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1 **Generation of Virus Like Particles for Epizootic Hemorrhagic Disease Virus**

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11

12 **Abstract**

13 *Epizootic hemorrhagic disease virus* (EHDV) is a distinct species within the genus
14 *Orbivirus,* within the family *Reoviridae*. The epizootic hemorrhagic disease virus
15 genome comprises ten segments of linear, double stranded (ds) RNA, which are
16 packaged within each virus particle. The EHDV virion has a three layered capsid-
17 structure, generated by four major viral proteins: VP2 and VP5 (outer capsid layer);
18 VP7 (intermediate, core-surface layer) and VP3 (innermost, sub-core layer). Although
19 EHDV infects cattle sporadically, several outbreaks have recently occurred in this
20 species in five Mediterranean countries, indicating a potential threat to the European
21 cattle industry. EHDV is transmitted by biting midges of the genus *Culicoides,* which
22 can travel long distances through wind-born movements (particularly over water),
23 increasing the potential for viral spread in new areas /countries. Expression systems to
24 generate self-assembled virus like particles (VLPs) by simultaneous expression of the
25 major capsid-proteins, have been established for several viruses (including bluetongue
26 virus). This study has developed expression systems for production of EHDV VLPs, for
27 use as non-infectious antigens in both vaccinology and serology studies, avoiding the
28 risk of genetic reassortment between vaccine and field strains and facilitating large scale
29 antigen production. Genes encoding the four major-capsid proteins of a field strain of

30 EHDV-6, were isolated and cloned into transfer vectors, to generate two recombinant
31 baculoviruses. The expression of these viral genes was assessed in insect cells by
32 monitoring the presence of specific viral mRNAs and by western blotting. Electron
33 microscopy studies confirmed the formation and purification of assembled VLPs.
34

35 **Keywords:** Epizootic hemorrhagic disease virus; baculovirus expression system; insect
36 cells; virus like particles.
37

## 38 1. Introduction
39

40 *Epizootic hemorrhagic disease virus* (EHDV) is a distinct virus species within the genus
41 *Orbivirus,* belonging to the family *Reoviridae*. Epizootic hemorrhagic disease virus
42 particles are non-enveloped, approximately 62-80 nm in diameter with a similar
43 structure to bluetongue virus (BTV), which is classified as the prototype of this genus
44 (Mertens et al.; 2005; Savini et al., 2011; Attoui et al.; 2012). The EHDV genome is
45 made of ten segments of linear dsRNA which encode seven structural proteins (VP1-
46 VP7) and probably, as recently discovered for BTV four non-structural proteins (NS1-
47 NS4) (Belhouchet et al., 2011; Mecham and Dean, 1988; Ratinier et al., 2011; Stewart
48 et al., 2015). The ten segments of the EHDV genome are enclosed, along with three
49 minor enzymatic proteins (VP1, VP4 and VP6) within the three-layered, icosahedral
50 virus capsid composed of the four 'major' structural proteins. The sub-core shell is
51 formed by 120 copies of VP3, the core-surface layer comprises 780 copies of VP7,
52 while the outer-capsid contains 180 copies of VP2 and 360 copies of VP5. VP3 is part
53 of the viral 'scaffolding' and contributes to the correct size and structure of the virus.
54 VP7 is the major core protein and is widely used as an orbivirus serogroup/species-
55 specific antigen in serological assays (Luo and Sabara, 2005; Mecham and Wilson,

56 2004). VP2, which is the outermost 'exposed' EHDV protein, is responsible for
57 receptor-binding on target cells and determines virus-serotype through the specificity of
58 its interactions with neutralizing antibodies. According to some reports, VP5, which is a
59 membrane permeabilization protein, may also contribute to the specificity of the
60 neutralizing antibody response, and like VP5 of BTV it has been shown to enhance the
61 immune response induced by VP2 alone. On the basis of antigenic and genetic analyses
62 of the two outer capsid proteins (VP2 and VP5), the EHDV serogroup has been
63 proposed to comprise seven serotypes, although there is not yet a widely accepted
64 consensus on their exact number (Anthony et al., 2010, 2009a, 2009b, 2009c; Maan et
65 al., 2010; Viarouge et al., 2015).
66 White-tailed deer (*Odocoileus virginianus*) is the species most severely affected by
67 EHDV, while mule deer and pronghorn antelope are less severely affected. Although
68 black-tailed deer, red deer, wapiti, fallow deer, roe deer, elk, moose, and bighorn sheep
69 do not appear to be severely affected, they may seroconvert, indicating that they can
70 become infected (MacLachlan and Osburn, 2004; Noon et al., 2002a, 2002b). Recently
71 EHDV has also been isolated in yaks (Van Campen et al., 2013). Goats do not seem to
72 be susceptible to EHDV infection and sheep can be infected experimentally but rarely
73 develop clinical signs, and a recent study has excluded a role in transmission to more
74 susceptible species (Kedmi et al., 2011a).

75 Cattle were first found to be susceptible to infection by an EHDV-2 strain, named as
76 Ibaraki virus, in Japan during 1959. This strain has subsequently been associated with
77 periodic outbreaks in cattle in East Asia (Hirashima et al., 2015; Kitano Y., 2004). In
78 recent years EHDV-6 and 7 have been involved in several outbreaks in cattle: EHDV-6
79 in Turkey, Algeria, Morocco, Reunion Island and French Guiana (Albayrak et al., 2010;
80 Cêtre-Sossah et al., 2014; Temizel et al., 2009; Viarouge et al., 2014); EHDV-7 in Israel

81 in 2006 (Eschbaumer et al., 2012; Wilson et al., 2015; Yadin et al., 2008). During 2007
82 and most recently, EHDV-2 caused morbidity in dairy cattle in Ohio and Kentucky
83 (Garrett et al., 2015) (ProMed Archive Number: 20150923.3666422). EHDV-6 was
84 identified for the first time in the USA, during 2006 and by 2008 was widespread,
85 although the new isolates were all reassortants containing genome segments derived
86 from the parental strains of EHDV-2 and 6 (Allison et al., 2010, 2012; Anbalagan et al.,
87 2014a). EHDV has recently also been isolated in Brazil (Favero et al., 2013). The
88 extended host range of EHDV-6 and 7 in cattle has caused major concerns for
89 international authorities and in 2008 the disease was included in the Office International
90 Epizootics (OIE) list of multispecies / transboundary diseases (OIE, 2012).

91 Analogous to BTV and several other members of the genus *Orbivirus*, EHDV is
92 transmitted by midges of the genus *Culicoides* (Brown et al., 1992; Paweska et al.,
93 2005) which can travel long distances by wind-borne movements particularly over
94 water, making it possible for infected vectors to move from North Africa and Middle
95 East to Southern Europe (Kedmi et al., 2010a; Lorusso et al., 2014). However, there has
96 been little interest so far in developing vaccines to control the disease or EHDV
97 circulation. To date an autogenous vaccine that can be used only in captive wild deer,
98 has been developed in USA. In Japan, both live modified and inactivated vaccines have
99 been developed to control Ibaraki disease in cattle (Ohashi et al., 1999).

100 Virus like particles (VLPs) are considered as a safer and effective alternative to live
101 attenuated vaccines, and VLPs have been generated and tested as non infective
102 immunogens both for human and animal diseases (including bluetongue), triggering the
103 same immunological responses as the whole virus. VLP have the advantage of being
104 immunogens with the same structural characteristics as the native virus particle but are

105 free of the viral genetic material and cannot therefore pose a risk of transmission (Lua et
106 al., 2014; Roy and Noad, 2008; Stewart et al., 2012; Zhao et al., 2013).

107 VLPs of BTV were generated several years ago using baculovirus expression systems
108 and more recently by expression in plants (French et al., 1990; Hewat et al., 1994; Le
109 Blois et al., 1991; Pérez de Diego et al., 2011; Roy, 1990; Roy et al., 1994; Stewart et
110 al., 2010; Thuenemann et al., 2013). Although their use as immunogens, as well as tools
111 to understand BTV replication has been well established, so far nothing similar has been
112 done for EHDV.

113 We report the generation of VLPs derived from a strain of EHDV-6 (MOR2006/05),
114 isolated from disease outbreaks in the Morocco during 2006. These VLPs could be used
115 as antigens to generate a safe and effective vaccine. The omission of non-structural
116 proteins from these VLPs, would allow development of serological assays that could be
117 used to distinguish infected from vaccinated animals (DIVA). This is not currently
118 possible with the live modified vaccines or conventional inactivated vaccines that have
119 been developed for the orbiviruses. The same VLPs, or a combination of individual
120 virus-structural proteins produced by recombinant technology, might be also used as
121 purified antigens for serological diagnosis.

