Antiviral Research 81 (2009) 25–36

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Biochemical and structural characterization of RHDV capsid protein variants produced in *Pichia pastoris*: Advantages for immunization strategies and vaccine implementation

Omar Farnós[a,*,1], Erlinda Fernández[a,1], Maylin Chiong[a], Francisco Parra[b], Marisdania Joglar[a], Lídice Méndez[a], Elsa Rodríguez[a], Galina Moya[c], Dalia Rodríguez[d], Ricardo Lleonart[a], Ernesto M. González[e], Alena Alonso[g], Pastor Alfonso[f], Marisela Suárez[a], María P. Rodríguez[a], Jorge R. Toledo[a]

[a] *Animal Biotechnology Division, Center for Genetic Engineering and Biotechnology (CIGB), P.O. Box 6162, CP 10600 Havana, Cuba*
[b] *Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain*
[c] *Physical-Chemistry Division, Center for Genetic Engineering and Biotechnology (CIGB), P.O. Box 6162, CP 10600 Havana, Cuba*
[d] *Centro Nacional de Epizootiología, Diagnóstico e Investigación, Ave. 51 No. 33212, Havana, Cuba*
[e] *Plant Biotechnology Division, Center for Genetic Engineering and Biotechnology (CIGB), P.O. Box 6162, CP 10600 Havana, Cuba*
[f] *National Center for Animal and Plant Health (CENSA), Apdo 10, San José de Las Lajas, Havana, Cuba*
[g] *Centro de Aplicaciones Tecnológicas y Desarrollo Nuclear (CEADEN), Havana, Cuba*

ARTICLE INFO

Article history:
Received 16 May 2008
Received in revised form 25 August 2008
Accepted 28 August 2008

Keywords:
RHDV
Calicivirus
Recombinant vaccine
VLP
Multimeric protein
Long-term immunity

ABSTRACT

Rabbit hemorrhagic disease virus (RHDV) VP60 capsid protein was recently expressed at approximately 1.5 g L^{-1} associated with the disruption pellet of *Pichia pastoris*, thus requiring an additional process of extraction by solubilization. Consequently, the expression of a soluble variant of VP60 was undertaken in order to attain an easier approach for vaccine production. The VP60 gene was cloned without secretion signal under the transcriptional control of the *AOX1* yeast promoter. The antigen obtained was intracellular and soluble at approximately 480 mg L^{-1}. Its characterization by size-exclusion HPLC, ultracentrifugation, and electron microscopy, showed the presence of high molecular weight structures similar in mass, size and buoyant density to native RHDV. The antigenic profile was similar to that from authentic virions as determined with monoclonal antibodies directed against RHDV conformational epitopes. These analyses, conducted on VP60 obtained insoluble in *P. pastoris* revealed the formation of protein aggregates rather than the presence of ordered multimeric structures. An immunization trial was conducted in which the soluble VP60 was employed by subcutaneous (s.c.) injection either purified by a single chromatographic step or contained within raw disruption supernatant, emulsified in Montanide 888. The insoluble variant was administered as a yeast extract powder by oral and s.c. routes. The earliest IgG response, titers and persistence of antibodies were studied by competition ELISA and hemagglutination inhibition (HI) assays. All rabbits immunized with the yeast-derived antigens developed a strong RHDV-specific response (including the "RHDVa" subtype) that lasted over one year after the primary immunization. Early HI titers up to 1/40 960 were generated. The immune response was similar to that induced by VP60 from Sf9 cells and superior to the response elicited with inactivated RHDV. Overall it was found that the soluble VP60 multimers, safely and easily produced in *P. pastoris*, are a valuable candidate for the rational implementation of a low-cost, scalable subunit vaccine against RHDV.

1. Introduction

Following its emergence in China in 1984 (Liu et al., 1984), rabbit hemorrhagic disease (RHD) has been responsible for the death or the slaughtering of millions of free-living and domestic rabbits from many countries in the world. At present, the disease remains as an important cause involved in the decline of wild rabbit populations throughout certain regions of Europe and Asia (Calvete, 2006; van de Bildt et al., 2006; Yang et al., 2008). Moreover, in recent years various outbreaks have been recorded from diverse and rather unusual geographical areas, including domestic rabbits in countries from the American region (Farnós et al., 2007; McIntosh et al., 2007). The disease has also become endemic in those areas in which eradica-

* Corresponding author. Tel.: +53 7 271 6022x5156; fax: +53 7 273 1779.
E-mail addresses: omar.farnos@cigb.edu.cu, ofv_1999@yahoo.com (O. Farnós).
[1] These authors contributed equally to this work.

0166-3542/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2008.08.007

tion of its viral aetiological agent has been unfeasible. Some other issues make difficult the control of the disease such as the possible existence of non-pathogenic modes of transmission of the virus, the description of strange variants that include a phylogenetically distant subtype and recombination events influencing RHDV evolution and epidemiology (Capucci et al., 1996, 1998; Moss et al., 2002; White et al., 2004; Marchandeau et al., 2005; Forrester et al., 2007, 2008; Tian et al., 2007).

RHD provokes high mortality and a large number of pathologies in adult rabbits (Parra and Prieto, 1990; Tunon et al., 2003; Ferreira et al., 2006). The causative agent is the rabbit hemorrhagic disease virus (RHDV), a non-enveloped and icosahedral calicivirus with a capsid composed of a structural protein (VP60) of approximately 60 kDa (Parra et al., 1993). The virus is highly resistant to the environment, contagious and infects rabbits by nasal, conjunctival and oral routes (Xu and Chen, 1989).

RHDV does not propagate in cell culture systems in vitro. Therefore, aiming to produce a scalable recombinant vaccine, several approaches have been conducted in which the VP60 capsid protein has been expressed in heterologous hosts as *Escherichia coli* (Boga et al., 1994), insect cultured cells (Laurent et al., 1994; Sibilia et al., 1995; Marín et al., 1995; Gromadzka et al., 2006), *Saccharomyces cerevisiae* (Boga et al., 1997), plants (Castañón et al., 1999; Fernández-Fernández et al., 2001; Gil et al., 2006), recombinant animal-derived viruses (Bertagnoli et al., 1996a,b; Fischer et al., 1997; Bárcena et al., 2000) and larvae of *Trichoplusia nt* (Pérez-Filgueira et al., 2007). The protein has shown its capacity to induce protective immune responses in vaccinated rabbits. As part of these efforts, the VP60 capsid protein from the Spanish isolate AST/89 was recently expressed at approximately 1.5 g L^{-1} in *Pichia pastoris* MP36 mutant-transformed cells (Farnós et al., 2005). The recombinant protein was antigenically similar to the native protein and capable to induce a potent RHDV-specific immune response in orally or parenterally immunized rabbits. The antigen was able to elicit a specific lymphoproliferative response, a mixed Th1/Th2 cytokine expression profile and both local (IgA) and systemic (IgG) humoral immune responses to RHDV (Farnós et al., 2006). However, a potential limitation for this antigen is the fact of being produced insoluble, associated to the yeast membranous system. Although oral vaccination employing the cell debris fraction or the whole transformed yeast appeared as an effective immunization method, the use of a formulation to be administered by the parenteral route inexorably implies the laborious solubilization process of the antigen. Therefore, the expression of a soluble form of VP60 in this *P. pastoris* mutant was undertaken, aiming to facilitate the recovery of the protein, the obtainment of high expression levels, a superior resemblance to RHDV and an easier approach for the production of a cost-effective subunit vaccine.

In this study, a soluble VP60 variant from RHDV was obtained at high levels in *P. pastoris* followed by the characterization of its structural, biochemical and antigenic features. A comparative study with the insoluble VP60 obtained associated with the cell debris of *P. pastoris* was accomplished, in order to gain clarity on the advantages and limitations of each protein for vaccination of rabbits. An immunization trial was conducted using different preparations and administration routes to study the generation of the earliest IgG specific response, the time course of antibody titers and their persistence during one year. The measurement of titers by ELISA or HI was developed using a representative isolate of the virus or a strain recently isolated in the American region (Farnós et al., 2007) identified as an antigenic variant, previously designated as "RHDVa" (Capucci et al., 1998). The humoral immune response was compared with the response generated by inactivated-RHDV and with VP60 expressed by the baculovirus/Sf9 system. Finally, we analyzed the impact of these results in vaccine production issues and in the design of rational immunization strategies. We also discussed the practical value of these antigens based on the experience of human and veterinary vaccines currently produced employing this yeast strain.

2. Materials and methods

2.1. Yeast and virus strains

E. coli XL-1 Blue (Bullock et al., 1987) was used for propagation of the expression vectors. *P. pastoris* MP36 (his3) strain (Yong et al., 1992) was used as host for the yeast expression vectors pPSVP60 and pNAOVP60. *P. pastoris* PVP11 (Farnós et al., 2005) and PVP12 strains were the clones transformed with plasmids pPSVP60 and pNAOVP60, respectively. Both strains were selected to produce the recombinant variants of VP60. RHDV CUB5-04 strain (DQ841708) was isolated in Havana, Cuba, during the epizootic occurred between years 2004 and 2005 and was used as an alternative to RHDV Bs.89 (X87607) (Italian Reference strain) in competition enzyme-linked immunosorbent assay (ELISA) and in hemagglutination inhibition experiments. CUB5-04 has been identified as a member of the distinct "RHDVa" subtype while Bs.89 constitutes a "classical" representative isolate of the virus.

2.2. Construction of the expression plasmids and growth conditions

The *P. pastoris* strain MP36 transformed with plasmid pPSVP60, encoding the entire VP60 capsid protein gene from RHDV AST/89 has been previously described (Farnós et al., 2005). In the resulting strain (PVP11), the recombinant VP60 was expressed under the *P. pastoris AOX1* promoter regulation and the sucrose-invertase signal peptide from *S. cerevisiae*. It was obtained associated to the cell debris fraction after cell disruption and centrifugation. In this work, the VP60 coding region from RHDV AST/89 strain was cloned into the pNAO *P. pastoris* expression vector, which contains the alcohol oxidase (AOX) promoter from *P. pastoris* followed by two gene fragments from *Saccharomyces cerevisiae*; the glyceraldehyde 3-phosphate dehydrogenase transcription terminator (*GAP*t) and the histidine 3 (*HIS3*) gene as selection marker. It also contains the 3′ region of the *AOX1* gene. The vector carries a bacterial replication origin and the ampicillin resistant marker. The vector pNAO was designed without the use of a secretion signal in order to express recombinant proteins in the intracellular medium. For cloning, the VP60 gene was amplified from the expression plasmid pAcyMIVP60 (Marín et al., 1995) using the oligonucleotide primers that carry NcoI and EcoRI restriction sites (underlined) 5′CCATGGAGGGCAAAGCCCGCACAGCG3′ and 5′ATGAATTCTCAGACATAAGAAAAGCCATTG3′, complementary to the 5′ and 3′ regions of the gene sequence, respectively. The transformant PVP12 was obtained after electroporation of *P. pastoris* MP36 strain, conducted as previously described (Becker and Guarente, 1991), using 0.2 cm electroporation cuvettes at 1500 V, 25 μF, 200 Ω and a BioRad Gene Pulser with Pulse Controller (BioRad, USA). All the His+ transformants were selected in Yeast Nitrogen Base (YNB) minimal medium (Difco Laboratories, USA) supplemented with 2% glucose. Genomic DNA from yeast cells was purified (Rothstein, 1985) and transformant genotypes were analyzed by Southern blot (Southern, 1975; Sambrook et al., 1989). PVP11 and PVP12 *P. pastoris* strains were cultured in a 5-L bioreactor (Marubishi, Japan) containing the supplemented saline medium (Rodríguez et al., 1994). In both cases the media were inoculated with transformed cells at OD_{530} = 0.2 and cells were grown during 16 h to an OD_{530} = 5. The bioreactor was operated in a fed-batch mode at 30 °C, pH 5.5, rotational speed of 700 rpm and aeration rate

of 1 vvm. Upon depletion of glycerol as the carbon source (20 h), 1% (v/v) methanol was added to the culture to induce the *AOX1* promoter and production of recombinant VP60. After 4 h, methanol was added continuously to maintain 0.5% within the culture. After 96 h, the cultures were harvested.

