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# Variability of UL49 in bovine herpesvirus type 1 (BoHV-1) from Uruguayan isolates

## Variabilidad de UL49 en herpesvirus bovino tipo 1 (BoHV-1) en aislamientos de Uruguay

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## SUMMARY

*Bovine herpesvirus* type 1 (BoHV-1) is the etiological agent of Infectious Bovine Rhinotracheitis (IBR). Depending on the genotype, it produces a wide variety of clinical syndromes, such as conjunctivitis, abortion and balanoposthitis. Vaccination against BoHV-1 is a common practice, so in order to differentiate infected from vaccinated animals, many BoHV-1 proteins were studied to develop marked vaccines. VP22, the major tegument protein codified by UL49 gene, is a non essential protein for virus multiplication, but induces a relevant immune response in the host. Therefore the variability of the gene UL49 of three BoHV-1 Uruguayan isolates (Uy1999, Uy2002 and Uy2004), was analyzed. Recombinant VP22 was expressed in a prokaryotic system and was recognized by sera of BoHV-1 immunized rabbits by Western blot analysis. Uy1999 (BoHV-1.1), Uy2002 and Uy2004 (BoHV-1.2), have 93.8%, 95% and 88.2% nucleotide similarity to the international Los Angeles BoHV-1.1 reference strain. The phylogenetic analysis showed that Uy1999 is grouped with BoHV-1.1 strains, while Uy2002 and Uy2004 isolates are grouped distant from other BoHV-1.1. The use of VP22 deleted vaccines and a serological technique able to detect antibodies against it, is an excellent alternative to be used in bovine vaccination programmes.

Key words: (UL49), (variability), (BoHV isolates), (Uruguay).

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## RESUMEN

*Herpesvirus bovino* tipo 1 (BoHV-1) es el agente responsable de la Rinotraqueitis Infecciosa Bovina (IBR) y de un amplia variedad de síndromes, como conjuntivitis, aborto y balanopostitis, según el genotipo que se encuentre presente. La vacunación contra BoHV-1 es una tarea de rutina en el manejo de ganado, por lo tanto se han estudiado varias proteínas de BoHV-1 con el objetivo de desarrollar vacunas marcadas que permitan la diferenciación entre animales vacunados e infectados. VP22 es la proteína principal del tegumento del virus y es codificada por el gen UL49. No es esencial para el ciclo de replicación viral pero induce una respuesta inmune significativa en el huésped. Por consiguiente se analizó la variabilidad del gen UL49 en tres aislamientos uruguayos de BoHV-1 (Uy1999, Uy2002 y Uy2004), se expresó una VP22 recombinante que fue reconocida por sueros de conejos inmunizados con BoHV-1 mediante su análisis por Western Blot. Los resultados obtenidos muestran que Uy1999 (BoHV-1.1), Uy2002 y Uy2004 (BoHV-1.2), tienen 93.8%, 95% y 88.2% de similitud en la secuencia de nucleótidos al compararlos con la cepa de referencia internacional Los Ángeles BoHV-1.1. El análisis filogenético mostró que Uy1999 está agrupado con cepas BoHV-1.1, mientras que Uy2002 y Uy2004 están agrupadas distantes de los otros BoHV-1.1. El uso conjunto de vacunas a las cuales se les ha eliminado VP22 y de técnicas capaces de detectar anticuerpos contra la proteína eliminada es una excelente alternativa a usar en los programas de vacunación bovina.

Palabras clave: (UL49), (VP22), (Herpesvirus Bovino), (Uruguay).

