Developing Virus-Like Particles (VLPs) and Heterologous VLPs Vaccines for Epizootic Hemorrhagic Disease Virus (EHDV) Serotypes

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Abstract

Epizootic Hemorrhagic Disease Virus (EHDV) is an insect-transmitted pathogen of ruminants, causing periodic and significant losses in wild and captive deer populations and less frequently, a bluetongue-like disease in cattle. The serogroup of EHDV within the *Orbivirus* genus of the *Reoviridae* family consists of seven serotypes, in which emerging serotypes pose an increasing risk either regionally or globally, due to the insect vectors. To date, no vaccine against EHDV is commercially available, apart from the live-attenuated vaccine for EHDV-2 (IBAV). In this study, Virus-Like Particles (VLPs) of EHDV-1 and heterologous VLPs of EHDV-2 were generated using baculovirus multigene expression system for the synthesis of the two outer and two inner capsid proteins, essential for the formation of VLPs. The assembly of EHDV-1 recombinant structural proteins into Core-Like particles (CLPs, two proteins) and (VLPs, four proteins) was confirmed by EM analysis. The biological activity of the raised antisera to neutralise EHDV-1 was efficiently confirmed by neutralisation assay at 1:64 dilution. Cross neutralising activities were also detected against EHDV-2 and EHDV-6 serotypes at 1:8 dilution. Results presented in this study validate the potential efficacy of the VLP as a neutralising vaccine and strongly suggest its use as vaccine candidate.

Additionally, an alternative approach was also initiated in this research to develop a rational vaccine against EHDV-2 using the reverse genetics system (RG). Towards this, it was first established that *in vitro* synthesised transcripts from purified EHDV-2 cores could generate infectious virus upon cell transfection. Note that both the generation of core transcripts and recovery of infectious virus of EHDV were not demonstrated previously. Subsequently a complete set of 10 T7 transcripts was

synthesised, however, it was not possible to recover any infectious virus, likely to be some unwarranted mutations. Nevertheless, these transcripts will be further investigated for future RG studies.

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Abbreviations

aa	amino acid
AcMNPV	*Autographa californica* multicapsid nucleopolyhedrosis virus
AHS	African Horse Sickness
AHSV	African Horse Sickness Virus
APS	ammonium persulphate
ATP	Adenosine triphosphate
Bacmid	Bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BEVS	Baculovirus expression vector system
BHK	Baby Hamster Kidney cells
BmNPV	*Bombyx mori* nucleopolyhedrosis virus
bps	base pair(s)
BSA	Bovine serum albumin
BSR	derivative of BHK
BT	Bluetongue
BTV	Bluetongue virus
°C	degree Celsius
cDNA	complementary Deoxyribonucleic acid
CLPs	Core-Like Particles
CPE	cytopathic effect
cryo-EM	cryo-electron microscopic
DEPC	Diethylpyrocarbonate
DIVA	differentiating infected from vaccinated animals

DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	days post infection
dsRNA	Double-stranded Ribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EHD	Epizootic Hemorrhagic Disease
EHDV	Epizootic Hemorrhagic Disease Virus
EM	electron microscope
F	forward
FCS	Fetal bovine serum
FLAC	Full-length amplification of Cdna
GFP	Green fluorescent protein
h	hour(s)
hpi	hours post infection
hpt	hours post transfection
HPV	Human papillomavirus
IBAV	Ibaraki virus
ICTV	International Committee on Taxonomy of Viruses
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	Kilodalton
L	Large
LB	Luria Bertani
LBA	Luria Bertani Agar

M	Molar
mA	Milliampere
µg	microgram
min	minute(s)
ml	millilitre
µl	microliter
mM	Millimolar
mRNAs	messenger RNAs
MOI	multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
nfH_2O	nuclease-free H_2O
NBT	nitroblue tetrazolium
ng	Nanogram
NLS	N-lauroyl sarcosine
OD_{600}	optical density
OIE	World Organisation for Animal Health
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pAcYM1	baculovirus transfer vector
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PNK	polynucleotide kinase
Poly	polyhedrin locus
Polyh	polyhedrin promoter

PRRs	pattern recognition receptors
PTA	phosphotungstic acid
RBCs	red blood cells
R	Reverse
RG	reverse genetics
RNA	Ribonucleic acid
SAP	shrimp alkaline phosphatise
SDS	sodium dodecyl sulphate
S	Small / Segment
SAM	S-adenosylmethionine
sec	Second(s)
*Sf*9 or *Sf*21	*Spodoptera frugiperda*
ssRNA	single-stranded RNA
TC	transcription complex
$TCID_{50}$	tissue culture infectious dose 50
TEMED	N,N,N',N'-Tetramethylethylenediamine
USDA	The United State National Deer Farmers Association
UV	ultraviolet
VLPs	Virus-Like Particles
V/V	volume/volume
W/V	weight/volume
x g	gravitational force
Zeo^R	Zeocin resistance

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Chapter 1

Introduction

1.1. *Reoviridae* Family

The *Reoviridae* is the largest of the double-stranded RNA (dsRNA) families and the most diverse with respect to host range including vertebrates, plants and insects (Table 1) (Hill et al., 1999). Genera of this family are distinguished by their genome organisation, particle structures as well as host range. As a group III viruses their genome is comprised of dsRNA as indicated by physiochemical properties and nuclease insensitivity. However, isolation of such genomic dsRNA demonstrates a segmented nature which is consistent across family members (Gomatos and Tamm, 1963, Langridge and Gomatos, 1963). The number of dsRNA segments is variable across species, falling between 10 and 12. This was initially confirmed for Bluetongue Virus (BTV) by early results from electronic microscopy (EM) (Vasquez and Kleinschmidt, 1968), followed by evidence from polyacrylamide gel electrophoresis (PAGE) which showed that the dsRNA genome is released from disrupted particles as 10 discrete segments, which can be divided into large (L), medium (M) and small (S) sizes (Shatkin et al., 1968, Watanabe et al., 1968). Initially, studies using sequence analysis of the full-length cDNA of the genome segments and *in vitro* translation indicated that each segment encodes for a single protein, on only one of the complementary RNA strands; however it was later demonstrated that some segments may encode for up to three proteins due to the presence of more than one in-frame initiation codon (Lee and Roy, 1986, Mertens et al., 1984). More recently, the presence of a frame shifted internal coding sequence has been observed for the NS4 protein of BTV which is translated from within the VP6 coding region (Ratinier et al., 2011).

Morphologically, the mature virion sizes ranges from 60 to 85 nm with no capsid envelope. The structural proteins are arranged in two concentric layers of icosahedral symmetry, an inner capsid or "core" and outer capsid.

Using cyro-electron micrographic (Cryo-EM) reconstruction and EM studies have shown that members of *Reoviridae* family can be further divided into two distinct subfamilies, turreted and non-turreted, based on the structures of their core particles (Hill et al., 1999). The *Sedoreovirinae* subfamily includes genera containing non-turreted (smooth) core particles that are almost spherical in appearance, including *Cardoreovirus*, *Mimoreovirus*, *Orbivirus*, *Phytoreovirus*, *Rotavirus* and *Seadornavirus*. The *Spinareovirinae* subfamily includes genera containing viruses with large spikes or turrets at the 12 icosahedral vertices of the virus or core particles including *Aquareovirus*, *Coltivirus*, *Cypovirus*, *Dinovernavirus*, *Fijivirus*, *Idnoreovirus*, *Mycoreovirus, Orthoreovirus* and *Oryzavirus* (Table 2) (Roy, 2013) . Such grouping of *Reoviridae* family members is supported partially by the similarities of enzyme motifs between the turreted and non-turreted groups (Nibert and Kim, 2004, Attoui et al., 2005). Despite these structural differences, all *Reoviridae* members distinctively synthesize viral messenger RNAs (mRNAs) within an enclosed capsid, setting the mechanistic details of their replication cycle apart from other virus families.

Table 1.

Taxonomic classification of dsRNA viruses. Adapted from (Hill et al., 1999).

Family	Genera	Hosts
Reoviridae	15	Mammals, Birds, Reptiles, Fish, Insects, Plants, Fungi
Birnaviridae	3	Birds, Fish, Insects
Totiviridae	3	Fungi, Protozoa
Partitiviridae	3	Fungi, Plants
Chrysoviridae	1	Fungi, Plants
Hypoviridae	1	Fungi
Cystoviridae	1	Bacteria

Table 2.

Virus genera and orbivirus serogroups. Adapted from *Fields Virology, Sixth Edition* (Roy, 2013).

***Reoviridae* viruses**		
Genus	**Genome segments**	**Hosts**
Subfamily *Sedoreovirinae*		
Cardoreovirus	12	Crustaceans
Mimoreovirus	11	Marine protists
Orbivirus	10	Mammals, Birds, Arthropods
Phytoreovirus	12	Plants, Insects
Rotavirus	11	Mammals, Birds
Seadornavirus	12	Humans, Insects
Subfamily *Spinareovirinae*		
Aquareovirus	11	Fish, Molluscs
Coltivirus	12	Mammals, Arthropods
Cypovirus	10	Insects
Dinovernavirus	9	Insects
Fijivirus	10	Plants, Insects
Idnoreovirus	10	Insects
Mycoreovirus	11-12	Fungi
Oryzavirus	10	Plants, Insects
Orthoreovirus	10	Mammals, Birds, Reptiles

1.2. Orbiviruses

The *Orbivirus* genus is one of 15 genera within the *Reoviridae* family, which includes vertebrate, arthropod, and plant pathogens. In Latin, *Orbis* means ring shape referring to the surface of the core particles which can be observed by negative staining under EM. Unlike the reoviruses and rotaviruses, orbiviruses are arthropod-borne viruses causing serious diseases in domestic and wild animals. Additionally, orbivirus particles are fairly fragile and lose their infectivity in low acidic conditions (pH= 6), while they are relatively resistant to solvents and detergents (Borden et al., 1971). Based on serologic reactivities, 21 serogroups of orbiviruses are distinguished, and within each group many serotypes are differentiated by neutralization tests (Table 3) (MacLachlan, 1994, Mertens et al., 2004, Maan et al., 2007).

These viruses are morphologically identical and transmitted to vertebrates primarily by arthropod vectors which, depending on the individual virus, can be certain species of *Culicoides* midges (Fig. 1), mosquitoes, black flies, sandflies or ticks (Tabachnick, 2004, Wilson et al., 2008, Purse et al., 2005). Although orbiviruses do not have the ability to transfer directly between vertebrates, they can replicate in both vertebrates and arthropods. The global and seasonal distribution of individual virus species is limited to the distribution of competent biological vectors and appropriate climate changes (Shope et al., 1960, Purse et al., 2005).

Economically, the most important viruses within this genus are BTV and African horse sickness virus (AHSV), which are responsible for Bluetongue disease (BT) in sheep and cattle and African horse sickness disease (AHS) in horses (Maclachlan and Guthrie, 2010). However, several others including EHDV pose an increasing risk either regionally or globally, due to the insect vectors.

Table 3.

Orbiviruses serogroups and serotypes. Adapted from (Mertens et al., 2004).

Serogroups	**Serotypes**
African Horse Sickness	**9**
Bluetongue	**26**
Changuinola	13
Chenuda	8
Chobar Gorge	2
Corriparta	6
Epizootic Hemorrhagic Disease virus	**7**
Equine encephalosis	7
Eubenangee	4
Ieri	3
Great Island	37
Lebombo	1
St. Croix River	1
Palyam	11
Peruvian Horse Sickness	1
Orungo	4
Umatilla	4
Wallal	3
Warrego	3
Wongorr	8
Wad Medani	2

Fig. 1. Blood-Feeding *Culicoides* Midges (Wilson et al., 2008).

1.3. Epizootic Hemorrhagic Disease Virus

EHDV belongs to the *Orbivirus* genus and shares many morphological and genetic characteristics with other members of the genus, particularly BTV and AHSV (Verwoerd et al., 1979, Roy and Gorman, 1990, Knudson and Monath, 1990). EHDV is an insect-transmitted pathogen of ruminants, causing periodic and significant losses in wild and captive deer populations and less frequently, a bluetongue-like disease in cattle (Omori et al., 1969). The current report by the International Committee on the Taxonomy of Viruses (ICTV) designates that there are ten predicted serotypes/strains of EHDV (Mertens et al., 2005). However, based on the recent molecular and serological analysis of the outer capsid proteins VP2 and VP5, seven serotypes of EHDV have been recognised, wherein the EHDV serotype 3 (Ib Ar 22619) and serotype 1 were similar and could be considered as one serotype, and EHDV-318 was serologically identical to EHDV-6 and Ibaraki virus (IBAV) is similar to EHDV-2 (Anthony et al., 2009b).

Recently, genomic studies and phylogenetic analysis comparing the whole genome of EHDV strains showed that the core proteins can be used to group EHDV into “eastern” (i.e. Asia and Australia) and “western” (i.e. America, Africa and Middle East) strains (Anthony et al., 2009b, Anthony et al., 2010). Depending on these studies, strain designation would depend on serological and molecular analysis of the genetic variations within the serotypes

1.4. Epidemiology and susceptible hosts

Historically, EHDV has been associated with disease in wild cervids, particularly white-tailed deer. Clinical signs of Epizootic Hemorrhagic Disease (EHD) have been observed in North America, Australia, Asia and more recently in the countries surrounding the Mediterranean Basin (Fig. 2). Two serotypes; EHDV-1 (New Jersey strain), first described in North America in 1955 when a large number of white-tailed deer died and EHDV-2 (IBAV), originally isolated in southern Alberta Canada in 1962, are known to be endemic (Shope, 1955).

In 2006, a virus was recovered from dead white-tailed deer in Indiana and Illinois, which was later confirmed as EHDV serotype 6 by serological and genetic tests (Allison et al., 2010). In 2007, additional isolates of EHDV-6 were detected in Missouri (in white-tailed deer) followed by detection in Kansas and Texas in 2008 (Allison et al., 2010), and Missouri and Michigan in 2009. Such spreading of the virus suggested that a reassortment strain EHDV-6 had appeared with higher pathogenicity and was geographicallly endemic in a widespread region of the United States.

Until 2006, it was believed that EHDV does not cause significant clinical signs in cattle with the exception of IBAV strain, which has been observed to infect cattle

causing severe disease (Omori et al., 1969). Several outbreaks of which occurred in Japan 1959, Korea and Taiwan, wherein thousands of herd of cattle were affected (Omori et al., 1969). However, in conjunction with this American outbreak, in 2006 and 2007, EHDV-6 and EHDV-7 seroprevelance was documented in the Mediterranean Basin by Algeria, Tunisia, Morocco, Israel, Jordan as well as the Island of Reunion (Breard et al., 2004, Gibbs and Lawman, 1977, Temizel et al., 2009). Each of the above strains was pathogenic and caused diseases in cattle. EHDV prevelance in the Mediterranean Basin can be traced back to 1951 in Israel, where a disease similar to BTV was described in cattle and sheep (Komarov and Goldsmith, 1951).

Although no cases of EHDV infection have been reported in Europe, the recent outbreaks “wherein cattle were the main host” in the countries bordering Europe signifcantly increases the risk of its invasion into central and northern Europe. The scenario for EHDV can be thought of as similar to that of BTVs emergence in Europe which occurred in the late 1990s wherein some BTV serotypes were circulating in Algeria, Tunisia, Turkey and Israel. The virus then crossed the Mediterranean basin and entered Europe.

Animal hosts of EHDV are prevelant in Europe allowing for potential emergence. European sheep breeds were found to be susceptible to EHDV infection. However, though the viraemia could been detected, the infection was subclinical, allowing sheep to potentially act as reservoirs in outbreaks (Gibbs and Lawman, 1977, Moore, 1974). The role of goats as a host for EHDV is uncertain although antibodies could be detected in goats in the regions where EHDV is found (al-Busaidy and Mellor, 1991). Pigs are not susceptible to EHDV and little is known about the susceptibility of dogs (Gibbs and Lawman, 1977, Howerth et al., 1995).

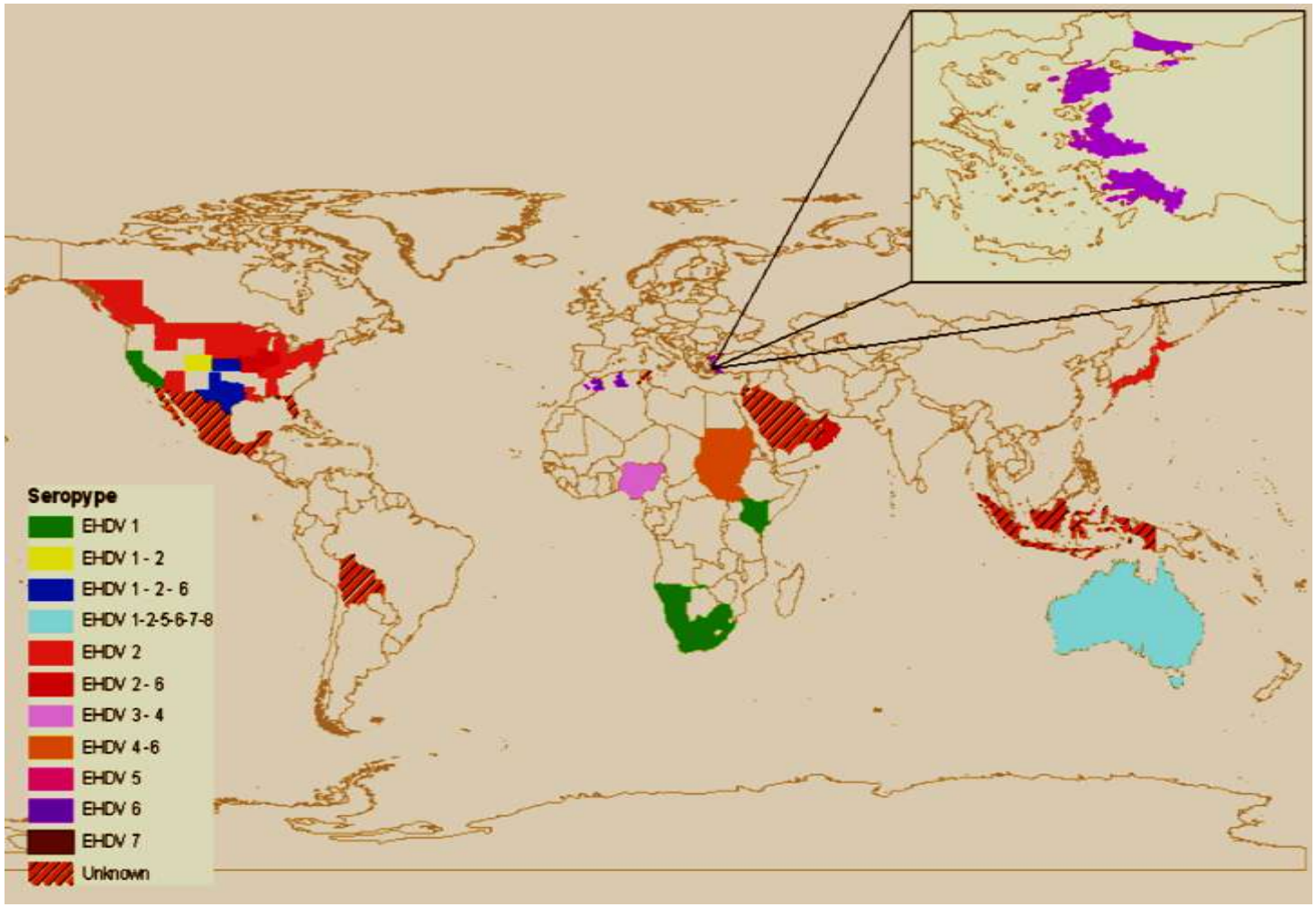

Fig. 2. Map with the localization of the EHDV infection worldwide. Where no geographical details as district/province etc, are not reported, the whole country was indicated as infected. Adapted from European Food Safety Authority (EFSA) Journal 2009;7(12):1418.

1.5. Pathogenicity

EHDV pathogenicity is similar to that of BTV which has been extensively studied and thoroughly reviewed by MacLachlan (McLaughlin et al., 2003). During blood feeding of the hematophagous *Culicoides* species, animals are innoculated with infectious particles which bind to the red blood cells (RBC) using glycophorin A as a receptor causing primary viraemia (Eaton and Crameri, 1989). The virus is then disseminated through the blood stream to other sites of replication such as the lymph nodes (McLaughlin et al., 2003). As EHDV has tropism towards the endothelial cells of the lymphatic vessels (Tsai and Karstad, 1970), the virus replicates there causing secondary viraemia, reaching the reticulum endothelia system comprising of liver, spleen and lymph node. The virus then undergoes another cycle of replication before spreading to the whole body via blood stream (Gibbs and Lawman, 1977, Aradaib et al., 1997).

During viraemia, the virus is cell-associated particularly to RBCs where the virus is present in higher titres and for longer period of time (Gibbs and Lawman, 1977, Aradaib et al., 1997). The duration of viraemia has been studied in deer, cattle and calves naturally and experimentally infected with EHDV. Gamma interferons were detected in the infected animals from 4 days post infection (dpi) until the appearance of detectable antibodies at 10 dpi, while neutralizing antibodies were detected between 10-14 dpi (Gibbs and Lawman, 1977).

EHD disease has several clinical forms depending on the host type and immunity. The disease can be presented subclinically, wherein the animal shows no symptoms in spite of viremic load. This mainly occurs in sheep or goats (Thompson et al., 1988). However, in cattle infected with EHDV-2 (IBAV) or EHDV-6, the disease is characterised with fever, anorexia and difficulties in swallowing leading to emaciation

(Inaba, 1975). Oedema haemorrhages, erosion and ulceration in the mouth and lips can also be seen (Fig. 3 and Fig. 4) (Temizel et al., 2009).

In white-tailed deer, there are three phases of the disease; pre-acute, acute and chronic. In the first phase, animals exhibit clinical signs similar to those observed in cattle, accompanied by severe and rapid oedema of the head and neck (Fig. 5). During this stage of disease, deer death is rapid, mostly within 8-36 hours (h) and at times without clinical symptoms (Omori et al., 1969). As the virus disseminates throughout the body, the disease progresses to the acute phase. In the acute phase, the clinical signs of pre-acute might be accompanied by haemorrhages in many tissues including skin, heart and the gastrointestinal tract.

Generally, animals in the acute phase develop ulcers or erosions of the tongue and dental pad. At this stage, the virus can be fatal and the animal could die within 72 h. The morbidity rate in the white-tailed deer is higher, up to 29% and a mortality rate of 20% (Gaydos et al., 2004), while in cattle the morbidity varies from 1-18% but the mortality is usually low, around 2.2% (Yadin et al., 2008).

In the chronic stage, deer are usually in poor health for several weeks but gradually recover. Post recovery, the individual might become lame or develop rings on hooves, ulcers scars, or erosions in the rumen, mainly due to the growth interruptions (Fig. 5) (Work et al., 1992).

Fig. 3. **Erosive lesion on pulvinus dentalis of cow seropositive for EHDV (Temizel et al., 2009).** **http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2662652/figure/F1/**

Fig. 4. **Swollen conjunctiva of cow seropositive for EHDV (Temizel et al., 2009).** http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2662652/figure/F1/

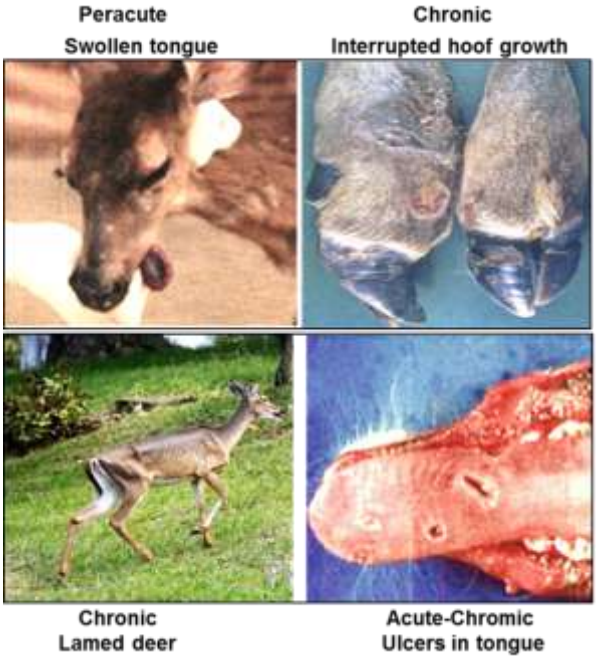

Fig. 5. **Lesions of white-tailed deer infected with EHDV during all forms of disease (Medical and veterinary entomology).** http://vet.uga.edu/population_health_files/HD-brochure-web.pdf

1.6. Economic impact

It is important to determine the economic impact of an infection as this influences the way in which the control strategies are selected. For a long time, EHD disease was considered as a disease of minor importance due to its non-agricultural host range. Recent outbreaks, however, in the Reunion Island, North Africa, Turkey and Israel wherein cattle were the main host have led to its consideration as an important emerging pathogen. Post these outbreaks, the economic losses in the industry of dairy cattle have become a significant concern especially for the USA and Europe.

During these epidemics, the economic impact of the EHD in cattle was characterized by a significant reduction in milk production and increased mortality (Kedmi et al., 2010). In total, a loss of $95 per cow could be attributed to EHDV infection, which equates 2% of the total annual production of these cows. This was a notable loss even when compared to the most costly production diseases in the dairy herd (Kedmi et al., 2010). In addition, economic losses were recorded due to abortion, infertility and inferior milk quality. Regarding morbidity and mortality, the highest morbidity rates of cattle infected with EHDV-6 occurred in Morocco (18%), while the rate of fatality cases was about 2.2% (OIE and WAHID, 2004). In comparison, EHDV-2 infected cattle in Japan scored the lowest percentage of both morbidity and mortality with up to 1.16% and 0.06%, respectively (Table 4) (Omori et al., 1969).

As for wildlife, it is difficult to determine the morbidity and mortality rates. Generally, in areas of outbreaks, studies showed that white-tailed deer were the most severely affected animal with the disease, wherein the estimated number of infection was 29% while mortality rate was 20% (Gaydos et al., 2004). However, the survival rate was considerably higher in mule deer, black-tailed deer and pronghorn antelope (Work et al., 1992).

Conversely, the economic impact might not only be due to the direct effect. For instance, the international movement of livestock and/or their germplasm is restricted from EHDV prevalent to EHDV free countries; unless the animals are certified to be clear of EHDV infection by serology or virus isolation (Aradaib et al., 1994). Such restrictions could lead to economic losses for EHDV-endemic countries that rely on the sale of livestock for foreign exchange (Abdy et al., 1999).

Table 4.

Morbidity and mortality of EHDV infection in cattle. Adapted from European Food Safety Authority (EFSA) Journal 2009;7(12):1418.

Serotype	Location	Apparent morbidity	Apparent mortality	Reference
IBAV (EHDV-2)	Japan	1.96%	0%	(Omori et al., 1969)
IBAV (EHDV-2)	Japan	1.16%	0.06%	(Omori et al., 1969)
EHDV-2	USA	-	-	(House et al., 1998)
NK	Reunion Island	3.23%	0%	(Breard et al., 2004)
EHDV-7	Israel	-	-	(Yadin et al., 2008)
EHDV-6	Turkey	-	-	(Temizel et al., 2009)
EHDV-6	Morocco	18%	2.2%	(OIE and WAHID, 2004)
EHDV-6	Algeria	8%	0.5%	(OIE and WAHID, 2004)

NK Not known

1.7. Monitoring and control measures

Control and prevention of the disease caused by viral infection should be based on two possibilities; the countries are free of the disease and want to prevent it from entering their territory, or they already have the infection but want to avoid its spreading. However, it is more difficult to manage and eradicate a viral vector-borne disease due to uncontrollable factors such as vector, climate and geographic features. Generally, controlling the disease using insecticides is expensive and not a

viable option in large areas, particularly when the reintroduction of the vector via wind is likely to occur. Another option is the use of insect secure tents to reduce the contact between the vector and vulnerable hosts.

For EHDV, no official monitoring or control programmes were established initially. This was because no significant economic losses on dairy cattle were reported from the infection with EHDV. The only losses reports were on farmed and wild deer as EHD of deer is considered a compulsory notifiable disease according to Annex I of Council Directive 92/119/EEC. This scenario changed after the recent outbreaks caused by exotic serotypes EHDV-6 and EHDV-7 in Mediterranean basin countries, Morocco, Algeria, Tunisia, Turkey and Israel, wherein cattle were the main host. To control the invasion of EHDV, the EHDV outbreak countries have applied measures, such as monitoring of wild reservoirs, quarantine, vector control in infected premises and increasing the awareness of veterinarians and farmers.

1.7.1. Vaccination

To control EHDV infection, vaccination is the primary strategy along with the control of arthropod vectors. Several vaccines for BTV have been developed and used in endemic regions for the prevention of virus infection (Roy et al., 1994, Matsuo et al., 2011). The development of a vaccine against EHDV is an urgent concern particularly for EHDV strains which are responsible for severe clinical indications in domestic ruminants in the USA or Turkey, which might be imported into Europe. Autogenous inactivated vaccines were prepared in North America from EHDV-1 and EHDV-2 isolates originated from infected or dead animals. However, the use of this vaccine was limited to the original and adjacent herds. Such vaccines have to be tested for safety and purity but not for efficacy; additionally, their application should be approved by The United State National Deer Farmers Association.

No vaccine for EHDV is commercially available, with the exception of the live-attenuated and inactivated vaccines to control Ibaraki disease caused by IBAV (Aradaib et al., 2009). This vaccine was used following the outbreaks in Japan in the 1980s and proved to be safe and effective, at least up to the 1997 epidemic, during which abortion, stillbirths and other new clinical signs never observed in the previous outbreak were documented (Ohashi et al., 1999).

One of the major challenges for *Orbivirus* vaccine researchers is the development of vaccines that can afford a wider protective immune response against many serotypes of each virus, and to develop diagnostic method to distinguish between vaccinated and infected animals (DIVA). However, it should be taken into consideration that the attenuated virus could revert to virulence and spread accidentally to animals via the *Culicoides* vectors (Aradaib et al., 2009), such as the insufficient attenuation of BTV-16 which allowed it to circulate in Corsica. In addition, the use of a modified vaccine, containing two or more serotypes may enable these viruses to reassort in nature, giving rise to virulence reversion. Furthermore, the genome reassortment can occur with circulating pathogen virus.

1.8. Virion morphology

Genetic studies and phylogenetic analysis have revealed that the members of *Orbivirus* genus in the *Reoviridae* family are closely related to each other particularly for BTV and EHDV (Mecham and Dean, 1988). BTV, as a prototype of the orbiviruses, has been extensively studied genetically, structurally and at the molecular level. Thus, it can act as a model for EHDV and other orbiviruses.

BTV particles are complex structures, non-enveloped and composed of seven proteins organised in two concentric shells that enclose 10 dsRNA viral genome

segments (Fig. 6) (Roy et al., 2009). EM studies of the negatively stained and purified particles indicated an icosahedral double capsid structure of the mature virion and the cores with the absence of the outer layer (Fig. 7A and C) (French et al., 1990). The outer shell of the mature BTV-1 virion is composed of VP2 (112 kDa) (Urakawa et al., 1994) and VP5 (59 kDa) (Loudon et al., 1991) that enclose a transcriptionally active core (Verwoerd et al., 1972). The outermost layer of the core is formed by VP7 (38 kDa) (Wade-Evans, 1990), which interacts with the underlying sub-core capsid shell composed of VP3 (103 kDa) (Gould, 1987). The VP3 shell interacts with both the viral RNA genome and the transcription complex (TC) composing of minor proteins VP1, VP4 and VP6.

The process of BTV assembly was previously investigated using baculovirus system that accommodated the expression of the entire size range of the structural proteins. Moreover, using baculovirus multiple expression system, the function of the structural proteins in the formation of specific virion structures was delineated. In insect cells, the production of CLPs was achieved by dual expression of the core proteins VP3 and VP7 (Fig. 7D) (French and Roy, 1990). Further expression studies enabling production of double-shelled VLPs (French et al., 1990) by coinfection of insect cells with two baculoviruses, one expressing CLP proteins VP3 and VP7 and the other expressing VP5 and VP2 outer capsid proteins (Fig. 7B). These data indicated that the outer and inner capsid proteins had the ability to form VLPs lacking the viral genome and TC.

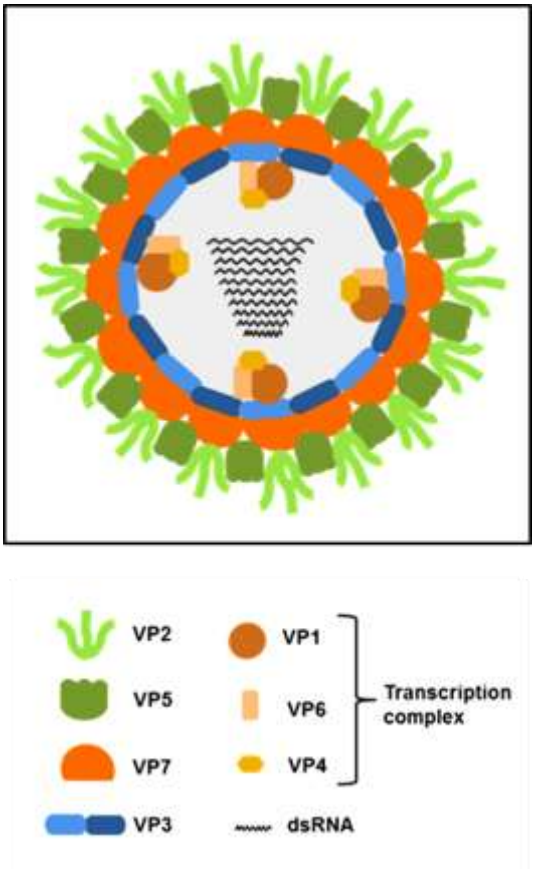

Fig. 6. BTV cartoon based on Cryo-EM study. The BTV particle is composed of an outer layer of (VP2 and VP5) proteins, an outer core layer of VP7 proteins and inner core layer of the VP3 proteins. Along with VP3 are the VP1, VP4 and VP6 proteins. The 10 segments of the virus dsRNA are enclosed within the capsid. Adapted from (Roy et al., 2009).

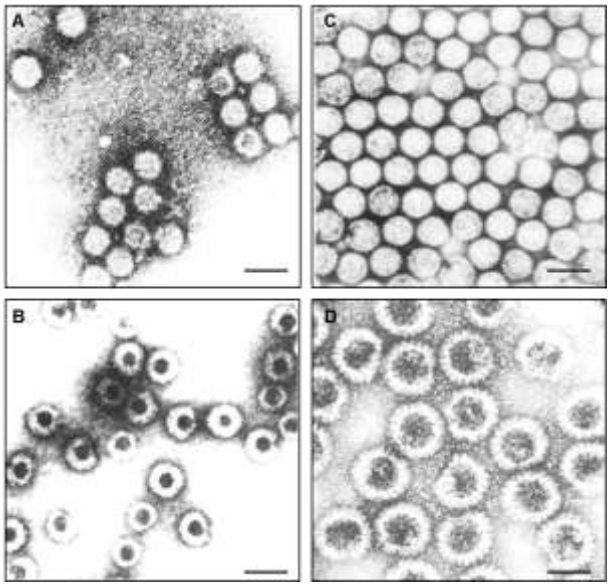

Fig. 7. Electron micrographs of baculovirus-expressed particles. (A) Authentic BTV particles. (B) Empty BTV double-shelled VLPs. (C) BTV core particles and (D) CLPs formed by VP7 and VP3. Adapted from *French et al* (French et al., 1990).

1.9. The outer capsid proteins

The outer layer consists of 60 triskelion trimers, made up of 180 molecules per particle of VP2 and a total of 360 molecules of VP5 exist per particle, forming 120 globular structures. VP2 represents the “sail-shaped” spikes which are located above of VP5 trimers and it is the most exposed protein (Fig. 8) that mainly interacts with the environment and mediates the initial attachment of the virus to the host cell receptors, thereby facilitating the entry (Hassan and Roy, 1999, Hassan et al., 2001, Huismans et al., 1987). Sequencing data and immunological analysis has demonstrated that VP2 is the most variable protein attributing to the serotype specificity (Maan et al., 2007, Maan et al., 2011). Moreover, sequence alignments of VP2 from several serotypes showed specific regions of higher variation suggesting that these regions might serve as epitope(s) targeted by neutralizing antibodies (Ghiasi et al., 1987). Studies show that VP2 has a strong affinity to the glycophorin A of RBCs, explaining its role in virus transmission by *Culicoides* species in vertebrates during blood feeding (Hassan and Roy, 1999). Furthermore, virion particles lacking VP2 but retaining VP5 lose the ability to bind to vertebrate cells and erythrocytes (Huismans et al., 1983).

VP5, the globular and the second capsid protein, has an essential role in the host cell membrane penetration by the virus. Both the structural features and the location of VP5 in the virion shell supports its involvement in the permeabilization activity of the membrane. VP5 has the characteristics of fusion proteins of enveloped viruses. This has been demonstrated by its ability to induce cell-to-cell syncytium formation, like other enveloped viruses when it is expressed as a membrane anchored protein (Forzan et al., 2004). Each VP2 and VP5 proteins make separate contact with the

underlying VP7 protein layer, suggesting that VP2 and VP5 proteins were independently assembled on the surface of the newly generated core particles.

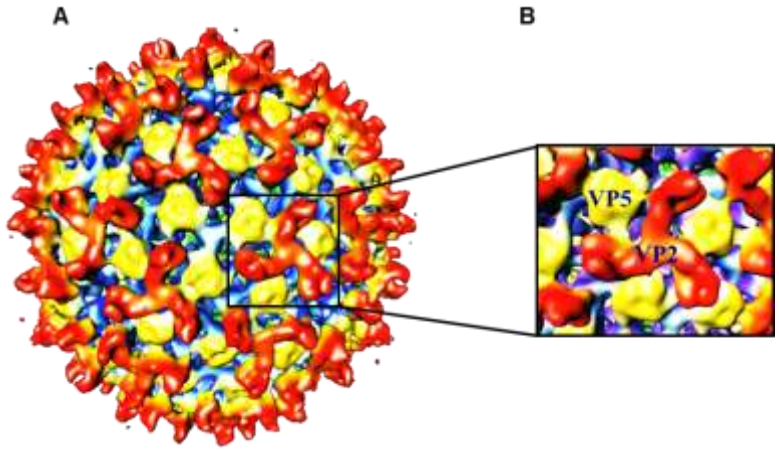

Fig. 8. Cryo-EM picture showing the structure of BTV-10 virion. (A) Triskelion shaped trimers of VP2 (red) are exposed on the outer surface. VP5 trimers (yellow) sit under VP2 trimers between the triskelion arms. (B) High magnification picture shows the position of VP2 and VP5 in the outer capsid (Nason et al., 2004).

1.10. The inner core proteins

The structure of purified core particles derived from virus via proteolytic treatment has been extensively studied by Cryo-EM and X-ray crystallography due to their stability (Fig. 9 and 10) (Prasad et al., 1992, Hewat et al., 1992, Grimes et al., 1998, Grimes et al., 1995). The core surface has a diameter of 73 nm with icosahedral symmetry, wherein the outer layer is made up of 780 molecules of VP7 protein forming 260 trimers that appear as knobbly projection on the surface of the core. Studies show that VP7 is the main immuno-dominant viral protein which could be involved in cell entry for insect cells for which BTV cores have a high specific infectivity (Prasad et al., 1992). As VP7 is antigenically specific to each of the *Orbivirus* species, it represents a suitable target for the design of group-specific diagnostic assays.

The underlying layer of the core is made up of 120 copies of VP3 protein, arranged in 60 dimers, in contrast to the VP7 layer, has a spherical structure with an angular appearance due to the variation in the thickness of the protein shells. The maximal diameter of VP3 shell is 59 nm with a central space of 38 nm internal diameter. VP3 dimers serves as a scaffold for the deposition of the VP7 layer (Grimes et al., 1998).

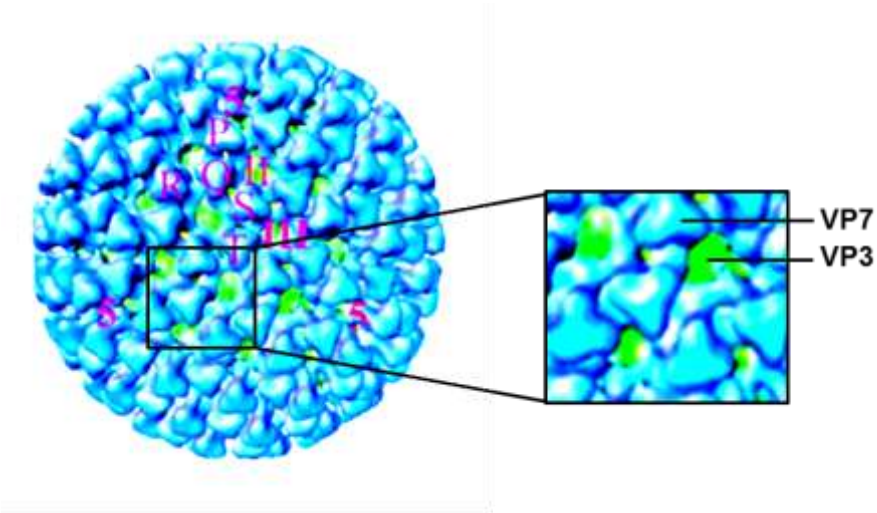

Fig. 9. BTV-10 core structure. Reconstitution of the core obtained by Cryo-EM showing VP7 (blue) and VP3 (Shapiro et al.) (Nason et al., 2004)

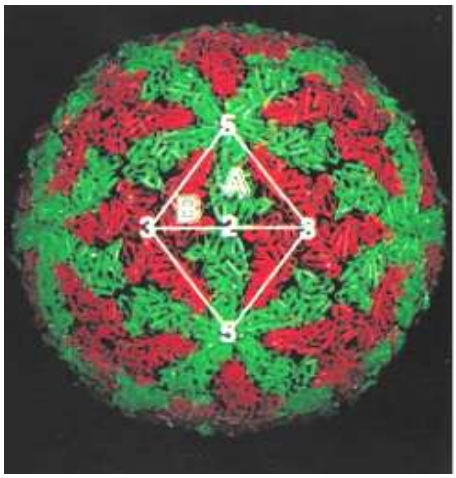

Fig. 10. X-ray crystallography of the core particle of BTV-1 showing the VP3 scaffold. The inner layer of VP3 (T2) molecules is coloured red and green (Grimes et al., 1998).

1.11. Minor proteins and genome structure

The Cryo-EM reconstructions and X-ray structures of both virion and core revealed a significant density internal to the VP3 layer. This density was occupied by the minor proteins (VP1, VP4 and VP6) that form the TC as well as layers of dsRNA which are organized in a highly ordered structure (Gouet et al., 1999). The genome segments are numbered from 1 to 10 according to their migration on SDS-PAGE (Fig. 11). Full details about each segment, encoded proteins and function of each protein are summarized in (Table 5) *Fields Virology, Fifth edition*, *Orbiviruses chapter (Roy, 2007)*.

Table 5.

BTV-10 and EHDV-1 coding assignments. Adapted from *Fields Virology, Fifth edition, Orbiviruses chapter* (Roy, 2007, Anthony et al., 2009a).