## 122 2. Material and Methods

### 123 2.1 Virus propagation

124 The virus used in this study EHDV-6 (MOR2006/05) was obtained from the Orbivirus
125 Reference Collection (ORC) at The Pirbright Institute (Pirbright, UK)
126 (http://www.reoviridae.org/dsRNAvirusproteins /ReoID/EHDV-Nos.htm). Virus was
127 propagated in BHK-21 cells (clone 13 obtained from European Collection of Animal
128 cell Cultures (ECACC – 84100501), supplied with Dulbecco's minimum essential

129 medium (DMEM) supplemented with antibiotics (100 units/ml penicillin and 100 μg/ml
130 streptomycin) and 2 mM glutamine. Infected cell cultures were incubated until they
131 showed widespread (100%) cytopathic effects (CPE). Viruses were harvested, aliquoted
132 and used for the dsRNA extraction, or stored in the ORC at -80°C.

133 **2.2 Preparation of viral dsRNA:**

134 Intact genomic dsRNA of EHDV-6 was extracted from infected cell cultures using a
135 guanidinium isothiocyanate procedure as described by Attoui et al., (2000). Briefly, the
136 infected cell pellet was lysed in 1 ml of commercially available TRIZOL® reagent
137 (Thermo Fisher Scientific, MA, USA), then 0.2 volume of chloroform was added,
138 mixed by vortexing and the mixture was incubated on ice for 10 minutes (min). The
139 supernatant containing total RNA was separated from cellular debris and DNA by
140 centrifuging at 10,000 *g* at 4°C for 10 min. Single stranded RNA (ssRNA) was
141 precipitated by adding 2 M LiCl and then incubating at 4°C overnight, followed by
142 centrifugation at 10,000 *g* for 5 min. The supernatant containing dsRNA was mixed
143 with an equal volume of isopropanol containing 750 mM ammonium acetate and
144 incubated at -20°C for a minimum of 2 hours (hrs). The dsRNA was pelleted by
145 centrifugation at 10,000 *g* for 10 min, washed with 70% ethanol, air dried and
146 suspended in nuclease free water. The RNA was either used immediately or stored at -
147 80°C.

148 **2.3 Reverse transcription, PCR amplification and cloning of EHDV cDNAs**

149 Genome segments encoding VP2, VP3, VP5 and VP7 of EHDV-6 (MOR2006/05) were
150 reverse-transcribed using a 'full-length amplification of cDNA' (FLAC) technique
151 described by Maan et al (2007). Briefly, a 35 base self-priming oligonucleotide 'anchor-
152 primer', with a phosphorylated 5` terminus was ligated to the 3` ends of the viral
153 dsRNAs using the T4 RNA ligase, followed by reverse transcription with the RT system

154 (Promega, MA, USA). The resulting cDNAs were amplified using a high fidelity KOD
155 polymerase (Merck Millipore, Darmstadt, Germania,) and 5-15-1-NOT-S primers (5`-
156 GCAGTTTAGAATCCTCAGAGGTC-3`), then analyzed by agarose gel electrophoresis
157 and purified using an illustra GFX™ PCR DNA and Gel Band Purification Kit (GE
158 Healthcare, Little Chalfont, UK). The amplified cDNAs of Seg-6 and Seg-7, encoding
159 for VP5 and VP7 respectively, were cloned into the Strataclone blunt-end PCR cloning
160 vector 'pSC-B-amp/kan' supplied in the StrataClone Blunt PCR cloning kit (Agilent
161 Technologies, CA,USA) and the recombinant plasmid vectors were transformed into
162 Solopack® competent cells (Agilent Technologies, CA,USA). Amplicons of Seg-2 and
163 Seg-3 (encoding for VP2 and VP3 respectively) were cloned into the 'pCR®-Blunt'
164 vector supplied with the Zero Blunt® PCR Cloning Kit. Clones containing the desired
165 inserts were identified by colony touch PCR using M13 universal primers and GoTaq®
166 Hot start polymerase (Promega, MA, USA). Plasmid DNA was isolated using the
167 QIAprep® Spin Miniprep kit (Qiagen, Heidelberg, Germany), quantified using a
168 NanoDrop® ND-1000 Spectrophotometer (Labtech, Uckfield, UK) and stored at -20ºC
169 until further use. Sequencing was done using an automated ABI 3730 DNA sequencer
170 (Thermo Fisher Scientific, MA, USA).

171 **2.4 Cloning of EHDV capsid proteins ORFs into baculovirus transfer vectors**

172 The cDNA sequences coding for the viral proteins were cloned into pAcUW51®
173 transfer vector (BD Bioscience, CA, USA), which enables the simultaneous expression
174 of two viral proteins by the baculovirus promoters, polyhedrin (Ph) and p10, having
175 opposite orientations. By this approach two different transfer vectors have been
176 generated: one carrying the VP2/VP5 genes (pU2/5) and another carrying the VP3/VP7
177 open reading frames (ORFs) (pU3/7). Sequences for the VP3 and VP5 ORFs were

excised from the original vectors by EcoRI digestion and ligated into EcoRI digested pAcUW51®, so that they could both be inserted downstream of the p10 promoter.

The VP2 and VP7 ORFs were amplified by PCR using cloning plasmids containing either the VP2 or VP7 sequences as templates, which were generated as described in section 2.3. Both cDNAs were inserted downstream the Ph promoter. Primers were designed to introduce BamHI sites at both ends of the amplicons. The primers used for the VP2 ORF (with BamHI sites underlines) are: VP2 BamHI Fwd2: CGCGGATCCAGGTTAAATTGTTCCAGGATGGATAGCGTT; VP2 BamHI Rev2: CGCGGATCCGTAAGTGTGTTGTTCCAGGTAATCTCTGTC. The following primers were used for the VP7 ORF: BamHI Fwd2: CGCGGATCCGTTAAAATTTGGTGAAGATGGAC; VP7 BamHI Rev2: CGCGGATCCGTAAGTTTGAATTTGGGAAAACGTAC.

After restriction digestion with BamHI, amplicons were ligated into the BamHI site of the transfer vector pAcUW51®. Recombinant plasmids were identified by restriction digestion and verified by sequence analysis.

### 2.5 Generation of recombinant baculoviruses

Recombinant baculoviruses were generated by homologous recombination between transfer vectors encoding the EHDV-6 capsid proteins genes and the linearised baculovirus DNA, as previously described (French et al., 1990; Kitts et al., 1990; Possee, 1997). This part of the work has been carried out using two different insect cell lines both belonging to *Spodoptera frugiperda* (Sf): transfection, viral amplification and plaque assay procedures were performed on Sf21 cells; virus infection for the production of VLPs was done using Sf9 cells.

201 In particular, the pU2/5 and pU3/7 transfer vectors and linearised Baculogold® AcNPV
202 DNA (*Autographa californica nuclear polyhedrosis virus*) (BD Bioscience, CA, USA)
203 were cotransfected in Sf21 (*Spodoptera frugiperda)* cells using the cellfectin®
204 transfection reagent, following the manufacturer's instructions (Thermo Fisher
205 Scientific, MA, USA). As a control a baculovirus expressing the CAT gene
206 (Chloramphenicol acetyltransferase) was generated by cotransfecting the transfer vector
207 pENTR-CAT DNA along with the linearised baculodirect N-terminal DNA following
208 the manufactures procedures (Thermo Fisher Scientific, MA, USA). Cells were grown
209 as monolayer in TC-100 medium (Thermo Fisher Scientific, MA, USA) at 28°C. At day
210 5 post infection (p.i.) cytopathic effect was detectable, supernatant was harvested and a
211 plaque assay was performed. Recombinant plaques were selected by their *lacZ*-negative
212 phenotype, resulting in the formation of white plaques when stained with 1% x-gal (5-
213 bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma-Aldrich,MO, USA) . Isolated
214 plaques were harvested and amplified to generate viral stocks, namely BacpU2/5 and
215 BacpU3/7, that were stored at 4°C in the dark.