## INTRODUCTION

Besides causing Infectious Bovine Rhinotracheitis (IBR) Bovine herpesvirus-1 (BoHV-1) is also responsible for conjunctivitis (BoHV-1.1), abortion (BoHV-1.1 and BoHV-1.2a), and balanoposthitis (BoHV-1.2b)[1]. Since vaccination is an effective measure to decrease IBR outbreaks, conventional live attenuated or inactivated vaccines have been used as control strategies[24], but these do not allow differentiating infected from vaccinated animals. Hence, many countries have programs based on the use of marked vaccines, DIVA (Differentiating Infected from Vaccinated Assays), in which genes not essential for virus replication are deleted. Serological techniques to detect antibodies against the deleted protein must be developed, in order to be able to differentiate between infected and vaccinated animals[11, 15, 25, 26]. The functions of VP22, the major tegument protein of BoHV-1 encoded by the UL49 gene, are not related to virus replication cycle, but VP22 is required for accumulation and optimal protein synthesis, the reorganization of microtubules in infected cells and the nucleic acid incorporation into the virion[8, 22]. Previously Liang et al[12] showed that BoHV-1 UL49 is an important virulence factor, and also suggested that deletion of this non-essential viral gene may be useful in developing recombinant BoHV-1 vaccines. Therefore, vaccination with strains not expressing VP22 such as the Japanese 758-43 BoHV-1 strain[6], could be a useful strategy for cattle vaccination campaigns.

The goals of this study were to analyze the genetic variability of UL49 gene sequence of three BoHV-1 Uruguayan isolates and express recombinant VP22 in a prokaryotic system, in order to obtain a protein to be used in diagnostic immunoassays, allowing the detection of VP22-negative vaccinated animals. Even though DIVA vaccines are not used in Uruguay, this strategy has allowed eradication of the disease in many countries. Differentiated vaccines may be used

in Uruguay, where the prevalence is still high, therefore greatly reducing the need to slaughter latently infected animals.

## MATERIALS AND METHODS

### Cells and virus

Virus was multiplied in *Madin Darby Bovine Kidney* (MDBK) cell lines with Minimum Essential Medium with Eagle salts (E-MEM; SIGMA, USA) with 10% Fetal Calf Serum (FCS) (Probiomont, Montevideo, Uruguay). The virus used in this study were the Uruguayan isolates Uy1999 (BoHV-1.1), Uy2002 (BoHV-1.2) and Uy2004 (BoHV-1.2)[19], and the BoHV-1.1 reference strains 758[18] and Los Angeles (LA)[14]. The following sequences were used for phylogenetic analysis purposes: BoHV-5, swine herpesvirus (SHV-1) and human herpesvirus 5 (HHV-5) (GenBank accession nos. AY261359.1, BK001744.1, y GU179001.1, respectively).

### Extraction of viral DNA and amplification of UL49 gene by Polymerase Chain Reaction (PCR)

Viral DNA was extracted from MDBK cultures infected with the BoHV-1 Uruguayan isolates (Uy1999; Uy2002 and Uy2004), using a commercial kit (MinElute Gel Extraction Kit- QIAGEN®), following the manufacturer's instructions. The primers used for PCR were designed based on the UL49 gene sequences of BoHV-1 available at GenBank (www.ncbi.nlm.nih.gov). Forward primer (FP): 5 -GGCTGATTGACCGCAACGC- 3 and Reverse primer (RP): 5 -GGGTGGAACAGGCAGGTGAA- 3 were designed to target a 776 bp fragment of the UL49 gene[6].

### DNA sequencing and sequence analysis

Amplified viral DNA was purified using MinElute Gel Extraction Kit (QIAGEN®). The quality of all DNA preparations was checked in agarose gel electrophoresis[21]. Sequencing was carried out with the BigDye Terminator (Applied Biosystems), following the manufacturer's protocol. Sequence determination was performed in a MegaBACE 500 automatic sequencer (GE Healthcare, USA). Each product was sequenced four times in both directions using the FP and RP described above.

The quality of DNA sequences was checked and overlapping fragments were assembled using the BioEdit package 7.0.5[5], Vector NTI 8.0, AlignX and ContigExpress (InforMax, Inc.). Assembled sequences with high quality were aligned using ClustalX[23] with default gap penalties. Homology analysis was performed with the NCBI database and BLAST[3]. Proteins were aligned using ClustalW[23]. Alignment gaps were removed from the analysis by pairwise distance computation (pairwise-deletion option) leading to approximate 200 aligned amino acid positions. Neighbor joining analyses were carried out with the data sets using an amino acid JTT model by means of MEGA3 software[9]. We also assessed nodal support using a nonparametric bootstrap technique (2,000 replicates).

