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Serological characterisation of *Lagovirus* virus-like particles originating from native and mutated VP60 of rabbit haemorrhagic disease virus 2 and European brown hare syndrome virus

Martyna Krejmer-Rąbalska[1]✉, Marta Peplińska[1], Bogusław Szewczyk[1], Andrzej Fitzner[2]

[1]Laboratory of Recombinant Vaccines, Intercollegiate Faculty of Biotechnology,
University of Gdańsk and Medical University of Gdańsk, 80-307 Gdańsk, Poland
[2]Department of Foot and Mouth Disease, National Veterinary Research Institute, 24-100 Puławy, Poland
martyna.krejmer-rabalska@ug.edu.pl

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Abstract

Introduction: Since lagoviruses cannot be cultivated *in vitro*, using expression systems is an alternative and promising way of producing diagnostic viral antigens. It opens up their use as active immunogens for vaccine production. **Material and Methods:** Virus-like particles (VLPs) were produced in a baculovirus expression system in *Spodoptera frugiperda* 9 (Sf9) insect cells based on wild-type and mutated variants of the virus capsid VP60 protein from a Polish strain of European brown hare syndrome virus (EBHSV) and wild-type and mutated versions of this protein from a Polish strain of rabbit haemorrhagic disease virus 2 (RHDV2). The mutations were the substitution of an arginylglycylaspartic acid (Arg-Gly-Asp/RGD) motif in the P2 subdomain and, in the S or P2 domain, the substitution of three lysines. The VLPs were purified with sucrose gradient ultracentrifugation. **Results:** Protein production was confirmed by Western blot analysis using rabbit or hare sera and ELISA tests with different types of monoclonal antibody. The haemagglutination properties of some VLPs were also evaluated. Electron microscopy of wild-type EBHSV, wild-type RHDV2 and the four VP60 variants produced in this experiment revealed the formation of characteristic VLP structures. **Conclusion:** For the first time, mutated VLPs of RHDV2 with an RGD motif in the VP60 sequence were obtained, which could potentially be used to deliver cargo to eukaryotic cells. Virus-like particles based on the VP60 proteins of EBHSV and RHDV with a three-lysine substitution in the S or P2 domains were also obtained. Potential exists for VLPs of EBHSV and RHDV2 as vaccine candidates.

Keywords: lagovirus, RHDV2, EBHSV, virus-like particles (VLPs), diagnostics.

Introduction

European brown hare syndrome (EBHS) and rabbit haemorrhagic disease (RHD) are two highly contagious, fatal and highly transmissible viral haemorrhagic plagues that emerged in the early 1980s in Europe and Asia, affecting hares and rabbits, respectively (2, 17). The rapid spread of RHD has caused severe economic losses in rabbit farming worldwide, which were particularly evident until the introduction of effective inactivated vaccines based on a virus extracted from the liver homogenates of infected rabbits. In addition, because of the high mortality rate and active and sometimes heterospecific transmission, EBHS and RHD contributed to a significant decline in hare and wild rabbit populations throughout Europe. This led to an imbalance in the natural ecological systems, which caused a real threat of extinction of some other species of wild animal (1, 2, 5, 13).

Both hepatotropic pathogens of lagomorphs belong to the *Caliciviridae* family, currently comprising eleven genera of non-enveloped, single-stranded, positive-sense genomic RNA viruses affecting mammals (*Lagovirus*, *Norovirus*, *Nebovirus*, *Recovirus*, *Sapovirus*, *Valovirus* and *Vesivirus*), birds (*Bavovirus* and *Nacovirus*) and fish (*Minovirus* and *Salovirus*) (38). The *Lagovirus* genus contains two pathogenic species: rabbit haemorrhagic disease virus (RHDV; genogroup GI), the prototype of the genus, and European brown hare syndrome virus (EBHSV, genogroup GII). In addition to genogroups,

there are numerous genotypes because of the significant genetic diversity within these virus species, and non-pathogenic caliciviruses of both rabbits and hares have also been identified within the *Lagovirus* genus (2, 9, 27). Similar morphology is noted in RHDV and EBHSV virions and is confirmed by some degree of antigenic similarity (2, 8, 17, 21). Among the pathogenic viruses responsible for RHD, two additional distinct genetic and antigenic forms have been distinguished since the identification of the first (RHDV) in 1984 – RHDVa and RHDV2 – which can now be classified into two serotypes encompassing RHDV/RHDVa in one case and RHDV2 in the other. Rabbit haemorrhagic disease virus 2 (GI.2) appeared in 2010 in France and most likely evolved from a non-pathogenic rabbit calicivirus (2, 14, 25). This virus, also termed RHDVb, has been shown to overcome protective immunity in rabbits immunised with vaccines homologous to RHDV and cause lethal disease in rabbits of all ages, including young animals under two months old. Moreover, while observations from several countries where RHDV2 infections were diagnosed initially indicated significantly lower morbidity and mortality than RHDV/RHDVa infections (30–40%) in susceptible farmed rabbits (12, 25), in subsequent years, significantly higher 70–80% mortality due to RHDV2 was also demonstrated, similar to that of RHDV/RHDVa infections (7). Unlike classical RHDV, RHDV2 has a highly specific feature to overcome the host-species barrier and cause EBHS-like disease in several hare and non-lagomorph species (1, 5, 6, 20, 26, 32, 34, 37).