216 **2.6 Detection of viral mRNA in infected cells**

217 To check whether the recombinant baculoviruses were expressing EHDV genes,
218 infected Sf9 cells were tested for the presence of EHDV mRNA. Sf9 cells were infected
219 separately with each of the recombinant viruses using a multiplicity of infection (MOI)
220 of 5. The cells were harvested after 48 hrs and total RNA extraction and DNase I
221 digestion were performed using the RNeasy® mini kit, following the manufacturer's
222 protocol (QIAGEN, Heidelberg, Germany). Total RNA was eluted in 40 µl of RNase-
223 free water. RNAs obtained from each infection (BacpU2/5 and BacpU3/7) were
224 transcribed into cDNAs. Reactions were carried out using Superscript III® Reverse
225 Transcriptase (Thermo Fisher Scientific, MA, USA) following the manufacturer's

226 instructions. The cDNAs were used as templates in PCR reactions using primers
227 specific either for VP2,VP5, VP3 and VP7 (Tab 1). Amplicons were run on a 1%
228 agarose gel. Markers used are the following : M1: MassRuler™ High Range DNA
229 ladder, M2: MassRuler™ Low Range DNA ladder (Thermo Fisher Scientific, MA,
230 USA).

231 **2.7 SDS/PAGE and western blotting of recombinant EHDV-6 protein expression**

232 Sf9 cells were grown as suspension culture in Sf900 II serum free medium (Thermo
233 Fisher Scientific, MA, USA) and plated on a six well plate at$1x10^6$ per well. Infection
234 with recombinant baculoviruses along with the Bac-CAT control was carried with
235 MOIs of 5. Cells were harvested at 72 hrs post infection and lysed. Samples were run
236 on a precast 4-12% Bolt® Bis-Tris polyacrylamide gel (Thermo Fisher Scientific, MA,
237 USA) . The gels were either stained with Coomassie brilliant blue or were subjected to
238 western blotting using both a rabbit polyclonal antibody against EHDV-1 and a mouse
239 polyclonal anti-EHDV-1 VP7 kindly donated by Prof. Polly Roy (London School of
240 Hygiene and Tropical Medicine, UK). Peroxidase conjugated goat anti rabbit and a goat
241 anti mouse secondary antibodies (Thermo Fisher Scientific, MA, USA) were used.
242 EHDV-6 infected cell lysate was run next to the samples (courtesy of The Pirbright
243 Institute, UK) as a positive control. As additional positive control purified CLPs kindly
244 donated by Prof. Matsuo were also run (Matsuo et al., 2015). Sharp Mass V plus
245 (Euroclone, Milan, Italy) was used as marker.

246 **2.8 Generation and purification of VLPs**

247 EHDV VLPS were purified as described for BTV (French et al., 1990; Stewart et al.,
248 2010). Infections were carried out on Sf9 cells grown to a density of $1x10^6$/ml, in a total
249 volume of 100 ml (total cell number $1x10^8$). Each virus was used with a MOI of 5 and

250 cells were harvested at 68-72 hrs post infection, when cell mortality had reached about
251 70-80%. Samples were centrifuged at 2,000 g and supernatants removed. Cells were
252 resuspended in lysis buffer (10 mM Tris-HCl pH 8,5; 50 mM NaCl; 10 mM EDTA) and
253 homogenised in ice by a dounce homogenizer in presence of a protease inhibitor
254 cocktail. Lysates were centrifuged once more to remove all cellular debris and the
255 supernatant loaded on top of a 40-66% sucrose gradient (in 0.02 M Tris-HCl pH 8,5).
256 Samples were centrifuged in a 80K Beckman ultracentrifuge (Beckman Coulter, CA,
257 USA) on a SW41Ti rotor at 28,000 rpm, for 3 hrs at 4°C. After centrifugation a band
258 was visible on the expected position at the interface between 40-66% gradient. The band
259 was collected from the top, diluted into 0.02 M Tris-HCl pH 8.5, loaded on top of a 2 ml
260 cushion consisting of 20% sucrose (in 0.02 M Tris-HCl pH 8.5) and run as before but
261 for only 30 min. The pellet was finally resuspended in 100 µl of 0.02 M Tris-HCl pH
262 8.5.

263 **2.9 Electron microscopy of VLPs**

264 A two step protocol was used for the specimen preparation: VLP samples in 20 mM
265 Tris-HCl pH 8.5, were adsorbed for 30 min onto carbon-coated 300 mesh grids
266 (Electron Microscope Science, Hatfield, PA, USA) washed three times with sterile
267 deionised/distilled water and stained for 30 min with 3% uranyl acetate solution in
268 ethanol, followed by draining of grids. All grids were analyzed using a Zeiss Libra 120
269 Plus transmission electron microscope, operating at 120 kV and equipped with an in-
270 column omega filter and 16-bit CCD camera 2k x 2k bottom mounted (Zeiss,
271 Oberkichen, Germany).

272

273

274 **3. Results**

275 **3.1 Generation of recombinant baculoviruses**

276 The regions of the EHDV genome encoding VP2, VP5, VP3 and VP7 were amplified by
277 PCR from a EHDV-6 field isolate (MOR2006/05) and individually cloned into two
278 different vectors to generate four recombinant plasmids. Amplicons of Seg-6 and Seg-7,
279 coding for VP5 and VP7 were cloned into pSC-B-amp/kan', while amplicons of Seg-2
280 and Seg-3, coding for VP2 and VP3 were cloned into the 'pCR®-Blunt' vector. The viral
281 genes were then inserted into the pAcUW51® transfer vector, which allows
282 simultaneous expression of two genes, thereby generating pU2/5 and pU3/7. The correct
283 insertion of the viral genes into pAcUW51® was confirmed by sequence analysis.
284 Recombinant baculoviruses, BacpU2/5 and BacpU3/7, were generated by homologous
285 recombination, after co-transfecting each recombinant transfer vector along with the
286 linearised baculovirus DNA in Sf21 cells. The presence of cytopathic effect on the
287 transfected cells and the plaque assay in the presence of x-gal indicated that almost
288 100% of the plaques were made by recombinant viruses. Single plaques were harvested
289 and the virus was amplified by passage in Sf21 cell cultures.

290 **3.2 Expression of EHDV proteins in recombinant-baculovirus infected cells**

291 The ability of the recombinant baculoviruses to express the EHDV proteins was
292 monitored by checking for the presence of viral mRNAs in infected Sf9 cells. RT-PCR
293 analysis using primers specific for each viral gene readily detected mRNAs of the
294 expected sizes for the EHDV-6 VP3, VP5 and VP7 genes in recombinant baculoviruses
295 infected cells. While an amplicon of 1126 bp was generated for VP3, for VP5 and VP7
296 the entire viral gene was amplified producing a single band of 1641 and 1162 bp
297 respectively (Fig 1A). The VP2 mRNAs was more difficult to detect on the first PCR
298 using the cDNA as template and the couple of primers VP2A (Table 1). On the other

299 hand, when this product was used as template for a semi-nested PCR using primers
300 defined as VP2B (Table 1) we were able to detect an amplicon of 751 bp as expected
301 (Fig 1B). As a negative control, in order to show specificity of the primers for the viral
302 genes, uninfected Sf9 cells were also lysate, total RNA extracted and used as template
303 for the generation of cDNA. However, the synthesis of all four viral proteins was
304 subsequently confirmed by the presence of protein bands of the expected sizes on SDS-
305 PAGE stained with Coomassie-blue. Proteins bands were visible with molecular weights
306 of 100 and 38 KDa (for VP3 and VP7 respectively), and with 110 and 60 KDa for VP2
307 and VP5 (Fig 2). In order to harvest infected cells when protein expression was highest,
308 Sf9 cells were infected at different MOIs and analysed at different time points.
309 Optimum conditions were identified as an MOI of 5 for each virus stock, with cell
310 harvested at 3 days p.i. Data obtained confirmed that the four inner and outer capsid
311 proteins of EHDV-6 were all expressed in the insect cells.

312 **3.3 Purification and isolation of EHDV VLPs from insect cells**

313 Assembly of complete VLPs requires simultaneous expression of the viral inner and
314 outer capsid proteins. Therefore it was necessary to co-infect insect cells with both
315 recombinant baculoviruses. In order to provide high cell density, infection was
316 performed on suspension cultures. Each virus was used at a MOI of 5 and the cells
317 harvested between 68-72 hrs post infection. The presence of all four viral capsid
318 proteins was confirmed by western blotting of the purified particles. Although the
319 antibodies used were raised against EHDV-1 proteins, they were able to identify EHDV-
320 6 proteins as shown in figure 3. EM analysis of purified VLPs showed particles of the
321 expected size and morphology for EHDV VLPs. We also observed smaller particles
322 resembling core like particles (CLPs) generated by the assembly of only VP3 and VP7
323 to form the viral inner core structure (Fig 4). The observed morphology of the VLPs

324 was similar to those generated for BTV (French et al., 1990; Hewat et al., 1994; Stewart
325 et al., 2010; Thuenemann et al., 2013). The data obtained indicated that the viral
326 proteins expressed by the recombinant baculoviruses where able to assemble so to
327 generate properly sized and shaped VLPs. This could also indirectly indicate that the
328 folding and the molar ratio of each protein of each recombinant protein was maintained.