### UL49 gene cloning and sequencing in the PCR2.1-TOPO® vector

Gene UL49 PCR product was cloned in the PCR2.1-TOPO® vector (Invitrogen) and sequenced using BigDye Terminator (Applied Biosystems, CA, USA). Sequences were studied by Bioedit v7.0.5 program[5], and later through BLASTn and BLASTx programs, in order to confirm the sequences identity.

### UL49 gene cloned in pET44b vector and recombinant UL49 expression

UL49 gene was extracted from the PCR2.1-TOPO vector using *Eco*RI restriction enzyme and purified in 0.8% agarose gel, using MinElute Gel Extraction Kit (QIAGEN®), following the manufacturer's instructions. pET44b vector (Novagen®) was digested with *Eco*RI and ligated to the UL49/*Eco*RI fragment with T4 DNA ligase (Invitrogen). The transformation was performed in *E. coli* One Shot™ TOP10 competent cells (Invitrogen), following the manufacturer's instructions. Recombinant plasmids were extracted from the

transformed colonies according to Sambrook & Russell[21]. Sequencing, *Eco*RI restriction enzyme digests and PCR were performed to confirm the presence of the insert in the vector (pET44b/UL49).

The recombinant VP22 protein (rVP22) was expressed in *E. coli* BL21 (GeneChoice, Inc.) using the recombinant pET44b/UL49 plasmid. Bacteria were grown in Luria Bertani (LB) media, with 100 μg $mL^{-1}$ ampicillin at 37 °C until 0.7 $OD_{600}$; 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) (Invitrogen) was added to induce the protein.

To determine the optimal expression, bacterial cultures were sampled at different times (1-to 16 hours). After centrifugation and proteins were analyzed in 10% SDS-PAGE gel[10].

**Purification of rVP22**

*E. coli* BL21/pET44b/UL49 was stimulated *overnight* with IPTG, then−centrifuged 10 minutes at 10,000 x g and resuspended in 20mM Tris-HCl buffer pH 6. Bacterias were sonicated, then protease inhibitors, 1 mM of phenylmethanosulfonyl fluoride (PMSF) and 0,1 mM of nα−ρTosyl L-lysine chloromethyl Ketone (TCLK) were added. Bacteria were centrifuged for 10 minutes at 14,000 x g and resuspended in elution buffer (0.2M-$NaH_2PO_4$, 0.2M of $Na_2HPO_4$, 300mM−NaCl, pH6). Proteins were purified through an affinity chromatography column (His Trap, GE Healthcare, USA).

**Antigenic characterization of recombinant VP22 (rVP22) by Western-blotting**

Recombinant VP22 was run through 0.8% polyacrylamide gel and transferred to nitrocellulose (Probind 45, Pharmacia Biotech®). The transferred nitrocellulose was blocked with PBS, 0.1% Tween 20 (v/v) and 10% low fat powdered milk, for 1 hour shaking at room temperature. The treated nitrocellulose was incubated at 37ºC for 1 hour with two rabbits sera, previously immunized with wild BoHV-1.1 strain 758[18]. Conjugate anti-rabbit IgG Peroxidase (Santa Cruz Biotechnology Inc), was used according to the manufacturer's instructions.

## RESULTS

**UL49 gene sequencing and analysis**

When compared to the UL49 gene amplified from the BoHV-1.1 LA reference strain with isolates Uy1999, Uy2002 and Uy2004, had 93%, 95%, 88% similarity at the nucleotide level, and 93%, 87%, 94% at the amino acid level (table 1).

Phylogenetic analysis showed that the isolate Uy1999 was grouped with BoHV-1.1 as LA reference strain, but Uy2002 and Uy2004 were clustered as BoHV-1.2 (Fig 1). The differences between both groups were due to nucleotide changes: as the first nucleotide change was a transversion from adenine (A) to thymine (T); this non synonymous change generated an aa substitution, a threonine (T) for a serine (S) at position 199. The other change was a nucleotide transition between purines, from an adenine (A) to a guanine (G). This generated a substitution from a serine (S) to a glycine (G) at position 232 of the protein. While Uy1999 was more related to the LA BoHV-1.1 reference strain, Uy2002 and Uy2004 (both BoHV-1.2) had some amino acid similarities to the BoHV-5 reference strain (Fig 2).