Segment No.	Protein	No. of Amino Acids of BTV-10	No. of Amino Acids of EHDV-1	Location in Virion Particle	Function
1	VP1	1,302	1,302	Inner core	• RNA polymerase
2	VP2	956	971	Outer shell (spike)	• Receptor binding • Virus entry • Hemagglutinin • specific neutralisation
3	VP3	901	899	Subcore layer (scaffold)	• Scafolld for VP7 trimers • Interacts with TC and genomic RNA
4	VP4	654	644	Inner core	• Capping enzyme guanylytransferase, methyltransferases 1 and 2, RNA5‘ triphosphatase • Inorganic pyrophosphatase NTPase
5	VP5	526	527	Outer shell (globular)	• Virus penetration • Fusogenic
6	NS1	552	551	Nonstructural	• Protein synthesis enhancer • Involved in virus maturation and trafficking • Forms tubules
7	VP7	349	349	Core surface layer	• Forms surface of core. • Responsible for core entry into

					insect cells
8	NS2	357	373	Nonstructural	• Phosphorylated • Recruits BTV single-stranded RNA (ssRNA) • Forms cytoplasmic inclusion bodies • Site for core assembly
9	VP6	328	360	Inner core	• Viral helicase • Adenosine triphosphatase (ATPse) • Form hexamer in presence of BTV RNA, RNA packaging
	NS4	77-79	-	Inner core	• Virus-host interaction • Counteract the antiviral response of the host (Ratinier et al., 2011)
10	NS3 NS3A	229 216	228 -	Nonstructural	• Glycoproteins • Membrane proteins • Interact with host membrane proteins calpactin and Tsg 101 • Aids virus trafficking and release

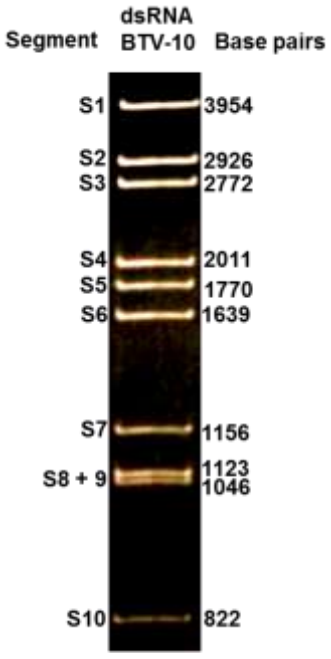

Fig. 11. BTV virion constituents. The 10 segments of dsRNA profile of purified core particles. (Sizes are calculated based on molecular weight for BTV serotype 10). Adapted from *Fields Virology, Fifth edition, Orbiviruses Chapter* (Roy, 2007).

1.12. Comparison of expression systems

The study of viruses, virion structures and viral vaccine production has been frequently impeded by difficulties in the large-scale production of virus particles, recovery and purification. Several protein expression systems are available for recombinant proteins expression for the development of viral vaccines. The most commonly described protein expression systems are bacterial, *Escherichia coli* (*E.coli*) (Hunt, 2005, Terpe, 2006), *Saccharomyces cerevisiae* (Holz et al., 2002), *Pichia pastoris* (Macauley-Patrick et al., 2005), baculovirus/insect cells (Brondyk, 2009, Jarvis, 2009), mammalian cell lines (Almo and Love, 2014) and cell-free systems (Jackson et al., 2004). Each of which has advantages and disadvantages. Such differentiation depends on the yield, speed of expression and complexity of the target recombinant protein/s.

Both the bacterial (*E.coli*) and eukaryotic (yeast) expression systems remain the preferred systems for the expression of heterologous proteins due to the rapid expression and the ease of scaling up. However, the disadvantage of these systems is that they do not effectively support the expression of complex proteins, such as those which are post-translationally modified or large complex protein folds (Baneyx, 1999, Mattanovich et al., 2012). On the other hand, mammalian cells can provide expression of the targeted proteins with correct glycosylation and native post-translational modification; but they are expensive at larger scale (Wurm, 2004).

However, since 1983, when baculovirus expression technology was introduced, the system has become one of the most versatile and powerful eukaryotic vector systems for recombinant protein expression for several reasons (Pennock et al., 1984, Smith et al., 1983). Firstly, substantial amounts of correctly folded recombinant proteins can be produced in high-density suspension culture (Bishop, 1990, Marek et

al., 2011). Secondly, as the insect cells do not need supplements derived from mammalian cells, the threat of opportunistic pathogens is minimized. Finally, baculovirus infects only a few species of lepidopteran, making it safe for vaccine applications. Such characteristics of baculovirus expression vector system (BEVS) have proven to be very useful for the correct expression and generation of VLPs. Therefore, several studies have been carried out to develop BEVS for simultaneous expression of multiple foreign proteins from a single recombinant baculovirus (Noad et al., 2009).

1.13. Baculovirus expression technology and its modifications

Baculoviruses belong to the *Baculoviridae* family that contains over 1000 members, most of which infect arthropods. These viruses have a large, double stranded, covalently-closed, circular DNA genome between 80 Kbp and 180 Kbp in size (van Oers and Vlak, 2007). The *Autographa californica* multicapsid nucleopolyhedrosis virus (AcMNPV) and *Bombyx mori* nucleopolyhedrosis virus (BmNPV), are the two mainly used species for recombinant protein expression, in which lepidopterans is the biological host (Blissard and Rohrmann, 1990).

There are two forms of the virus, unoccluded and an occluded forms. In the unoccluded form, virions are membrane-bound and exist singly, while in the occluded form, multiple virus particles or virions are occluded within a crystalline matrix or polyhedron, termed as inclusion body (Blissard and Rohrmann, 1990). Polyhedrin protein constitutes the main component of the matrix, allows for the survival of the virus under extreme environmental conditions (Smith et al., 1983, van der Beek et al., 1980). This protein matrix dissolves in the alkaline environment of the larvae midgut releasing the un-occluded virions that fuse to the epithelial cell

membranes (Volkman and Summers, 1977). These are taken up by the cell through endosomes, trafficked to the nucleus wherein the virus transcription and translation occur (Volkman and Summers, 1977). Upon infection, the virus spreads systemically, causing liquefaction of the host by the viral enzyme protease (Hawtin et al., 1997). This allows the dissemination of viral inclusion bodies into the environment where the virus thrives until another host uptake it.

Molecular studies have shown that the most abundantly expressed protein near the end of baculovirus infectious cycle is polyhedrin (Smith et al., 1983). Polyhedrin protein typically represents over half of the total protein found in the AcMNPV infected cells at this time (Miller et al., 1983). Furthermore, the polyhedrin gene is not essential for virus replication in culture and can be replaced with a foreign gene wherein the polyherin (*polyh)* promoter would drive its high expression (Smith et al., 1983). Therefore, such ability of baculovirus to produce high level of polyhedrin protein (or foreign proteins) has been exploited by researchers to express specific proteins of interest to the biomedical sector.

1.14. Baculovirus expression vector system for the production of VLPs

The expression of a single protein can be easily achieved for the viruses that consist of one structural protein such as the commercially available VLP-based human vaccines against human papillomavirus (HPV) (Koutsky et al., 2002, Harper et al., 2004, Harper et al., 2006). However, several viral capsids consist of more than one structural protein could be problematic. In the initial studies to produce VLPs of multi-complex proteins using BEVS, a dual baculovirus expression vector was constructed which contains two *polyh* promoters placed in opposite orientations to minimize the homologous sequence recombination (Emery and Bishop, 1987), which allowed the

expression of the two recombinant proteins (French and Roy, 1990). Although successful, this process of VLPs formation suffers from several drawbacks. Firstly, the efficiency of coinfection is generally low and needs accurate determination of multiplicity of infections (MOI) of both recombinant baculoviruses, in order to get a correct expression of all proteins (Bertolotti-Ciarlet et al., 2003). Consequently, any problem in the expression of one of the proteins may stop the whole process of assembly. Secondly, due to recombination, the stability of DNA elements within baculoviruses is low and thereby making the simultaneous expression of the proteins from two dual recombinant baculoviruses very inefficient (French and Roy, 1990, French et al., 1990).

To solve the instability and inefficiency problems of the dual vectors, further studies identified another strong promoter of the very late genes in the *p10* locus wherein high expression levels of recombinant genes can be obtained (Vlak et al., 1990). The combination of both *p10* and *polyh*, led to a more stable dual expressing vectors (Weyer and Possee, 1991). With this combinatorial method, triple and quadruple transfer vectors were generated (Belyaev et al., 1995, Belyaev and Roy, 1993); but were difficult to be manipulated by molecular techniques.

More recent studies have shown that the genomic baculoviral DNA is easier to manipulate as a bacterial artificial chromosome (Bacmid) that can then be propagated in the *E. coli* strain (Oppenheim et al., 2004, Copeland et al., 2001). This has permitted researchers to generate viruses that can express up to 8 subunits using the multi-loci expression recombinant vector. One of the molecular mechanisms of manipulation is the use of the recET system for the introduction of multiple expression cassettes into the baculovirus genome at different loci (Noad et al., 2009). This approach ensured that every cell in the cell culture would be infected

with the recombinant baculovirus, expressing specific genes at the same ratio, leading to sustainable levels of proteins expression at larger passages (Noad et al., 2009).

1.15. Immunological value of VLPs

To control microorganism invasion, viral pathogens are recognised by pattern recognition receptors (PRRs) of the immune system and protective defences against them are induced. Several studies demonstrated that a specific recombinant viral capsid protein contains epitopes 'which are targeted of broadly neutralising antibodies' has the ability to protect hosts from infection. For example, gp350/220, is the predominant virion envelope protein of Epstein-Bar virus (EBV) (Cohen et al., 2011), the HIV-1 envelope proteins (gp120 and gp41) (Pancera et al., 2010) and VP2 protein the major serotype determining antigen of BTV (Inumaru and Roy, 1987, Hassan and Roy, 1999, Huismans et al., 1987). Therefore, considerable efforts have been devoted to generate soluble forms of these proteins for vaccination purpose.

Although promising, the variable success of the soluble protein trials showed that soluble protein vaccines are generally limited in immunogenicity and do not elicit long-lasting protection (Kingsman and Kingsman, 1988, Link et al., 2012, Roy et al., 1990). According to *Roy et al* studies with BTV, partial protection was afforded in vaccinated animals with 100 µg of BTV-10 VP2 at least for 75 days (Roy et al., 1990), while only 50 µg of VP2 in combination with 25 µg of VP5 was sufficient to protect animals against virulent virus challenge indicating that lower doses of VP2 could achieve similar level of protection when the both outer capsid proteins were presented in the vaccination cocktail (Roy et al., 1990). In contrast to these subunit antigens, only 10 µg of VLPs were sufficient to afford complete protection for up to 14 months post-vaccination challenge (Roy et al., 1992, Roy et al., 1994). This is

due to the particulate structure of the VLPs, organisation of multivalent epitopes and the correct antigen conformation (Deml et al., 1997, Grgacic and Anderson, 2006, Chackerian 2007, Noad and Roy, 2003). The presence of such regular array structure mimicking those of infectious viruses increases the recognition of VLPs as safe and effective vaccines due to their ability to stimulate innate and adaptive immune response against pathogens, leading to specific B cells activation inducing rapid and robust antibody response (Spohn et al., 2007, Rohn et al., 2006, Da Silva et al., 2007, Stewart et al., 2013).

1.16. BTV and other successful VLPs vaccines

For most viral infections, vaccines are the most cost efficient long standing protection against the disease and the spread of the infectious viruses. Generally, the main purpose of vaccination is to produce suitable immune responses against antigens present on the virus surface or the virus-infected cells by eliciting innate and humoral immunities.

At present, many vaccines against various viral diseases have been developed and commercially approved. These vaccines are mainly based on live attenuated viruses (such as measles, mumps, rubella, oral polio, smallpox, varicella and yellow fever) (Dhillon and Curran, 2008, Martin and Minor, 2002, Saijo et al., 2006) or inactivated viruses (like influenza, polio, rabies and hepatitis A) (Dumas et al., 1997, Centers for Disease and Prevention, 2013) or subunit vaccines (for instance hepatitis B) (Purcell and Gerin, 1975); however each of these vaccines has disadvantages regarding safety, efficiency and low levels of immunogenicity. The recombinant DNA technology has enabled the engineering of VLPs based safer vaccines in yeasts (like papillomavirus VLPs), insect or mammalian cells via specific expression vectors;

though insect cells in combination with the baculovirus expression system have proven to be one of the most effective technologies for the development of VLPs.

VLPs are highly efficient subunit vaccines that mimic the organization and conformation of native viruses but lack the viral genome. The possibility of developing VLPs has originated from the ability of capsid proteins of the virion to self-assemble into structures that are morphologically identical to the infectious particles. So far, several baculovirus derived VLPs have been generated for human and animal enveloped or non-enveloped, DNA or RNA viruses in which their structure ranges from single to multiple capsid proteins (Table 6).

VLPs for some members of the *Reoviridae* family, particularly BTV the prototype of orbiviruses, comprising the four structural proteins (VP2, VP3, VP5 and VP7) have been successfully produced using a baculovirus expression system (French et al., 1990). In 1990, *French and Roy* were able to generate the first VLPs for BTV-10 by coinfection of insect cells with dual baculoviruses, one expressing the outer capsid proteins VP2 and VP5 and another expressing the CLP proteins VP7 and VP3 (French et al., 1990). The BTV-10 VLPs elicited strong neutralizing antibody titres in vaccinated guinea pigs (French et al., 1990). Due to this success, VLPs were developed for the four serotypes of BTV-1, -2, -13 and 17. The purified VLPs particles were tested singly (BTV-10, -17) and in combination (BTV-1, -2, -10, -13 and 17). Vaccinated sheep were challenged with homologous (BTV-10, -13 and 17) or specific heterologous virulent viruses (BTV-4, -11 and 16) (Stewart et al., 2010, Stewart et al., 2012). A complete protection was obtained in vaccinated animals against the vaccine serotypes, while heterologously challenged sheep were partially protected (Roy et al., 1994).

Studies showed that the multi-layered capsid of VLPs is made up of highly conserved cores and variable outer capsids which specify the serotype (Yamaguchi et al., 1988). This has been utilized to rapidly produce VLPs with the outer capsid proteins from different serotypes by coating the conserved inner layers. Based on this fact, vaccines against each of the BTV serotypes 2, 4 and 9 were generated, in which protection at low dose was shown (Stewart et al., 2010). Likewise, the developed VLPs could be rapidly applied for emerging viruses i.e. BTV-8 which was responsible for the outbreak in Europe (Stewart et al., 2013).

Table 6.

Baculovirus derived VLPs that have been tested as vaccines. Adapted from *Roy and Noad* (Roy and Noad, 2009).

Family	**VLPs**	**Proteins Expressed**	**Vaccine Tested In**
Papillomaviridae	Papillomavirus	1	Humans (licensed)
Caliciviridae	Norwalk and Norwalk-like viruses, Feline calicivirus	1	Mice, Cats, Humans
Hepeviridae	Hepatitis E virus	1	Mice, Cynomologous Monkeys
Parvoviridae	Porcine parvovirus, mink enteritis parvovirus, Canine parvovirus, B19, Adeno-associated virus	1	Pigs, Dogs, Mink
Circoviridae	Chicken anemia virus, porcin circovirus	1, 2 (Chicken anaemia virus)	Chickens
Polyomaviridae	SV40,JC virus, murine, polyomavirus	1	Mice, Rabbits (*in vitro*)
Picornaviridae	Polio virus	1 (polyprotein)	-
Reoviridae	**Bluetongue virus** **Rota virus**	**4 (Bluetongue)** **2-3 (Rota)**	**Sheep (Bluetongue), Mice, Pigs (Rota)**
Flaviviridae	Hepatitis C virus	3	Mice, Baboons
Retroviridae	HIV, SIV, FIV, Visna virus, FeLV, BLV, Rous Sarcoma virus	2	Mice, Guinea Pigs
Paramyxoviridae	Newcastle disease virus	1	Chickens

Coronaviridae	SARS coronavirus	3	Mice (*in vitro*)
Bunyaviridae	Hantavirus	3	Mice
Orthomyxoviridae	Influenza A virus	2-4	Mice
Birnaviridae	Infectious Bursal Disease virus	1	Chickens

1.17. Aims of the studies

EHDV, the causative agent of EHD disease, belongs to the *Orbivirus* genus, *Reoviridae* family. As already outlined, EHDV imposes an emerging agricultural threat due to recent outbreaks in large number of domestic cattle. Currently No vaccine is available to control the viral infection, except the live-attenuated vaccine developed and used in Japan. Since VLPs have been shown to be highly immunogenic and safe vaccines, this study aimed to develop VLPs for EHDV serotypes. Baculovirus multiple genes expression system was used for the simultaneous expression of the four structural proteins, responsible for VLPs formation. To achieve this target, several steps were undertaken.

Single gene expression in AcMNPV

Due to the lack of published literature regarding the synthesis of EHDV structural proteins, initially the individual expression of the outer and inner capsid proteins of EHDV serotypes 1, 2 and 7 was investigated. DNA clones were produced and verified to assess the ability of utilising baculovirus multi-loci expression system.

Development of EHDV-1 VLPs

The advantages of VLPs as vaccines have been demonstrated for several types of enveloped and nonenveloped viruses, in particular, VLPs of BTV have been produced efficiently and shown to be highly efficacious (Stewart et al., 2012, Stewart et al., 2013). In contrast, VLPs of EHDV have not been reported to date, however this aim seems plausible given the very close phylogenetic relationship between BTV

and EHDV both at structural and genetic levels. This project examined the possibility of generating EHDV-1 VLPs using baculovirus multi-genes expression system for simultaneous expression of the recombinant targeted proteins. The possibility of generating conserved CLPs proteins as a platform for the formation of VLPs of heterologous serotypes was also examined.

Development of heterologous VLPs against EHDV serotypes

The recent epidemics caused by the emergent serotypes of EHDV that were more virulent to cattle, highlighted the need for a rapid generation of a safe vaccine. Previous research for BTV has demonstrated the ability to construct heterologous VLPs utilising the conserved CLP proteins VP7 and VP3 as a background, and exchanging the outer layer proteins related to the pathogenic strain (Belyaev and Roy, 1993, Stewart et al., 2010). Since no previous studies have addressed this issue for EHDV, here, the generation of heterologous VLPs of EHDV serotypes was undertaken using the conserved proteins of EHDV-1 CLP as a structural scaffold.

Generation of EHDV-2 vaccine using RG system

A RG system has been established and proved to be efficient for the development of defective virus vaccines, particularly, for BTV. EHDV-2 has been considered to be highly pathogenic for both wild and domestic animals. Therefore, as an initial attempt, in this work, an alternative strategy towards the development of a highly attenuated vaccine against EHDV-2 based on a RG system was carried out.

Chapter 2

Materials and Methods

2.1. Baculovirus expression system

2.1.1. Polymerase Chain Reaction (PCR) amplification

Standard procedures were used for PCR according to Sambrook and Russell (Sambrook and Russell, 2001). To express EHDV structural proteins in baculovirus vector, the coding regions of EHDV-1 S2, S5 and S7, EHDV-2 S2 and S5, EHDV-7 S2 were amplified using specific primers (Table 7). Primers were designed with appropriate restriction enzymes according to the published gene sequence (Genbank accession numbers Table 8).

Melting temperature of each primer was calculated using the Sigma-Aldrich software http://www.sigmaaldrich.com/configurator/servlet/DesignTool?prod_type=STANDARD. Prior to amplification and to increase the efficiency of ligation of S2 into baculovirus transfer vector pAcYM1, primers were phosphorylated using T4 Polynucleotide Kinase (T4 PNK) (Thermo Scientific) in a total volume of 10 µl containing: 0.5 µl forward (F) or reverse (R) primers; 1.0 µl 10X Buffer A; 10 mM ATP and 0.5 µl PNK enzyme (10U/µl). The mixture was incubated at 37°C for 20 minutes (min) followed by incubation at 75°C for 10 min to inactivate the enzyme.

Standard PCR reactions were carried out using KOD Hot Start DNA polymerase (Novagen) in a total volume of 50 µl containing: 30 µl PCR nuclease free water (nfH_2O), 5 µl 10X PCR buffer, 5 µl dNTPs (final concentration 0.2 mM), and 2 µl $MgSO_4$ (final concentration 1 mM), up to 100 ng template DNA, 3 µl of each (F and R) primers (5 pmol/µl, final concentration 0.3 µM) and 1 µl KOD Hot Start DNA Polymerase (1 U/µl). The PCR cycle conditions were carried out according to the manufacturer's recommendations which are presented in (Table 9). PCR products were excised and purified from 1% agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, UK). Briefly, the excised DNA band from gel was

transferred into a 1.5 ml microcentrifuge tube and 10 µl of membrane binding solution per 10 mg of gel slice was added. The mixture was incubated at 50-65°C until complete dissolve. The dissolved DNA mixture was transferred into the minicolumn, incubated at room temperature for 1 min and centrifuge at 16,000 *x g* for 1 min. After centrifugation, 700 µl membrane wash solution were added and centrifuged using the same previous conditions. To elute the DNA, 25 µl nfH_2O were used and the eluted PCR product was kept at -20°C for further experiments.

Table 7.

Primers used for cloning relevant inserts into pAcYM1 vector.

Oligonucleotide name	Sequence (5'-3')	Competent
EHDV-1 primers		
***Bgl*II-S2 (F)[a]**	CTA*AGATCT*CATAAATATGGAGGACATTAACCTA	***Bgl*II** **PH enhancer** **Start codon**
***Bgl*II-S2 (R)[b]**	CTA*AGATCT*TCAGTTCATTAATTTTG	***Bgl*II**
***Bam*HI-S5 (F)[a]**	CTA*GGATCC*ATAAATATGGGCAAGTTCATT AAAC	***Bam*HI** **PH enhancer** **Start codon**
***Bam*HI-S5 (R)[b]**	CTA*GGATCC*TCACGCGTATCGCATAAATTCG	***Bam*HI**
***Bam*HI-S7 (F)[a]**	GTT*GGATCC*CATAAATATGGACACGATTGCTGCGGAG	***Bam*HI** **Start codon**
***Bam*HI-S7 (R)[b]**	CTA*GGATCC*TCAGTTCATTAATTTTG	***Bam*HI**
pRN306-*Bam*HI-S3 (F)[a]	GCTCGGTACCCG*GGATCC*CAAAATGGCAGATCCACCAGAT	**pRN306** ***Bam*HI** **PH enhancer** **Start codon**
pRN306-*Xba*I-S3 (R)[a]	CTGCAGGTCGAC*TCTAGA*ATTACTAGGTATAGATATCGGT	**pRN306** ***Xba*I** **PH enhancer**
EHDV-2 primers		
***Sma*I-S2 (F)[a]**	CTA*CCCGGG*TAAATATGGAGGAGATTTTCTTTA	***Sma*I** **PH enhancer** **Start codon**
***Sma*I-S2 (R)[b]**	CTA*CCCGGG*TCACTAGTGATTGTAA	***Sma*I**

***Bam*HI-S5 (F)[a]**	**CTA*GGATCC*ATAAAT ATGGGCAAGATTATCAAGAAATT**	***Bam*HI** **PH enhancer** **Start codon**
***Bam*HI-S5 (R)[b]**	**TCA*GGATCC*CGCGTAACGCATAAACT**	***Bam*HI**
EHDV-7 primers		
***Nru*I-S2 (F)[a]**	**CTA*TCGCGA*CATAAATATGGAGGAGATTTTCTTTAGCGTAATT GA**	***Nru*I** **PH enhancer** **Start codon**
***Nru*I-S2 (R)[b]**	**CTA*TCGCGA*TCATTAGTTTTGAGATTATCT**	***Nru*I**
***Bam*HI-S5 (F)[a]**	**CTAG*GGATCC*ATAAATATGGGCAAGATTATCAAGAGATTGA**	***Bam*HI** **PH enhancer** **Start codon**
BamHI-S5 (R) [b]	**CTA*GGATCC*CTCAATCTCTCTCCAATTAAGCTTA**	***Bam*HI**
Primers used to clone EHDV-7 S5 into pRN296 transfer vector		
pRN296-BamHI-S5 (F)[a]	**GCTCGGTACCCG*GGATCC*CAAAATGGCGGCCGCGGGAATTC GAT**	**pRN296** ***Bam*HI** **PH enhancer** **Start codon**
pRN296-BamHI-S5 (R)[b]	**CTGCAGGTCGAC*GGATCC*ATTATCATGATTTAAGGAGTT**	**pRN296** ***Bam*HI** **PH enhancer**

(F)[a]: Forward
(R)[b]: Reverse

Table 8.

EHDV-1, EHDV-2 and EHDV-7 Genbank accession numbers.

EHDV Gene	Accession number	Size (bps)
EHDV-1 S2	NC_013397	2968
EHDV-1 S5	NC_013400	1803
EHDV-1 S7	NC_013402	1162
EHDV-1 S3	NC_013398	2768
EHDV-2 S1	AM744997	3942
EHDV-2 S2	AM744998	3002
EHDV-2 S3	AM744999	2768
EHDV-2 S4	AM745000	1983
EHDV-2 S5	AM745001	1803
EHDV-2 S6	AM745002	1641
EHDV-2 S7	AM745003	1162
EHDV-2 S8	AM745004	1186

EHDV-2 S9	**AM745005**	**1143**
EHDV-2 S10	**AM745006**	**810**
EHDV-7 S2	**AM745048**	**3002**
EHDV-7 S5	**AM745051**	**1769**

Table 9.

Cycling conditions used for PCR reactions.

Stage	Stage name	Temperature °C	Duration (min:sec)	Cycles
First	**Initial denaturation**	**95**	**2:00**	**1**
Second	**Denaturation**	**95**	**0:20**	**30**
	Annealing	**50-60**	**0:30**	
	Extension	**70**	**0:20/kbp**	
Third	**Final extension**	**70**	**7:00**	**1**
Forth	**Final extension**	**4**	**10:00**	**1**

2.1.2. The preparation of plasmid and Bacmid DNA

To purify high-copy plasmids and Bacmid DNA from the bacterial culture, Invitrogen PureLink™ HiPure Plasmid DNA Purification Kit was used following the manufacturer's procedure (Invitrogen™, UK). Briefly, the overnight culture resulted from the inoculation of a freshly streaked single bacterial colony in 50 ml Luria Bertani (LB) supplemented with 100 µg/ml ampicillin or (50 µg/ml kanamycin and 15 µg/ml chloramphenicol) for Bacmid DNA, was centrifuged at 4,000 *x g* for 10 min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 4 ml of buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µl/ml RNase A). After complete resuspension, 4 ml of buffer P2 (200 mM NaOH; 1% SDS) were added to lyse the cell and the mixture was incubated at room temperature for 5-10 min. After incubation, 4 ml of chilled buffer P3 (3 M potassium acetate, pH 5.5) were added and incubated on ice for 10 min to enhance the precipitation of genomic DNA. At this stage, the precipitation material also contained proteins and cell debris. To separate the plasmid DNA, the mixture was centrifuged at 4,500 *x g* for 25 min at 4°C and the

clear supernatant was collected and applied onto a pre-equilibrated column to which the plasmid DNA binds. After washing, 5 ml of the elution buffer (1.25 M NaCl, 100 mM Tris-HCl, pH 8.5) were used to elute the bound DNA followed by 1 volume of isopropanol precipitation. After centrifugation, the pellet was washed with 70% ethanol and allowed to air dry. The DNA pellet was resuspended in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) and quantified by NanoDrop®ND-1000 Spectophotometer (NanoDrop Techlonogies, Inc, USA).

2.1.3. Construction of transfer vectors for baculovirus system

To construct recombinant baculovirus single gene expression vectors, the coding regions of EHDV-1 S2, S5 and S7, EHDV-2 S2 and S5, and EHDV-7 S2 and S5 were cloned into a baculovirus transfer vector pAcYM1 previously linearised by *Bam*HI or *Sma*I (Thermo Scientific) restriction digestion.

For the generation of baculovirus multigene expression vectors, a previously developed system was used (Noad et al., 2009). In this method, lambda red and bacteriophage PI *Cre* recombinase systems were combined to generate a recombinant AcMNPV in which foreign genes were inserted into different loci. Three transfer vectors; pRN296, pRN260 and pRN306 were used with a *polyh* promoter, *Cre* recombinase gene (*Loxp71* and *Loxp66*), *LacZα* gene and baculovirus sequence specific to each locus. A specific baculovirus sequence in each vector is required for locus-specific homologous recombination. These vectors also contain *Bam*HI or *Bam*HI-*Xba*I cloning sites for the insertion of the foreign gene. For instance, pRN296-*Bam*HI was used to clone the coding region of EHDV-1 S5, EHDV-2 S5 and EHDV-7 S5, while EHDV-1 S7 coding region was ligated into pRN260-*Bam*H1 and EHDV-1 S3 was inserted into pRN306-*Bam*HI-*Xba*I.

As shown in diagram 12 (Fig. 12), a linear DNA containing both cassettes was amplified by PCR (Fig. 12B) and inserted into Bacmid$_{1629}$ already incorporated into the competent *E. coli* cells (E3K2) chromosome (Fig. 12C). Bacmid$_{1629}$ is a modified baculovirus DNA by *Kitts and Possee* (Kitts and Possee, 1993). The key feature of the parental genome is the presence of a novel sequence in the polyhedrin locus that contributes a unique *Bsu*36I site. The linearised viral DNA cannot be replicated until it is mixed with a transfer vector which then used to cotransfect insect cells. In addition, *Bsu*36I digestion allows the deletion of a portion of *orf*1629, which is an essential viral gene located downstream of polyhedrin (Vialard and Richardson, 1993). Recent studies using recombinatorial cloning methodology that utilises RecE and RecT genes products (Muyrers et al., 1999), *orf*1629 knockout has performed in a bacmid version of the baculovirus genome held in *E.coli* (Zhao et al., 2003). Such modification of the viral genome has increased the efficiency of recombinant baculovirus vector production in large volumes without the need for further isolation of the virus through large-scale DNA transfection. For the integration of the next gene, the selectable marker should be flipped out by activating *Cre* recombinase expression (Fig. 12D).

Table 10.

Primers used for generating appropriate gene inserts for the development of multiple genes Bacmid by homologous recombination. Adapted from (Kanai et al., 2013).

Primers to amplify baculovirus sequence specific to each locus				
Plasmid	**Locus**	**Promoter**	**Forward**	**Reverse**
pRN260	***egt***	***polyh***	**ATATGCGGAACGTACAAACG**	**TACATGTAGTAATTTGAATACGG**
pRN296	***odv-ec56***	***polyh***	**TCGAGGGGCCGTTGTTGG**	**CATGACGCCGCTGCCGTG**
pRN306	***39K***	***polyh***	**GCAAGGGCGCATTCACAGCAACCG**	**GACTGTGGTGCTTACCAACGC**

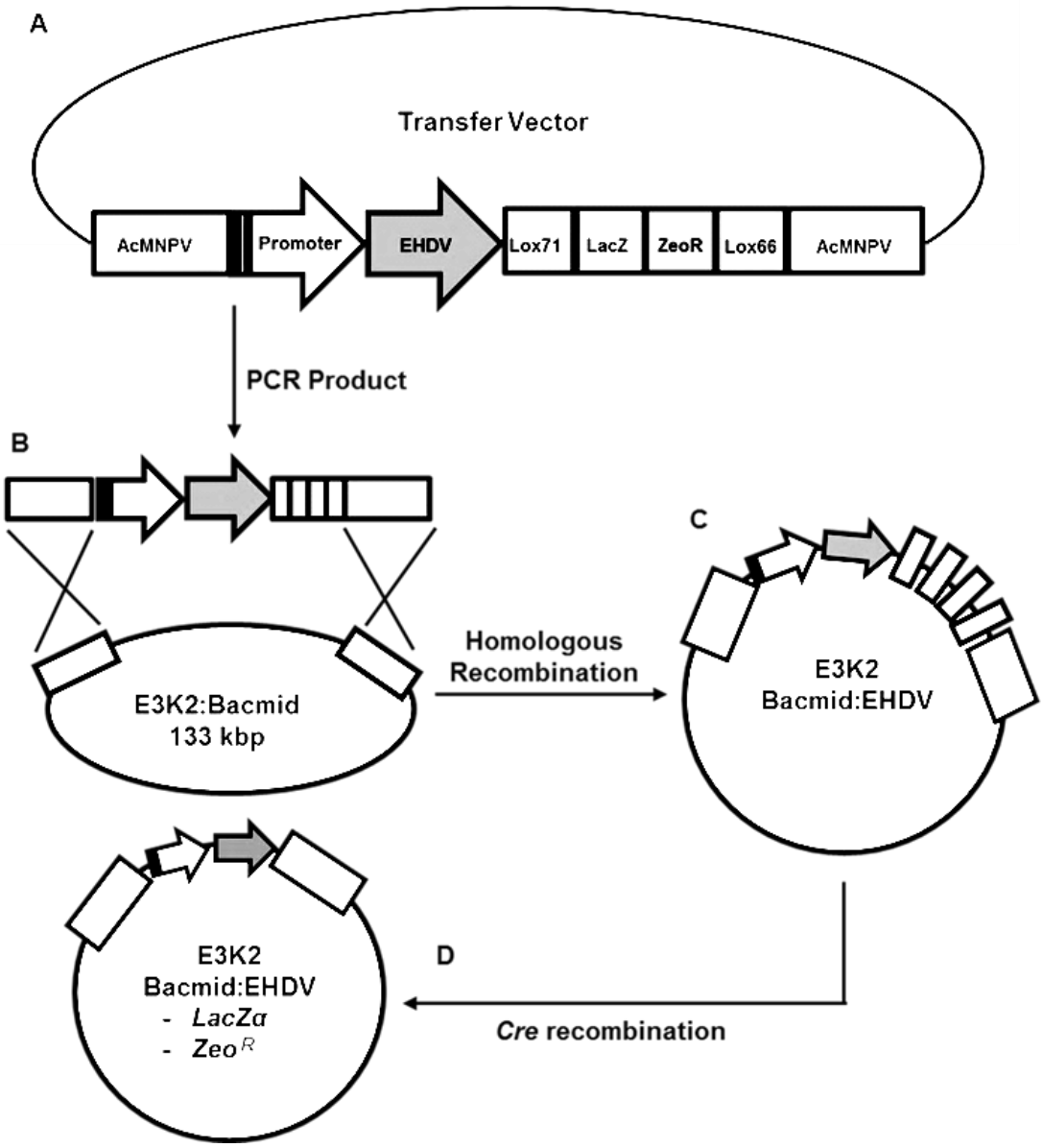

Fig. 12. Schematics showing the generation of baculovirus multi-gene expression vector. (A) Transfer vector map containing specific AcMNPV sequence, expression cassette (*p10* or *polyh* promoter and foreign gene) and selectable cassette *(LacZα*, *Zeo*^{R} flanked by *Cre* recombinase gene Loxp71 and Loxp66). (B) Upper panel, PCR amplification of the insertion and selectable cassettes flanked by AcMNPV sequence. Bottom panel, competent cells (E3K2) containing Bacmid$_{1629}$ genome. (C) Insertion of the PCR fragment in Bacmid$_{1629}$ by homologous recombination. (D) The removal of selectable marker by activation of *Cre* recombinase genes. Adapted from (Kanai et al., 2013).

Prior to ligation, the complete digestion of the vectors was verified by electrophoresis on 1% agarose gel (Sigma-Aldrich Ltd, UK). The linearised vectors were then dephosphorylated by incubation with Shrimp Alkaline Phosphatase enzyme (SAP) (Thermo Scientific). Briefly, 2-4 µg of the linear plasmid was mixed with 2 µl 10X

reaction buffer, 1 µl (1 U) SAP enzyme and nfH_2O up to the total volume of 20 µl. The reaction mixture was incubated at 37°C for 1 h followed by incubation for 15 min at 65°C to stop the reaction. Ligation reactions were carried out using (TAKARA) ligation mix. The amount of the insert required for ligation of (50-100) ng of vector was calculated according to the following equation:

$$\frac{\text{ng of vector X size of insert (Kb)}}{\text{size of vector (Kb)}} \text{ X molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The ligation mixtures were incubated at room temperature for 1-3 h and then transformed into the chemically competent cells (DH5α). The initial selection of the transformed colonies was throughout ampicillin resistance conferred by the vectors. To determine the ampicillin resistance clones had the target gene of interest in the correct orientation, restriction digestion with specific enzymes or PCR amplification using primers from the insert and from the vector was performed (Table 11). The complete sequence of the coding regions was carried out with specific universal (Table 12) or internal primers (Table 13) by Source BioScience and the results were compiled using BioEdit Software.

Table 11.

Molecular methods used to determine the orientation of the insert in the recombinant constructs.

Vector	Insert	Method for determining the orientation	
		Restriction Digestion	PCR
pAcYM1	EHDV-1 S2	*EcoR*V	PH (F) + EHDV-1 S2 (R)
	EHDV-1 S5	*Xma*I + *Xho*I	-
	EHDV-1 S7	-	PH (F) + EHDV-1 S7 (R)
	EHDV-2 S2	*Hind*III	-
	EHDV-2 S5	-	PH (F) + EHDV-2 S5 (R)
	EHDV-7 S2	-	PH (F) + EHDV-7 S2 (R)
pRN296	EHDV-1 S5	-	PH (F) + EHDV-1 S5 (R)
	EHDV-2 S5	-	PH (F) + EHDV-2 S5 (R)
	EHDV-7 S5	-	PH (F) + EHDV-7 S5 (R)

pRN260	**EHDV-1 S7**	***Nru*I**	**PH (F) + EHDV-1 S7 (R)**
pRN306	**EHDV-1 S3**	**-**	**EHDV-1 S3 (F) and (R)**

PH (F): polyhedrin forward primer
(R): Reverse primer

Table 12.

Universal primers for sequencing.

Oligonucleotide name	Sequence 5'-3'
M13 (F)	**GTTTTCCCAGTGACGAC**
M13 (R)	**CAGGAAACAGCTATGAC**
Polyhedrin PH (F)	**ACCATCTCGCAAATAAATAAG**
Polyhedrin PH (R)	**ACAACAATTGTCTGTAAATC**
C9	**5' P-GACCTCTGAGGATTCTAAAC/iSp9/TCCAGTTTAG AATCC-OH3'**
FLAC2	**5' P-GAGTTAATTAAGCGGCCGCAGTTTAGAATCCTC AGAGGTC 3'**

Table 13.

EHDV-1, EHDV-2 and EHDV-7 internal primers used for sequencing.

Oligonucleotide name	Sequence 5'-3'	Position (nucleotide)
EHDV-1 S2	**CTACATCCGCGGATAGCGGA**	**400-420**
	GGATGAGAGATCAGAATGT	**821-840**
	GGTGAGAGAAGATTATTT	**1242-1260**
	GGGACAAAAAAACCGCAGA	**1661-1680**
	GAAATCAACTACGGAGGA	**2152-2170**
	CAAATGGTAGAATTATACTAT	**2569-2590**
EHDV1 S3	**GAGGCATGGAAATAGCGGA**	**471-490**
	GCGACGTGCTTACCGACA	**890-908**
	TGAAAACAGGGAGCCAGCG	**1381-1400**
	CCGCTAATCGAGTCGGTCTAT	**1799-1820**
	GCAAGATTGCAGGCAAATAT	**2255-2275**
EHDV-1 S5	**CATGATAAAATTTGACCCAA**	**400-420**
	CCACAGACACTTGTGCAAAT	**890-910**
	GTATTTGGGAAAATGATGAAT	**1308-1329**
EHDV-1 S7	**CTACATCAATCGTATCCTCGA**	**465-486**
	CAATGTTTACTCATATAAGA	**820-840**
EHDV-2 S1	**CTATTAAGGTTTGGGCAGCAT**	**447-468**
	ATGTGGGGAGTAGGCGCA	**821-839**
	CGCGCAAGTCTCAGAAGGAA	**1228-1248**

	CGTCACCGGAGTACAAGAAAT	1659-1680
	GAGTATTACCAGTCTCAACT	2055-2075
	GATAATATCATCTGAGCGA	2494-2513
	GTTTCTTCTCGAAGATGGCAC	2961-2982
	CGTGATGCGTGGCTTCATAA	2409-3429
EHDV-2 S2	CAGAGTCGAATTACATAGGGT	542-563
	GCGAAATATAGAATGGGA	1156-1174
	GTGGCTTCTTCCTCTACCGA	1734-1764
	GCGAGGAGGAGATCCGAGTGAT	2150-2172
	CTACAAAAGGTATAATTGCGGT	2681-2703
EHDV-2 S3	GCCTTTATCTTGCATAACAT	434-454
	GATAATGAATATAGCGACGT	877-897
	CAGTATGATTGTAACGTAT	1307-1326
	CTTTTTCGATGCCAGACGAT	1632-1652
	GATGCTGACTGATCAGGTGTA	2101-2122
	GATTACCAGACAAATGAGA	2411-2430
EHDV-2 S4	GACATTGAATGATGCGAAT	421-440
	TCTACATCAGGTTTGTTCTC	890-910
	TACCTTACGAATAGGTGC	1238-1256
	CGTATGGGCTTAGACGTCT	1643-1662
EHDV-2 S5	GATTTAGATGATGTGTAT	402-420
	GTGATCCTACGGACACCA	879-897
	GTATGTATTTTACGAAGATTT	1232-1253
EHDV-2 S6	CTATTTGGGAGAGACTTA	390-408
	GAGGGCGGATATAAGTTGAA	783-803
	CTACATTGCGTGGCGTCACA	1167-1187
EHDV-2 S7	GACCGACAACCATTTGGTTA	380-400
	CAGGCACAAATTTTCAATGT	806-820
EHDV-2 S8	GAGTTACCCGGGGTCCAGAGAT	445-467
EHDV-2 S9	GTACGGAGTGAGTATTCCTGT	460-481
EHDV-2 S10	CATAATACAACAACGGAGGCGA	452-474
EHDV-7 S2	TGATTTCAAGGGAGAGCAG	338-407
	ATTCGGATAAAATCATAACG	882-902
	AGACACGATATATAAGTG	1312-1330
	TCAAGAAAGAGAATGAAATG	1716-1736
	GCGAGGAGGACATACGCAT	2151-2170
	AAACGAAAAGAAATAATTG	2564-2583
EHDV-7 S5	ATGATTAAATATGACCCAAC	400-420
	TATGTGACTCAGCTGTTTG	802-821
	TACAACAGGAATGGAAGC	1242-1260

2.1.4. Preparation of chemically competent *E.coli* cells

Preparation method of the DH5α strain of *E.coli* used for transformation of the DNA constructs, was based on the modified Inoue protocol (Inoue et al., 1990, Sambrook and Russell, 2001). In brief, 5 µl of the bacterial stock stored at -80°C was inoculated

in 5 ml LB broth (Sigma-Aldrich, UK) and incubated at 37°C with vigorous shaking at 200-250 rounds per minutes (rpm) overnight. The resulting culture was used for further inoculation of 200 ml LB medium which was incubated at 37°C with shaking at 200-250 rpm until the optical density (OD_{600}) of 0.4 was reached. The cell culture was then incubated on ice for 10 min, transferred into pre-chilled 50 ml conical centrifuge tubes (Fisher Scientific) and centrifuged at 2,000 *g* for 10 min at 4°C (Beckman JA-10 rotor). The pellet formed after centrifugation was gently resuspended in 60 ml of ice-cold transformation buffer (55 mM $MnCl_2.4H_2O$; 15 mM $CaCl_2.2H_2O$; 250 mM KCl; 10 mM PIPES, pH 6.7) and centrifuged using the same condition described previously. The supernatant was discarded and the pellet was carefully resuspended in 20 ml of ice-cold transformation buffer. Subsequently, the bacterial suspension was mixed gently in the presence of 1.5 ml dimethyl sulfoxide (DMSO) (Sigma-Aldrich Ltd, UK), incubated on ice for 10 min, aliquoted in 100 µl and stored at - 80°C for further use.