2.3. Characterization of recombinant VP60 variants

2.3.1. Western blot and protein quantification

Samples from both induction processes were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) under reducing (1% glycerol, 0.4% SDS, 0.1% β-mercaptoethanol, 12.5 mM Tris–HCl, pH 6.6) and non-reducing (1% glycerol, 0.4% SDS, 12.5 mM Tris–HCl, pH 6.6) conditions and were electrotransferred onto nitrocellulose membranes (Amersham, UK) using a TransBlot^{TM} transfer semi-dry (BioRad, USA). Immune detection was achieved with a 1:200 dilution of a polyclonal anti-RHDV hyperimmune serum obtained from rabbits immunized with VP60 purified from native virions (AST/89 strain). The nitrocellulose membrane was blocked for 1 h with 5% skimmed milk (Oxoid, UK) at 37 °C, incubated with the hyperimmune serum diluted in phosphate buffered saline (PBS) for 1 h and washed with PBS-0.1% Tween-20 (PBST). The membrane was incubated with a goat anti-rabbit IgG-horseradish peroxidase conjugate (Amersham, UK) for 1 h, subsequently washed, and diaminobenzidine was used for visualization of protein bands. The High-Range Rainbow Molecular Weight Markers (Amersham, UK) was used for visualization of markers on Western blot membranes. The expression levels of soluble VP60 were measured by sandwich ELISA using hyperimmune serum anti-RHDV at a dilution of 1:3500 as capture antibody, the monoclonal antibody 6H6 (dilution 1:500) for antigen detection, and a curve of different concentrations of standard VP60 (Marín et al., 1995) expressed in the baculovirus/Sf9 system. Reactions were developed for 3–5 min in the dark using 0.4 mg mL^{-1} orthophenylenediamine (OPD) (Sigma, St. Louis, USA) diluted in 0.005 M citric acid/0.1 M Na_2HPO_4 containing 0.015% H_2O_2 (BDH, UK). The reaction was stopped with 50 μL per well of 2.5 M H_2SO_4. Measurements were performed at 492 nm in a SensIdent Scan ELISA reader (Merck, Germany).

2.3.2. Enzymatic deglycosylation

The recombinant VP60 obtained in PVP11 strain (expressed under the *ss*SUC2) was denatured at 100 °C for 10 min in 1% SDS and 5% β-mercaptoethanol and was cooled to 25 °C. Nonidet P-40 detergent (NP-40) was added to a final concentration of 1% before PNGase F addition. PNGase F digestion was carried out in 20 mM sodium phosphate buffer, pH 8.6, at a ratio of 5 U mg^{-1} protein, and incubated at 37 °C during 16 h. Enzymatic deglycosylation was verified by Western blot.

2.3.3. Antigenic profile

The antigenic characterization of recombinant VP60 variants was conducted by sandwich ELISA, using a panel of horseradish peroxidase-conjugated monoclonal antibodies directed against conformational epitopes, provided by the O.I.E. (Office Internationale des Epizootes) reference laboratory for the disease (Brescia, Italy). Hyperimmune serum obtained from one animal vaccinated with RHDV AST/89 strain was used at 1:500 in the assay for the coating of plates. The characteristics of mAbs employed for the sandwich are as follows: mAb 1H8 recognizes an external conformational epitope from correctly assembled RHDV or virus-like particles. mAb 6H6 recognizes an epitope present in both assembled and unassembled particles while mAb 6G2 is directed against a buried epitope located at the N-terminal part of VP60 protein. The assay was performed in duplicate for each sample analyzed. In addition, mAb 3B12, which recognizes an epitope present only in "RHDVa" was used for the assessment of immune responses against this distinct subtype as described further in Section 2.11.1.

2.4. Purification of soluble VP60

The method to purify the recombinant VP60 obtained associated to the disruption pellet of PVP11 strain was previously described (Farnós et al., 2005). To purify the soluble VP60 protein intracellularly expressed from PVP12 strain, yeast cells were collected from the bioreactor and centrifuged at 10,600 × *g*. The pellet was resuspended at 350–450 g L^{-1} (wet-weight) in disruption buffer (50 mM phosphate buffer pH 7 containing 0.3 M NaCl) and disrupted in a glass bead Dynomill KDL disintegrator (Willy A. Bechofen Maschinenfabrik, Basel, Switzerland) equipped with a cold water-cooling jacket at a flow rate of 20 mL min^{-1}. Twenty millilitres of raw disruption supernatant containing the soluble VP60 were applied to a chromatography column (2.6 cm × 100 cm) of Sephacryl S300 HR (Pharmacia, Sweden) equilibrated with PBS. The sample was run at a flow rate of 5 mL min^{-1}. Fractions were analyzed by immunodot and Western blot. Highly purified VP60 was also obtained by size-exclusion chromatography-HPLC as described below.

2.5. Ultrafiltration experiments

Ultrafiltration experiments with soluble VP60 were carried out using membranes of 100,000 (YM-100) and 300,000 (XM-300) kDa cut-off in a concentration apparatus (AMICON, USA). Previous to ultrafiltration, samples were subjected to centrifugation at 10,000 × *g* for 30 min and filtrated through 0.2 μm filters. Concentrated samples were further analyzed by immunodot, SDS-PAGE and Western blot.

2.6. Equilibrium and rate-zonal sucrose density gradients centrifugation

For the study of recombinant VP60 protein in equilibrium sucrose density gradients, five hundred microliters of either VP60 from PVP11 or PVP12 strains were applied to a linear 20–70% (w/v) sucrose density gradient in PBS, and centrifuged at 30,000 rpm for 24 h in a HIMAC centrifuge (Hitachi, model SCP70H, Japan). Five hundred microliter aliquots of each fraction were collected from the tube, until a total of 20 aliquots. For rate-zonal sucrose density gradients, five hundred microliters of samples containing VP60 from *P. pastoris* PVP12 strain were applied to a linear 10–30% (w/v) sucrose density gradient and centrifuged at 30,000 rpm for 90 min. The refractive index of each fraction was measured using an Abbe-3L refractometer. To detect the recombinant VP60, fractions were assayed by immunodot with mAb 1H8 and quantified by sandwich ELISA as described. Sedimentation coefficient was estimated using the previous known value of RHDV as a pattern in a parallel run.

2.7. Analysis in size-exclusion chromatography

The recombinant VP60 obtained in the soluble fraction of disrupted PVP12 as well as the recombinant protein solubilized from PVP11 strain were characterized by high performance size-exclusion chromatography (sec-HPLC). Samples were run in a TSK G5000 PW column (600 mm × 7.5 mm) system. Fifty microliters of disruption supernatant or purified VP60 solution were injected to the column and the elution was performed in PBS at a flow rate of 0.2 mL min^{-1}, at 280 nm. A chromatography lot of HBsAg (>1500 kDa) and sodium azide samples were used as controls.

2.8. Electron microscopy

The yeast cells were fixed in 1% glutaraldehyde and 4% paraformaldehyde at 4 °C for 1 h, rinsed in 0.1 M sodium cacodylate pH 7.4, post-fixed in 1% OsO_4 at 4 °C for 1 h and dehydrated in increasing concentration of ethanol. The embedding was done as described by Spurr (1969) with minor modifications. Ultrathin sections, made with an ultramicrotome (NOVA, LKB) were placed on 400 mesh grids, stained with 2% uranylacetate and Reynold's lead citrate, and examined with a JEOL/JEM 2000 EX transmission electron microscope.

2.9. Spray-drying process for powder production from the cell debris fraction of PVP11 strain

A laboratory-scale spray drier device was employed to atomize the initial aqueous material containing the cellular debris obtained after mechanical disruption of PVP11 yeast cells. The starting material was converted into powder particles that were collected at the bottom portion of the dryer.

2.10. Immunogenic capacity

To asses the immunogenic capacity of VP60 antigens using different routes, schemes and formulations, an experiment was conducted employing thirty-one New Zealand female rabbits of 8 weeks age and 2 kg, seronegative to RHDV, which were randomly distributed into seven groups of 5 animals each, respectively, with two of these groups comprising three animals each. The first group of 5 animals was immunized at day 0 by subcutaneous (s.c.) injection at five points on the back with 50 μg of VP60 from PVP12 strain purified by size-exclusion chromatography, emulsified in the oil-based adjuvant Montanide 888 (SEPPIC, France). These animals were boosted at day 21 of the experiment. The second group was immunized with untreated disruption supernatant from PVP12 strain containing the same VP60 concentration, employing an identical scheme. The third group was orally immunized at days 0, 21 and 40 with the yeast-based powder containing the whole cell debris fraction (with about 0.5 mg of VP60 per dose) obtained after disruption and spray drier treatment. The same powder resuspended in PBS, containing 50 μg of VP60 per dose, was utilized to immunize the fourth group of rabbits employing the same scheme and adjuvant as in the first two groups. The fifth (composed of three animals) and the sixth groups were included as positive controls and were immunized with 50 μg of VP60 expressed in Sf9 cells and with the RHDV inactivated vaccine Cunipravac-RHD™ following the manufacturer's instructions, respectively. A negative control group of three animals was simultaneously injected with Montanide 888 as placebo. The formulations were prepared in 1 mL of final volume. Blood samples were extracted from the marginal ear vein at days 0, 14, 40, 60, 90 and monthly until one year in order to analyze the level, specificity and time-course of the antibody response against RHDV.