**Recombinant pET44b /UL49 plasmid construction and rVP22/BoHV expression**

UL49 gene cloning in pET44b vector was confirmed by PCR, restriction *Eco*RI enzyme analysis and sequencing of the cloned fragment. The rVP22/BoHV soluble protein was expressed in *E. coli* BL21, after induction of 0.4 mM IPTG in an overnight incubation.

**Evaluation of the reactivity of rVP22 protein with antibodies of BoHV-1 immunized rabbits.**

Rabbit serum immunized with BoHV-1 recognized rVP22 as demonstrated by Western-blotting (Fig 3).

| *Sequences* | *BoHV-1.1 LA* | *Uy2002* | **Uy1999** | **Uy2004** | **NC001847.1 (BoHV-1)** | **NC005261.2 (BoHV-5)** | **NC006151.1 (SuidHV-1)** | **NC006273.2 (HHV-5)** |
|---|---|---|---|---|---|---|---|---|
| **BoHV-1.1 LA** | **ID** | *95* | *93,8* | *88,2* | *82* | *73,5* | *49,2* | *19,3* |
| **Uy2002** | *87,5* | **ID** | *97,1* | *92,9* | *85,7* | *75,3* | *47,4* | *20* |
| **Uy1999** | *93,3* | *94* | **ID** | *93,7* | *85,9* | *77* | *46,6* | *20* |
| **Uy2004** | *94,5* | *92,9* | *97,2* | **ID** | *82,6* | *72,6* | *44,7* | *19,1* |
| **NC001847.1 (BoHV-1)** | *93* | *91,8* | *96,8* | *96,9* | **ID** | *70,3* | *42,4* | *21,7* |
| **NC005261.2 (BoHV-5)** | *66,5* | *68,9* | *69,3* | *70,9* | *72,2* | **ID** | *46,9* | *21,1* |
| **NC006151.1 (SuidHV-1)** | *27* | *29,7* | *28,3* | *28,1* | *27,8* | *25,8* | **ID** | *19,2* |
| **NC006273.2 (HHV-5)** | *8* | *8,5* | *8,4* | *8,5* | *8,7* | *7,7* | *5,7* | **ID** |

**TABLE 1.** Percentage homology of Herpesvirus UL49 variants. Above "ID" percentage of nucleotide similarity is shown and below the percentage of amino acid similarity is shown. BoHV-1 LA: international reference strain of BoHV-1.1 Los Angeles; Uy2002: Uruguayan isolate previously classified as BoHV-1.2 (Puentes et al., 2007); Uy1999: Uruguayan isolate previously classified as BoHV-1.1 (Puentes et al. , 2007); Uy2004: Uruguayan isolate previously classified as BoHV-1.2a (Puentes et al., 2007); NC001847.1, NC006151.1 and NC006273.2 sequences, bovine herpesvirus-1, Pig Herpesvirus-1 and human herpesvirus -1 respectively, deposited in GenBank. Table constructed with the help of the program BioEdit 7.0.