2.1.5. Transformation of competent *E.coli* cells

An aliquot (100 µl) of DH5α competent cells was incubated on ice until complete thawing, and used for transformation of the DNA plasmid or the ligation mixture. After DNA was added and 30 min of incubation on ice, the competent cells were heat shocked at 42°C for 42 seconds (sec) and incubated again on ice for further 5 min. the LB medium (300 µl) was added to the cells which were then incubated at 37°C for 30 min in a shaking incubator. Following incubation, 100 µl of the bacterial culture was spread on LB agar plates supplemented with 100 µg/ml of ampicillin (Sigma-Aldrich Ltd, UK) and incubated at 37°C overnight.

2.1.6. Electrocompetent *E.coli* cells

For the construction of multiple gene expression recombinant baculoviruses (Noad et al., 2009), a Bacmid E3K2 modified from bMON14272 (Luckow et al., 1993) maintained in the *E.coli* strain EL350 (Lee et al., 2001) was prepared and purified as mentioned in DNA Bacmid and plasmid preparation (see section 2.1.2). The electrocompetent bacterial cells were prepared and stored at - 80°C using standard methods (Sambrook and Russell, 2001). In brief, a single bacterial colony was inoculated in 3 ml LB broth supplemented with 50 µg/ml kanamycin and 15 µg/ml chloramphenicol and incubated at 32°C overnight. An aliquot (100 µl) of the overnight culture was further inoculated in 10 ml LB containing the same antibiotics as above and incubated at 32°C until the OD_{600} of 0.4. To induce the expression of *Cre* recombinase genes, the culture was incubated at 42°C for 1 h. Subsequently, the bacterial culture was incubated on ice for 10 min followed by centrifugation at 1.800 *g* for 10 min at 4°C (Beckman JA-10 rotor). The supernatant was discarded and the pellet resuspended carefully in 10 ml pre-chilled autoclaved distilled water, incubated on ice for further 10 min, centrifuged as above and resuspended again with the same volume of distilled water in the presence of 10% DMSO. Finally, the electrocompetent cells were centrifuged and the pellet resuspended with 1 ml chilled distilled water in 10% DMSO and divided into 200 µl aliquots in micro tube which were stored at - 80°C for further use.

2.1.7. Lambda red recombination and *Cre* recombination in *E.coli*

For lambda red recombination, 50 ng of the amplified PCR product (Table 10) containing specific EHDV gene was transformed into 100 µl of EL350 cells by electroporation at 2500 V in a microelectroporation chamber. Immediately, 900 µl LB

broth was added and the cells were recovered by incubation at 32°C for 2 h before plating on LB-agar (LBA) plates supplemented with 50 µg/ml kanamycin, 15 µg/ml chloramphenicol, 15 µg/ml Zeocin, 40 µg/ml Xgal and 0.2 mM IPTG. Plates were incubated overnight at 32°C and blue colonies were isolated and screened for the presence of the targeted genes.

In order to flip out the selectable marker (Fig. 12), the expression of *Cre* recombinase was induced by culturing the positive recombinant Bacmid clones (*Zeo*^{R} and ***LacZα*** positive) at 32°C in LB supplemented with 0.1% arabinose for 2 h. EL350 bacterial cells were then plated and cultured in the presence of kanamycin (50 µg/ml), chloramphenicol (15 µg/ml), Xgal (40 µg/ml) and IPTG (0.2 mM). To confirm the loss of the zeocin resistance gene, white colonies were re-streaked onto the same selection plates with/without zeocin. The generated colonies were then used for further cloning.

2.1.8. Insect cell culture

Standard protocols for working with insect cell cultures and baculovirus amplification were followed according to the methods and recommendations described by King and Possee (Possee et al., 1999). Two strains of insect cell lines derived from the fall army worm *Spodoptera frugiperda* (*Sf*21 and *Sf*9), were used for the expression of foreign genes by infection with the recombinant AcMNPV. *Sf*21 cells derived from ovarian tissue were used for the generation of recombinant baculoviruses and for virus titration. As *Sf*9 cells grow faster at higher cell densities than *Sf*21 cells, *Sf*9 cells were used for the expression of recombinant proteins.

*Sf*21 cells were grown at 28°C in suspension in round-bottom flasks on a magnetic stirrer at 100 rpm in TC-100 medium (Lonza Ltd, UK) supplemented with 10% Feotal

calf serum (FCS) (Sigam-Aldrich Ltd, UK) and 1X antibiotic/antimycotic (100U penicillin, 100 µg/ml streptomycin and 250 µg/ml amphotericin B, AA) (Sigma-Aldrich Ltd, UK). *Sf*9 cells were grown at 28°C in conical flasks in Insect-XPRESS medium (Lonza Ltd, UK) in the presence of 100U/ml (AA). Both cells were maintained at cell densities between 0.5 x10^6 and 2 x 10^6 cells/ml and their viability was monitored by staining of 100 µl of cell culture with trypan blue (1:1) (Sigma-Aldrich Ltd, UK).

2.1.9. Generation of single and multiple recombinant proteins in AcMNPV

To generate the recombinant baculoviruses expressing single (Table 14) and multi-protein complexes (Table 15), *Sf*21 cells were seeded at a density of 1.0 x 10^6 to 1.5 x 10^6 per well in 6 well plates and incubated for 20 min at room temperature to allow the cells to settle down. For cotransfection, 2.0 µg of the $Bacmid_{1629}$ DNA was incubated with 500 ng of the purified recombinant plasmids, 10 µl Insect GeneJuice transfection reagent (Novagen) and 100 µl TC-100 medium. The transfection mixture was incubated at room temperature for 20 min. *Sf*21 cells were washed twice with TC-100 medium and 1.0 ml of it was added to the cells. After incubation, the reaction mixture was added drop-wise to the cells and incubated at 28°C for 5 h. TC-100 supplemented with 10% FCS (1 ml) was added to the cotransfected cells which were further incubated at 28°C for 4 days. The supernatant containing the recombinant baculovirus progeny was harvested and stored at 4°C for further use. As a control of the recombination efficiency, *Sf*21 cells were cotransfected with $Bacmid_{1629}$ and the pRN43-GFP vector which drives the expression of green fluorescent protein (GFP) under the control of *polyh* promoter. The expression of GFP protein which was synthesised by the recombinant baculovirus, was examined daily and confirmed by visualisation under the light and ultraviolet (UV) microscopy (Fig. 18).

Table 14.

Individual expression of the recombinants proteins for EHDV serotypes.

Baculovirus DNA	Recombinant vector
Bacmid$_{1629}$-*Bsu*36I	**pAcYM1-EHDV1.VP2**
	pAcYM1-EHDV1.VP5
	pAcYM1-EHDV1.VP7
	pAcYM1-EHDV1.VP3
	pAcYM1-EHDV2.VP2
	pAcYM1-EHDV2.VP5
	pAcYM1-EHDV7.VP2
	pRN43-GFP

Table 15.

Baculovirus multiple genes expression of the recombinant proteins of EHDV serotypes.

Baculovirus DNA	Recombinant vector
Bacmid$_{1629}$:EHDV1.VP7.VP3	**pAcYM1-*Alw*NI**
Bacmid$_{1629}$:EHDV1.VP7.VP3.VP5	**pAcYM1-*Alw*NI**
Bacmid$_{1629}$:EHDV1.VP7.VP3.VP5	**pAcYM1-EHDV1.VP2- *Alw*NI**
Bacmid$_{1629}$:EHDV2.VP5	**pAcYM1-EHDV2.VP2- *Alw*NI**

2.1.10. Isolation and propagation of recombinant baculoviruses

For the isolation of individual recombinant clones from the recovered viruses after cotransfection, plaque assays were performed in *Sf*21 insect cells. Briefly, *Sf*21 insect cells were seeded in 6 well plates at a density of 1 x 10^6 cells per well. Each virus sample was serially diluted (10^{-1} to 10^{-6}) in serum-free medium and 200 µl of each dilution was added directly onto the cells. To allow virus absorption to the cells, plates were incubated at room temperature on an orbital shaker for 1 h. The

inoculums were removed after incubation and 2 ml of 1.5% (w/v) molten low melting point agarose (Sigma-Aldrich Ltd, UK) in TC-100 media supplemented with 5% FCS were added to each well. The TC-100 supplemented with 10% FCS was added on the top of agarose after incubation for 10 min at room temperature to allow the agarose to set. Plates were further incubated at 28˚C for 4-5 days. Consequently, the medium was removed and the cells were stained with 2 ml of filtered 1:20 dilution of neutral red (Sigma-Aldrich Ltd, UK) in 1X phosphate-buffered saline (PBS). Staining was performed at 28˚C for 5 h or overnight for better visualisation of the plaques. Individual and well separated plaques were isolated using sterile glass Pasteur pipettes, transferred into 300 µl of TC-100 medium containing 2% FCS and stored at 4˚C. To amplify a single plaque, three rounds of amplifications were carried out in 6 well plates by infecting *Sf*21 at a density of 1 x 10^6 per well and incubated at 28˚C until the cytopathic effect (CPE) was observed in the cells, which occurred 42-72 hours post infection (hpi). The supernatant was harvested by centrifugation of the cell culture at 9000 *g* for 5 min and stored at 4˚C while the pellet was washed once with 1X PBS and used to analyse the expression of recombinant proteins. Once the expression was correctly determined, a virus stock was prepared by infecting *Sf*9 cells at a low MOI (0.01-0.1) plaque forming unit per cell [pfu/cell]) and stored at 28˚C until 100% CPE was detected. The recombinant baculoviruses in the culture medium were harvested by centrifugation at 9000 *g* for 10 min and stored in media with 2% FCS at 4˚C. High titre of working virus stocks were generated by inoculating 1 ml of the previously harvested virus into 100 ml of Insect-XPRESS media containing *Sf*9 cells at a concentration of 1 x 10^6 cells/ml which was incubated at 28˚C until 100% CPE. The harvested baculoviruses were then stored at 4˚C for subsequent experiments.

2.1.11. Confirmation of the expression of recombinant proteins

2.1.11.1. SDS-PAGE

A standard SDS-PAGE procedure was performed to detect the expression of proteins that derived either from transfection or infection of insect cells or mammalian cells (Laemmli, 1970). To verify the expression of the proteins using baculovirus stocks, suspensions of *Sf*9 cells at density of 1.2 x 10^6 cells/ml were infected at MOI of 3 to 5 and incubated for 48-60 h at 28˚C. Infection conditions including incubation time and MOI were optimised for each recombinant protein. Cells infected with recombinant baculoviruses were harvested by centrifugation at 1500 *g* for 15 min at 4˚C and washed twice with 1X PBS to remove the residual FCS that might affect the visualization of the proteins, and centrifuged at 10.000 *g* for 3 min. Samples were resuspended in a sufficient amount of 1X protein loading buffer (40% glycerol; 4% SDS; 0.02% bromophenol blue; 50 mM Tris-HCl, pH 6.8; 10% β-mercaptoethanol), and incubated at 100°C in a heat block for 5 min. The heated protein samples were then loaded on a discontinuous 10% polyacrylamide separating gel (1.5 M Tris-HCl, pH 8.8; 0.1% (w/v) SDS; 1% (v/v) TEMED; 10% (w/v) APS) and 5% (w/v) polyacrylamide stacking gel (13 mM Tris-HCl, pH 6.8; 0.1% (w/v) SDS; 1% (w/v) TEMED; 10% (w/v) APS). The running buffer was made up of (200 mM glycine; 25 mM Tris-HCl, pH 8.8; 0.1% (w/v) SDS). All gels were electrophoresed at 50 mA per gel at a 180 V limit and protein samples were stained with coomassie brilliant-blue (40% methanol; 10% glacial acetic acid; 0.2% (w/v) coomassie brilliant blue) for 45 min followed by incubation in a destaining buffer (40% (v/v) methanol; 10% (v/v) acetic acid). The time of destaining was depended on the visualisation of the separated protein bands. Thereafter, SDS-PAGE gels

were dried between two sheets of cellophane at 40°C using the GelAir dryer (Bio-Rad Laboratories Ltd, UK).

2.1.11.2. Western blotting

For protein detection and antibody specificity tests, proteins were analysed by SDS-PAGE and transferred to Filters Immobilon-P transfer membranes (Fisher Scientific Ltd, UK). Briefly, after SDS-PAGE, the gels were incubated for 5 min in western blotting buffer (12 mM Tris base; 96 mM glycine, pH 8.3; 20% methanol). Membranes were incubated in 100% (v/v) methanol for 1 min followed by incubation in western blotting buffer for 3 min. Whatman filter papers (8 sheets of 3 MM paper) were also soaked in western blot buffer for 5 min. A transfer sandwich was assembled as follows (from the anode electrode plate to the cathode electrode plate): 4 filter papers, transfer membrane, SDS-PAGE gel and 4 filter papers. The transfer of proteins to the membranes were performed for 2 h at a constant current of 1.2-2.5 mA/cm^2 using a semi-dry transfer blotting according to the manufacturer's procedure. As transference was completed, membranes were blocked in blocking buffer (10% dry skimmed milk powder; 1% Tween-20 in 1X PBS) for 1 h at room temperature then probed for 2 h in the presence of EHDV polyclonal as primary antibodies diluted in the same blocking buffer (Table 16). To remove the unbound antibody, the membrane was washed three times with blocking buffer followed by further incubation of the membrane for 1 h with the appropriate alkaline phosphatase-conjugated anti-species IgG (Sigma-Aldrich Ltd, UK) (Table 17). Membranes then were washed three times with the washing buffer (0.5% Tween-20 in 1X PBS) to remove the unbound secondary antibody. The development of the colour was carried out by incubating the membrane blot in 10 ml of alkaline phosphatase buffer (100 mM Tris; 100 mM NaCl; 5 mM $MgCl_2$) using 5-Bromo-4-

chloro-3-indolylphosphate/Nitro blue tetrazolium (BCIP/NBT) substrate system (Sigma Aldrich Ltd, UK).

Table 16.

Primary antibodies used in western blotting.

Protein/epitope	Source	Supplier	Dilution
EHDV-1 VLPs (VP2, VP5, VP7, VP3)	Rabbits	ThermoFisher Scientific, Pierce antibodies	1:10.000
EHDV-1 CLPs (VP7, VP3)	Rabbits	ThermoFisher Scientific, Pierce antibodies	1:10.000
EHDV-1 VP2	Guinea pigs	ThermoFisher Scientific, Pierce antibodies	1:5.000
EHDV-12 VP2	Guinea pigs	ThermoFisher Scientific, Pierce antibodies	1:5.000

Table 17.

Secondary antibodies used in western blotting.

Primary detected	Source	Supplier	Dilution
Anti-rabbit IgG (whole molecule)- Alkaline Phosphatase	Goat	Sigma-Aldrich Ltd, UK	1.10.000
Anti-guinea pig IgG (whole molecule)- Alkaline Phosphatase	Goat	Sigma-Aldrich Ltd, UK	1.5.000

2.1.12. Purification of the recombinants expressing EHDV-1 CLPs

The EHDV-1 CLPs were purified from *Sf*9 cells infected with a single recombinant baculovirus Bacmid$_{1629}$:EHDV1.VP7.VP3 expressing both the inner core proteins EHDV-1 VP7 and VP3. Briefly, 100 ml of *Sf*9 cells were cultivated in Insect-XPRESS media in a conical flask at 28°C to a density of 1 X 10^6 cells/ml and infected at MOI of 5. After incubation at 28°C for 72 h, the infected cells were harvested by centrifugation at 5000 *g* for 5 min at 4°C. The pellet was washed twice with cold PBS

and cell pellet was lysed in 1 ml ice-cold lysis buffer (150 mM NaCl; 50 mM Tris-HCl, pH 8.0; 0.5% NP-40; 1 mM EDTA; 0.75% (w/v) deoxycholate) for 1 x 10^7 of the cells. For complete lysis, the lysis mixture was incubated on ice for 10 min and homogenised with a glass Dounce homogeniser (10 strokes). Supernatants were collected by centrifugation at 4000 *g* for 10 min at 4°C, kept in ice and then loaded on top of pre-formed 25% (w/v) and 50% (w/v) discontinuous sucrose gradient in SW40 ultracentrifuge tubes. These gradients were formed with 2 ml 50% (w/v) and 4 ml 25% (w/v) in sucrose in 150 mM NaCl; 50 mM Tris-HCl, pH 8.0; 0.5% NP-40; 1 mM EDTA. Samples were centrifuged at 40.000 *g* for 90 min at 10°C in SW40 rotor (Beckman). At the end of the spin, two bands were visualised at both the 0-25% and 25-50% sucrose interfaces. Protein content in each fraction was analysed by 10% SDS-PAGE.

The sample collected from 25-50% sucrose interface which contained the CLPs proteins, was concentrated by further ultracentrifugation. In brief, the sample was diluted in 20 mM Tris-HCl, pH 8.0 and centrifuged at 45,000 *g* for 40 min at 10°C in TLS-55 rotor (Beckman). After centrifugation, the pellet was resuspended in 100 µl of 20 mM Tris-HCl, pH 8.0 and kept at 4°C overnight. To verify the presence and purity of EHDV-1 CLPs proteins VP7 and VP3, 5 µl of the concentrated sample was analysed on 10% SDS-PAGE. The concentration of the CLPs proteins were determined by Bradford reagent (Sigma-Aldrich Ltd, UK) for further work.

2.1.13. Purification of EHDV-1 VLPs and heterologous VLPs of EHDV-2

For purification of EHDV-1 VLPs consisting of VP2, VP5, VP7 and VP3 and heterologous VLPs made up of the inner core protein of EHDV-1 CLPs (VP7 and VP3) and the outer capsid proteins of EHDV-2 (VP2 and VP5), 4 X 175cm^2 flasks with *Sf*9 cells grown as monolayer cultures were infected with the appropriate

recombinant baculoviruses at a MOI of 5 pfu/cell. The cells were harvested by centrifugation at 5000 *g* at 4°C after 72 h of infection when the CPE was 100%. The cell were lysed in a total of 25 ml of chilled 2X lysis buffer (200 mM Tris-HCl, pH 8.8; 100 mM NaCl; 20 mM EDTA; 0.2% NP-40) and incubated on ice for 10 min. After gentle homogenisation with a glass Douncer (10 times), cell lysates were centrifuged at 4000 *g* for 5 min at 4°C. The supernatants were collected and pellets were resuspended in 25 ml of 1X lysis buffer and the same steps were repeated. After centrifugation, the supernatants collected from the two lysis steps were pooled and loaded onto 4 ml 40% (w/v) and 3.5 ml 75% (w/v) sucrose discontinuous gradients in 50 mM NaCl; 100 mM Tris-HCl, pH 8.8; 1 mM EDTA, pH 8.0; 0.02% NLS. Samples were centrifuged at 28,000 *g* at 16°C for 2 h. Each interface and pellet were carefully collected. A sample of each fraction was run on 10% SDS-PAGE to detect the presence of the four proteins VP2, VP5, VP7 and VP3. For further purification, the interfaces were diluted slowly 1 in 5 in 100 mM Tris-HCl, pH 8.8; 50 mM NaCl; 1 mM EDTA, pH 8.0; 0.02% NLS and loaded onto 3 ml 40% per 2 ml 70% (w/v) sucrose cushions prepared as before, then centrifuged at 28,000 *g* for 2 h at 16°C. VLPs proteins were detected by 10% SDS-PAGE and coomassie brilliant blue staining. The samples were kept at 4°C for further work.

2.1.14. Antibodies production

To determine the total concentration of the EHDV-1 CLPs and VLPs proteins after purification, Bradford reagent (Sigma-Aldrich Ltd, UK) was used. A standard curve within the linear range was generated using a range of concentrations (1 to 5 µg/µl) of bovine serum albumin (BSA) protein. The protein concentration of the CLPs and VLPs samples was determined by comparison to that of a series of the BSA standard protein which was measured by NanoDrop®ND-1000 Spectophotometer

(NanoDrop Techlonogies, Inc, USA). For antibody production, 2.5 mg/ml of the EHDV-1 CLP proteins resuspended in 10 mM Tris-HCl, pH8.0 and 2.0 mg/ml of EHDV-1 VLPs were used to inoculate Rabbits by a commercial company (ThermoFisher Scientific, Pierce antibodies).

Additionally, polyclonal antibodies against VP2 protein of EHDV-1 and EHDV-2 were raised in Guinea pigs by ThermoFisher Scientific. In brief, after separating the above proteins by electrophoresis on 10% SDS-PAGE, the gels were washed twice with autoclaved pre-chilled 1X PBS and then soaked with the pre-cold 250 mM KCl and incubated at 4°C until white bands of the specific protein were visualised. A total of 1 mg of the detected proteins (based on BSA concentration) were excised from gel and incubated in 10 mM Tris-HCl, pH 8.0 supplemented with protease inhibitors and kept at - 20°C. The specificity of each antibody was analysed by western blotting.

2.1.15. Electron Microscopy

Aliquots (2 µl) of purified CLPs and VLPs were adsorbed onto copper 400-mesh Formvar carbon-coated grids (TAAB Laboratories Equipment Ltd, UK) for 2 min. After washing twice with 10 mM Tris-HCl, pH 8.0 (for CLPs) and 10 mM Tris-HCl pH 8.8 (for VLPs) 30 seconds apart, the particles were stained with filtered 2% (w/v) phosphotungstic acid (PTA), pH 6.8. After blotting the stain, the grids were air-dried for 25 min and examined under JEOL 1200 EX transmission microscope.

2.1.16. Neutralisation assay

The antibodies raised against purified EHDV-1 VLPs were assayed for their neutralising activity against EHDV-1 virus. In addition, cross neutralising reactivities were tested against EHDV-2 and EHDV-6. In brief, 96 well plates were seeded with 5×10^3 BSR cells/well and incubated overnight at 37°C with 5% CO_2. The serum was

heat inactivated at 42°C for 20 min prior to use and two-fold serial dilution in Dulbecco Modified Essential Medium (DMEM) (Sigma-Aldrich Ltd, UK), was prepared in a total volume of 100 µl in triplicate. The amount of EHDV-1 virus was calculated as being equivalent to the tissue culture infectious dose 50 ($TCID_{50}$) in 100 µl per well. At the containment lab, the virus and the serum were added to the cells and incubated at 35°C in 5% CO_2 for 3-4 days. For fixing, 50 µl of 10% (w/v) formaldehyde in PBS were added to each well and incubated for at least 3 h at room temperature. For visualisation, 200 µl of crystal violet staining were used, incubated for 5 min and washed with water. After drying, plates were observed for signs of neutralisation. The neutralisation activity was calculated as the higher dilution of sera that was able to inhibit virus replication. All dilutions were performed in triplicate and the assay was repeated at least three times.

2.2. Reverse Genetics (RG) system

2.2.1. Mammalian cell line

BSR cells, a clone derived from the Baby Hamster Kidney cells (BHK) (Sato et al., 1977) were used for virus propagation, titration and transfection with single-stranded (ssRNA) derived from cores or T7 clones. BSR cells were grown in DMEM supplemented with 5% FCS in the presence of 50U/ml antibiotic/antimycotic and L-glutamine (Sigma-Aldrich Ltd, UK). The cells were grown as monolayers in vented tissue culture flasks at 37°C in the presence of 5% CO2. The sub-culturing of BSR cells was carried out by disrupting the monolayer by trypsinisation using trypsin-EDTA solution diluted in PBS (Sigma-Aldrich Ltd, UK).

2.2.2. EHDV purification, propagation and titration

The EHDV isolates used in all experiments were from cell culture adapted strains belonging to serotype 1 (New Jersey), serotype -2 (Alberta, CANADA), serotype 6 (The Reunion Island, France) and serotype 7 (strain CSIRO 775). Virus purification, titration and recovery were performed in a P3 level containment laboratory using the safety procedures of Category III level (CATIII-DEFRA). For the isolation of individual virus plaques and titration, plaque assay was performed. In brief, 6 well plates were seeded with BSR cells to 80-90% confluency in DMEM supplemented with 5% FCS. EHDV stocks were ten-fold serially diluted in serum-free DMEM and 200 µl of each dilution was added to the cells after washing them twice with FCS-free DMEM medium and incubated at 35°C for 1 h. Following incubation, the inoculum was removed and 2 ml of 1.5% (w/v) molten low melting point agarose diluted in 1X DMEM supplemented with 2% FCS was added to the cells. The overlay was allowed to set for 5-10 min at room temperature and plates were incubated at 35°C for 2-3 days until plaques were visible.

To isolate individual virus clones, cells were stained with a solution of 2% (v/v) neutral red diluted in PBS incubated at 35°C for 4-5 h. Subsequently, single plaques were picked and amplified in BSR monolayers for the generation of virus stocks. For virus titration, plaque assays were fixed with 10% (w/v) formaldehyde in PBS for at least 4 h at room temperature. The agarose overlay was removed carefully and the monolayer cells were stained with 400 µl of 0.2% (w/v) crystal violet in 20% (v/v) ethanol for 3 min. The excess stain was washed away with ultra-pure deionised water and the plates were allowed to dry. The titre of EHDV virus was calculated as pfu/ml after counting the formed plaques.

2.2.3. Purification of dsRNAs from EHDV-2 infected BSR cells

BSR cells were grown to 80% confluency in a 175 cm^2 flask. Prior to infection, cells were washed twice with FCS-free DMEM medium and EHDV-2 at an MOI of 1.0 was added to the monolayer. The virus was allowed to absorb for 1 h at 35°C and 13 ml DMEM supplemented with 2% FCS were added. The infected BSR cells were incubated at 35°C until complete CPE was detected. Consequently, the infected culture and cells were harvested and transferred to a 15 ml sterile centrifuge tube. To clarify the sample, the culture was centrifugated at 2,500 *g* for 5 min at 4°C. The pellet was lysed in 10 ml Tri reagent (Sigma-Aldrich Ltd, UK) and incubated at room temperature for 30 min to 1 h. After incubation, the sample was safely removed from the containment lab and proteins were removed by the addition of 1 ml chloroform (Sigma-Aldrich Ltd, UK) mixed by vortex and centrifugated. The upper phase was collected and the RNA were precipitated by the addition of an equal amount of isopropanol followed by centrifugation at 6,000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol, air dried and resuspended in 400 µl of Diethylpyrocarbonate (DEPC) treated water. To specifically precipitate ssRNAs, 135 µl of 8 M LiCl (Sigma-Aldrich Ltd, UK) was added, mixed by inversion and incubated at 4°C overnight. The dsRNA was separated from ssRNA by centrifugation at 9,000 *g* for 20 min at 4°C. The supernatant was carefully collected, as it had the dsRNA, and was further precipitated by isopropanol as described. After centrifugation, the pellet was resuspended in 30 µl DEPC-treated water and the pattern of the segmented dsRNA as well as its concentration was determined by electrophoresis on 8% non-denaturing polyacrylamide gel.

2.2.4. Reverse transcription-PCR (RT-PCR)

The cDNA copies of the 10 genome segments of EHDV-2 were amplified from dsRNA extracted and purified from infected BSR cells using the method of full-length amplification of cDNA (FLAC) in a sequence independent manner. A modified 35-mer oligonucleotide 'anchor primer' (Maan et al., 2007) has a modification consisting in a C9 (phosphoramidite) spacer between a self-annealing sequence and phosphorylated 5' (Table 12) (Eurogenetic). The C9 primer was ligated into the unfractionated viral dsRNA in a total volume of 20 µl as follows: dsRNA (5 µg); C9 primer (10 µg/µl); 10X T4 RNA Ligase Buffer (2 µl) (Fermantas); 10X BSA (2 µl); 10 mM ATP (2 µl) and T4 RNA ligase enzyme (0.5 µl) (New England Bio Labs) and incubated at 4°C overnight.

After ligation, the dsRNAs were separated on 1% agarose gel electrophoresis to excise the 10 segments of dsRNA genome. This step was performed to remove the excess C9 primer prior to DNA synthesis in order to prevent any mispriming by the free primer molecule. The ligated dsRNA was purified using Invitrogen PureLinkTM HiPure DNA Purification Kit (Invitrogen^{TM}, UK) and precipitated in 0.3 M NaOAc and 50% Isopropanol. After centrifugation, the RNA pellet was air dried and resuspended in 10 µl nfH_2O.

The synthesis cDNA from gel-purified genome segments was performed using 10U of RevertAid Premium Reverse transcriptase enzyme (Thermo Scientific) at 55°C for 1.5 h in the presence of 1X RT buffer (Thermo Scientific), RNase inhibitors (RNasin Plus) (0.5U/µl) and 10 mM dNTPs. The cDNA was then used directly for PCR amplification using 10 µM of 5' phosphorylated FLAC2 primer (Table 12); 10 mM dNTPs; 1X Kapa buffer and Kapa DNA polymerase (1U/µl) (KAPA Biosystems). The

PCR amplification was detected on 0.8% agarose gel electrophoresis and the product was purified for further experiments.

2.2.5. Construction of cDNA T7 plasmid clones

Initially, to construct cDNA T7 plasmid clones for the 10 segments of EHDV-2 genome to use as templates for ssRNAs generation, sequence analysis of each segment was carried out by Source BioScience, UK. Briefly, amplification products of the full length segments were ligated into a *Sma*I linearised and dephosphorylated pUC19 vector. The ligation reactions were transformed into DH5α competent cells and colony PCR with FLAC2 primer was performed to detect the presence of each segment in the ampicillin resistant clones. The complete sequence of the 10 segments was obtained using M13 (Table 12) and internal primers specific to each one (Table 13) and the results were compared with the exact gene sequence (GenBank accession numbers Table 8). Base on the data obtained from sequencing, primers with T7 promoter and specific restriction enzyme site were designed to amplify the full length of EHDV-2 10 segments (Table 18). After ligation, the presence of the inserts in the constructs was analysed by PCR using specific primers and by digestion with the exact enzyme inserted downstream each segment (Table 18). The results were visualised on 1% agarose gel electrophoresis.

Table 18.

EHDV-2 T7 primers used for cloning the 10 genome segments into pUC19.

EHDV-2 T7 primers		
T7-S1 (F)[a]	**TAATACGACTCACTATAGTTAAAATGCAATGGTCGCAATTA**	**T7 polymerase Conserved region**
***Bsm*BI-S1 (R)[b]**	**CGTCTCTGTAAGTATAATGCGGGT**	***Bsm*BI Extra nucleotide**
T7-S2 (F)[a]	**TAATACGACTCACTATAGTTAAATTGTCCCCAGAATGGAGGA**	**T7 polymerase Conserved region**

***Bsa*I-S2 (R)[b]**	GGTCTCTGTAAGTTTGTTGTTCCCAGTA	***Bsa*I Extra nucleotide**
T7-S3 (F)[a]	TAATACGACTCACTATAGTTAAATTTCCGGAGCGATGGCA	**T7 polymerase Conserved region**
***Bsa*I-S3 (R)[b]**	GGTCTCTGTAAGTGTATTCCCGGT	***Bsa*I Extra nucleotide**
T7-S4 (F)[a]	TAATACGACTCACTATAGTTAAAACATGCCGGAGCCACAT	**T7 polymerase Conserved region**
***Bsa*AI-S4 (R)[b]**	TACGTAGTAAGTGTAACACGCA	***Bsa*AI Extra nucleotide**
T7-S5 (F)[a]	TAATACGACTCACTATAGTTAAAAAGTTCTTCGTCGAC	**T7 polymerase Conserved region**
***Bsa*I-S5 (R)[b]**	GGTCTCTGTAAGTGTAAAGTTCGAA	***Bsa*I Extra nucleotide**
T7-S6 (F)[a]	TAATACGACTCACTATAGTTAAAAAGGAGGCACGTTCTTGCATC	**T7 polymerase Conserved region**
***Bbs*I-S6 (R)[b]**	GAAGACTTGTAAGTGTAAGGAGTT	***Bbs*I Extra nucleotide**
T7-S7 (F)[a]	TAATACGACTCACTATAGTTAAAATTTGGTGAAGATTGA	**T7 polymerase Conserved region**
***Bsa*I-S7 (R)[b]**	GGTCTCTGTAAGTTGAATTTGGGAAGA	***Bsa*I Extra nucleotide**
T7-S8 (F)[a]	TAATACGACTCACTATAGTTAAAAATTCCTTGTGCAAT	**T7 polymerase Conserved region**
***Bsm*BI-S7 (R)[b]**	CGTCTCTGTAAGTGTAAATCCCTT	**BsmBI Extra nucleotide**
T7-S9 (F)[a]	TAATACGACTCACTATAGTTAAAAATTGCGCATGTCAGCT	**T7 polymerase Conserved region**
***Bbs*I-S9 (R)[b]**	GAAGACTTGTTAAATTAAATTGCGCGCGT	**BbsI Extra nucleotide**
T7-S10 (F)[a]	TAATACGACTCACTATAGTTAAAAAGAGGTTGGCATCATGCT	**T7 polymerase Conserved region**
***Bbs*I-S10 (R)[b]**	GAAGACTTGTAAGTGTGTCGAGGAT	**BbsI Extra nucleotide**

(F)[a]: Forward
(R)[b]: Reverse

2.2.6. Synthesis of EHDV-2 transcripts from cDNA plasmid clones

The T7 plasmid templates for *in vitro* transcription were generated for the 10 segments of EHDV-2 genome (as mentioned in the construction of cDNA plasmid clones) by digestion with a specific enzyme (Table 18). The complete linearisation of each recombinant plasmid was confirmed by 1% agarose gel electrophoresis

followed by precipitation with isopropanol in the presence of 0.15 M NaOAc. DNA pellets were washed twice with 70% (v/v) ethanol and dissolved in 10 mM Tris-HCl, pH 8.0. In order to produce capped ssRNA T7 transcripts, mMESSAGE mMACHINE T7 Ultra Kit (Ambion) was used. In brief, the transcription reactions were assembled at room temperature in the following order: T7 2X NTP/ARCA (5 µl); 10X T7 reaction buffer (1 µl); T7 linear DNA template (1 µg); T7 enzyme mix (1 µl) and RNasin Plus (0.25 µl). The reactions were mixed thoroughly, incubated at 37°C for 2 h followed by the addition of 1 µl of TURBO DNase to remove the DNA template and further incubated at 37°C for 15 min. The complete set of T7 EHDV-2 10 transcripts were extracted once with phenol-chloroform, followed by one extraction with chloroform. Microspin G-25 columns (GE Healthcare) were used to remove unincorporated ribonucleoside triphosphate by size fractionation according to the manufacturer's instructions. The T7 transcripts were precipitated by adding equal volume of isopropanol, 0.5 µl glycogen and 0.3 M NaOAc and incubated at 4°C overnight. The ssRNA pellets were washed twice with 70% (v/v) ethanol and dissolved in 10 µl sterile DEPC treated water and stored at -80°C to be used in further experiments.

2.2.7. Denaturing agarose gel electrophoresis

The purified EHDV-2 ssRNAs were analysed by electrophoresis on 1% denaturing agarose in morpholinepropanesulfonic acid (MOPS) buffer in the presence of 6.3% formaldehyde according to the standard methods (Sambrook and Russell, 2001). Briefly, 5 µl of the 2X RNA loading buffer (Thermo Scientific) was mixed with 0.5 µl aliquots of ssRNAs and heated at 72°C for 2 min. The ssRNA samples were immediately loaded on agarose gel and run for 30 min at 100 V. As ladder, 1 µg of the RNA marker (Thermo Scientific) was included.

2.2.8. Purification of EHDV-2 virus cores

In order to purify EHDV-2 cores, 6 x 175 cm^2 flasks were seeded with BSR cells in 25 ml DMEM supplemented with 5% FCS and incubated at 37°C, 5% CO_2 until 75% confluency. The cells were infected with EHDV-2 at an MOI of 1.0 in a total volume of 5 ml serum-free media and incubated at room temperature for 1 h. After incubation, 20 ml of DMEM with 5% FCS were added and cells incubated at 35°C, 5% CO2 until 100% CPE was observed. The transcriptionally active cores of EHDV-2 were purified according to the modified methods of *Mertens et al* (Mertens et al., 1987) and *Van Dijk and Huismans* (Van Dijk and Huismans, 1980). Briefly, the medium culture and infected cells were harvested and centrifuged at 2,500 *g* for 10 min. The pelleted cells were lysed in a 30 ml pre-chilled lysis buffer (100 mM Tris-HCl, pH 8.8; 50 mM NaCl; 10 mM EDTA; 0.1% NP-40) at 4°C for 10 min followed by centrifugation at 2,500 *g* for 5 min at 4°C to pellet the nuclei and intact cells. The supernatant was kept and the pellet was lysed as above using 25 ml of the lysis buffer. To remove the outer capsid proteins VP2 and VP5, the supernatants were pooled and α-Chymotrypsin was added to 60 µg/ml, and the samples were incubated at 35°C for 1 h. The sample was then made 0.2% (w/v) for *N*-lauroyl sarcosine (sodium sault, NLS) and incubated at room temperature for 1 h. Samples were loaded onto a sucrose cushion consisting of 40% (w/v) sucrose; 0.6 M $MgCl_2$; 20 mM Tris-HCl, pH 8.0 and ultracentrifugation was carried out in SW28 rotor (BeckMan), at 28,000 *g* at 20°C for 2 h. Subsequently, the pellet was gently resuspended in 200 µl 20 mM Tris-HCl, pH 8.0; 0.5% NP-40 and incubated at 4°C overnight. A second ultracentrifugation spin was performed through a sucrose cushion consisting of 40% (w/v) sucrose; 20 mM Tris-HCl, pH 8.0 at 28,000 *g* and 20°C for 2 h. After spinning, the pellet was resuspended with 200 µl 20 mM Tris-HCl, pH 8.0 and stored at 4°C.

An aliquot of 10 µl of the purified cores were inactivated with 1X protein loading buffer and load on 10% SDS-PAGE.

2.2.9. *In vitro* synthesis and purification of core derived EHDV-2 mRNA

In order to establish the infectivity of core transcripts and with the final goal of developing a reverse genetics system for EHDV, full length authentic mRNAs derived from purified core were prepared. In brief, purified core particles were mixed at 40 µg/ml in transcription mix containing 100 mM Tris-HCl, pH 8.0; 4 mM ATP; 2 mM GTP; 2 mM CTP; 2 mM UTP; 0.5 mM S-adenosylmethionine (SAM); 6 mM dithiothreitol (DTT); 9 mM $MgCl_2$; 0.5U/µl RNAsin Plus (Promega) and 0.6 mM $MnCl_2$. The transcription mixture was then incubated at 30°C for 5 h. For the purification of EHDV-2 core-derived mRNAs, a standard method described by *Boyce and Roy* was performed (Boyce and Roy, 2007). Briefly, cores were removed by performing ultracentrifugation in TLS-55 rotor at 45,000 *g* for 25 min at 4°C twice. The supernatant was collected carefully to avoid contamination with the pelleted cores. The supernatant from the second ultracentrifugation was made 2 M LiCl, using 8 M LiCl stock (Sigma), and incubated overnight at 4°C to precipitate the ssRNA. Subsequently, the samples were centrifuged at 15,000 *g* at 4°C for 20 min. The pellet was then resuspended in 200 µl Proteinase K (10 µg/ml in OptiMEM I [Invitrogen]) and incubated at 35°C for 30 min. After incubation, the samples were deproteinised by extraction with phenol-chloroform twice, treated with 0.1% SDS (w/v) and re-extracted with chloroform twice. The upper phase was collected and analysed by denaturing agarose gel electrophoresis as described in denaturing agarose gel electrophoresis.

2.2.10. Recovery of infectious EHDV-2 by transfection of BSR cells with the core-derived or the complete set of synthetic T7 transcripts

For the recovery of the infectious virus EHDV-2 from the ssRNAs, derived either from transcribing cores or from the T7 transcripts of the 10 segments, cell cultures were transfected as per protocol described by *Boyce et al* (Boyce and Roy, 2007). A total of 1.0-1.5 µg of the transcripts were mixed in Opti-MEM® I media in the presence of 0.1U/µl RNasin® Plus (Promega). The RNA mixture was incubated at room temperature for 30 min followed by incubation at 4°C for 10 min. The transfection reagent Lipofectamine 2000 (Invitrogen) was prepared according to the manufacturer's instructions (2.5 µl Lipofectamine 2000/1 µg ssRNA) in Opti-MEM® I supplemented with 0.1U/µl RNasin® Plus, and incubated at room temperature for 10 min. After incubation, the appropriate amount of transfection reagent was added to the ssRNAs mixture and the reactions were incubated at room temperature for 20 min.

Confluent BSR monolayers in 6- or 12- well plates were washed once with serum-free DMEM and the transfection mixture was added directly on the top of the cells. Plates were incubated at 35°C for 5 h and 1.5 ml DMEM supplemented with 5% FCS was added and incubated overnight at 35°C. As it has been shown that a second transfection increases the yield of virus recovery (Matsuo and Roy, 2009), 18 h post first transfection, a second transfection was performed using the same conditions mentioned above. At 5 hours post transfection (hpt), the culture medium was replaced with a 1 or 2 ml overlay consisting of DMEM, 2% FCS and 1.5% (w/v) 4% agarose low melting point (Sigma-Aldrich Ltd, UK). Recovery experiments were incubated at 35°C in 5% CO_2 for 72 h to allow plaques to appear. Subsequently, individual plaques were isolated, amplified and dsRNAs were extracted and purified

to detect the pattern of the segmented genome from the recovered infectious virus in comparison to that of those obtained from infected BSR cells. The results were visualised by electrophoresis on 1% agarose gel.

Chapter 3

Expression of the outer capsid proteins of EHDV serotypes

3.1. INTRODUCTION

In recent years, a dramatic increase in EHDV associated morbidity and mortality in animal farms, particularly in cattle, was recognised. The geographical distribution of these outbreaks was also increasing due to the emergent of more virulent serotypes to domestic ruminants (Yadin et al., 2008, Temizel et al., 2009, Breard et al., 2004, Anthony et al., 2009). Several studies have shown that among the most effective strategies to overcome the viral infection and prevention of spreading, vaccines have been shown to be the optimal solution.

To date, no vaccine against EHDV is commercially available, apart from the live attenuated vaccine for EHDV-2 (IBAV) (Omori et al., 1969). Therefore, developing EHDV-1 VLPs, and heterologous VLPs of EHDV-2 and EHDV-7 using EHDV-1 CLP proteins as background, were the main aims of this study. This was based on the use of baculovirus expression system that offers advantage of expression of several large proteins simultaneously, by a single recombinant virus.

In this study, prior to utilising the advanced baculovirus multiple genes expression system, individual expression of the outer and inner capsid proteins of EHDV-1 VP2, VP3, VP5 and VP7, EHDV-2 VP2 and VP5 and EHDV-7 VP2 were assessed. The transfer replicon, pAcYM1, which has the *polyh* promoter of AcMNPV, was used for this purpose. This was based on previous reports that demonstrated high expression level of the targeted genes from recombinant baculoviruses using pAcYM1 (Matsuura et al., 1987, Romanowski et al., 1985, Emery and Bishop, 1987, Urakawa and Roy, 1988). For the individual expression of EHDV-1, -2 and 7 recombinant structural proteins, insect cells derived from *Sf*21 and *Sf*9 were used.

