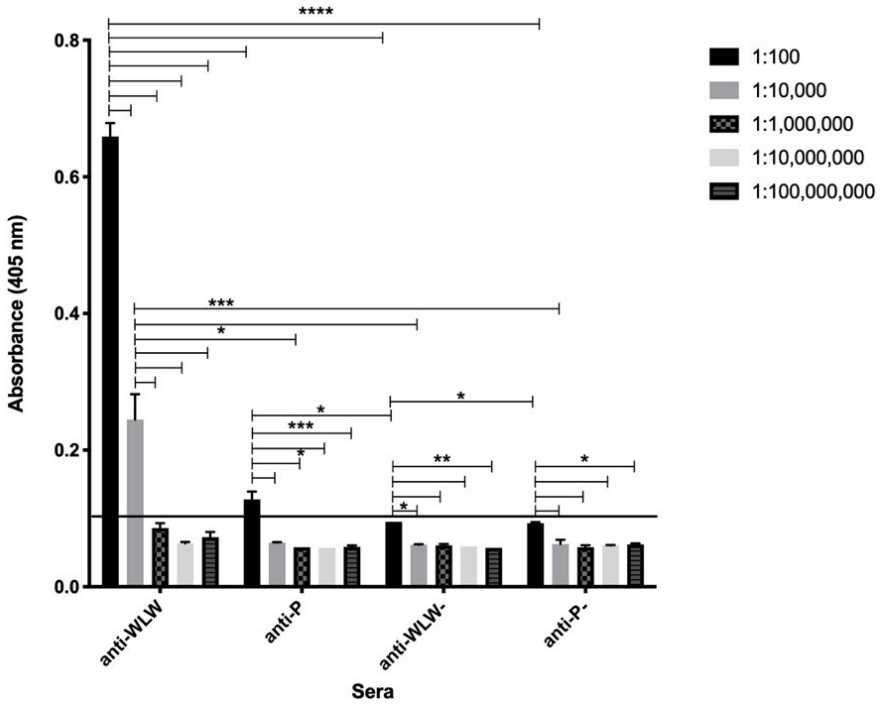

Fig. 10. Anti-wart serum reactivity against WWL at titres of 1:100–1:10,000 and at a titre of 1:100 on day 42 in anti-peptide serum against WWL. The solid line indicates the cut-off point at 0.08; **** – extremely significant difference (Tukey test $P < 0.0001$); *** – highly significant difference (Tukey test $P < 0.0002$) (n = 2); ** – significant difference (Tukey test $P < 0.0021$); * – significant difference (Tukey test $P < 0.0332$) (n = 2)

Once it was corroborated that, despite being a hapten, it induced a considerable antibody titre, the synthetic peptide was verified as an epitope by observing whether this immunisation induced antibodies capable of detecting BPV particles in WWL. Reactivity was measured in sera from mice immunised with the synthetic peptide against WWL up to a titre of 1:10,000 on days 14 and 35 (Fig. 9D, $P < 0.05$). The same was observed with positive control mouse sera against the synthetic peptide with reactivity up to a titre of 1:10,000 on days 7, 14 and 35 (Fig. 9C, $P < 0.05$), which indicates that the synthetic peptide derived from the L1 protein of BPV-1 was recognised by antibodies produced after induction by the immunisation and is indeed exposed in the capsid because of its position in the C-terminal region. The results of both assays (Figs 9C and 10) showed a peak in absorbance on day 14 (after the second dose was administered), indicating that more intensive specific antibody production in the IgG subclass is induced with subsequent doses through stimulation of adaptive immunity. Although on day 35 the absorbance peaks seemed to be shorter than those on day 14, the day on which the third dose was administered, statistically there was no difference between these days ($P < 0.05$).

Besides the strength of antibody induction, the duration and maintenance of immunity are also important. To evaluate these factors, sera from positive control mice and mice immunised with synthetic peptide groups were assayed against WWL on day 42 by the same technique, and reactivity was observed at a 1:100 titre in anti-peptide sera and at a 1:10,000 titre in positive control sera with a highly significant difference to reactivity in negative control sera (Fig. 10) ($P < 0.001$).

Discussion

In Mexico there are limited reports of the incidence of bovine papillomatosis, and genotyping has been reported only in one state, Tamaulipas, where BPV-1 and BPV-2 were identified by amplifying an E7 gene fragment. This approach makes possible detection of a protein expressed in all infected dermal layers, not necessarily in cells permissive to the virus. The genotype was confirmed later, in the second step of sequencing, the results of which were not demonstrated (22). In contrast, we targeted L1 and L2 genes, genotype-specific sequences, and performed another sequencing for double-checking. These proteins are expressed only in keratinocytes or permissive cells in which the virus is assembled (24, 26), which highlights the importance of the sampling methodology. Bovine papillomaviruses 1 and 2 were identified in samples from Nuevo León, Veracruz, Chiapas and Tabasco, which corroborates what Rojas *et al.* (23) found in Tamaulipas. The BPV-1 and BPV-2 variants were recognised in previous research as the most prevalent, promoting the appearance of warts on the skin and in the genital areas (22, 25). The genotype of two of the wart samples obtained could not be identified, at least not with the primers available for BPV-1/2/4; these warts may have belonged to a different BPV genotype.