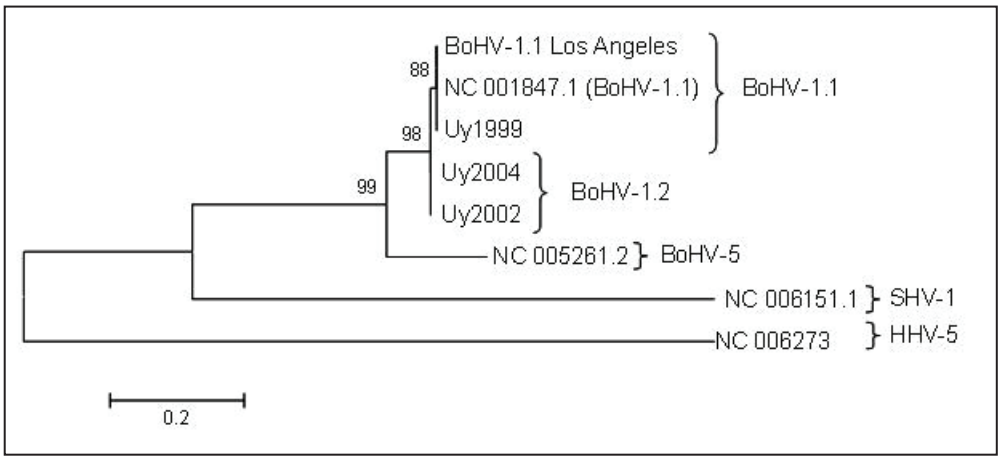


**FIGURE 1.** Phylogenetic tree based on the method of "Neighbor joining" to the amino acid sequence from VP22 of Uy1999, Uy2002 and Uy2004 isolations and reference strains of bovine herpesvirus 1 (BoHV-1.1) and 5 (BoHV-5), Pig herpesvirus 1 (SHV-1) and human herpesvirus 5 (HHV-5). As shown, isolate Uy1999 was grouped with strains BoHV-1.1. Uy2002 and Uy2004 isolates were grouped distant of the other BoHV-1.1.

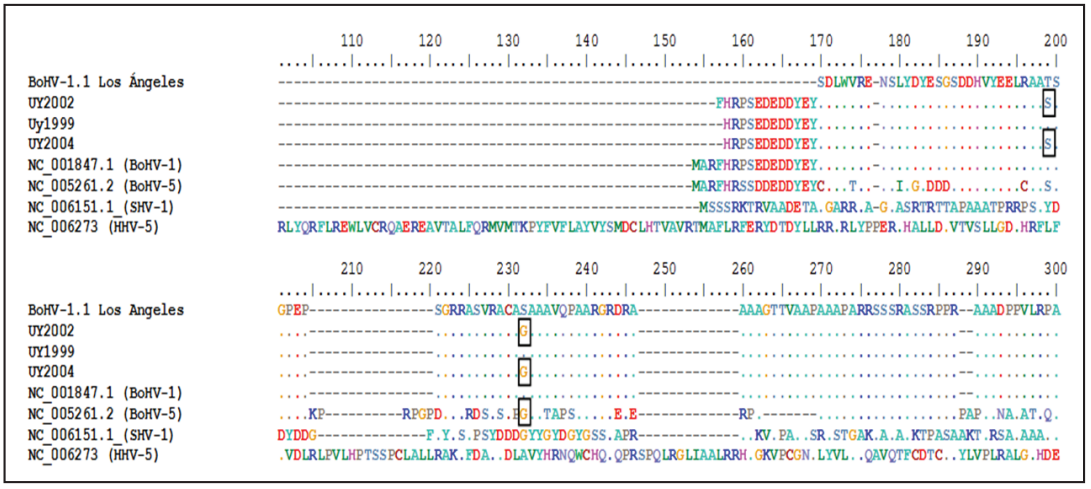


**FIGURE 2.** Alignment of the sequences of Uruguayan isolations of BoHV (Uy1999, Uy2002 and Uy2004) and reference strains of bovine herpesvirus 1 (BoHV-1.1) and 5 (BoHV-5), swine herpesvirus 1 (SHV-1) and human herpesvirus 5 (HHV-5). In relation to the reference strain of BoHV-1.1 Angels (BoHV-1.1 LA) at position 199 there was a change to a serine and threonine and at position 232 a change to serine by a glycine in Uy2002 and Uy2004. Amino acids in this position are more related to BoHV-5 strain. Alternatively Uy1999 behaves like a BoHV-1.1 strain.