European brown hare syndrome virus mainly affects brown hares (*Lepus europaeus*) and mountain hares (*Lepus timidus*) and is also widely distributed in Italian hares (*Lepus corsicanus*). Although EBHSV has evolved into several genetic groups, it has not produced distinct antigenic forms and functions as a single serotype (2, 23).

Calicivirus genomic RNAs of 6.4–8.5 kilobases are covalently linked at the 5′ end with a viral genome-linked protein (acting in the translation initiation process) and polyadenylated at the 3′ termini. Their genome is organised in two or three open reading frames (ORFs). The ORF1 of lagoviruses encodes a large polyprotein cleaved into six non-structural proteins and a major structural protein, the latter being viral protein 1 (VP1), also termed VP60. Viral protein 60 has 576 amino acids (aa) and a molecular mass of approximately 55–70 kDa and is composed of three domains: the N terminal arm, S (shell) and P (protrusion) divided into P1 and P2 subdomains (39). Ninety dimers of the major structural protein form the characteristic icosahedral capsids with 27–40 nm diameter.

The VP60 capsid protein of lagoviruses self-assembles into virus-like particles (VLPs) that morphologically resemble native virions but lack the genetic material of a virus. In the past, the production of VLPs based on EBHSV (23), RHDV (31) and RHDV2 (30) was reported, as well as production of chimeric RHDV/RHDV2 VLPs (3, 11). Despite many attempts to adapt RHDV to cell lines, no confirmed method enables effective *in vitro* multiplication (21). Hopes for success culturing lagoviruses in the future were born of the promising results of culturing human noroviruses in epithelial cells (36). A substitution of Arg-Gly-Asp (the RGD motif) made in the VP60 protein of RHDV allows virus cultivation in the rabbit kidney 13 (RK13) cell line (40). It was therefore decided to produce VLPs made of the VP60 protein of RHDV2 with an RGD motif. In addition, VP60 protein variants of both EBHSV and RHDV2 were constructed with three-lysine substitutions in the S domain or P2 subdomain in order to analyse VLP formation.

The importance of the use of VLPs generated in protein expression systems should be emphasised as a way to study the biology of animal lagoviruses, deliver antigens for vaccines and laboratory diagnostics and survey immunological relationships with natural hosts. This article presents the production, purification and immunodetection with different antibodies of VLPs based on a wild-type VP60 protein of Polish strains of EBHSV and RHDV2 and of some mutated variants of this protein of both viruses.

Material and Methods

RHDV, RHDVa, RHDV2 and RHDV VLP control antigens. Polish reference strains of native RHDV (GI.1c) (KGM 1988), native RHDVa (GI.1a) (GRZ 2004), native RHDV2 (GI.2) (LIB 2018 and ZWO 2021) as well as RHDV VLPs (recombinant RHDV VP60 based on RNA of a classical RHDV strain (SGM1988)) were included in the analyses.

Reference sera. Convalescent rabbit sera were collected two weeks after infection with RHDV2 (S1/201609 from a rabbit which had not been vaccinated against RHD with an RHDV-derived preparation, and S3/201609 and S6/201609 from rabbits which had been vaccinated against RHD with such a preparation). Hyperimmune rabbit and hen antisera were obtained from animals immunised with purified native RHDV (GI.1c) SGM1988 strain (15). Serum positive for EBHSV (S213/III) was taken from a brown hare shot during a hunt in the 2020/2021 season in south-eastern Poland (16).

Sequence of the *VP60* gene for RHDV2 and EBHSV recombinant capsid protein (VP60) production. Synthetic genes (cloned into pFastBac1 plasmids) named RHDV2-wild type (WT) and EBHSV-WT were ordered (Invitrogen GeneArt, Thermo Fisher Scientific, Waltham, MA, USA) on the basis of translated RNA amino acid sequences acquired from the GenBank database. These sequences were of Polish strains with accession Nos MN853659 for RHDV2 LIB 2018 and MK440613 for EBHSV G104.