3.2. RESULTS

3.2.1. Cloning and expression of EHDV-1 structural proteins

3.2.1.1. Cloning of EHDV-1 S2 into pAcYM1

The EHDV-1 S2 gene that encodes the outer capsid protein VP2 of EHDV-1 (New Jersey strain) was previously cloned into pUC4K vector. However, to subclone the S2 coding region, specific primers with *Bgl*II restriction sites (Table 7) were designed and utilised to amplify the open reading frame (ORF) of EHDV-1 S2 gene. To identify the efficiency of amplification, the PCR product was examined by 1% agarose gel electrophoresis (Fig. 13) showing an exact band of the correct size of EHDV-1 S2 segment (2968 bps) (Fig. 13, lane 3). Moreover, no bands were detected in the negative control without DNA template (Fig. 13, lane 2).

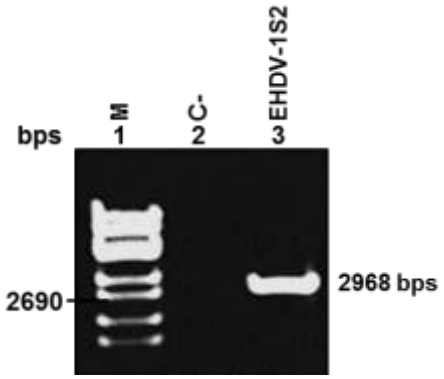

Fig. 13. PCR amplification of the coding region of EHDV-1 S2 segment. The PCR was visualised by gel electrophoresis on 1% agarose gel, stained with ethidium bromide. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-1 S2.

The amplified ORF of EHDV-1 S2 gene was then ligated into a *Bam*HI site of the linearised and dephosphorylated pAcYM1vector (Fig. 14B, lane 2). To produce the recombinant plasmid pAcYM1-EHDV1.S2 (Fig. 14A), the ligation reaction was transformed into the *E.coli* DH5α competent cells and plated onto LBA plates. Bacterial colonies were grown in LB media supplemented with ampicillin and the

plasmid DNA was extracted. The purified recombinant plasmid was digested with the endonuclease enzyme *Bgl*II (Fig. 14B, lanes 3-7), and positive clones would release a fragment of size ~ 2700 bps. This was visualised in clone #1 (Fig. 14B, lane 3) after electrophoresis on a 1% agarose gel as two DNA bands; one to the predicted size of the vector pAcYM1 (~9171 bps) and another with the same length of EHDV-1 S2 (~2968 bps) gene, while other 4 colonies were negative (Fig. 14, lanes 4-7).

Fig. 14. Molecular analysis of the presence of EHDV-1 S2 in the recombinant pAcYM1-EHDV1.S2. (A) Restriction map of pAcYM1-EHDV1.S2 showing *Bgl*II sites. (B) *Bgl*II digestion of the recombinant pAcYM1-EHDV1.S2. Lane 1: DNA ladder, lane 2: pAcYM1-*Bam*HI and lanes (3-7) pAcYM1-EHDV1.S2 clones digested with *Bgl*II # 1-5. Colony # 1 was positive (lane 3).

For the synthesis of EHDV-1 VP2 structural protein, the encoding region should be integrated in a correct orientation to the *polyh* promoter in the recombinant pAcYM1-EHDV1.S2. Thus, depending on the restriction map of pAcYM1-EHDV1.S2, *EcoR*V endonuclease enzyme was chosen as it had two restriction sites in the recombinant; one in pAcYM1 and another in EHDV-1 S2 (Fig. 15A). Consequently, a correct position of EHDV-1 S2 in the recombinant was visualised on an agarose gel as shown in Figure 15B (Fig. 15B, lane 2). Further confirmation was carried out by PCR amplification as an alternative molecular method using PH (F) primer from pAcYM1 and the specific (R) primer for EHDV-1 S2. Post electrophoresis of the PCR product,

a single band to the size of EHDV-1 S2 gene was observed (Fig. 15C, lane 3). An amplification reaction was undertaken without DNA template as a negative control revealing no fragments on the 1% agarose gel as shown in Figure 12C (Fig. 15C, lane 2).

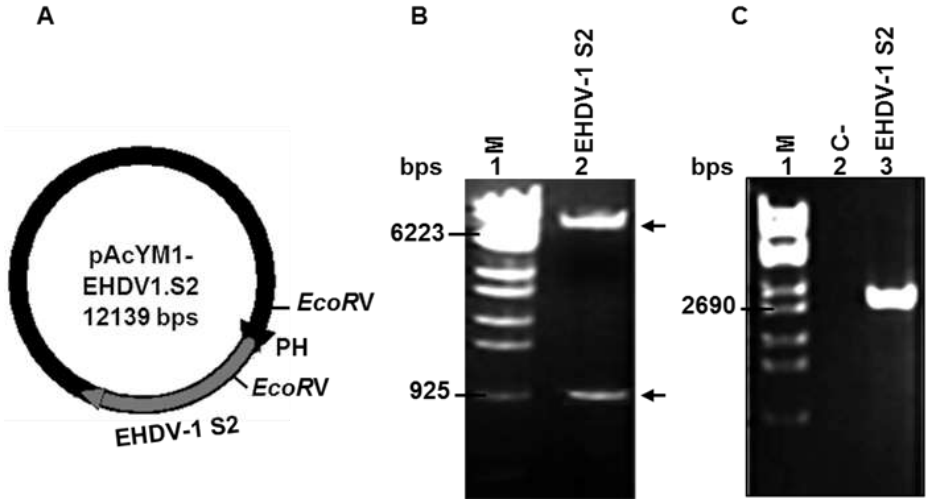

Fig. 15. Determining the orientation of EHDV-1 S2 in the recombinant pAcYM1-EHDV1.S2. (A) Restriction map with *Eco*RV sites. (B) *Eco*RV digestion of pAcYM1-EHDV1.S2. Lane 1: DNA ladder and lane 2: pAcYM1-EHDV1.S2 (clone # 1). (C) PCR with PH (F) and EHDV-1 S2 (R) primers. Lane 1: DNA ladder, lane 2: negative control and lane 3: correct orientation of EHDV-1 S2 # 1 in the recombinant.

3.2.1.2. Expression of EHDV-1 VP2 recombinant protein in baculovirus expression system

Baculovirus BAC10:KO_{1629} was utilised for the synthesis of the outer capsid protein EHDV-1 VP2. Therefore, the parental viral DNA was digested with the endonuclease enzyme *Bsu*36I, then mixed with the recombinant plasmid pAcYM1-EHDV1.S2 in the presence of transfection reagent cellfectin® II. However, the digestion of Bac10:KO_{1629} resulted linear baculovirus DNA incapable of replication in insect cells. Accordingly, cotransfection of *Sf*21 insect monolayers with the linearised Bac10:KO_{1629} and the recombinant plasmid pAcYM1-EHDV1.S2 restored *orf1629* and recircularised the virus DNA by allelic replacement. After cotransfection, two

rounds of baculoviral amplification were undertaken; followed by plaque assay for the isolation of the recombinant clonal population. Plaques were visualised by staining *Sf*21 cells with 10% neutral red and three independent plaques were picked and amplified three times in *Sf*9 insect monolayers to increase the titre of the recombinant baculovirus expressing EHDV-1 VP2 protein (Fig. 16).

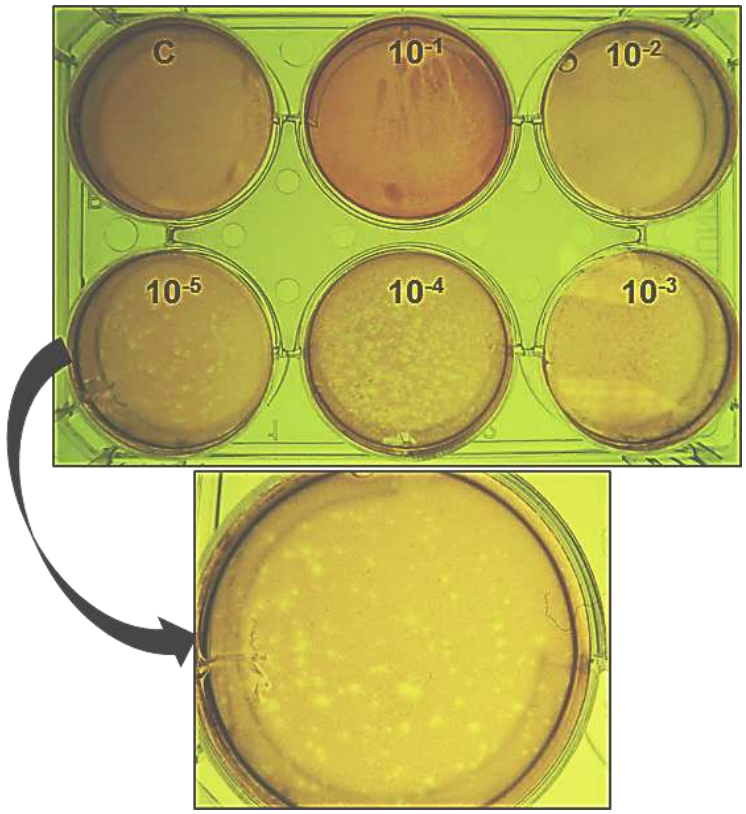

Fig. 16. Plaque assay of the recombinant baculovirus expressing EHDV-1 VP2 in *Sf*21 insect cells stained with 10% neutral red.

The synthesis of the recombinant protein EHDV-1 VP2 was analysed by 10% SDS-PAGE followed by coomassie blue staining, which showed high level of expression (Fig. 17A, lane 4). To analyse the antigenic properties of EHDV-1 VP2, the proteins were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with EHDV-1 polyclonal primary antibody raised in Rabbits, followed by the specific secondary antibody. Protein band was visualised after colour

development displaying a specific band with the correct size of EHDV-1 VP2 (112 kDa) as shown in figure 17B, lane 4 (Fig. 17B, lane 4).

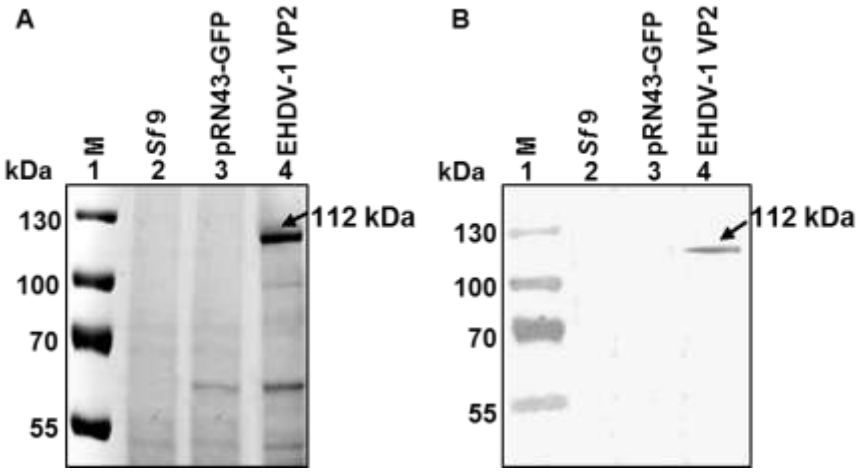

Fig. 17. Analysis of the recombinant EHDV-1 VP2 protein expression from clonal populations. (A) 10% SDS-PAGE analysis. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with the recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP2 (black arrow). (B) Western blotting using EHDV-1 polyclonal antibody. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with the recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP2 (black arrow).

As a positive control of the recombination efficiency, *Sf*21 insect cells were cotransfected with the linearised Bac10:KO_{1629} and the plasmid construct pRN43-GFP. The expression of the recombination GFP protein was confirmed by visualisation under the light (Fig. 18A) and fluorescence (Fig. 18B) microscopy at 24, 48 and 72 hpt.

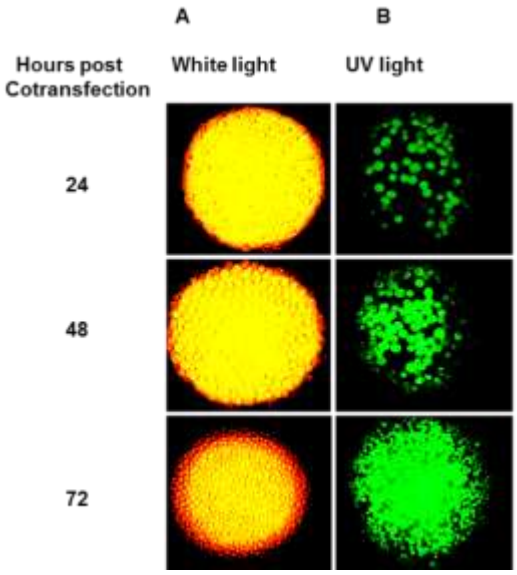

Fig. 18. GFP produced from insect cells transfected with recombinant AcMNPV pRN43-GFP. (A) GFP of transfected cells under a light microscopy at 24, 48 and 72 hpi. (B) The transfection of pRN43-GFP recombination under fluorescence microscopy at 24, 48 and 72 hpi, (100X amplification).

3.2.1.3. Cloning of EHDV-1 S5 into pAcYM1

To clone EHDV-1 S5 into pAcYM1, the coding sequences of S5 gene was amplified from pUC19-EHDV1.S5 (available in the lab) using specific sense and antisense primers for EHDV-1 S5 (Table 7). The PCR amplicon of the EHDV-1 S5 was identified after gel electrophoresis displaying an exact band of the expected size of EHDV-1 S5 (1640 bps) (Fig. 19, lane 3), whilst a negative control in lane 2 yielded no band (Fig. 19, lane 2).

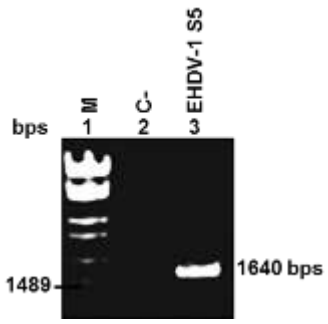

Fig. 19. PCR amplification of the coding region of EHDV-1 S5. The PCR product was visualised by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-1 S5.

The PCR amplicon of EHDV-1 S5 was excised from agarose gel and cloned into *Bam*HI site of the linearised and dephosphorylated pAcYM1 vector. *E.coli* DH5α competent cells were transformed with the ligation reaction mixture. A total of 4 ampicillin resistance colonies were screened by digestion with *Bam*HI to detect the presence of EHDV-1 S5 in the pAcYM1-EHDV1.S5 construct (Fig. 20B). The restriction digestion products were observed by agarose gel electrophoresis. Each of the four selected colonies had the insert of size corresponding to EHDV-1 S5 (1640 bps) (Fig. 20B, lanes 2-5).

To determine the orientation of EHDV-1 S5 in the recombinant pAcYM1-EHDV1.S5, the plasmid DNAs were extracted and purified from positive clones, followed by double restriction digestion with *Xma*I and *Xho*I (Fig. 20C). Based on the restriction analysis of the recombinant pAcYM1-EHDV1.S5 sequence, *Xho*I site was presented in pAcYM1 at 1900 bps and *Xma*I at 295 bps within the EHDV-1 S5 insert. The correct position of the targeted gene in pAcYM1-EHDV1.S5 was identified by gel electrophoresis for clone # 5 (Fig. 20D, lane 5) that produced the two predicted bands of 8354 bps and 2474 bps sizes. Whereas, the integration of the insert in the other three clones was in the wrong orientation, as seen in lanes 2, 3 and 4 of figure 21D (Fig. 20D, lanes 2-4).

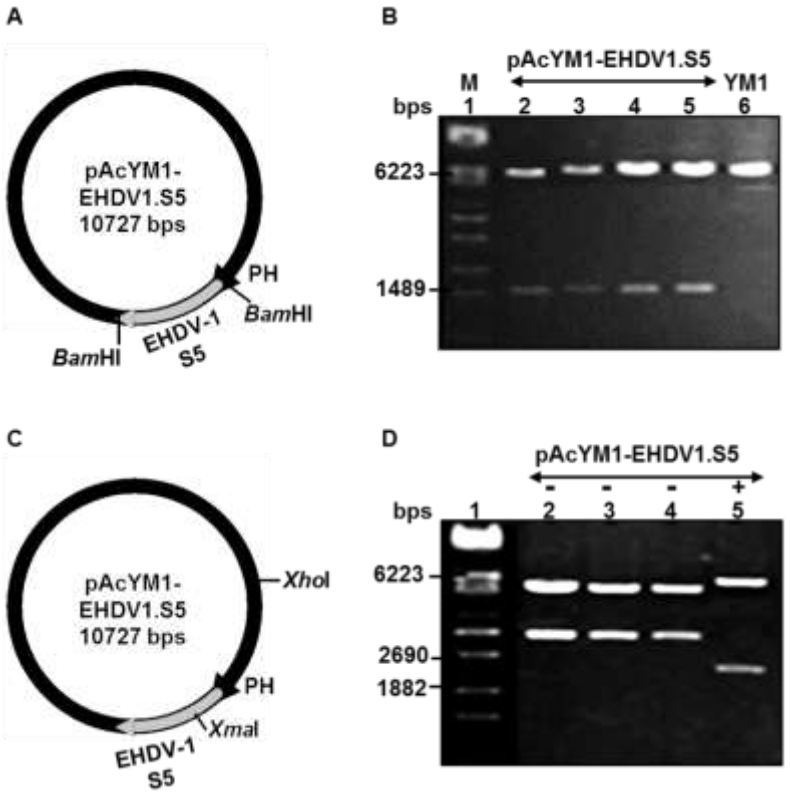

Fig. 20. Molecular analysis to detect the presence and the orientation of EHDV-1 S5 in the recombinant pAcYM1-EHDV1.S5. (A) Restriction map of pAcYM1-EHDV1.S5 showing *Bam*HI sites. (B) *Bam*HI digestion of the construct pAcYM1-EHDV1.S5. Lane 1: DNA ladder, lanes 2-5: pAcYM1-EHDV1.S5 and lane 6: pAcYM1 (*Bam*HI). Colonies # 2, 3, 4 and 5 are positive. (C) Restriction map of pAcYM1-EHDV1.S5 with *Xma*I and *Xho*I sites. (D) Digestion of pAcYM1-EHDV1.S5 with *Xma*I and *Xho*I. Lane 1: DNA ladder, lanes 2-5: pAcYM1-EHDV1.S5. Clone # 5 is in correct orientation.

3.2.1.4. Expression of EHDV-1 VP5 recombinant protein in baculovirus expression system

A recombinant baculovirus was generated by the cotransfection of *Sf*21 insect cells with the circular recombinant pAcYM1-EHDV1.S5 and the *Bsu*36I linearised BAC10:KO$_{1629}$. After the amplification of specific clones, the synthesis of recombinant EHDV-1 VP5 protein was analysed by 10% SDS-PAGE, stained with coomassie blue (Fig. 21A, lane 4) and also detected by immunoblotting. A band similar to the correct size of EHDV-1 VP5 protein was detected on a nitrocellulose membrane utilizing EHDV-1 polyclonal antibody (Fig. 21B, lane 4).

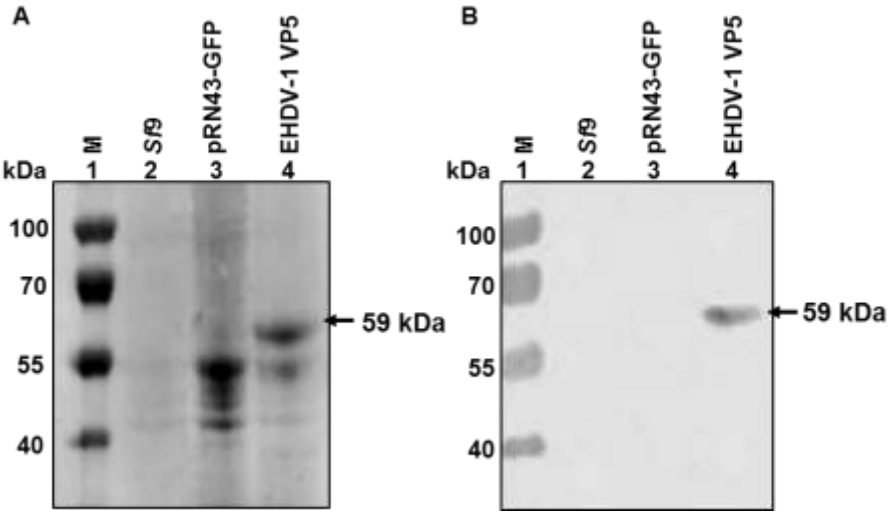

Fig. 21. Analysis of expression of recombinant EHDV-1 VP5 protein from clonal populations. (A) 10% SDS-PAGE analysis. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP5 (Black arrow). (B) Western blotting using EHDV-1 polyclonal antibody. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP5 (~59 kDa) (Black arrow).

3.2.1.5. Cloning of EHDV-1 S7 into pAcYM1

To clone EHDV-1 S7 into pAcYM1 transfer vector, the available plasmid construct pUC19-EHDV1.S7 containing the complete sequence of EHDV-1 S7 gene was used as a template. To amplify S7, the gene coding region was amplified by PCR using EHDV-1 S7 specific (F and R) primers (Table 7). The results of PCR amplification were examined by gel electrophoresis displaying a single band with the anticipated size of EHDV-1 S7 (1162 bps) (Fig. 22, lane 3). Similarly, an amplification reaction was performed with no template and run on 1% agarose gel electrophoresis as a negative control (Fig. 22, lane 2).

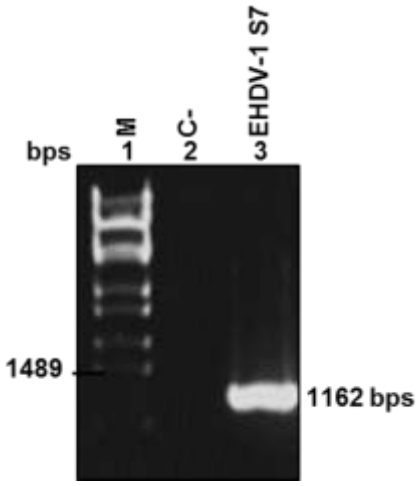

Fig. 22. PCR amplification of the coding region of EHDV-1 S7. The PCR product was visualised by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-1 S7.

The excised EHDV-1 S7 amplicon was ligated into pAcYM1 vector previously digested with *Bam*HI enzyme and dephosphorylated. After the transformation of the ligation mixture into bacterial competent cells, clones with the positive integration of the insert were examined by *Bam*HI restriction digestion (Fig. 23A). Positive plasmid containing the recombinant pAcYM1-EHDV1.S7 was visualised on agarose gel as two DNA fragments of the same size of pAcYM1 vector and EHDV-1 S7 gene (Fig. 23B, lane 3). The orientation of the integrated gene in the recombinant plasmid was determined by PCR using PH (F) and EHDV-1 S7 (R) primers. The results were visualised by agarose gel electrophoresis and a single specific fragment up to the exact size of EHDV-1 S7 (1162 bps) was shown as in Figure 24, lane 2 (Fig. 24, lane 2).

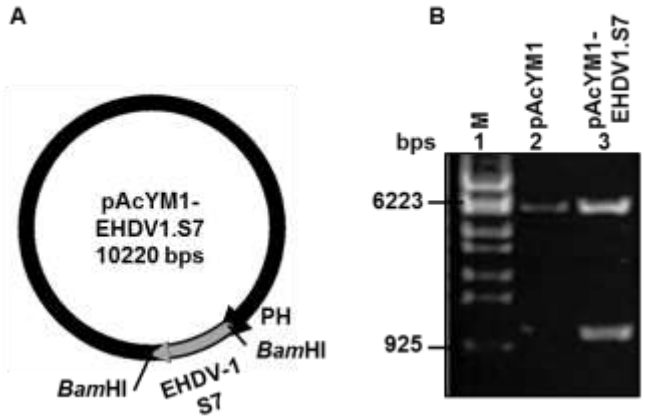

Fig. 23. Molecular analysis of the presence of EHDV-1 S7 in the construct pAcYM1-EHDV1.S7. (A) Restriction map of pAcYM1-EHDV1.S7. (B) *Bam*HI digestion of the recombinant pAcYM1-EHDV1.S7. Lane 1: DNA ladder, lane 2: pAcYM1-*Bam*HI and lane 3: the recombinant pAcYM1-EHDV1.S7.

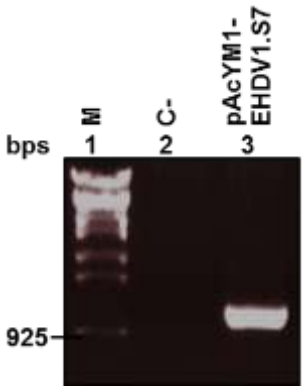

Fig. 24. Molecular analysis to determine the orientation of EHDV-1 S7 in the recombinant pAcYM1-EHDV1.S7 by PCR with PH (F) and EHDV-1 S7 (R) primers. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-1 S7 in the construct pAcYM1-EHDV1.S7.

3.2.1.6. Expression of EHDV-1 VP7 recombinant protein in baculovirus expression system

For individual synthesis of the outer layer core protein EHDV-1 VP7 from baculoviral clone, *Sf*21 insect cells were cotransfected with pAcYM1-EHDV1.S7 and the linear BAC10:KO_{1629}. After 3-4 days of incubation at 27°C, recombinant baculovirus BAC10:KO_{1629}:EHDV1.VP7 was generated and three plaques were isolated and amplified. To examine the expression of EHDV-1 VP7 recombinant protein, the total

cell extract was analysed on a 10% SDS-PAGE, wherein high level of the recombinant protein synthesis was observed in lane 3 (Fig. 25A, lane 3). As a negative control, uninfected *Sf*9 insect cell lysates were used (Fig. 25A, lane 2) which showed no specific band corresponding to the size of EHDV-1 VP7. The AcMNPV pRN43-GFP recombinant was used as a positive control showing a protein band corresponding to the size of baculovirus envelope gp64 protein (Fig. 25A, lane 4). Further confirmation was carried out by western blot using polyclonal antibody for EHDV-1. The results were visualised on a nitrocellulose membrane as a single band of size of 39 kDa correlating to the molecular weight of EHDV-1 VP7 (Fig. 25B, lane 3).

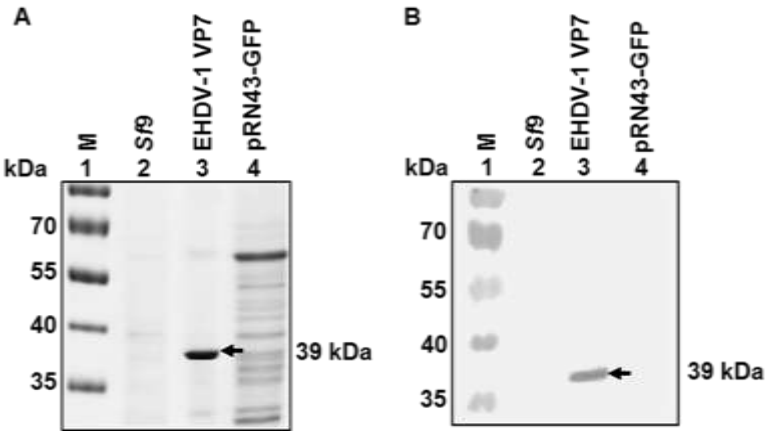

Fig. 25. Analysis of expression of recombinant EHDV-1 VP7 protein from clonal populations. (A) 10% SDS-PAGE analysis. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP7 (Black arrow) and lane 4: *Sf*9 cells insect infected with recombinant AcMNPV pRN43-GFP (B) Western blotting using EHDV-1 polyclonal antibody. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP7 (39 kDa) (Black arrow) and lane 4: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP.

3.2.1.7. Expression of EHDV-1 VP3 recombinant protein in baculovirus expression system

The recombinant pAcYM1-EHDV1.S3 plasmid was already available in the lab, thus the expression of the inner core protein VP3 was accomplished by cotransfection of

*Sf*21 insect cells with the linearised Bac10:KO_{1629} and the circular pAcYM1-EHDV1.S3. After 3 rounds of amplification of the individual clonal population, EHDV-1 VP3 expression was detected by both 10% SDS-PAGE gel, (Fig. 26A, lane 4) and western blot using polyclonal antibodies against EHDV-1 (Fig. 26B, lane 4). In both methods, two relatively close protein bands to the size of EHDV-1 VP3 recombinant were visualised. Additionally, no non-specific bands were detected in the negative and positive controls, corresponding to the size of EHDV-1 VP3 protein (Fig. 26A, lanes 2 and 3) (Fig. 26B, lanes 2 and 3).

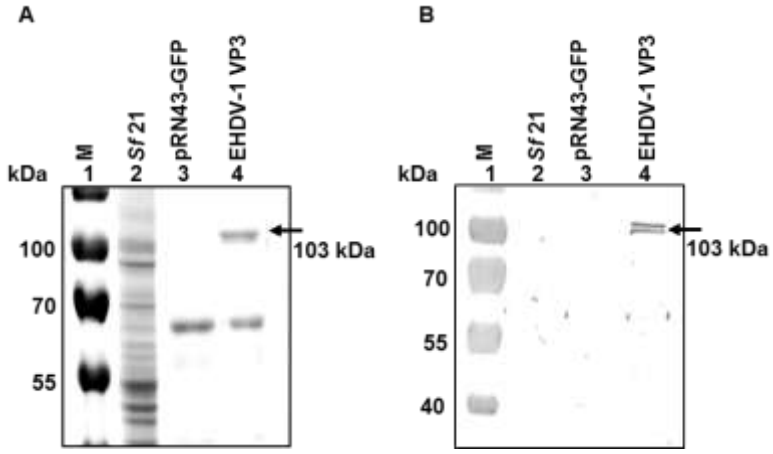

Fig. 26. Expression of recombinant EHDV-1 VP3 protein from clonal populations. (A) 10% SDS-PAGE analysis. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP3 (B) Western blotting using EHDV-1 polyclonal antibody. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP3 (103 kDa) (Black arrows).

3.2.2. Cloning and Expression of EHDV-2 outer capsid proteins

3.2.2.1. Cloning of EHDV-2 S2 and S5 into pAcYM1

The cDNAs of EHDV-2 S2 (3002 bps) and EHDV-2 S5 (1641 bps) provided from our collaborator's, Dr. Richt, Kansas State University (KSU), were cloned into the pGEM-T vector. Prior to subcloning the two genes into pAcYM1 transfer vector, both of S2

and S5 were sequenced to determine the absence of any unwarranted stop codons, insertions or deletions in the coding regions in comparison to the published sequence. Subsequently, to amplify the coding regions of EHDV-2 S2 and S5, the (F and R) primers were designed with *Sma*I and *Bam*HI restriction sites, respectively (Table 7). To improve the efficiency of ligation, particularly for EHDV-2 S2, primers were phosphorylated by adding a phosphate group to the 5' end using T4 PNK enzyme. After amplification, the PCR product containing the coding regions of EHDV-2 S2 and S5 were visualised on 1% agarose gel electrophoresis as single specific bands of sizes of EHDV-2 S2 (Fig. 27A, lane 3) and EHDV-2 S5 (Fig 27B, lane 3). No amplification was observed with negative controls (Fig. 27A, lane 2) and (Fig. 27B, lane 2).

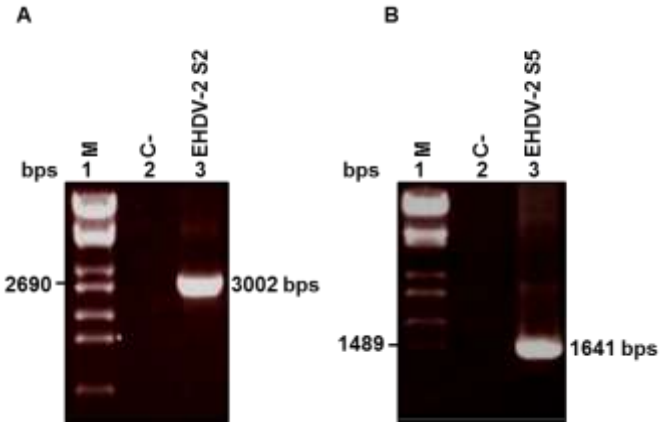

Fig. 27. PCR amplification of the coding region of EHDV-2 S2 and S5. The PCR product was visualised on a 1% agarose gel electrophoresis, stained with ethidium bromide. (A) Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-2 S2. (B) Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-2 S5.

The amplified genes were excised, purified from gel and ligated into pAcYM1 transfer vector. The ligation reactions were then transformed into the competent cells DH5α and plated onto ampicillin plates. To detect the presence of the inserts EHDV-2 S2 and S5 in the recombinants pAcYM1-EHDV2.S2 and pAcYM1-EHDV2.S5 plasmids, colony PCR using specific primers for each gene was performed. In total,

four ampicillin resistance colonies of pAcYM1-EHDV2.S2 and three of pAcYM1-EHDV2.S5 were picked and screened (Fig. 28, A and B). Accordingly, each of the selected colonies had the targeted genes EHDV-2 (S2 and S5) in the recombinants pAcYM1-EHDV2.S2 (Fig. 28A, lanes 4-7) and pAcYM1-EHDV2.S5 (Fig. 28B, lanes 2-4), respectively.

To detect the direction of EHDV-2 S2 in pAcYM1-EHDV2.S2, the recombinant plasmids were extracted, purified and digested with *Hind*III endonuclease enzyme. The *Hind*III enzyme has two restriction sites in the vector pAcYM1 at (5498 and 4457) bps and one in the insert EHDV-2 S2 at 2935 bps (Fig. 29A). For correct orientation, three fragments of sizes 10,600 bps, 1041 bps and 471 bps were expected. This was the case for pAcYM1-EHDV2.S2 clone # 4 (Fig. 29B, lane 7).

With respect to EHDV-2 S5, the orientation in the recombinant plasmid pAcYM1-EHDV1.S5 was determined by PCR using PH (F) primer from pAcYM1 and EHDV-2 S5 (R) primer (Fig. 30A). Defined DNA bands corresponding to the size of EHDV-1 S5 in the positive control pGEM-T-EHDV2.S5 (Fig. 30B, lane 3) were visualised in the three recombinant clones (Fig. 30B, lanes 4-6), while no fragment was observed in the negative control (Fig. 30B, lane 2).

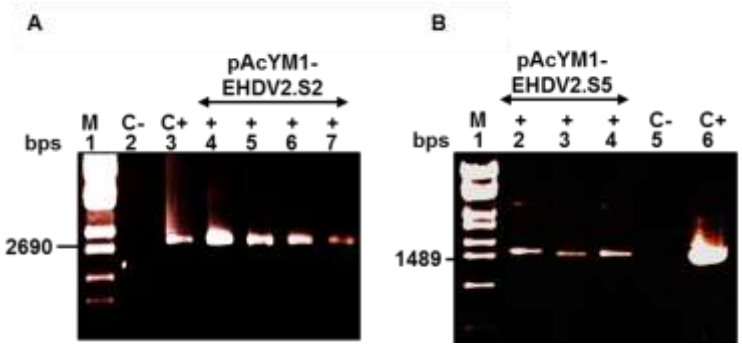

Fig. 28. Analysis of the presence of EHDV-2 S2 and S5 in the recombinants pAcYM1-EHDV2.S2 and pAcYM1-EHDV2.S5. (A) Colony PCR with EHDV-2 S2 (F&R) primers. Lane 1: DNA ladder, lane 2: negative control, lane 3: positive control (pGEMT-EHDV2.S2) and lanes (4-7) pAcYM1-EHDV2.S2 clones (All are positive). (B) Colony PCR with EHDV-2 S5 (F&R) primers. Lane 1: DNA ladder, lanes (2-4) pAcYM1-EHDV2.S5 clones (All are positive), lane 5: negative control and lane 6: positive control (pGEMT-EHDV2.S5).

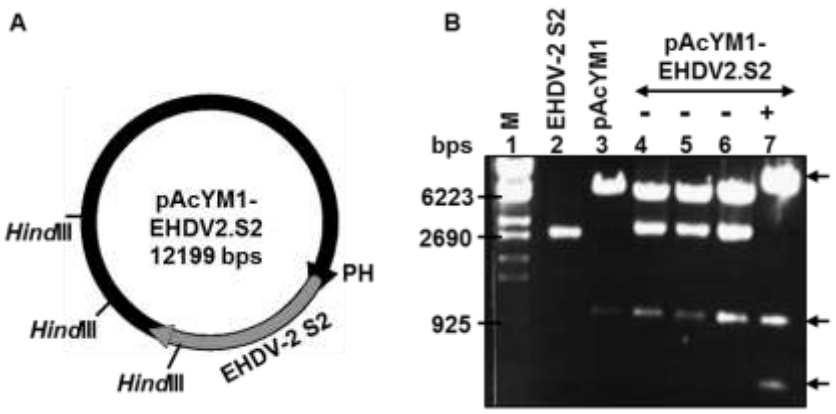

Fig. 29. Molecular analysis of the orientation of EHDV-2 S2 in the construct pAcYM1-EHDV2.S2. (A) Restriction map of the construct pAcYM1-EHDV2.S2 with *Hind*III site. (B) *Hind*III digestion of the recombinant pAcYM1-EHDV2.S2. Lane 1: DNA ladder, lane 2: EHDV-2 S2, lane 3: pAcYM1 and lanes (4-6) negative orientation of EHDV-2 S2 in the recombinant and lane 7: correct orientation of EHDV-2 S2 in pAcYM1-EHDV2.S2.

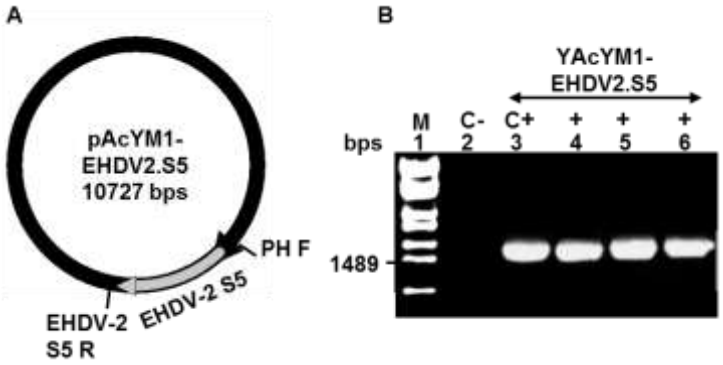

Fig. 30. Molecular analysis of the orientation of EHDV-2 S5 in the construct pAcYM1-EHDV2.S5. (A) Analysis map of the construct pAcYM1-EHDV2.S2 showing PH (F) and EHDV-2 S5 (R) primers. (B) Colony PCR of the recombinant pAcYM1-EHDV2.S5 with EHDV-2 S5 (R) and PH (F) primers. Lane 1: DNA ladder, lane 2: negative control, lane 3: pGEM-T-EHDV2.S5 positive control and lanes (3-6) pAcYM1-EHDV2.S5 recombinants (All are in correct orientation).

3.2.2.2. Single expression of the EHDV-2 VP2 and VP5 recombinant proteins in baculovirus expression system

To synthesise EHDV-2 VP2 and VP5 recombinant proteins, monolayers of *Sf*21 insect cells were cotransfected with the circular recombinants pAcYM1-EHDV2.S2 or pAcYM1-EHDV1.S5 and the *Bsu*36I linearised BAC10:$KO_{1629.}$ After the second

round of amplification, plaque assays were performed, stained with 10% neutral red and individual clones were isolated and amplified.

The successful expression of both EHDV-2 VP2 and VP5 recombinants were analysed on 10% SDS-PAGE followed by coomassie blue staining. Protein bands with expected sizes of EHDV-2 VP2 (~ 114 kDa) (Fig. 31A, lane 4) and EHDV-2 VP5 (~ 59 kDa) (Fig. 31B, lane 5) were observed equivalent to that of the positive controls EHDV-1 VP2 (Fig. 31A, lane 5) EHDV-1 VP5 (Fig. 31B, lane 4). The uninfected *Sf*9 insect cells were used as a negative control (Fig. 31A and B, lane 2), and the recombinant baculovirus AcMNPV pRN43-GFP was used as a positive control of the cotransfection efficiency (Fig. 31A and B, lane 3).

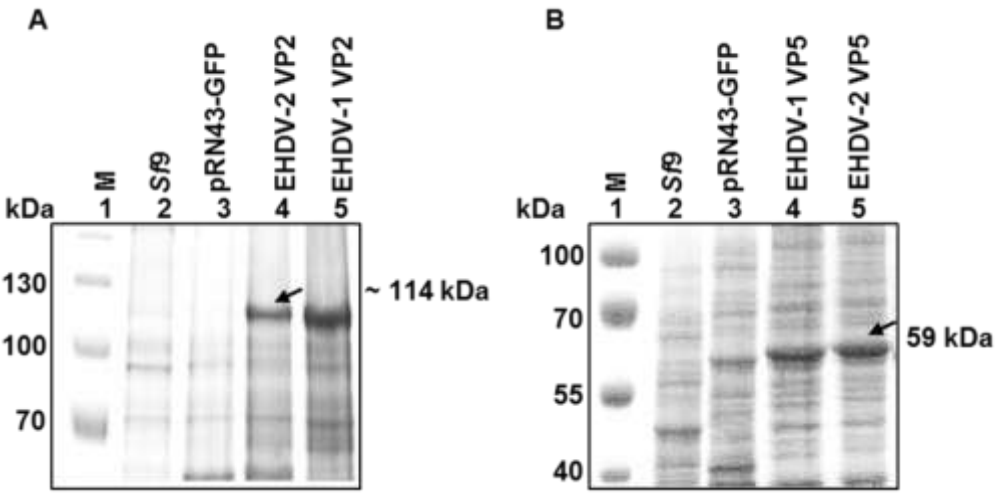

Fig. 31. 10% SDS-PAGE analysis of recombinant EHDV-2 VP2 and VP5 proteins from clonal populations. (A) Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP, lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-2 VP2 (black arrow) and lane 5: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP2 (Positive control). (B) Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP, lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP5 (Positive control) and lane 5: lysate from cells infected with recombinant baculovirus expressing EHDV-2 VP5 (black arrow).

3.2.3. Antibodies development against the recombinant protein VP2 of EHDV-1 and EHDV-2

Specific polyclonal antisera were commercially produced in Guinea Pigs for each of the recombinant proteins; EHDV-1 VP2 and EHDV-2 VP2. The specificity of each antibody was tested by blotting on a nitrocellulose membrane using the suitable secondary antibodies. Post colour development, a defined protein band corresponding to the size of EHDV-1 VP2 (~ 112 kDa) (Fig. 32A, lane 4) and EHDV-2 VP2 (~ 114 kDa) (Fig. 32B, lane 4) were detected. Negative (*Sf*9 insect cells) and positive (AcMNPV pRN43-GFP) controls gave no bands with the antisera resulting in non-specific reactivities, as shown in (Fig. 32A, lanes 2 and 3) and (Fig. 32B, lanes 2 and 3).