2.11. Assessment of specific immune responses against RHDV

2.11.1. Competition enzyme-linked immunosorbent assay

Serum samples from each day collected were evaluated in duplicate at a single dilution (1:10) with a serological test for RHD manufactured at the OIE-Reference Laboratory, Brescia, Italy, following the manufacturer's directions. MaxiSorp F96 immunoplates (Nunc, Denmark) were coated with anti-RHDV hyperimmune rabbit serum at a dilution 1:400 in 50 μL of 0.1 M carbonate buffer, pH 9.6, for 16 h at 4 °C. The plates were washed twice with PBST, blocked for 1 h at 25 °C with 100 μL per well of 5% skimmed milk (Oxoid, UK), and test and control sera were added in duplicate to each well, followed by the addition of RHDV (Bs.89 reference strain) diluted 1:200. After incubation with shaking at 37 °C, and washes, the horseradish peroxidase-conjugated mAb 1H8 was added at a dilution 1:400. Reactions were developed for 3 min in the dark using 0.4 mg mL^{-1} of orthophenylenediamine (OPD) (Sigma, St. Louis, USA) diluted in 0.005 M citric acid/0.1 M Na_2HPO_4 containing 0.015% H_2O_2 (BDH, UK). The reaction was stopped with 50 μL per well of 2.5 M H_2SO_4. Measurements were performed at 492 nm in a SensIdent Scan ELISA reader (Merck, Germany). The assay measures the ability of antibodies in the test serum to compete with binding of RHDV to the polyclonal serum absorbed onto the solid phase. Sera were considered negative when the absorbance value at 492 nm decreased by less than 15% of the reference value (negative serum). They were considered positive when decreased by more than 25%. When "RHDVa" CUB5-04 isolate (diluted 1:20) was used instead of RHDV Bs.89 strain, the horseradish peroxidase-conjugated mAb 3B12 was added at a dilution 1:500. When titration experiments were performed, test and control sera from days 0, 60 and 365 were used at a starting dilution of 1:80 for day 60, and 1:10 for days 0 and 365. Titers were expressed as the reciprocal of the highest dilution in which the absorbance value decreased by more than 25% with respect to the equivalent dilution in the placebo group.

2.11.2. Hemagglutination inhibition assay

Serum hemagglutination inhibition titers were measured prior to vaccination, and at each time point of the experiment. The HI assay was performed using 2% human type O erythrocytes at pH 6.8 according to the O.I.E. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2004). Positive controls (sera from rabbits immunized with inactivated-RHDV isolated from the epizootic occurred in Cuba between the years 2004 and 2005) and negative controls (sera collected from seronegative rabbits at day 0 of the experiment) were included in the assay. Briefly, round-bottom microtiter plates were used for making double serum dilutions into wells starting from 1:20, in PBS. Afterwards, 25 μL of RHDV inactivated antigen-containing 8 HA units were added to each well followed by incubation at 25 °C for 45 min. Human red blood cells were added and were allowed to settle at 25 °C for 45 min. The HI titers of each sample were expressed as the reciprocal of the highest dilution at which no hemagglutination was observed.

2.12. Statistical analysis

Mean antibody titers were determined from serum of individual animals in each group using a competition ELISA. An analysis of variance was employed for each time point selected. Mean titers were compared using the Bonferroni's Multiple Comparison test. Inhibition percents from day 0 to 365 were also calculated using a competition ELISA. Values were compared with the Kruskal–Wallis and Dunns tests. All tests were conducted using the statistical software GraphPad Prism v4.0.

3. Results

3.1. Expression of the recombinant VP60 capsid protein in P. pastoris MP36-derived strains

RHDV VP60 capsid protein from the Spanish isolate AST/89 was recently expressed at approximately 1.5 g L^{-1} in *P. pastoris* PVP11 strain. In this construction, the recombinant protein was produced under the *P. pastoris AOX1* promoter regulation employing the sucrose-invertase signal peptide from *S. cerevisiae*. The protein obtained was insoluble, associated with the cell debris fraction of the transformed yeast after mechanical disruption (Farnós et al.,

2005). In the current design, the VP60 coding region was cloned under the transcriptional control of the *P. pastoris AOX1* promoter and the glyceraldehyde 3′-phosphate dehydrogenase terminator (GAPt) from *S. cerevisiae*, though lacking a secretion signal with the aim to produce a soluble, intracellular form of the protein. The gene was amplified by PCR using the pAcyMIVP60 vector as template and the amplification product was cloned to produce the pNAOVP60 expression plasmid. The expression cassette obtained by pNAOVP60 SalI digestion was used to transform by electroporation the His^- *P. pastoris* MP36 strain. Eighty His^+ transformants were selected on minimal medium and chromosomal DNA from selected clones was analyzed by Southern blot to confirm the integration of the cassette into the yeast genome. Ten recombinant clones were methanol-induced in a 200 mL shake flask culture experiment for 96 h. One recombinant that preliminarily showed the highest expression levels was selected and designated as PVP12. The analysis of this clone showed a methanol utilization slow (Mut^s) phenotype that was achieved by a double crossover event at the endogenous *AOX1* structural gene of the yeast.

Culture of PVP12 strain was conducted in a 5 L bioreactor. The induction phase lasted over 100 h employing a gradual increase in the methanol-feeding rate. The biomass increased from about 45 to approximately 290 wet-weight g L^{-1} during this phase. Samples from the induction process were analyzed at periodic intervals revealing the expression of the recombinant protein only in the liquid phase after yeast cells disruption and separation. The characterization of the intracellular/soluble VP60 was conducted using the raw disruption supernatant or the protein purified by high performance size-exclusion chromatography. The protein proved to be antigenically similar to the native capsid protein as determined by Western blot with rabbit polyclonal antibodies raised against VP60 purified from homologous RHDV. The protein detected under reducing conditions showed the expected size of about 60 kDa and was obtained at approximately 480 mg L^{-1} as quantified by sandwich ELISA using VP60 expressed in the baculovirus/Sf9 system as standard. The study under non-reducing conditions did not show a detectable band, thus suggesting the formation of high molecular weight structures which were incapable to enter the gel (Fig. 1). The latter differed with the putative protein aggregates of about 300 kDa observed for VP60 expressed under the *SUC2* signal peptide studied under non-reducing conditions. This insoluble antigen was solubilized from the cell debris fraction as described elsewhere (Farnós et al., 2005) and used for comparative purposes in the experiments designed. It was obtained with a final purity in the range of 65–70% after a combination of washed pellet procedures, extraction with urea, renaturalization and acid precipitation of contaminants.

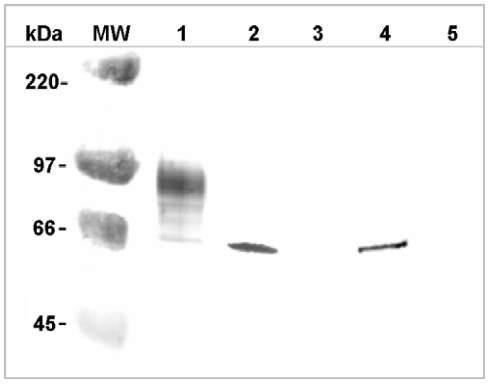

Fig. 1. Expression of the recombinant VP60 in *P. pastoris* PVP12 strain studied by Western blot using a hyperimmune anti-RHDV serum. Lane 1 corresponds to VP60 obtained insoluble associated to the cell debris fraction of PVP11 strain, studied under reducing conditions. Lanes 2, 3 and 4 correspond to cell lysates, disruption pellet and disruption supernatant of PVP12-induced cells, respectively, analyzed under reducing conditions. The protein showed the expected size of about 60 kDa. Lane 5 corresponds to PVP12 disruption supernatant run under non-reducing conditions, in which no band could be detected. MW and numbers in the left indicate the migration of standard molecular weight markers.

3.2. Characterization of structural and antigenic differences in VP60 variants produced in P. pastoris

The recombinant VP60 expressed in PVP12 strain was found only in the soluble fraction after cell disruption. No recombinant protein was detected in the pellet fraction. In order to characterize the released antigen, raw disruption supernatant was analyzed by sec-HPLC in a TSK G5000 PW column. As a control the highly particulated HBsAg, which was also expressed in *P. pastoris* MP36-transformed cells (Hardy et al., 2000), was analyzed under equivalent conditions. The analysis by immunodot of all fractions from the disruption supernatant confirmed the presence of the protein as a putative multimer only in the first maximum collected with a retention time (RT) of approximately 33 min. The chromatographic pattern revealed that the protein eluted as a uniform peak close to the particulate form of HBsAg, which corresponds to a known molecular mass of about 2×10^6 Da and a RT of 32 min (Fig. 2A–C). The pattern obtained pointed as well to the homogeneity of the self-assembled multimers. The recombinant VP60 solubilized from the disruption pellet of PVP11 strain exhibited a similar pattern after the analysis in the same column. This protein eluted with a RT of 31 min, slightly inferior to that of HBsAg, thus also indicating the formation of high molecular weight structures (Fig. 2D). In the same run, the monomeric VP60 from PVP11 was collected with a RT of approximately 53 min.

Samples from raw disruption supernatant of PVP12 cells were subjected to ultrafiltration experiments using membranes of 10^5 and 3×10^5 Da cut-off. The VP60 antigen solubilized from the cell debris fraction of *P. pastoris* PVP11 strain was included in the experiment. Quantification by sandwich ELISA showed that 100% and approximately 80% of the soluble VP60 expressed without the signal peptide was effectively retained by the membranes of 10^5 and 3×10^5 Da cut-off, respectively. On the contrary, the antigen solubilized from PVP11 strain was retained only by the 10^5 Da cut-off membrane, while no significant quantities of the protein were retained by the second filter. This could be in agreement with the fact that protein aggregates rather than ordered capsid-like structures were expressed in *P. pastoris* PVP11 strain. Such aggregates, with an apparent molecular weight of 250–300 kDa, were easily observed in SDS-PAGE or Western blot under non-reducing conditions, before or after solubilization of VP60 from the cell debris fraction (not shown). They were separated from the monomeric fraction by using membranes of 100,000 Da cut-off.