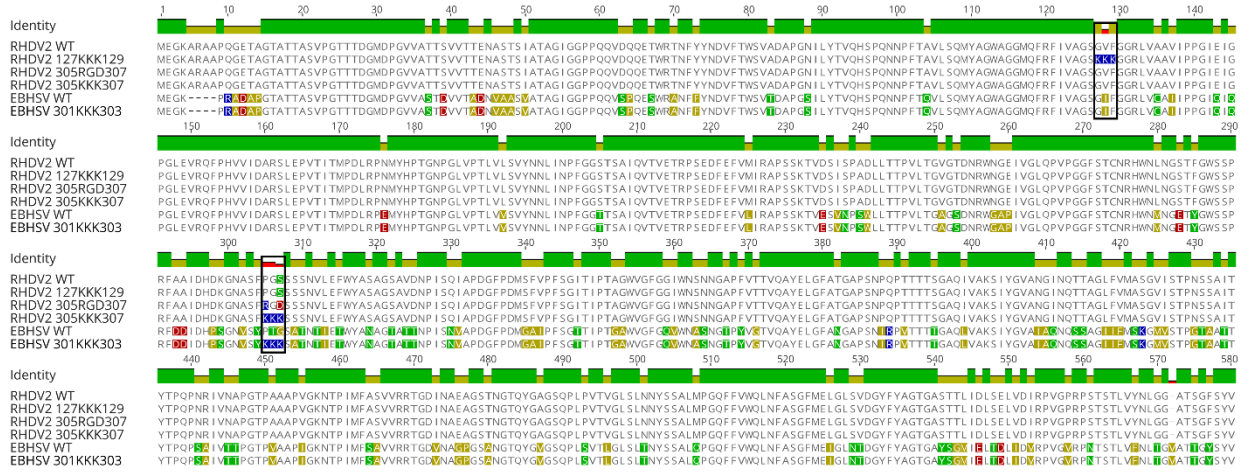

Fig. 1. The alignment of six amino acid sequences of virus capsid protein 60 used in this study. These were rabbit haemorrhagic disease virus 2 (RHDV2)-wild type (WT), -127KKK129, -305RGD307 and -305KKK307 and European brown hare syndrome virus (EBHSV)-WT and -301KKK303. The position of the three-lysine (KKK) (or Arg-Gly-Asp (RGD)) substitution is marked with a black rectangle. Amino acids 127–129 in RHDV2 are in the S domain, while aa 305–307 in RHDV2 and aa 303–305 in EBHSV are in the P2 subdomain

Mutant version. Three mutated variants were constructed of the VP60 of RHDV2 (127KKK129, 305RGD307 and 305KKK307) and one of the VP60 of EBHSV (301KKK303) (Fig. 1). Substitution sites were chosen based on the VP60 protein structure from RHDV (39) and from EBHSV, in the latter case where only partial structure is available in the literature (28). Alignment was analysed in Geneious Prime software (Biomatters, Auckland, New Zealand) using the blocks of amino acid substitution matrix 62 (BLOSUM62). The RGD motif is an amino-acid sequence recognised by integrins present in cell membranes. Substitution of this motif into the P2 subdomain potentially allows RHDV with its VP60 changed with an RGD to enter RK13 cells in culture (40). Three-lysine substitutions in the S domain or P2 subdomain of VP60 were constructed to introduce a basic positive charge into the VP60 protein for better synthetic RNA packing into VLPs. The general approach was using simple acid–base interactions between VLPs as vehicles and RNA as cargo in the preparation of antigens for vaccination studies. The change in the S domain is designed to be located in the inner part of a VLP, whilst in the P2 subdomain the change is in the outer part.

Mutated versions of the *VP60* gene were constructed using a site-specific mutagenesis kit (Q5 Site Directed Mutagenesis Kit; New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol using a PCR reaction and specific primers. The reaction conditions were initial denaturation at 98°C for 30 s; 27 cycles at 98°C for 10 s, 61°C (for KKK substitution) or 68°C (for RGD substitution) for 20 s and 72°C for 3 min 15 s; and final extension at 72°C for 2 min. The primers sequences were as follows (5′→3′): RHDV2-127KKK129 forward AAAAGGTGGTCGTTTGGTGGCT, reverse TTTTTG GAACCAGCCACGATGAAAC; RHDV2-305RGD307 forward AGACTCCAGCTCCAACGTGCTC, reverse CCACGGAAGCTAGCGTTGCCCTTG; and EBHSV-305KKK307 forward AAAATCTGCTACCAACACC ATC, reverse TTTTTGTAAGACACATTGCCGGAG. The mutant RHDV2-305KKK307 was ordered as a synthetic gene (Invitrogen GeneArt). The nucleotide sequences of all the constructed mutants were confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

Recombinant bacmids. The pFastBac1 plasmids containing full-length wild-type and mutated *VP60* genes from RHDV2 and EBHSV were used for site-specific recombination in the DH10Bac *E. coli* strain using the Bac-to-Bac system according to the manufacturer's protocol (Invitrogen) (29). The success of the insertion was confirmed by PCR with universal primers for bacmid inserts and agarose gel electrophoresis. Then all six recombinant bacmid DNA constructs harbouring *VP60* genes (RHDV2-WT, -127KKK129, -305RGD307 and -305KKK307 and EBHSV-WT and -301KKK303) were used to transfect the *Spodoptera frugiperda* 9 (Sf9) insect cell line. Baculoviruses were harvested 3 d post transfection and then amplified by being passaged twice to higher titres, and the baculoviruses from the second passage were used in further experiments.

