VIRUS DE LA LEUCOSIS BOVINA (VLB) Y EVIDENCIAS DE SU POTENCIAL ZOONÓTICO

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Anexo 2. Corredor, A.P.; Gonzales, J.; Baquero, L.A.; Curtidor, H.; Olaya-Galán, N.N.; Patarroyo, M.A.; Gutierrez, M.F.; González, J.; Baquero, L.A.; Curtidor, H.; et al. In silico and in vitro analysis of boAP3d1 protein interaction with bovine leukaemia virus gp51. *PloS One* 2018, *13*, 1–18, https://doi.org/10.1371/journal.pone.0199397

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Research Paper

Prevalence and molecular epidemiology of bovine leukemia virus in Colombian cattle

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ABSTRACT

Bovine leukemia virus (BLV) is one of the five agents considered most significant for cattle. It is important to determine the prevalence and molecular epidemiology of BLV throughout the country in order to gain a more thorough understanding of the current situation of BLV and to reveal the possibility of masked genotypes that the primers used by OIE are unable to identify. Blood samples were collected at random from 289 cows distributed in 75 farms across the country. PCR amplification of *env, gag* and *tax* gene segments was performed. The obtained amplicons were sequenced and then subjected to phylogenetic analyses. A total of 62% of the cows present at 92% of the farms were BLV-positive for *gag* fragment. Genotype 1 was exclusively detected by *env* gene segment when analyzed using previously reported primers. However, *tax* gene analysis revealed circulation of genotype 6 variants, which were also detected based on *env* gene analysis with newly designed primers. These results indicate that current genotyping approaches based on partial *env* sequencing may bias BLV genetic variability approaches and underestimate the diversity of the detected BLV genotypes. This report is one of the first molecular and epidemiological studies of BLV conducted in Colombia, which contributes to the global epidemiology of the virus; it also highlights the substantial impact of BLV on the country's livestock and thus is a useful resource for farmers and government entities.

1. Introduction

Viruses are one of the main causes of health problems, of which bovine leukemia virus (BLV) is one of the five agents considered most significant for cattle. Colombia had previously reported a seroprevalence of animals of 42.7%, a figure that is consistent with worldwide reports, which range between 5 and 90% (Lee et al., 2016; Murakamia et al., 2011; Ortiz et al., 2016; Polat et al., 2016).

BLV belongs to *Retroviridae* family and Deltaretrovirus genus with two copies of a single-strand positive-RNA with a length of 8714 nucleotides. Its genome contains 8 open reading frames with 3 gene segments (*gag, pol, env*) encoding the structural proteins and enzymes necessary for viral replication, a pX region encoding the Tax and Rex auxiliary proteins, which perform regulatory functions, and two long terminal regions (LTRs) at the terminal ends of the genome (Lee et al., 2016; Ochirkhuu et al., 2016; Polat et al., 2017b, 2016; Rosewick et al.,

Abbreviations: BLV, bovine leukemia virus; ORFs, open reading frames; LTRs, long terminal regions; gp51, extracellular portion envelope protein; gp30, transmembrane region envelope protein; RFLP, restriction fragment length polymorphism; OIE, World Organization for Animal Health; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AIC, Akaike information criterion

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Table 1
Primers used for amplification*, sequencing* and construction of plasmid DNA┼ for the BLV *gag, tax* and *env* gene segments. NA (not applicable).

Region and position on the genome	Primer sequence 5`- 3` (Forward and reverse)	PCR product (bp)	Annealing Temperature (°C)	Minimum detectable concentration (ng/ul)	Reference
*gag**┼	AACACTACGACTTGCAATCC	385	59.3	2.3	(Buehring et al.,
1068–1453	GGTTCCTTAGGACTCCGTCG				2014)
*tax**┼	CTTCGGGATCCATTACCTGA	373	56.5	0.042	
7197–7570	GCTCGAAGGGGGAAAGTGAA				
*env**	CCCACAAGGGCGGCGCCGGTTT	509	62.8	159×10^{-9}	(Fechner et al.,
5107–5636	AACAACAACCTCTGGGAAGGGT				1997)
*env**	TGTCCCTAGGAAAYCAAC	750	56	159×10^{-4}	Current study
4938–5688	AGATTAACCAGGGAGATAGG				
env┼	ATGAGATGCTCCCTGTCCCTAG	801	57.6	NA	(Corredor et al.,
4925–5726	ACGTCTGACCCGGGTAGG				2018)

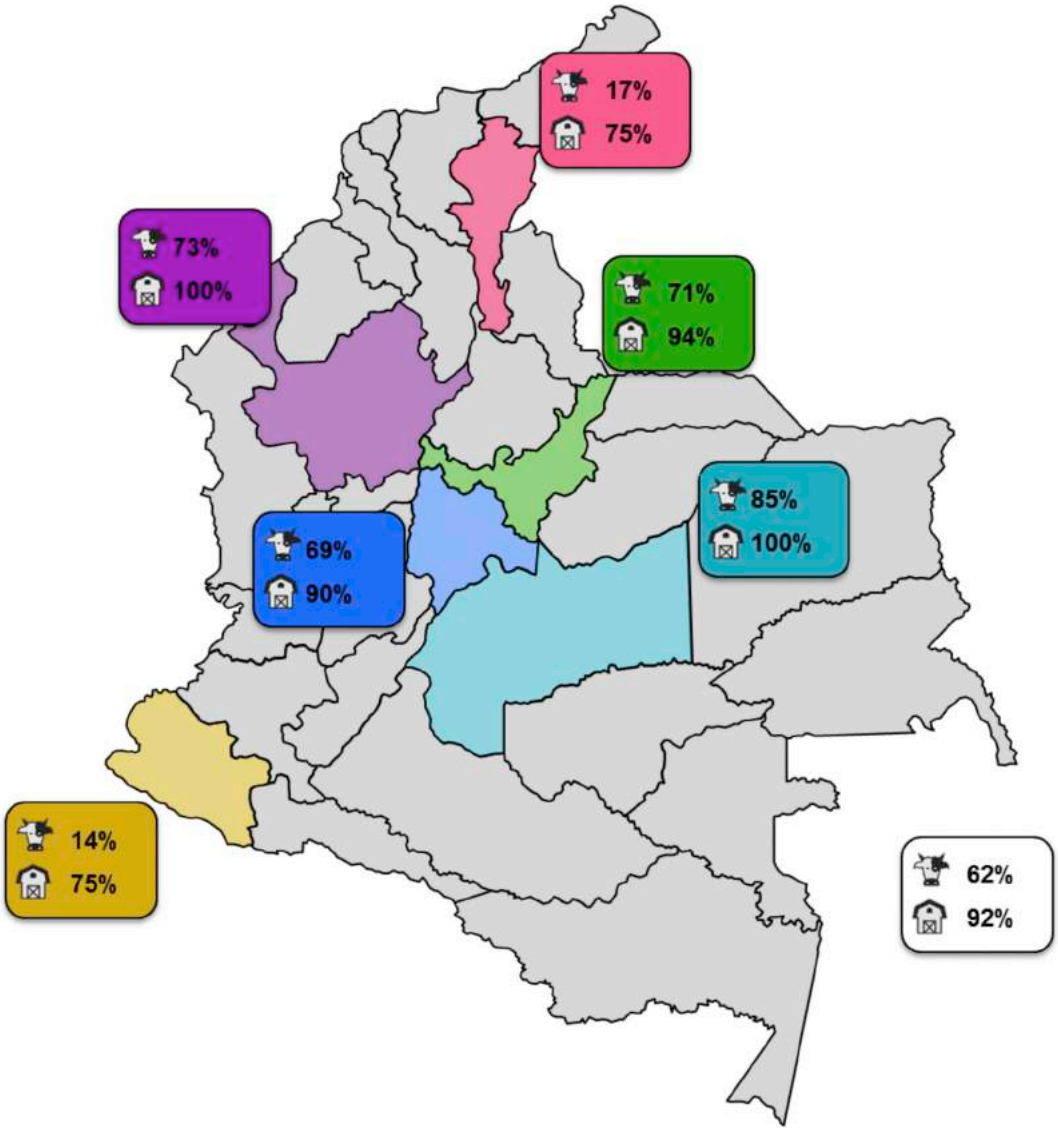

Fig. 1. Prevalence of BLV by detection of the *gag* and *tax* gene segments in the Colombian cattle population. Map of Colombia showing prevalence per animal and per farm, distributed in six regions of Colombia: Cundinamarca 69 and 90% (dark blue), Boyacá 71 and 94% (green), Antioquia 73 and 100% (purple), Meta 85 and 100% (turquoise), Nariño 14 and 75% (yellow) and Cesar 17 and 75% (pink), respectively. The overall prevalence of BLV in Colombia was 62% per animal and 92% per farm. The Colombian map was prepared by the authors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2013). The *gag* gene consists of 1183 nucleotides and encodes the capsid protein p24-CA, the *env* gene has a length of 1547 nucleotides and encodes the envelope glycoproteins, which are comprised by the extracellular portion (gp51) and the gp30 transmembrane region (gp30) (Corredor et al., 2018; Polat et al., 2016). gp51 is essential for recognition and entry of the virus into the host cell and is one of the most immunogenic viral proteins. The pX region has 3304 nucleotides and encodes the Tax protein, which fulfills regulatory functions

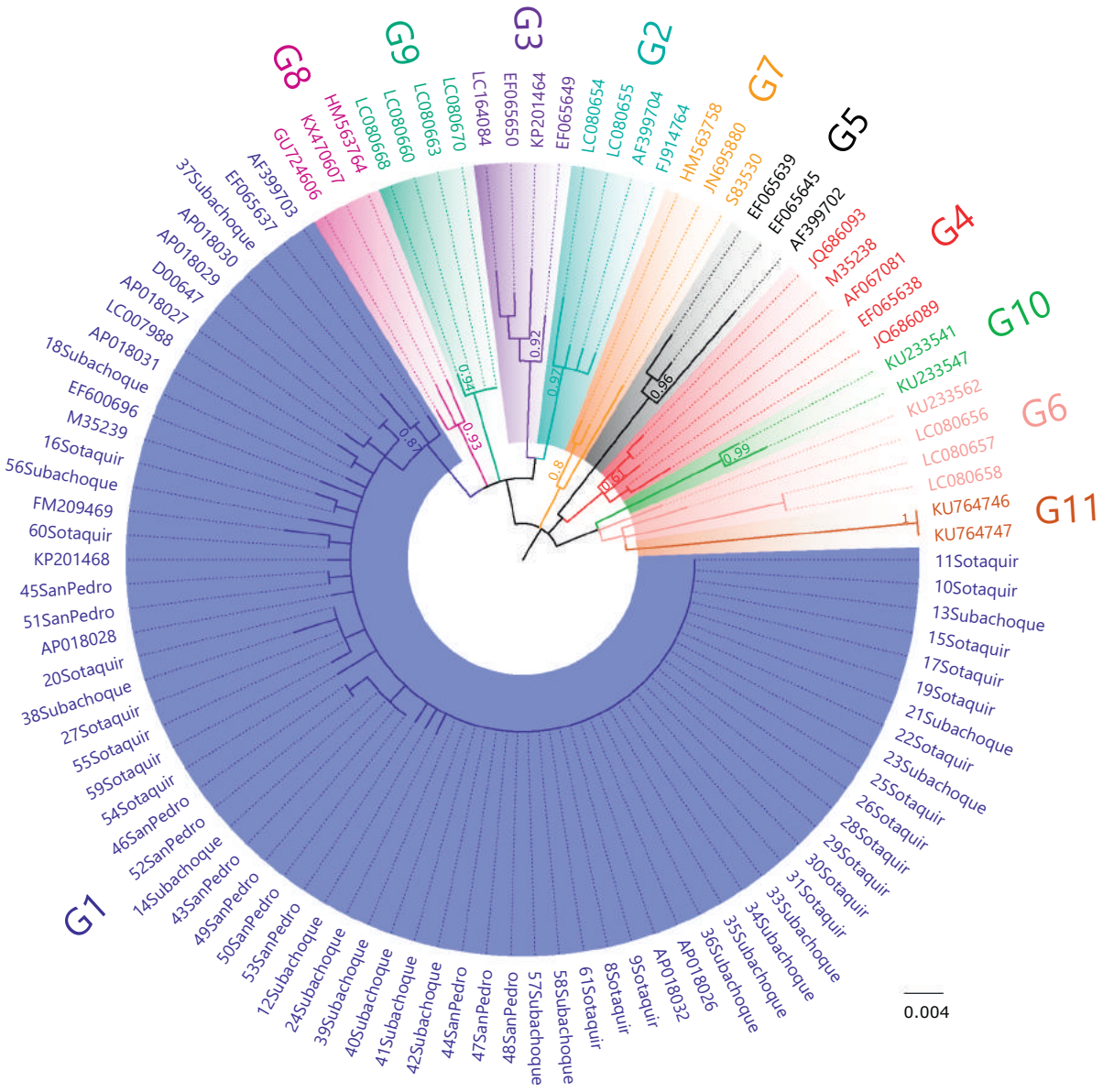

Fig. 2. Maximum-likelihood phylogenetic tree analysis of the *env* gene of BLV circulating in Colombia. The ML method based on the General Time Reversible model maximum composite likelihood (MCL) approach was used, and the topology with a superior log likelihood value was selected. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3005)). The rate variation model allowed some sites to be evolutionarily invariable ([+*I*], 39.13% sites). The analysis involved 100 nucleotide sequences. A total of 423 positions were included in the final dataset.

(Rosewick et al., 2013).

At present, 11 BLV genotypes have been reported in different regions of the world (Ababneh et al., 2012; Bazzucchi et al., 2019; Gautam et al., 2018; Lee et al., 2016; Ochirkhuu et al., 2016; Polat et al., 2017b, 2016; Yu et al., 2019). Frequently, BLV genotype detection is based on a 444 bp segment amplification, as indicated in the World Organization for Animal Health (OIE) manual for viral diagnosis (Fechner et al., 1997; OIE, 2018). Both in this document and in several others, Fechner primers are used, and they indicate that genotype 1 is the most prevalent worldwide (Lee et al., 2005; Polat et al., 2017b). However, the topology of some trees displays different clusters in the same genotype, leading to the question of whether the amplification of the 444 bp segment of the *env* region efficiently identifies all the current genotypes, or instead mimics the presence of other circulating genotypes (Buehring et al., 2014; Felmer et al., 2005; Ochirkhuu et al., 2016).

In Colombia there are very few studies on the seroprevalences of this virus and even less on prevalence and genotype. Consequently, the aim of this study was to identify the circulating genotype of BLV throughout the country in order to gain a more thorough understanding of the current situation of BLV and to reveal the possibility of masked genotypes that the primers used by OIE are unable to identify.

2. Materials and methods

2.1. Sample collection and DNA extraction

The samples were randomly collected, taking into consideration the areas with greatest bovine cattle production in the country, based on the bovine census carried out by the Colombian Agricultural Institute (ICA) in 2014, which counted 22,574,780 heads of cattle distributed among 495,072 properties. The number of samples to be studied was determined based on the sampling formula to estimate a proportion using the WinEpiscope tool available online (http://www.winepi.net/).