329 **4. Discussion**

330 In recent years, diseases transmitted by arthropod vectors such as mosquitoes and
331 *Culicoides* have become an increasingly important concern for animal and human
332 health. The presence and dissemination of Arthropod-borne viruses in countries where
333 they were not formerly present can be caused either through the movement of animals
334 by trade, shifting of the geographical range of vectors driven by global warming, or
335 possibly by the introduction/movement of even small numbers of infected adult vectors
336 (Carpenter et al., 2011). The reported co-circulation of BTV and EHDV in several
337 countries during 2011 and 2012, indicates that both viruses can be present
338 simultaneously within the same host and vector populations (Sailleau et al., 2012; Toye
339 et al., 2013; Viarouge et al., 2014). Although EHDV has long been regarded as a
340 causing only infrequent outbreaks in cattle, clinical disease similar to bluetongue has
341 been reported on several occasions, since 2005, in five Mediterranean countries as well
342 as in the USA (Breard et al., 2013). Recent sequence analyses of EHDV isolates from
343 cattle with clinical disease in Indiana (USA) indicate high identity levels to the EHDV-2
344 reference strain isolated from a white tailed deer, suggesting that similar viruses can
345 circulate in both species, posing a threat to both wildlife and cattle (Anbalagan and
346 Hause, 2014b). Furthermore, when the EHDV-7 strain isolated from infected cattle in
347 Israel was used to experimentally infect white tailed deer, they developed clinical signs
348 similar to EHD caused by north American EHDV strains (Ruder et al., 2012).

349 Serological and epidemiological investigations of Israeli cattle, after the 2006 EHD
350 outbreaks, found high seroprevalence for EHDV and BTV which clustered together
351 (Kedmi et al., 2011b).

352 The economic losses due to EHDV infection of dairy cattle during the outbreak in
353 Israel in 2006 were estimated as US$2.5 million due to reduced milk and meat
354 production (Kedmi et al., 2010b), therefore a possible outbreak of EHDV in susceptible
355 and naïve European cattle would be economically important.

356 In the event of a major new outbreak, the availability of diagnostic reagents (proteins
357 and antibodies) assays and vaccines would represent key components of any control
358 programs. Unlike modified live vaccines, the use of EHDV VLPs as a immunogen, like
359 inactivated vaccines, would avoid the risk of vaccine strain transmission and genetic
360 reassortment between vaccine strains and wild type viruses circulating in the field.
361 However, unlike the inactivated vaccines, a VLP based vaccine would be compatible
362 with the use of serological DIVA assays for continued surveillance adopting a viral non
363 structural protein based ELISA since those are not present in VLPs.

364 We report the generation of EHDV VLPs from a field strain of EHDV-6 (MOR2006/05)
365 isolated in Morocco in 2006, that caused outbreaks in cattle in several countries and was
366 reported to reassort with other EHDV strains. The genome segments coding for
367 structural viral proteins VP2,VP3,VP5 and VP7 were successfully isolated, transcribed
368 into cDNAs and cloned into baculovirus transfer vectors, to generate two recombinant
369 baculoviruses, each expressing two viral proteins. The correct expression of EHDV
370 genes was initially assessed by monitoring the synthesis of each viral mRNA in infected
371 Sf9 cells and was then confirmed by western blot analysis. The correct assembly of
372 these proteins into VLPs was finally verified by EM showing a correct VLP
373 morphology and size.

We have demonstrated that expression of all four viral structural proteins allows them to self-assemble into non-infectious and morphologically correct particle antigens that could be used safely without risk of infection, for diagnostic test development, or vaccination strategies. This approach could be also used to explore the replication strategies of the virus *in vivo* and *in vitro,* by inserting mutation into the structural viral proteins VP2, VP3, VP5 or VP7 to elucidate their roles in virus infection.

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403 **References**


404 Albayrak, H., Ozan, E., Gur, S., 2010. A serologic investigation of epizootic
405 hemorrhagic disease virus (EHDV) in cattle and gazella subgutturosa subgutturosa
406 in Turkey. Tropical Animal Health and Production. 42, 1589–1591.

407 Allison, A. B., Goekjian, V.H., Potgieter, A.C., Wilson, W.C., Johnson, D.J., Mertens,
408 P.P.C., Stallknecht, D.E., 2010. Detection of a novel reassortant epizootic
409 hemorrhagic disease virus (EHDV) in the USA containing RNA segments derived
410 from both exotic (EHDV-6) and endemic (EHDV-2) serotypes. Journal of General
411 Virology. 91, 430–439.

412 Allison, A.B., Holmes, E.C., Potgieter, A.C., Wright, I.M., Sailleau, C., Breard, E.,
413 Ruder, M.G., Stallknecht, D.E., 2012. Segmental configuration and putative origin
414 of the reassortant orbivirus, epizootic hemorrhagic disease virus serotype 6, strain
415 Indiana. Virology 424, 67–75.

416 Anbalagan, S., Cooper, E., Klumper, P., Simonson, R.R., Hause, B.M., 2014a. Whole
417 genome analysis of epizootic hemorrhagic disease virus identified limited genome
418 constellations and preferential reassortment. Journal of General Virology. 95, 434–
419 441.

420 Anbalagan, S., Hause, B.M., 2014b. Characterization of epizootic hemorrhagic disease
421 virus from a bovine with clinical disease with high nucleotide sequence identity to
422 white-tailed deer isolates. Archives of. Virology. 159, 2737–2740.

423 Anthony, S.J., Darpel, K.E., Maan, S., Sutton, G., Attoui, H., Mertens, P.P.C., 2010.
424 The evolution of two homologues of the core protein VP6 of epizootic
425 haemorrhagic disease virus (EHDV), which correspond to the geographical origin
426 of the virus. Virus Genes 40, 67–75.

427 Anthony, S.J., Maan, N., Maan, S., Sutton, G., Attoui, H., Mertens, P.P.C., 2009a.
428 Genetic and phylogenetic analysis of the core proteins VP1, VP3, VP4, VP6 and
429 VP7 of epizootic haemorrhagic disease virus (EHDV). Virus Research. 145, 187–
430 99.

431 Anthony, S.J., Maan, N., Maan, S., Sutton, G., Attoui, H., Mertens, P.P.C., 2009b.
432 Genetic and phylogenetic analysis of the non-structural proteins NS1, NS2 and
433 NS3 of epizootic haemorrhagic disease virus (EHDV). Virus Research. 145, 211–
434 9.

435 Anthony, S.J., Maan, S., Maan, N., Kgosana, L., Bachanek-Bankowska, K., Batten, C.,
436 Darpel, K.E., Sutton, G., Attoui, H., Mertens, P.P.C., 2009c. Genetic and
437 phylogenetic analysis of the outer-coat proteins VP2 and VP5 of epizootic
438 haemorrhagic disease virus (EHDV): comparison of genetic and serological data to
439 characterise the EHDV serogroup. Virus Research. 145, 200–10.

440 Attoui, H., Billoir, F., Cantaloube, J.F., Biagini, P., de Micco P., de Lamballerie X.2000.
441 Strategies for the sequence determination of viral dsRNA genomes. Journal of
442 Virological Methods. 89, 147-58.

443 Attoui, H., Mertens, P.P.C., Becnel, J., Belaganahalli, M., Bergoin, M., Brussaard, C.P.,
444 Chappell, J.D., Ciarlet, M., del Vas, M., Dermody, T.S., Dormitzer, P.R., Duncan,
445 R., Fcang, Q., Graham, R., Guglielmi, K.M., Harding, R.M., Hillman, B., Maan, S.,
446 M, et al, 2012. Reoviridae, in: King, A., Adams, M., Carstens, E., Lefkwitz, E.
447 (Eds.) (Ed.), Virus Taxonomy, Ninth Report of the International Committee on
448 Taxonomy of Viruses. Elsevier Academic Press, London,UK, pp. 497–650.