Confirmation of the expression of the recombinant wild-type (WT) and mutated variants of RHDV2 and EBHSV VP60: VLP production and purification from the medium. Insect cells of the Sf9 line cultivated in flasks with shaking were infected with recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 1 and harvested after 5 d. The collected samples were centrifuged at 1,000 × *g* for 30 min to separate cells and the remaining baculoviruses from the medium. Next, the cleared medium was pelleted by ultracentrifugation at 100,000 × *g* for 18 h at 4°C in an SW28 swinging-bucket rotor (Beckman Coulter, Palo Alto, CA, USA). The pellet containing VP60 VLPs was suspended in 1× phosphate-buffered saline (PBS). The VLPs were purified on a sucrose gradient (60%–10% sucrose in 1× PBS) by ultracentrifugation at 80,000 × *g* for 1 h 45 min at 4°C in

an SW41Ti swinging-bucket rotor (Beckman Coulter). Afterwards, 1mL sucrose fractions were collected and tested for VP60 protein presence and purity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis and Coomassie brilliant blue staining. Controls (mock and baculovirus harbouring the beta-glucuronidase gene) were prepared in the same way as the analysed samples. Protein concentrations in the samples were assessed by the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific).

Confirmation of the expression of the recombinant wild-type (WT) and mutated variants of RHDV2 and EBHSV VP60: Western blotting. Samples containing VP60 VLPs were tested in 12% SDS-PAGE in denaturing conditions and transferred onto polyvinylidene difluoride membranes. Western blot analysis was performed with three different rabbit sera, namely S1/201609, S3/201609 and S6/201609, and a hare serum, S213/III. The anti-RHDV2 S1/201609 serum was used at a dilution of 1:3,000, and S3/201609 and S6/201609 were at 1:15,000 for all four recombinant RHDV2-derived proteins. The anti-EBHSV S213/III serum was diluted 1:10,000 for both the relevant recombinant proteins.

Confirmation of the expression of the recombinant wild-type (WT) and mutated variants of RHDV2 and EBHSV VP60: transmission electron microscopy of analysed VLPs. To show that the substitutions introduced in the VP60 protein facilitated VLP formation, all six types of VLP produced in the baculovirus expression system – purified VLPs in sucrose fractions (or diluted with double-distilled H_2O) – were adsorbed onto carbon-coated grids. Negative staining was performed using 2% uranyl acetate. Samples were examined with a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR, USA).

Detection of RHDV and EBHSV lagovirus antigens by ELISA. The samples of recombinant RHDV2-WT, -305RGD307 and EBHSV-WT VLPs were tested using two commercially available ELISA kits (codes 72575 and 80415; IZSLER, Brescia, Italy) according to the manufacturer's protocol. The latter contains a set of monoclonal antibodies for detecting and typing RHDV, RHDVa and RHDV2 antigens. Briefly, the wells of an immunoplate were coated with rabbit IgG anti-RHDV using standard carbonate buffer. After washing using 1× PBS with Tween-20 (PBS-T) (pH 7.4), the test and control samples were added to the coated wells with ELISA buffer (PBS-T + yeast extract). All the samples were tested in a 1:10 dilution. After 1 h incubation at 37°C with agitation followed by washing with 1× PBS-T, the appropriate wells were incubated (1 h at 37°C) with single monoclonal antibodies (MAbs) specific to RHDV, RHDVa and RHDV2 and with cross-reactive MAbs that react with each type of RHDV. All the wells received anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugate in the next step. The presence of antigen was detected after 5 min incubation with o-phenylenediamine dihydrochloride substrate. The test samples' absorbance values (OD 492 nm) were compared to the positive and negative controls and RHD reference viruses.

To detect the presence of EBHSV antigen in the test sample, the reagents of an ELISA kit for EBHS serology (code 72574; IZSLER) were used. Briefly, the test sample was incubated on a plate coated with IgG anti-EBHS and then reacted with EBHSV-specific MAbs (5F5) conjugated to HRP as a tracer.

Assessment of haemagglutinating properties. The haemagglutinating properties of the recombinant VP60 proteins were tested using a haemagglutination assay (HA). Samples diluted between 1:10 and 1:20,480 in PBS (pH 6.5) were incubated for 2 h at 2–8°C with 0.75% human group O red blood cells suspended in PBS.