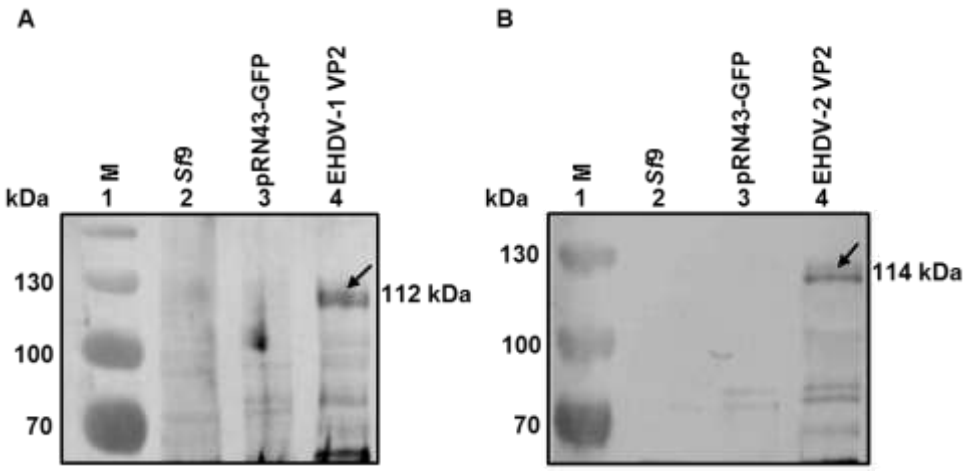

Fig. 32. Immunoblotting analysis of specificity of the polyclonal antibodies for the recombinant VP2 of EHDV-1 and EHDV-2. (A) Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP2 (112 kDa) (Black arrow) (B) Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 4: lysate from cells infected with recombinant baculovirus expressing EHDV-2 VP2 (114 kDa) (Black arrow).

3.2.4. Isolation of EHDV-7 outer capsid proteins

Prior to isolate viral genomic RNAs for cloning purpose, EHDV-7 virus was cloned to ensure the purity of the virus that was obtained from a laboratory of a collaborator.

Therefore, in order to isolate individual clones of EHDV-7 (strain CSIRO 775), plaque assay was performed in confluent BSR monolayers. After staining with 10% neutral red, five plaques were selected and the clonal populations were amplified. The virus cultures harvested from the infection with plaques # 1 and 2, were used to extract dsRNA. Following extraction, the dsRNA were purified as described in Materials and Methods, and analysed on 0.8% agarose gel electrophoresis in order to visualise the profile of the ten segments (Fig. 33, lanes 3 and 4). As a positive control, dsRNA of EHDV-2 previously purified were used. As in Figure 33, lane 2, different mobilities were seen for S2, S5, S6 and S10 (Fig. 33, lane 2).

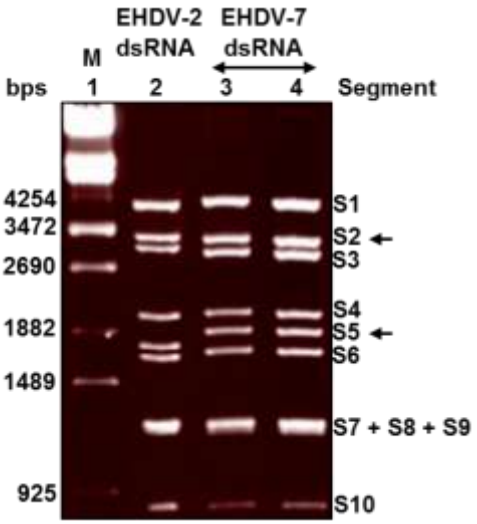

Fig. 33. Agarose gel electrophoresis analysis of the segmented EHDV-7 dsRNA genome on 0.8% agarose gel electrophoresis. Lane 1: DNA ladder, lane 2: EHDV-2 dsRNA (control positive) and lanes 3 and 4: EHDV-7 dsRNA isolated from individual plaque (Lane 3: plaque # 1 and Lane 4: plaque # 2).

To ligate EHDV-7 S2 (3002 bps) (Fig. 33, lane 4) (upper black arrow) into pAcYM1 transfer vector, the dsRNA band was excised from gel and purified as described in Materials and Methods. For subsequent amplification of the coding region of S2 gene, specific (F and R) primers with *Nru*I endonuclease enzyme were designed

(Table 7). The EHDV-7 S2 amplification was detected by electrophoresis on 1% agarose gel; a single band of size of EHDV-7 S2 (3002 bps) was detected (Fig. 34A, lanes 3 and 4). The PCR reaction with no DNA template was used as negative control and gave no amplification (Fig. 34A, lane 2).

To generate a baculovirus multiple gene expression vector containing EHDV-7 genes, EHDV-7 S5 (1769 bps) (Fig. 33, lane 4) (lower black arrow) was purified from gel and the (F and R) primers were designed with *Bam*HI restriction sites. The PCR product of EHDV-7 S5 was observed by electrophoresis on agarose gel (Fig. 34B, lanes 3 and 4) displaying DNA fragment with the estimated length (1769 bps) of EHDV-7 S5. No amplification was identified from the negative control as shown in lane 2 (Fig. 34B, lane 2).

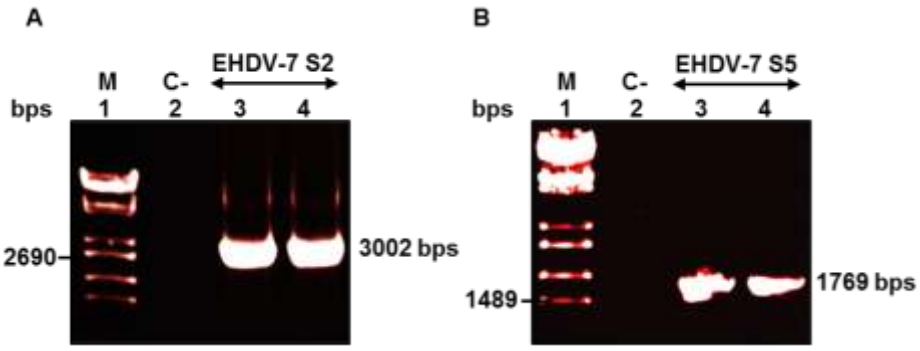

Fig. 34. PCR amplification of the coding region of EHDV-7 S2 and S5. (A) The PCR product was visualised by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide. Lane 1: DNA ladder, lane 2: negative control and lanes 3 and 4: EHDV-7 S2. (B) The PCR product was visualised by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide. Lane 1: DNA ladder, lane 2: negative control and lanes 3 and 4: EHDV-7 S5.

3.2.4.1. Cloning of EHDV-7 S2 into pAcYM1

The amplified coding regions of EHDV-7 S2 was ethanol precipitated followed by ligating into the *Sma*I site of the linearised and dephosphorylated pAcYM1 (Fig. 35, lane 3). After transformation into DH5α competent cells, the integration and orientation of EHDV-7 S2 in the recombinant pAcYM1-EHDV7.S2 plasmid were

analysed simultaneously. This was performed by colony PCR on seven ampicillin resistance colonies using PH (F) primer and EHDV-7 S2 (R) primer. The amplified products were detected by electrophoresis on 1% agarose gel, demonstrating a positive integration and accurate direction of EHDV-7 S2 in the recombinant pAcYM1-EHDV7.S2 in clone # 3 (Fig. 36, lane 4), whereas other 6 colonies were found to be negative (Fig. 36, lanes 2, 3, 5, 6, 7 and 8).

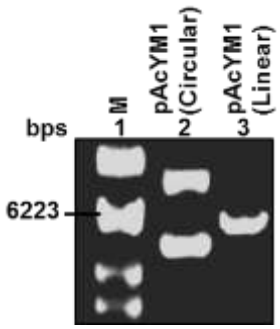

Fig. 35. Molecular analysis of the transfer vector pAcYM1. Lane 1: DNA ladder, lane 2: pAcYM1 (circular) and lane 3: *Sma*I linearised pAcYM1.

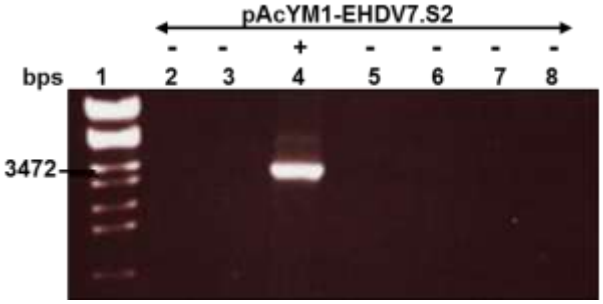

Fig. 36. Colony PCR with PH (F) and EHDV-7 S2 (R) primers to check the presence and the orientation of EHDV-7 S2 in the recombinant pAcYM1-EHDV7.S2. Lane 1: DNA ladder and lanes (2-8) pAcYM1-EHDV2.S2 recombinants (lane 4: is positive).

3.2.4.2. Expression of EHDV-7 VP2 recombinant protein in baculovirus expression system

The recombinant EHDV-7 VP2 protein expressed well in *Sf*21 insect cells post cotransfection with the linearised BAC10:KO_{1629} and the circular recombinant

pAcYM1-EHDV7.S2. To analyse the synthesis of EHDV-7 VP2 protein, cell lysate of *Sf*9 infected cells with the recombinant baculovirus expressing the above protein was visualized on 10% SDS-PAGE gel stained with coomassie blue. Intense protein bands were observed corresponding to the estimated sizes of EHDV-7 VP2 (~ 114 kDa) (Fig. 37, lanes 4 and 5), and to that of the positive controls EHDV-1 VP2 (112 kDa) (Fig. 37, lane 7) and EHDV-2 VP2 (~ 114 kDa) (Fig. 37, lane 6).

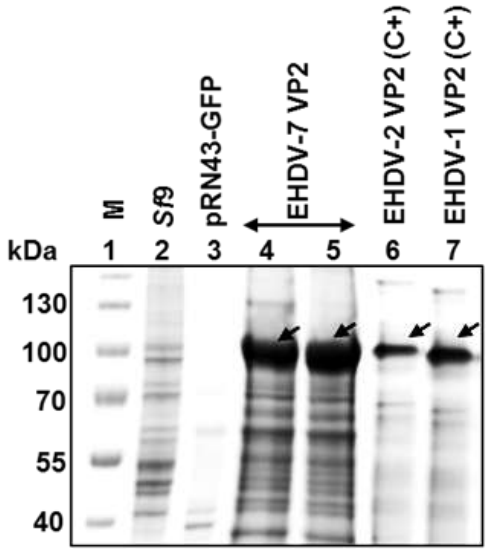

Fig. 37. 10% SDS-PAGE Analysis of the recombinant EHDV-7 VP2 protein from clonal populations. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP, lanes 4 and 5: lysate from cells infected with recombinant baculovirus expressing EHDV-7 VP2, lane 6: lysate from cells infected with recombinant baculovirus expressing EHDV-2 VP2 (control positive) and lane 7: lysate from cells infected with recombinant baculovirus expressing EHDV-1 VP2 (control positive). All recombinant proteins are indicatied in black arrows.

In conclusion, in this chapter it was possible to achieve all the clones of EHDV-1 (S2, S5 and S7), EHDV-2 (S2 and S5) and EHDV-7 (S2 and S5), that are necessary for the formation of VLPs. All clones were also shown to be able to express in baculovirus expression system. Moreover, antisera against the most variable protein VP2 of EHDV-1 and EHDV-2 were commercially raised in Guinea Pigs and confirmed to be specific by western blotting.

3.3. DISCUSSION

Currently, the only vaccine available to control EHD caused by EHDV is a live-attenuated vaccine (Aradaib et al., 2009). This vaccine was developed and used in Japan against EHDV-2 (IBAV) following outbreaks that occurred in 1980, during which it was demonstrated to be effective and safe, at least until 1997, due to the emergence of more virulent serotypes (Ohashi et al., 1999). Thus, to combat EHD, the development of a safer and more effective vaccine for EHDV serotypes is economically important, particularly, following the outbreaks in cattle in different countries bordering to Europe. However, generating such universal vaccine is hampered by the divergent and immunologically distinct seven serotypes for EHDV.

As a safe type of subunit vaccine, the aim of this study was to develop VLPs of EHDV. The choice of VLP as a viable vaccine was based on several studies showing a complete protective neutralising antibody response in vaccinated animals with BTV VLPs (Roy, 1995a), that proved to be closely related to EHDV both genetically and structurally (French et al., 1990, Ritter and Roy, 1988). However, the production of VLPs containing multiple interacting capsid proteins is more challenging in comparison to those that are formed by one ‘papillomavirus’ or two ‘influenza virus’ major capsid proteins, as more qualifications are needed.

As a preliminary step for the production of EHDV VLPs and heterologous VLPs, it was essential to determine the possibility of an individual expression of each structural protein VP2, VP5, VP7 and VP3 of EHDV serotypes 1, 2 and 7 using baculovirus vector BAC10:KO$_{1629}$. The plasmid replicon, pAcYM1, containing AcMNPV *polyh* promoter, was utilized to generate recombinant viruses that express high level products of the targeted genes. This was based on previous studies of many genes, for which high expression was obtained with a recombinant baculovirus

using the pAcYM1 transfer vector (Matsuura et al., 1987, Romanowski et al., 1985, Emery and Bishop, 1987, Urakawa and Roy, 1988).

No published studies are available showing the cloning of the three EHDV structural genes in to pAcYM1 apart from EHDV-1 S3 (2768 bps) (Le Blois et al., 1991). However, other work demonstrated the cloning of EHDV-1 S7 gene into pUC plasmids (pUC8 or pUC19) (Iwata et al., 1992), and EHDV-2 S5 into pGEM-T Easy (3015 bps) transfer vector (Ohashi et al., 2002). Cloning the targeted genes into pUC or pGEM-T Easy vectors is not as difficult as to that of pAcYM1 due to their small sizes. In this work, after several attempts, a successful subcloning of the full-length ORF of EHDV-1 S5 (1803 bps), EHDV-1 S7 (1162 bps) and EHDV-2 S5 (1803 bps) were achieved into pAcYM1 vector.

Cloning S2 coding region of EHDV-1 and EHDV-2 serotypes into pAcYM1 was not so straightforward technique. S2 has large molecular weight, making it more difficult to manage and harder to clone. Moreover, the ligation of S2 ORF of the three EHDV serotypes into pAcYM1 was not previously described for this vector, although there was report of cloning the coding region of Ibaraki (EHDV-2) S2 into pGEM-T Easy vector (Ohashi et al., 2002). Additionally, cloning of EHDV-7 S2 into pAcYM1 and EHDV-7 S5 into pRN296 vector was very difficult as the FLAC method was not suitable to amplify cDNAs from dsRNAs of both genes.

To overcome these challenges, several molecular techniques were performed. The 5’ and 3’ termini of the amplified S2 PCR product was phosphorylated using T4 DNA polynucleotide or digested with specific endonuclease enzymes presented in the designed primers, however both were inefficient. Thus, alternative method was used to prepare the insert for ligation. The (F and R) primers of S2 of EHDV-1, EHDV-2 and EHDV-7 were phosphorylated prior to the PCR reaction which proved to be

successful for ligation. Furthermore, chemically competent *E.coli* cells suitable for high efficiency transformation were prepared and used following the protocol by *Tang et al* (Tang et al., 1994).

Due to the nature of sticky ends of the PCR product, it could be inserted into a plasmid in either of two orientations. Thus, determining the orientation of the targeted gene in the recombinant was rather essential, as the wrong direction would lead to a non-functional protein. This issue was overcome by applying a number of molecular methods. Firstly, single restriction endonuclease digestion was performed for EHDV-1 S2 in pAcYM1-EHDV1.S2 with *EcoR*V, while *Hind*III used for EHDV-2 S2 pAcYM1-EHDV1.S2. Double restriction digestion with *Xma*I and *Xho*I was carried out for EHDV-1 S5 in pAcYM1-EHDV1.S5. Secondly, PCR as an alternative strategy was undertaken using PH (F) primer from the vector pAcYM1 and specific (R) primer from the insert. Thirdly, colony PCR as a rapid and convenient high-throughput method was applied for analysing the presence or absence of the DNA insert in the recombinants and determining its orientation.

Post transformation of the recombinant plasmid pAcYM1-EHDV1.S2, pAcYM1-EHDV-2.S2 into DH5α competent cells, small and large colonies were developed on ampicillin plates; however, only small colonies proved to be potentially correct. Due to the slow growth rate, small colonies developed after 2 days, which also allowed satellite colonies to develop. The DNA yield was extremely low from a correct colony when was scaled up for maxi-preparation. Based on these observations, it was likely that the insert S2 was toxic to the bacterial cells resulting in the instability of the vector. In addition, the ligation of S2 ORF of the three EHDV serotypes into pAcYM1 was not previously described for this vector, although there was report of cloning the coding region of Ibaraki (EHDV-2) S2 into pGEM-T Easy vector (Ohashi et al., 2002).

Prior to protein expression, examining the full-length sequence of each insert was necessary for the production of VLPs and heterologous VLPs. The results were verified by BlastN and shown to be corresponding to the published sequence of EHDV structural coding regions. After alignment with PubMed published data, no deletions, insertions or frame shifts were revealed.

The individual expression of the recombinant proteins of EHDV-1 VP2, VP3, VP5 and VP7, EHDV-2 VP2 and VP5 and EHDV-7 VP2 were successfully achieved in *Sf*21 insect cells using baculovirus expression system. None of the expressed proteins was previously reported either in baculovirus or in yeast or *E.coli* except for EHDV-1 VP3, for the formation of heterologous CLPs which made up of EHDV-1 VP3 and BTV-10 VP7 (Le Blois et al., 1991). The expression of VP2 protein of EHDV serotypes was quite difficult not just due to its large size (over 112 kDa) but also maybe due to the sequences of the genes. For instance, VP3 also has large size (103 kDa), but was shown to be amenable to expression by baculovirus both for EHDV and BTV (Le Blois et al., 1991, French and Roy, 1990). Therefore, in this work, the expression of VP2 of EHDV serotypes was not only possible by baculovirus expression system, but also proved to be highly efficient.

In this project, the modified DNA baculovirus BAC10:KO_{1629} was used for recombination due to variant distinct factors. Basically, BAC10:KO_{1629} has an insertion in the *orf*1629, which is essential for its replication. Such knockout in the viral genome prevents completion of baculovirus replication unless complemented by recombination from a transfer vector. Other studies showed that applying this modification, the recombination protocols were shortened and made all recovered viruses as recombinants and thus allowing high throughput protein expression strategies (Zhao et al., 2003, Kitts and Possee, 1993). Moreover, work by *Kitts and*

Possee introduced a *Bsu*36I restriction enzyme in the wild type (Hawtin et al., 1997) Baculovirus DNA generating a unique site for linearisation. This point mutation reduced the background of the WT baculovirus in comparison to the recombinant ones (Kitts and Possee, 1993). However in this case it does not matter about background virus as the Bacmid has a knockout in *orf*1629, linearization in our protocol is performed as it has been shown to improve recombination efficiency between Bacmid and transfer vector (Zhao et al 2003).

To examine the successful recombination and the level of recombinant protein produced, the construct pRN43GFP was used as a positive control. The GFP protein has been reported to be successfully expressed in different host organisms and cells such as *Sf*21 and *Sf*9 cells (Reilander et al., 1996). Furthermore, unlike other reporter tags such as luciferase, β-galactosidase, or fluorescent-tagged antibodies, GFP does not require fixation techniques that are toxic to the cells under investigation (Chalfie et al., 1994). The expression of GFP was monitored 48 and 72 hpt which was correlated with the expression of the very late genes of baculovirus coding for products including polyhedrin protein.

For single gene expression, the recombinant baculoviruses; BAC10:KO_{1629}:EHDV1.(S1, S5, S7), BAC10:KO_{1629}:EHDV2.(S2, S5) and BAC10:KO_{1629}:EHDV7.S2 were successfully generated in *Sf*21 insect cells. Plaque assays were performed from the first or second rounds of amplification to purify clonal population of the generated recombinant baculoviruses, which correlated with the expressed recombinant proteins. For instance, no plaques were visualised after the first amplification, particularly for VP2 of EHDV-1, -2 and 7 and VP5 of EHDV-1 and 2. Therefore, the need for higher number of amplifications were required. Such an outcome might not be solely due to the size of these proteins, but may also be

due to the sequence and the function of the proteins. For example, VP5 (~ 59 kDa) size is less than half of VP2 (~ 112 kDa). However, it has an essential function that allows the protein to cause permeabilisation in host cell membrane (Forzan et al., 2004). Such function tends to slow cell growth rate, reduce cell density, and kill the host cells in some cases. In contrast, clear plaques post neutral red staining were observed using baculovirus collected from the second transfection of *Sf*21 insect cells with, EHDV-1 (VP7 and VP3) which were more stable and easier to be expressed.

The positive control GFP was also detected for protein expression as an indication to the changes of the viral titre. Generally, more GFP was recognised at 48 hpi indicating that the time required to increase the expression of the protein might be decreased with each round of amplification. This was consistent with *Pennock GD* findings as remarkable high levels of the foreign gene (β-galactosidase) were achieved at 48 hpi (Pennock et al., 1984).

Successful expression of the outer and inner capsid proteins of EHDV three serotypes was achieved. Generally, no significant variations of the expressed proteins were found between the three serotypes, though VP2 expression was more difficult compared to the other structural proteins (VP3, VP5 and VP7). The high specificity of antisera raised against VP2 of EHDV-1 and EHDV-2 was confirmed to be high by western blotting.

Singly EHDV structural protein expression is well tolerated in the system chosen and indicates that multigene expression may well be successful, allowing this study to be taken further.

Chapter 4

The use of multiple genes expression vector to generate VLPs of EHDV-1

4.1. INTRODUCTION

In North America, EHDV-1 (New Jersey strain) was found to be the causative agent of a highly fatal disease in wild deer, which known to be endemic (Shope et al., 1960). The significant morbidity and mortality, particularly, in white tailed deer, was of concern of wildlife managers because of clinical disease in populations. Additionally, the pathological signs and clinical lesions produced by EHDV, are indistinguishable from that formed by BTV, making these viral diseases of interest to veterinary pathologists (Wilson, 1994). Therefore, in this study, the main aim was to develop EHDV-1 VLPs, which would be used as a candidate vaccine against the virulent virus.

Several VLPs for orbiviruses, BTV in particular, have been generated successfully by baculovirus expression system (French et al., 1990). The VLPs for BTV-10 were shown to elicit strong neutralising antibodies in guinea pigs (French et al., 1990). Following this success, VLPs were developed for a number of BTV serotypes; -1, -2, -13 and 17 and heterologous VLPs of BTV-4, -11 and 16 (Stewart el al., 2010, Stewart et al., 2012). Further, high level of protection was demonstrated in VLP-vaccinated animals against virulent virus challenges (Roy et al., 1994).

Baculovirus multiple gene expression system, with some further improvement, was mainly used for the generation of EHDV-1 VLPs. In previous reports, the simultaneous expression of multiple gene products has enabled efficient production of protein complexes including VLPs (Roy and Noad, 2009). To date, several drawbacks have affected the expression of multiple large proteins (>100 kDa), such as the need to produce large recombinant plasmids, repeat use of promoters and homologous sequences. These issues were addressed by the production of

baculovirus multiple gene expression system, which allowed the integration of up to 8 genes at different loci into baculovirus genome (Noad et al., 2009).

4.2. RESULTS

4.2.1. The use of baculovirus multiple genes expression system to develop EHDV-1 CLPs

4.2.1.1. Generation of the recombinant Bacmid$_{1629}$:EHDV1.S7

The recombinant plasmid pRN260-EHDV1.S7 was constructed by cloning the ORF of EHDV-1 S7 (1162 bps) into pRN260 vector under *polyh* promoter control. The linearisation of pRN260 plasmid was confirmed by electrophoresis on 1% agarose gel (Fig. 38, lane 3). As shown in Figure 39, lanes 4 and 6, positive insertion was detected in two ampicillin resistance colonies ; two bands of estimated sizes of EHDV-1 S7 (1162 bps) and the vector pRN260 (5500 bps) were obtained upon *Bam*HI digestion of the corresponding plasmid DNA (Fig. 39B, lanes 4 and 6).

The orientation of EHDV-1 S7 in recombinant pRN260-EHDV1.S7 plasmid (Fig. 40A), was determined by restriction digestion with *Nar*I endonuclease enzyme. A correct orientation was identified in one of the recombinant clones as evident from the presence of two DNA fragments corresponding to the sizes of 5593 bps and 1990 bps on agarose gel (Fig. 40B, lane 3). Further confirmation of EHDV-1 S7 direction in the recombinant plasmid was carried out by PCR using PH (F) and EHDV-1 S7 (R) primers. The PCR product was visualised by gel electrophoresis as a single amplified fragment corresponding to the size of EHDV-1 S7 (Fig. 40C, lane 3).

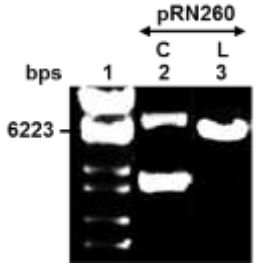

Fig. 38. ***Bam*HI digestion of pRN260 transfer vector.** Lane 1: DNA ladder, lane 2: pRN260 (circular) and lane 3: pRN260 (linear).

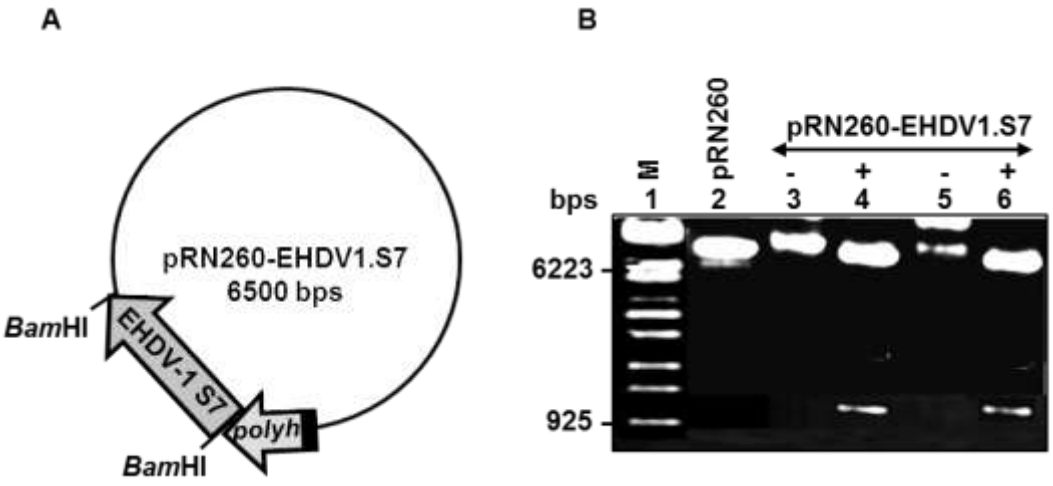

Fig. 39. Molecular analysis to detect the presence of EHDV-1 S7 in the recombinant pRN260-EHDV1.S7. (A) Restriction map of pRN260-EHDV1.S7 showing *Bam*HI sites (B) *Bam*HI digestion of the construct pRN260-EHDV1.S7. Lane 1: DNA ladder, lane 2: pRN260 and lanes 3-6: pRN260-EHDV1.S7. Colonies # 4 and 6 are positives.

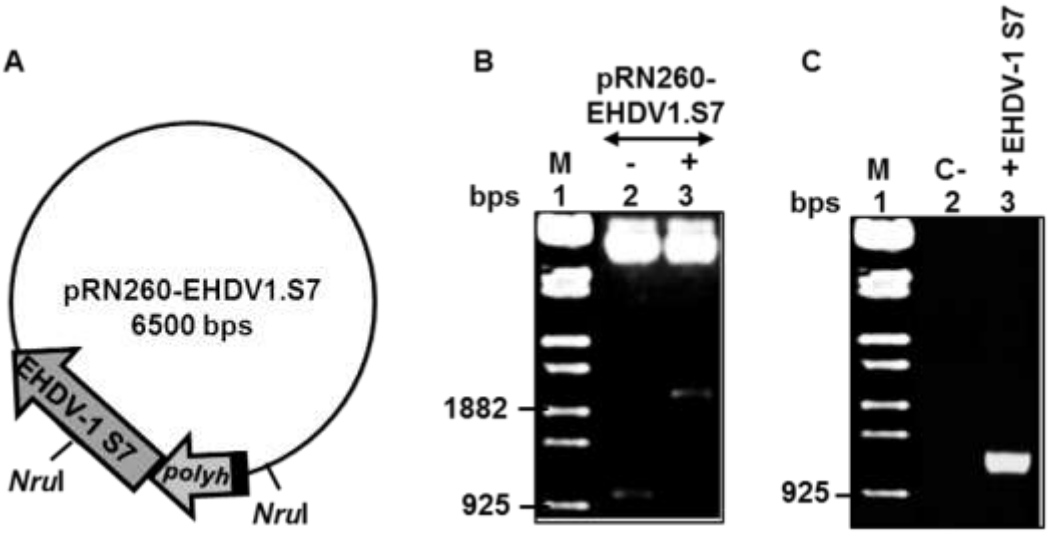

Fig. 40. Molecular analysis to detect the orientation of EHDV-1 S7 in the recombinant pRN260-EHDV1.S7. (A) Restriction map of pRN260-EHDV1.S7 with *Nru*I (B) *Nru*I digestion of pRN260-EHDV1.S7. Lane 1: DNA ladder, lane 2 and 3: pRN260-EHDV1.S7. Clone # 3 is in correct orientation (C) PCR with PH (F) and EHDV-1 S7 (R) primers. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-1 S7 in the construct pRN260-EHDV1.S7.

For lambda red recombination of the cloning cassette in the recombinant plasmid pRN260-EHDV1.S7, each of the selectable and expression markers were amplified using specific primers pRN260-Exact (F and R) (Table 10) (Fig. 41A). The amplified

PCR product was observed after electrophoresis on 1% agarose gel, showing one band corresponding to the size of 3472 bps (Fig. 41B, lane 2).

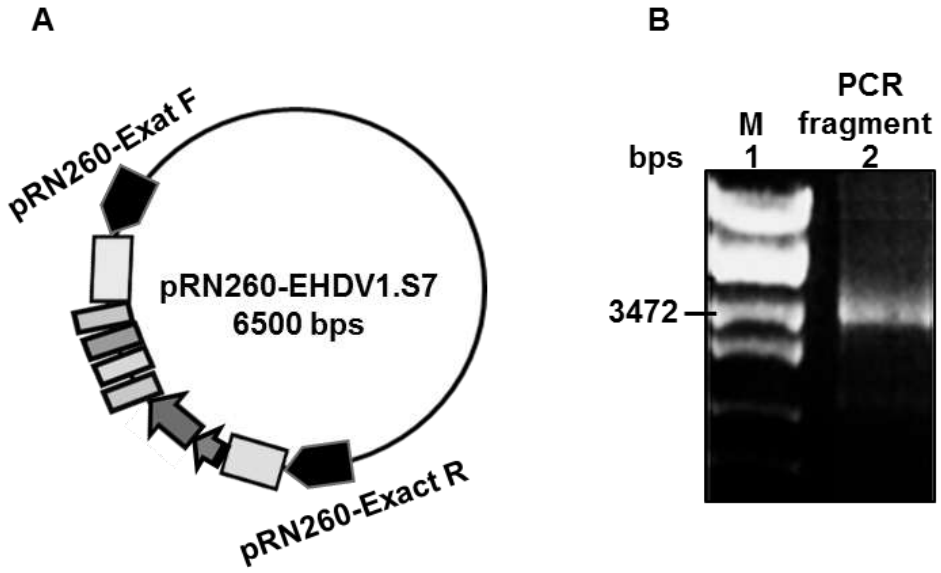

Fig. 41. Amplification of the selectable and expression cassettes flanked by AcMNPV sequences. (A) pRN260-EHDV1.S7 map showing pRN260-Exact (F and R) primers. (B) PCR amplification with pRN260-Exact (F and R) primers. Lane 1: DNA ladder and lane 2: PCR product.

After excising and purifying the PCR fragment from agarose gel, it was electroporated into the E3K2 bacterial competent cells, to be integrated into the baculovirus genome at *egt* locus. To detect the construction of the recombinant Bacmid$_{1629}$:EHDV1.S7, several blue colonies were screened with EHDV-1 S7 specific primers (Fig. 42A). Subsequently, the positive insertion was observed by 1 % agarose gel as one band corresponding to the size of EHDV-1 S7 (Fig. 42B, lane 4). No amplification was obtained from Bacmid$_{1629}$ (WT), which was used as a negative control (Fig 42, lane 2). The recombinant plasmid pRN260-EHDV1.S7 was used as a positive control as shown in lane 3 (Fig. 42B, lane 3).

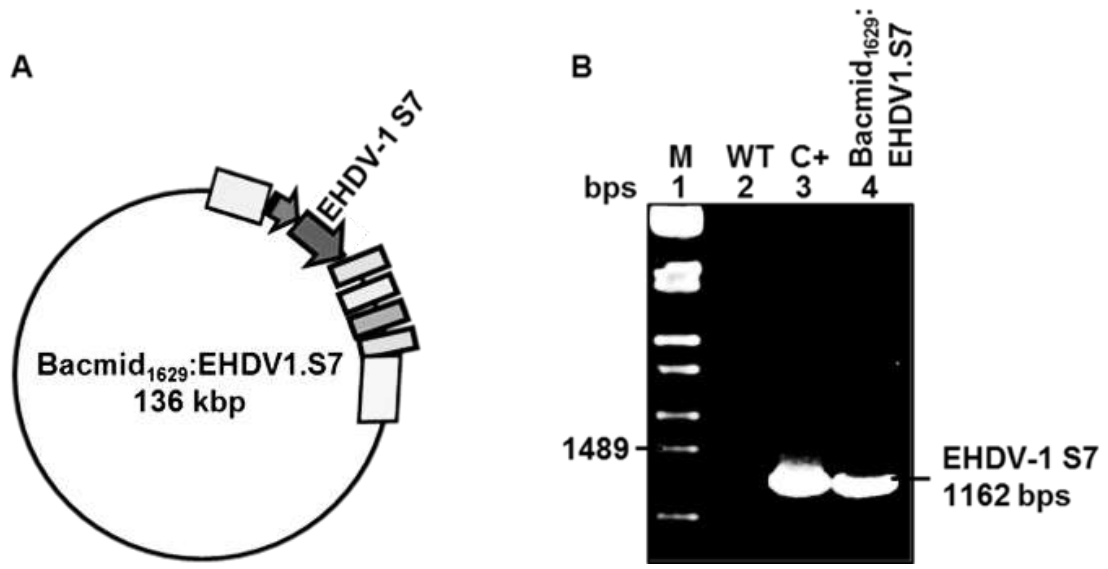

Fig. 42. Molecular analysis to detect the presence of EHDV-1 S7 in the Bacmid$_{1629}$:EHDV1.S7. (A) Analysis map of the Bcamid$_{1629}$:EHDV1.S7 in E3K2 cells showing the presence of the expression, selectable cassettes and specific AcMNPV sequence for homologous recombination. (B) PCR amplification of EHDV-1 S7 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: EHDV-1 S7 in the recombinant pRN260-EHDV1.S7 (positive control) and lane 4: EHDV-1 S7 in the recombinant Bacmid$_{1629}$: EHDV1.S7.

For the incorporation of the next gene encoding for the second inner core protein EHDV-1 VP3 into the recombinant Bacmid$_{1629}$:EHDV1.S7, the selectable marker needed to be flipped out (Fig. 43A). In order to confirm the presence of the EHDV-1 S7 gene in the recombinant Bacmid$_{1629}$:EHDV1.S7 after flipping out the selectable marker, several white colonies were screened. The results in Figure 43, lanes 4-6 (Fig. 43, lanes 4-6) show a single DNA fragment similar to the size of the positive control EHDV-1 S7 in the recombinant plasmid pRN260:EHDV1.S7 (Fig. 43, lane 3). No signs of amplification similar to that of EHDV-1 S7 were detected in lane 2 in which Bacmid$_{1629}$ was used as negative control (Fig. 43B, lane 2).

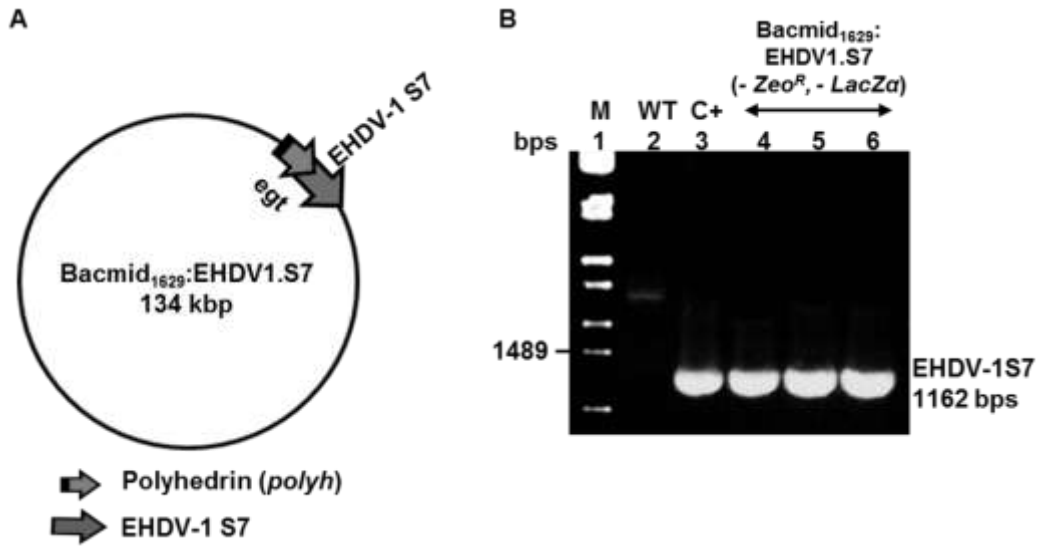

Fig. 43. Molecular analysis to determine the presence of EHDV-1 S7 after flipping out the selectable marker. (A) Analysis map showing the recombinant Bacmid$_{1629}$:EHDV1.S7 at *egt* locus after flipping out the selectable cassette. (B) PCR amplification of EHDV-1 S7 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: EHDV-1 S7 (positive control) and lanes 4-6: EHDV-1 S7 in the recombinant Bacmid$_{1629}$: EHDV1.S7.

4.2.1.2. Generation of the recombinant Bacmid$_{1629}$:EHDV1.S7.S3

To integrate EHDV-1 S3 (2768 bps) gene into the recombinant Bacmid$_{1629}$:EHDV1.S7 at *39k* locus, EHDV-1 S3 ORF was successfully subcloned into the pRN306 transfer vector. For fast and directional ligation, primers were designed with specific 15 bps sequences overlapping with the vector at its ends (Table 7). The positive ligation was examined by colony PCR of several colonies using EHDV-1 S3 primers. In total, three recombinant plasmids had the insert in correct direction to the *polyh* (Fig. 44, lanes 3-5), and one colony was shown to be negative (Fig. 44. lane 2).

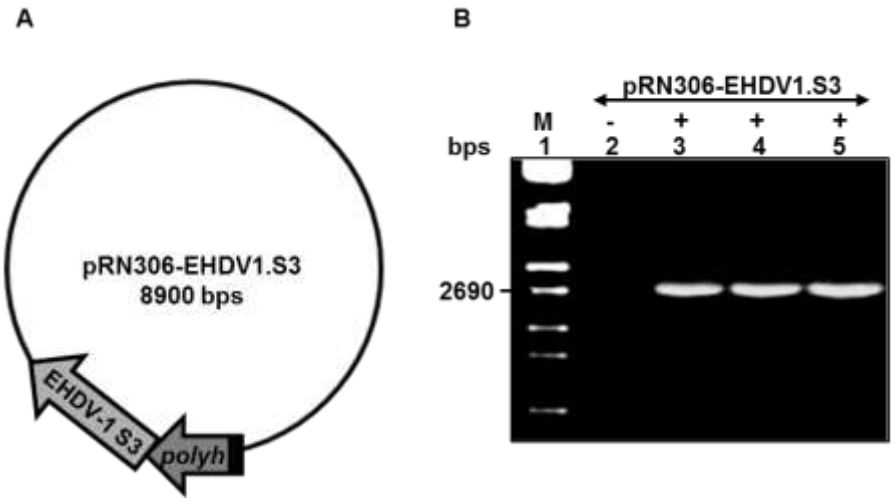

Fig. 44. Molecular analysis to detect the presence of EHDV-1 S3 in the recombinant pRN306-EHDV1.S3. (A) Restriction map of pRN306-EHDV1.S3 (B) Colony PCR using EHDV-1 S3 (F and R) specific primers. Lane 1: DNA ladder, lane 2-5: pRN306-EHDV1.S3. Colony # 2 is negative. Colonies # 3, 4 and 5 are positives.

For the generation of the recombinant plasmid pRN306-EHDV1.S3, pRN306-ec (F and R) primers (Table 10), were used to amplify both the selectable and expression cassettes (Fig. 45A). Amplification results were visualised by agarose gel electrophoresis as specific band of the size of ~4000 bps (Fig. 45B, lanes 3 and 4). Following excision and purification of the PCR product, the amplified fragment was electroporated into E3K2 electrocompetent cells containing the recombinant Bacmid$_{1629}$:EHDV1.S7. (Fig. 46A). The presence of targeted gene EHDV-1 S3 in the recombinant Bacmid$_{1629}$:EHDV1.S7.S3 was identified by screening 11 blue colonies. Gel electrophoresis displayed a total of 5 positive colonies (Fig. 46B, lanes 3, 4, 6, 7 and 8), and the others were negative (Fig. 46B, lanes 5, 9-13).

The resistant marker was flipped out effectively and PCR reaction was performed to test the existence of EHDV-1 S3 in the baculovirus recombinant at the *39k* site (Fig. 47A). The results were visualised by 1 % agarose gel electrophoresis as DNA bands (Fig. 47, lanes 4 and 5) corresponding to the size of the positive control EHDV-1 S3

in the recombinant plasmid pRN306-EHDV1.S3 (Fig. 47, lane 3). The Bacmid$_{1629}$ was used as a negative control, and showed no amplification (Fig. 47, lane 2).

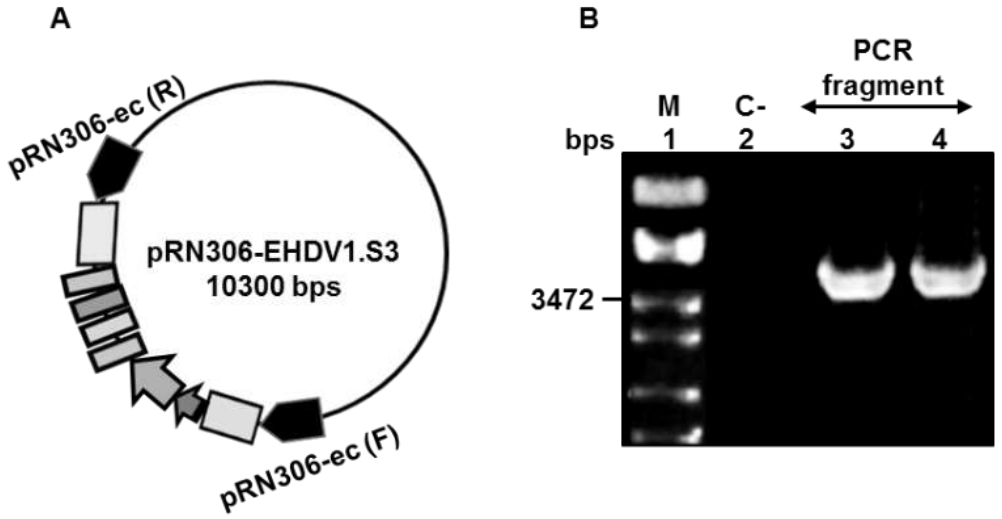

Fig. 45. Amplification of the selectable and expression cassettes flanked by AcMNPV sequences. (A) pRN306-EHDV1.S3 map showing pRN306-ec (F and R) primers. (B) PCR amplification with pRN306-ec (F and R) primers. Lane 1: DNA ladder, lane 2: negative control and lanes 3 and 4: amplification of the cassettes.