The VP60 antigen purified from the disruption supernatant of PVP12 strain was analyzed by equilibrium (20–70% [w/v]) and rate-zonal (10–30% [w/v]) sucrose density gradients ultracentrifugation. In both cases, the sucrose gradient was fractionated after centrifugation and the individual fractions were assayed by immunodot for the detection of VP60, which was quantified by ELISA. In the equilibrium sucrose gradient, one fraction was collected containing most of the recombinant antigen that migrated to a position corresponding to a buoyant density in the range of 1.29–1.32 g mL^{-1} (Fig. 3). The RHDV sample run under similar conditions migrated to a higher buoyant density of approximately 1.35 g mL^{-1}, which was expectable due to its nucleic acids content. The sedimentation coefficient for VP60, calculated from the 10–30% [w/v] sucrose gradient experiment and the use of RHDV as a control of 153S, was estimated in about 120S. The latter indicated that soluble particles similar in size and shape, but with a slight difference in buoyant

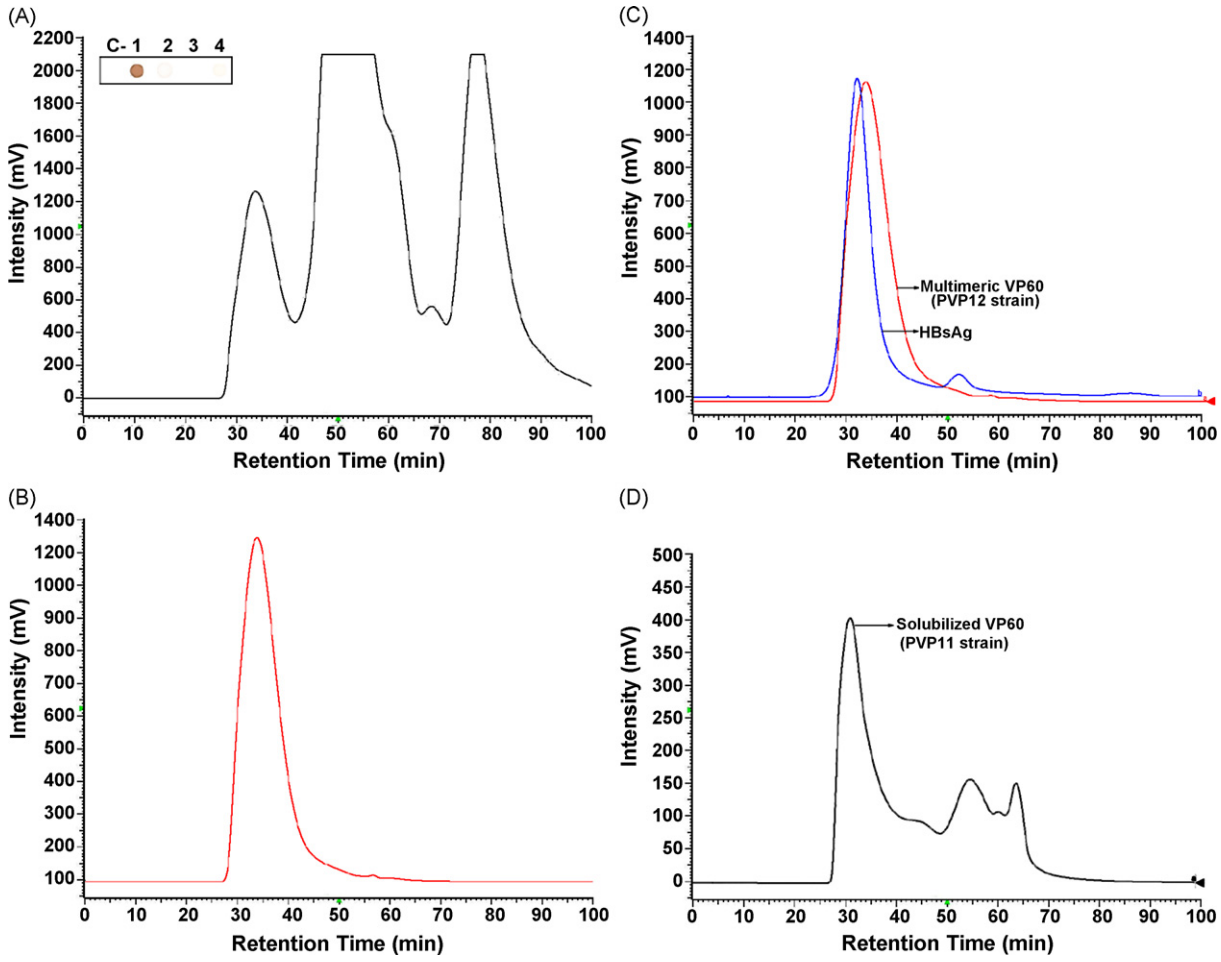

Fig. 2. Analysis by sec-HPLC in a TSK G5000 PW column of VP60 from *P. pastoris* PVP12 and PVP11 strains. (A) Raw disruption supernatant from PVP12 strain, in which VP60 was detected by mAb 1H8 with a retention time (RT) of approximately 33 min. The inner box shows VP60 detection in the first of the maximums collected. c- indicates raw disruption supernatant from wild-type yeast. (B) Purified VP60 protein. (C) The particulate form of HBsAg expressed in *P. pastoris* MP36-transformed cells (molecular mass of about 2×10^6 Da, RT = 32 min) was analyzed under the same conditions to confirm the multimeric structure of VP60. (D) VP60 solubilized from the disruption pellet of *P. pastoris* PVP11 strain. As determined by immunodot, this protein eluted with a RT = 31 min. Monomeric VP60 was detected in the same run with a RT of approximately 53 min.

density to RHDV were intracellularly produced by *P. pastoris* PVP12 strain.

The study of the antigenic profile displayed by both the soluble and insoluble VP60 recombinant variants was also conducted with

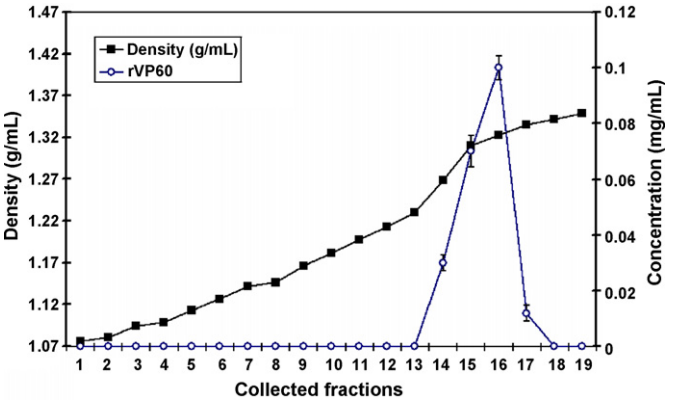

Fig. 3. Analysis of VP60 from *P. pastoris* PVP12 strain in equilibrium sucrose (20–70% [w/v]) density gradient ultracentrifugation experiments. After centrifugation, the sucrose gradient was fractionated and the individual fractions were assayed by immunodot for the detection of VP60, which was quantified by ELISA. Most of the recombinant protein migrated to a position corresponding to a buoyant density in the range of 1.29–1.32 g mL^{-1}.

the use of mAbs 1H8, 6H6 and 6G2 that recognize conformational epitopes in the RHDV Bs.89 strain. The recombinant VP60 expressed in PVP11 strain under the ss*SUC2* was specifically recognized in immunodot by mAbs 6H6 and 6G2, and was recognized only by mAb 6H6 in a sandwich ELISA. An identical result was obtained after enzymatic deglycosylation of this protein with PNGase F, suggesting that N-glycosylation was not responsible for the apparent lack of the epitope recognized by mAb 1H8. In addition to the fact that N-glycans were added to the amino acid structure of VP60 from PVP11 strain, O-glycans should be also present as the deglycosylation assay produced a recombinant protein size of about 63–65 kDa (Fig. 4). On the other hand, the soluble VP60 released after *P. pastoris* PVP12 cells disruption was detected in immunodot by all the corresponding mAbs and was recognized in sandwich ELISA by mAbs 1H8 and 6H6 (Fig. 5). A further experiment was conducted to analyze the recognition of the above-mentioned epitopes on VP60 after spray-drier treatment of the pellet fraction from PVP11. Both mAbs 6H6 and 6G2 reacted against the recombinant protein in immunodot or ELISA when assayed immediately after drying or three years later, in which case the powder was maintained at 4 °C. The multimeric VP60 obtained from PVP12 strain also retained the antigenic properties after ultrafiltration and chromatographic experiments.

Both the soluble form of VP60 obtained from *P. pastoris* PVP12 cells and VP60 solubilized from PVP11 disruption pellet were subjected to electron microscopy. The analysis of yeast cells from

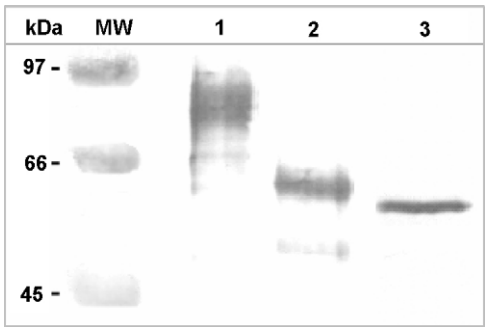

Fig. 4. Western blot after deglycosylation to determine the presence of N-glycans in the amino acid structure of VP60 obtained from PVP11 strain. The pattern obtained before (lane 1) and after (lane 2) deglycosylation suggested that O-glycans could be also present since an unexpected protein size of about 63–64 kDa was obtained. Lane 3 shows the 60-kDa recombinant VP60 from PVP12. MW and numbers in the left indicate the migration of standard molecular weight markers.

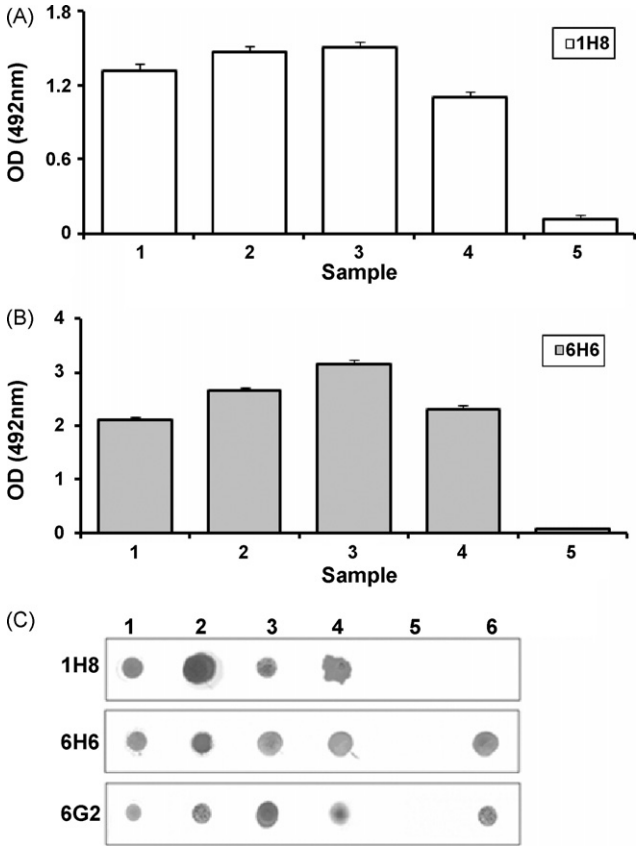

Fig. 5. Detection by sandwich ELISA (A and B) and immunodot (C) of RHDV conformational epitopes in the recombinant VP60 variants produced in *P. pastoris*. Equivalent quantities of the proteins were analyzed using the monoclonal antibodies 1H8 (A and C), 6H6 (B and C) or 6G2 (C), directed against the reference RHDV Bs.89 strain. As capture antibody, a polyclonal anti-RHDV serum was used. ELISA determinations were performed in duplicate and standard deviation bars are indicated. The sample numbers in each panel indicate the following: 1. RHDV (Bs.89 strain). 2. Raw disruption supernatant from *P. pastoris* PVP12 strain. 3. Same sample as 2, subjected to filtration through a 300,000 Da cut-off membrane. 4. VP60 expressed by the baculovirus/Sf9 system. 5. Cell lysates from MP36 *P. pastoris* strain. No significant differences were found in the absorbance values among samples 1 to 4 in ELISA. Sample 6 was included only in the immunodot and corresponds to insoluble disruption pellet from PVP11 strain.