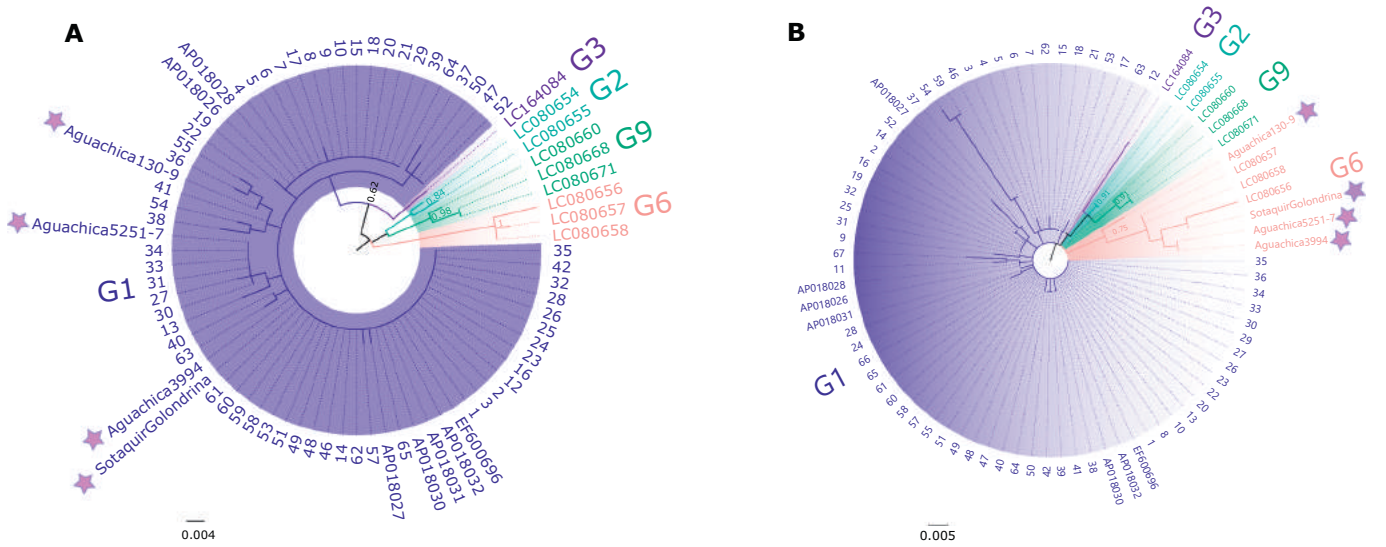

Fig. 3. Maximum-likelihood phylogenetic tree analysis of the *gag* and *tax* genes of BLV circulating in Colombia. The evolutionary history was inferred using the ML method based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; (+G, parameter = 0.1621) for *gag* and (+G, parameter = 0.1749) for *tax*)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 43.30% sites for *gag* and [+I], 42.83% sites for *tax*). A total of 306 and 314 positions were included in the final dataset for *gag* and *tax*, respectively. Fig. 3A, phylogenetic tree of the *gag* gene segments with 80 sequences: 64 Colombian and 16 reference sequences. Note that all Colombian samples are grouped into genotype 1, including the four G6 samples. Fig. 3B, phylogenetic tree constructed with the *tax* gene segments with 83 sequences: 67 Colombian and 16 reference sequence segments. Note that four Colombian samples are included in the genotype 6 cluster and are shown with an * both in *gag* and *tax* trees.

The sample size (n) was estimated using the 42% prevalence reported by (Ortiz et al., 2016), which yielded an n value of 289. Blood samples were taken from the coccygeal vein of healthy cows distributed throughout the country in 6 different regions, between 2015 and 2016. Mononuclear cells were separated from blood samples using density gradient centrifugation with LymphoSep (MP®); after recovering the buffy coat, total DNA was extracted in order to look for proviral DNA with the High Pure PCR Template Preparation Kit (Roche®).

2.2. PCR sensitivity tests

Previously amplified amplicons belonging to the segments of *gag* (381 bp) and *tax* (396 bp) genes of BLV were purified with a PCR Wizard kit (Promega®), followed by the insertion of each of them into the pELMO vector (Ramos et al., 2017) and transforming them into *E. coli* TOP10 cells (Invitrogen®). The plasmid DNA was used as an amplification template in the sensitivity tests. Serial dilutions of each plasmid were performed with initial concentrations of 230 ng/µl and 411 ng/µl for *gag* and *tax*, respectively. Subsequently, the respective PCR assays were performed at each dilution level to determine the detection limit of the technique corresponding to the maximum amplified dilution. For the *env* region, a segment of 801 bp was amplified as described above, but the sequence was cloned into the pEXP5-CT/TOPO (Invitrogen) vector with an initial concentration of 159 ng/µl.

2.3. Detection of BLV gene segments

Initially, the constitutive gene bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. It was amplified in order to ensure that the DNA was long enough to be able to contain the virus segments. The primers (5´CCT TCA TTG ACC TTC ACT ACA TGG TCT A 3′ and 5´ GCT GTA GCC AAA TTC ATT GTC GTA CCA 3′) were those reported by (Buehring et al., 2014) and the amplified segment was 857pb long. Multiple PCR was performed to amplify both the GAPDH and *gag* gene segments simultaneously. Afterwards, individual PCRs were performed to amplify the *gag, tax* and *env* genes. The PCR conditions and the primers used are found on the Table 1. For all the PCR assays described below, the PCR master mix (Roche®) was prepared according to the manufacturer's instructions. Plasmid DNA containing each gene segment (*gag, tax,* and *env*) was used as a positive control.

Once positive samples for BLV were identified by *gag* gene amplification, PCR was carried out to detect a 373 bp segment of the *tax* region and another PCR was done in order to detect the circulating genotype with a segment of 530 bp of the *env* gene. The newly designed pair of primers for *env* overlap with the region that was first amplified using Fechner's set of primers, because the Fechner primer amplified the region between nt 5107 and nt 5636, whereas the new set of primers amplified the segment between nt 4938 and nt 5688.

The amplicons obtained from the PCRs were purified with a High Pure PCR Product Purification kit (Roche®) according to the manufacturer's instructions. Subsequently, sequencing was performed by the Sanger sequencing service of Macrogen Korea, the coverage for each sequence obtained was 4× for *env* gene and 2× for *gag* and *tax* gene. The sequences obtained in this study were deposited in the GenBank database with accession numbers MH041897 to MH042017, MH057402 to MH057465 and MH057466 to MH057532 for *env, gag* and *tax*, respectively.

2.4. Phylogenetic analysis

In order to identify the circulating genotype of BLV, the sequences obtained in this study were compared to complete BLV genome sequences available in GenBank. 64 *gag* and 67 *tax* sequences from this study were compared to the 16 complete sequences of BLV reported in the GenBank. These 16 sequences are from different genotypes. For the *env* gene, in the first PCR with Fechner primers, 53 Colombian sequences were compared to 49 partial sequences reported in the Genbank, and for the second PCR with the new primers, 121 Colombian sequences were compared to 49 partial sequences, which included the first 11 BLV genotypes described. These GenBank sequences were randomly selected, but from different regions around the world.

The combined multiple alignment of all Colombian sequences was performed with the ClustalW program implemented in Mega 7.

Once aligned, the best evolutionary model that described our sequence data was assessed using the “Find Model” interface in the Mega7

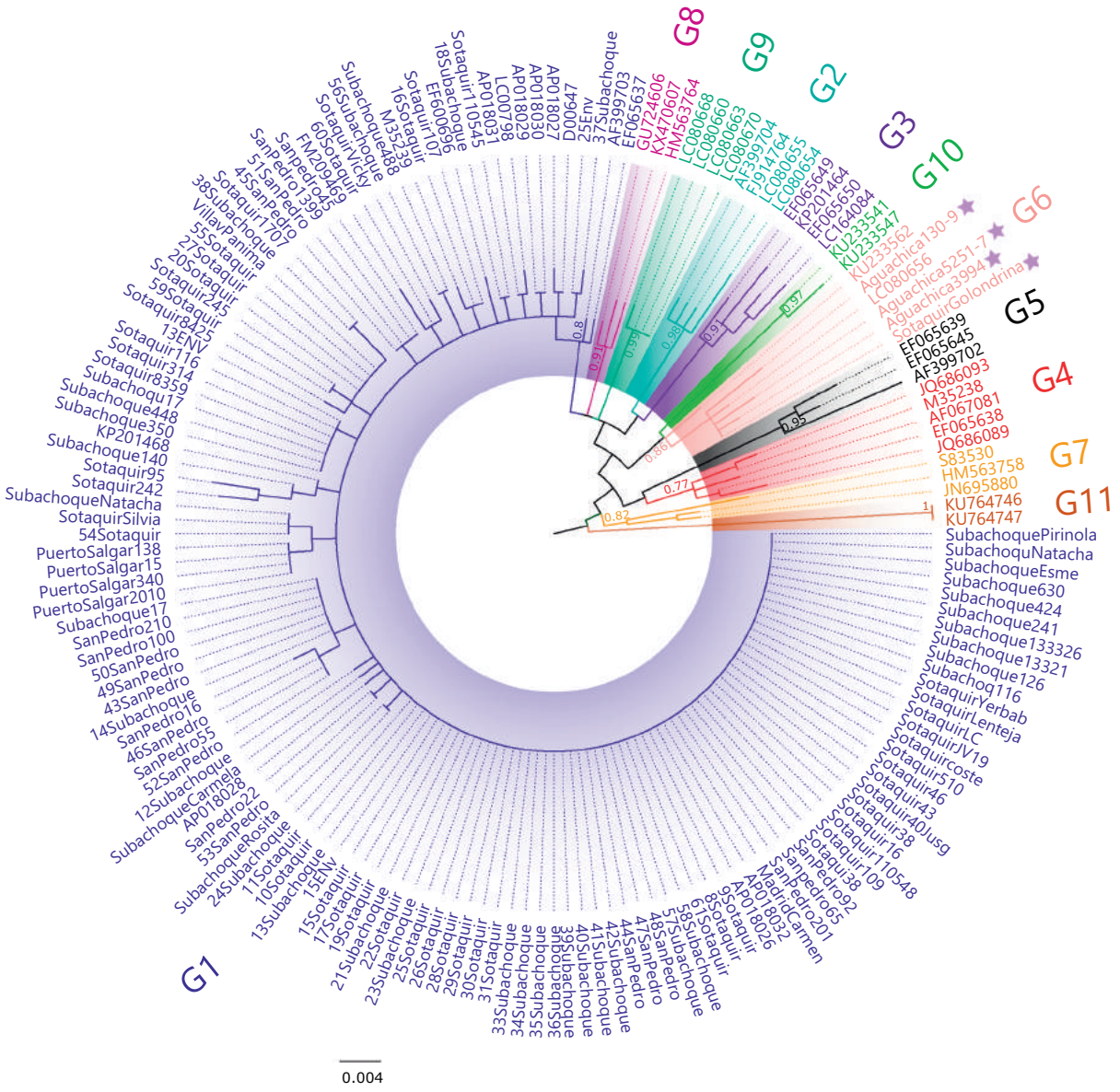

Fig. 4. Maximum-likelihood phylogenetic tree analysis of the *env* gene of BLV circulating in Colombia. The evolutionary history was inferred using the ML method based on the Tamura-Nei model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3588)). The analysis involved 168 nucleotide sequences, 124 Colombian and 44 reference sequences from Genbank. Genotype 6 samples are shown with an *.

package based on the Akaike information criterion (AIC). Using this model, maximum likelihood trees were constructed using the MEGA 7.0 software. As a measure of the robustness of each node, the bootstrapping method (1000 pseudo-replicates) was employed. The phylogenetic trees were edited using the FigTree program v1.4.1, which is available online (http://tree.bio.ed.ac.uk/software/figtree/).

For the *env* sequences, phylogenetic inference was also performed by maximum likelihood analysis using the RAxML program. Phylogenetic tree inference using maximum likelihood/rapid bootstrapping was run on XSEDE (RAxML - HPC2 on XSEDE (8.2.10) (Stamatakis, 2014) with 1000 bootstrap replicates using the MrBayes program (Huelsenbeck et al., 2015). The phylogenetic inference was performed in a Bayesian framework with GTR + G, two runs and three chains.

3. Results

3.1. Prevalence of BLV

In order to determine the prevalence of BLV in Colombia, *gag* and *tax* segments were amplified. All samples were positive for the GADPH gene and 179 out of 289 samples were positive for *gag* and *tax* segments. Consequently, the observed prevalence of BLV based on both gene segments was 62% per animal and 92% per farm. Across the country, this prevalence was distributed by department as follows: Cundinamarca 69 and 90%, Boyacá 71 and 94%, Antioquia 73 and 100%, Meta 85 and 100%, Nariño 14 and 75% and Cesar 17 and 75% (Fig. 1).

3.2. Viral genotype

To determine the circulating genotypes in Colombia, phylogenetic

analyses were performed. The *env* region of the BLV genome was amplified, but only 53 samples could be sequenced and phylogenetically analyzed, as shown in the phylogenetic tree in Fig. 2, where all these samples were grouped within the genotype 1 clade, with a support bootstrap value of 87% and the phylogenetic inference was performed in a Bayesian framework to confirm the ML analyses (data not shown).

For the *gag* and *tax* segment, amplification was achieved in the same 179 samples and the corresponding sequence was obtained only in 131 of them for both segments.

Even though *gag* and *tax* sequences are not used for genotyping, these sequences were studied, and for *gag* segment all the Colombian samples were grouped in the same clade, which corresponded to genotype 1 (Fig. 3A). However, for the *tax* region, four of the sequences (marked as Sotaquira Golondrina, Aguachica130–9, Aguachica 5251–7 and Aguachica3934) were clustered separately from the genotype 1 clade, together with genotype 6 representatives (Fig. 3B). These four sequences seemed to have phylogenetic discordances between *tax* and *env* regions. Therefore, a new set of primers was designed, considering the current genetic variability of BLV strains, as deduced by available sequences of all BLV genotypes reported in GenBank. With this new set of primers, a 750 bp region of the *env* gene was amplified, which was 244 nt longer than the sequence amplified by the set of primers currently used for genotyping purposes.

3.3. Evidence of circulation of genotype 6

To analyze a phylogenetic discordance between *env* and *tax* regions, the 131 samples that were positive for *gag* and *tax* segments were amplified with the new set of primers designed for the *env* gene. The results showed that the four samples that were grouped within genotype 6 by *tax* gene were also clustered together with genotype 6 strains when analyzed by this new *env* gene segment. Altogether, the analyses performed indicate the circulation of both BLV genotypes 1 and 6 in Colombia, with 117 out of 121 samples belonging to genotype 1, while the 4 remaining correspond to genotype 6 (Fig. 4).

4. Discussion

Molecular epidemiological studies of BLV worldwide, as well as in Colombia, enable the identification of circulating strains in specific regions, in order to propose and build public policies aimed at controlling and, in the future, eradicating this virus, which in addition to producing problems for cattle, has also been reported as a possible risk factor involved in human's pathologies (Buehring et al., 2017; 2015; Ceriani et al., 2018; Olaya et al., 2016; Schwingel et al., 2019). On the other hand, the variability of genotypes as well as results of structural and functional properties of its envelope proteins provide useful information for research focused on the production of vaccines using specific strains. In the case of Colombia, based on this study's results and previous reports (Benavides et al., 2017; Úsuga-Monroy et al., 2018), it will be appropriate to include conserved epitopes shared between genotypes 1,2,3 and 6. In addition, phylogenetic studies are important for understanding the geographical distribution of the virus, and thus identify conserved and hypervariable regions, including specific mutations that could be related to different levels of virulence or pathogenicity.

Currently, the OIE and others use primers reported by Fechner et al., 1997 (Lee et al., 2005; Polat et al., 2017a) to detect BLV circulating genotypes, and such primers were used initially in this study. However, when carrying out the phylogenetic analysis, all the Colombian isolates were grouped under genotype 1, a result that differs from the sequences obtained from other gene regions (*gag* and *tax*). With the *tax* region, 4 of the positive samples of BLV were grouped under genotype 6; the *tax* region is not usually used for genotyping, since it is considered a polymorphic region in retrovirus. This suggests that the primers reported by Fechner present disadvantages for identifying genotypes of the virus reported after 1996, and thus, a new set of primers was designed in the current study. The new primers were designed by bioinformatic analyses including 69 sequences of the complete genome of the virus available in the GenBank representing the genotypes reported to date, selecting a conserved region from among all the genotypes in the *env* gene. As a result, a fragment of 750 bp was amplified and sequenced, finding that, in fact, the Fechner primers did not enable the identification of other genotypes, and indicating that genotypes 1 and 6 are simultaneously circulating in Colombia (Fig. 4).

Even though the *tax* segment is not typically used for genotyping, its amplification provided good information, which led to extending the *env* segment, with the aim of having the four samples display a change of position in the tree. The finding that the *tax* sequences produce warnings regarding the genotypes should be addressed in future studies, in order to verify the hypothesis that the *tax* gene is useful for genotyping purposes. Even though the Fechner primers were not able to detect G6, when the amplification region is extended over the *env* segment, the resolution of the result was increased, enabling better determination of the genotypes, while ruling out recombination and coinfection effects.

In this study, finding of genotype 1 was expected because this is the most prevalent genotype worldwide. However, evidence of G6 in Colombia is a novel finding. Overall, G6 has been reported in four Asian countries (Philippines, Thailand, Jordan, and India) and five South American countries and in Italy (Argentina, Brazil, Bolivia, Peru and Paraguay) (Bazzucchi et al., 2019; Gautam et al., 2018; Polat et al., 2017a). Different genotypes distributed in the world and the emergence of new genotypes in specific areas, as reported here, suggests that importing and exporting processes in the cattle industry contribute to increased viral prevalence and virus diversity, which in the case of silent diseases such as enzootic bovine leukosis are transferred unnoticed between cattle from different regions. This suggests the necessity of establishing global policies of control and diagnosis.