Phylogenetic analysis indicates that BPV sequences are highly conserved, as the distance from the common ancestor is the same for all clades. Similarities between sequences were shown by a likelihood test (8), in which all sequences showed 92% to 99% homology with the sequences recorded in the NCBI database from other countries. This high value confirms its identity, since its homology percentage is greater than 30% (20).

In the present study, BPV-2 was only found in co-infections with BPV-1 in 59.38% of the samples. The southern states, except for Chiapas with 18.75%, showed higher percentages of co-infection compared with Nuevo León's 25%: Tabasco had 93.75% and Veracruz 100%. This could be attributed to the breeding purpose of farms in southern states, which opens the possibility of direct and indirect infection from mother/father to the foetus, considering that transplacental infection may occur and that the virus has been reported in monocytes, blood, and semen (pellets and pre-seminal fluid) (15, 23). In the northern states, on the other hand, cattle are finished in feedlots for export to the United States and Canada.

We hypothesise that BPV-1 infection could increase susceptibility to BPV-2 co-infection by immunosuppressing cattle, as the former is the most common worldwide, and the latter was only found in co-infection with BPV-1. One of the possible mechanisms by which BPV-1 could immunosuppress the animal is by modulating TLR4: E2 and E7 proteins have been demonstrated to downregulate TLR4 in equine fibroblasts (30), and HPV E6 downregulates TLR9 action (5). The cattle incoming from southern countries carries more genotypic diversity in their viruses, and the incidence of genotypes other than BPV-1/2 is reported (18). This suggests infection by other genotypes, and the failure of our assays on two of our representative samples from farms in Tabasco and Chiapas to amplify any BPV-1/2/4 genetic material despite the cauliflower wart morphology strongly indicating BPV infection (14) points to another infecting genotype not evaluated in the present study.

Since amino acid and nucleotide L1 sequences are highly conserved (14), they are a good target for a prophylactic vaccine. We decided to employ the amino acid L1 sequences reported in online databases for *in silico* predictions, specifically the C-terminal region sequences which we sequenced and found conserved when compared with international ones. B cell epitope prediction was carried out with the complete amino acid sequence of L1, and it was found that the C-terminal region (the last 400 amino acids) could be immunogenic, coinciding with the viral capsid model (16). The L1 amino acid 413 was taken as the first amino acid of the candidate peptide, thereby skipping amino acids 300–400 which are reported as non-efficient at promoting a humoral response (12). The candidate peptides were selected by being exposed in their viral capsid location, with linear conformation, and an epitope of a maximum of 30 amino acids in length was sought to fit the length of a B cell epitope within the L1 amino acidic sequence (19); the selected sequence, composed of 17 amino acids, fits in this range. It is worth mentioning that this strategy of focusing on the L1 protein as target for a vaccine has been already used in the human VLP vaccine Gardasil-9.

Anti-peptide sera demonstrated a strong production of antibodies, similar to the positive control that showed a titre of 1:1,000,000 on day 35, whereas anti-peptide sera against WWL reacted up to a titre of 1:10,000, which is why the peptide can be considered an immunogenic epitope. This showed reactivity at a higher titre, as shown by similar investigations, where sera from rabbits immunised with recombinant HPV-6b-L1 (7) showed reactivity against a synthetic peptide from the same protein (amino acids 417–437) only up to 1:200 titre. Furthermore, reactivity was observed up to day 42 of the anti-peptide sera to WWL, coinciding with the finding of Campo (2) who also evaluated reactivity over time. An important aspect to consider is whether immunisation with a single peptide will be sufficient to endow the immune system with the capability to detect the virus. Campo found that sera from calves vaccinated with individual peptides (3 different regions from the L2 protein) responded with titres capable of detecting the peptide in ELISA, but also responded to recombinant L2a protein, confirming that the humoral response could be induced by immunisation with a single synthetic peptide (2). In addition, the synthetic peptide output by *in silico* simulations in BLASTp could have dual specificity against BPV-1 and BPV-2, which offers the perspective of testing reactivity against WWL from samples positive for both genotypes in future ELISA probes with sera of immunised mice and bovines. The present experiments were performed with WWL from samples that were positive for BPV-1 by PCR genotyping. In future investigations, we could include more than one peptide to obtain a stronger immune response and increase protection against other viral genotypes.

This study demonstrates that the local BPV-1 and BPV-2 prevalence correlates with the worldwide incidence. Moreover, the nucleotide sequences of the L1 and L2 proteins were shown to be highly conserved despite being in exclusively local clades. Synthetic peptides induced considerable production of antibodies specific for BPV-1 and are thus considered an antigenic epitope and candidate for a vaccine in a bovine model.

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