Results

Western blot analysis. Two representative Western blot results are presented in Figs 2 and 3. Both the wild-type (RHDV2-WT) and mutated variants (RHDV2-127KKK129, -305KKK307 and -305RGD307) of RHDV2 VP60 VLP reacted specifically with all three rabbit sera (Fig. 2). The RHDV2-305RGD307 protein was the most efficiently produced among all the tested variants and was produced comparably to RHDV2-WT protein in the baculovirus expression system. The production efficiency measurements of the remaining two protein variants (RHDV2-127KKK129 and -305KKK307) were comparable and lower in the same conditions (m.o.i. 1, 5 d post infection).

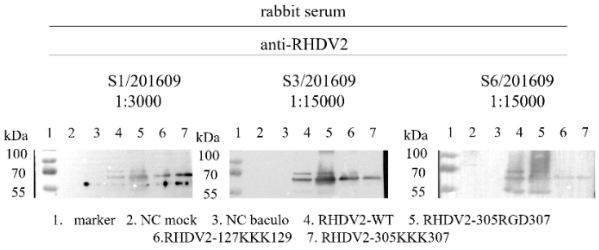

Fig. 2. Western blot analysis of rabbit haemorrhagic disease virus 2 (RHDV2) virus-like particles (VLPs) based on virus capsid protein 60 (purified from the medium) produced in a baculovirus expression system. Lanes: 1 – Page Ruler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA); 2 – non-infected control (NC) medium (mock); 3 – medium from cells infected with baculovirus harbouring the beta-glucuronidase gene as a negative control; 4 – wild type (WT) RHDV2 VLPs; 5 – P2 subdomain Arg-Gly-Asp (RGD motif)–substituted RHDV2-305RGD307 VLPs; 6 – S-domain three-lysine (KKK)–substituted RHDV2-127KKK129 VLPs; 7 – P2 subdomain–substituted RHDV2-305KKK307 VLPs. S1/201609 – serum from a rabbit not vaccinated against RHD which survived an outbreak caused by RHDV2; S3/201609 and S6/201609 – sera from two rabbits vaccinated against RHD which survived an outbreak caused by RHDV2 infection

The same positive reaction was observed for EBHSV-WT and -301KKK303 with anti-EBHSV S213/III serum. Both protein variants were specifically recognised by anti-EBHSV serum (Fig. 3). The production of the EBHSV-301KKK303 variant was lower in the same conditions (m.o.i. 1, 5 d post infection) than the production of the EBHSV-WT variant. In Fig. 3 it is shown that only one protein form in the EBHSV-301KKK303 variant was detected but that several forms of EBHSV-WT were present.

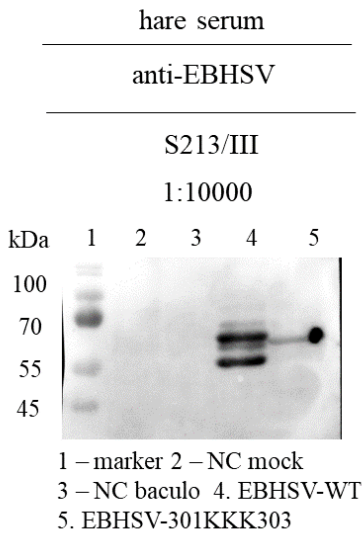

Fig. 3. Western blot analysis of European brown hare syndrome virus (EBHSV) virus-like particles (VLPs) based on virus capsid protein 60 (purified from the medium) produced in a baculovirus expression system. Lanes: 1 – Page Ruler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA); 2 – non-infected control (NC) medium (mock); 3 – medium from cells infected with baculovirus harbouring the beta-glucuronidase gene as a negative control; 4 – wild-type (WT) EBHSV VLPs; 5 – P2 subdomain three-lysine (KKK)–substituted EBHSV-301KKK303 VLPs. S213/III – serum from an EBHSV-positive hare

Electron microscopy. Transmission electron photomicrographs of EBHSV-WT and -301KKK303 (Fig. 4A) and RHDV2-WT, -127KKK129, -305KKK307 and -305RGD307 (Fig. 4B) samples show the formation of structures characteristic of VLPs. The diameter of these structures was around 27–40nm. Most of the analysed VLPs represented typical lagovirus morphology with depressions visible in the photographs, the EBHSV-301KKK303 mutant being the only exception. The introduction of an RGD motif into the VP60 protein (RHDV2-305RGD307) from RHDV2 did not disturb virus-like particle formation and these particles resembled RHDV2-WT VLPs, and neither did KKK substitutions when introduced into VP60 in the same position (RHDV2-305KKK307). The quantity of the RHDV2-305RGD307 VLPs was high and similar to that of wild-type VP60-based particles (RHDV2-WT VLPs).

ELISA. Specific RHDV and EBHSV antigens were detected using the pool of cross-reactive MAbs in the tested samples of recombinant RHDV2-WT, -305RGD307 and EBHSV-WT VLPs and in the positive controls of native RHDVs and EBHSV (Fig. 5).