One of the most relevant findings of this study was the detection of genotype 6 by *tax* region. So far, no other studies have used this ORF for genotyping, and there are few available sequences for this region in databases that are representative of all viral genotypes that would enable more in-depth studies in terms of comparing the relationship between *env* and *tax* genes for the effects of genotyping. Further studies are needed to confirm its application for genotyping, but at least this study's results suggest that this gene, in addition to the *env* region, could be useful for genotyping.

On the other hand, in addition to identifying the circulating genotypes, it is also important to discuss the prevalence reported in this study, which found a positive prevalence of 62% in animals and 92% of farms, which is one of the highest prevalence rates reported in Latin America. In Colombia, the latest report published by Ortiz et al. found a lower seroprevalence (42% per animal and 67% by farm) (Ortiz et al., 2016) compared to this study, using the same cattle population.

One of the main differences between the two studies lies in the diagnostic method. Here, prevalence was determined by PCR tests, which directly detect fragments of the viral genome, whereas the study by Ortiz et al. used the ELISA commercial kit, which detects antibodies in the host (Ortiz et al., 2016). Even if the presence of antibodies could be interpreted as presence of the virus, according to some authors the sensitivity of ELISA kits might be lower than the detection level of a PCR test (Lee et al., 2005; Trono et al., 2001). Now, in terms of antibodies production, it is possible that the amount of antibodies in sera samples could be below the detection limits of ELISA, which implies that false negative samples could be reported, especially in the case of viruses with slow replication rates such as BLV, giving as a result low levels of antibodies in blood due to immune response evasion (Frie and Coussens, 2015; Lee et al., 2005).

Even though there no records available on the importance of the cattle industry in our country and in Latin America, Ritcher et al. found that BLV has substantial effects on mortality, morbidity, premature

birth, culling, stillbirths, abortion and reinfection, which have a significant influence on the monetary level of direct losses (Richter et al., 2017). It would therefore be relevant for governmental entities and policymakers to consider adopting enzootic bovine leukosis as an official control for the disease, to facilitate the diagnosis of infected animals by members of the cattle industry, and furthermore to control the dissemination of the virus through the future promotion of eradication programs in order to minimize the impact that BLV has on cattle and eventually on humans who consume bovine-derived food products (Olaya-Galán et al., 2017).

Studies like this one are very important for science and for One Health approaches, in which interfaces between animals-humans and ecosystems should be considered (Kelly et al., 2016). In the case of BLV, it has been shown that the virus not only is present in cattle but also in other species such as sheep, buffalo, goats, alpacas and humans (Buehring et al., 2014; Mesa et al., 2013; Nekoei et al., 2015; Robinson et al., 2016). Although the effect BLV might have on humans is not yet clear, evidence of its presence has been reported, and it has been proposed as a potential risk factor for breast cancer development, although this hypothesis has been rejected by others (Barez et al., 2015; Gillet and Willems, 2016; Zhang et al., 2016). In addition, due to the fact that BLV is considered one of the main viral agents associated with economic impact in livestock production, which is distributed worldwide (Richter et al., 2017) and seems to have a zoonotic behavior, there are enough arguments to continue searching for vaccine candidates and strategies aimed at controlling viral infection by preventing risk factors within farms as well as the implementation of good livestock production practices for each specific region (Olaya-Galán et al., 2017; Olaya et al., 2016; Ortiz et al., 2016).

5. Conclusions

The epidemiological data provided here demonstrated a higher prevalence of BLV in Colombia compared to the seroprevalence previously reported. In addition, the findings contribute to the epidemiology of the virus by identifying genotype 1 and 6 in Colombia. Furthermore, the new primers that were designed in this study will be available for future studies willing to amplify all the current circulating genotypes, as an update of the proposal of Fechner et al. Finally, although *tax* is not commonly used for genotyping, phylogenetic analyses showed that this gene does contribute to the genotype identification and may be useful for BLV genotyping as well.

Ethics approval and consent to participate

This paper is part of the project called “Búsqueda y relación filogenética del virus de la leucosis bovina (BLV) en tejido mamario humano y en linfocitos de bovinos” financed by Colciencias and approved in virtual session of the ethics committee of Science Faculty of Pontificia Universidad Javeriana (June 9, 2014). For the blood samples, the informed consent was taken orally by two of the researchers, Alfredo Sanchez and Diego Ortiz, who were the veterinarians who visited the farms and spoke with the owners to request the samples. Additionally, through Pontificia Universidad Javeriana, the research team requested authorization for taking these samples from the Institutional Committee for the Care Use of Animals, (CICUAL, by its acronym in Spanish), which approved the veterinarians because of their status as VECOL staff members, who are responsible for taking samples and requesting informed consent from farm owners. The informed consent was taken at each farm where cattle was tested, with no objections noted.

Consent for publication

Not applicable.

Availability of data and materials

All relevant data are included in the paper.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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RESEARCH ARTICLE

In silico and *in vitro* analysis of boAP3d1 protein interaction with bovine leukaemia virus gp51

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Abstract

The envelope glycoprotein 51 (gp51) is essential for bovine leukaemia virus (BLV) entry to bovine B-lymphocytes. Although the bovine adaptor protein 3 complex subunit delta-1 (boAP3D1) has been proposed as the potential receptor, the specific ligand-receptor interaction has not yet been completely defined and boAP3D1 receptor and gp51 3D structures have not been determined. This study was thus aimed at a functional annotation of boAP3D1 cellular adaptor protein and BLV gp51 and, proposing a reliable model for gp51-AP3D1 interaction using bioinformatics tools. The boAP3D1 receptor interaction patterns were calculated based on models of boAP3D1 receptor and gp51 complexes' 3D structures, which were constructed using homology techniques and data-driven docking strategy. The results showed that the participation of 6 key amino acids (aa) on gp51 (Asn170, Trp127, His115, Ala97, Ser98 and Glu128) and 4 aa on AP3D1 (Lys925, Asp807, Asp695 and Arg800) was highly probable in the interaction between gp51 and BLVR domains. Three gp51 recombinant peptides were expressed and purified to validate these results: the complete domain (rgp51), the N-terminal portion (rNgp51) and the C-terminal fragment (rCgp51); and binding assays to Madin-Darby bovine kidney (MDBK) cells were then carried out with each recombinant. It was found that rNgp51 preferentially bound to MDBK cells, suggesting this domain's functional role during invasion. The rNgp51-MDBK cell interaction was sensitive to trypsin (98% reduction) and chymotrypsin treatment (80% reduction). These results highlighted that the N-terminal portion of gp51 interacted *in vitro* with the AP3D1 receptor and provides a plausible *in silico* interaction model.

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Introduction

The bovine leukaemia virus (BLV) is a retrovirus from the same genus as the human T-cell leukaemia-lymphoma virus (HTLV) [1], displaying tropism mainly to cattle B-lymphocytes [2], where it has been associated with a slow infection similar to human acquired immunodeficiency syndrome (AIDS), known as enzootic bovine leukosis. This disease is characterised by having no evident symptomatology in 65% of infected animals, causing persistent lymphocytosis in 30% of them and leukaemia or lymphoma in 5% to 10% [3–5].

The pertinent literature has reported that this virus infects cells other than B-lymphocytes and even cells from species other than cattle [6–10]; it has also been reported recently that it is present in women's mammary gland cells, suggesting the virus' association with breast cancer [11,12]. The cellular protein candidate for viral receptor, allowing virus entry to these cells, must be studied to advance understanding of how BLV can infect cells other than B-lymphocytes, such as human epithelial cells and sheep T-lymphocytes.

The BLV envelope (Env) protein, comprising a 51 kDa molecular weight surface (SU) domain (called gp51), a transmembrane (TM) domain (known as gp30) and a cytoplasmatic (CP) domain, has been involved in virus binding to and penetration of cells [13,14].

Two studies have been published to date referring to the BLV cell receptor. Two receptor (BLVR)-related clones (BLVcp1 and BLVcp1/5') were found in the first experimental approach, encoding a plasmatic membrane protein whose extracellular domain binds BLV gp51 and increased the susceptibility of cells to recombinant BLV infection [15,16]. A later study proposed that BLVR was related to the adaptor-related protein complex-3 (AP-3) which participates in intracellular protein transport [17]; the MDBK cell line was used for the experiments in both studies. There are currently 75 complete BLV genome sequences in GenBank; only two proteins, a capsid (CA) [18] and a transmembrane protein [19] have been resolved by crystallography. Using computational tools to understand the function of the proteins involved in binding is therefore an important step in resolving concerns about BLV biology.

Generally speaking, *in silico* approaches have been of key importance in assessing protein-protein interactions [20,21]; such methods were used here for identifying functionally important protein regions. Some *in vitro* approaches have been used regarding BLV to identify the cellular receptor [15–17]. The present study describes the functional annotation of BLV gp51 and boAP3D1 proteins and predicts their interaction (GenBank Accession No M35242.1. and No NP_776423). BLV Env and boAP3D1 protein tertiary structures were here modelled and analysed for identifying domains and binding sites and identify and functionally characterise infection pathway components which could lead to a better understanding of BLV pathogenesis and provide pharmacological targets.

Three gp51 recombinant proteins were constructed for determining their Madin-Darby bovine kidney (MDBK) cell binding capability, under the premise that AP3D1 is a cell membrane molecule present in these cells. This was done as a first validation of the *in silico* results which showed an interaction between gp51 and boAP3D1; these results were quite promising according to *in vitro* tests, opening the way forward for further studies aimed at clarifying the receptor involved in BLV infection and also solving gaps in tropism, pathogenesis and maybe identifying future vaccine targets.

Materials and methods

Computational analysis of primary structure

The BLV Env and AP3D1 proteins primary sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) accession numbers M35242.1 (FLK-BLV isolate used as BLV

reference strain), NP_776423.3 (AP3D1 bovine boAp3F1).ProtParam [22] was used for calculating protein physical-chemical properties, such as molecular weight, theoretical pI, aa composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

The ProtScale tool in the ExPASy server was used for boAP3D1 and Env protein aa scale representation (Kyte & Doolittle hydrophobicity scale) [23], having an aa scale defined by a numerical value assigned to each type of aa. The most frequently used scales are hydrophobicity or hydrophilicity scales and secondary structure conformational parameter scales; there are many other scales based on aa chemical and physical properties. The ProtScale tool provides 57 predefined scales entered from the literature [22].

Functional annotation

BLV Env and AP3D1 conserved domains were analysed by sequence similarity search with close orthologous family members available in various protein databases using the web-tools CDD-BLAST [24], INTERPROSCAN [25,26] and COGs [27,28] for this purpose. PROSITE [29] was used for identifying patterns and profiles.

Secondary structure prediction

The PDBSum [30] and CDD_BLAST servers [24] were used for computing and analysing protein sequence secondary structural features. The NSP server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html) gave average results from the consensus of 5 algorithms using two basic methods: probability parameters determined by relative frequencies and Bayesian probabilities. GlobPlot tools [31] were used for identifying boAPd1 and gp51 intrinsically disordered proteins (IDPs).

3D structure prediction

The Env and boAP3D1 proteins' 3D structure was predicted by using measures for each type of aa in local structural environments and defined in terms of solvent accessibility and protein secondary structure. Coat protein complex I (COPI) was used for Env; COPI is involved in traffic between the Golgi apparatus and the endoplasmic reticulum [32]. Several criteria were taken into account when selecting the template for modelling, such as crystal resolution, sequence similarity (% identity), conserved regions/domains and sequence coverage. Despite the 3D structure of the BLV Env transmembrane region being available, the main goal of the present study was to characterise the surface (SU) domain (gp51), since this is directly involved in the interaction with the cell receptor and thus, following the above-mentioned criteria, COPI turned out to be the best template choice.

The clathrin-associated AP2 adaptor complex was used for boAP3D1 as it plays roles in many vesicle trafficking pathways within cells [33]. COPI (PDB ID 5A1U) and AP2 adaptor complex (PDB ID 2VGL) crystal structures were the templates selected for obtaining the 3D structures of Env and boAP3D1, respectively. GROMOS96 force field (http://www.gromacs.org) [34] was used for quality and reliability assessment once the 3D model had been obtained and energy minimisation performed. Structural evaluation and stereochemical quality was evaluated.

Molecular docking simulations

Scripps Research Institute (http://www.scripps.edu/mb/olson/doc/autodock) Autodock software (v4.2) (Autodock, Autogrid, Autotors, Copyright- 1991e2000) was used for Env protein

and AP3D1 docking analysis. A searching grid extended over the selected target protein to delimitate the docking area was used to run Autodock. Polar hydrogens were added to ligand moieties, Kollman charges assigned and atomic solvation parameters added. Gasteiger polar hydrogen charges were assigned and nonpolar hydrogens were merged with the carbons; internal degrees of freedom and torsions were set. AP3D1 was docked with target protein, being this molecule considered a rigid body. Affinity maps for all atom types and an electrostatic map were computed (0.375 E grid spacing). The Lamarckian genetic algorithm selected in Autodock was used for the search.

Refinement and complex validation

MacroModel (softwarehttps://www.schrodinger.com/macromodel) was used for screening docking solutions for energy minimisation to avoid steric overlaps and clashes. A 0.05 kJ/A˚ -mol was set as convergence criterion for gradient minimisation for protein–protein complex and docking performance quality test.

Computing binding free energy

Distance-scaled, finite ideal-gas reference (DFIRE) state energy software [35] was used for assessing the complex's (Env and AP3D1) binding free energy and estimating binding affinity. PyMOL (the PyMOL Molecular Graphics System, version 2.0 Schrödinger, LCC) was used for polar contact assessment.

Mapping protein-protein interactions

PyMOL (the PyMOL Molecular Graphics System, version 2.0 Schrödinger, LCC) was used for visualising and mapping interactions between BLV Env and AP3D1 aa.

Obtaining recombinant protein gp51

BLV DNA was extracted from a blood sample collected from a serologically positive bovine. For this purpose, a LymphoSep density gradient (MP Biomedicals) was used for obtaining peripheral blood mononuclear cells (PBMCs). A High Pure PCR Template Preparation kit (Roche) was then used for extracting total DNA for obtaining proviral DNA, following the manufacturer's indications.

PCR amplification of the *gag* gene was used for confirming BLV presence in the sample, using previously reported primers [11]. The proviral DNA was then used as template in PCR reactions for which specific primers were designed for amplifying gp51 fragments from the FLK reference sequence deposited in the NCBI database (accession number M35242). Such regions were gp51 Nt aa 35–173 (Fwd `5' ATGAGATGCTCCCTGTCCCTAG 3'` and Rev `5' TAAAGAAAAGGTGATCAGGGG 3'`), gp51 Ct aa 173–301 (Fwd `5' ATGTTACATAAGATCC CTGATCCC` and Rev `5' ACGTCTGACCCGGGTAGG 3'`) and the complete gp51 (aa 35–301), using the gp51 Nt forward and gp51 Ct reverse primers. A Wizard PCR Clean-Up System kit (Promega) was used for purifying PCR products; amplicon quality was then evaluated on 1.5% agarose gels. The purified products were ligated into pEXP5-CT/TOPO expression vector (Invitrogen) and each recombinant construct was used for transforming *E. coli* TOP-10 cells (Invitrogen). Several recombinant clones were grown for plasmid DNA extraction with an UltraClean mini plasmid prep purification kit (MO BIO Laboratories). Insert integrity and correct orientation were confirmed by Sanger sequencing (Macrogen, Seoul, South Korea). ClustalW NPS software [36] was used for determining similarity between FLK reference strain *gp51* gene sequences and that isolated from bovine sera.

Once the *gp51* sequence was confirmed, the pEXP5-gp51 (complete, NT and CT) recombinant plasmids were transformed in *E. coli* BL21-DE3 cells (Invitrogen), following the manufacturer's recommendations. Once the cells had reached a 0.5 DO_{600}, 1 mM IPTG (Sigma-Aldrich) was added to induce molecule expression for 4h at room temperature with constant shaking at 250 rpm. *E. coli* BL21-DE3 bacteria were recovered by spinning and the cell pellet was used for extracting recombinant proteins in denaturing conditions.

After verifying expression by Western blot, all recombinant proteins were purified from whole cell lysate supernatants by affinity chromatography using Ni^{2+}-NTA resin (Qiagen). The mixture was left overnight at 4˚C and then passed through a chromatography column; exhaustive dialysis was carried out twice to obtain the three recombinant proteins in a functional conformation. The first was carried out inside the column before elution, using decreasing concentrations of urea buffer (3, 1.5, 0.75, 0.37 M in PBS 1X adding 1mM reduced glutathione, 0.1 mM oxidised glutathione). The fractions obtained after elution were dialysed with PBS 1X pH 7.2 for 72 h at 4˚C to eliminate remaining urea and enable proper recombinant refolding. This procedure has been described by Singh, S. *et al*., as being effective for obtaining a proper conformation and function for proteins expressed in *E. coli* and extracted as denatured protein [37]. We therefore think that the three fragments so obtained had the proper conformation and were functionally active; however, additional assays are required to confirm correct recombinant folding.

