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Generation of virus-like particles for emerging epizootic haemorrhagic disease virus: Towards the development of safe vaccine candidates

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ABSTRACT

Epizootic haemorrhagic disease virus (EHDV) is an insect-transmitted pathogen which causes high mortality in deer populations and may also cause high morbidity in cattle. EHDV belongs to the Orbivirus genus and is closely related to the prototype Bluetongue virus (BTV). To date seven distinct serotypes have been recognized. However, a live-attenuated vaccine is commercially available against only one serotype namely EHDV-2, which has been responsible for multiple outbreaks in North America, Canada, Asia and Australia. Here we expressed four major capsid proteins (VP2, VP3, VP5 and VP7) of EHDV-1 using baculovirus multiple gene expression systems and demonstrated that three-layered VLPs were assembled mimicking the authentic EHDV particles but lacking the viral genomic RNA segments and the transcriptase complex (TC). Antibodies generated with VLPs not only neutralized EHDV-1 infection in cell culture but also showed cross neutralizing reactivity against two other serotypes, EHDV-2 and EHDV-6. For proof of concept, we demonstrated that EHDV-2 VLPs could be generated rapidly by expressing the EHDV-2 variable outer capsid proteins (VP2, VP5) together with EHDV-1 VP3 and VP7, the two inner capsid proteins, which are highly conserved among the 7 serotypes. Data presented in this study validate the VLPs as a potential vaccine and demonstrate that a vaccine could be developed rapidly in the event of an outbreak of a new serotype.

1. Introduction

Epizootic haemorrhagic disease (EHD) is a vector-borne, non-contagious viral disease of domestic and wild ruminants, primarily white-tailed deer (*Odocoileus virginianus*) and occasionally in cattle. The virus, with seven distinct serotypes (EHDV-1-EHDV-7), is endemic in many parts of North America, Australia, and certain Asian countries. More recently, it has emerged in the countries surrounding the Mediterranean Basin. Until 2006, it was believed that EHDV does not cause significant clinical disease in cattle with the exception of EHDV-2/Ibaraki virus (IBAV), which emerged in Japan 1959 when the virus infection killed 4000 cattle and caused high morbidity in 39,000 cattle in the affected areas [1,2]. However, the recent outbreaks of EHDV-6 in the US and EHDV-6 and EHDV-7 in the Mediterranean Basin as well as the French Island of Reunion were also highly pathogenic in cattle, demonstrating that not only EHDV-2 but other serotypes can also be pathogenic in domestic animals with considerable economic consequences [3–6]. Further, the emergence of these serotypes in Mediterranean Basin also significantly increases the risk of invasion into central and northern Europe. EHDV infection in animals induces a wide range of clinical signs. In white-tailed deer, infection can be severe with high morbidity and mortality [7], while in cattle, the disease is generally subclinical. However, infected cattle could experience a reduction in milk production, infertility and abortion [5,8]. To date, the only commercially available vaccine is a live-attenuated vaccine to control Ibaraki disease caused by IBAV (EHDV-2) [9]. The major problem associated with this vaccine is an inability to differentiate between vaccinated and naturally infected animals (DIVA). Thus, a safe and efficacious vaccine with DIVA compliance is needed.

EHDV is an orbivirus, closely related to Bluetongue virus (BTV), the prototype of the Orbivirus genus in the *Reoviridae* family. BTV and EHDV particles are non-enveloped capsid structures, made up of seven structural proteins that are organized in two concentric shells enclosing 10 dsRNA segments of viral genome [10,11]. At the amino acid level the capsid proteins of EHDV and BTV are highly homologous indicating that these two viruses are structurally similar [12,13].

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The outer shell of BTV (and most likely also of EHDV) is composed of 180 molecules of VP2 and 360 molecules of VP5, both of which attach onto the VP7 layer, the surface layer of the capsid, termed as 'core'. The VP7 layer, which is composed of 260 VP7 trimers (780 molecules), encapsidates a further protein layer of 120 VP3 molecules. The interior of the core also includes three viral enzymes (VP1, VP4 and VP6) that are closely associated with the 10 double-stranded (ds) RNA segments of viral genome [14–17]. While VP2 and VP5 are highly variable at the sequence level among serotypes, the core proteins are highly conserved [18]. The two outer capsid proteins are responsible for attachment and penetration of the host membrane; they take no further parts in the generation of new viral genomes in the host cytoplasm as both proteins are detached and degraded during the entry process. However, consistent with their locations, each is responsible for triggering strong neutralizing antibody response. These have been documented extensively for BTV and to a lesser extent for other orbiviruses, including EHDV.

The expression of the four structural proteins (VP2, VP3, VP5 and VP7) of BTV in insect cells, utilizing baculovirus multiple expression vectors, leads to the assembly of 3-layered virus-like particles (VLPs), which structurally mimic the authentic virion particles but lack the viral genome and polymerase complex [19,20]. When used for immunization, BTV VLPs have been shown to afford complete protection against virulent strains challenge with no side effects in BTV-susceptible sheep [21–23], indicating that such vaccines could be highly desirable for EHDV serotype.

In this study, we have cloned the genes encoding the four major capsid proteins of EHDV-1 and demonstrated their expression as multilayered recombinant VLPs which induced strong neutralizing antibodies in model animals. Further, the possibility of generation of VLPs for other serotypes as a rapid response to the emergent serotypes has also been documented.