The absorbance values (OD 492 nm) in the analysed samples ranged from slightly less than 0.8 in the case of EBHSV-WT VLPs (at an initial concentration of 0.2 mg/mL) to 1.2–1.4 in the RHDV2-WT and -305RGD307 variants (both at an initial concentration of 0.16 mg/mL). In the reaction with three single monoclonal antibodies (specific to RHDV, RHDVa and RHDV2), only recombinant RHDV2-WT VLPs were detected. No reactivity of these proteins was observed with anti-RHDV or anti-RHDVa MAbs. The RHDV, RHDVa and RHDV2 control antigens and the reference sample RHDV 2005 VLPs reacted only with the corresponding single MAbs.

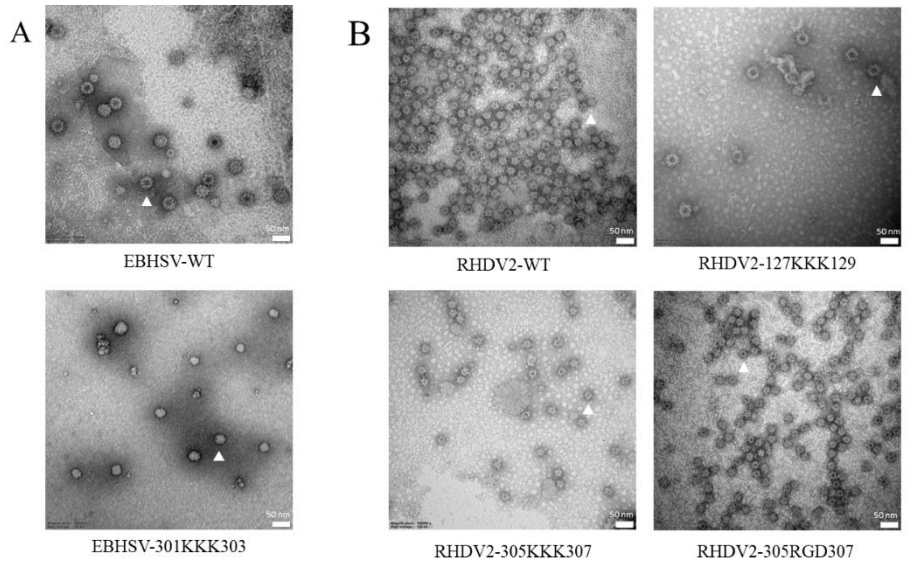

Fig. 4. Transmission electron photomicrographs of virus-like particles (VLPs) based on virus capsid protein 60 of European brown hare syndrome virus (EBHSV) and rabbit haemorrhagic disease virus 2 (RHDV2) with uranyl acetate negative staining. White triangles indicate a representative wild-type (WT) EBHSV and P2 subdomain three-lysine (KKK)–substituted EBHSV-301KKK303 VLP (A); and RHDV2-WT, S domain–substituted RHDV2-127KKK129, P2 subdomain–substituted RHDV2-305KKK307 and P2 subdomain Arg-Gly-Asp (RGD motif)–substituted 305RGD307 VLP (B). Scale bar – 50 nm

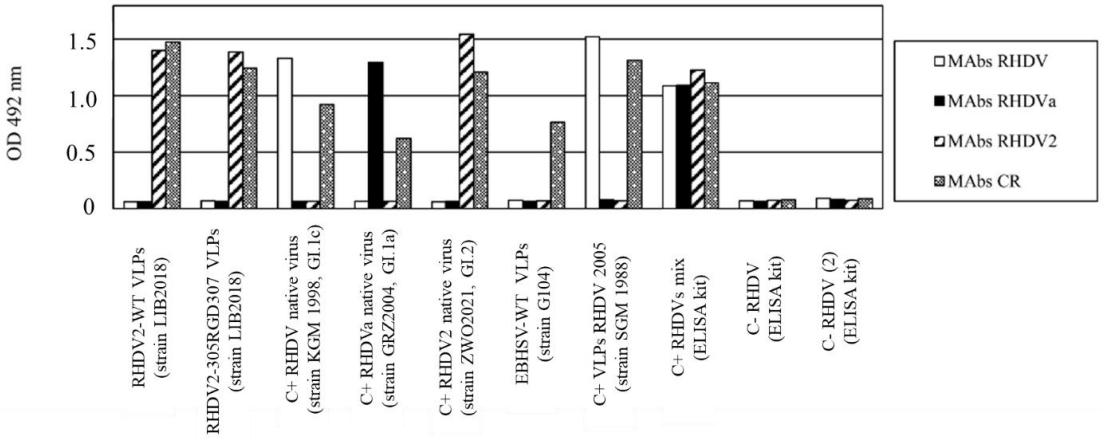

Fig. 5. Detection and typing of virus-like particles (VLPs) based on virus capsid protein 60 (VP60) as wild-type recombinant rabbit haemorrhagic disease virus 2 (RHDV2-WT) and P2 subdomain Arg-Gly-Asp (RGD motif)–substituted-305RGD307 VP60s (based on the Polish LIB2018 strain), and wild-type recombinant European brown hare syndrome virus (EBHSV-WT) VP60 (based on the Polish G104 strain) using a differential ELISA test and RHDV-EBHSV test
OD – optical density; MAbs – monoclonal antibodies; CR – cross-reactive