All the fractions collected were analysed by 12% SDS-PAGE and Western blot; those presenting just one band were dialysed in 1X PBS at pH 7.2. A BCA protein assay micro kit (Thermo scientific) was used for quantifying the proteins which were ultra-filtered and concentrated with Amicom Ultra-4 centrifugal filters (Merck Millipore).

Verifying protein expression involved separating purified recombinant proteins (rgp51, rNgp51 and rCgp51) (10 μg) by 12% SDS-PAGE and then transferring it to a nitrocellulose membrane and incubating with a peroxidase conjugated (1:4,500) monoclonal anti-histidine antibody (A7058, Sigma-Aldrich) recognising these recombinant proteins' histidine tail. The membranes were revealed with a peroxidase substrate kit (Vector Laboratories), according to the manufacturer's recommendations. The proteins' molecular masses were determined by linear regression using the XL-OptiProtein (New England Biolabs) molecular mass marker as reference.

Evaluating recombinant protein capability to bind to MDBK cells

MDBK (ATCC, #CCL-22 derived from bovine kidney) cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Sigma, D5523) containing L-glutamine and 1,000 mg/L glucose, supplemented with 3.7 gm/L sodium bicarbonate and 10% foetal calf serum.

The next step involved radiolabelling 15μg complete (rgp51) or N-terminal (rNgp51) or Carboxy-terminal (rCgp51) recombinant proteins with 4 μL $Na^{125}I$ (100 cpm/mL; ARC) and Iodination Beads (Pierce-Thermo Scientific), following the manufacturer's instructions. Following 12 min of incubation, radiolabelled recombinant protein was separated by size-exclusion chromatography on a Sephadex G-25 column (Pharmacia). Each eluted fraction was then analysed by gamma counter (Packard Cobra II).

Binding assays involved 1.2×10^6 MDKB cells being incubated with 150 and 300 nM concentrations of each radiolabelled recombinant protein at room temperature for 90 min in the absence (total binding) or presence (non-specific binding) of 13μM of the same unlabelled recombinant protein. The cells were spun through a 60:40 dioctyl phthalate -dibutyl phthalate cushion (1.015 g/ml density, 10,200 x *g* for 1.5 min) and a gamma counter (Packard Cobra II) was used for quantifying cell-associated radioactivity.

Each recombinant protein's binding activity was also evaluated in a binding assay with enzyme-treated cells. Briefly, cells were independently treated with 1mg/mL trypsin (Sigma T-1005) or 1 mg/mL chymotrypsin (Sigma C-4129) for 60 min at 37˚C. Following incubation, enzyme-treated cells were washed twice with HBS buffer and used in a typical binding assay. Untreated cells were used as positive binding control.

Results

Computational analysis of primary structure

Table 1 gives boAP3D1, BLV Env and recombinant proteins' physicochemical properties. The Env protein consists of 515 residues, 33 of which form part of the signal peptide, 36 are positively charged and 33 negatively charged. The boAP3D1 protein has 1,207 residues; 173 of them are positively charged and 179 negatively charged. Grand average of hydropathy (GRAVY) was also calculated, thereby determining that the proteins were hydrophilic as the resulting value was negative, favouring protein solubility in water. Fig 1 shows Kyte & Doolittle hydrophobicity for boAP3D1 and gp51 proteins. The physicochemical properties of the three recombinant proteins (rgp51, rNgp51 and rCgp51) are also described.

Aligning bovine and human AP3D1 protein sequences with ClustalOmega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed 15 differences in the BLVR domain whilst no differences between human and bovine peptide were found in the adaptin domain. The red line in Fig 2 represents the BLVR domain.

Functional annotation

The BLV Env protein is 515 aa long; it is located on cell membrane and has three domains. The extracellularly-located initial domain consists of a 33 aa-long signal peptide followed by an extracellular region from aa 34 to 438 (SU or gp51). The intermediate portion is a transmembrane region from aa 439 to 460 (TM or gp30) and the final portion is a cytoplasmic region from aa 461 to 515. Fourteen aa (48, 50, 77, 98, 99, 112, 113, 120, 122, 135, 136, 186, 187, 292 and 298) which can be considered binding sites are found all along the SU. Fig 3A shows the 14 binding aa in yellow and the 9 glycosylation sites (aa 129, 203, 230, 251, 256, 271, 287, 351 and 398).

The 1,207 aa-long boAP3D1 protein has two domains: the adaptin domain (residues 32–583) and the BLVR domain (residues 661–807) which is a disorganised region, like others all along the protein. Fig 3B shows the scheme for this protein, having 2 binding sites in positions

Table 1. boAP3D1, BLV Env and recombinant proteins physicochemical properties.

Property	Value				
	boAP3D1	BLV Env	rgp51	rNgp51	rCgp51
Amount of aa	1,207	515	268	140	129
Molecular weight KDa	136	54	30	16	14
Theoretical pI	6.65	8.19	7.74	7.10	8.06
Total amount of negatively charged residues (Asp+Glu)	179	33	19	12	7
Total amount of positively charged residues (Arg+Lys)	173	36	20	12	8
Ext. coefficient M−1 cm−1	75,845	120,735	74,410	39,795	34,615
Instability index (all are unstable protein)	49.79	49.17	51.57	45.22	58.02
Aliphatic index	88.86	95.93	74.57	65.32	86.98
Grand average of hydropathicity (GRAVY)	-0.490	-0.092	-0.346	-0.458	-0.194

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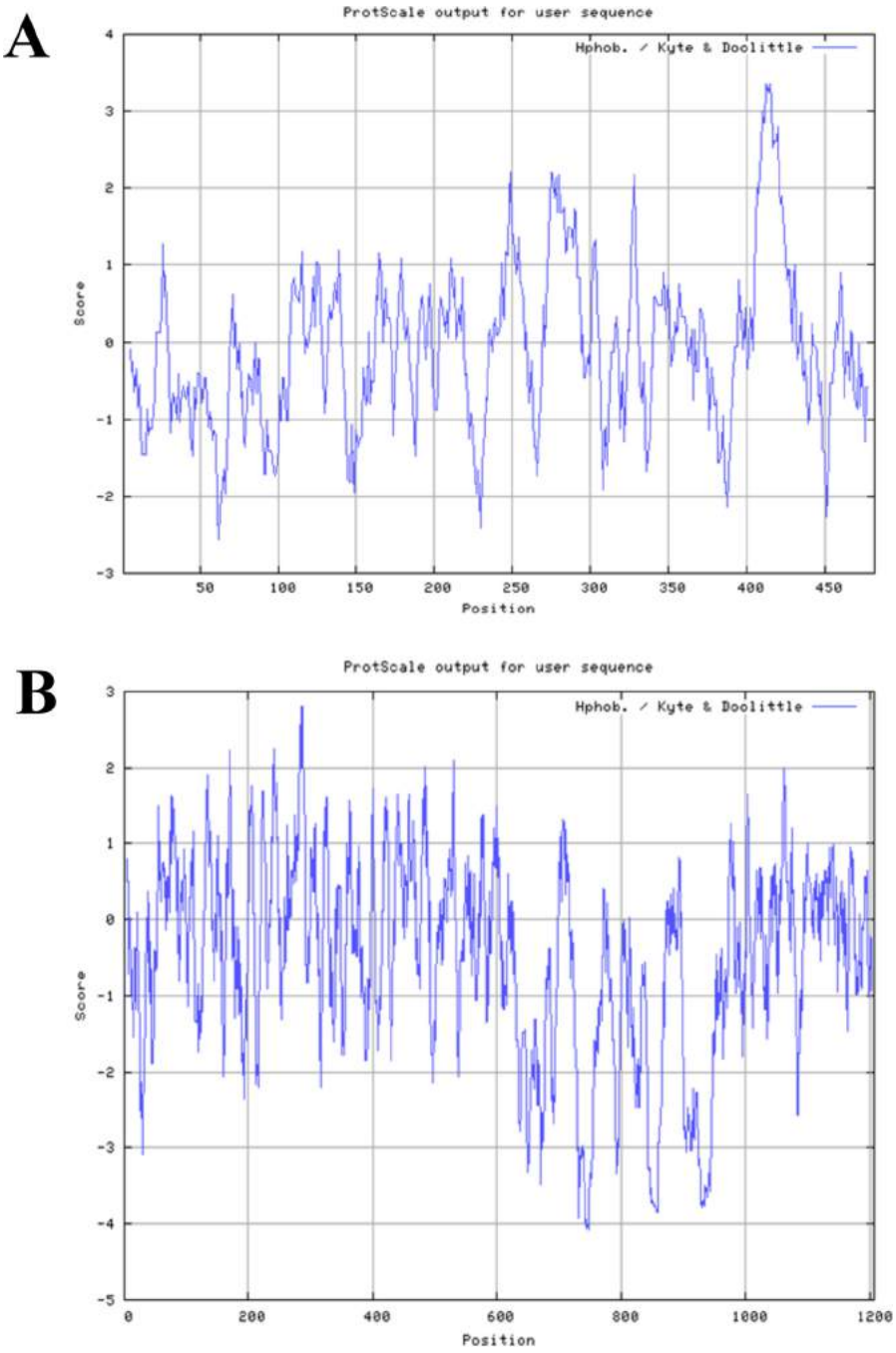

Fig 1. Kyte & Doolittle hydrophobicity for BLV Env (A) and boAP3D1 (B) proteins. Despite differences regarding the amount of aa in both proteins, their physicochemical characteristics were comparable.

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1 and 474 and 16 conserved sites in the BLVR domain (D661, E662, S686, S688, L726, E728, E729, D739, D767, E779, E783, E785, E786, S788, D797 and A801).

Secondary structure prediction

The BLV envelop protein's secondary structure consists of 37 α-helices, 41 helix-helix interaction regions, 77 β-turns and 22 γ-turns. The boAP3D1 protein has 3 β-sheets, 3 β-hairpins, 2

Fig 2. ClustalOmega sequence alignment of boAP3D1 and huAP3D1. "-" represents identical aa, the red line represents the BLVR domain.

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β-bulges, 7 strands, 4 α-helices, 2 helix-helix interaction regions, 15 β-turns and 2 γ-turns. The boAP3D1 protein has 10 disordered regions in positions 3–8, 39–64, 80–99, 139–157, 181–189, 200–208, 223–228, 255–264, 443–456 and 472–482 and a globular domain between aa 158 to 442.

The AP3D1 intrinsically disordered protein (IDPs) is distributed in 12 regions in positions 120–128, 190–198, 632–637, 680, 695, 714–718, 784–802, 873–878, 943–947, 964–970, 1033–1051 and 1182–1190 and four globular domains between aa 1–679, 696–783, 803–1032 and 1052–1207.

Analysis performed with the PROSITE [29] database predicted that the boAP3D1 gp51 binding domain would be located between aa 660 and 807 (Fig 3B, shown in red).

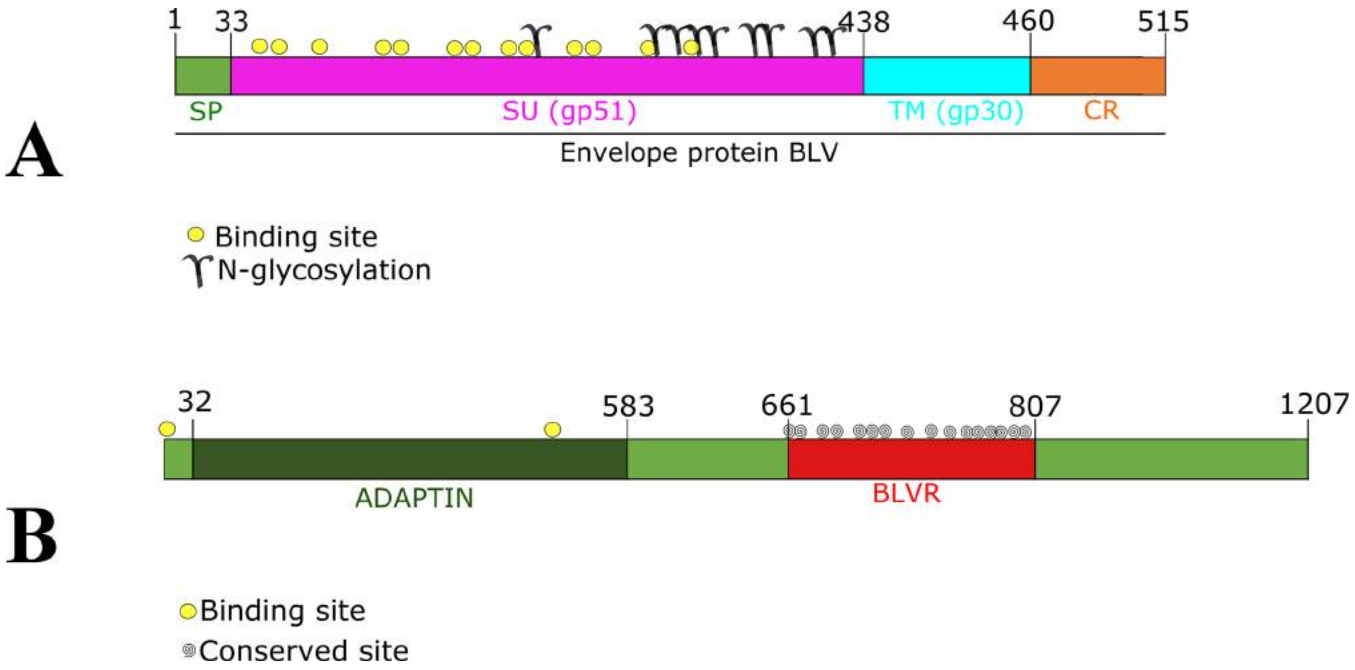

Fig 3. Schematic representation of Env BLV and boAP3D1 proteins. 3A. BLV Env protein. Signal peptide 1 to 34, SU (gp51) 34 to 438, TM (gp30) 439 to 460 and cytoplasmic region 461 to 515. Binding sites are shown by yellow circles (48, 50, 77, 98, 99, 112, 113, 120, 122, 135, 136, 187, 292 and 298), N-glycosylation sites (129, 203, 230, 251, 256, 271, 287, 351 and 398). 3B. boAP3D1 protein has 1,207 aa with two domains: one from aa 32 to 583 for adaptin (dark green) and a second (BLVR) from aa 661 to 807 (red). AP3D1 has 2 binding sites in positions 1 and 474 (yellow dots) and 16 conserved sites in the BLVR domain (D661, E662, S686, S688, K726, E728, E729, K739, D767, E779, E783, A785, L786, S788, D797 and A801).