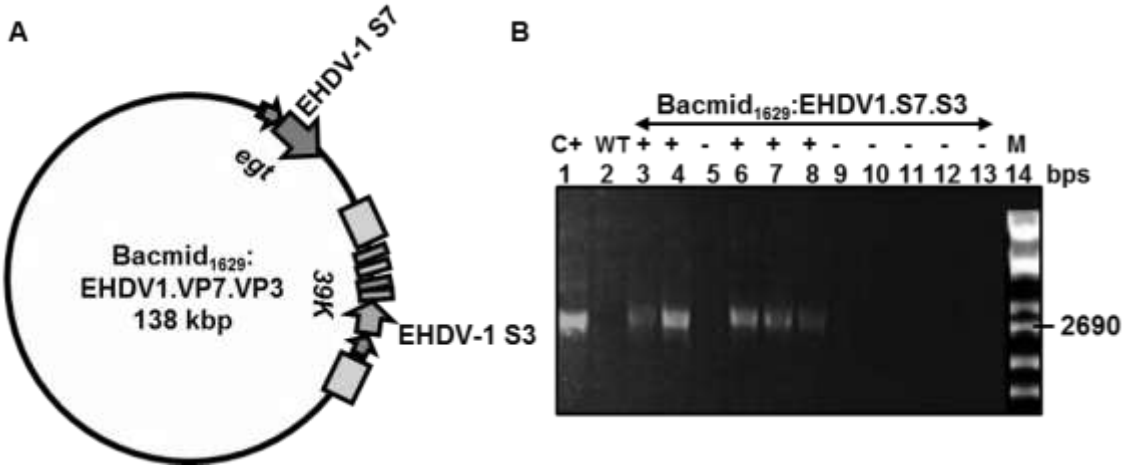

Fig. 46. Molecular analysis to detect the presence of EHDV-1 S3 in the recombinant Bacmid$_{1629}$:EHDV1.S7.S3. (A) Analysis map of the Bcamid$_{1629}$:EHDV1.S7.S3 in E3K2 cells showing the presence of the expression, selectable cassettes and specific AcMNPV sequence for homologous recombination. (B) PCR amplification of EHDV-1 S3 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: pRN306-EHDV1.S3, lanes 3-13: EHDV-1 S3 in the recombinant Bacmid$_{1629}$:EHDV1.S7.S3 (Lanes 3, 4, 6, 7 and 8 are positives) and lane 14: DNA ladder.

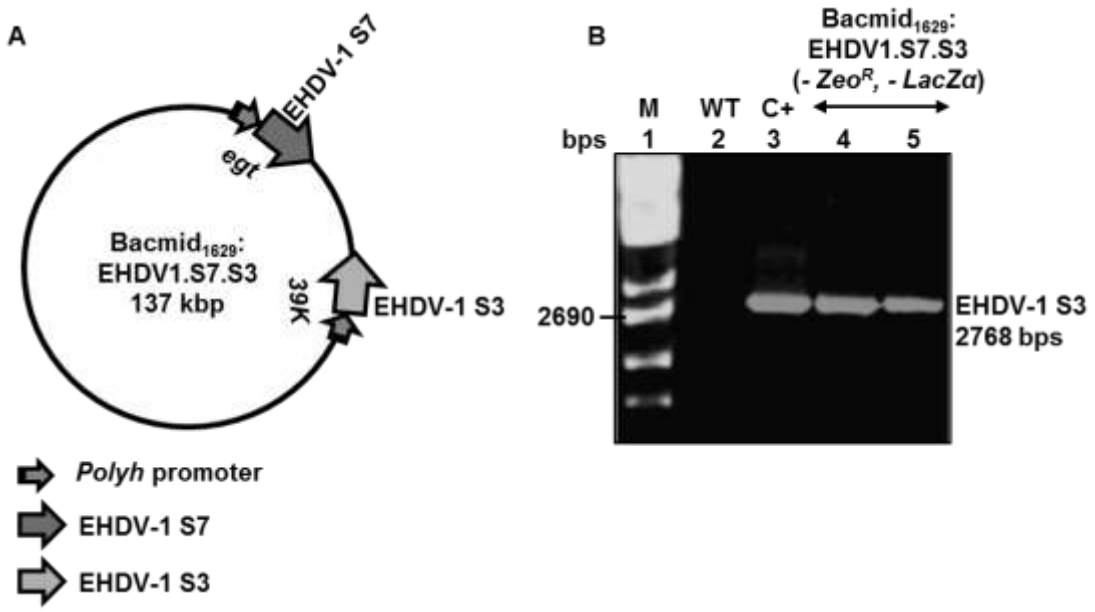

Fig. 47. Molecular analysis to determine the presence of EHDV-1 S3 after flipping out the selectable marker. (A) Analysis map of the recombinant Bacmid$_{1629}$:EHDV1.S7.S3 after flipping out the selectable cassette. (B) PCR amplification of EHDV-1 S3 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: EHDV-1 S3 (positive control) and lanes 4-5: EHDV-1 S3 in the recombinant Bacmid$_{1629}$:EHDV1.S7.S3.

4.2.1.3. Expression, purification and assembly of EHDV-1 CLP Particles in AcMNPV

The expression of the inner capsid proteins EHDV-1 (VP7 and VP3) was tested in *Sf*21 insect cells by cotransfecting them with the circular recombinant Bacmid$_{1629}$:EHDV1.S7.S3 and the empty *Alw*NI transfer vector pAcYM1 (Fig. 48). The linearised pAcYM1 vector was used to recover the viral genome by homologous recombination enabling the replication in *Sf*21 insect cells.

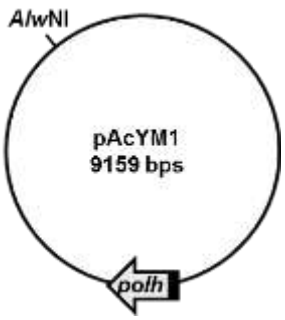

Fig. 48. Restriction map of the vector pAcYM1 with *Alw*NI site.

Following cotransfection, plaque assay was performed and several individual plaques were isolated. The clonal populations of the recombinant baculovirus were amplified three times in *Sf*9 insect cells to increase the titre of the virus and protein production. To analyse the assembly of VP3 and VP7 of EHDV-1 into CLPs, 25-50% sucrose discontinuous gradient centrifugation was performed. Two protein bands were observed at both 0-25% and 25-50% sucrose interface corresponding to the correct sizes of EHDV-1 (VP3 and VP7) (Fig. 49A). Fractions of the sucrose gradient were collected from 0-25%, 25%, 25-50%, as well as from the pellet. The expression of the recombinant proteins of EHDV-1 [VP3 (103 kDa), VP7 (39 kDa)] were detected by 10% SDS-PAGE gel, particularly, in the 25-50% sucrose fraction (Fig. 49B, lane 4). Additionally, aggregation of the cellular proteins was visualised in the samples obtained from the fraction 25-50% sucrose (Fig. 49, lane 4) and from the pellet (Fig. 49, lane 5). Additionally, non-specific protein bands were also found in all fractions (Fig. 49, lanes 2-5).

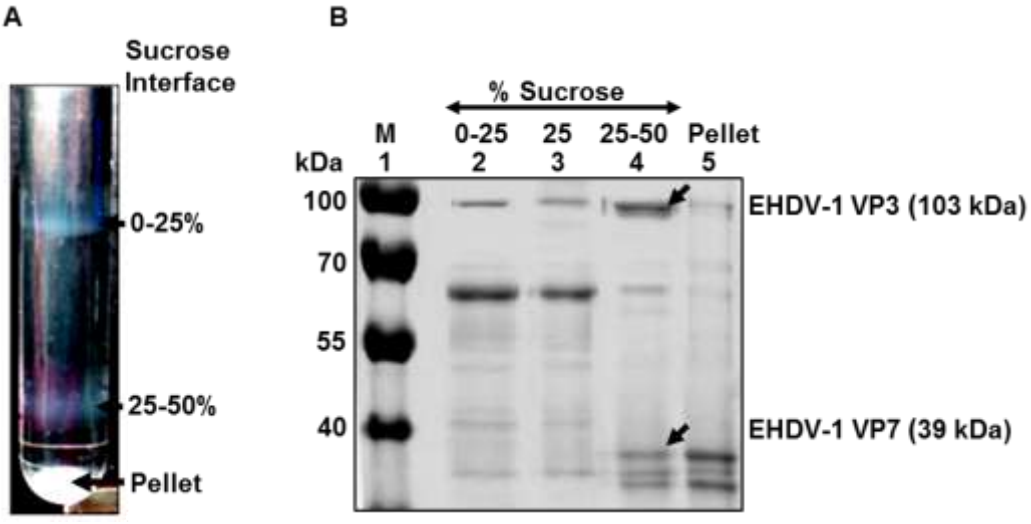

Fig. 49. 10% SDS-PAGE analysis of EHDV-1 CLPs. (A) Sucrose gradient centrifugation of EHDV-1 CLPs. Two bands were visible at both the 0-25% and 25-50% sucrose interface (B) 10% SDS-PAGE analysis of EHDV-1 CLPs. Lane 1: protein ladder, lane 2: sample collected from 0-25% sucrose interface, lane 3: sample collected from 25% sucrose interface, lane 4: sample collected from 25-50% sucrose interface showing EHDV-1 [VP3 (103 kDa) and VP7 (39 kDa)] (black arrows) and lane 5: sample collected from the pellet.

To remove the non-specific bands and to concentrate EHDV-1 CLPs, a second gradient was carried out to purify the sample collected from 25-50% sucrose fraction. The analysis of the purified CLPs proteins by 10% SDS-PAGE demonstrated the presence of the inner layer recombinant proteins VP7 and VP3 of EHDV-1 at correct molecular ratios (Fig. 50, lane 2).

To identify whether the bands composing of VP7 and VP3 was assembled into CLP particles, EM analysis was performed. The EM micrographs of the negatively stained samples showed a spiky morphology (Fig. 51A) similar in size and appearance to the authentic EHDV purified cores (Fig. 51B)

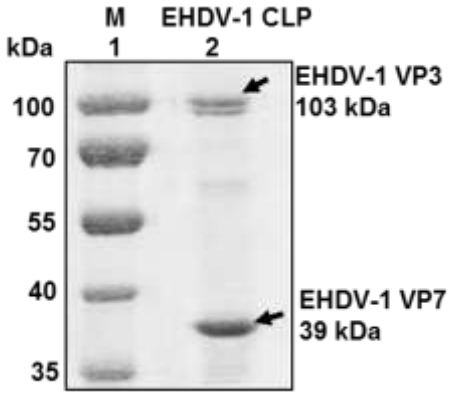

Fig. 50. 10% SDS-PAGE analysis of the purified and concentrated sample of EHDV-1 CLPs from 20-25% sucrose fraction. Lane 1: protein ladder and lane 2: purified EHDV-1 CLPs.

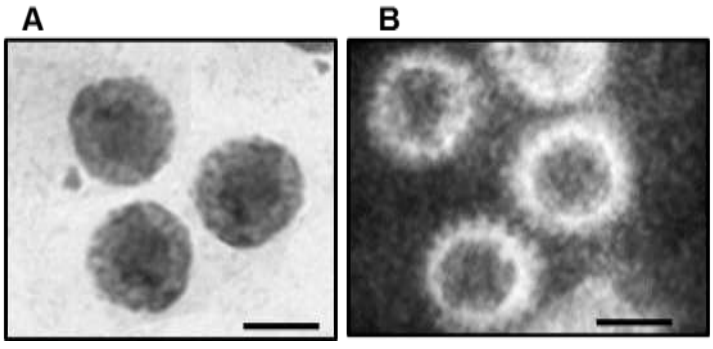

Fig. 51. Electron micrographs of EHDV-2 cores (A) and EHDV-1 CLPs (B). The bars indicate 100 nm.

In total, 2 ml (2.5 mg/ml) of the purified and concentrated EHDV-1 CLP samples were sent to Sigma-Aldrich to raise polyclonal antibody in Rabbits, and sera were collected post inoculation at different days (0, 35 and 56 + 58). The specificity of the developed antisera was tested by western immunoblotting, in which two specific protein bands were visualised with equal sizes of EHDV-1 VP3 (103 kDa) and EHDV-1 VP7 (39 kDa) (Fig. 52, lane 3). The AcMNPV pRN43-GFP recombinant protein was used as a negative control, and EHDV-2 core particles as positive control. Two protein bands were observed in lane 4 from EHDV-2 cores (Fig. 52, lane 4), similar to the size of EHDV-1 CLP recombinant proteins VP7 and VP3. To detect whether there was interaction between BTV-10 CLP proteins and the produced antisera against EHDV-1, western blotting was performed. As shown in Figure 52, lane 5, protein bands corresponding to the estimated sizes of BTV-10 VP3 (~ 103 kDa) and BTV-10 VP7 (~ 38 kDa) were detected (Fig. 52, lane 5). No non-specific protein bands were found in the negative control in lane 2 corresponding to the sizes of either VP3 or VP7 (Fig. 52, lane 2).

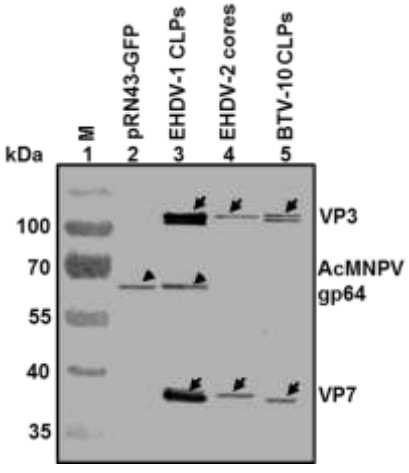

Fig. 52. Immunoblotting analysis of EHDV-1 CLPs proteins VP3 and VP7. Lane1: protein ladder, lane 2: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP, lane 3: lysate from cells infected with recombinant Bacmid$_{1629}$:EHDV1.VP3.VP7 expressing EHDV-1 VP3 and VP7 proteins, lane 4: EHDV-2 cores showing EHDV-2 VP3 and VP7 proteins and lane 5: BTV-10 CLPs VP3 and VP7 proteins. All recombinant proteins are indicated in black arrows. AcMNPV gp64 protein is indicated in head arrow.

4.2.2. The use of baculovirus multiple genes expression system to develop EHDV-1 VLPs

4.2.2.1. Generation of the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3

Next step towards the production of EHDV-1 VLPs by baculovirus multiple synthesis method was the integration of the coding sequences of the outer capsid protein of EHDV-1 VP5 into the recombinant Bacmid$_{1629}$:EHDV1.S7.S3. Therefore, EHDV-1 S5 ORF was subcloned into the *Bam*HI linearised and dephosphorylated pRN296 transfer vector (Fig. 53, lane 3). The correct cloning was determined by restriction digestion with *Bam*HI enzyme (Fig. 54A), and observed by agarose gel as two bands of sizes of pRN296 (5930 bps) and EHDV-1 S5 (1640 bps) (Fig. 54B, lane 3). The PCR amplification using PH (F) and S5 (R) primers was performed to determine the correct orientation of the insert in the recombinant plasmid pRN296-EHDV1.S5. A single band corresponding to the estimated size of EHDV-1 S5 was visualised in Figure 55, lane 3 post 1% gel electrophoresis (Fig. 55, lane 3).

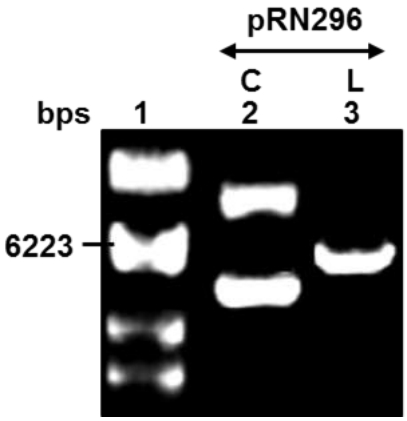

Fig. 53. ***Bam*HI digestion of pRN296 transfer vector.** Lane 1: DNA ladder, lane 2: pRN296 (circular) and lane 3: pRN296 (linear).

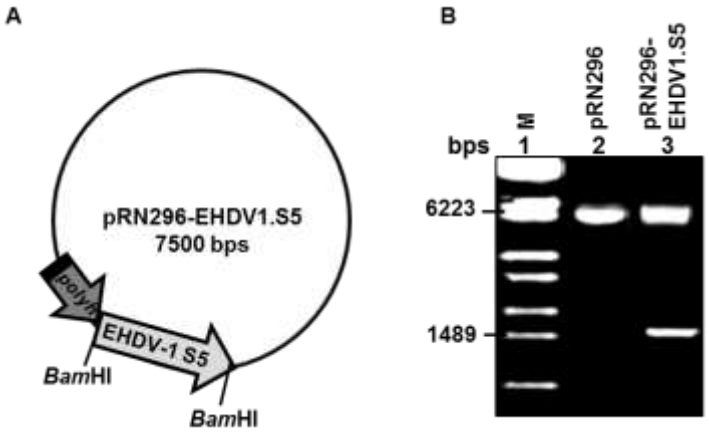

Fig. 54. Molecular analysis to detect the presence of EHDV-1 S5 in the recombinant pRN296-EHDV1.S5. (A) Restriction map of pRN296-EHDV1.S5 showing *Bam*HI sites. (B) *Bam*HI digestion of the construct pRN296-EHDV1.S5. Lane 1: DNA ladder, lane 2: pRN296 (Linear) and lane 3: pRN296-EHDV1.S5.

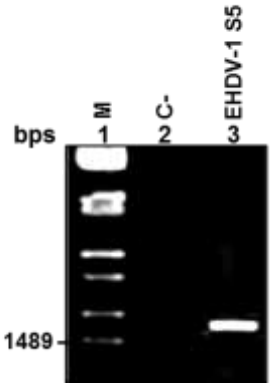

Fig. 55. Molecular analysis to detect the orientation of EHDV-1 S5 in the recombinant pRN296-EHDV1.S5 using PH (F) and EHDV-1 S5 (R) primers. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-1 S5 in the construct pRN296-EHDV1.S5.

Prior to the insertion of EHDV-1 S5 into the recombinant Bacmid$_{1629}$:EHDV1.S7.S3, PCR amplification of the selectable and synthesis markers was carried out using pRN296-ec primers (Table 10). The amplified product was identified on agarose gel as a DNA band similar to the size of 4770 bps (Fig. 56, lane 3). No DNA fragments were detected in the negative control without template in Lane 2 (Fig. 56, lane 2).

After electroporation, the PCR fragment containing EHDV-1 S5 failed to be integrated into the recombinant Bacmid$_{1629}$:EHDV1.S7.S3 at *odv-e56* locus. Therefore, EHDV-1 S5 gene was inserted into an empty Bacmid$_{1629}$ genome at *odv-e56* locus (Fig. 57A). The initial selection of the positive recombinant was based on blue-white screening followed by PCR amplification. Amplified DNA fragment was shown in Figure 57B, lane 9 (Fig. 57B, lane 9), corresponding to the size of the positive control EHDV-1 S5 in the recombinant plasmid pRN296-EHDV1.S5 (Fig. 57B, lane 10). Thereafter, each of EHDV-1 (S3 and S7) genes were successfully incorporated in baculovirus genome at *39k* and *egt* loci, respectively, generating Bacmid$_{1629}$:EHDV1.S5.S7.S3 (Fig. 58).

The PCR amplification was carried out to identify the presence of the all three genes coding for the capsid proteins; EHDV-1 (VP5, VP7 and VP3) in the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S7. Gel electrophoresis in Figure 59A, Lane 4 (Fig. 59A, lane 4), showed amplification equivalent to the size of that seen from the positive control EHDV-1 S5 in the recombinant plasmid pRN296-EHDV1.S5. In parallel, according to the data observed in Figure 59B, lane 4 by gel electrophoresis, the presence of EHDV-1 S7 in the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3 was detected (Fig. 59B, lane 4). Moreover, a positive incorporation was found for EHDV-1 S3 using specific primers (Fig. 59C, lane 4). No amplification similar to the estimated sizes of the three tested genes was observed in the negative control, Bacmid$_{1629}$ (WT).

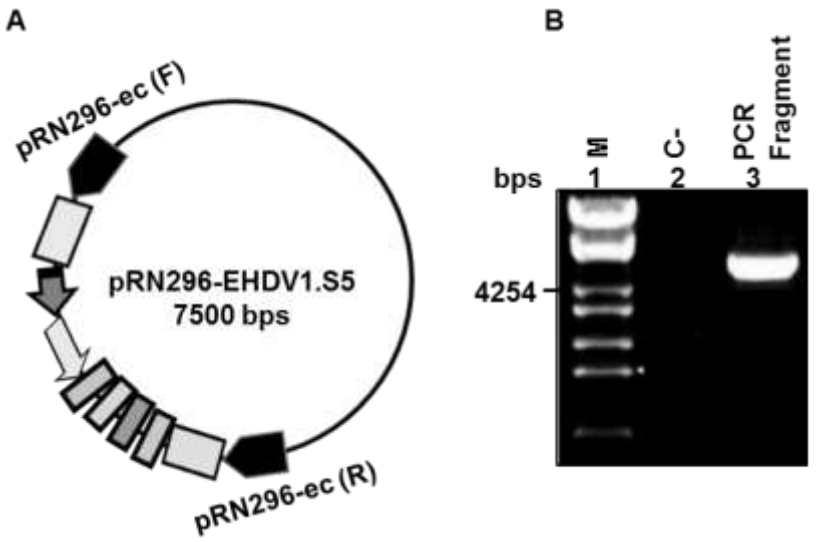

Fig. 56. Amplification of the selectable and expression cassettes flanked by AcMNPV sequences. (A) pRN296-EHDV1.S5 map showing pRN296-ec (F and R) primers. (B) PCR amplification with pRN296-ec (F and R) primers. Lane 1: DNA ladder, lane 2: negative control and lane 3: amplification of the cassettes.

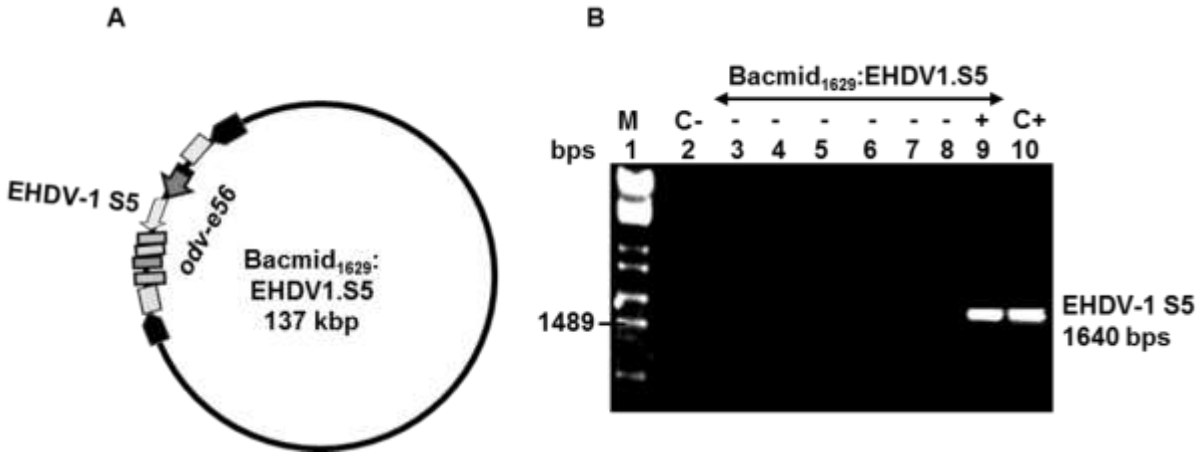

Fig. 57. Molecular analysis to detect the presence of EHDV-1 S5 in the recombinant Bacmid$_{1629}$:EHDV1.S5. (A) Analysis map of the Bacmid$_{1629}$:EHDV1.S5 in E3K2 cells showing the presence of the expression, selectable cassettes and specific AcMNPV sequence for homologous recombination. (B) PCR amplification of EHDV-1 S5 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lanes 3-11: EHDV-1 S5 in the recombinant Bacmid$_{1629}$:EHDV1.S5 (Lane 9 is positive) and lane 12: pRN296-EHDV1.S5 (positive control).

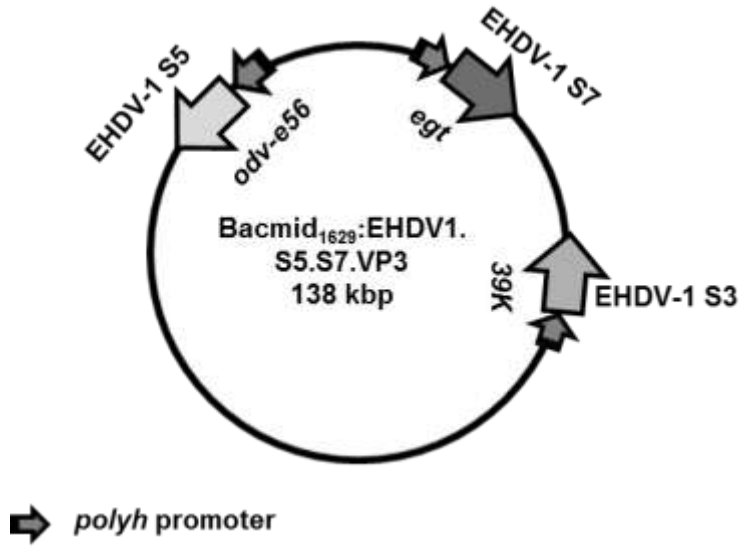

Fig. 58. Molecular analysis map of the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3 showing the presence of EHDV-1 (S5 at *odv-e56*, S7 at *egt* and S3 at *39k*) loci after flipping out the selectable markers.

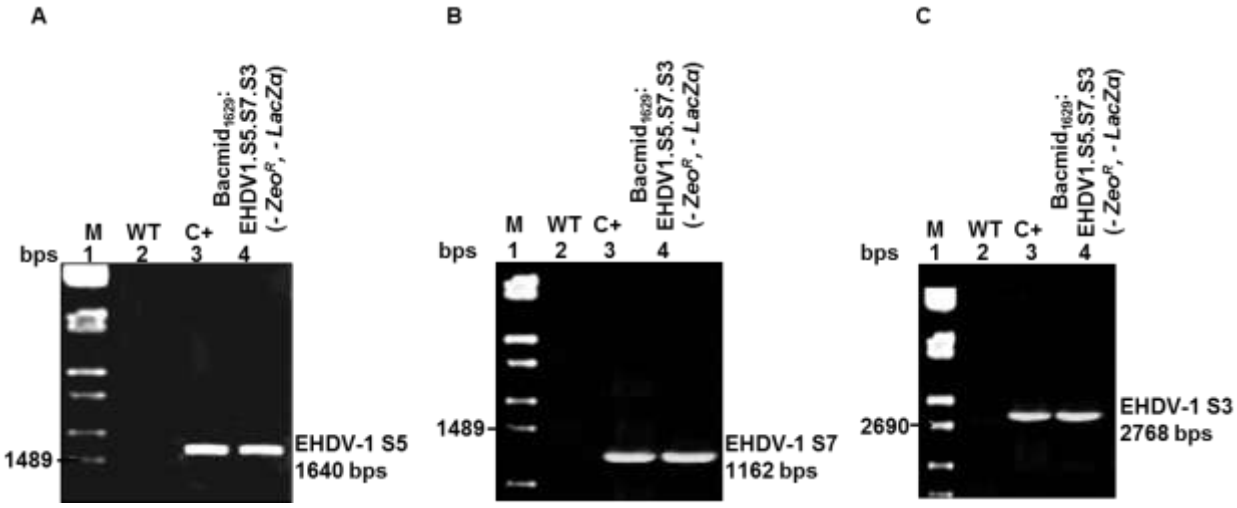

Fig. 59. Molecular analysis to detect the presence of EHDV-1 S5, S7 and S3 after flipping out the selectable marker. (A) PCR amplification of EHDV-1 S5 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: EHDV-1 S5 (positive control) and lane 4: EHDV-1 S5 in the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3. (B) PCR amplification of EHDV-1 S7 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: EHDV-1 S7 (positive control) and lane 4: EHDV-1 S7 in the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3. (C) PCR amplification of EHDV-1 S3 using specific (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ (WT), lane 3: EHDV-1 S3 (positive control) and lane 4: EHDV-1 S3 in the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3.

4.2.2.2. Expression and purification of EHDV-1 triple proteins (VP5, VP7 and VP3) in AcMNPV

To express the outer and inner capsid proteins from the recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3, *Sf*21 insect monolayers were cotransfected with the circular Bacmid$_{1629}$:EHDV1.S5.S7.S3 and the linearised empty pAcYM1 vector to recover baculovirus genome. The 25-50% sucrose discontinuous gradients purification was accomplished and three protein bands corresponding to the correct sizes of EHDV-1 [VP3 (103 kDa), VP5 (59 kDa) and VP7 (39 kDa)] were seen at 25-50% fraction on a 10% SDS-PAGE (Fig. 60, lane 4). In addition, non-specific protein band similar in size to that found in the fraction collected from 0-25% sucrose were visualised (Fig. 60, lane 4, head arrow). In contrast, several cellular protein bands were detected in the samples collected from 0-25% sucrose fraction (Fig. 60, lane 2) and 25% sucrose fraction (Fig. 60, lane 3) and the pellet (Fig. 60, lane 5).

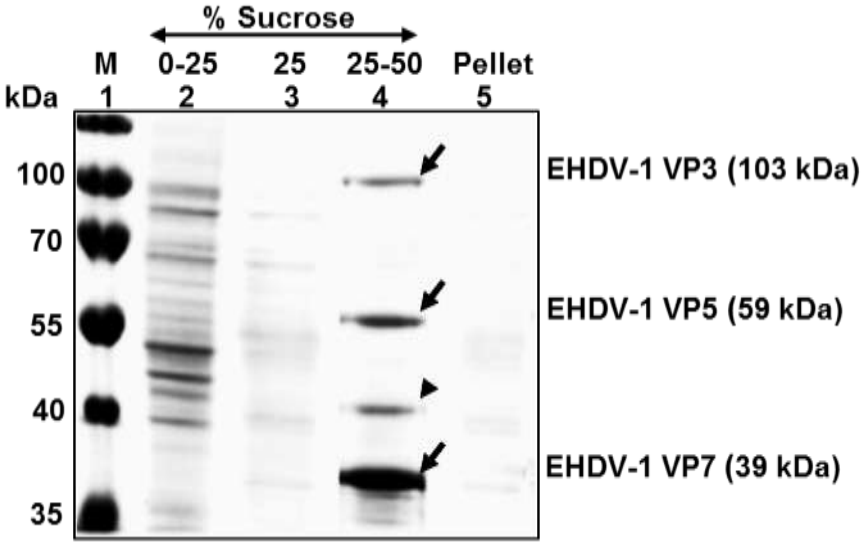

Fig. 60. 10% SDS-PAGE analysis of EHDV-1 Triple recombinant proteins VP5, VP7 and VP3. Lane 1: protein ladder, lane 2: sample collected from 0-25% sucrose interface, lane 3: sample collected from 25% sucrose interface, lane 4: sample collected from 25-50% sucrose interface showing EHDV-1 [VP3 (103 kDa), VP5 (59 kDa) and VP7 (39 kDa)] and lane 5: sample collected from the pellet. All recombinant proteins are indicated with black arrows. Non-specific protein band is indicated in head arrow.

4.2.3. Generation, purification and assembly of EHDV-1 VLP particles in AcMNPV

The EHDV-1 VLPs consisting of the four structural proteins VP2, VP3, VP5 and VP7 was expressed in *Sf*21 insect cell monolayers as follows. The cells were cotransfected with the *AIwn*I linearised recombinant plasmid pAcYM1-EHDV1.S2 and the circular recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3. To demonstrate that all four proteins of EHDV-1 were correctly expressed, cell lysates infected with 8 clones of baculovirus were analysed by 10% SDS-PAGE. In Figure 61, lane 7, SDS-PAGE analysis shown four unique proteins bands and the sizes corresponded to the expected sizes of the structural proteins of EHDV-1 [VP2 (112 kDa), VP3 (103 kDa), VP5 (59 kDa) and VP7 (39 kDa)] (Fig. 61, lane 7), respectively. The expression of the complete set of the four recombinant proteins was not detected from other cell lysates of other 7 clones (Fig. 61, lanes 3-6, 8-10)

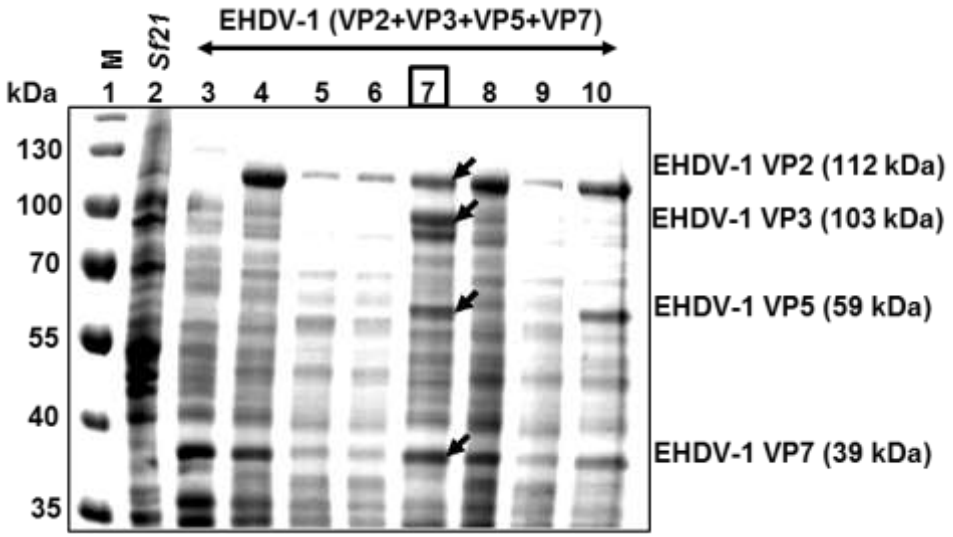

Fig. 61. 10% SDS-PAGE analysis of the expression of EHDV-1 VLPs proteins from clonal populations. Lane 1: protein ladder, lane 2: *Sf*21 insect cells, lanes 3-10: lysate from cells infected with recombinant baculovirus expressing EHDV-1 (VP2, VP3, VP5 and VP7) proteins. Lane # 7 is correct (black arrows).

To examine whether the expressed recombinant proteins (Fig. 61, lane 7) could be assembled into EHDV-1 VLPs, *Sf*9 insect cell monolayers were infected with the recombinant baculovirus expressing the four structural proteins of EHDV-1. The infected cells were harvested at 64-72 hpi, and the EHDV-1 VLPs were purified using 40-75% sucrose discontinuous gradients. Purification protocol was optimised to maintain the integrity of the particles by using cell lysis buffer with pH and ionic strength similar to that used for the virion purification. The 10% SDS-PAGE analysis of the purified VLPs of EHDV-1 showed that all four capsid proteins EHDV-1 [VP2 (112 kDa), VP5 (59 kDa), VP3 (103 kDa), and VP7 (39 kDa)] were present (Fig. 62, lane 3). As positive controls, lysates from cells expressing individual structural proteins of EHDV-1 were used (Fig. 62. VP2, lane 4; VP3, lane 5; VP5, lane 6 & VP7, lane 7).

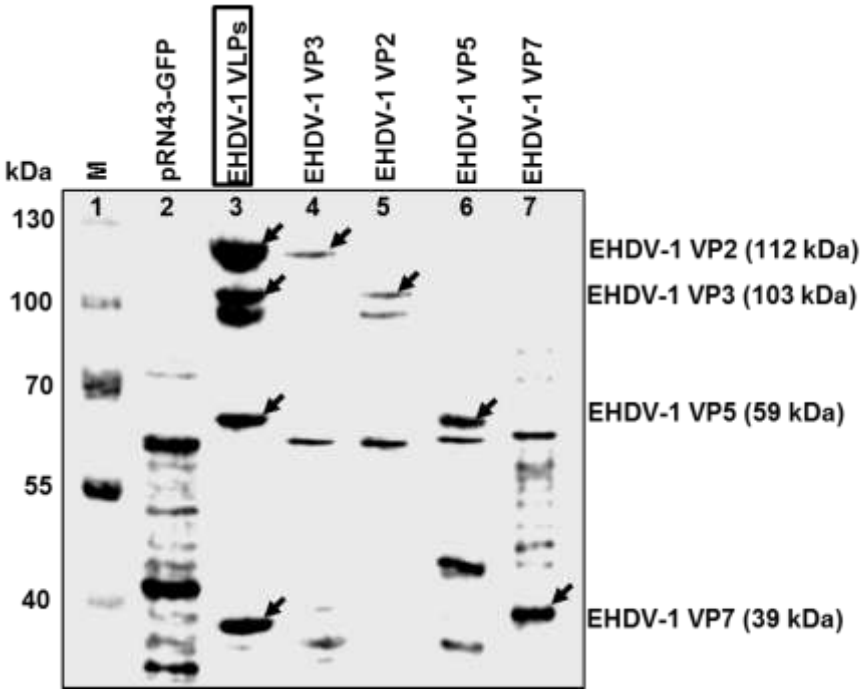

Fig. 62. 10% SDS-PAGE analysis of EHDV-1 VLPs proteins. Lane 1: protein ladder, lane 2: lysate from cells infected with recombinant pRN43-GFP, lane 3: purified EHDV-1 VLPs from 40-75% sucrose fraction (VP2, VP3, VP5 and VP7), lane 4: EHD-1 VP2 (positive control), lane 5: EHDV-1 VP3 (positive control), lane 6: EHDV-1 VP5 (positive control) and lane 7: EHDV-1 VP7 (positive control). Each EHDV-1 proteins is indicted by black arrows.

Samples of the purified EHDV-1 VLPs were prepared and then sent to a commercial company to raise polyclonal antibodies in Rabbits. After 2 doses of inoculation, the specificity of the antisera raised commercially against EHDV-1 VLP proteins were tested by western blotting. Protein bands equivalent to the estimated sizes of the EHDV-1 (VP2, VP3, VP5 and VP7) recombinant proteins were observed on a nitrocellulose membrane (Fig. 63, lane 4). Two negative controls were used and no distinct protein band was detected corresponding to the size of any of the four structural capsid proteins in the uninfected *Sf*9 insect cells (Fig. 63, lane 2), or that infected with the recombinant AcMNPV pRN43-GFP (Fig. 63, lane 3).

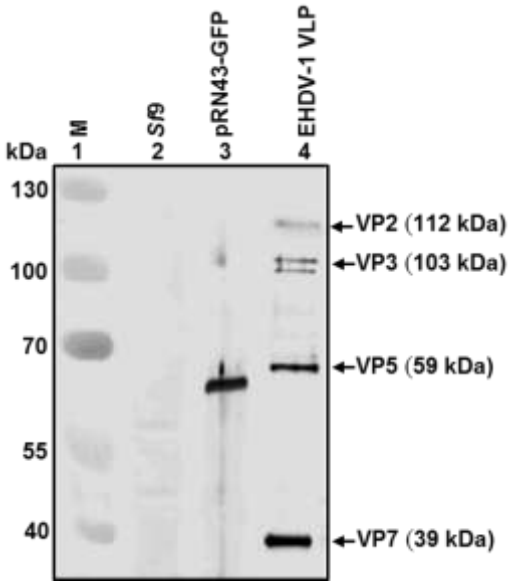

Fig. 63. Immunoblotting analysis of the synthesis of the EHDV-1 VLP proteins from clonal populations. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP and lane 3: lysate from cells infected with the recombinant Bacmid$_{1629}$:EHDV1.VP2.VP5.VP3.VP7 expressing EHDV-1 [VP2 (112 kDa), VP3 (103 kDa), VP5 (59 kDa) and VP7 (39 kDa)] (black arrows).

EM analysis was carried out to examine whether the four expressed recombinant proteins from purified sample of infected *Sf*9 cells, were assembled into EHDV-1 VLPs. The negatively stained sample in Figure 64 (Fig. 64) exhibiting similar in size

and morphology of EHDV-1 VLP particles to that reported for BTV-10 VLPs (Fig. 4B), but lack the dsRNA viral genome.

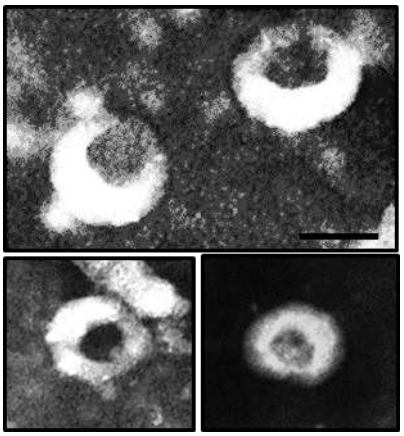

Fig. 64. Electron micrographs of EHDV-1 VLP particles. The bar indicates 100 nm.

4.2.4. Analysis of the neutralising antibody response

Since the VLPs were generated for vaccine purpose, it was necessary to test whether the polyclonal antisera raised against EHDV-1 VLPs would neutralise EHDV-1 *in vitro* in BSR cells. Serum neutralisation with $TCID_{50}$ was performed as described in the Material and Methods, and neutralisation of EHDV-1 was detected at dilution of 1:64 (Table 19). As controls, FSC serum was added to the BSR monolayers infected with EHDV-1 showing 100% CPE, while uninfected cells were used as negative control. Additionally, cross neutralising activities were tested against EHDV-2 and EHDV-6 which detected at a low dilution of 1:8 for both serotypes (Table 19).

Table 19: EHDV-1 VLPs antisera neutralization titre with $TCID_{50}$ in BSR cells infected with EHDV-1, EHDV-2 and EHDV-6.

EHDV serotype	EHDV-1 VLPs Sera dilution
EHDV-1	**1:64**
EDHD-2	**1:8**
EHDV-6	**1:8**

In summary, using baculovirus multiple gene expression system, the genes encoding for the four structural proteins of EHDV-1 (VP2, VP5, VP3 and VP7), were successfully subcloned into specific transfer vector and integrated into baculovirus genome at different loci. The assembly of the expressed proteins; EHDV-1 VP7 and VP3 into CLPs and VP2, VP5, VP7 and VP3 into VLP particles, respectively, were tested by EM analysis. Antisera further raised against EHDV-1 CLP and VLP proteins, demonstrated their specificity in western immunoanalysis. Furthermore, neutralisation activities against EHDV-1, -2 and 6 serotypes were tested using EHDV-1 VLPs antibodies, and neutralising data demonstrated that at high antibody titre, low cross neutralising activities were presented.

4.3. DISCUSSION

Several reports of multigene expression by recombinant baculovirus have depended on the use of homologous recombination or locus specific transposition (Tn7 transposition), to obtain the required recombinant baculoviruses (Berger et al., 2004, Latham and Galarza, 2001, Stewart et al., 2010). The major target of these studies was to develop a highly immunogenic and safe vaccine, like the new-generation HPV vaccines (Koutsky et al., 2002, Harper et al., 2004). The efficacy of a current baculovirus multiple loci cloning system has been demonstrated via the production of VLPs for influenza A and BTV-1 (Noad et al., 2009), for which VLPs immunogenicity has been shown to be efficient against both infectious viruses (Roy et al., 1994, Galarza et al., 2005).