449 Belhouchet, M., Jaafar, F., Firth, A.E., Grimes, J.M., Mertens, P.P.C., Attoui, H., 2011.
450 Detection of a fourth orbivirus non-structural protein. PLoS One 6, 1-16.

451 Breard, E., Belbis, G., Viarouge, C., Riou, M., Desprat, A., Moreau, J., Laloy, E.,
452 Martin, G., Sarradin, P., Vitour, D., Batten, C., Doceul, V., Sailleau, C., Zientara,
453 S., 2013. Epizootic hemorrhagic disease virus serotype 6 experimentation on adult
454 cattle. Research in Veterinary Science. 95, 794–798.

455 Brown, S., Gorman, B., Tesh, R., Knudson, D., 1992. Isolation of bluetongue and
456 epizootic hemorrhagic disease viruses from mosquitoes collected in Indonesia.
457 Veterinary Microbiology. 32, 241–252.

458 Carpenter, S., Wilson, A., Barber, J., Veronesi, E., Mellor, P., Venter, G., Gubbins, S.,
459 2011. Temperature Dependence of the Extrinsic Incubation Period of Orbiviruses
460 in Culicoides Biting Midges. PLoS One 6, 1–8.

461 Cêtre-Sossah, C., Roger, M., Sailleau, C., Rieau, L., Zientara, S., Bréard, E., Viarouge,
462 C., Beral, M., Esnault, O., Cardinale, E., 2014. Epizootic haemorrhagic disease
463 virus in Reunion Island: Evidence for the circulation of a new serotype and
464 associated risk factors. Veterinary Microbiology. 170, 383–390.

465 Eschbaumer, M., Wernike, K., Batten, C. A., Savini, G., Edwards, L., Di Gennaro, A.,
466 Teodori, L., Oura, C. a L., Beer, M., Hoffmann, B., 2012. Epizootic hemorrhagic
467 disease virus serotype 7 in European cattle and sheep: Diagnostic considerations
468 and effect of previous BTV exposure. Veterinary Microbiology. 159, 298–306.

469 Favero, C., Matos, A., Campos, F., Candido, M., Costa, E., Heinemann, M., Barbosa-
470 Stancioli, E., Lobato, Z., 2013. Epizootic Hemorrhagic Disease in Brocket Deer.
471 Brazilian Emerging Infectious Diseases. 19, 346–348.

472 French, T.J., Marshall, J.J., Roy, P., 1990. Assembly of double-shelled, viruslike
473 particles of bluetongue virus by the simultaneous expression of four structural
474 proteins. Journal of Virology. 64, 5695–5700.

475 Garrett, E., Po, E., Bichi, E., Hexum, S., Melcher, R., Hubner, A., 2015. Clinical disease
476 associated with epizootic hemorrhagic disease virus in cattle in Illinois. Journal of
477 the American Veterinary Medical Association. 247, 190–195.

478 Hewat, E.A., Booth, T.F., Roy, P., 1994. Structure of correctly self-assembled
479 bluetongue virus-like particles. Journal of Structural Biology. 112, 183–91.

480 Hirashima, Y., Kato, T., Yamakawa, M., Shirafuji, H., Okano, R., Yanase, T., 2015.
481 Reemergence of Ibaraki disease in southern Japan in 2013. Journal of Veterinary
482 Medical Science.Epub ahead of print. doi.org/10.1292/jvms.15-0039.

483 Kedmi, M., Galon, N., Herziger, Y., Yadin, H., Bombarov, V., Batten, C., Shpigel,
484 N.Y., Klement, E., 2011b. Comparison of the epidemiology of epizootic
485 haemorrhagic disease and bluetongue viruses in dairy cattle in Israel. Veterinary
486 Journal. 190, 77–83.

487 Kedmi, M., Herziger, Y., Galon, N., Cohen, R.M., Perel, M., Batten, C., Braverman, Y.,
488 Gottlieb, Y., Shpigel, N., Klement, E., 2010a. The association of winds with the
489 spread of EHDV in dairy cattle in Israel during an outbreak in 2006. Preventive
490 Veterinary Medicine. 96, 152–60.

491 Kedmi, M., Levi, S., Galon, N., Bomborov, V., Yadin, H., Batten, C., Klement, E.,
492 2011a. No evidence for involvement of sheep in the epidemiology of cattle virulent
493 epizootic hemorrhagic disease virus. Veterinary Microbiology. 148, 408–12.

494 Kedmi, M., Van Straten, M., Ezra, E., Galon, N., Klement, E., 2010b. Assessment of
495 the productivity effects associated with epizootic hemorrhagic disease in dairy
496 herds. Journal of Dairy Science. 93, 2486–2495.

497 Kitano Y., 2004. Ibaraki disease in cattle, in: Coetzer J.A.W., T.R.C. (Ed.), Infectious
498 Diseases of Livestock, Vol 2. Oxford University Press Southern Africa, Cape
499 Town, pp. 1221–1226.

500 Kitts, P. A., Ayres, M.D., Possee, R.D., 1990. Linearization of baculovirus DNA
501 enhances the recovery of recombinant virus expression vectors. Nucleic Acids
502 Research.18 (19), 5667-5672.

503 Le Blois, H., Fayard, B., Urakawa, T., Roy, P., 1991. Synthesis and characterization of
504 chimeric particles between epizootic hemorrhagic disease virus and bluetongue
505 virus: functional domains are conserved on the VP3 protein. Journal of Virology.
506 65, 4821–4831.

507 Lorusso, A., Sghaier, S., Ancora, M., Marcacci, M., Di Gennaro, A., Portanti, O.,
508 Mangone, I., Teodori, L., Leone, A., Camma', C., Petrini, A., Hammami, S.,
509 Savini, G., 2014. Molecular epidemiology of bluetongue virus serotype 1
510 circulating in Italy and its connection with northern Africa. Infection Genetics and
511 Evolution. 28, 144–9.

512 Lua, L.H.L., Connors, N.K., Sainsbury, F., Chuan, Y.P., Wibowo, N., Middelberg,
513 A.P.J., 2014. Bioengineering virus-like particles as vaccines. Biotechnology and
514 Bioengineering. 111, 425–440.

515 Luo, L., Sabara, M.I., 2005. Production of a recombinant major inner capsid protein for
516 serological detection of epizootic hemorrhagic disease virus. Clinical and
517 Diagnostic Laboratory Immunology. 12, 904–909.

518 Maan, N.S., Maan, S., Nomikou, K., Johnson, D.J., Harrak, M. El, Yadin, H., Incoglu,
519 S., Yesilbag, K., Allison, A.B., Stallknecht, D.E., Batten, C., Anthony, S.J.,
520 Mertens, P.P.C., 2010. RT-PCR Assays for Seven Serotypes of Epizootic
521 Haemorrhagic Disease Virus & Their Use to Type Strains from the Mediterranean
522 Region and North America. Plos one. 5,1-13.

523 Maan, S., Rao, S., Maan, N.S., Anthony, S.J., Attoui, H., Samuel, A.R., Mertens, P.P.C.
524 2007. Rapid cDNA synthesis and sequencing techniques for the genetic study of
525 bluetongue and other dsRNA viruses. Journal of Virological Methods. 143, 132-9.

526 MacLachlan N.J. & Osburn B.I, 2004. Epizootic haemorrhagic disease of deer, in: R.C,
527 C.J.A.W.& T. (Ed.), Infectious Diseases of Livestock, Vol 2. Oxford University
528 Press Southern Africa, pp. 1227–1230.

529 Matsuo, E., Saeki, K., Roy, P., Kawano, J. 2015 Development of reverse genetics for
530 Ibaraki virus to produce viable VP6-tagged IBAV. FEBS Open bio. 5, 445-53.

531 Mecham, J.O., Dean, V.C., 1988. Protein coding assignment for the genome of
532 epizootic haemorrhagic disease virus. Journal of General Virology. 69, 1255-1262.

533 Mecham, J.O., Wilson, W.C., 2004. Antigen Capture Competitive Enzyme-Linked
534 Immunosorbent Assays Using Baculovirus-Expressed Antigens for Diagnosis of
535 Bluetongue Virus and Epizootic Hemorrhagic Disease Virus. Journal of Clinical
536 Microbiology. 42, 518–523.