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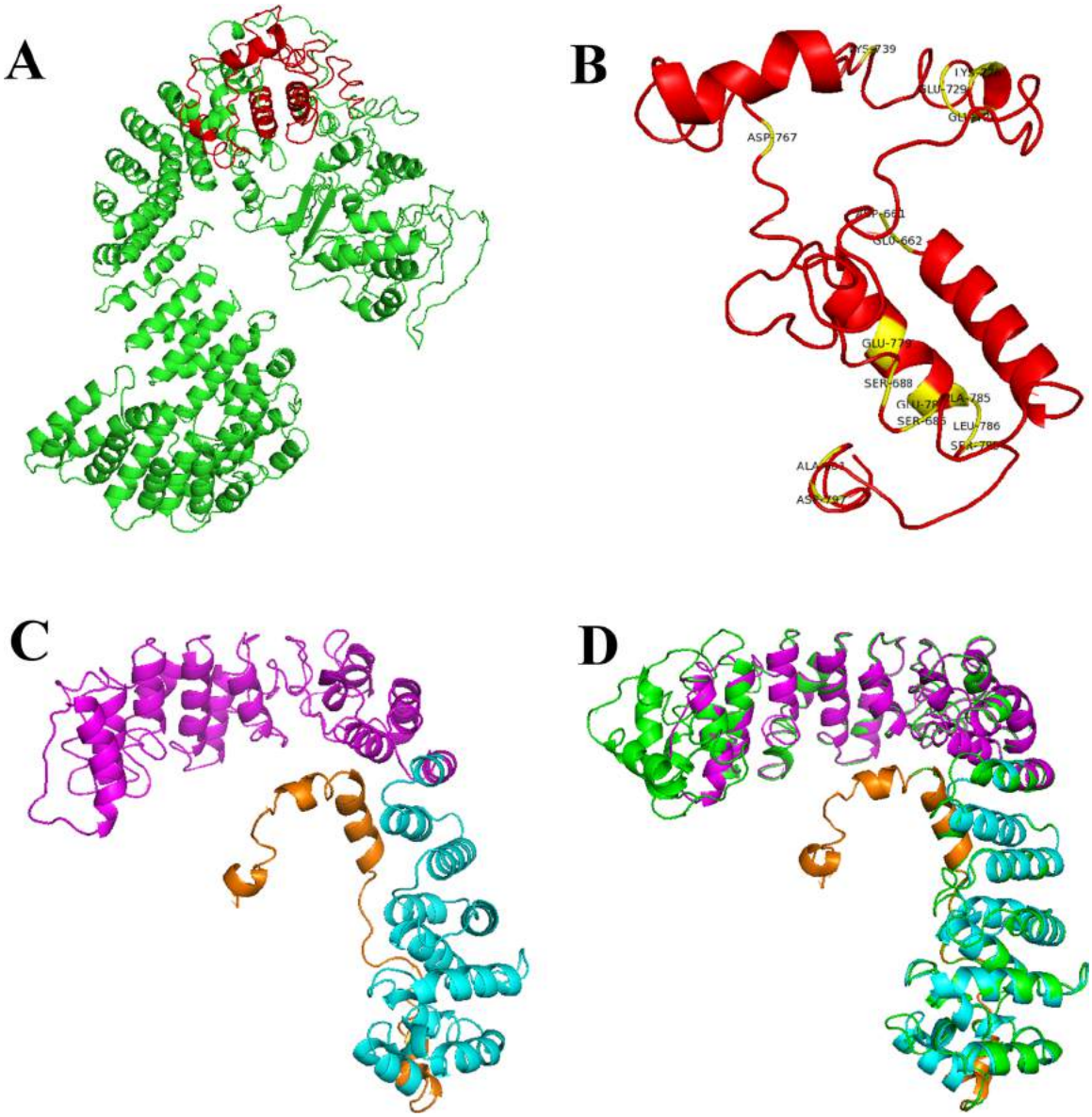

Fig 4. Modelling boAP3D1 and Env proteins. 4A. boAP3D1 structure is coloured green, and in red the BLVR domain. 4B. The BLVR domain (red) with conserved sites highlighted in yellow (D661, E662, S686, S688, K726, E728, E729, K739, D767, E779, E783, A785, L786, S788, D797 and A801). 4C. BLV Env protein structure predicted by I-TASSER, gp51 (SU) is shown in magenta, gp30 (TM) in cyan and cytoplasmic domain in orange. 4D. The COPI coat triad structure (5A1U in green) overlaps with our BLV Env protein model.

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3D models predicted for env and boAP3d1

Fig 4 shows the predicted model for both proteins; Fig 4A shows boAP3D1 structure where green represents the binding domain (BLVr) and Fig 4B shows this domain in red and conserved areas in yellow (i.e. aa D661, E662, S686, S688, K726, E728, E729, k739, D767, E779, E783, A785, L786, S788, D797 and A801). Fig 4C shows Env 3D structure; magenta represents the gp51 domain (SU), cyan gp30 (TM), orange the cytoplasmic domain and yellow conserved residues A15, Y17, R44, R65, R66, E78, P79, D87, F89, Q102, G103, Q153, L154, S259 and R265.

The predicted model for the Env protein by Ramachandran plot [38] showed that 68.4% of the residues were located in the most favoured regions, 24.6% residues in additional allowed regions, 2.6% residues in generously allowed regions and 4.3% residues in disallowed regions. DFIRE was -607.58, reflecting the model's quality; lower energy would have indicated that the model was closer to the native conformation. A perfect structure overlap with 5A1U was shown in this study, suggesting the model's high quality (Fig 4D).

Molecular docking simulations, complex refinement and validation and binding free energy computation

PyMOL was used for visualising molecular docking between gp51 and boAP3D1 (Fig 5). The part of the boAP3D1 protein making direct contact with gp51 was the BLVR domain (Fig 5A, red). The BLV-gp51 residues interacted with the proposed receptor (boAP3D1). The interface areas (A^2) were 1,495 for boAP3D1 and 1,443 for gp51. This model obtained a score of -131.7, 14 cluster size, 23.6 RSMD for the overall lowest-energy structure, -50.4 Van der Waals energy, -470.4 electrostatic energy, -27.7 solvation energy restraints, 405.2 violation energy, 2,120.2 buried surface area and -1.9 Z-Score.

Protein-protein interaction prediction

Possible interactions in the complex regarding Asn170 and Lys925 (Fig 5B), Trp127 and Asp807 (Fig 5C), His115 and Asp695 (Fig 5D), Ala97, Ser98, Glu128 and Arg800 participation in boAP3D1 (BLVR domain) and Env (gp51 domain) were analysed (Fig 5E). Table 2 gives the values calculated for binding free energy between these aa, i.e. 4 salt bridge interactions, 14 hydrogen bonds and 167 no direct contact points.

An additional *in silico* test showed the effect of mutating the predicted interaction residues in the binding energies between boAP3D1 and gp51. Two experimentally testable predictions were thus made; mutations leading to drastic chemical shifts were made in gp51 amino acids predicted as being crucial in binding to boAP3D1 (His115, Glu128 and Asn170 were replaced by Ala) and ClustalOmega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for multiple sequence alignment to assess whether the predicted interaction residues were conserved amongst the 10 BLV gp51 genotypes reported so far.

As a result, binding energies' native spectrum changed (-12.9 to -9.8 ΔG (Kcal mol^{-1}), with a difference of 3.1). This could have led to decreased stability and loss of interaction. On the other hand, key amino acids in the interaction found here were highly conserved amongst different BLV genotypes (highlighted in yellow in Fig 6).

Evaluating recombinant proteins' MDBK cell binding capability

gp51 contains 9 N-glycosylation sites, most of them towards the C-terminus. Although the most appropriate expression system for expressing a glycoprotein would have been a eukaryotic one, previous studies by Rizzo *et al*., published in 2016 [39] have shown that gp51 interaction with its receptor was mediated by specific domains but sugars did not play any role in such interaction, as syncytia formation with maintenance of BLV particle infectivity remained when N-glycosylation sites of gp51 were mutated. A prokaryotic system was thus used here, taking its advantages into account, in terms of cost and ease of use.

Fig 7 shows gp51 recombinant fragments' purification. Western blot detection is shown with Coomassie blue stained anti-his tag monoclonal antibody, i.e. the expected weight and purity of each recombinant fragment.

Binding assays determined rgp51 protein binding to MDBK cells. It was found that rgp51 had higher MDBK cell binding at low protein concentration (black bars) (Fig 8A); however,

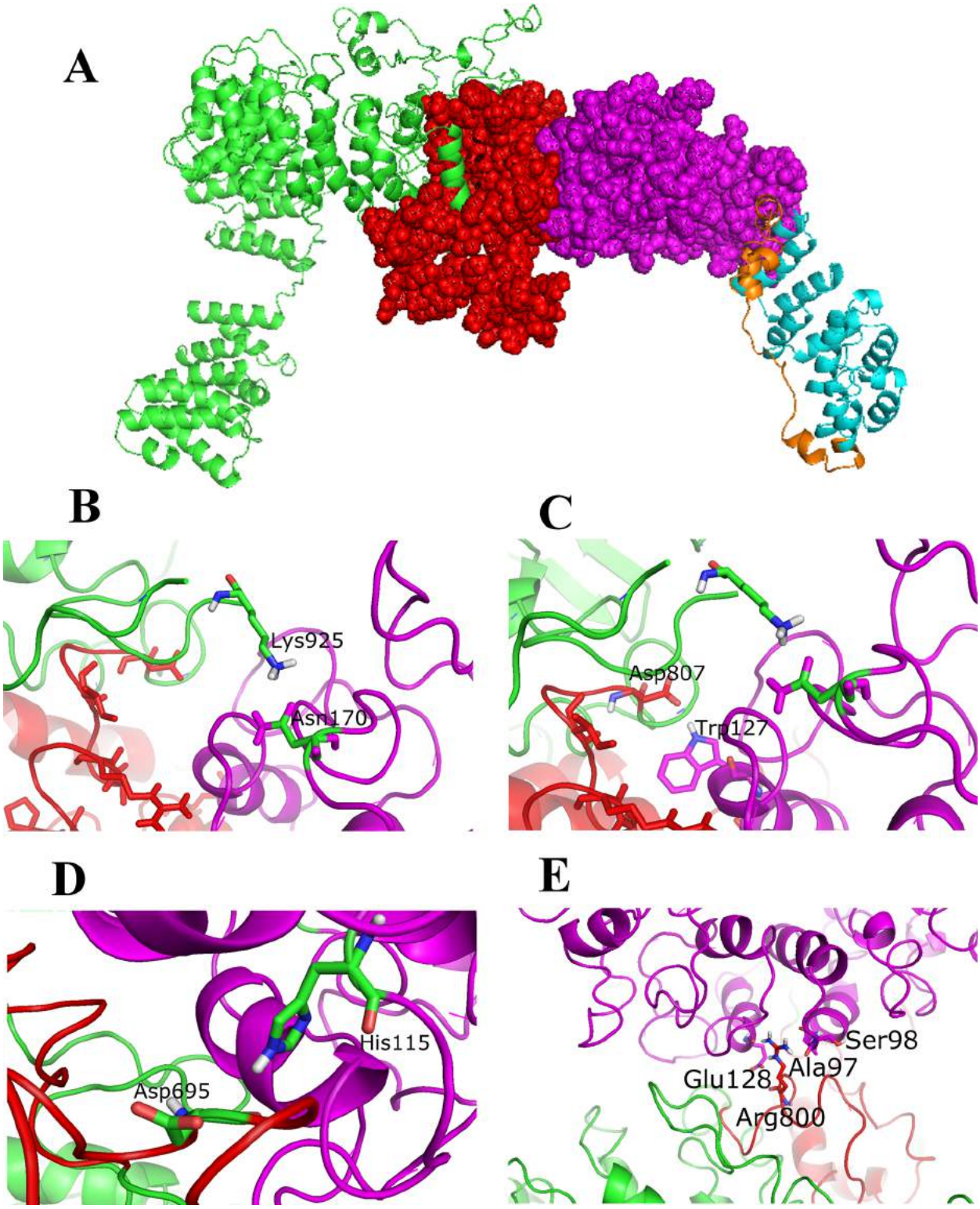

Fig 5. boAP3D1 and gp51 docking. 5A. Overview. 5B. Asn170 (gp51) and Lys925 (boAP3D1). 5C. Trp127 (gp51) and Asp807 (boAP3D1). 5D. His115 (gp51) and Asp695 (boAP3D1), hydrogen bonds, non-bonded contacts. 5E. Ala97, Ser98, Glu128 (gp51) and Arg800 (boAP3D1), hydrogen bonds, non-bonded contacts and salt bridges.

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Table 2. Binding free energy results for gp51 and boAP3D1 interaction.

gp51	AP3D1	Hydrogen bonded	Non-bonded contacts	Salt bridges
		Distance (A)		
Asn170	Lys925	2.8	3.69	-
Trp127	Asp807	2.89	3.77	-
His115	Asp695	2.90	3.46	-
Ala97	Arg800	2.76	3.30	-
Ser98		3.20	3.20	-
Glu128		2.97	2.97	2.97

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when protein concentration was increased (blue bars), a clear preferential binding was observed for rNgp51, whilst rgp51 and rCgp51 interaction remained the same.

Taking into account that binding experiments showed N-terminal fragment (rNgp51) preferential binding to MDBK cells, further experiments only involved this recombinant; furthermore, *in silico* analysis had also shown that the key interacting residues (Ala97, Ser98, His115, Trp127, Glu128 and Asn170) all lay within such fragment (aa 35 to 173). The gp51 N-terminal domain binding to MDBK cells was thus concentration dependent, suggesting this domain's functional role during invasion; rNgp51-MDBK cell interaction was sensitive to trypsin and chymotrypsin treatment, binding becoming reduced by 98% and 80%, respectively (Fig 8B).

Discussion

Bovine enzootic leukosis (LBE) is an infection affecting cattle and seems to have been restricted to such species to date [2]; however, there is evidence of it passing to humans, and some authors have proposed a relationship with breast cancer in women [11,12,40,41]. The possibility of a zoonosis is supported by the virus appearing in milk and meat from cows proving seropositive for BLV which could be acting as vectors of viral transmission to humans [42]. The possibility of being a zoonotic virus and its impact on public health mean that studies are needed in the search for the viral receptor enabling infection in two genetically distinct species (i.e. cattle and humans).

Studies *in silico* (like that described in this article) are necessary as a basis for initiating experimental studies verifying gp51 interaction with AP3D1, complementing existing information about BLV's cellular receptor to achieve infection.

According to Env primary and secondary sequence characterisation, the primary sequence is extremely conserved amongst different BLV strains (0.0 e-value) (data not shown). High sequence identity with human (88%), mouse (88%), sheep (99%) and goat (99%) sequences was found for boAP3D1 (0.0 e-value), suggesting a common ancestor regarding this protein.

PSI-BLAST predicted 88% identity between bovine and human protein for AP3D1 and 99% with sheep and goat protein (data not shown). Aligning bovine and human AP3D1 protein sequences with ClustalOmega software (https://www.ebi.ac.uk/Tools/msa/clustalo/)

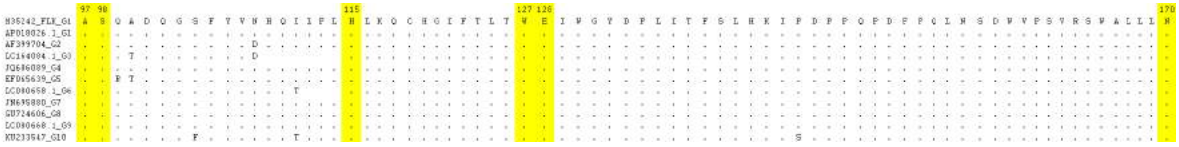

Fig 6. ClustalOmega sequence alignment of different BLV gp51 sequences (genotypes 1 to 10). "-" represents identical aa. The six key amino acids in the interaction are shown in yellow (Ala97, Ser98, His115, Trp127, Glu128 and Asn170).

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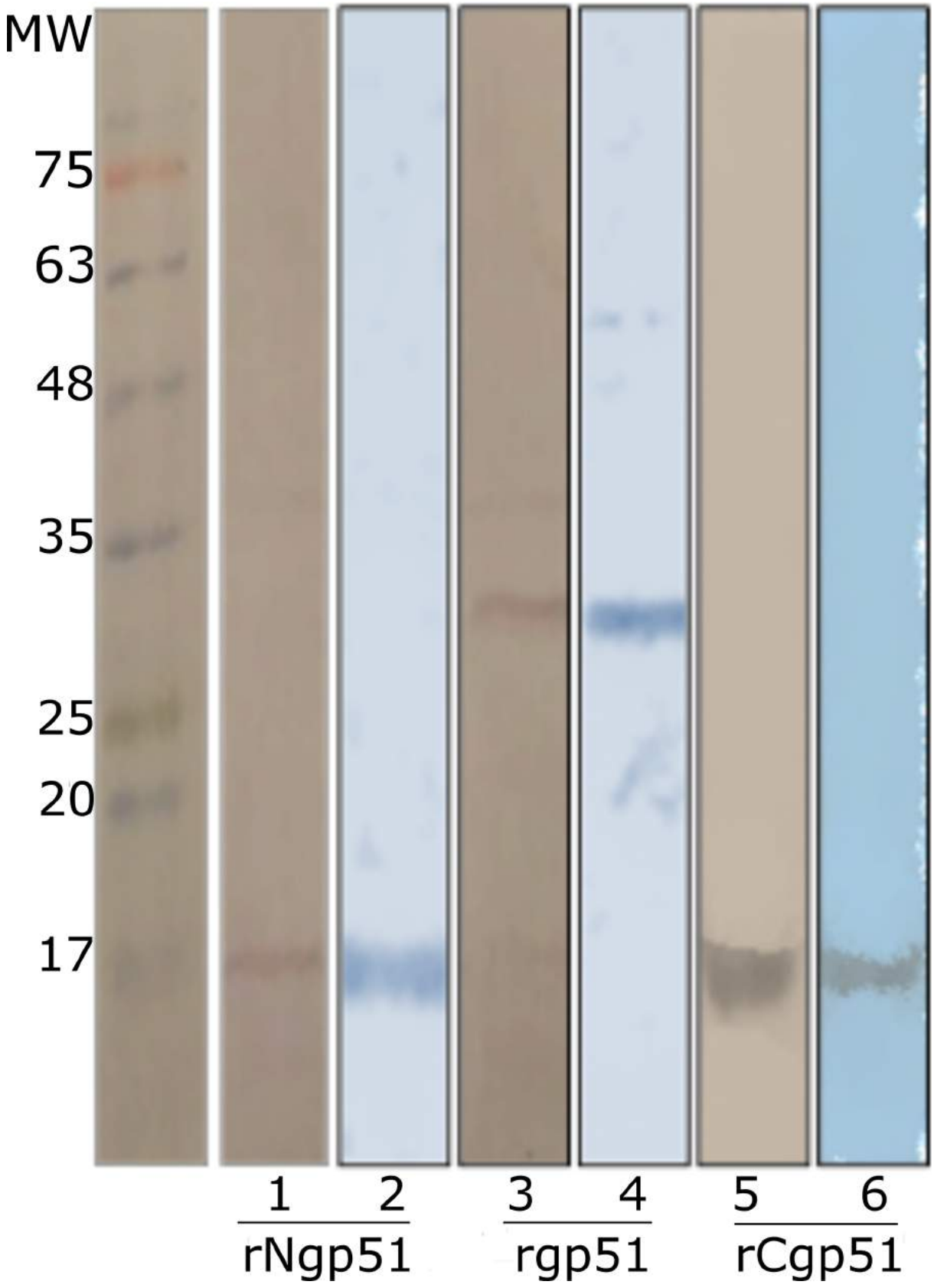
MW
75
63
48
35
25
20
17
1
2
3
4
5
6
rNgp51
rgp51
rCgp51

Fig 7. gp51 recombinant fragment purification. Lanes 1, 3 and 5 show Western blot detection with an anti-his monoclonal antibody. Lanes 2, 4 and 6 show Coomassie blue stained purified recombinant proteins. The proteins' molecular weight marker is indicated in the first lane (molecular masses for the three recombinants agreed with expected ones: 30, 16 and 14 kDa for rgp51, rNgp51 and rCgp51, respectively).

