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Generation of virus like particles for epizootic hemorrhagic disease virus

Mario Forzan [a,*], Sushila Maan [b], Maurizio Mazzei [a], Manjunatha N. Belaganahalli [b], Lucia Bonuccelli [a], Monica Calamari [c], Maria Luisa Carrozza [d], Valentina Cappello [e], Mariagrazia Di Luca [f], Patrizia Bandecchi [a], Peter P.C. Mertens [b], Francesco Tolari [a]

[a] Dipartimento di Scienze Veterinarie, Università di Pisa, Italy
[b] The Pirbright Institute, Pirbright, UK
[c] Dipartimento di Biologia, Università di Pisa, Italy
[d] Scuola Normale Superiore, Pisa, Italy
[e] Istituto Italiano di Tecnologia, Center for Nanotechnology Innovation, NEST, Pisa, Italy
[f] NEST, Istituto Nanoscienze-CNR and Scuola Normale Superiore, Pisa, Italy

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ABSTRACT

Epizootic hemorrhagic disease virus (EHDV) is a distinct species within the genus *Orbivirus*, within the family *Reoviridae*. The epizootic hemorrhagic disease virus genome comprises ten segments of linear, double stranded (ds) RNA, which are packaged within each virus particle. The EHDV virion has a three layered capsid-structure, generated by four major viral proteins: VP2 and VP5 (outer capsid layer); VP7 (intermediate, core-surface layer) and VP3 (innermost, sub-core layer). Although EHDV infects cattle sporadically, several outbreaks have recently occurred in this species in five Mediterranean countries, indicating a potential threat to the European cattle industry. EHDV is transmitted by biting midges of the genus *Culicoides*, which can travel long distances through wind-born movements (particularly over water), increasing the potential for viral spread in new areas/countries. Expression systems to generate self-assembled virus like particles (VLPs) by simultaneous expression of the major capsid-proteins, have been established for several viruses (including bluetongue virus). This study has developed expression systems for production of EHDV VLPs, for use as non-infectious antigens in both vaccinology and serology studies, avoiding the risk of genetic reassortment between vaccine and field strains and facilitating large scale antigen production. Genes encoding the four major-capsid proteins of a field strain of EHDV-6, were isolated and cloned into transfer vectors, to generate two recombinant baculoviruses. The expression of these viral genes was assessed in insect cells by monitoring the presence of specific viral mRNAs and by western blotting. Electron microscopy studies confirmed the formation and purification of assembled VLPs.

1. Introduction

Epizootic hemorrhagic disease virus (EHDV) is a distinct virus species within the genus *Orbivirus*, belonging to the family *Reoviridae*. Epizootic hemorrhagic disease virus particles are non-enveloped, approximately 62–80 nm in diameter with a similar structure to bluetongue virus (BTV), which is classified as the prototype of this genus (Mertens et al., 2005; Savini et al., 2011; Attoui et al., 2012). The EHDV genome is made of ten segments of linear dsRNA which encode seven structural proteins (VP1-VP7) and probably, as recently discovered for BTV four non-structural proteins (NS1-NS4) (Belhouchet et al., 2011; Mecham and Dean, 1988; Ratinier et al., 2011; Stewart et al., 2015). The ten segments of the EHDV genome are enclosed, along with three minor enzymatic proteins (VP1, VP4 and VP6) within the three-layered, icosahedral virus capsid composed of the four 'major' structural proteins. The sub-core shell is formed by 120 copies of VP3, the core-surface layer comprises 780 copies of VP7, while the outer-capsid contains 180 copies of VP2 and 360 copies of VP5. VP3 is part of the viral 'scaffolding' and contributes to the correct size and structure of the virus. VP7 is the major core protein and is widely used as an orbivirus serogroup/species-specific antigen in serological assays (Luo and Sabara, 2005; Mecham and Wilson, 2004). VP2, which is the outermost 'exposed' EHDV protein, is responsible for receptor-binding on target cells and determines virus-serotype through the specificity of its interactions with neutralizing antibodies. According to some reports, VP5, which is a membrane permeabilization protein, may also contribute to the specificity of the neutralizing antibody response, and like VP5 of BTV it has been shown to enhance the immune response induced by VP2 alone. On the basis of antigenic and genetic analyses of the two outer capsid proteins (VP2 and VP5), the EHDV serogroup has been proposed to comprise seven serotypes, although there is not yet a widely accepted

* Corresponding author.

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consensus on their exact number (Anthony et al., 2010, 2009a, 2009b, 2009c; Maan et al., 2010; Viarouge et al., 2015).