537 Mertens P.P.C.,Duncan, R.Attoui,H.,Dermody, T.S. 2005. Reoviridae., in: Fauquet CM,
538 M.A. Mayo, J. Maniloff, U.D. and L.A.B. (Ed.), Virus Taxonomy Eighth Report of
539 the International Committee on Taxonomy of Viruses. Elsevier/Academic Press,
540 London, pp. 447–454.

541 Noon, T.H., Wesche, S.L., Cagle, D., Mead, D.G., Bicknell, E.J., Bradley, G. A.,
542 Riplog-Peterson, S., Edsall, D., Reggiardo, C., 2002a. Hemorrhagic disease in
543 bighorn sheep in Arizona. Journal of Wildlife Diseases. 38, 172–176.

544 Noon, T.H., Wesche, S.L., Heffelfinger, J., Fuller, A., Bradley, G. A, Reggiardo, C.,
545 2002b. Hemorrhagic disease in deer in Arizona. Journal of Wildlife Diseases. 38,
546 177–181.

547 Ohashi, S., Yoshida, K., Watanabe, Y., Tsuda, T., 1999. Identification and PCR-
548 restriction fragment length polymorphism analysis of a variant of the Ibaraki virus
549 from naturally infected cattle and aborted fetuses in Japan. Journal of Clinical
550 Microbiology. 37, 3800–3803.

551 Office International Epizooties (OIE), 2012. Manual of Diagnostic Tests and Vaccines
552 for Terrestrial Animals. Vol1 & 2, 7th ed.

553 Paweska, J.T., Venter, G.J., Guillet, P., Hamblin, C., 2005. A comparison of the
554 susceptibily of Culicoides imicola and C.bolitinos to oral infection with eight
555 serotypes of epizootic haemorrhagic disease virus. Medical and Veterinary
556 Entomology. 19, 200–207.

557 Pérez de Diego, A.C., Athmaram, T.N., Stewart, M., Rodríguez-Sánchez, B., Sánchez-
558 Vizcaíno, J.M., Noad, R., Roy, P., 2011. Characterization of protection afforded by
559 a bivalent virus-like particle vaccine against bluetongue virus serotypes 1 and 4 in
560 sheep. PLoS One 6, 1–7.

561 Possee, R.D., 1997. Baculoviruses as expression vectors. Current Opinion in
562 Biotechnology. 8, 569–572.

563 Ratinier, M., Caporale, M., Golder, M., Franzoni, G., Allan, K., Nunes, S.F.,
564 Armezzani, A., Bayoumy, A., Rixon, F., Shaw, A., Palmarini, M., 2011.
565 Identification and characterization of a novel non-structural protein of bluetongue
566 virus. PLoS Pathogens. 7, 1-14

567 Roy, P., 1990. Use of baculovirus expression vectors: development of diagnostic
568 reagents, vaccines and morphological counterparts of bluetongue virus. FEMS
569 Microbiology Immunology. 2, 223–234.

570 Roy, P., Bishop, D., LeBlois, H., Erasmus, B., 1994. Long-lasting protection of sheep
571 against bluetongue challenge after vaccination with virus-like particles: evidence
572 for homologous and partial heterologous protection. Vaccine 12, 805–11.

573 Roy, P., Noad, R., 2008. Virus-like particles as a vaccine delivery system: Myths and
574 facts. Human Vaccines. 4, 5–12.

575 Ruder, M.G., Allison, A.B., Stallknecht, D.E., Mead, D.G., McGraw, S.M., Carter,
576 D.L., Kubiski, S. V, Batten, C. a, Klement, E., Howerth, E.W., 2012. Susceptibility
577 of white-tailed deer (Odocoileus virginianus) to experimental infection with
578 epizootic hemorrhagic disease virus serotype 7. Journal of Wildlife Diseases. 48,
579 676–85.

580 Sailleau, C., Zanella, G., Breard, E., Viarouge, C., Desprat, A., Vitour, D., Adam, M.,
581 Lasne, L., Martrenchar, A., Bakkali-Kassimi, L., Costes, L., Zientara, S., 2012. Co-
582 circulation of bluetongue and epizootic haemorrhagic disease viruses in cattle in
583 Reunion Island. Veterinary Microbiology. 155, 191–197.

584 Savini, G., Afonso, a., Mellor, P., Aradaib, I., Yadin, H., Sanaa, M., Wilson, W.,
585 Monaco, F., Domingo, M., 2011. Epizootic heamorragic disease. Research in
586 Veterinary Science. 91, 1–17.

587 Stewart, M., Bhatia, Y., Athmaran, T.N., Noad, R., Gastaldi, C., Dubois, E., Russo, P.,
588 Thiéry, R., Sailleau, C., Bréard, E., Zientara, S., Roy, P., 2010. Validation of a
589 novel approach for the rapid production of immunogenic virus-like particles for
590 bluetongue virus. Vaccine 28, 3047–3054.

591 Stewart, M., Dovas, C.I., Chatzinasiou, E., Athmaram, T.N., Papanastassopoulou, M.,
592 Papadopoulos, O., Roy, P., 2012. Protective efficacy of Bluetongue virus-like and
593 subvirus-like particles in sheep: Presence of the serotype-specific VP2,
594 independent of its geographic lineage, is essential for protection. Vaccine 30,
595 2131–2139.

596 Stewart, M., Hardy, A., Barry, G., Pinto, R., Caporale, M., Melzi, E., Hughes, J.,
597 Taggart, A., Janowicz, A., Varela, M., Ratinier, M., Palmarini, M., 2015.
598 Characterisation of a second open reading frame in genome segment 10 of
599 bluetongue virus.Journal of General Virology. Epub ahead of print. doi:
600 10.1099/jgv.0.00026

601 Temizel, E.M., Yesilbag, K., Batten, C., Senturk, S., Maan, N.S., Mertens, P.P.C.,
602 Batmaz, H., 2009. Epizootic hemorrhagic disease in cattle, Western Turkey.
603 Emerging Infectious Diseases. 15, 317–319.

604 Thuenemann, E.C., Meyers, A.E., Verwey, J., Rybicki, E.P., Lomonossoff, G.P., 2013.
605 A method for rapid production of heteromultimeric protein complexes in plants:
606 Assembly of protective bluetongue virus-like particles. Plant Biotechnology
607 Journal. 11, 839–846.

608 Toye, P.G., Batten, C. A., Kiara, H., Henstock, M.R., Edwards, L., Thumbi, S., Poole,
609 E.J., Handel, I.G., Bronsvoort, B.M. deC, Hanotte, O., Coetzer, J. A. W.,
610 Woolhouse, M.E.J., Oura, C. A.L., 2013. Bluetongue and Epizootic Haemorrhagic
611 Disease virus in local breeds of cattle in Kenya. Research in Veterinary Science.
612 94, 769–773.

613 Van Campen, H., Davis, C., Flinchum, J.D., Bishop, J. V, Schiebel, A., Duncan, C.,
614 Spraker, T., 2013. Epizootic hemorrhagic disease in yaks (Bos grunniens). Journal
615 of Veterinary Diagnistic Investigastion. 25, 443–6.

616 Viarouge, C., Breard, E., Zientara, S., Vitour, D., Sailleau, C., 2015. Duplex Real-Time
617 RT-PCR Assays for the Detection and Typing of Epizootic Haemorrhagic Disease
618 Virus. PLoS One. 10,1-11.

619 Viarouge, C., Lancelot, R., Rives, G., Bréard, E., Miller, M., Baudrimont, X., Doceul,
620 V., Vitour, D., Zientara, S., Sailleau, C., 2014. Identification of bluetongue virus
621 and epizootic hemorrhagic disease virus serotypes in French Guiana in 2011 and
622 2012. Veterinary Microbiology. 174, 78–85.

623 Wilson, W., Ruder, M., Klement, E., Jasperson, D., Yadin, H., Stallknecht, D., Mead,
624 D., Howerth, E., 2015. Genetic Characterization of Epizootic Hemorrhagic Disease
625 Virus Strains isolated from Cattle in Israel. J. Gen. Virol. Feb 20.

626 Yadin, H., Brenner, J., Bumbrov, V., Oved, Z., Stram, Y., Klement, E., Perl, S.,
627 Anthony, S., Maan, S., Batten, C., Mertens, P., 2008. Epizootic haemorrhagic
628 disease virus type 7 infection in cattle in Israel. Veterinay Record. 162, 53–56.

629 Zhao, Q., Li, S., Yu, H., Xia, N., Modis, Y., 2013. Virus-like particle-based human
630 vaccines: Quality assessment based on structural and functional properties. Trends
631 in Biotechnology.