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revealed 15 differences in the BLVR domain whilst no differences between human and bovine peptide were found in the adaptin domain. The red line in Fig 2 represents the BLVR domain. (Fig 2); however, docking analysis (Fig 5) showed that they were not involved in the interaction with gp51 (Fig 2, in red). Most substitutions were conservative and just one of them was drastic (between Ala and Thr); such residues have different chemical properties, but such position was not predicted to be relevant for the interaction.

Properties supposed from the proteins' tentative functions (i.e. homology, main domains, structural similarities and physical-chemical characterisation inferred by the predictions) provide useful information and should be verified experimentally.

The boAP3D1 envelope protein binding region and BLV entry to target cells requires virus-encoded glycoprotein gp51 to interact with cell receptors to facilitate virus entry. It has been proposed that gp51 plays an important role in virus entry to target cells during the viral cycle [43]. The protein's most exposed aa should interact with specific receptors on target cells. A previous study reported that a protein similar to AP3D1 enabled BLV fusion and entry, but no specific boAP3D1 binding regions were established [15–17]. Regarding BLV and boAP3D1 docking assay results, it was found here that the AP3D1 interacting region with gp51 (located from aa 660–803) was the same region called the BLVR domain in previous studies (Figs 3 and 5).

Furthermore, considering BLV Env proteins, it has been shown that a region mediating interaction with a tentative receptor is located in gp51 or SU domain between aa 83–158. Some receptor binding domains (RBDs), zinc ion linker and binding and glycosylation sites have previously been described in this specific region [13] (Fig 3A). These regions could thus be crucial for a first interaction between host and viral proteins thereby mediating viral attachment. Fig 5 shows the most relevant interactions between both proteins' specific aa as follows: Asn170-Lys925 (5B), Trp127-Asp807 (5C), His115-Asp695 (5D). Some interactions were mediated by hydrogen bonds, non-bonded contacts, as in Ala97, Ser98, Glu128 and Arg800 (5E), and hydrogen bonds, non-bonded contacts and salt bridges. Table 2 gives the distances between interactions. Interestingly, when alanine replacement led to drastic chemical shifts in

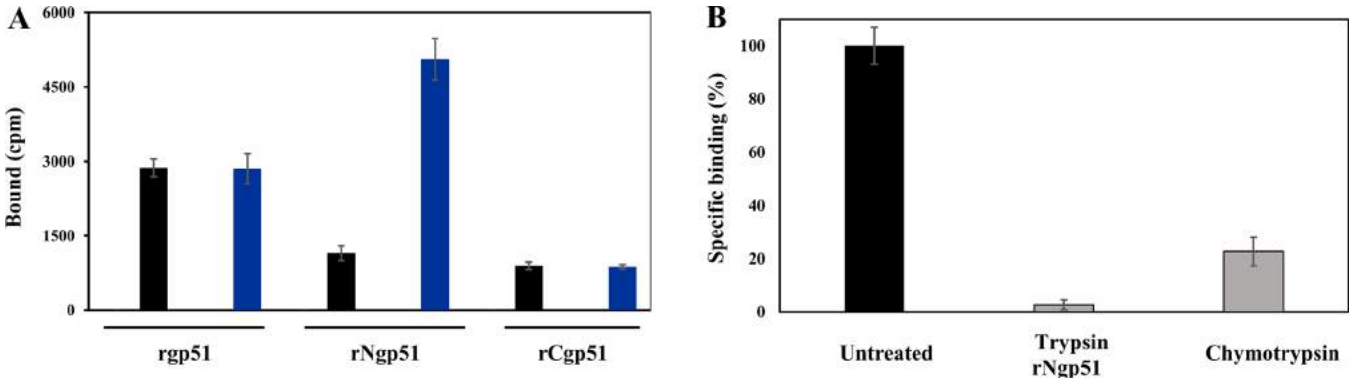

Fig 8. gp51 recombinant protein binding assays. 8A. rgp51 MDBK cell binding. Low rgp51 protein concentration (black bars) gave greater MDBK cell binding; however, rgp51 and rCgp51 binding did not change when protein concentration was duplicated (blue bars), whilst rNgp51 increased. 8B. MDBK cell interaction with rNgp51. The black bars represent enzymatically-treated MDBK and rNgp51 binding to the proposed receptor (AP3D1), followed by trypsin and chymotrypsin treatment, resulting in reduced in rNgp51 (98%) and MDBK binding (80%).

https://doi.org/10.1371/journal.pone.0199397.g008

some gp51 amino acids predicted as crucial in binding to boAP3D1 (His115, Glu128 and Asn170), the binding energy became drastically changed, thereby supporting the importance of such residues in the interaction.

The present study has suggested a possible hypothesis for viral entry to cells being AP3D1 protein-mediated as there is relevant similarity between this protein in different species and it could thus be said that this virus may have a binding pattern which is not species-specific at all and is using a ubiquitous receptor for achieving viral entry [8,44].

As other studies have proposed that the virus could enter other cells, it leads to a novel possibility involving a co-receptor being involved in cell infection [45,46] as AP3D1 can be found in a wide range of cells, so an additional molecule would seem to be required for BLV to acquire target cell and/or host specificity.

More recent studies have found the virus in other cell systems, particularly in the brains of cattle suffering neurological syndromes, suggesting different target cells being ultimately susceptible to viral infection and suggesting that this virus might be associated with other conditions than classically studied pathology [7]. These studies have presented a different perspective in which cattle are not really the only host which might be affected, as well as only lymphocyte cells being susceptible to infection by the virus.

However, it is clear that AP3D1 is not B-lymphocyte-specific, as shown by the BioGPS search (http://biogps.org) fed with experimental data accounting for this protein's presence in different cell types (including human kidney cells), and the *in vitro* experiments in this work showing recombinant gp51 binding (NT fraction and complete protein) to MDBK cells (Fig 8A), whose binding was inhibited by enzymatic treatment (Fig 8B). The above is supported by these cells containing AP3D1 as, according to the present work, this protein's similarity seems to locate it on the membrane (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2238969/); the Locate (http://locate.imb.uq.edu.au/) and QuickGO databases (https://www.ebi.ac.uk/QuickGO/) predicted the same.

This study adopted an in-depth bioinformatics and *in vitro* approach, searching for an explanation for gp51 interaction and its receptor. This is the first *in silico* approach to understanding BLV interaction with its host, further confirmed experimentally.

Conclusions

Knowledge concerning the boAP3D1 cellular adopter protein and BLV gp51 has been expanded through these results. Molecular modelling and protein docking methods were useful for obtaining boAP3D1-receptor and gp51 complex 3D structures, showing these proteins' interaction in detail. These models suggested the receptor-ligand interactions which could be occurring in BLV infection, leading to viral binding and fusion regarding viral entry. Further *in vitro* analyses confirmed that both the N-terminal region and the whole gp51 bound to MDBK cells, being the binding of the former region the strongest. Future studies aimed at assessing the potential use of the interactions here described for developing drugs or vaccines are thus recommended.

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Writing – review & editing: Adriana Patricia Corredor, Janneth González, Manuel Alfonso Patarroyo, María Fernanda Gutiérrez.

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ANEXO 3

Resúmenes de ponencias en congresos nacionales e internacionales

ACTA BIOLÓGICA COLOMBIANA

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PP262

VIRUS DE LA LEUCOSIS BOVINA: FACTOR DE RIESGO ASOCIADO A CÁNCER DE MAMA EN MUJERES COLOMBIANAS.

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Introducción. Los virus históricamente se han asociado con la formación de tumores, con tasas aproximadas del 15 al 20% de enfermedades cancerígenas asociadas con la presencia de virus. El cáncer de mama es una de las causas más frecuentes de cáncer en Colombia y en el mundo. El virus de la leucosis bovina (VLB) ha sido identificado en tejido mamario, el cual es un retrovirus oncogénico que infecta al ganado bovino, causando una linfocitosis persistente y en algunos casos puede llegar a desarrollar leucemias en los animales. En estudios previos, el VLB se ha propuesto como un potencial factor de riesgo para el desarrollo del cáncer de mama en diferentes regiones. En Colombia, hay evidencias previas de la presencia del virus en tejido mamario, pero hasta el momento no se ha establecido su asociación con el cáncer.

Objetivo. Se evaluó la presencia del VLB y su asociación con el cáncer de mama en una población de mujeres colombianas. Así mismo, se comparó la presencia del virus con marcadores de pronóstico de la enfermedad (HER2, KI67 y receptores hormonales).

Métodos. Se tomaron 168 muestras pareadas de sangre y tejido mamario provenientes de cirugías de mama (con y sin cáncer), y se les determinó la presencia del virus por medio de PCR anidada, PCR *in situ* e inmunohistoquímica.

Resultado. La presencia del virus fue mayor en la población con cáncer (65%), y se encontró una asociación significativa con el cáncer de mama por medio de una regresión logística multinomial con un OR de 2.63 (IC 95% 1.065-5.25 95%, p = 0.034), comparado con patologías benignas de la mama. No se encontró correlación entre los marcadores de pronóstico de la enfermedad y la presencia del virus.

Conclusión. Este estudio apoya hallazgos previos donde se plantea al VLB como un potencial factor de riesgo para el desarrollo del cáncer de mama.

Palabras clave: virus de la leucosis bovina (VLB), cáncer de mama.

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nCounter) in 641 pts (Pembro n=461 and Ipi n=180) and 134 pts had both TMB and GEP data available. All pts with TMB data and 458 pts with GEP data had PD-L1 expression data (PD-L1 IHC 22C3 pharmDx, MEL score). Statistical testing was prespecified prior to merging biomarker and clinical outcome data. Relationships between biomarkers and best overall response (BOR) were assessed by logistic regression and by area under the ROC (AUROC) curve; progression free survival (PFS) and overall survival (OS) by Cox proportional hazards regression. Prespecified cutoffs were -0.318 for GEP and 175 (mutations/exome) for TMB. Clinical data cutoff was Dec 04, 2017. Results: TMB, GEP and PD-L1 MEL were significantly associated with BOR, PFS and OS in the Pembro arm ($p<0.050$). Response rates to Pembro therapy were greater in pts who had high levels of both TMB $\geq$175 and GEP $\geq$-0.318 or TMB $\geq$175 and PD-L1 MEL $\geq$2 compared with those with low levels of these biomarkers (54% vs 14% and 51% vs 33%, respectively). TMB, GEP and PD-L1 MEL remained significantly associated with response to Pembro for BOR and PFS, and in general for OS, when assessed in joint models. TMB showed low correlations with GEP (0.14; $p=0.057$) and PD-L1 (0.22; $p=0.001$); GEP and PD-L1 were moderately correlated (0.60; $p<0.001$). In Ipi-treated pts, TMB was not significantly associated with BOR ($p=0.428$) and trended toward significance for PFS (0.098) and OS ($p=0.099$); GEP was also not significantly associated with BOR ($p=0.188$) but was significantly associated with PFS ($p=0.012$) and OS ($p<0.001$). AUROCs (95% CI) were 0.64 (0.55, 0.73) and 0.53 (0.35, 0.70) for TMB, and 0.64 (0.59, 0.7) and 0.56 (0.45, 0.67) for GEP with Pembro and Ipi. Note the observed TMB relationship with Ipi is based on an assessment of limited data in that arm and definitive conclusions cannot be made. Conclusion: TMB and inflammatory biomarkers (GEP and PD-L1) showed statistically significant and independent associations with BOR, PFS and OS in Pembro-treated pts with advanced melanoma. Only GEP showed significant associations with PFS and OS for Ipi, potentially indicating a prognostic value.

#4218 Validation of a new blood-based biomarker strategy for the early detection of lung cancer. Michael N. Kammer, Amanda K. Kussrow, Sanja L. Antic, Rina Nguyen, Heidi Chen, Darryl J. Bornhop, Pierre P. Massion. *Vanderbilt University, Nashville, TN.*

Rationale: The management of indeterminate pulmonary nodules (IPNs) remains a challenging problem. Our new assay methodology, the Free Solution Assay (FSA), measured by the Compensated Interferometric Reader (CIR), consisting of a diode laser, capillary and CCD, provides 40-fold lower limits of quantitation (LOQ) than ELISA for known candidate serum protein biomarkers, while speeding assay development, accuracy, and sensitivity. We hypothesized that lowering the LOQ of previously investigated biomarkers can increase the biomarker discriminatory power, enabling patients in an intermediate risk group (15-80%) to be reclassified into a low (<15) or high (>80) risk group. Methods: In this retrospective case-control study, FSA-CIR was used to measure the serum concentration of CYFRA 21-1 in two patient cohorts, a training cohort (N=274) of patients with IPNs collected at Vanderbilt University Medical Center and an external validation cohort (N=103) collected at the University of Pittsburgh Medical Center. Patient malignancy was determined by tissue biopsy or 2-year follow-up CT scan showing no signs of nodule growth. Baseline risk for cancer was calculated using nodule size. The added value of CYFRA 21-1 was assessed by comparing the difference in risk for lung cancer after incorporating CYFRA 21-1 into the model. Results: The limit of detection (LOD) of 6pg/mL and an average LOQ for standards was determined to be 60pg/mL. Patient samples in the training cohort were found to have CYFRA 21-1 concentrations ranging 100 pg/mL to 10 ng/mL, with a median of 0.79 (0.28-1.22, interquartile range) ng/mL in the control population and 1.90 (1.33-3.35) ng/mL in the case population, providing a ROC-AUC of 0.86 across three histological subtypes (adenocarcinoma, squamous cell carcinoma, and small cell lung cancer). The CYFRA 21-1 + nodule size risk model correctly reclassified 28 (25%) [DB1] of intermediate-risk benign nodules into the low-risk group and 28(24%) of intermediate-risk malignant nodules into the high-risk group. The independent validation cohort's controls had a median of 0.35 (0.20-0.56) ng/mL, while cases had 0.97(0.66-1.22) ng/mL, providing a ROC-AUC of 0.84 and correct reclassification of 12(34%) of intermediate-risk benign nodules and 6(15%) of intermediate-risk malignant nodules. The CYFRA 21-1 + nodule size risk model correctly classified 90.6% in the low-risk group and 87.0% in the high-risk group. Conclusions: FSA-CIR measurements requiring only a few microliters of serum allowed for reclassification of patients in the intermediate risk group in both the training and validation cohorts. The results suggest that CYFRA 21-1 measured by FSA-CIR represents a strong candidate biomarker for risk stratification of patients with IPNs.