Table 1. Reactivity of recombinant virus-like particles (VLPs) based on virus capsid protein 60 (VP60) as wild-type European brown hare syndrome disease virus (EBHSV-WT), rabbit haemorrhagic disease virus 2 (RHDV2)-WT and P2 subdomain Arg-Gly-Asp (RGD motif)–substituted RHDV2-305RGD307 VP60s – reactivity with a single monoclonal antibody specific for EBHSV and pool of monoclonal antibodies (MAbs) specific for EBHSV-RHDV in ELISA

Sample	ELISA 5F5 MAb at OD 492 nm	ELISA CR MAbs at OD 492 nm
EBHSV-WT VLPs (G104 strain)	1.654	1.768
RHDV2-WT VLPs (LIB2018 strain)	0.065	1.628
RHDV2-305RGD307 VLPs	0.056	1.595
RHDV KGM1988 strain, native virus, positive control	0.057	0.935
RHDV2 native virus ZWO2021 strain, positive control	0.065	1.558
EBHSV native virus G104 strain, positive control	0.926	0.916

CR – cross-reactive; OD – optical density

The EBHSV-WT VLPs and the native EBHSV (G.II) positive control (G104 strain, in the form of 10% liver homogenate) were detected with the appropriate HRP-conjugated EBHSV-specific MAbs (the 5F5 reagent of the code 72574 IZSLER ELISA kit). The recombinant RHDV2-WT VLPs, an archived sample of RHDV 2005 VLPs and the native RHDV, RHDVa and RHDV2 antigens were not detected by EBHSV-specific MAbs (Table 1).

Haemagglutination assay. The recombinant RHDV2-WT VLPs were positive in the HA and confirmed their ability to agglutinate type O human red blood cells, as did the RHDV and RHDV 2005 VLP native controls used in the assay. Haemagglutination titres of 2,560 and 10,240 were demonstrated for the RHDV2-WT and -305RGD307 variants, respectively. No haemagglutination in three of the five EBHSV-WT recombinant protein samples was found, and only a low haemagglutination titre (in the range of 160 to 320) was demonstrated in the other two samples under the same reaction conditions.

Discussion

Virus-like particles are self-assembled protein structures morphologically similar to native, infectious viruses. They have been produced for many DNA and RNA viruses with different capsid protein structures using different expression systems. These particles have many advantages, the most important of which are the high immunogenicity and specificity of the expressed viral antigen associated with their likeness to structural proteins of the pathogen, and the safety of their use due to non-infectivity. For these same reasons, VLPs can be used in the development of vaccines and diagnostic tests as a key element of a DIVA (differentiating infected from vaccinated animals) strategy that enables differentiation between vaccinated and unvaccinated animals (4).

The EBHSV, classical RHDV and RHDV2 capsid structural proteins have been already expressed in a baculovirus system (3, 11, 18, 23, 24, 30, 31). The recombinant VP60 proteins produced were able to assemble into VLPs that mimicked the original infectious lagoviruses. They were antigenic, induced seroconversion in immunised laboratory animals and, in the case of RHDV proteins, protected vaccinated rabbits in a challenge test (31). In this study, we obtained and characterised two recombinant proteins expressed on the basis of a synthetic *VP60* capsid gene of Polish strains of pathogenic EBHSV and RHDV2 lagoviruses. It was evident that both recombinant proteins assembled into VLPs demonstrated morphological and antigenic properties characteristic of native viruses.

The results of the HA reaction, which is the binding of viral antigens located on the surface of the capsid to histo-blood group antigens present in human red blood cells, confirmed the strong haemagglutination properties of the recombinant RHDV2-WT antigen and the absence or weakness of haemagglutination activity of EBHSV-WT particles. This is generally consistent with the ability of all pathogenic RHDVs (except the rare HA-negative strains or samples containing degraded capsid protein) to agglutinate human erythrocytes and with the low reactivity in the HA of native EBHSV strains (8, 10, 33, 35).

The unique reaction of RHDV2 (-WT and -305RGD307) VLPs with anti-RHDV2 MAbs and the absence of cross-reactivity with anti-RHDV and -RHDVa MAbs in the typing ELISA test as shown in Fig. 5 clearly indicate a faithful reproduction of the icosahedral virion structure. This result demonstrated the presence of specific epitopes localised on the surface of the capsid. The same level of specificity was shown by the EBHSV-WT VLPs, which reacted only with anti-EBHSV MAbs. In addition, the results of the ELISA tests prove the presence of EBHSV-specific epitopes in unchanged form in the VLPs formed by the recombinant EBHSV capsid protein, and indirectly confirm the antigenic and probably genetic stability of the EBHSV field strain inducing anti-EBHSV antibodies.