In expression studies, two specific loci; *polh* and/or *p10* in the baculovirus genome were frequently used. For heterologous protein expression, *v-cath* was also reported in a few studies (Berger et al., 2004, Fitzgerald et al., 2006), but foreign gene expression from other loci remained mostly uncharacterised, until 2009. In 2009, seven genetic loci (i.e. *egt*, *orf51, gp37, 39k, iap2, odv-e56* and *ctx*) were identified by *Noad et al* (Noad et al., 2009) in addition to *polh* and *p10*. The expression level of foreign gene products from these new loci was similar to that obtained from the latter two loci. A recent study evaluated the utility of those loci for the expression of a large protein, VP2 of AHSV which is ~110 kDa in size, and the level of expression was determined by comparing with that of *polh* and *p10* (Kanai et al., 2013). The synthesis of this recombinant protein was found to be high from *odv-e56, egt, p10* and *39k* loci (Kanai et al., 2013). It is notable to mention that expression cassettes in each of the loci, have either *polh* or *p10* promoters, the two known strong promoters.

In this study, a similar approach was undertaken to develop EHDV-1 VLPs by simultaneous expression of the outer and inner capsid proteins from a single recombinant baculovirus. To address this aim, successful subcloning of the gene encoding the three structural proteins of EHDV-1 (VP5, VP7 and VP3) was performed into transfer vectors, each targeting a specific genetic locus at baculovirus genome (Noad et al., 2009, Kanai et al., 2013). As subcloning of the large and the most variable gene S2, was difficult, the previously generated recombinant plasmid pAcYM1-EHDV1.S2 was used to recover the recombinant baculovirus. Although achievable, the overall progress was affected by several drawbacks which eventually were all solved.

Prior to the development of EHDV-1 VLPs, EHDV-1 CLPs (VP7 and VP3) and EHDV-1 VLPs composed of the three structural proteins (VP5, VP7 and VP3) were generated. This was to detect the expression of each gene after its integration into the baculovirus genome in order to determine whether the correct stepwise assembly of VLPs could be achieved. High expression level of EHDV-1 CLP recombinant proteins was achieved from *egt* and *39k* loci as shown by SDS-PAGE gel analysis. Purification of cores indicated that correct assembly was successful. A similar molar ratio of the protein components to that predicted from the core structure of BTV-10 was observed, this was similar to the pattern seen for BTV-10 CLPs and heterologous CLPs composed of BTV-10 (VP7) and EHDV-1 (VP3) (Le Blois et al., 1991). In contrast to the extensive work performed to produce CLPs and/or VLPs for BTV, to date no published reports exist regarding the formation of EHDV CLPs or VLPs using individual or dual expression vectors.

To further validate the purification data, the assembly of the recombinant proteins into EHDV-1 CLPs was analysed by EM of purified CLPs. The EM micrographs

confirmed to assembly of EHDV-1 CLPs of the same size, morphology and organisation of VP3 and VP7 as found in the authentic EHDV-2 cores. Here, the available EHDV-2 cores were purified from EHDV-2/Alberta strain and used as positive control, in which a nuclear-protein centre surrounded by two distinct layers formed by VP3 and VP7 were identified by electron micrographs. These results were consistent with that shown by *French and Roy* (French and Roy, 1990) for BTV-10 suggesting that the structure of EHDV cores, which is closely similar to BTV, is maintained by the inner capsid proteins. Moreover, in contrast to the core proteins, none of the minor proteins, non-structural proteins or dsRNAs are necessary, indicating that these viral components do not play any role in the assembly of VP7 and VP3 proteins into CLPs in *Sf*9/*Sf*21 insect cells. This is not surprising due to its close phylogenetic relationship to BTV.

No antiserum directed specifically against EHDV-1 CLPs proteins has previously been described. This issue was addressed in this work, by raising polyclonal antibodies against EHDV-1 CLPs commercially by subcutaneous inoculation of rabbits with the purified and concentrated samples. The specificity of the antibodies were tested by western immunoblotting in which two unique protein bands were observed at low dilution of 1:10,000, indicating high specific antibody titres were elicited *in vivo* and thus indicated the high immunogenic nature of the CLPs. Each identified protein size was similar with the estimated sizes of the inner core structural proteins EHDV-1 VP7 and VP3.

Genetic analysis of S3 and S7 of EHDV serotypes both on the nucleotide levels and amino acid sequences exhibited high degree of similarity (Anthony et al., 2009). Such high conservation was also detected by similarity with BTV, particularly for VP3 protein, as *Le Blois et al* (Le Blois et al., 1991) previously demonstrated that EHDV-1

VP3 protein could replace the corresponding BTV protein in a heterologous CLPs. These results suggest that between BTV and EHDV the structure of core particles and their protein constituents are highly conserved in molecular interactions and folding. Here, antisera developed for EHDV-1 CLPs had shown strong interactions with the VP3 and VP7 of EHDV-2 cores as well as with BTV-10 CLPs. On serological bases, these outcomes were consistent with the above reports demonstrating that the inner capsid proteins VP3 and VP7 are not only highly preserved within EHDV serotypes but also react well with those of BTV serotypes. Taken together, these results indicated successful expression, assembly and purification of EHDV CLPs was achieved using the multi-locus baculovirus expression platform.

To validate the next step of assembly of EHDV-1 VLPs, the gene sequences coding for the second outer capsid protein EHDV-1 VP5 needed to be integrated first into the recombinant baculovirus Bacmid$_{1629}$:EHDV1.S7.S3. However, after several attempts, it was still not possible to achieve. An attempt to integrate the ORF of S5 at *odv-e56* locus into Bacmid$_{1629}$:EHDV1.S7 or Bacmid$_{1629}$:EHDV1.S3, were both unsuccessful. However, the successful insertion was accomplished at *ovd-e56* locus into Bacmid$_{1629}$, followed by the integration of S7 and S3 of EHDV-1 in a sequential manner. These data suggest that the gene encoding VP5 protein is probably less stable and harder to be integrated into baculovirus DNA in the presence of other genes.

This research presented an appropriate order of cloning the structural genes essential for the formation of the orbiviruses VLPs. After excising the selectable marker and prior to the insertion of the next gene into baculovirus genome, detecting the presence of the previous integrated gene was crucial step for the completion of this work to ensure correct expression of the targeted VLPs.

The simultaneous expression of the 3 capsid proteins was analysed by SDS-PAGE gel, showing high expression level of EHDV-1 (VP5, VP7 and VP3). In order to generate EHDV-1 VLPs, the expression of all four major structural proteins was achieved by cotransfection *Sf*21 insect cells with the linear pAcYM1-EHDV1.S2 and the circular recombinant Bacmid$_{1629}$:EHDV1.S5.S7.S3. Although, variability in the expression level of the gene products was shown between clones, utilising this method, a consistent yield of recombinant proteins could be achieved from each cell in the culture and in the same ratio (Bertolotti-Ciarlet et al., 2003). This was confirmed by SDS-PAGE gel showing that EHDV-1 (VP2, VP5, VP7 and VP3) recombinant proteins were expressed well in an optimal proportion from the purified *Sf*9 cell lysate. The successful assembly of the recombinant proteins into VLPs was morphologically examined by EM using BTV VLPs as positive control. The particles exhibited a thick outer shell, morphologically similar to that of double-capsid authentic particles (French et al., 1990). Such appearance permitted an easy differentiation between the particles of VLPs and that of CLPs.

Commercially, antisera were raised against the purified EHDV-1 VLPs, and their specificity was analysed by western immunoblotting at a dilution of 1:5,000, in which the antibody titre *in vivo* was not as strong as that of EHDV-1 CLPs. Strong interactions were shown for both VP7 and VP5 proteins. Additionally, high reaction was also observed for VP2 but less for VP3 proteins. Hypothetically, such variable affinity to each protein might be associated with the number of molecules per particles that reported for BTV; 180 molecules of VP2, 360 molecules for VP5, 120 molecules for VP3 and 780 molecules for VP7.

The biological ability of the raised antisera to neutralise EHDV-1 was confirmed by neutralisation assay with reasonably high titre (1:64), which is higher than the typical

neutralisation level in natural convalescent sera (1:20) collected from deer infected with either EHDV-1 or EHDV-2 (Dubay et al., 2004). This data indicates that EHDV-1 VLPs will likely to afford complete protection against EHDV-1 infection in animals.

Further, cross neutralising activities were also detected against the genetically related EHDV-2 and EHDV-6 serotypes at low dilution of 1:8 which is less than that of the respective serotype. These data were consistent with earlier studies for orbiviruses, BTV in particular, demonstrating that although VP2 is the most variable protein and is the serotype specific antigen (Huismans and Erasmus, 1981), certain domains of VP2 of different serotypes are conserved based on sequence analysis.

Overall, new data were presented in this work regarding the development of EHDV-1 CLPs and EHDV-1 VLPs, using baculovirus as multiple gene expression system. To date, no work has been published addressing the assembly of the recombinant structural proteins into CLPs and VLPs, which was successfully confirmed in this study by EM analysis. In addition, the raised antisera were confirmed to neutralise EHDV-1, and cross neutralising reactivities were shown against EHDV-2 and EHDV-6 serotypes. These results validate the potential efficacy of the VLPs produced in this study as a neutralising vaccine and strongly suggest its use as vaccine candidate.

Chapter 5

Generation of heterologous VLPs of EHDV serotypes

5.1. INTRODUCTION

For a long time, EHDV-1 and 2 were thought to be the only two serotypes responsible for causing EHD disease particularly in white-tailed deer. In 2006, this assumption had changed when neutralisation assay and serotype-specific (RT-PCR) of isolates recovered from white-tailed deer belonged to neither EHDV-1 nor EHDV-2 (Allison et al., 2010). Serological tests and genetic analysis showed the causative agent to be EHDV-6, which was identified in Australia and became pathogenic in cattle in Morocco, Algeria, Turkey and the USA (Temizel et al., 2009, Breard et al., 2004).

Genetic characteristics of the emerging serotypes EHDV-6 and EHDV-7 responsible for the recent outbreaks, revealed a reassortment between outer capsid proteins (VP2 and VP5) of EHDV-6 and EHDV-7 and core proteins (VP3 and VP7) of EHDV-2 which supposed to be the parental serotype (Allison et al., 2010). Furthermore, the comparison analysis of the inner core proteins sequence of the EHDV seven serotypes indicated a high conservation among these proteins, with identity up to 80% for VP7 and 90% for VP3. These data were consistent with that found in orbiviruses, especially for BTV and AHSV.

To overcome the disease caused by the virulent strain, earlier studies reported the rapid generation of heterologous VLPs of BTV-2, -4 and 9, based on the used on the inner structural conserved proteins of specific serotype (Stewart et al., 2010). Therefore, in the present study, the high sequence identity between EHDV inner capsid proteins would be exploited to generate heterologous VLPs of EHDV-2 and EHDV-7 by exchanging the outer capsid proteins (VP2 and VP5) of the latter two

serotypes, and coating them onto EHDV-1 CLPs which would be used as a foundation.

5.2. RESULTS

5.2.1. The use of baculovirus multiple gene expression system for the synthesis of EHDV-2 outer capsid proteins

5.2.1.1. Cloning of EHDV-2 S5 into pRN296 transfer vector

To generate the recombinant Bacmid$_{1629}$:EHDV2.S5 expressing the second variable structural protein, VP5, the ORF of EHDV-2 S5 had to be subcloned into pRN296 transfer vector. Therefore, S5 (1641 bps) was amplified by PCR using specific primers from the previously constructed recombinant pAcYM1-EHDV2.S5. The PCR product was visualised on agarose gel as a DNA fragment corresponding to the size of 1641 bps (Fig. 65, lane 2). The purified gene was then ligated into pRN296 under the control *polyh* promoter (Fig. 66A). The accurate construction of the recombinant plasmid pRN296-EHDV2.S5 was determined by PCR screening of several ampicillin resistance colonies, using EHDV-2 S5 primers (Table 7). Three colonies were shown to contain the targeted gene (Fig. 66B, lanes 3, 4 and 6), corresponding to the size of the positive control EHDV2.S5 in the recombinant plasmid pAcYM1-EHDV2.S5 (Fig. 66B, lane 2). Another amplification was carried out to identify the orientation of EHDV-2 S5 using PH (F) and EHDV-2 S5 (R) primers. A correct orientation of S5 gene was detected in two recombinants (Fig. 67, lanes 3 and 5) when visualised on 1% agarose gel.

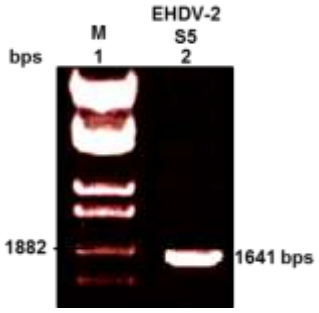

Fig. 65. Molecular analysis of the PCR amplification of EHDV-2 S5 using EHDV-2 S5 (F and R) primers. Lane 1: DNA ladder and lane 2: EHDV-2 S5 (1641 bps).

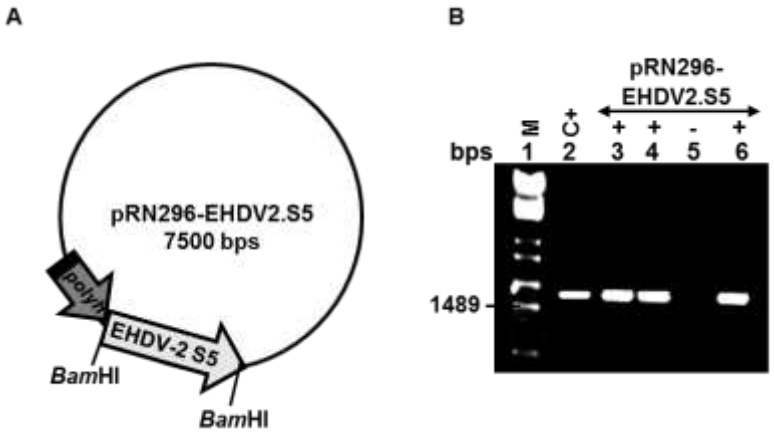

Fig. 66. Molecular analysis to detect the presence of EHDV-2 S5 in the recombinant pRN296-EHDV2.S5 using EHDV-2 S5 (F and R) primers. Lane 1: DNA ladder, lane 2: positive control (pAcYM1-EHDV2.S5) and lanes 3-6: EHDV-2 S5 in the construct pRN296-EHDV2.S5. Clones in lanes # 3, 4 and 6 are positives.

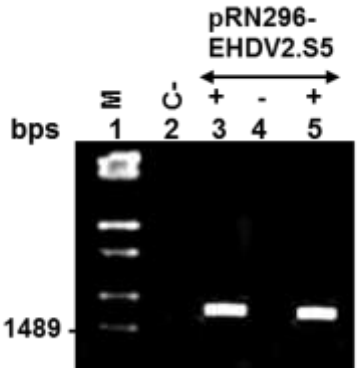

Fig. 67. Molecular analysis to detect the orientation of EHDV-2 S5 in the recombinant pRN296-EHDV2.S5 using PH (F) and EHDV-2 S5 (R) primers. Lane 1: DNA ladder, lane 2: negative control and lanes 3-5: EHDV-2 S5 in the construct pRN296-EHDV2.S5. Colonies in lanes 3 and 5 are positives.

5.2.1.2. Generation of the recombinant Bacmid$_{1629}$:EHDV2.S5

To integrate EHDV-2 S5 into the recombinant baculovirus, both expression and selectable markers were amplified using pRN296-ec (F and R) primers (Table 10) (Fig. 68A). Amplification products were identified by agarose gel as one band corresponding to the estimated size of 4800 bps (Fig. 68B, lane 2). The amplified DNA fragment was then integrated into Bacmid$_{1629}$ genome at *odv-e56* locus, and the correct integration was tested by PCR amplification using EHDV-2 S5 primers. Gel

electrophoresis in Figure 69, lane 4 displayed the DNA amplicon corresponding to the predicted size of EHDV-2 S5 from the positive control pRN296-EHDV2.S5 (Fig. 69. lane 3). Negative control, the WT Bacmid$_{1629}$, used, showed no amplification in Figure 69, lane 2 (Fig. 69, lane 2).

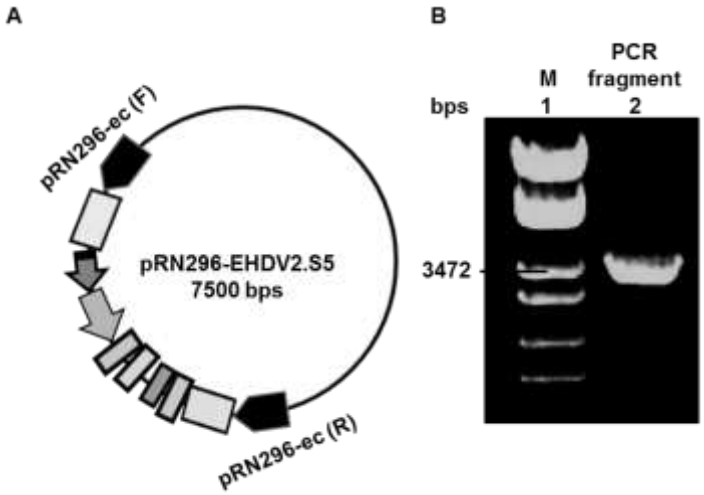

Fig. 68. Amplification of the selectable and expression cassettes flanked by AcMNPV sequences. (A) pRN296-EHDV2.S5 map showing pRN296-ec (F and R) primers. (B) PCR amplification with pRN296-ec (F and R) primers. Lane 1: DNA ladder and lane 2: amplification of the cassettes.

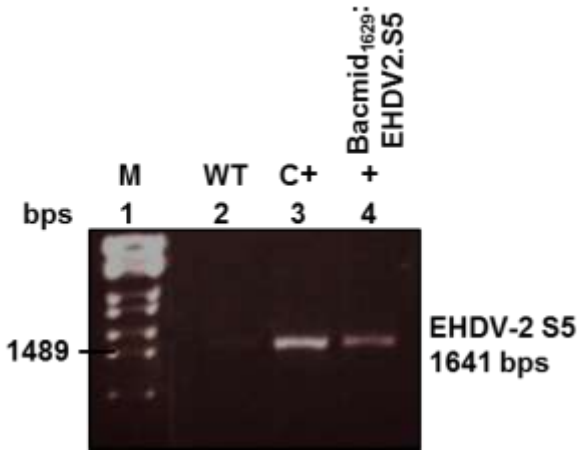

Fig. 69. Molecular analysis to detect the integration of EHDV-2 S5 in the recombinant Bacmid$_{1629}$:EHDV2.S5 using specific EHDV-2 S5 (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ wild type, lane 3: EHDV-2 S5 in the recombinant pRN296-EHDV2.S5 (positive control) and lane 4: Bacmid$_{1629}$:EHDV2.S5.

For simultaneous synthesis of EHDV-2 VP2 and VP5 capsid proteins, the selectable cassette was flipped out by the addition of 0.1% arabinose to the bacterial culture (Fig. 70A). Following flipping the marker out, colony PCR was performed on two clones to determine whether the targeted gene was presented in the Bacmid$_{1629}$:EHDV2.S5 recombinant. The resulted data were verified on agarose gel as one specific band of size of EHDV-2 S5 (Fig. 70B, lane 3), and no amplification corresponding to S5 was identified in another clone (Fig. 70B, lane 2).

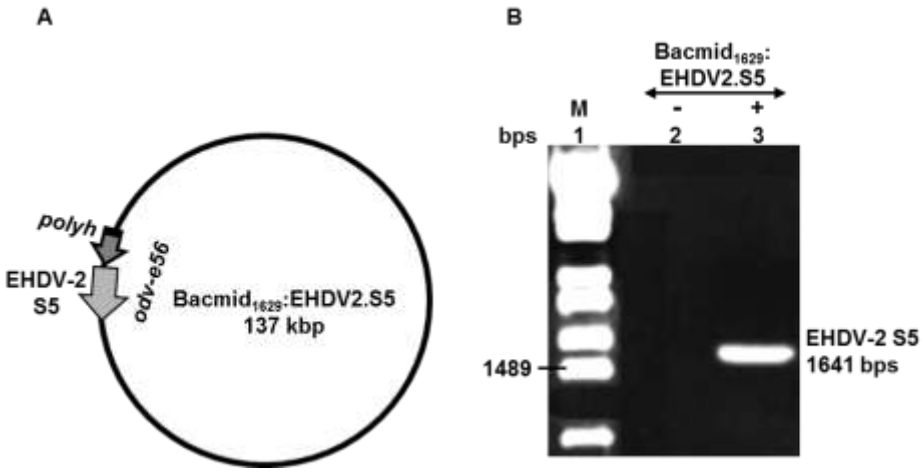

Fig. 70. Molecular analysis to determine the presence of EHDV-2 S5 after flipping out the selectable marker. (A) Analysis map of the recombinant Bacmid$_{1629}$:EHDV2.S5 after flipping out the selectable cassette. (B) PCR amplification of EHDV-2 S5 using specific (F and R) primers. Lane 1: DNA ladder, lanes 2 and 3: Bacmid$_{1629}$: EHDV2.S5. Clone in lane 3 is positive.

5.2.1.3. Dual expression of EHDV-2 VP2 and VP5 recombinant proteins in baculovirus expression system

To produce dual recombinant baculovirus expressing EHDV-2 outer layer proteins, *Sf*21 insect monolayers were cotransfected with *Bsu*36I linearised recombinant Bacmid$_{1629}$:EHDV2.S5 and the recombinant plasmid pAcYM1-EHDV2.S2. The efficient expression of the recombinant proteins VP2 and VP5 were analysed by 10% SDS-PAGE. As seen in Figure 71, lane 4, two intense protein bands corresponding to the sizes of EHDV-2 VP2 (114 kDa) and EHDV-2 VP5 (59 kDa) (Fig. 71, lane 4),

from the infected lysate of *Sf*9 insect cells were identified. As positive controls, the individual synthesis of EHDV-2 VP2 (Fig. 71, lane 5), EHDV-1 VP2 (112 kDa) (Fig. 71, lane 6), EHDV-2 VP5 (59 kDa) (Fig. 71, lane 7) and EHDV-1 VP5 (59 kDa) (Fig. 71, lane 8), were used. Negative controls, uninfected *Sf*9 insect cells (Fig. 71, lane 2) and the recombinant AcMNPV pRN43-GFP (Fig. 71, lane 3), were utilised.

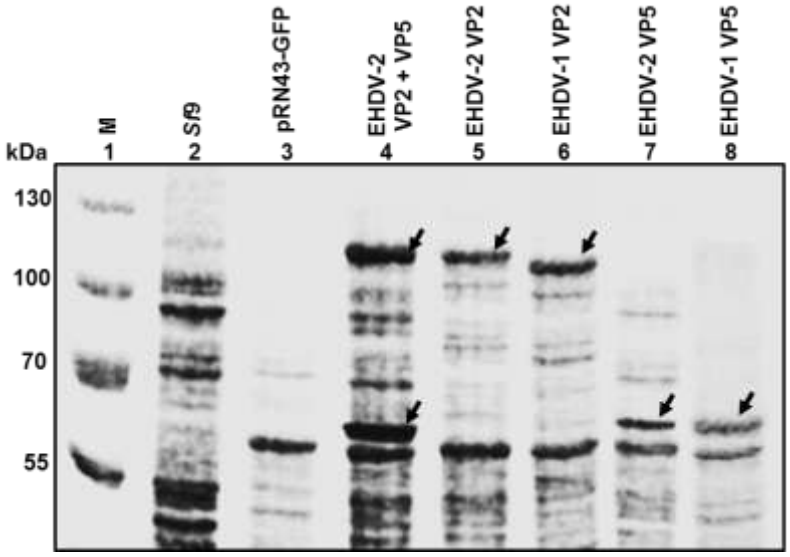

Fig. 71. 10% SDS-PAGE analysis of the recombinants EHDV-2 VP2 and VP5 expression in Bacmid$_{1629}$:EHDV2.VP2.VP5 from clonal populations. Lane 1: protein ladder, lane 2: *Sf*9 insect cells, lane 3: *Sf*9 insect cells infected with the recombinant AcMNPV pRN43-GFP, lane 4: lysate from cells infected with dual baculovirus expressing EHDV-2 VP2 and EHDV-2 VP5, lane 5: individual expression of EHDV-2 VP2 (positive control), lane 6: individual expression of EHDV-1 VP2 (positive control), lane 7: individual expression of EHDV-2 VP5 (positive control) and lane 8: individual expression of EHDV-1 VP5 (positive control). All recombinant proteins are indicated in black arrows.

For further confirmation of the synthesis of the most variable protein EHDV-2 VP2, western blot with the raised antisera against this recombinant protein was performed. Post blotting, a defined band equivalent to the estimated size of EHDV-2 VP2 (114 kDa) was visualised on a nitrocellulose membrane (Fig. 72, lane 3), similar to that detected from the individual expression of EHDV-2 VP2 protein (Fig. 72, lane 4). No precise protein band of the size of VP2 was found in *Sf*9 insect cell lysate infected with the recombinant AcMNPV pRN43-GFP (Fig. 72, lane 2).

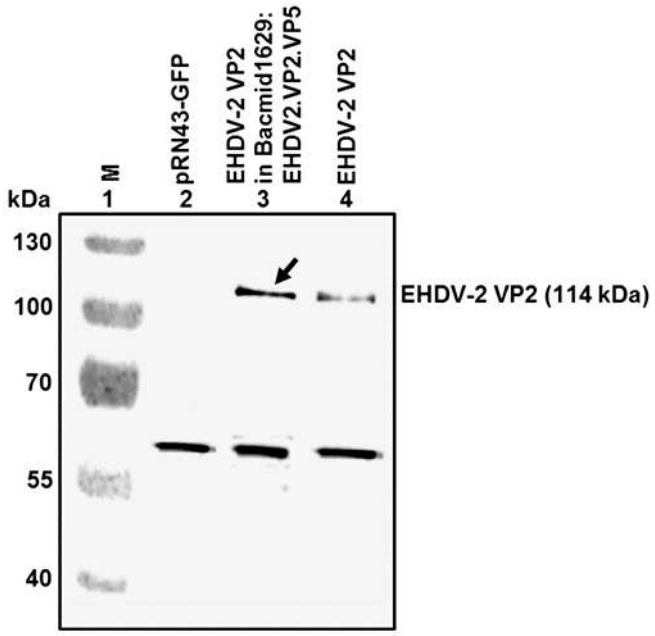

Fig. 72. Immunoblotting analysis of the expression of EHDV-2 VP2 in the dual baculovirus Bacmid$_{1629}$:EHDV2.VP2.VP5. Lane 1: protein ladder, lane 2: *Sf*9 insect cells infected with recombinant AcMNPV pRN43-GFP, lane 3: lysate from *Sf*9 insect cells infected with purified dual recombinant baculovirus expressing EHDV-2 VP2 (114 kDa) (black arrow) and lane 4: individual expression of EHDV-2 VP2 (114 kDa) (positive control).

5.2.2. Generation of heterologous VLPs of EHDV-2

To develop heterologous VLPs composing of EHDV-1 and EHDV-2, monolayers of *Sf*21 insect cells were coinfected with dual recombinant baculoviruses each synthesising two proteins; EHDV-1 VP3 and VP7 and EHDV-2 VP2 and VP5. Several rounds of amplifications were performed in *Sf*9 insect cells from clonal populations to increase the titre of baculovirus. To detect whether the four structural proteins were expressed in correct ratios, 10% SDS-PAGE analysis was undertaken. The detection of each of the recombinant proteins of VP2 (114 kDa) and VP5 (59 kDa) of EHDV-2, VP3 (103 kDa) and VP7 (39 kDa) of EHDV-1 was shown in Figure 73, lane 3 (Fig. 73, lane 4), similar to the sizes of EHDV-1 protein components that were used as positive control (Fig. 73, lane 3).

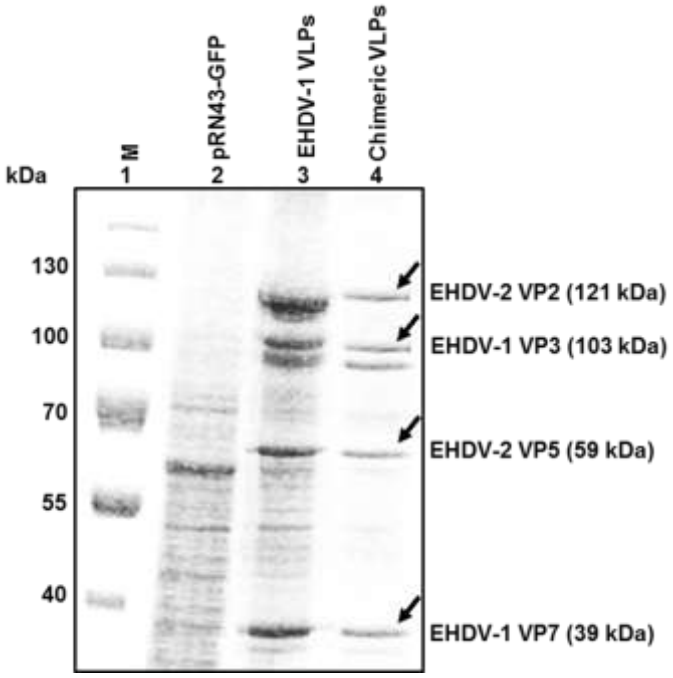

Fig. 73. 10% SDS-PAGE analysis of EHDV-2 heterologous VLPs. Lane 1: protein ladder, lane 2: *Sf*9 insect cells infected with the recombinant AcMNPV pRN43-GFP, lane 3: EHDV-1 VLPs and lane 4: Heterologous VLPs of EHDV-2 (red arrows).

5.2.3. The use of baculovirus multiple genes expression system for the synthesis of EHDV-7 outer capsid proteins

5.2.3.1. Cloning of EHDV-7 S5 into pRN296 transfer vector

The PCR amplification of EHDV-7 S5 coding region (1769 bps) generated in chapter 3, was successfully cloned into pRN296 *Bam*HI linearised and dephosphorylated vector. The cloned recombinant DNA was analysed by agarose gel electrophoresis which showed DNA fragment of the size of 1769 bps in lane 7 (Fig. 74, lane 7). Further PCR amplification was undertaken using PH (F) and EHDV-7 S5 (R) primers to identify the orientation of EHDV-7 S5 in the recombinant plasmid pRN296-EHDV7.S5. Figure 75, lane 3 of agarose gel electrophoresis showed one band equivalent to the size of EHDV-7 S7 (Fig. 75. lane 3). PCR reaction without DNA template was performed as a negative control, showing no bands in lane 3, Figure 75 (Fig. 75, lane 3).

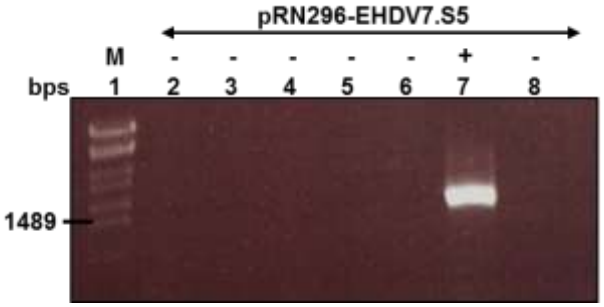

Fig. 74. Molecular analysis to detect the presence of EHDV-7 S5 in the recombinant pRN296-EHDV7.S5 using EHDV-7 S5 (F and R) primers. Lane 1: DNA ladder and lanes 2-8: pRN296-EHDV7.S5. Clone in lane # 7 was positive.

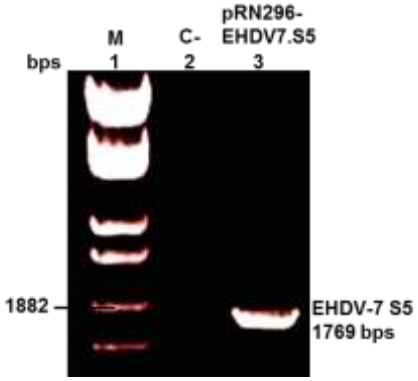

Fig. 75. Molecular analysis to detect the orientation of EHDV-7 S5 in the recombinant pRN296-EHDV7.S5 using PH (F) and EHDV-7 S5 (R) primers. Lane 1: DNA ladder, lane 2: negative control and lane 3: EHDV-7 S5 in the construct pRN296-EHDV7.S5.

5.2.3.2. Generation of the recombinant Bacmid$_{1629}$:EHDV7.S5

To incorporate EHDV-7 S5 into Bacmid$_{1629}$, the expression marker containing S5 gene and *polyh* promoter, *lacZα* and *Zeo^R genes* were amplified by pRN296-ec primers (Table 10). After electroporation of the PCR fragment into E3K2 electrocompetent cells, the presence of the recombinant Bacmid$_{1629}$:EHDV7.S5 was identified in a number of blue colonies. Using EHDV-7 S5 primers, three positive amplicons were visualised by agarose gel electrophoresis (Fig. 76, lane 4, 6 and 7), having DNA fragment similar to the size of EHDV-7 S5 in the positive control

pRN296-EHDV7.S5 (Fig. 76, lane 3). As a negative control, the WT Bacmid$_{1629}$, was used showing no amplification (Fig. 76, lane 2).

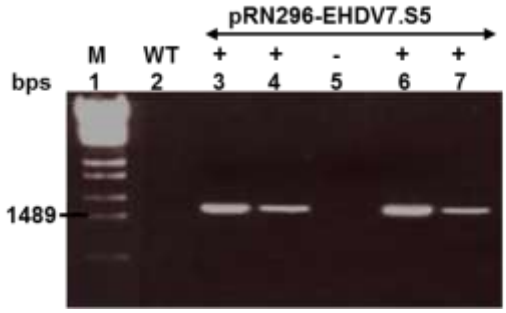

Fig. 76. Molecular analysis to detect the integration of EHDV-7 S5 in the recombinant Bacmid$_{1629}$:EHDV7.S5 using specific EHDV-7 S5 (F and R) primers. Lane 1: DNA ladder, lane 2: Bacmid$_{1629}$ wild type and lanes 3-7: EHDV-7 S5 in the recombinant pRN296-EHDV7.S5. Clones in lanes # 3, 4, 6 and 7 are positives.

To conclude, the ORFs of EHDV-2 S2 and S5 encoding for the outer capsid proteins, essential in the formation of heterologous VLPs, were successfully inserted into baculovirus at *polh* and *odv-e56* loci. High expression level of both recombinant proteins; VP2 and VP5 in the dual Bacmid$_{1629}$:EHDV2.VP2.VP5, was efficiently achieved in *Sf*21 insect monolayers. The expression of EHDV-2 VP2 recombinant protein was further confirmed by western blotting using antisera raised against VP2. A successful formation of EHDV-2 heterologous VLPs were performed and further analysed by SDS-PAGE. An initial attempt to produce EHDV-7 heterologous VLPs using EHDV-1 CLPs as scaffold was undertaken.

5.3. DISCUSSION

For the control of EHD, commercial vaccines are not widely available except the live attenuated vaccine, which was developed for IBAV/EHDV-2 infectious virus in Japan and proved to be safe until 1997 (Ohashi et al., 1999). Since 2006 several outbreaks of severe disease in cattle were documented in the USA and European boarding counties, via multiple strains of EHDV. Hence, the overlapping outbreaks of different EHDV serotypes, particularly EHDV-2 (Omori et al., 1969, Campbell and St George, 1986), EHDV-6 and EHDV-7 (Yadin et al., 2008, Temizel et al., 2009), highlighted the necessity for rapid VLPs production against multiple serotypes.

Developing VLPs for the seven immunogically distinct serotypes of EHDV is far more challenging for several reasons. Generating VLPs require the synthesis of four viral structural proteins (VP2, VP5, VP7 and VP3), two of the genes coding for VP2 and VP3 proteins are large in their sizes (~3 kb). Transfer vectors containing the four viral genes are more than 12 kb in length. More importantly, in practice, re-cloning S2 and S5 genes is not an easy approach due to their toxicity features to the host cell. Thus, the rational solution was the development of heterologous VLPs for EHDV serotypes.

In this study, the aim was the use of baculovirus as a multiple gene expression system for simultaneous synthesis of heterologous VLPs for EHDV. As earlier shown in the previous chapter, to integrate the four structural genes into baculovirus genome at different loci, a specific order should be followed, in which S5 gene should be the first then S3 and S7. However, subcloning the latter gene into specific vectors and inserting them into baculovirus is difficult and time consuming. Therefore, heterologous VLPs were produced for EHDV-2 using EHDV-1 CLPs as a background. In a similar manner, an attempt was carried out to generate a

heterologous VLPs of EHDV-7. The choice of the three serotypes was based on their economic importance and genetic relationships at both the amino acid and nucleotide levels (Allison et al., 2010).

Previous report demonstrated the assembly of heterologous VLPs of BTV-1, -2, -10, -11, -13 and -17 serotypes by coinfection of *Sf*21/9 insect cell lines with three recombinant baculoviruses (Loudon et al., 1991). Though achievable, the resultant recombinant proteins were not expressed in each infected cell in the same expected ratio (Bertolotti-Ciarlet et al., 2003). This was consistent with the results obtained in this work when coinfection with three viruses; EHDV-1 CLPs, EHDV-2 VP2 and VP5 was performed in *Sf*9 insect cells. No expression was seen from EHDV-2 VP2 and only low expression of EHDV-2 VP5 was detected, although, both inner core proteins of EHDV-1 were highly expressed.

Thus, to achieve this target, the previously synthesised single-shelled CLPs of EHDV-1 was subsequently used to prepare the modified VLPs. This was based on the fact that the inner core proteins are relatively well preserved among *Orbivirus* members in comparison to the outer capsid proteins. Moreover, *Belyaev and Roy* have shown that the outer capsid proteins from different BTV serotypes could be coated onto a heterologous inner core protein using baculovirus expression vectors (Belyaev and Roy, 1993). Following such findings, an improved strategy was recently developed for the generation of heterologous VLPs for BTV-2, -4 and 9 serotypes (Stewart et al., 2010), in which BTV-10 core proteins (VP3 and VP7) were used for the generation of heterologous vaccines by replacing the most variable outer capsid proteins (VP2 and VP5) of BTV-2, -4 and -9 (Stewart et al., 2010).

For the formation of dual recombinant baculovirus, VP5 was inserted into the *odv-e56* locus as published literature reported that this site was proven to be suitable for high levels of expression (Noad et al., 2009, Kanai et al., 2013). In order to validate the stable expression of EHDV-2 outer capsid proteins (VP2 and VP5) in insect cells a dual expression virus was recovered and expression level detected on SDS-PAGE, which demonstrated bands of the correct predicted sizes for EHDV-2 VP2 and VP5 viral proteins. For further confirmation, EHDV-1 (VP2 and VP5) proteins that individually expressed using baculovirus were used. A slight difference in terms of size was detected by SDS-PAGE between VP2 of EHDV-1 and that of EHDV-2, but not for VP5 proteins, which is consistent with the molecular weight of these proteins; EHDV-1 VP2 (~ 112 kDa), EHDV-2 VP2 (~ 114 kDa) and ~ 59 kDa for EHDV-1 and EHDV-2 VP5. These results demonstrate the variability in the outermost VP2 proteins of EHDV presumably due to host selective pressure of the humoral immune response, consistent with variability reported in BTV serotypes. Despite the extensive protein expression work that has been undertaken in the baculovirus system for BTV (the closely similar virus to EHDV) (French et al., 1990, French and Roy, 1990), very little literature is available describing the expression of the recombinant proteins for EHDV serotypes. In this report, the antigenic properties of the most variable recombinant protein EHDV-2 VP2 in the purified dual baculovirus Bacmid$_{1629}$:EHDV2.VP2.VP5 were examined by blotting with specific antibodies produced previously. Strong signals of the expressed EHDV-2 VP2 protein were visualised exhibiting similar reactivity to that of VP2 from EHDV-2 virions, indicating that the expressed recombinant VP2 protein in baculovirus system retained the nature of the epitope of the authentic virion.

The incorporation of outermost shell proteins of EHDV-2 (VP2 and VP5) into EHDV-1 CLPs (VP7 and VP3) was analysed by coinfecting the *Sf*9 insect with the dual recombinant baculoviruses. Each of these recombinants expressed two of the capsid proteins: $Bacmid_{1629}$:EHDV2.VP2.VP5 and $Bacmid_{1629}$:EHDV1.VP7.VP3. The results obtained from purified *Sf*9 infected cell lysate, showed the presence of four protein bands equivalent to the size of EHDV-2 (VP2 and VP5), EHDV-1 (VP7 and VP3) in correct ratios when compared to EHDV-1 and EHDV-2 authentic virions. Such findings gave an opportunity for determining the conservative nature of the interacting sites of the structural outer capsid proteins between EHDV-1 and EHDV-2 serotypes.

A preliminary step was conducted in this project to study the possibility of developing heterologous VLPs composed of EHDV-1 CLPs (VP7 and VP3) and EHDV-7 outer capsid proteins (VP2 and VP5). Using baculovirus multiple loci expression system, the coding region of the EHDV-7 S5 was subcloned into transfer vector pRN296 and successfully inserted into baculovirus genome at *odv-e56* locus, however due to time restrictions further study that needed to be performed to validate this heterologous VLP was not possible.

Overall, new data were presented in this report showing a successful generation of the heterologous VLPs of EHDV serotypes. This was achieved by using EHDV-1 CLPs, due to their conservative nature among orbiviruses, which coated by the outer capsid proteins of EHDV-2 (VP2 and VP5). High level synthesis of the recombinant proteins EHDV-2 (VP2 and VP5) was identified from a dual expression baculovirus coinfection. Post coinfection of *Sf*9 insect cells, the authenticity of the structural proteins forming heterologous VLPs EHDV-2 (VP2 and VP5), EHDV-1 (VP7 and VP3), was confirmed by SDS-PAGE in terms of sizes. Further confirmation of

antigenic reactions of the recombinant EHDV-2 VP2 protein in the dual Bacmid$_{1629}$:EHDV2.VP2.VP5, were identified by immunoblotting. Moreover, an initial step to produce heterologous VLPs of EHDV-7 by exchanging the variable outer capsid proteins EHDV-7 (VP2 and VP5) on the conserved EHDV-1 CLPs was established.

Chapter 6

The Recovery of EHDV-2 from RNA transcripts

6.1. INTRODUCTION

EHDV is genetically closely related to BTV, which has been considered as a model for other members of *Orbivirus* genus. Since both EHDV and BTV have similar properties of viral structure and replication, the RG system for EHDV can also contribute to vaccine development, similar to BTV. Hence, using RG system, the aim of this study was to develop a rationally designed attenuated vaccine against EHDV-2, the causative agent of several outbreaks in both cattle and deers (Omori et al., 1969, Campbell and St George, 1986).

The first indication that RG system could be possible for the members of *Reoviridae* family was reported by *Roner and Joklik* (Roner and Joklik, 2001). They reported the ability of recovering the infectious reovirus using core transcripts, synthesised *in vitro*, combining with a transcript of one of the virus genome segments (Roner and Joklik, 2001). Later, *Boyce et al* has shown that the recovery of BTV-1 virus entirely from ssRNA extracted from cores could be achieved without using helper vector (Boyce and Roy, 2006). In this study, aimed at generating a RG system for EHDV-2, the possibility of recovering the infectious EHDV-2 from core transcripts *in vitro* synthesised would be examined in BSR monolayers.