PVP12 showed a homogeneous population of high-density structures resembling multimers or particles of VP60 with a diameter average of approximately 30 nm. These structures, with relative uniformity in size and shape, were observed only in cells from PVP12 and localized in vacuoles or inside autophagic bodies. Such structures were not detected in cells from *P. pastoris* MP36 wild-type strain (Fig. 6). On the other hand, the recombinant VP60 solubilized from the disruption pellet of *P. pastoris* PVP11 strain appeared to form protein aggregates (Fig. 6C) that were similar to those found for Bm86 and Bm95 vaccine antigens from the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*, which were previously expressed in the same *P. pastoris* MP36 strain (Rodríguez et al., 1994; García-García et al., 2000).

3.3. Immunization experiments and assessment of RHDV-specific immune responses

To investigate the immunogenicity of the soluble variant of VP60 expressed in *P. pastoris* PVP12 strain, New Zealand rabbits were immunized as previously detailed. The anti-RHDV specific antibodies were first assayed using a single-dilution inhibition ELISA, which test their ability to compete in the liquid phase with binding of RHDV to specific polyclonal antibodies bound to the solid phase. As shown in Table 1, all the animals from the group immunized with the soluble VP60 purified from the disruption supernatant of PVP12 cells developed antibody levels with inhibition values over 90% at 14 days post immunization. At this point, similar levels were obtained in 3 of 5 animals injected with raw disruption supernatant from PVP12 strain; in 2 of 5 injected with VP60 contained in the disruption pellet of PVP11 strain; and in all animals from the group immunized with VP60 from Sf9 cells. Table 2 shows a long-term follow up analysis expressed as the mean of inhibition values determined from serum of individual animals in each group, starting at day 0 of the experiment until one year after the primary immunization. Except for the group immunized by the oral route with the insoluble VP60 variant contained within the cell debris fraction, all vaccinated animals maintained a long-lasting and homogeneous specific humoral immune response with inhibition levels superior or close to 90%. The group immunized by the oral route developed lower levels, which were maintained for a shorter period of time. This group received three doses, in contrast with the other groups, taking into account the possible antigen degradation in gastrointestinal associated lymphoid tissues, and aiming to maximize systemic immune responses, as supported by data obtained from previous assessments (Farnós et al., 2005). The antibody titers were also determined at days 0, 60 and 365 in all the experimental groups using RHDV and competition ELISA (Fig. 7). The results coincided with data previously obtained in the one-year follow-up survey. At day 60, the groups immunized with the soluble VP60 showed the higher titers that ranged from 1/544 to 1/1312. These titers were comparable with titers from the animals injected with the baculovirus-derived VP60. At this time point, statistical significant differences ($p < 0.05$) were only found between the animals orally immunized with VP60 or with inactivated RHDV and the rest of the groups that received the recombinant proteins. Animals from the placebo group remained seronegative. At day 365, although a decline in specific titers was noticed, the higher levels were also detected in animals vaccinated with the soluble VP60 variant (titers over 1/100) and with VP60 from Sf9 cells. Statistical significant differences were found between the animals orally immunized with VP60 and the rest of the immunized groups. Finally, the specificity of antibodies against the distinct "RHDVa" subtype was determined. This pathogenic variant was employed instead of "classical" RHDV in a similar competition enzymatic assay. With the exception of animals immunized with the RHDV-inactivated vaccine, all the rabbits

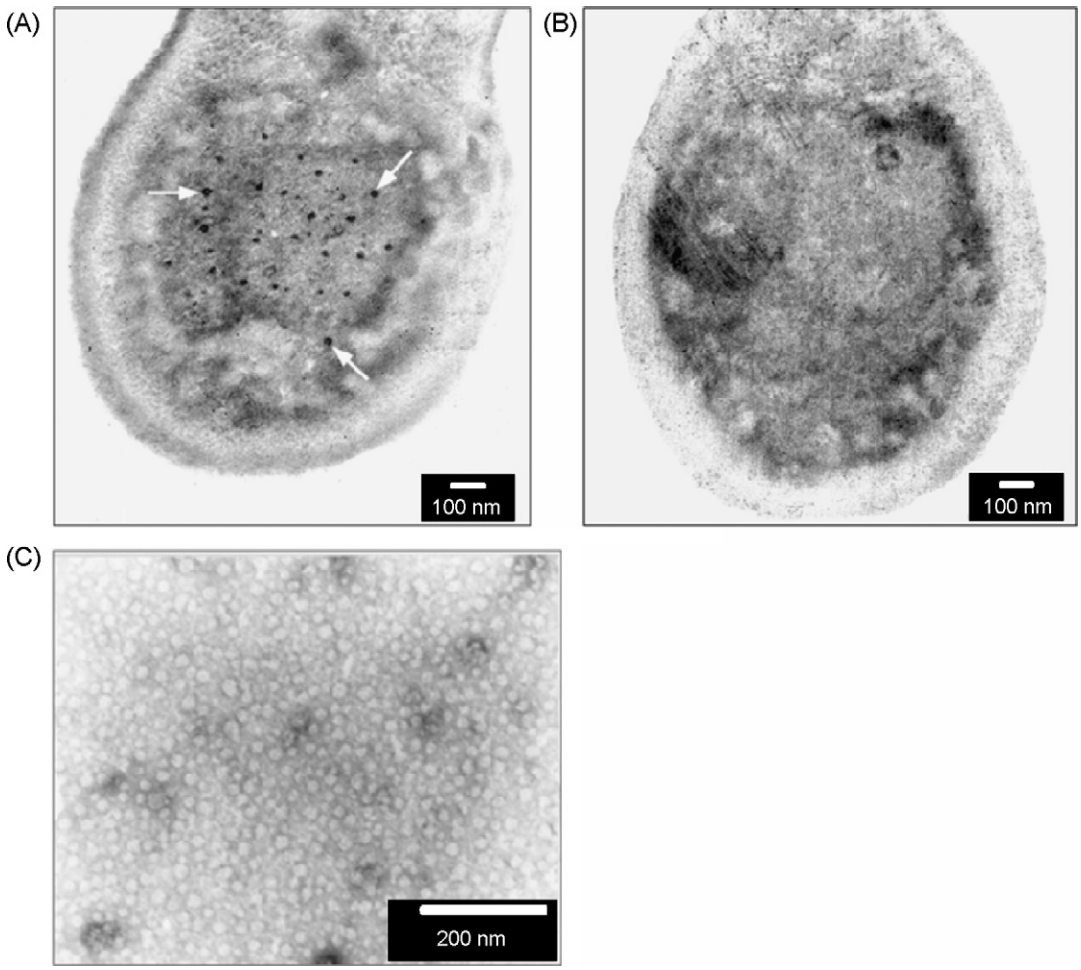

Fig. 6. Analysis by electron microscopy of recombinant PVP12 yeast cells. (A) The study showed high-density structures resembling multimers or particles of VP60 with a diameter of approximately 30 nm. These structures, signalled with arrows, were relatively uniform in size and shape and were observed only in PVP12 cells. (B) The latter structures were not detected in cells from *P. pastoris* wild-type MP36 strain. Panel (C) shows the formation of protein aggregates in VP60 solubilized from the cell debris fraction of PVP11 disrupted cells.

immunized with the recombinant VP60 variants produced in *P. pastoris* or in Sf9 cells developed high levels of specific anti-"RHDVa" antibodies from days 14 to 365 (Table 3). Rabbits injected with the RHDV-inactivated vaccine developed antibodies with a diminished capacity to compete in the liquid phase of the ELISA for binding to "RHDVa". This result was later confirmed by their complete lack of hemagglutination inhibition activity in vitro against "RHDVa" (Table 4). On the contrary, all the animals immunized with the recombinant variants of VP60 by using the oral or subcutaneous approaches developed early HI titers that increased until levels ranging from 1/160 to 1/40 960 in the last determination practiced. Positive controls in the HI assay reached HI values of 1/2560. The variations observed between animals in the same group suggested that such differences were independent of the formulation or the route employed. In summary, the highest HI titers, which correlate with the in vivo protective capacity against RHDV, were obtained in the groups of rabbits subcutaneously immunized with VP60 produced in *P. pastoris* and with VP60 obtained in Sf9 cells.

Table 1
Antibody levels in individual animals against RHDV measured 14 days after the primary immunization and expressed as percents (%) of inhibition determined in a competition ELISA.

Rabbit number	Group 1 (VP60-PVP12/purified, via s.c.)	Group 2 (VP60-PVP12/disruption supernatant, via s.c.)	Group 3 (VP60-PVP11 disruption pellet, via oral)	Group 4 (VP60-PVP11 disruption pellet, via s.c.)	Group 5 (VP60 baculovirus/Sf9, via s.c.)	Group 6 (RHDV-inactivated vaccine, via s.c.)	Group 7 (Placebo)
1	93.3	89.5	0.8	91.1	93.7	90.6	0
2	92.9	92.9	0.0	73.1	93.9	43.0	0
3	93.2	93.1	0.2	83.2	93.8	63.4	0
4	93.3	39.6	0.0	78.3		28.4	
5	93.1	40.2	0.0	91.5		61.4	

The result is expressed as the inhibition value for each animal, calculated as follows: %inhibition = [OD(neg. control serum) − OD(serum sample)] × 100/OD(neg. control serum). RHDV from the reference strain Bs.89 was used in the experiment. Sera were considered negative when the absorbance value at 492 nm decreased by less than 15% of the negative control serum. They were considered positive when decreased by more than 25%.

Table 2
Long-term follow up (from day 0 of the experiment to one year after the primary immunization) of antibody levels against RHDV measured as percent (%) of inhibition in a competition ELISA.