2. Materials and methods

2.1. Virus stocks and cell lines

BSR cells, a clone derived from the Baby Hamster Kidney cells (BHK), were cultured in Dulbecco's modified medium (DMEM) supplemented with 5% foetal calf serum (FCS) in the presence of 50 U/ml antibiotic/antimycotic and L-glutamine (Sigma–Aldrich Ltd, UK). EHDV-1 (New Jersey, US), EHDV-2 (Alberta, Canada) and EHDV-6 (The Reunion Island, France) were propagated and titrated in BSR cells both by plaque assay and $TCID_{50}$/ml. Insect cells (*Spodoptera frugiperda Sf*21 and *Sf*9) were cultured at 28 °C in suspension in TC-100 (Lonza Ltd, UK) or Insect-Xpress medium, respectively (Lonza Ltd, UK) supplemented with 10% FCS (Sigma–Aldrich Ltd, UK).

2.2. Construction of transfer vectors for baculovirus system

For single expression of the structural proteins, bacmid BAC10:KO_{1962} which has a mutation in the orf1629 and pAcYM1 transfer vector were used [24,25]. For the generation of baculovirus multiple gene expression vector, a modified bacmid from bMNO14272 maintained in *Escherichia coli* strain EL350 was used as described previously [26,27]. Three transfer vectors, pRN306, pRN260 and pRN296 were utilized for cloning the coding regions of EHDV-1 S3, S7 and/or EHDV-2 S5. Each vector is composed of a *ph* promoter, *Cre* recombinase gene (*Loxp*71 and *Loxp*66), LacZα gene and a baculovirus locus specific sequence for homologous recombination. The transfer vector pAcYM1 was used to subclone S2 of EHDV-1 and EHDV-2 from the available recombinant plasmids pUc4K-EHDV1.S2 and pGEM-T-EHDV2.S2.

2.3. Generation and amplification of recombinant baculoviruses

To purify high-copy bacmid DNA from the bacterial culture, Invitrogen PureLinkTM HiPure Plasmid DNA Purification Kit (InvitrogenTM, UK) was used. For single and multiple gene expression, 2 μg of Bacmid DNA together with 500 ng of specific recombinant transfer vectors were used for transfection. The confluent *Sf*21 insect cells in the presence of 10 μl Insect GeneJuice (Novagen) and 100 μl TC-100 medium according to the manufacturer's instructions. For baculovirus amplification, the supernatant containing the recombinant baculovirus progeny was harvested 72 h post cotransfection and subjected to 4 rounds of amplification in fresh *Sf*9 insect cells. The virus infection was analyzed daily by light microscopy and confirmed further by plaque assay.

2.4. Protein expression

Suspensions of *Sf*21 cells at density of 1.2×10^6 cells/ml were infected at multiplicity of infection (MOI) of 2–5 and incubated for 72 h at 28°C. Samples were harvested and analyzed by SDS-PAGE followed by coomassie blue staining or western blot analysis using specific EHDV-1 polyclonal antibodies.

2.5. Particles purification

For the expression and purification of the recombinant core-like particles (CLPs, composed of VP3 and VP7) of EHDV-1, *Sf*9 insect cells were infected with the recombinant dual baculovirus Bac_{1629}:EHDV1.VP7.VP3 at a MOI of 3–5, incubated at 28 °C and harvested 72 hpi. CLPs were purified as previously described [18]. For EHDV-1 VLPs, the recombinant proteins were generated by infection of *Sf*9 insect cells with the quadruple recombinant baculovirus consisting of S2, S3, S5 and S7 of EHDV-1. For the generation of heterologous VLPs of EHDV-2, *Sf*9 insect monolayers were coinfected with the dual baculoviruses; Bac_{1629}:EHDV1.S3.S7 and Bac_{1629}:EHDV2.S2.S5. Homologous and chimeric VLPs were purified according to the methods described by Stewart et al. [28]. As positive controls, EHDV-1 cores and virions were purified according to the modified methods described previously [29,30].

2.6. Antibodies production

For polyclonal antibody production, 2.5 mg/ml of the CLPs quantified by Bradford reagent (Sigma–Aldrich Ltd, UK) and 2.0 mg/ml of VLPs were supplied to a commercial company. Immunization and work with vertebrate animals (rabbits) was performed at ThermoFisher Scientific in accordance with the UK Animals (Scientific Procedures) Act 1986 (UK Government 1986) using the principles outlined in the Home Office guidance 'Antibody Production: Principles for Protocols of Minimal Severity' (Home Office 2000). The work adhered to the principles of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (see https://www.nc3rs.org.uk/arrive-guidelines).

Animals were immunized first with 500 μg of antigen in 10 sites followed by boosting twice with 250 μg antigen at 14 and 28 respectively in the presence of Incomplete Fruend's adjuvant.

2.7. Electron microscopy

Aliquots (2 μl) of purified EHDV-1 cores, CLPs and VLPs were adsorbed onto copper 400-mesh Formvar carbon-coated grids (TAAB Laboratories Equipment Ltd, UK) for 2 min and stained with filtered 2% (w/v) phosphotungstic acid (PTA), pH 6.8. Grids were examined under JEOL 1200 EX transmission microscope.

2.8. Neutralizing and cross neutralizing antibody response

Antibodies raised against purified EHDV-1 VLPs were collected at (0, 35 and 56–58 days) and analyzed for their neutralizing activity

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