White-tailed deer (*Odocoileus virginianus*) is the species most severely affected by EHDV, while mule deer and pronghorn antelope are less severely affected. Although black-tailed deer, red deer, wapiti, fallow deer, roe deer, elk, moose, and bighorn sheep do not appear to be severely affected, they may seroconvert, indicating that they can become infected (MacLachlan and Osburn, 2004; Noon et al., 2002a, 2002b). Recently EHDV has also been isolated in yaks (Van Campen et al., 2013). Goats do not seem to be susceptible to EHDV infection and sheep can be infected experimentally but rarely develop clinical signs, and a recent study has excluded a role in transmission to more susceptible species (Kedmi et al., 2011a).

Cattle were first found to be susceptible to infection by an EHDV-2 strain, named as Ibaraki virus, in Japan during 1959. This strain has subsequently been associated with periodic outbreaks in cattle in East Asia (Hirashima et al., 2015; Kitano, 2004). In recent years EHDV-6 and 7 have been involved in several outbreaks in cattle: EHDV-6 in Turkey, Algeria, Morocco, Reunion Island and French Guiana (Albayrak et al., 2010; Cêtre-Sossah et al., 2014; Temizel et al., 2009; Viarouge et al., 2014); EHDV-7 in Israel in 2006 (Eschbaumer et al., 2012; Wilson et al., 2015; Yadin et al., 2008). During 2007 and most recently, EHDV-2 caused morbidity in dairy cattle in Ohio and Kentucky (Garrett et al., 2015) (ProMed Archive Number: 20150923.3666422). EHDV-6 was identified for the first time in the USA, during 2006 and by 2008 was widespread, although the new isolates were all reassortants containing genome segments derived from the parental strains of EHDV-2 and 6 (Allison et al., 2010, 2012; Anbalagan et al., 2014a). EHDV has recently also been isolated in Brazil (Favero et al., 2013). The extended host range of EHDV-6 and 7 in cattle has caused major concerns for international authorities and in 2008 the disease was included in the Office International Epizootics (OIE) list of multispecies/transboundary diseases (OIE, 2012).

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

Virus like particles (VLPs) are considered as a safer and effective alternative to live attenuated vaccines, and VLPs have been generated and tested as non-infective immunogens both for human and animal diseases (including bluetongue), triggering the same immunological responses as the whole virus. VLP have the advantage of being immunogens with the same structural characteristics as the native virus particle but are free of the viral genetic material and cannot therefore pose a risk of transmission (Lua et al., 2014; Roy and Noad, 2008; Stewart et al., 2012; Zhao et al., 2013).

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

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

2. Material and methods

2.1. Virus propagation

The virus used in this study EHDV-6 (MOR2006/05) was obtained from the Orbivirus Reference Collection (ORC) at The Pirbright Institute (Pirbright, UK) (http://www.reoviridae.org/dsRNAvirusproteins/ReoID/EHDV-Nos.htm). Virus was propagated in BHK-21 cells (clone 13 obtained from European Collection of Animal cell Cultures (ECACC – 84100501)), supplied with Dulbecco's minimum essential medium (DMEM) supplemented with antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and 2 mM glutamine. Infected cell cultures were incubated until they showed widespread (100%) cytopathic effects (CPE). Viruses were harvested, aliquoted and used for the dsRNA extraction, or stored in the ORC at −80 °C.

2.2. Preparation of viral dsRNA

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

2.3. Reverse transcription, PCR amplification and cloning of EHDV cDNAs

Genome segments encoding VP2, VP3, VP5 and VP7 of EHDV-6 (MOR2006/05) were reverse-transcribed using a 'full-length amplification of cDNA' (FLAC) technique described by Maan et al. (2007). Briefly, a 35 base self-priming oligonucleotide 'anchor-primer', with a phosphorylated 5′ terminus was ligated to the 3′ ends of the viral dsRNAs using the T4 RNA ligase, followed by reverse transcription with the RT system (Promega, MA, USA). The resulting cDNAs were amplified using a high fidelity KOD polymerase (Merck Millipore, Darmstadt, Germania,) and 5-15-1-NOT-S primers (5′-GCAGTTTAGAATCCTCAGAGGTC-3′), then analyzed by agarose gel electrophoresis and purified using an illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK). The amplified cDNAs of Seg-6 and Seg-7, encoding for VP5 and VP7 respectively, were cloned into the Strataclone blunt-end PCR cloning vector 'pSC-B-amp/kan' supplied in the StrataClone Blunt PCR cloning kit (Agilent Technologies, CA, USA) and the recombinant plasmid vectors were transformed into Solopack® competent cells (Agilent Technologies, CA,USA). Amplicons of Seg-2 and Seg-3 (encoding for VP2 and VP3 respectively) were cloned into the 'pCR®-Blunt' vector supplied with the Zero Blunt® PCR Cloning Kit. Clones containing the desired inserts were identified by colony touch PCR using M13 universal primers and GoTaq® Hot start polymerase (Promega, MA, USA). Plasmid DNA was isolated using the QIAprep® Spin Miniprep kit (Qiagen, Heidelberg,