## DISCUSSION

Two Uruguayan genetic variants of BoHV-1 subtypes were characterized previously as BoHV-1.1 (Uy1999 strain) and BoHV-1.2a (Uy2004 strain). The third isolate (Uy2002) could not be characterized as any of the regular subtypes previously described[19]. According to the enzyme restriction pattern this isolate is closer to subtype BoHV-1.2b[16]. Phylogenetic analysis of the gC carboxiterminal region from these BoHV-1 isolates grouped Uy1999 as BoHV-1.1 and Uy2002/Uy2004 as BoHV-1.2[4]. In the present experiment a more conserved tegument viral protein was analyzed which is under less immunological pressure than the gC studied in previous work[7]. Genetic analysis of UL49 in these isolates allowed us to group Uy1999 into a BoHV-1.1 cluster, while Uy2002 and Uy2004

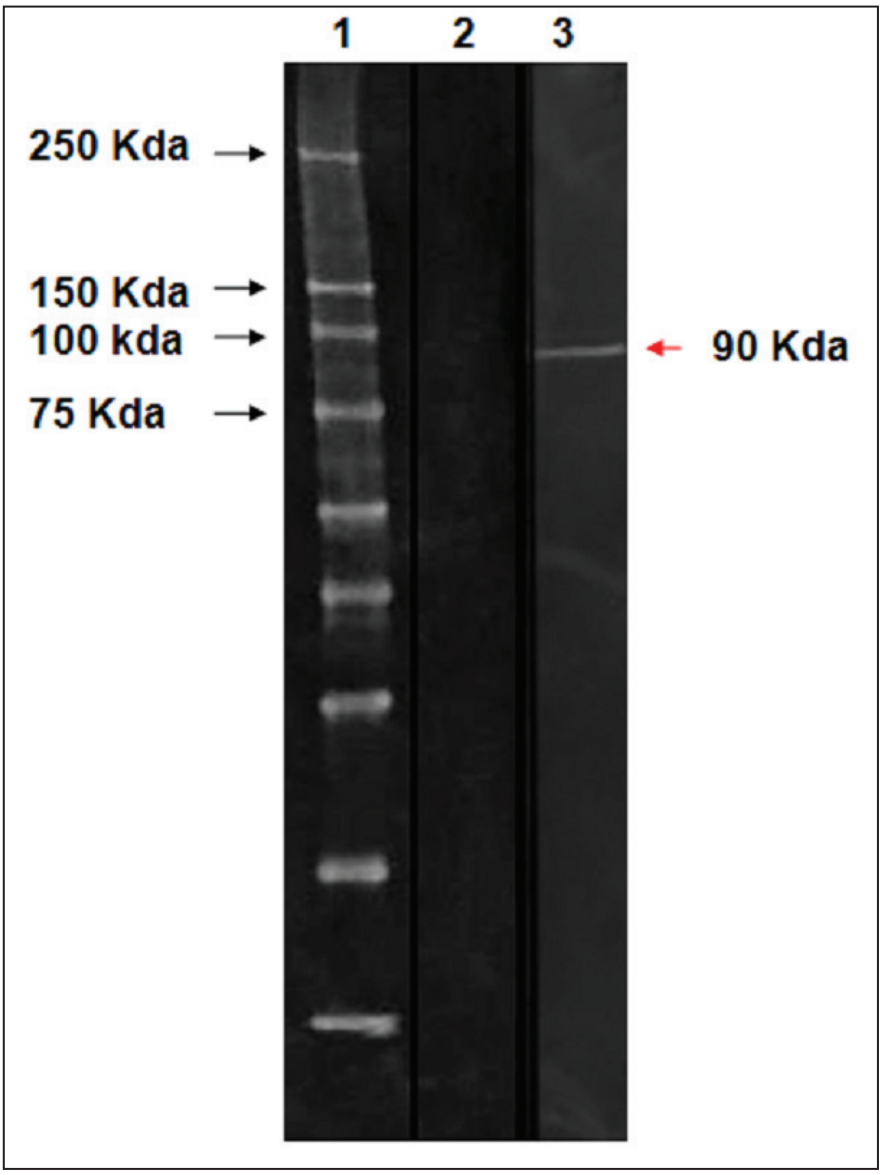


**FIGURE 3.** Immunoreactivity of rabbit serum with the cloned VP22. lane 1: molecular weight marker, 2: Serum from rabbit immunized with the vaccine strain 758-43 (with deletion of VP22), 3: Serum from rabbit immunized with BoHV-1 reference (Los Angeles strain). The red arrow shows a band of approximately 90 kDa corresponding to the immunoreaction with recombinant VP22 with the serum from the immunized animal with a reference strain for BoHV-1.

were very similar and were grouped distant of BoHV-1.1., these findings confirm previous observations[4,19].