632

633

634 **Figures Legend**

635

636 **Fig 1 Analysis of viral mRNAs**.

637 Analysis of RT-PCR samples on a 1% agarose gel; (A): PCR products generated from

638 VP3 (1116 bp),VP5 (1640 bp)and VP7 (1162) ORF cDNAs using specific primers; (B):

639 PCR product for the VP2 ORF (750 bp) using higher concentration of cDNA template.

640

641 **Fig 2 Protein expression studies.**

642 Samples were run on a precast 4-12% Bolt® Bis-Tris polyacrylamide gel (Thermo

643 Fisher Scientific, MA, USA) and the proteins stained with Coomassie-blue. As a

644 positive control purified CLPswere run next to the samples.

645 **Fig 3 Western blotting on purified VLPs**.

646 Purified EHDV VLP particles were run on a 4-12% Bolt® Bis-Tris precast

647 polyacrylamide gel (Thermo Fisher Scientific, MA, USA) and, after blotting, proteins

648 were detected by polyclonal antibodies as described in materials and methods. As

649 positive controls, EHDV-6 infected cell lysate and purified CLPs were run next to the

650 samples.

651

652 **Fig.4 EM studies on purified VLPs.**

653 Purified VLP particles were stained and visualized as described in material and

654 methods. Size bars are of 200 nm on upper panel and 50 nm on lower panels. Black

655 arrow indicates CLP and white arrow purified VLPs.

656

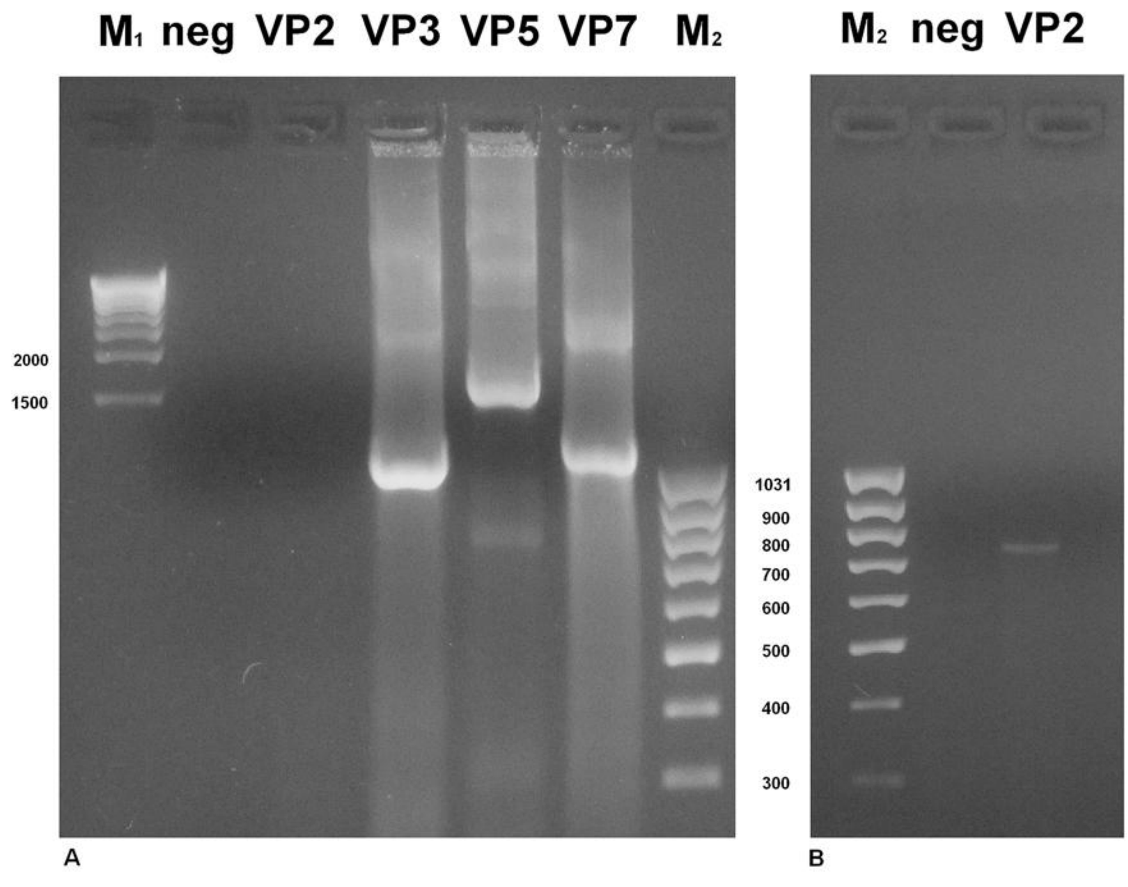
| Gene | | Primers | Size (bp) |
|---|---|---|---|
| VP2 | **VP2A** | | |
| | 129F | 5’ GCTTATAGTGGTGAAAAGGAAGG 3’ | 1318 |
| | 1547R | 5’CGATGCCGCCCACATCCGAG 3’ | |
| | **VP2B** | | |
| | 796F | 5’AGTGAAGCGTATCGAGAGGTTGCT 3’ | 751 |
| | 1547R | 5’CGATGCCGCCCACATCCGAG 3’ | |
| VP3 | 826F | 5’GTGCTTACCGACAGGAGAATACGTCT 3’ | 1126 |
| | 1952R | 5’AGGCTCTGGCGATACTTCCATAACA 3’ | |
| VP5 | BamHIF | 5’CGCGGATCCGTTAAAAAGATCCAGTGCCGTTGCAAAATGG 3’ | 1641 |
| | BamHIR | 5’CGCGGATCCGTAAGTTGAAGATCCGAATACCATCCGC 3’ | |
| VP7 | BamHIF | 5’CGCGGATCCGTTAAAATTTGGTGAAGATGGAC 3’ | 1162 |
| | BamHIR | 5’CGCGGATCCGTAAGTTTGAATTTGGGAAAACGTAC 3’ | |