The P2 subdomain of the VP60 protein of RHDV is the most exposed to the environment. It possesses seven loops (L1–L7) built of seven hypervariable regions distinguished in the amino acid sequence of the VP60 protein. As long as the L1 loop (aa 300–318) is the most exposed part of the protein (39), the changes that may occur in this region because of the virus' evasion of the host's immune system cannot block virus formation. We have confirmed that RGD or KKK substitution in aa 305–307 did not disrupt the formation of VLPs based on RHDV2 VP60 in the baculovirus expression system. Because the S domain is not as variable as the P domain (10), the changes made in the position aa 127–129 in the shell of RHDV2 VP60 from neutral Gly-Val-Phe amino acid residues to basic Lys-Lys-Lys (KKK) did not have an impact on particle formation. However, the quantity of VLPs with this substitution produced in insect cells was lower than that of RHDV2-WT VLPs in the same conditions. If these VLPs are to be used for mRNA packing, the production process needs to be further optimised.

In this study, we present for the first time the production of a mutant version of VLPs based on EBHSV VP60. In the past, epitope mapping and monoclonal antibody binding sites in the VP60 protein have been studied on the basis of different subtypes of EBHSV and RHDV (22, 23). A partial secondary structure of this protein of EBHSV, where only the protrusion domain is presented, is available in the literature (28). We have tested several variants of VP60 mutant (data not shown), and only one VP60 variant – EBHSV-301KKK303 – made the formation possible of spherical structures of 35–40 nm in diameter. However, they did not resemble native particles characteristic of lagoviruses (Fig. 4) (8, 23).

In Table 2, we summarise the serological properties of VP60 proteins as assessed by Western blotting, ELISA and HA. Although not all the properties were checked for each variant, we can conclude that the formation of VLPs can occur for all of them. This is an important observation in view of their applicability as vectors for transporting nucleic acid cargo. The mutations introduced to VP60 (apart from the RGD substitution in RHDV2-305RGD307) were chosen to enhance the ability to bind nucleic acids. These lysine-rich residues should efficiently bind nucleic acids with a highly negative charge. Non-infectious structures such as the VLPs formed in this research, if put to use carrying therapeutic mRNA or DNA, may profoundly influence future constructions of vectors and eliminate the drawbacks of the presently used vehicles for introducing nucleic acids into cells. Virus-like particles, which are the most studied and utilised as recombinant vaccines (for example those against hepatitis B, papillomatosis and influenza as reviewed by Gupta *et al.* (19)), combined with mRNA vaccine production methods may open a new road for vaccination against single or multiple infectious agents. Further investigations using lagovirus-based VLPs as vehicles in vaccination studies will be undertaken in our laboratory.

Table 2. Summary of virus-like particles (VLPs) based on wild-type or mutated variants (with Arg-Gly-Asp (RGD motif) or three-lysine (KKK) substitutions) in the VP60 protein from Polish strains of rabbit haemorrhagic virus 2 (RHDV2) or European brown hare syndrome virus (EBHSV) produced in a baculovirus expression system in *Spodoptera frugiperda* 9 insect cells

Sample	Western blot (expression)	Formation of VLP	ELISA	HA
RHDV2-WT VLPs	+++	+++	+++	+++
RHDV2-305RGD307 VLPs	+++	+++	+++	+++
RHDV2-127KKK129 VLPs	+++	+	+/–	n/a
RHDV2-305KKK307 VLPs	+++	++	+/–	n/a
EBHSV-WT VLPs	+++	+++	+++	+
EBHSV-301KKK303 VLPs	+	+	+/–	n/a

HA – haemagglutinin assay; WT – wild type; EBHSV – European brown hare syndrome virus; +++ – high signal; ++ – medium signal; + – low signal; +/– – very low signal; n/a – not analysed

Conclusion

The presented results show that recombinant VP60 proteins based on Polish strains of RHDV2 and EBHSV in the form of VLPs can be used as a source of highly specific and safe antigens. These proteins could be applied in the laboratory diagnosis of RHD and EBHS and the production of vaccines that could be used in the prevention of RHD on rabbit farms and in wild animal breeding centres, *e.g.* in hare reintroduction programmes. Additionally, the formation of VLP structures like RHDV2 and EBHSV was shown for VP60 protein variants with basic amino acid residue substitution, which in further studies could contribute to better nucleic acid binding by non-enveloped VLPs based on lagoviruses. This novel approach in antigen preparation for vaccination research can be developed, and bring lagoviruses into use as vehicles laden with RNA as cargo.

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