Based on nucleotide variability of UL49 in isolates Uy1999, Uy2002 and Uy2004, 90% similarity was seen with the international reference strain BoHV-1.1 LA. This demonstrates high levels of homology between the UL49 gene of BoHV-1.1 and BoHV-1.2 isolations. However, as we have shown in Fig. 2, there are some aa changes in Uy2002 and Uy2004. The UL49 from BoHV-1.1, 1.2, 5, Uy2002 and Uy2004 shared two amino acids at the positions 45 and 62 (numbered by the BoVH-1 NC_001847.1) with BoHV-5 instead of BoHV-1 as could be expected. These two aa changes (ST; GS) found in both Uy2002 and Uy2004 may not be responsible for significant alterations in

the protein, since the aa involved have similar physicochemical characteristics. Therefore, we would not expect to find immunogenic differences in the epitopes recognized. Because VP22 is not essential for viral replication[13], the immunization of animals with the VP22 deleted BoHV-1 mutant strains, and a diagnostic test with VP22 as antigen, should allow us to differentiate between vaccinated and infected animals[6].

We have shown that recombinant VP22 protein was recognized by antibodies from rabbits immunized with the wild type BoHV-1 virus. A protein of approximately 90Kda was detected, corresponding to the VP22 protein with 33 amino acids and the NusTag protein with 495 amino acids (54.8kDa) included in the pET-44 vector. This suggests that rVP22 can be used in diagnostic assays such as ELISA, because it is a highly conserved protein and likely to be cloned.

Many European countries have BoHV-1 control and eradication programs. In them, marked vaccines with deletion of genes that code for immunogenic proteins are used, so differentiation of infected and vaccinated animals is possible (DIVA strategy)[11,17,24,26]. The efficacy of marked vaccines has been established, as BoHV-1 seroprevalence has decreased in herds where these types of vaccines were used[3,15,20]. The success of the deleted vaccines depends on the protein silenced. It should be a non essential structural protein, present in the majority of the wild viruses and an inducer of long lasting humoral immune response[24]. On that basis VP22 deletion in marked vaccines can be a useful strategy in BoHV control programmes.

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## REFERENCES

1 Ackermann, M.; Muller, H.K.; Bruckner, L.; Kihm, U. Eradication of infectious bovine rhinotracheitis in Switzerland: review and prospects. *Vet Microbiol.* 1990; 23(1-4):365-70.

2 Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; *et al.* 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; 25(17):3389-402.

3 Ampe, B.; Duchateau, L.; Speybroeck, N.; Berkvens, D.; Dupont, A.; Kerkhofs, P.; Thiry, E.; Dispas, M. Assessment of the long-term effect of vaccination on transmission of infectious bovine rhinotracheitis virus in cattle herds hyperimmunized with glycoprotein E-deleted marker vaccine. *Am. J. Vet. Res.* 2012; 73(11):1787-93.

4 Esteves, P.A.; Dellagostin, O.A.; Pinto L.S.; *et al.* Phylogenetic comparison of the carboxy-terminal region of glycoprotein C (gC) of bovine herpesviruses (BoHV) 1.1, 1.2 and 5 from South America (SA). *Virus Res.* 2008; 131:16-22.

5 Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 1999; 41:95–98.

6 Kamiyoshi, T.; Murakami, K.; Konishia, M.; Izumi, Y.; Sentsui, H. The presence of a deletion sequence in the BHV-1 UL49 homolog in a live attenuated vaccine for infectious bovine rhinotracheitis (IBR). *Vaccine.* 2008; 26:477-485.

7 Knipe, D.M.; Howley, P.M. *Fields Virology.* 2007; Fifth edition. Vol. 2. Ed. Lippincott Williams & Wilkins. Chapter 66.