657

658 **Tab. 1** List of primers used for the PCRs performed on viral cDNAs

659

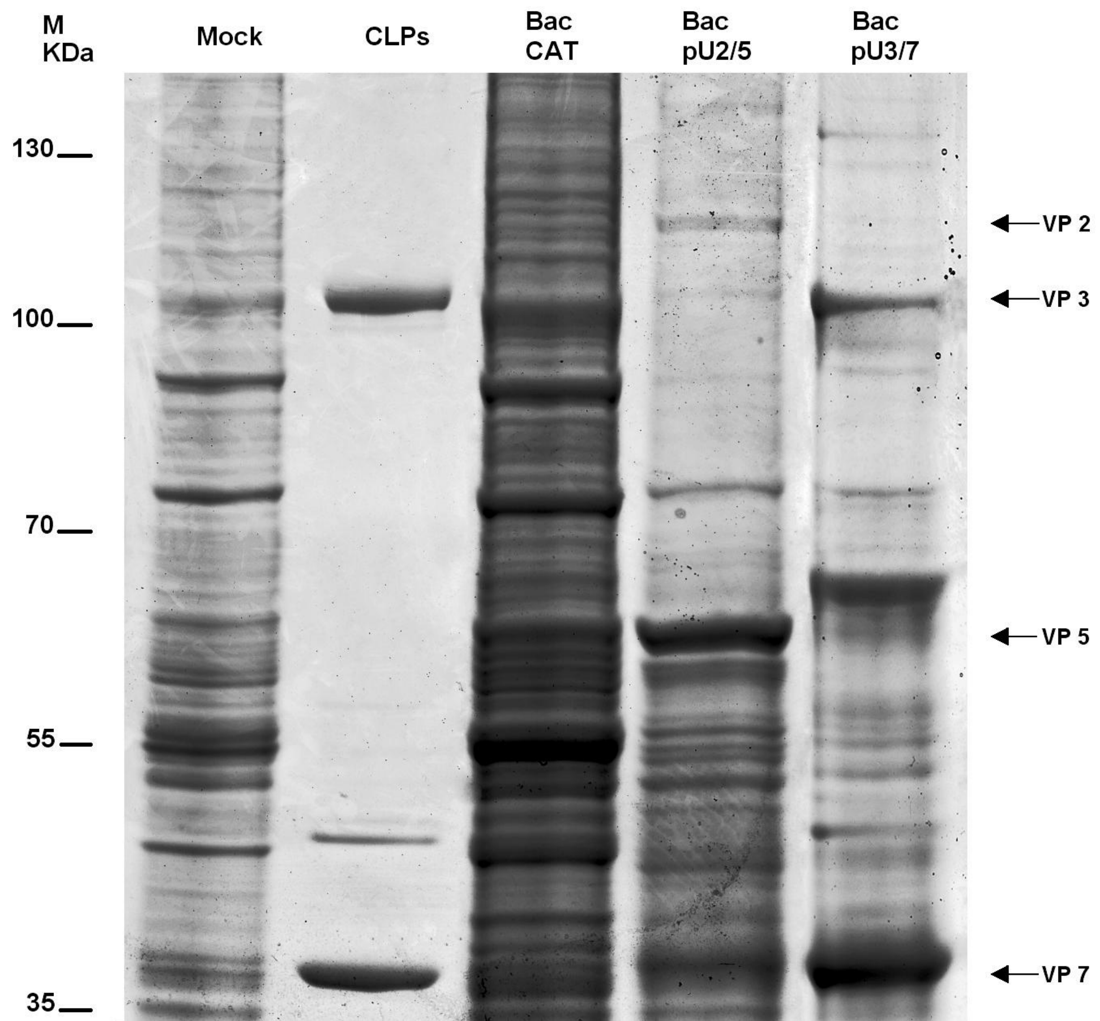
660




661 **Fig 1 Analysis of viral mRNAs**.

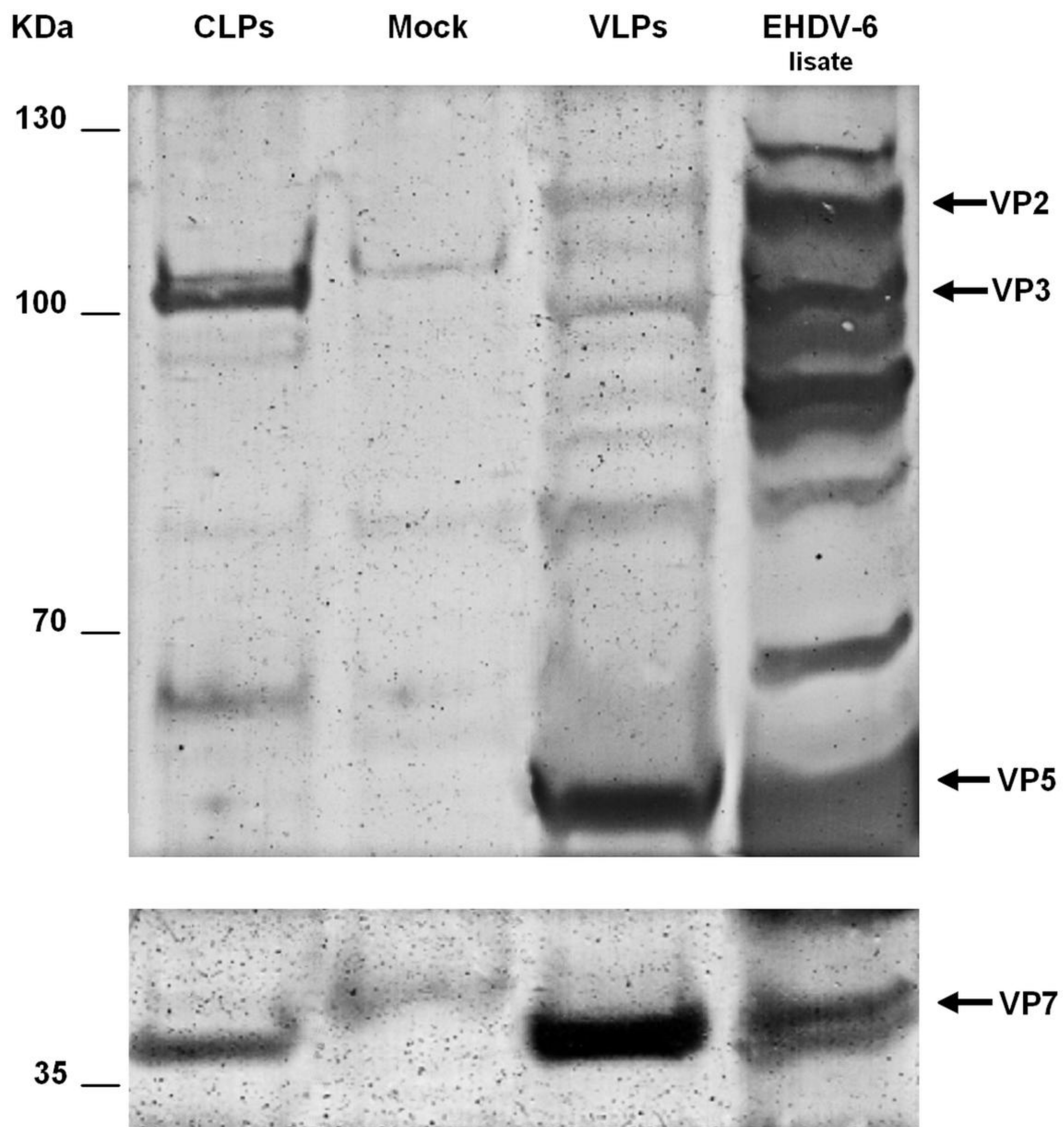
662

663




664 **Fig 2 Protein expression studies.**

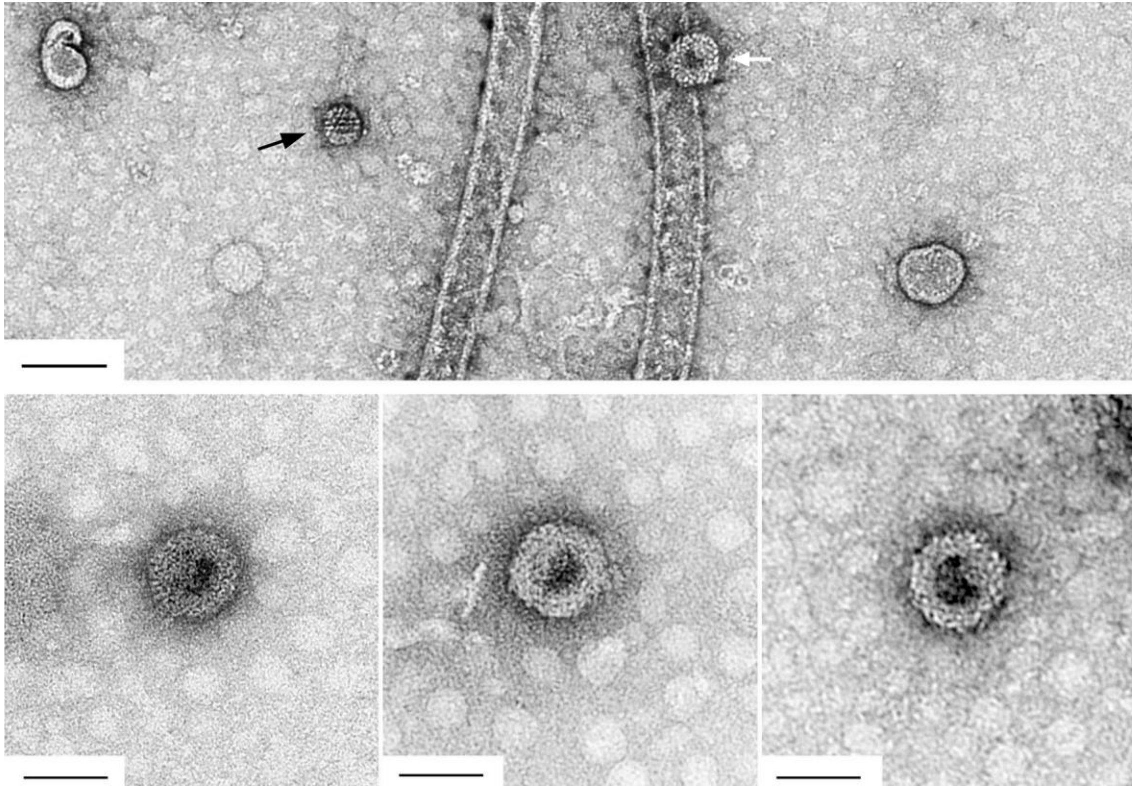
665




666

667 **Fig 3 Western blotting on purified VLPs**.

668



669

670 **Fig.4 EM studies on purified VLPs.**

671