#4219 Mitochondrial DNA damage: A promising tool to screen cervical cancer risk. Balaji Sadhasivam, Camille C. Gunderson, Elizabeth H. Hahn, Sarah E. Johnston, Yan D. Zhao, Vengatesh Ganapathy, Lurdes Queimado. *University of Oklahoma Health Science Center, Oklahoma City, OK.*

Background: Cervical cancer is the 4th most common female cancer, accounting for 500,000 new cases and 200,000 deaths across the world. 99% of cervical cancer is due to infection by human papillomavirus (HPV). The factors leading to HPV integration and induced carcinogenesis are not entirely clear, but high DNA damage levels have been proposed to play a role in HPV integration. Chronic HPV infection increases DNA damage and reduces DNA repair, leading to even higher DNA damage levels. Recently, we showed that the levels of nuclear DNA (nDNA) damage are a potential biomarker predictive of cancer risk. Mitochondrial DNA (mtDNA) damage has been reported to be more persistent than nDNA damage. We have previously demonstrated a good correlation between mtDNA and nDNA damage. Herein, we performed a pilot study to assess whether the levels of mtDNA damage can be used as a potential tool to screen cancer risk. Aims: (1) To measure determine whether the amount of mtDNA damage differs between cervical samples with distinct pathologies. (2) To determine whether the number of mtDNA lesions correlates with the grade of cervical dysplasia. Methods: Ethics Committee approval and written informed consent was obtained from all participants. Samples from 25 patients were collected from the endocervix during a pelvic exam in the Dysplasia Clinic. Samples for DNA damage studies were collected by cytobrush and DNA extracted as previously reported. Demographic and risk factor data were collected through detailed questionnaires. Clinicopathologic data was abstracted from the medical chart. The amount of mtDNA damage was quantified by a long-run real-time PCR-based DNA-damage quantification (LORDQ) assay. Data were analyzed by Student's t-tests. Logistic regression analysis was done modeling the probability of having present pathology risk. Results: Histopathological reports indicated that 9 cervical samples were normal (C0) and 16 had cervical dysplasia. Dysplasia samples were as follows: 9 low-grade squamous intraepithelial lesions (LSILs or C1) and 7 high-grade squamous intraepithelial lesions (HSILs or C2/3). The amount of mtDNA lesions per 10,000 bases were increased two-fold in LSILs or C1 cases and three-fold in HSILs or C2/3 cases when compared to C0. Regression analysis showed no significant correlation between age, race, smoking and drinking status compare with pathology risk. Conclusion: Our data show that mtDNA damage is the lowest in patients without cervical dysplasia and increases progressively with higher grades of dysplasia and correspondent cancer risk. This pilot study shows the feasibility of the approach and stresses the potential of using mtDNA damage to predict cervical cancer risk. Measuring mtDNA damage detection by LORDQ is less time consuming and more cost-effective than the measurement of nDNA damage. Larger population studies are urgently needed to fully assess the potential of this approach for cervical cancer risk.

#4220 Evidence of bovine leukemia virus genes detected in Colombian women with and without breast cancer: A zoonotic infection. Nury N. Olaya-Galán,[1] Sandra P. Salas-Cárdenas,[2] Adriana P. Corredor-Figueroa,[2] Gertrude C. Buehring,[3] HuaMin Shen,[3] Manuel A. Patarroyo,[1] Ma, Fernanda Gutierrez[2]. *[1]Universidad del Rosario, Bogota, Colombia; [2]Pontificia Universidad Javeriana, Bogota, Colombia; [3]University of California, Berkeley, CA.*

Bovine leukemia virus (BLV), is an oncogenic virus that infects cattle worldwide and is the causative agent of leukemias, lymphomas and persistent lymphocytosis. BLV has also been found in humans and has recently been proposed as a risk factor for developing breast cancer in the USA and Australia. In Colombia, there is evidence of infection in women but no correlation with breast cancer. This study was aimed at comparing the presence of the virus in breast tissue from different sources: necropsies of women without tumor development (normal breast tissue), and surgeries of benign and malignant tumors, to better understand the role of BLV in Colombia. A cross-sectional study was designed in which 315 participants were included. Paired samples of breast tissue and blood were obtained from surgeries and necropsies in Bogotá city. The presence of BLV was determined by nested PCR and in situPCR targeting different viral genes. For the nested PCR, DNA was extracted from fresh tissues and blood samples, and human GAPDH amplification was carried out to assess DNA quality for the PCRs. Afterwards, different BLV genes were detected by nested PCR. In situ PCR was directed to the tax region of the virus and was done to FFPE sections of the same samples. Positive samples were considered when at least one of the techniques was positive for the virus and were confirmed by Sanger sequencing. Correlation with other risk factors related with breast cancer (HER2, PR, ER and KI67) and the presence of the virus were determined. From the overall population, 40% of the samples were positive for BLV. In the breast cancer population, 37% of the samples were infected with the virus; similar

distributions were found in the other groups (pre-malignant, 33%; benign tumors, 27%). Interestingly, almost 60% of the samples were infected in the no-tumor group. 22% of the samples were positive for both breast tissue and blood from the same patients. All the positive samples were confirmed to have BLV by Sanger sequencing of different gene regions of the virus. When correlating viral presence with other risk factors of breast cancer, it was found that most of the positive BLV samples were also positive for progesterone (79%) and estrogen (93%) receptors and were negative for the HER2 mutation. It could thus be possible that the hormonal profile could be linked with the presence of the virus. This study shows that irrespective of the breast pathology, BLV can be found in Colombian women both in breast tissue and blood. Sanger sequencing showed that the virus found in humans is similar to previously reported sequences obtained from cattle with high identity percentages. Even when the biology of the virus remains unknown in humans, it is a big concern to find the presence of an oncogenic virus coming from animals. Further studies are needed to understand the viral mechanisms related with cancer in humans.

#4221 Development and evaluation of cell line-derived FFPE reference material for MSI assay validation. Takahiro Matsusaka, Eva-Maria Surmann, Sian Constantine, Hannah Child, Julie Wickenden. *Horizon Discovery Limited, Cambridge, United Kingdom.*

Microsatellite Instability (MSI) is defined by variance in the repeat count of microsatellite motifs and occurs in cells that are deficient in one or more mismatch repair proteins. MSI is present in varying cancer types, but is most commonly found in colorectal, gastric and endometrial cancer. Patients with early stage colorectal cancers that display MSI have a better prognosis and show a better response to chemotherapy compared to those with microsatellite stable tumors. MSI alongside additional markers such as tumor mutational burden is also a positive predictive biomarker for immune checkpoint inhibition Identification of MSI tumors in the diagnostic laboratory is traditionally performed by fluorescent multiplex PCR, evaluating the microsatellite length of two mononucleotide repeats and three dinucleotide repeats (recommended NCI Panel) or a commercially available kit, consisting of five mononucleotide markers alongside IHC staining for the four MMR proteins MSH2, MSH6, MLH1, and PMS2. With the increase in MSI testing related to the recent FDA approval of Keytruda®, several companies and laboratories are now developing newer and better PCR-based and next-generation sequencing assays to assess MSI. To control for error, we have developed a pair of cell line-derived MSI/MSS reference samples covering the most commonly used MSI biomarkers. MSI/MSS cell lines were mixed at biologically-relevant ratios and processed into FFPE to serve as a whole-process control. Our data support the suitability of this material on a variety of different platforms and with a high degree of consistency throughout various FFPE batches. In conclusion, our cell line-derived reference samples represent a commutable control to support MSI assay development and validation.

#4222 Omega-3 fatty acids produce DNA methylation changes in inflammation-related genes and pathways with implication of toll-like receptor signaling. David E. Frankhouser,[1] Sarah Woelke,[2] Michael Sovic,[2] Ralf Bundschuh,[2] Pearlly Yan,[2] Lisa D. Yee[1]. *[1]City of Hope, Duarte, CA; [2]The Ohio State University, Columbus, OH.*

Introduction: Omega-3 polyunsaturated fatty acids (n-3 PUFA) have been studied for potential health benefits in many diseases including breast cancer. The preventive effects of n-3 PUFAs may relate in part to inhibition of chronic, low-grade inflammation. Of interest is the role of n-3 PUFAs in modulating pro-inflammatory gene expression via epigenetic factors such as DNA methylation (DNAm). Methods: DNA methylation of PBMCs obtained at 0 and 6 months of n-3 PUFA supplementation was assessed via reduced representation bisulfite sequencing (RRBS). PBMC samples were obtained from women at high risk for breast cancer during a randomized clinical trial investigating the effects of different n-3 PUFA doses. The dosing arm selected for this study was 5 g of EPA+DHA/day, with baseline and 6 month samples (n=10). DNAm was quantified using Bismark from trimmed, pass filter reads and analyzed with MethylKit to determine n-3 PUFA treatment specific DNAm changes. Results Global DNAm showed no change after 6 months of n-3 PUFA treatment; however, we detected 30,974 differentially methylated CpGs (DMCs) across the genome. DMCs, both genome-wide and in gene promoters, where DNAm can regulate gene expression, were significantly enriched for hypermethylation events after treatment. Focusing the analysis on pro-inflammatory signaling mediators led to identification of candidate gene promoter DMCs involved in several inflammation-related pathways. Four pathways were significantly enriched for both DMCs and DMC hypermethylation events even when accounting for the genome-wide trend toward hypermethylated DMCs (hypergeometric test p-value < 0.001). The Toll-Like Receptor Pathway (TLR) genes harbored the most DNAm changes post n-3 PUFA treatment. Conclusion Our findings demonstrate that n-3 PUFA supplementation can result in inflammation-related changes stemming from PBMC methylome perturbation. The results suggest the TLR pathway as a potential mechanism for the anti-inflammatory effects of EPA and DHA. Functional studies are needed to confirm our current observation.

#4223 Conducting community oral cancer screening among South Asians in British Columbia. Leigha D. Rock,[1] Madhurima Datta,[1] Denise M. Laronde,[1] Anita Carraro,[2] Jagoda Korbelik,[2] Alan Harrison,[2] Martial Guillaud[2]. *[1]University of British Columbia, Vancouver, British Columbia, Canada; [2]BC Cancer, Vancouver, British Columbia, Canada.*

Introduction: Globally, more than 300,000 cases of oral cancer are diagnosed annually. South Asian countries, such as India, bear the brunt of this disease due to rampant use of chewing tobacco, betel quid and areca nut. BC has a high proportion of South Asian immigrants. Oral cancer has a high mortality rate (~50% 5-year survival) due to the advanced stage at which it is often diagnosed. It is purported that the majority of oral cancers develop from oral potentially malignant lesions (OPML). While lesions can be easily detected by oral health care providers, it can be challenging to differentiate benign lesions from OPML. DNA aneuploidy has been shown to be an effective marker to predict malignant transformation in OPML. Quantitative cytology (QC) studies DNA content (ploidy) and nuclear morphometric changes within the cell. The aim of this project is to assess the need for oral cancer screening in South Asians in BC and to validate QC as an adjunct screening device in a predominantly South Asian community screening setting to assess its effectiveness in identifying high-risk lesions among visually suspicious lesions. Methods: Demographic information (gender, age, country of birth, ethnicity, risk habit information and dental usage) were collected at the time of screening. Extraoral, intraoral and fluorescence visualization (FV) examinations were conducted. Buccal mucosal brushings were collected from each participant. Brushings were also collected from lesions or areas that had a loss of fluorescence. Thin-layer cytology slides were prepared and stained using Feulgen Eosin. Slides were scanned using the Cancer Imaging Scanner at BC Cancer using machine learning classification algorithms to identify single, in-focus epithelial nucleus. Cells are classified based on DNA ploidy and malignant associated changes. Results: 307 participants were screened of which 303 were eligible. More than 99% of the participants were South Asian or Asian. 104 (34%) lesions were documented: 45 (15%) were high risk (white or red lesions, lichen planus (LP)) and 59 (19%) were low risk (trauma, candidiasis, aphthous ulcers). Twenty participants (7%) were suspected to have high-risk OPMLs (not LP): 12 were referred directly to our Next Gen Oral Dysplasia clinic for biopsy, while 8 were reassessed at 3 weeks. Chewing tobacco was found to be strongly associated with lesion presence ($p<0.01$). QC analysis is ongoing for 320 samples. To date, 5 biopsies have been performed resulting in 1 mild dysplasia, 1 severe dysplasia and 3 hyperplasia. Conclusion: South Asians in BC were found to be at high risk for OPMLs. QC may help to improve the sensitivity and specificity of oral cancer screening by distinguishing false FV positive inflammatory lesions from high-risk lesions.

#4224 SGLT2 is a diagnostic target for early stage lung adenocarcinoma. Brendon Villegas, Eva Koziolek, Jie Liu, W. Dean Wallace, David Elashoff, Denise R. Aberle, David B. Shackelford, Jorge R. Barrio, Jane Yanagawa, Steven M. Dubinett, Claudio Scafoglio. *University of California, Los Angeles, Los Angeles, CA.*

Lung cancer is the leading cause of cancer-related death, and lung adenocarcinoma (LUAD) is the most frequent histology. Early diagnosis and treatment are essential; however, there are no targeted diagnostic and therapeutic approaches for early LUAD. An important hallmark of cancer is the increased glucose uptake via GLUT transporters. GLUT activity is imaged in cancer by positron emission tomography (PET) with 2-[^{18}F] fluorodeoxyglucose (FDG). FDG PET is a standard tool for staging advanced lung cancer, but has low sensitivity for early-stage LUAD. This is considered a consequence of slow growth and low requirement of glucose. However, we discovered a novel system of metabolic supply not detected by FDG PET: the sodium glucose transporter 2 (SGLT2)[1]. SGLT2 activity can be measured in vivo with the PET tracer methyl-4-[^{18}F] fluorodeoxyglucose (Me4FDG). We identified high expression of SGLT2 in human lung premalignancy and early-stage LUAD. Me4FDG detects early-stage, FDG-negative LUAD in mouse models. Targeting SGLT2 with FDA-approved inhibitors significantly reduces tumor growth and prolongs survival in genetic and patient-derived murine models, confirming an important role of SGLT2 in early-stage LUAD[2]. The reliance of early stage LUAD on SGLT2 opens exciting diagnostic and therapeutic opportunities. The National Lung Screening Trial showed a 20% reduction in lung cancer mortality in high risk individuals

8TH BAY AREA SYMPOSIUM ON VIRUSES-UC BERKELEY

Evidence of Bovine Leukemia Virus in blood and breast tissues of Colombian women: A zoonotic infection?

Olaya, Salas, Corredor, Shen, Buehring, Patarroyo, Gutierrez.

Bovine Leukemia Virus (BLV) infects cattle all around the world with prevalence rates between 20 and 90%. It is considered the causative agent of enzootic leukosis in cattle, and in advance stages also could develop into leukemia. This virus has also been found in humans in the USA and is proposed to be associated with breast cancer. In this study, the presence of BLV genes in paired blood and breast tissue samples from women in Colombia, South America was assayed using nested-PCR. From a total sample of 160 patients, 53 (33%) were positive for BLV in either breast tissue, blood, or both. Results were confirmed by Sanger sequencing. Although it is not known how the infected humans acquired the virus, it could be via a zoonotic infection through direct contact with cattle or consumption of bovine food products. Further studies are needed to determine the effect of BLV infection in humans.

BLV infects cattle worldwide but also has been found in the US in humans associated with breast cancer development. In this study BLV genes were found in paired blood and breast tissues from women in Colombia. Although it is not known how the infected humans acquired the virus, it could be via a zoonotic

LIBRO DE RESUMENES

XXIII CONGRESO LATINOAMERICANO DE MICROBIOLOGíA

XIV CONGRESO ARGENTINO DE MICROBIOLOGIA

IV CONGRESO LATINOAMERICANO DE MICROBIOLOGIA DE MEDICAMENTOS – CLAMME

REUNIóN DE LA SOCIEDAD LATINOAMERICANA DE TUBERCULOSIS Y OTRAS MICOBACTERIOSIS (SLAMTB)

Asociación Latinoamericana de Microbiología (ALAM)
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26 al 30 de septiembre de 2016 – Salón Metropolitano

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TRABAJOS CIENTÍFICOS

JU-1198

Virus de la Leucosis Bovina (VLB) presente en leche cruda y carne de consumo humano.