Days	ELISA inhibition percent (anti-RHDV)						
	Group 1 (VP60-PVP12/purified, via s.c.)	Group 2 (VP60-PVP12/disruption supernatant, via s.c.)	Group 3 (VP60-PVP11 disruption pellet, via oral)	Group 4 (VP60-PVP11 disruption pellet, via s.c.)	Group 5 (VP60 baculovirus/Sf9, via s.c.)	Group 6 (RHDV-inactivated vaccine, via s.c.)	Group 7 (Placebo)
0	0^a	0^a	0^a	0^a	0^a	0^a	0^a
14	93.1 ± 0.1^a	71.0 ± 25.4^a	0.1 ± 0.3^b	83.4 ± 12.4^a	93.7 ± 0.1^a	57.3 ± 19.4^c	0^d
40	93.9 ± 0.3^a	93.5 ± 1.3^a	72.9 ± 18.3^b	93.2 ± 0.6^a	95.0 ± 0.8^a	80.7 ± 17.2^a	0^c
60	91.6 ± 1.4^a	92.5 ± 0.5^a	82.2 ± 15.7^a	92.3 ± 1.4^a	91.9 ± 1.2^a	85.3 ± 10.0^a	0^b
90	92.2 ± 0.4^a	92.1 ± 0.9^a	53.1 ± 31.5^b	91.7 ± 1.3^a	91.2 ± 1.2^a	88.1 ± 5.2^a	0^c
180	92.5 ± 0.3^a	92.9 ± 0.2^a	58.9 ± 9.5^b	90.5 ± 0.6^a	93.5 ± 1.7^a	90.8 ± 1.4^a	0^c
270	92.8 ± 0.4^a	91.8 ± 3.1^a	60.8 ± 14.3^b	93.8 ± 1.2^a	93.6 ± 0.3^a	92.7 ± 3.5^a	0^c
365	91.6 ± 1.7^a	91.4 ± 0.6^a	55.8 ± 12.5^b	90.8 ± 3.1^a	92.1 ± 0.3^a	89.8 ± 3.9^a	0^c

The result is expressed as the mean ± S.D. of inhibition values from determinations in individual animals. The reference RHDV strain Bs.89 was used in the experiment. All groups receiving the recombinant proteins were immunized at days 0 and 21, except Group 3 that received an additional dose at day 40. Different letters within a row indicate statistical significant differences for $p < 0.05$, according to the Kruskal–Wallis and Dunns tests.

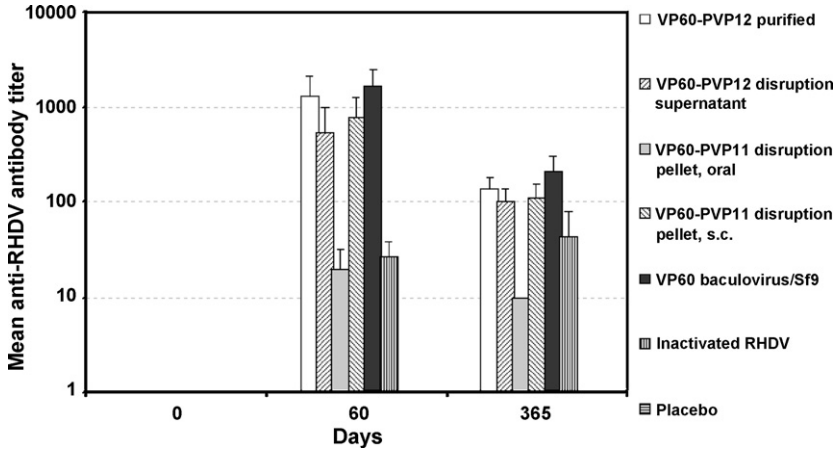

Fig. 7. Mean antibody titers to RHDV in rabbits immunized with the recombinant variants of VP60. Titers were determined using a competition ELISA and sera from days 0, 60 and 365 of the experiment. Titers were expressed as the reciprocal of the highest dilution in which the absorbance value decreased by more than 25% with respect to the equivalent dilution in the placebo group. Positive standard deviations bars are indicated in the figure. Statistical significant differences ($p < 0.05$) were found at days 60 and 365 between rabbits orally immunized with VP60 or injected with inactivated RHDV and the rest of immunized groups. Animals from the placebo group remained seronegative. An analysis of variance and a Bonferroni's Multiple Comparison test were used.

4. Discussion

An important number of approaches have been developed to date aiming to obtain a novel vaccine against RHDV unlike the traditional preparations produced from organs of infected rabbits. The capsid protein expressed in the systems assayed has been protective in most cases, including the capability to protect at very low doses, in the absence of adjuvants and by alternative approaches as administration by the oral route (Plana-Durán et al., 1996; Bárcena et al., 2000; Martín-Alonso et al., 2003; Pérez-Filgueira et al., 2007). Recently, the VP60 gene was expressed at about 1.5 g/L of culture in *P. pastoris* (Farnós et al., 2005) in a further attempt to produce a scalable vaccine against RHDV. The antigen obtained induced a potent RHDV-specific humoral and cell-

Table 3
Antibody levels against the distinct "RHDVa" subtype measured as percent (%) of inhibition in a competition ELISA.

Days	ELISA inhibition percent (anti-"RHDVa")						
	Group 1 (VP60-PVP12/purified, via s.c.)	Group 2 (VP60-PVP12/disruption supernatant, via s.c.)	Group 3 (VP60-PVP11 disruption pellet, via oral)	Group 4 (VP60-PVP11 disruption pellet, via s.c.)	Group 5 (VP60 baculovirus/Sf9, via s.c.)	Group 6 (RHDV-inactivated vaccine, via s.c.)	Group 7 (Placebo)
0	0^a	0^a	0^a	0^a	0^a	0^a	0^a
14	71.8 ± 5.8^a	52.4 ± 24.8^a	10.7 ± 9.8^b	29.6 ± 7.0^b	78.1 ± 9.7^a	12.0 ± 8.3^b	0^c
40	75.7 ± 2.7^a	73.1 ± 6.3^a	17.7 ± 11.2^b	69.8 ± 6.3^a	73.4 ± 1.9^a	30.7 ± 16.5^c	0^d
60	85.4 ± 5.2^a	84.8 ± 2.0^a	55.0 ± 14.1^b	79.8 ± 4.4^a	89.2 ± 1.4^a	57.4 ± 14.1^c	0^d
90	84.4 ± 1.6^a	83.8 ± 1.5^a	52.2 ± 6.3^b	80.4 ± 5.7^a	85.5 ± 2.7^a	61.5 ± 19.7^c	0^d
180	86.7 ± 0.9^a	84.2 ± 1.5^a	38.8 ± 16.8^b	83.8 ± 1.0^a	82.2 ± 1.2^a	60.4 ± 27.6^c	0^d
270	89.2 ± 0.8^a	85.5 ± 4.6^a	40.7 ± 7.9^b	84.9 ± 2.9^a	83.5 ± 4.7^a	60.9 ± 24.4^c	0^d
365	88.5 ± 1.8^a	81.5 ± 3.7^a	39.0 ± 11.4^b	84.6 ± 7.7^a	88.8 ± 0.3^a	59.4 ± 26.3^c	0^d

The results are shown from day 0 to one year after the primary immunization.
The result is expressed as the mean ± S.D. of inhibition values from individual animals. The Cuban "RHDVa" strain CUB5-04 was used in the experiment. All groups receiving the recombinant proteins were immunized at days 0 and 21, except Group 3 that received an additional dose at day 40. Different letters within a row indicate statistical significant differences for $p < 0.05$, according to the Kruskal–Wallis and Dunns tests.

Table 4
Hemagglutination inhibition titers detected in sera of rabbits immunized with the recombinant VP60 capsid protein variants obtained in *P. pastoris*, in Sf9 cells or vaccinated with inactivated RHDV.

Days	Hemagglutination inhibition titers against "RHDVa"						
	Group 1 (VP60-PVP12/purified, via s.c.)	Group 2 (VP60-PVP12/disruption supernatant, via s.c.)	Group 3 (VP60-PVP11 disruption pellet, via oral)	Group 4 (VP60-PVP11 disruption pellet, via s.c.)	Group 5 (VP60 baculovirus/Sf9, via s.c.)	Group 6 (RHDV-inactivated vaccine, via s.c.)	Group 7 (Placebo)
0	0	0	0	0	0	0	0
	1/80	0	0	1/80	1/160	0	0
	1/160	0	0	1/80	0	0	0
14	0	0	0	0	0	0	0
	0	0	0	0		0	
	0	0	0	0		0	
	1/320	1/160	0	1/320	1/160	0	0
	1/320	1/160	0	1/320	1/320	0	0
40	1/80	1/80	0	1/80	1/320	0	0
	0	0	0	0		0	
	0	0	0	0		0	
	1/40960	1/5120	1/320	1/640	1/640	0	0
	1/5120	1/640	1/160	1/2560	1/20480	0	0
60	1/5120	1/5120	1/160	1/5120	1/2560	0	0
	1/5120	1/2560	0	1/2560		0	
	1/2560	1/320	0	1/640		0	

The titer was expressed as the maximum dilution capable to completely inhibit the agglutination of human type O erythrocytes by RHDV. In the assay, hyperimmune sera against RHDV or "RHDVa" (with HI titers of 1/2560) were included as positive controls. All groups receiving the recombinant proteins were immunized at days 0 and 21, except Group 3 that received an additional dose at day 40.

mediated immune response (Farnós et al., 2006) although the scale up and further implementation of this system is limited by the fact that VP60 is obtained insoluble under the *S. cerevisiae SUC2* secretion signal. Nevertheless, various examples support VP60 production in *P. pastoris* as a medium to advance beyond the laboratory stages. For instance, the Gavac®plus vaccine against the cattle tick *R. microplus* consists of the Bm86 antigen expressed and purified from the same mutant in a similar number of analogous steps (Canales et al., 1997; de la Fuente et al., 1998, 2007; Valle et al., 2004). Other vaccines or vaccine candidates produced from this yeast strain are currently under wide use in human populations (e.g. subunit vaccine against HBV) (Pentón et al., 1994; Hardy et al., 2000; Jain et al., 2000; Estévez et al., 2007) or will enter phase I clinical trials (e.g. subunit candidates against HCV and dengue virus) (Muné et al., 2003; Valdés et al., 2007). Having these facts in mind, and considering that a veterinary vaccine for a species of moderate economical importance as rabbits should imply a simple process of antigen expression and separation, we produced the VP60 capsid protein in *P. pastoris* MP36 strain lacking a secretion signal. Although the expression levels reached were three times lower with respect to the insoluble VP60, several advantages appeared obvious for this soluble variant. First, the high molecular weight structures produced in PVP12 strain resembled multimers or virus-like particles as those of HBV or HCV produced in the same yeast (Tleugabulova et al., 1998; Acosta-Rivero et al., 2004). The capacity of this strain to yield ordered multimeric structures have been previously demonstrated by the expression of various antigens from human viruses (Hardy et al., 2000; Muné et al., 2003; Acosta-Rivero et al., 2004; Valdés et al., 2007). Such ordered structures were apparently absent in VP60 obtained insoluble in PVP11, which seemed to yield protein aggregates of a molecular weight close to 250–300 kDa. These aggregates coincided with the event observed for particulate antigens from tick gut cells expressed in *P. pastoris* MP36 strain under the *SUC2* secretion signal. These antigens were obtained in a non-secreted form, and associated to the yeast membranous system despite the correct processing of the signal peptide (Montesino et al., 1996; García-García et al., 2000). The presence of glycans N-linked to the insoluble VP60 indicated the signal processing and the translocation of the protein into the endoplasmatic reticulum, thus leaving unclear the protein association to the insoluble cell components. A second advantage of the soluble VP60 was its recognition by the three conformational-sensitive monoclonal antibodies used. On the opposite, VP60 from PVP11 was recognized by two of these mAbs. Of particular importance was the recognition of the soluble VP60 by mAb 1H8, which reacts against a protective epitope in RHDV (not present in "RHDVa") that is missing in unassembled viral particles (Capucci et al., 1995) or aberrant capsids.