8 Kotsakis, A.; Pomeranz, L.E.; Blouin, A.; Blaho, J.A. Microtubule reorganization during herpes simplex virus type 1 infection facilitates the nuclear localization of VP22, a major virion tegument protein. *Journal of Virology.* 2001; 75:8697-8711.

9 Kumar, S.; Tamura, K.; Nei, M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform*. 2004; 5, 150–163.

10 Laemmli, U.K. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature.* 1970; v. 227, n. 5259, p. 680-685.

11 Lehmann, D.; Sodoyer, R.; Leterme, S.; Crevat, D. Improvement of serological discrimination between herpesvirus-infected animals and animals vaccinated with marker vaccines. *Vet. Microbiol.* 2002; 86:59-68.

12 Liang, X.; Chow, B.; Babiuk, L.A. Study of immunogenicity and virulence of bovine herpesvirus 1 mutants deficient in the UL49 homolog, UL49.5 homolog and dUTPase genes in cattle. *Vaccine*. 1997; 15(10):1057-64.

13 Liang, X.; Pyne, C.; Li, Y.; Babiuk, L.A.; Kowalski, J. Delineation of the essential function of bovine herpesvirus 1 gD: an indication for the modulatory role of gD in virus entry. *Virology*. 1995; 207:429-441.

14 Madin, S.H.; York, C.J.; Vmckercher, D.G. Isolation of the infectious bovine rhinotracheitis virus. *Science.* 1956; 124, 721–722.

15 Mars, M.H.; de Jong, M.C.; Franken, P.; Van Oirschot, J.T. Efficacy of a live glycoprotein E-negative bovine herpesvirus 1 vaccine in cattle in the field. *Vaccine.* 2001; 19 (15-16):1924-1930.

16 Metzler, A.E.; Matile, H.; Gassmann, U.; Engels, M.; Wyler, R. European isolates of bovine herpesvirus 1: a comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. *Arch. Virol.* 1985; 85:57-69.

17 Muylkens, B.; Thiry, J.; Kirten, F.S.; Thiry, E. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet. Res.* 2007; 38:181-209.

18 Nishimado, M.; Fujii, H.; Okamoto, T.; *et al.* A collective outbreak of infections bovine rhinotracheitis in imported cattle. *J Jpn Vet Med Assoc.*1972; 25:600-4.

19 Puentes, R.; Alonzo, P.; Benavides, U.; *et al.* Primer aislamiento de Herpesvirus Bovino 1 Subtipo 2 (BoHV-1.2) en Uruguay. *Veterinaria (Montevideo)*. 2007; 42:9-13.

20 Raaperi, K.; Aleksejev, A.; Orro, T.; Viltrop, A. Dynamics of bovine herpesvirus type 1 infection in Estonian dairy herds with and without a control programme. Vet Rec. 2012 171(4):99.

21 Sambrook, J.; Russell, D.W. Molecular Cloning: A Laboratory Manual. *Cold Spring Harbor Laboratory Press*, 2001; New York.

22 Sciortino, M.T.; Taddeo, B.; Poon, A.P.; Mastino, A.; Roizman, B. Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells for expression prior to viral infection. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:8318–8323.

23 Thompson, J.D.; Higgins, D.G.; Gibson, T.J.; Clustal, W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; 22:4673-4680.

24 Van Drunen Littel-Van Den Hurk, S. Rationale and perspectives on the success of vaccination against bovine herpesvirus-1. *Vet Microbiol.* 2006; 31;113 (3-4):275-82.

25 Van Oirschot, J.T.; Kaashoek, M.J.; Rijsewijk, F.A.; Stegeman, J.A. The use of marker vaccines in eradication of herpesviruses. *J. Biotechnol.,* 1996; 44(1-3):75-81.

26 Van Oirschot, J.T.; Kaashoek, M.J.; Maris-Veldhuis, M.A.; Weerdmeester, K., Rijsewijk, F.A. An enzyme-linked immunosorbent assay to detect antibodies against glycoprotein gE of bovine herpesvirus 1 allows differentiation between infected and vaccinated cattle. *J. Virol. Methods.* 1997; 67(1):23-34.