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El Virus de la Leucosis Bovina (VLB), perteneciente a la familia *Retroviridae*, es el agente causal de la leucosis bovina, enfermedad que ha sido reportada a nivel mundial en ganado vacuno. A pesar que la mayoría de los animales infectados permanecen asintomáticos en el transcurso de su vida, los estados más avanzados de la enfermedad se manifiestan con una leucocitosis persistente en un 20% de los animales infectados y genera procesos neoplásicos en tan solo el 10% de esta población. En América Latina, la propagación del virus se ha encontrado a lo largo de todo el continente puesto que no existen estrategias de prevención y control que eviten la comercialización y distribución de ganado infectado tanto para consumo de sus productos como para procesos de reproducción. En humanos hay algunos reportes donde muestran la presencia de anticuerpos contra proteínas estructurales del virus, al igual que la presencia de segmentos génicos virales en tejido mamario neoplásico y no neoplásico, infiriendo una relación entre el virus y el desarrollo de cáncer de mama. Ahora bien, al tener estos antecedentes donde un virus bovino se encuentra en el humano, surge la inquietud de indagar cuál podría ser la vía de transmisión al humano y qué papel está cumpliendo allí.

Con base en estos antecedentes el objetivo de este estudio fue determinar la presencia de tres genes del VLB *(gag, env, tax*) en carnes listas para consumo humano y leches crudas obtenidas de ganado en distintos lugares del país. Para este fin, se recolectaron 50 muestras de leche cruda y 50 cortes de carne provenientes de almacenes de cadena. A las 100 muestras se les realizó extracción de ADN total. Las muestras fueron validadas por medio de la amplificación del gen GAPDH, el cual también fue considerado como control interno de amplificación. La detección de los fragmentos de interés, se realizó por PCR tradicional - anidada para cada uno de los genes. Los resultados obtenidos fueron visualizados en geles de agarosa y confirmados por secuenciación.

En primera instancia, en la PCR de diagnóstico para el virus (gen *gag*), se encontró que el 43% de las carnes y 48% de las leches analizadas fueron positivas para el VLB. A estas muestras positivas, se les detectó la presencia de los otros dos genes; donde se encontró que el 27% de las muestras positivas para VLB, contaban con la presencia de los tres genes, mientras que en un 50% de las mismas, se encontró el gen *tax* pero no *env.*

El diagnóstico del VLB suele realizarse a partir de muestras de sangre. En este estudio se detectó el VLB en muestras distintas a sangre, el cual es el primer estudio que sugiere que la transmisión al humano puede estar mediada por el consumo de productos derivados de bovinos con presencia del virus. Estos hallazgos dan pie a futuros estudios para determinar si realmente esta infección, hasta ahora restringida a bovinos, puede estar involucrada en procesos zoonóticos, así como su posible papel en el cáncer de mama.

JU-1199

Rol del sistema de dos componentes YhcYZ en la regulación por temperatura del gen *desA* de *Bacillus cereus* ATCC14579.

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Bacillus cereus es un patógeno oportunista transmitido por los alimentos, capaz de producir gastroenteritis y otras infecciones severas en humanos. Estas bacterias pueden vivir en hábitats muy variados y adaptarse a crecer en una gama muy amplia de temperaturas. *B. cereus* ATCC14579 se adapta a bajas temperaturas de crecimiento ajustando la fluidez de su membrana plasmática principalmente por incorporación de ácidos grasos insaturados (AGI). A 37°C contiene una alta proporción de Δ10-AGI en sus lípidos de membrana, mientras que a bajas temperaturas sintetiza Δ10- y Δ5-AGI. Estos AGI son sintetizados por las desaturasas DesA y DesB que introducen dobles enlaces en las cadenas de los ácidos grasos (AGs) de los fosfolípidos de membrana en las posiciones 5 y 10 respectivamente. En nuestro laboratorio demostramos que la expresión del gen *desA* se induce a bajas temperaturas de crecimiento mientras que la expresión de *desB* es constitutiva. Recientemente en *B. cereus* ATCC 14579 ha sido propuesto que el sistema de dos componentes YhcYZ (CasKR) juega un papel clave en la adaptación al frío regulando la síntesis de AGs, en particular la expresión de los genes *desA* y *desB*, sin embargo hasta el momento no hay resultados concluyentes sobre el mecanismo de dicha regulación. Dado que las proteínas YhcYZ están presentes en *Bacillus subtilis*, con altos porcentajes de identidad con respecto a las de *B. cereus,* el objetivo de este trabajo es evaluar en *B. subtilis* la participación de las mismas en la expresión del gen *desA* de *B. cereus*. Para ello se construyó una cepa de *B. subtilis* mutante en el operón *yhcYZ* por reemplazo génico, utilizando un casete de resistencia a espectinomicina (Sp). Esta cepa mutante *yhcYZ* - y la cepa de *B. subtilis* salvaje fueron transformadas con una construcción que contiene la zona promotora del gen *desA* fusionadas al gen *lacZ* de *Escherichia coli* y con ellas se procedió a estudiar la influencia del sistema de dos componentes YhcYZ sobre la expresión del promotor p*desA.* Para ello se realizaron ensayos en placa y en medio líquido de la actividad β-galactosidasa a 37°C, 25°C, 15°C y 10°C. Los resultados mostraron que cuando la cepas fueron cultivadas a 37°C los niveles de actividad β-galactosidasa fueron relativamente bajos, pero la actividad β-galactosidasa aumentó notablemente tanto en la cepa salvaje como en la mutante en el sistema YhcYZ a temperaturas bajas de crecimiento. Este resultado estaría indicando que la expresión del gen *desA* que codifica para la Δ5 desaturasa de *B. cereus* es activada por la temperatura de crecimiento y es independiente del sistema YhcYZ y que además existirían en *Bacillus* otros elementos regulatorios que deben ser investigados. La comprensión de los mecanismos de adaptación de *B. cereus* para crecer a distintas temperaturas podría contribuir a controlar la multiplicación en los alimentos refrigerados y prevenir brotes de intoxicación por alimentos.

JU-1200

Calidad microbiológica de hortalizas y frutas mínimamente procesadas comercializadas en la ciudad de Bahía Blanca, provincia de Buenos Aires, Argentina.

Anexo 4
Apropiación social de la ciencia

PROYECTO "PEQUEÑOS CIENTÍFICOS"
Aplicando el método científico en primera infancia

Como parte de mi formación doctoral me capacité en Estados Unidos durante mi pasantía en métodos de educación STEM (Science, Technology, Education and Math) para llevar la ciencia a la comunidad. Realicé un voluntariado en el Lawerence Hall of Science de la Universidad de Berkeley donde aprendí estrategias de enseñanza de la ciencia para estudiantes de colegio en diferentes niveles de formación, colaboré en el museo de ciencia y en ferias de ciencia llevadas a los colegios.

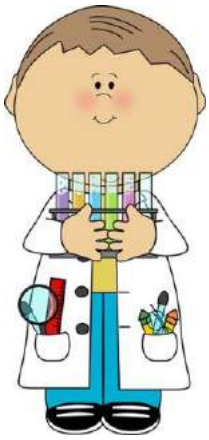
Basados en esta experiencia, desarrollé un proyecto en alianza con una institución de educación inicial en Bogotá para implementar lo aprendido como prueba piloto en primera infancia. En colaboración con el J.I Mi Dulce Encuentro implementamos el proyecto de "Pequeños Científicos" el cual ahora hace parte del Proyecto Educativo Institucional (P.E.I.) y lleva cuatro años de ejecución.

Los objetivos planteados para el proyecto es permitir que el niño/a de primera infancia de edades de 3-6 años a través de experiencias vivenciales y significativas desarrollen en sus primeras etapas de formación la capacidad de observación, análisis y pensamiento crítico a través de la exploración de conceptos de ciencia y del método científico enfocado a resolver preguntas e hipótesis sobre situaciones cotidianas que pueden ocurrir en su entorno.

Adjunto como ejemplo soporte de las bitácoras de laboratorio utilizadas para la ejecución del proyecto en las cuales se registran las estrategias en el aula para llevar a cabo el desarrollo del contenido temático según la unidad a desarrollar.

Así mismo adjunto registros fotográficos del proyecto en dónde los estudiantes aprenden a "observar, escuchar, pensar, explicar y sacar conclusiones" bajo un modelo de enseñanza constructivista, dinámico y vivencial adquiriendo habilidades que les permitirá a futuro aplicarlas en contextos como solución de problemas, análisis vivencial, seguimiento de instrucciones, aplicación de orden lógico y exploración de saberes (Ver video adjunto, anexo4_Proyecto STEM).

PROYECTO "PEQUEÑOS CIENTÍFICOS"



NIVEL: JARDIN Y TRANSICIÒN

BIENVENIDA A

NUESTRO LABORATORIO

EXPERIMENTO # 1

MARZO 17 Y 18 DE 2021

"MAGNETISMO: ¿Qué es fuerza magnética?"

MATERIALES

1. 1 Tarjeta adhesiva para nevera
2. 6 clips metálicos
3. Un cartón del mismo tamaño del adhesivo de la nevera
4. 6 botones plásticos medianos
5. Un plato plano
6. 6 tapas plásticas

PALABRAS CLAVES: Atracción, imán, metálico y no se atrae, fuerza magnética.

PROCESO

1 Llegará nuestro amigo el Conde Contar quien también tendrá la bata del laboratorio y nos dirá que todos vamos a ser pequeños científicos y que nos trae una linda sorpresa que es nuestra profe Luisita que juntos vamos hacer muchos experimentos y realizara la pregunta:

- ¿A qué venimos al laboratorio? A investigar.
- Y ¿Qué es investigar? Descubrir cosas nuevas

- Y ¿Cómo descubrimos cosas nuevas?
- Debemos observar, escuchar, pensar y explicar y todo eso que hacemos se llama hacer ciencia.

2 En segundo lugar, el Conde nos enseñará que los científicos observamos y analizamos antes de expresar una idea.

Iniciaremos nuestro experimento con un plato de seco en el cual vamos a poner 6 clips, vamos a coger solo uno y lo vamos a observar y llevaremos a los niños que describan detalladamente realizando preguntas como: ¿Tiene color ? , ¿Qué forma tiene?, ¿Cómo se siente? etc. En el mismo plato pondremos 6 botones plásticos y cogeremos uno de ellos el cual pasaremos a describir y por consiguiente la maestra realizara la misma indagación que con el clip haciendo una comparación de los objetos; luego vamos a traer una tarjetica adhesiva de nevera la cual observaremos y describiremos, ahora iniciaremos la exploraciones pondremos la tarjetica de nevera por el lado negro encima de los clips y los botones contaremos hasta 5 y cuando la profe indique todos juntos levantaremos la tarjetica al hacerlo vamos a plantear hipótesis de lo que creemos que sucedió, realizaremos este ejercicio una vez mas siempre indagando a los niños y niñas que sucede y por qué sucede.

3 Como tercer paso vamos a pasar a traer nuestro cartoncito que será del mismo tamaño de la tarjetica imantada la cual observaremos y describiremos realizando una corta comparación con la otra tarjeta, realizaremos el mismo procedimiento anterior donde pondremos el cartón sobre los clips y los botones, después de ver lo que sucede al levantar el cartón realizaremos hipótesis de que pudo suceder y por qué en el cartón no se pegaron los clips

4 Realizaremos una comprobación de las hipótesis y lo hallazgos encontrados por eso vamos a poner nuevamente la tarjeta imantada para verificar que pasa.

En ese momento damos el concepto que hay algo que pega los clips que no es pegante y que no deja que se pegue los botones porque son de otro material.

5 Como siguiente paso traeremos un imán, lo exploraremos, observaremos y describiremos en detalle.

Vamos a dar la instrucción que observaremos que sucede si ponemos el imán sobre los clips y los botones sin tocarlos después que cada uno lo realice pasaremos a plantear hipótesis buscando llegar a que el imán tiene una fuerza llamada fuerza magnética.

Para lograr este objetivo les pediremos a los auxiliares y los niños que se cojan de las manos y se jalaran con mucha fuerza, preguntamos que sentimos y por qué sucede, luego exploraremos nuevamente con el imán sobre los clips y los botones

6 Para finalizar realizaremos entre todas las conclusiones a las que llegamos, que descubrimos que los imanes tienen una fuerza magnética y que esa atrae los objetos metálicos.

El Conde realizara la conclusión.

BITACORA JARDIN Y TRANSICIÒN

En nuestro primer laboratorio podemos evidenciar la gran motivación de los chiquis, en el momento de realizar el laboratorio se hicieron cambios pues descubrimos que era importante explorar con la tarjeta imantada y el cartón al tiempo, eso permitió que los niños y las niñas llegaran más fácil a las conclusiones deseadas, por otro lado observamos que se debe dar las instrucciones paso a paso para que lo realicen correctamente. En el grado de transición lograron decir que la tarjeta imantada tiene imán y por eso atrae lo metálico.

INDICADORES DE LOGROS

1. Mantiene un periodo de concentración adecuado a su edad en cada una de las exploraciones.
2. Observa e intenta describir lo que ve.
3. Sigue instrucciones adecuadamente.
4. Reconoce que es un imán y que tiene fuerza magnética
5. Explora y realiza hipótesis de lo observado.
6. Crea sus propias exploraciones con curiosidad y atención.

PROYECTO "PEQUEÑOS CIENTÍFICOS"

NIVEL: PÁRVULOS Y PRE – JARDÍN

EXPERIMENTO # 1

"EL MAGNETISMO" FASE 1

MATERIALES

- Botella plástica transparente
- Clips
- Agua
- Imanes de nevera
- Tapas plásticas de gaseosa
- Material de ingeniería

PALABRAS CLAVES: Imán, metal.

PROCESO

1 En primer lugar tendremos nuestra experiencia significativa donde los niños y niñas observaran una botella de agua con clips en su interior. La maestra moverá la botella y observaremos y socializaremos que sucede, luego con imán por el exterior de la botella se atraerán los clips, los niños y niñas expresaran lo que piensan y por qué sucede.

2 En segundo lugar vamos a dividir cada curso en tres grupos, los cuales dos de de ellos estarán realizando una actividad con el material de ingeniería; él

otro grupo vamos a explorar con los imanes de nevera y clips pegando estos. También lo vamos a intentar con las tapas si también se pega a los imanes de la nevera.

EXPLICACION PARA LOS NIÑOS

Los imanes de la nevera que utiliza mamita, tienen una fuerza escondida que atrae los objetos metálicos y se llama fuerza magnética y lo que hace que exista esta fuerza magnética es algo que se llama imanes

EXPLICACIÓN

Los imanes tienen dentro de ellos una capa de una sustancia que es la que atrae los objetos metálicos porque son compatibles o iguales

BITACORA "PÁRVULOS"

Nuestra primera experiencia de laboratorio fue muy linda, ellos estaban muy motivados de estar en laboratorio de pequeños científicos, la única que estuvo un poco dispersa fue María Victoria pero pienso que hace parte de su adaptación pues aún le falta un poco en estar sentadita aunque en la parte del experimento su tiempo de concentración fue un poco más largo.

La actividad significativa los motivó mucho aunque realmente fue difícil construir el conocimiento entre todos pues aun no son muy participativos, realmente tuve que ayudarle a argumentar. De igual manera en el momento de explorar con los imanes y los clips, aunque descubrimos que la idea de trabajar con tapas también fue una distracción así que se decidió retirarlos

BITÁCORA "PRE- JARDÍN"

La experiencia con los chiquis de pre- jardín tuvo aspectos muy positivos, pues algunos chiquitos del año pasado recordaron algunos términos vistos.

Por otro lado los encontré muy motivados descubrieron que los clips se pegaban, pero también tuve que llevarlos a la conclusión.

En la parte de ingeniería realizaron cosas hermosas y con una intención de construcción se nota el trabajo del año pasado en los niños y niñas antiguos.

INDICADORES DE LOGROS

PARVULOS

1. Mantiene un periodo de concentración adecuado a su edad en cada una de las exploraciones.
2. Sigue instrucciones dadas dentro del laboratorio de pequeños científicos
3. Observa e intenta expresar lo que encuentra
4. Reconoce el imán como un elemento que tiene fuerza magnética

PRE-JARDIN

1. Mantiene un periodo de concentración adecuado a su edad en cada una de las exploraciones.
2. Observa y describe con detalle
3. Explora e indaga sobre lo que ve
4. Sigue instrucciones y reglas dentro del laboratorio
5. Reconoce el imán como un elemento que tiene fuerza magnética

Anexo 5
Acto de sustentación de la tesis doctoral

El acto de sustentación se realizó de manera virtual, con la participación de los jurados evaluadores Dra. Luisa Matheus (Universidad del Rosario, Bogotá – Colombia), Dra. Carolina Ceriani (UNICEN – Tandil, Argentina) y Dra. Maria Cristina Navas (Universidad de Antioquia, Medellín – Colombia).

Se realizó la defensa de tesis por transmisión en el canal de YouTube de la Universidad del Rosario, disponible en el siguiente enlace:

Defensa de tesis doctoral – Candidata Nury Olaya | Canal YouTube URosario
https://youtu.be/junvleA2Kys