In addition, similarities in shape, mass and buoyant density to RHDV found for VP60 from PVP12 were described by authors that previously expressed VP60 VLPs in insect cultured cells (Laurent et al., 1994; Sibilia et al., 1995; Bárcena et al., 2004; Gromadzka et al., 2006). Although both antigens from *P. pastoris* were capable to elicit high HI titers and anti-RHDV/"RHDVa" specific antibodies, the presence of a higher number of protective epitopes makes the intracellular protein a more universal antigen. Moreover, the relative small difference found in the IgG response and HI titers between the groups vaccinated with the soluble VP60 purified or contained within the raw disruption supernatant supports the idea that an extensive purification process (e.g. including a chromatographic step) might not be necessary for vaccine use. It could be possible to develop a single step of supernatant filtration through 100,000 or 300,000 Da cut-off membranes to obtain a suitable material for formulation in adjuvant. In this sense, recent approaches in the veterinary field consider vaccination strategies with the use of cellular fractions that do not interfere with the immune response expected (Shin et al., 2007; Xia et al., 2007). In our experiment, a high number of contaminants were present in the immunogen consisting of VP60 within the cell debris fraction administered to rabbits by subcutaneous injection. These animals were yet capable of generating high titers of specific antibodies with hemagglutination inhibition activity. In a different approach, a formulation consisting of the antigen within the yeast disruption pellet (from PVP11) orally administered seemed an interesting strategy for vaccination (Farnós et al., 2005; Xia et al., 2007) taking into account that VP60 by

this route has been protective against RHDV (Plana-Durán et al., 1996; Bertagnoli et al., 1996a; Bárcena et al., 2000; Farnós et al., 2005). Moreover, *P. pastoris* is an FDA-approved additive for animal food (http://www.fda.gov/cvm/FOI/food_add_permitted.htm) thus the results obtained must not discard its use by this route. Adjuvants and release systems for mucosal administration may enhance those responses that are stronger when using systemic routes (Lavelle and O'Hagan, 2006). Viral proteins from human pathogens expressed in this yeast strain have induced both systemic and mucosal immunity in various animal models (Aguilar et al., 1998, 2003; Lobaina et al., 2003).

The VP60 variant expressed as a soluble multimer was also capable to develop an early and long-lasting immune response maintained for one year. Although a decline in specific titers was noticed at this time, such pattern was also obtained with different approaches as the Sf9-expressed VP60 and inactivated RHDV. The latter is an expectable fact considering that no re-exposure to the antigen occurred in the whole period. Hence, the immunity achieved provides the opportunity to explore a revaccination schedule in no less than 12 months. On the other hand, both an experiment of minimum protective dose and analyses of costs of the antigen separation processes, are obligatory steps in a further developmental phase of this antigen. The specificity found against the "classical" Bs.89 strain and against the antigenic variant CUB5-04 pointed to a broad spectrum of protective capability of the multimers expressed in *P. pastoris*. In this sense, the lack of hemagglutination inhibition titers against "RHDVa" observed in the animals immunized with the RHDV-inactivated vaccine might be explained by a reduced cross-reactivity between the corresponding RHDV and "RHDVa" strains. This is supported by the fact that the inactivated vaccine generated similar IgG levels to those elicited by the rest of the antigens when assayed against the Italian Bs.89 strain. Such possible failure in cross-reactivity has been already described (Schirrmeier et al., 1999). In summary, this work presents the characterization of two different variants of the recombinant VP60 capsid protein from RHDV expressed at high levels in *P. pastoris*. The experiments conducted and the analysis on the suitability of each antigen for vaccination, lead to the conclusion that VP60 obtained as a soluble multimer in PVP12 strain is the most attractive candidate presented so far for an easy and inexpensive large-scale production and further implementation of a recombinant subunit vaccine against RHDV.

Acknowledgments

The authors would like to acknowledge the assistance of Dr. Viviana Falcón and Ivón Menéndez from the Electron Microscopy department of the CIGB. MSc. Carlos Montero, Dunia Sánchez, and technical personnel from CENSA are also acknowledged for inestimable cooperation related to immunization experiments in rabbits. We specially thank Dr. Lorenzo Capucci and the Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia, Italy, for kindly providing inactivated RHDV (Bs.89 strain) antigen and monoclonal antibodies.

References

Acosta-Rivero, N., Rodríguez, A., Musacchio, A., Falcón, V., Suárez, V.M., Chávez, L., Morales-Grillo, J., Dueñas-Carrera, S., 2004. Nucleic acid binding properties and intermediates of HCV core protein multimerization in *Pichia pastoris*. Biochem. Biophys. Res. Commun. 323, 926–931.

Aguilar, J.C., Pichardo, D., Muzio, V., Guillén, G., Veliz, G., Leal, M.J., Pentón, E., 1998. Formulations containing virus-like particles as mucosal adjuvant. CU 98/December, PCT/CU/99/00006.

Aguilar, J.C., Acosta-Rivero, N., Dueñas-Carrera, S., Morales, J., Pichardo, D., Urquiza, D., Guillén, G., Muzio, V., 2003. HCV core protein modulates the immune response against the HBV surface antigen in mice. Biochem. Biophys. Res. Commun. 310, 59–63.

Bárcena, J., Morales, M., Vázquez, B., Boga, J.A., Parra, F., Lucientes, J., Pagés-Mantés, A., Sánchez-Vizcaíno, J.M., Blasco, R.Y., Torres, J.M., 2000. Horizontal transmissible protection against Myxomatosis and Rabbit Haemorrhagic Disease by using a recombinant Myxoma virus. J. Virol. 74, 1114–1123.

Bárcena, J., Verdaguer, N., Roca, R., Morales, M., Angulo, I., Risco, C., Carrascosa, J.L., Torres, J.M., Castón, J.R., 2004. The coat protein of rabbit hemorrhagic disease virus contains a molecular switch at the N-terminal region facing the inner surface of the capsid. Virology 322, 118–134.

Becker, D.M., Guarente, L., 1991. High-efficiency transformation of yeast by electroporation. Methods Enzymol. 194, 182–187.

Bertagnoli, S., Gelfi, J., Le Gall, G., Boilletot, E., Vautherot, J.F., Rasschaert, D., Laurent, S., Petit, F., Boucrat-Baralon, C., Milon, A., 1996a. Protection against myxomatosis and rabbit viral hemorrhagic disease with recombinant myxoma viruses expressing rabbit hemorrhagic disease virus capsid protein. J. Virol. 70, 5061–5066.

Bertagnoli, S., Gelfi, J., Petit, F., Vautherot, J.F., Rasschaert, D., Laurent, S., Le Gall, G., Boilletot, E., Chantal, J., Boucraut-Baralon, C., 1996b. Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus. Vaccine 14, 506–510.

Boga, J.A., Casais, R., Marin, M.S., Martin-Alonso, J.M., Carmenes, R.S., Prieto, M., Parra, F., 1994. Molecular cloning, sequencing and expression in *Escherichia coli* of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). J. Gen. Virol. 75, 2409–2413.

Boga, J.A., Martín-Alonso, J.M., Casais, R., Parra, F., 1997. A single dose immunization with rabbit haemorrhagic disease virus major capsid protein produced in *Saccharomyces cerevisiae* induces protection. J. Gen. Virol. 78, 2315–2318.

Bullock, W.O., Fernández, J.M., Short, J.M., 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques 5, 376–379.

Calvete, C., 2006. Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease. Conserv. Biol. 20, 1232–1241.

Canales, M., Enríquez, A., Ramos, E., Cabrera, D., Dandie, H., Soto, A., Falcón, V., Rodríguez, M., de la Fuente, J., 1997. Large-scale production in *Pichia pastoris* of the recombinant vaccine Gavac™ against cattle tick. Vaccine 15, 414–422.

Capucci, L., Frigoli, G., Roenshold, L., Lavazza, A., Brocchi, E., Rossi, C., 1995. Antigenicity of the rabbit hemorrhagic disease virus studied by its reactivity with monoclonal antibodies. Virus Res. 37, 221–238.

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. J. Virol. 70, 8614–8623.

Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M.L., Brocchi, E., 1998. A further step in the evolution of rabbit hemorrhagic disease virus: the appearance of the first consistent antigenic variant. Virus Res. 58, 115–126.

Castañón, S., Marín, M.S., Martín-Alonso, J.M., Boga, J.A., Casais, R., Humara, J.M., Ordás, R.J., Parra, F., 1999. Immunization with potato plants expressing VP60 protein protects against Rabbit Haemorrhagic Disease Virus. J. Virol. 73, 4452–4455.

de la Fuente, J., Rodríguez, M., Redondo, M., Montero, C., García-García, J.C., Méndez, L., Serrano, E., Valdés, M., Enríquez, A., Canales, M., Ramos, E., Boué, O., Machado, H., Lleonart, R., de Armas, C.A., Rey, S., Rodríguez, J.L., Artiles, M., García, L., 1998. Field studies and cost-effectiveness analysis of vaccination with Gavac against the cattle tick *Boophilus microplus*. Vaccine 16, 366–373.

de la Fuente, J., Almazán, C., Canales, M., Pérez de la Lastra, J.M., Kocan, K.M., Willadsen, P., 2007. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim. Health Res. Rev. 8, 23–28.

Estévez, Z.C., Betancourt, A.A., Muzio-González, V., Baile, N.F., Silva, C.V., Bernal, F.H., Arias, E.P., Delhanty-Fernández, A., Olazábal, N.M., del Río-Martín, A., Batista, L.L., Véliz-Ríos, G., Hernández, H.H., Hernández, A.B., Lugo, E.P., de la Torre-Cruz, J., Batista-Marchec, B.L., Falcón, L.A., Brito, J.T., León, D.O., Saura, P.L., 2007. Immunogenicity and safety assessment of the Cuban recombinant hepatitis B vaccine in healthy adults. Biologicals 35, 115–122.

Farnós, O., Boué, O., Parra, F., Martín-Alonso, J.M., Joglar, M., Valdés, O., Navea, L., Naranjo, P., Lleonart, R., 2005. High-level expression and immunogenic properties of the recombinant rabbit hemorrhagic disease virus VP60 capsid protein obtained in *Pichia pastoris*. J. Biotechnol. 117, 215–224.

Farnós, O., Rodríguez, M., Chiong, M., Parra, F., Boué, O., Lorenzo, N., Colás, M., Lleonart, R., 2006. The recombinant rabbit hemorrhagic disease virus VP60 protein obtained from *Pichia pastoris* induces a strong humoral and cell-mediated immune response following intranasal immunization in mice. Vet. Mic. 114, 187–195.

Farnós, O., Rodríguez, D., Valdés, O., Chiong, M., Parra, F., Toledo, J.R., Fernández, E., Lleonart, R., Suárez, M., 2007. Molecular and antigenic characterization of rabbit hemorrhagic disease virus isolated in Cuba indicates a distinct antigenic subtype. Arch. Virol. 152, 1215–1221.

Fernández-Fernández, M.R., Mourino, M., Rivera, J., Rodríguez, F., Plana-Duran, J., García, J.A., 2001. Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. Virology 280, 283–291.

Ferreira, P.G., Costa-e-Silva, A., Oliveira, M.J., Monteiro, E., Cunha, E.M., Aguas, A.P., 2006. Severe leukopenia and liver biochemistry changes in adult rabbits after calicivirus infection. Res. Vet. Sci. 80, 218–225.

Fischer, L., Le Gros, F.-X., Mason, P.W., Paoletti, E., 1997. A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. Vaccine 15, 90–96.

Forrester, N.L., Trout, R.C., Gould, E.A., 2007. Benign circulation of rabbit haemorrhagic disease virus on Lambay Island. Eire. Virol. 358, 18–22.
Forrester, N.L., Moss, S.R., Turner, S.L., Schirrmeier, H., Gould, E.A., 2008. Recombination in rabbit haemorrhagic disease virus: possible impact on evolution and epidemiology. Virology 376, 390–396.
García-García, J.C., Montero, C., Redondo, M., Vargas, M., Canales, M., Boué, O., Rodríguez, M., Joglar, M., Machado, H., González, I.L., Valdés, M., Méndez, L., de la Fuente, J., 2000. Control of ticks resistant to the immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. Vaccine 18, 2275–2287.
Gil, F., Titarenko, E., Terrada, E., Arcalis, E., Escribano, J.M., 2006. Successful oral prime-immunization with VP60 from rabbit haemorrhagic disease virus produced in transgenic plants using different fusion strategies. Plant Biotechnol. J. 4, 135–143.
Gromadzka, B., Szewczyk, B., Konopa, G., Fitzner, A., Kesy, A., 2006. Recombinant VP60 in the form of virion-like particles as a potential vaccine against rabbit hemorrhagic disease virus. Acta Biochim. Pol. 53, 371–376.
Hardy, E., Martínez, E., Diago, D., Díaz, R., González, D., Herrera, L., 2000. Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*. J. Biotechnol. 77, 157–167.
Jain, A., Mathur, U.S., Jandwani, P., Gupta, R.K., Kumar, V., Kar, P., 2000. A multicentric evaluation of recombinant DNA hepatitis B vaccine of Cuban origin. Trop. Gastroenterol. 21, 14–17.
Lavelle, E.C., O'Hagan, D.T., 2006. Delivery systems and adjuvants for oral vaccines. Expert Opin. Drug Deliv. 3, 747–762.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Laurent, S., Vautherot, J.F., Madelaine, M.A., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into virus like particles and induces protection. J. Virol. 68, 6794–6798.
Liu, S.J., Xue, H.P., Pu, B.Q., Quian, N.H., 1984. A new viral disease in rabbits. Anim. Husb. Vet. Med. 16, 253–255.
Lobaina, Y., Garcia, D., Abreu, N., Muzio, V., Aguilar, J.C., 2003. Mucosal immunogenicity of the hepatitis B core antigen. Biochem. Biophys. Res. Commun. 300, 745–750.
Marchandeau, S., Le Gall-Reculé, G., Bertagnoli, S., Aubineau, J., Botti, G., Lavazza, A., 2005. Serological evidence for a non-protective RHDV-like virus. Vet. Res. 36, 53–62.
Marín, M.S., Martín-Alonso, J.M., Pérez-Ordoyo García, L.I., Argüello-Villares, J.L., Casais, R., Venugopal, K., Jiang, W., Gould, E.A., Parra, F., 1995. Immunogenic properties of rabbit haemorrhagic disease virus structural protein VP60 expressed by a recombinant baculovirus: an efficient vaccine. Virus Res. 39, 119–128.
Martín-Alonso, J.M., Castañon, S., Alonso, P., Parra, F., Ordas, R., 2003. Oral immunization using tuber extracts from transgenic potato plants expressing rabbit hemorrhagic disease virus capsid protein. Transgenic Res. 12, 127–130.
McIntosh, M.T., Behan, S.C., Mohamed, F.M., Lu, Z., Moran, K.E., Burrage, T.G., Neilan, J.G., Ward, G.B., Botti, G., Capucci, L., Metwally, S.A., 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. Virol. J. 4, 96.
Montesino, R., Cremata, J., Rodríguez, M., Besada, V., Falcón, V., de la Fuente, J., 1996. Biochemical characterization of the recombinant *Boophilus microplus* Bm86 antigen expressed by transformed *Pichia pastoris* cells. Biotechnol. Appl. Biochem. 23, 23–28.
Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. J. Gen. Virol. 83, 2461–2467.
Muné, M., Rodríguez, R., Ramírez, R., Soto, Y., Sierra, B., Rodríguez-Roche, R., Márquez, G., García, J., Guillén, G., Guzmán, M.G., 2003. Carboxy-terminally truncated Dengue 4 virus envelope glycoprotein expressed in *Pichia pastoris* induced neutralizing antibodies and resistance to Dengue 4 virus challenge in mice. Arch. Virol. 148, 2267–2273.
Parra, F., Prieto, M., 1990. Purification of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. 64, 4013–4015.
Parra, F., Boga, J.A., Marín, M.S., Casais, R., 1993. The amino sequence of VP60 from rabbit hemorrhagic disease virus supports its putative subgenomic origin. Virus Res. 27, 219–228.
Pentón, E., Muzio, V., González-Griego, M., 1994. The hepatitis B virus (HBV) infection and its prevention by a recombinant-DNA viral surface antigen (rec-HBsAg) vaccine. Biotecnología Aplicada 11, 1–11.
Pérez-Filgueira, D.M., Resino-Talaván, P., Cubillos, C., Angulo, I., Barderas, M.G., Bárcena, J., Escribano, J.M., 2007. Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. Virology 364, 422–430.
Plana-Durán, J., Bastons, M., Rodríguez, M.J., Climent, I., Cortés, E., Vela, C., Casal, I., 1996. Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch. Virol. 141, 1423–1436.
Rodríguez, M., Rubiera, R., Penichet, M., Montesino, R., Cremata, J., de la Fuente, J., 1994. High-level expression of the *B. microplus* Bm86 antigen in the yeast *Pichia pastoris* forming highly immunogenic particles for cattle. J. Biotechnol. 33, 135–146.
Rothstein, R., 1985. Cloning in yeast. In: Glover, D.M. (Ed.), DNA Cloning II. IRL Press, Oxford, pp. 45–46.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Schirrmeier, H., Reimann, I., Kollner, B., Granzow, H., 1999. Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterization of antigenic variants. Arch. Virol. 144, 719–735.
Shin, S.J., Shin, S.W., Kang, M.L., Lee, D.Y., Yang, M.S., Jang, Y.S., Yoo, H.S., 2007. Enhancement of protective immune responses by oral vaccination with *Saccharomyces cerevisiae* expressing recombinant *Actinobacillus pleuropneumoniae* ApxIA or ApxIIA in mice. J. Vet. Sci. 8, 383–392.
Sibilia, M., Boniotti, M.B., Angoscin, P., Capucci, L., Rossi, C., 1995. Two independent pathways of expression lead to self-assembly of the rabbit hemorrhagic disease virus capsid protein. J. Virol. 69, 5812–5815.
Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.
Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrast. Res. 26, 31–43.
Tian, L., Liao, J., Li, J.W., Zhou, W.R., Zhang, X.L., Wang, H.N., 2007. Isolation and identification of a non-haemagglutinating strain of rabbit hemorrhagic disease virus from China and sequence analysis for the VP60 gene. Virus Genes 35, 745–752.
Tleugabulova, D., Falcón, V., Sewer, M., Pentón, E., 1998. Aggregation of recombinant hepatitis B surface antigen in *Pichia pastoris*. J. Chromatogr. B Biomed. Sci. Appl. 716, 209–219.
Tunon, M.J., Sánchez-Campos, S., García-Ferreras, J., Álvarez, M., Jorquera, F., González-Gallego, J., 2003. Rabbit hemorrhagic viral disease: characterization of a new animal model of fulminant liver failure. J. Lab. Clin. Med. 141, 272–278.
Valdés, I., Hermida, L., Zulueta, A., Martín, J., Silva, R., Alvarez, M., Guzmán, M.G., Guillén, G., 2007. Expression in *Pichia pastoris* and immunological evaluation of a truncated Dengue envelope protein. Mol. Biotechnol. 35, 23–30.
Valle, M.R., Méndez, L., Valdez, M., Redondo, M., Espinosa, C.M., Vargas, M., Cruz, R.L., Barrios, H.P., Seoane, G., Ramírez, E.S., Boué, O., Vigil, J.L., Machado, H., Nordelo, C.B., Piñeiro, M.J., 2004. Integrated control of *Boophilus microplus* ticks in Cuba based on vaccination with the anti-tick vaccine Gavac. Exp. Appl. Acarol. 34, 375–382.
van de Bildt, M.W., van Bolhuis, G.H., van Zijderveld, F., van Riel, D., Drees, J.M., Osterhaus, A.D., Kuiken, T., 2006. Confirmation and phylogenetic analysis of rabbit hemorrhagic disease virus in free-living rabbits from the Netherlands. J. Wildl. Dis. 42, 808–812.
White, P.J., Trout, R.C., Moss, S.R., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., Hudson, P.J., 2004. Epidemiology of rabbit haemorrhagic disease virus in the United Kingdom: evidence for seasonal transmission by both virulent and avirulent modes of infection. Epidemiol. Infect. 132, 555–567.
Xia, M., Farkas, T., Jiang, X., 2007. Norovirus capsid protein expressed in yeast forms virus-like particles and stimulates systemic and mucosal immunity in mice following an oral administration of raw yeast extracts. J. Med. Virol. 79, 74–83.
Xu, Z.J., Chen, W.X., 1989. Viral haemorrhagic disease in rabbits: a review. Vet. Res. Commun. 13, 205–212.
Yang, L., Wang, F., Hu, B., Xue, J., Hu, Y., Zhou, B., Wang, D., Xu, W., 2008. Development of an RT-PCR for rabbit hemorrhagic disease virus (RHDV) and the epidemiology of RHDV in three eastern provinces of China. J. Virol. Methods 151, 24–29.
Yong, V., González, M.E., Herrera, L., Delgado, J., 1992. El gen *HIS3* de *Saccharomyces cerevisiae* complementa una mutación his$^-$ de *Pichia pastoris*. Biotecnología Aplicada 9, 55–61.