Infectious BTV has been entirely produced from cDNA, by generating *in vitro* T7-transcripts for each of the 10 RNA segments, and by transfecting the permissive cells with the generated transcripts (Boyce et al., 2008). Such successful findings allowed the generation of specific mutations that could be exploited in the development of an attenuated vaccine. Therefore, to investigate whether EHDV-2 could be recovered from the 10 synthetic T7 transcripts, exact copy of the 10 segments needed to be cloned into pUC19 transfer vector, wherein the segments

are flanked by T7 promoter at the 5` end and a restriction enzyme site at the 3` end, respectively.

6.2. RESULTS

6.2.1. Isolation of EHDV-2 virus

To ensure the purify of a clonal population of EHDV-2, Alberta/Canada (CAN1962) strain provided from a collaborator's laboratory, BSR monolayers were infected with EHDV-2 and individual plaques were isolated from varying dilutions of the viral stocks, and grown in BSR monolayer. To examine the migration pattern of the segmented genome, the dsRNAs were extracted and purified as discussed in the Materials and Methods. The profile and the purity of the genome segments were visualised on the nondenaturing 9% PAGE gel. Ethidium bromide staining showed 10 segments of the dsRNAs S (1-10) from large to small RNA segment, in order of their migration. Each RNA segment size was correlated to the estimated sizes of S1 (3942 bps), S2 (3002 bps), S3 (2768bps), S4 (1983 bps), S5 (1803 bps), S6 (1641 bps), S7 (1162 bps), S8 (1186 bps), S9 (1143 bps) and S10 (810 bps) (Fig. 77, lane 2). As a positive control, BTV-1 genomic RNAs were used (Fig. 77, lane 1).

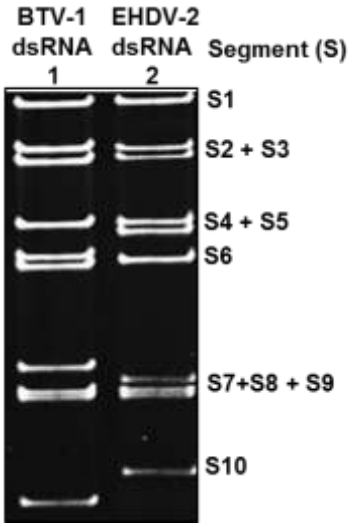

Fig. 77. 9% PAGE analysis of the 10 RNA segments of EHDV-2 showing the migration pattern of each segment. Lane 1: BTV-1 dsRNAs (positive control) and lane 2: EHDV-2 dsRNAs from plaque #1.

6.2.2. Purification of EHDV-2 cores and *in vitro* synthesis of EHDV-2 ssRNAs

To analyse whether EHDV-2 cores were transcriptionally active, confluent BSR monolayers were infected with EHDV-2 and the virus titre was analysed by plaque assay (Fig. 78). For the preparation and purification of EHDV-2 cores, BSR cells were infected with the virus at a MOI of 1.0. Following purification, 10% SDS-PAGE analysis of the purified cores was carried out showing the existence of all five proteins (VP1, VP3, VP4, VP6 and VP7) that forms the cores in the expected ratios with the absence of the outer capsid proteins, VP2 and VP5 (Fig. 79, lane 4). For the synthesis of EHDV-2 ssRNAs, core concentration was estimated using 0.2 µg/µl (Fig. 79, lane 2) and 0.5 µg/µl (Fig. 79, lane 3) of BSA protein. Using Bradford protein assay, the total concentration of the purified cores was estimated approximately equal to 0.3 µg/µl (Fig. 79, lane 4).

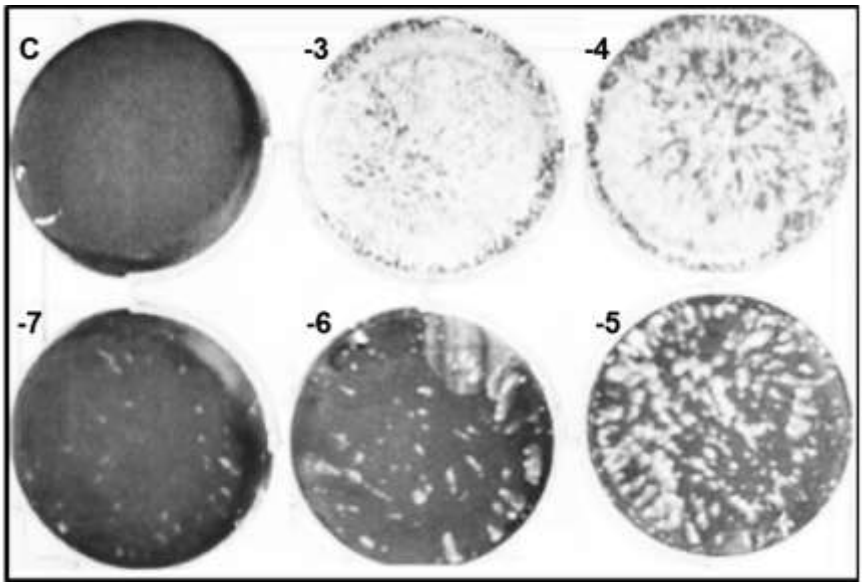

Fig. 78. Quantification of EHDV-2 virus in BSR cells. BSR monolayer fixed with 10% formaldehyde and stained with crystal violet 3 days post infection.

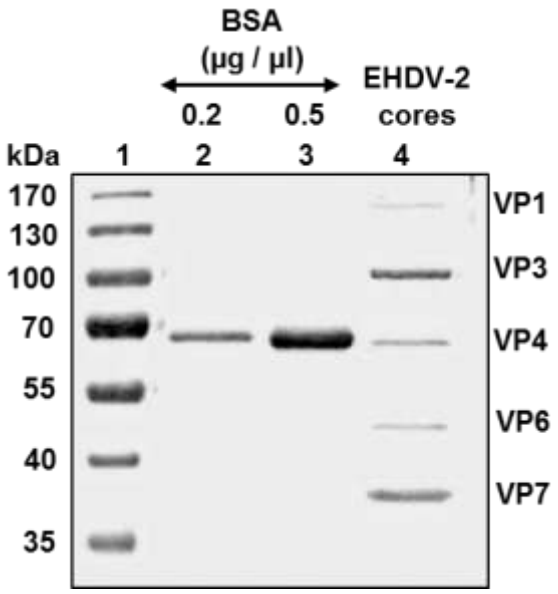

Fig. 79. 10% SDS-PAGE analysis of the purified EHDV-2 cores. Lane 1: protein ladder, lane 2: 0.2 µg/µl of BSA protein, lane 3: 0.5 µg/µl of BSA protein and lane 4: EHDV-2 core proteins.

In vitro, EHDV-2 ssRNAs were synthesised using the *in vitro* transcription conditions for the cores which are discussed in the Materials and Methods. To remove the infectious cores from the newly synthesised RNA transcripts, at the end of transcription process, ultracentrifugation was performed twice followed by two rounds of phenol-chloroform extraction to deproteinize the sample. The total RNA containing both the ssRNA transcripts and dsRNA was isolated. The ssRNAs were selectively precipitated by 2 M LiCl treatment as discussed in the Materials and Methods, and examined on 1% denaturing agarose gel electrophoresis using 0.5 µg/µl of the purified transcripts. Figure 80, lane 2 displays the 10 viral transcripts; no premature termination or degradations were observed (Fig. 80, lane 2).

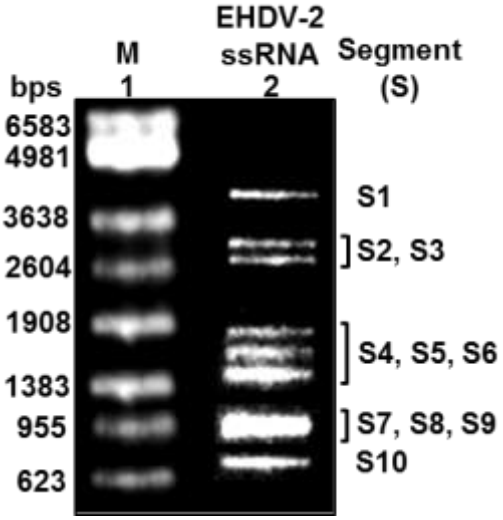

Fig. 80. Denaturing 1% agarose gel electrophoresis of purified EHDV-2 ssRNAs. Lane 1: ssRNAs marker (1 µg) and lane 2: 1.5 µg EHDV-2 ssRNAs

6.2.3. Recovery of the infectious EHDV-2 from BSR cells transfected with purified EHDV-2 ssRNAs

To examine the probability of recovering the infectious virus EHDV-2 from ssRNA transcripts, BSR cells were transfected twice, 18 hours apart, with a total of 1.0 µg or 1.5 µg of ssRNAs for each transfection as described for BTV (Matsuo and Roy, 2009). After 48 hours of incubation, CPEs similar to that caused by EHDV-2 infection, were detected in BSR cells transfected with 1.0 µg ssRNAs (Fig. 81B). A complete CPE was observed at 3 dpt, in the BSR monolayers transfected with 1.0 µg of EHDV-2 ssRNAs (Fig. 81D), while 100% CPE was detected in BSR cells transfected with 1.5 µg of ssRNAs just after 18 hours of the first transfection (Fig. 81C). The untreated BSR cells were used as negative control (Fig. 81A).

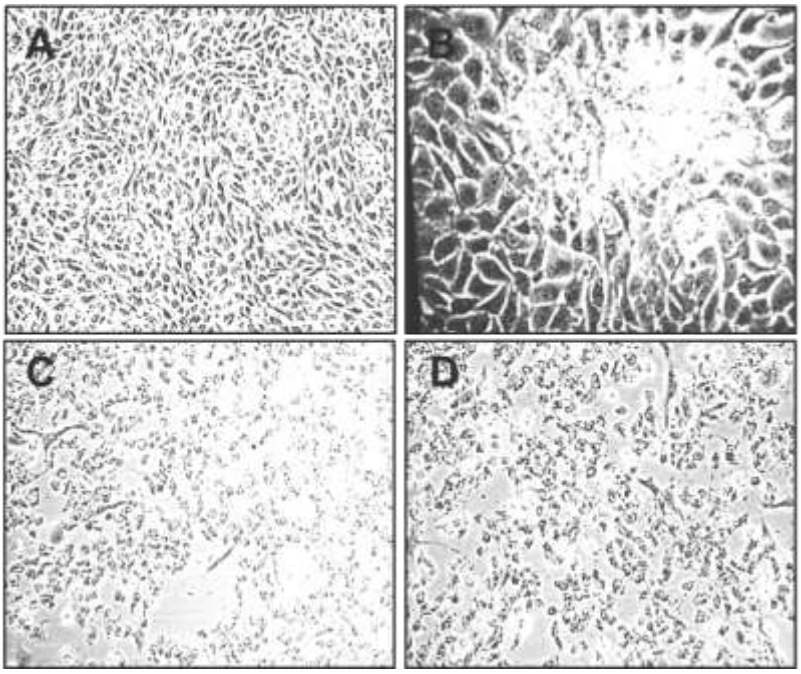

Fig. 81. The recovery of EHDV-2 virus from ssRNA transcripts. (A) Untreated BSR cells (B) BSR cells transfected with 1.0 µg of EHDV-2 ssRNAs at 48 hpt (C) BSR cells transfected with 1.5 µg of EHDV-2 ssRNA at 18 hpt and (C) BSR cells transfected with 1.0 µg of EHDV-2 ssRNA at 72 hpt.

To detect whether the viral ssRNAs were replicated to produce the genomic dsRNAs were extracted at 72 hpt and purified from BSR cells that had been transfected with 1.0 µg of EHDV-2 ssRNAs. The dsRNA genome profile was analysed by electrophoresis on 1% agarose gel (Fig. 82). Gels show 10 discrete segments of dsRNA derived from transfection (Fig. 82, lane 3), indistinguishable from that obtained from EHDV-2 virus (Fig. 82, lane 2), confirming that the dsRNAs were efficiently synthesised in the BSR cells transfected with EHDV-2 core transcripts (Fig. 82, lane 3).

Simultaneously, BSR cells were inoculated with 60 µl of 1 ml supernatant harvested from transfected cells, to determine the presence of the infectious virus. After 3 days of inoculation, a 100% CPE was detected through the entire culture confirming the presence of the infectious virus (Fig. 83B).

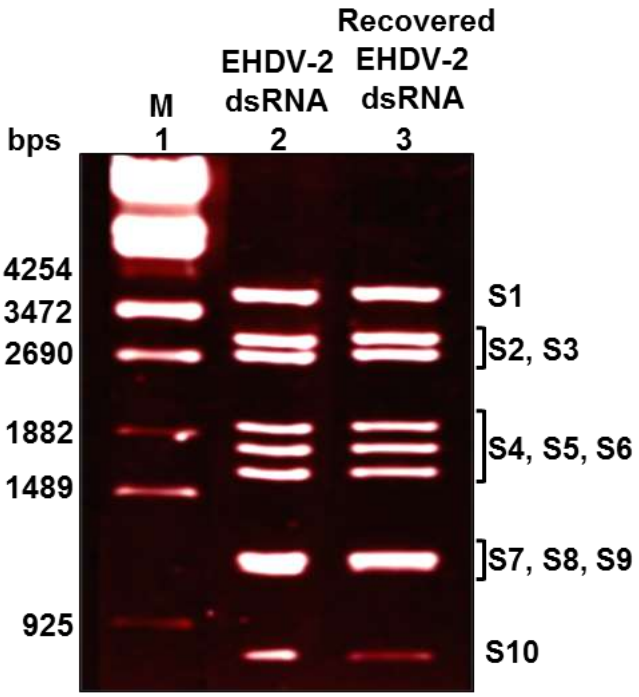

Fig. 82. 1% agarose gel electrophoresis of the synthesis of EHDV-2 dsRNAs from the recovered virus. Lane 1: DNA marker, lane 2: EHDV-2 dsRNAs extracted from BSR cells infected with EHDV-2 virus and lane 3: dsRNAs extracted from transfected BSR cells 72 hpi.

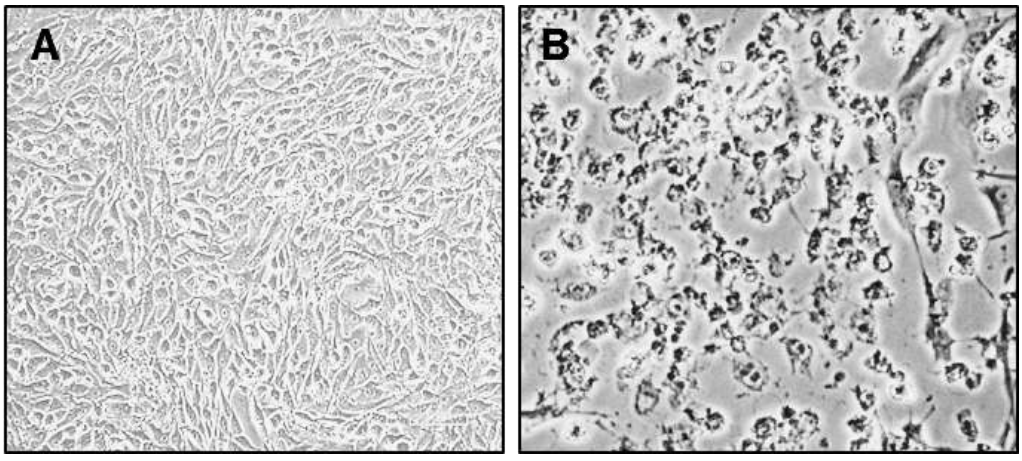

Fig. 83. Infection of BSR cells with the culture harvested from cells transfected with 1.0 µg ssRNA. (A) Uninfected BSR cells (B) 2.5 X10^5 BSR cells infected with 60 µl of the culture harvested from cells transfected with 1.0 ssRNA. The images were captured 3 days post infection (dpi).

The recovered virus was quantified and the pattern of the plaques were determined in the infected BSR monolayers overlaid with low melting agarose gel. Plaques produced from the recovered virus (Fig. 84A) had the same morphology as that obtained by viral infection (Fig. 84B). Individual plaques were picked and amplified by infecting monolayers of 10^6 BSR cells, followed by extraction of dsRNAs from one plaque. The separated dsRNAs analysed by 0.8% agarose gel electrophoresis demonstrated 10 dsRNA segments (Fig. 85, lane 2) similar in terms of sizes and mobility to that obtained from infection (Fig 85, lane 1).

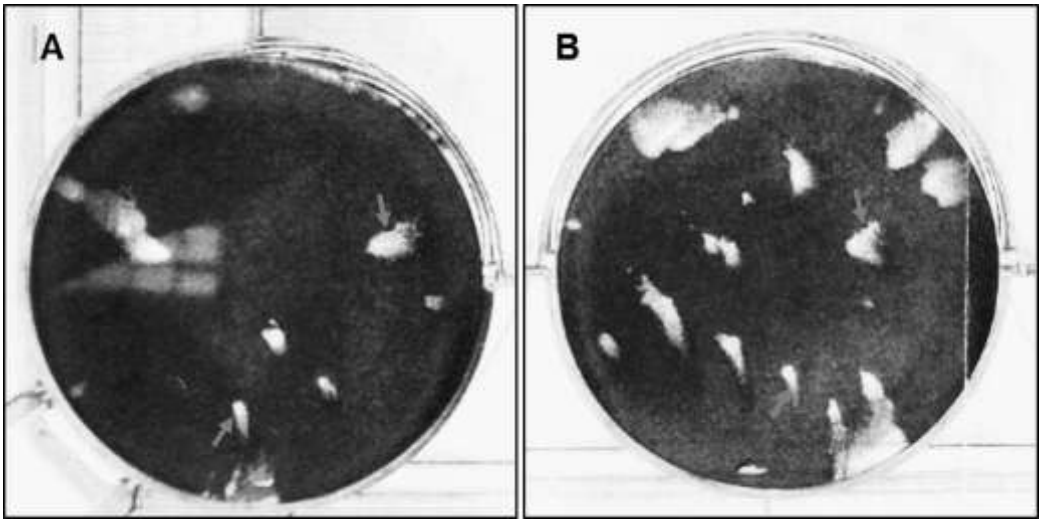

Fig. 84. Plaques morphology in BSR cells infected with the recovered EHDV-2 virus (A) and the wild type EHDV-2 (B) fixed with 10% formaldehyde and stained with crystal violet 3 dpi.

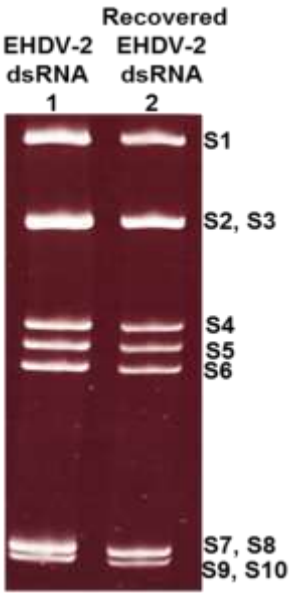

Fig. 85. 0.8% agarose gel analysis of the segmented pattern of EHDV-2 dsRNAs. Lane 1: EHDV-2 dsRNAs extracted from BSR cells infected with EHDV-2 virus and lane 2: dsRNAs extracted from BSR cells infected with the recovered EHDV-2.

6.2.4. The recovery of EHDV-2 from plasmid-derived T7 transcripts

6.2.4.1. Amplification of the 10 RNA segments of EHDV-2

Following the successful recovery of the infectious EHDV-2 from core transcripts, the recovery of the virus entirely from the 10 plasmid-derived T7 transcripts using RG system was examined. The cDNA copies of the 10 genomic segments of EHDV-2 were amplified from viral dsRNA in a sequence-independent manner using the method of FLAC. After ligating the self-priming anchor-primer C9 (Table 12) at both sides of the 10 segments of viral dsRNA, the unlinked oligos were removed by electrophoresis of dsRNA on 0.8 % agarose gel. For the synthesis of first-strand cDNA, the 10 segments of dsRNA were excised, purified from gel and subjected to the RT-PCR step using Premium RevertAid RT enzyme. Subsequently, the full-length cDNA copies of all 10 EHDV-2 genome segments were amplified using FLAC2 primer (Table 12). The PCR products were visualised on 1% agarose gel as

a single specific band corresponding respectively to the published sizes of EHDV-2 S (1-10) (Table 8) (Fig. 86, A–H).

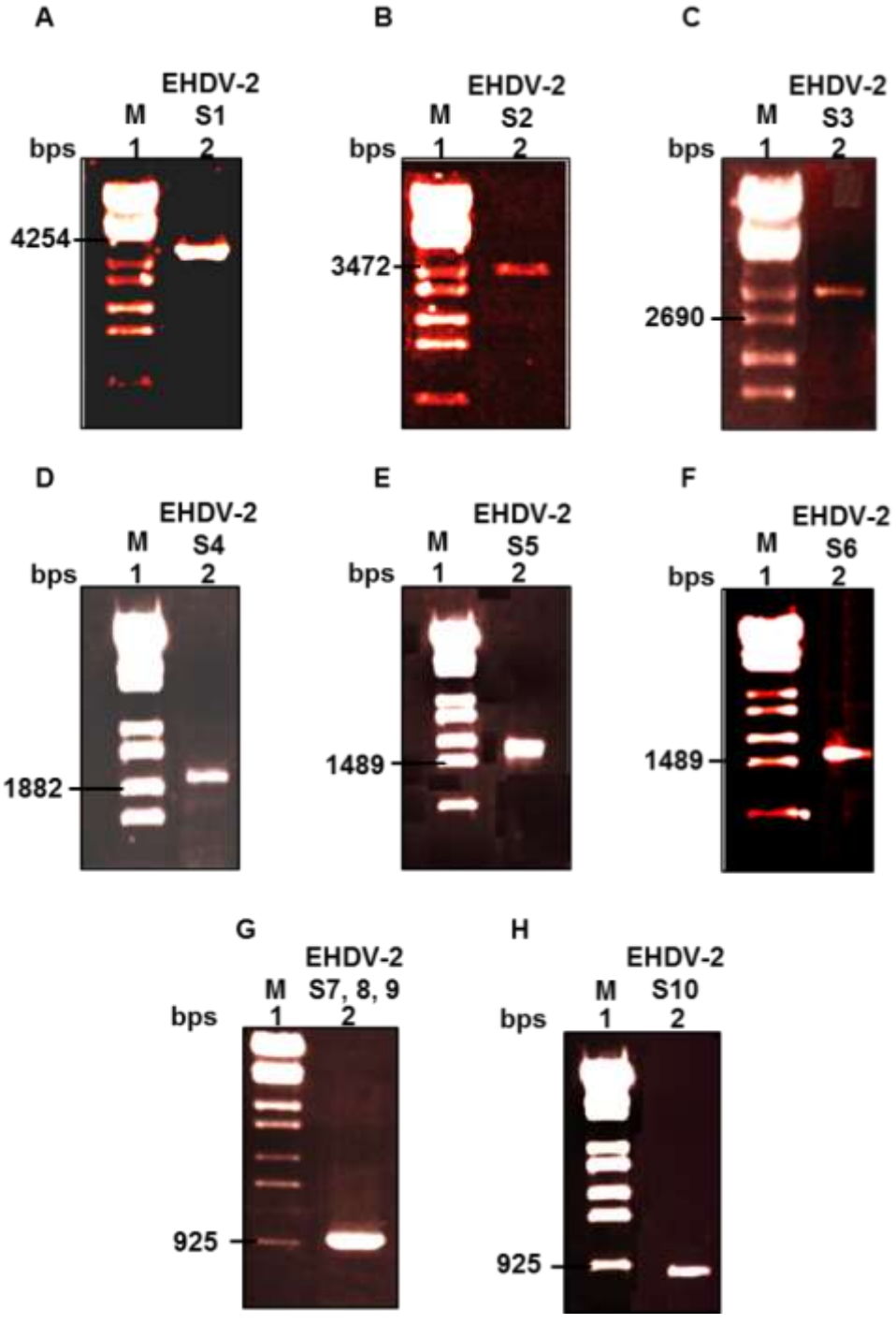

Fig. 86. 1% agarose gel analysis of the full-length cDNA copies from the 10 segments of EHDV-2 generated by FLAC2 primer. (A) EHDV-2 S1. Lane 1: DNA ladder and lane 2: EHDV-2 S1. (B) EHDV-2 S2. Lane 1: DNA ladder and lane 2: EHDV-2 S2. (C) EHDV-2 S3. Lane 1: DNA ladder and lane 2: EHDV-2 S3. (D) EHDV-2 S4. Lane 1: DNA ladder and lane 2: EHDV-2 S4. (E) EHDV-2 S5. Lane 1: DNA ladder and lane 2: EHDV-2 S5. (F) EHDV-2 S6. Lane 1: DNA ladder and lane 2: EHDV-2 S6. (G) EHDV-2 S (7+8+9). Lane 1: DNA ladder and lane 2: EHDV-2 S (7+8+9). (H) EHDV-2 S10. Lane 1: DNA ladder and lane 2: EHDV-2 S10.

6.2.4.2. Cloning of the 10 cDNAs of EHDV-2 into pUC19 vector

The amplified cDNA copies were purified from 1% agarose gel and cloned into a *Sma*I linearized pUC19 vector (Fig. 87A). Efficient linearisation of pUC19 was identified by gel electrophoresis (Fig. 87B, lane 3) in comparison to the circular vector (Fig. 87B, lane 2). To detect the generation of the 10 recombinants plasmids containing each of EHDV-2 segments, colony PCR was performed using FLAC2 primer. The correct integration of each segment was visualized by 1% agarose gel electrophoresis as single specific band corresponding to the estimated size of each segment as shown in Figure 88 (Fig. 88, A-F) and Figure 89 (Fig. 89, A-D).

The recombinant plasmids were extracted and sequenced using primers specific to the vector M13 (F and R) (Table 12) and the respective gene (Table 13). The results of the full sequences were confirmed by BlastN to be EHDV-2 S (1-10) (Table 8).

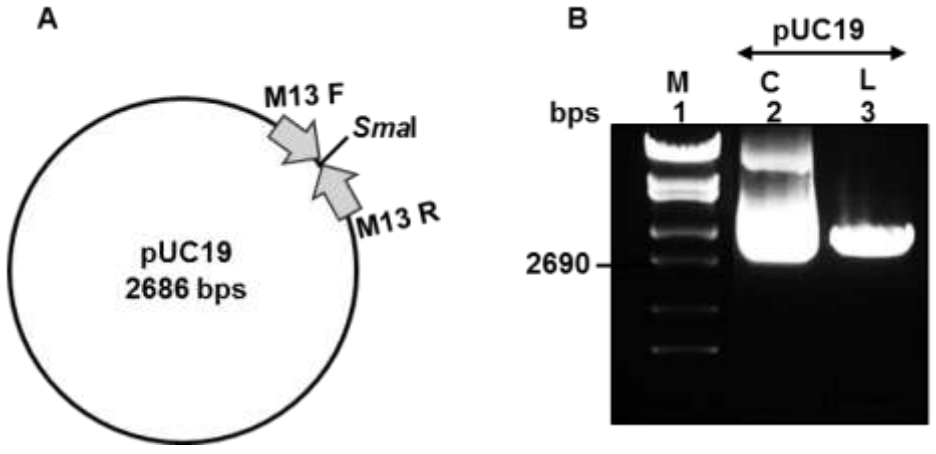

Fig. 87. Molecular analysis of pUC19 vector. (A) Map of pUC19 transfer vector showing M13 (F and R) primers and *Sma*I endonuclease enzyme site. (B) *Sma*I restriction digestion of pUC19 vector visualized by1% agarose gel electrophoresis. Lane 1: DNA ladder, lane 2: pUC19 (circular) and lane 3: pUC19 (linear).

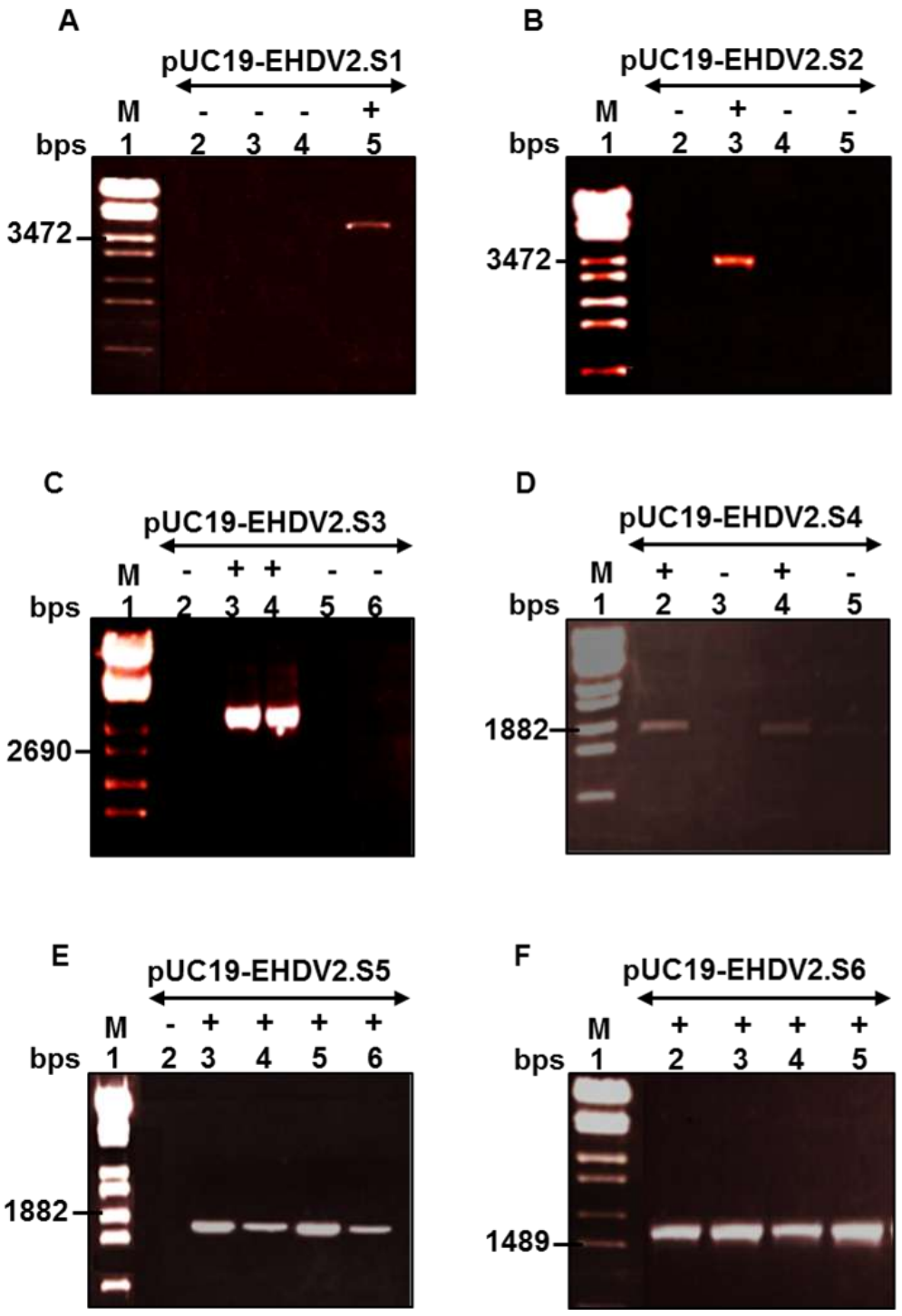

Fig. 88. Colony PCR with FLAC2 primer visualised by 1% agarose gel electrophoresis to detect the correct cloning of EHDV-2 (S1-S6) in pUC19 vector. (A) pUC19-EHDV2.S1. Clone in lane 5 is positive. (B) pUC19-EHDV2.S2. Clone in lane 3 is positive. (C) pUC19-EHDV2.S3. Clones in lanes 3 and 4 are positives. (D) pUC19-EHDV2.S4. Clones in lanes 2 and 4 are positives. (E) pUC19-EHDV2.S5. Clones in lanes 3, 4, 5, and 6 are positives and (F) pUC19-EHDV2.S6. All clones are positives.

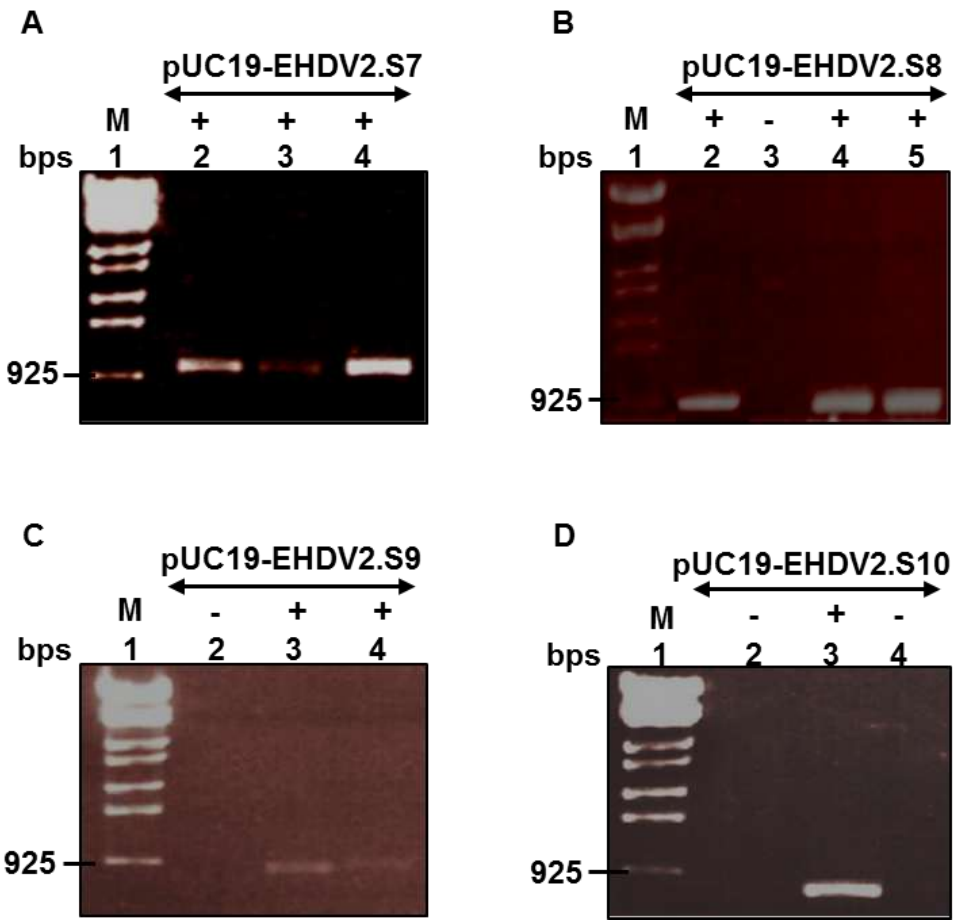

Fig. 89. Colony PCR with FLAC2 primer visualised by 1% agarose gel electrophoresis to detect the correct cloning of EHDV-2 (S7-S10) in pUC19 vector. (A) pUC19-EHDV2.S7. Clones in lanes 2 and 4 are positives. (B) pUC19-EHDV2.S8. Clones in lanes 2, 4 and 5 are positives. (C) pUC19-EHDV2.S9. Clones in lanes 3 and 4 are positives. (D) pUC19-EHDV2.S10. Clone in lane 3 is positive.

6.2.4.3. The construction of T7 clones of EHDV-2 10 segments

Once a complete set of the 10 EHDV-2 clones was achieved, primers with T7 promoter and reverse primer containing a specific endonuclease enzyme for each segment were designed (Table 18). To introduce the T7 promoter upstream of the genome segment and the restriction enzyme site downstream, each segment was amplified by PCR and the products were visualised by 1% agarose gel electrophoresis. Figure 90 shows 10 precise DNA fragments corresponding to the correct sizes of EHDV-2 genes (Fig. 90, lanes 2-11).

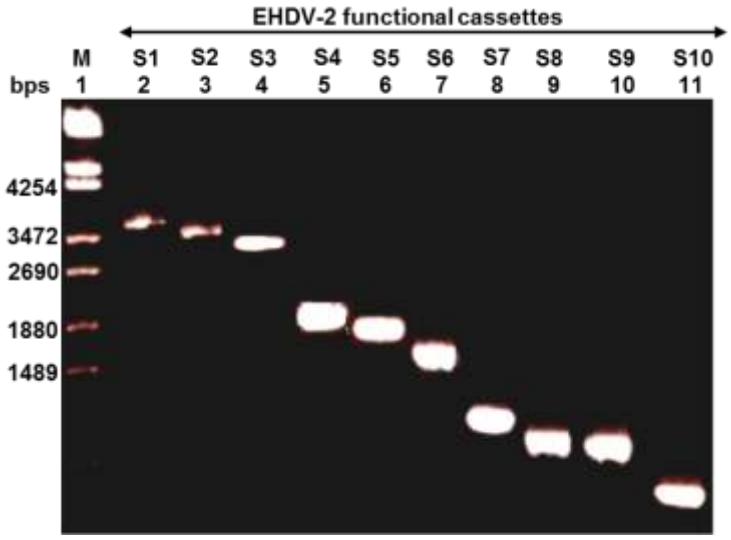

Fig. 90. Amplification of the functional cassettes containing T7 promoter, EHDV-2 segments and specific enzyme visualised by 1% agarose gel electrophoresis. Lane 1: DNA marker, lane 2: EHDV-2 S1, lane 3: EHDV-2 S2, lane 4: EHDV-2 S3, lane 5: EHDV-2 S4, lane 6: EHDV-2 S5, lane 7: EHDV-2 S6, lane 8: EHDV-2 S7, lane 9: EHDV-2 S8, lane 10: EHDV-2 S9 and lane 11: EHDV-2 S10.

The amplified EHDV-2 segments were subcloned into *Sma*I linearised pUC19 vector. The introduction of each enzyme in a correct orientation was confirmed by restriction digestion with the enzyme sites had been incorporated into the primers. For instance, *Bsm*bI digestion was performed to detect the integration of EHDV-2 S1 gene into pUC19-EHDV2.S1. The results were visualised on agarose gel as two fragments, one corresponding to the size of EHDV-1 S1 (3942 bps) and another to the size of pUC19 (2500 bps) (Fig. 91, lane 3). In lane 5, Figure 91, two bands were shown equivalent to EHDV-2 S2 (3002 bps) and pUC19 post digestion with *Bsa*I (Fig. 91, lane 5), while lane 4 revealing the circular recombinant pUC19-EHDV2.S2 (Fig. 91, lane 4). Additionally, the construct pUC19-EHDV2.S3 (Fig. 92, lane 2) was linearised by *Bsa*I enzyme and the data shown as one band in lane 3, Figure 92 (Fig. 92, lane 3). The presence of the *Bsa*AI enzyme site in the recombinant plasmid pUC19-EHDV2.S4 was shown in lane 5, Figure 92 (Fig. 92, lane 5). Recombinant plasmids; pUC19-EHDV2.S5 and pUC19-EHDV2.S6 were digested by *Bsa*I and

*Bbs*I, respectively. The efficient linearisaion was detected in lanes 7 and 9 post electrophoresis on a 1% agarose gel (Fig. 92, lanes 7 and 9).

The four small segments S (7-10), were linearised with *Bsa*I, *Bsm*bI, *Bsb*I and *Bsb*I, respectively, and data displayed by gel electrophoresis (Fig. 93, lane 3), (Fig. 93, lane 5), (Fig. 94, lane 3) and (Fig. 94, lane 5), correspondingly. After the successful generation of the 10 T7 clones, the existence of T7 promoter was defined by sequencing using M13 F primer.

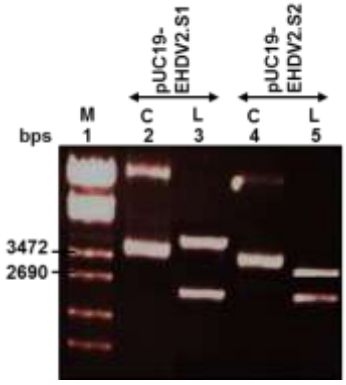

Fig. 91. Molecular analysis to determine the presence of EHDV-2 S1 and S2 in the recombinant plasmids by 1% agarose gel electrophoresis. Lane 1: DNA marker, lane 2: pUC19-EHDV2.S1 (circular), lane 3: *Bsm*bI digestion of pUC19-EHDV2.S1, lane 4: pUC19-EHDV2.S2 (circular) and lane 5: *Bsa*I digestion of pUC19-EHDV2.S2.

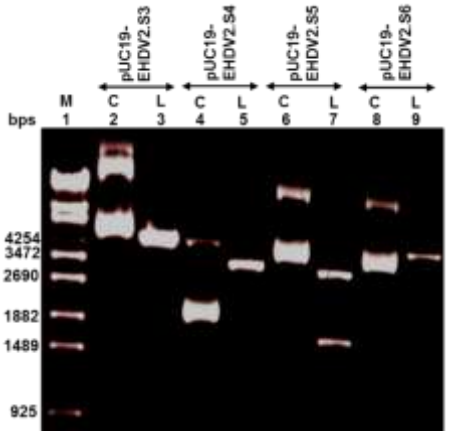

Fig. 92. Molecular analysis to determine the presence of EHDV-2 S3, S4, S5 and S6 in the recombinant plasmids by 1% agarose gel electrophoresis. Lane 1: DNA marker, lane 2: pUC19-EHDV2.S3 (circular), lane 3: *Bsa*I digestion of pUC19-EHDV2.S3, lane 4: pUC19-EHDV2.S4 (circular), lane 5: *Bsa*AI digestion of pUC19-EHDV2.S4, lane 6: pUC19-EHDV2.S5 (circular), lane 7: *Bsa*I digestion of pUC19-EHDV2.S5, lane 8: pUC19-EHDV2.S6 (circular) and lane 9: *Bbs*I digestion of pUC19-EHDV2.S6.

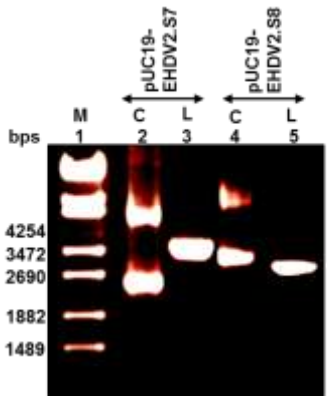

Fig. 93. Molecular analysis to determine the presence of EHDV-2 (S7 and S8) in the recombinant plasmids by 1% agarose gel electrophoresis. Lane 1: DNA marker, lane 2: pUC19-EHDV2.S7 (circular), lane 3: *Bsa*I digestion of pUC19-EHDV2.S7, lane 4: pUC19-EHDV2.S8 (circular) and lane 5: *Bsm*bI digestion of pUC19-EHDV2.S8.

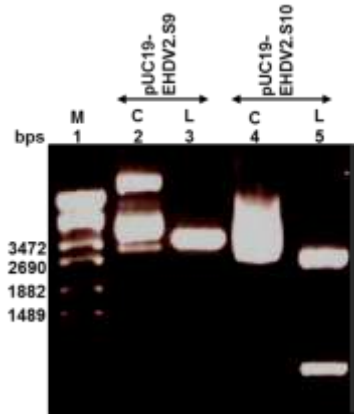

Fig. 94. Molecular analysis to determine the presence of EHDV-2 (S9 and S10) in the recombinant plasmids by 1% agarose gel electrophoresis. Lane 1: DNA marker, lane 2: pUC19-EHDV2.S9 (circular), lane 3: *Bbs*I digestion of pUC19-EHDV2.S9, lane 4: pUC19-EHDV2.S10 (circular) and lane 5: *Bbs*I digestion of pUC19-EHDV2.S10.

6.2.4.4. The synthesis of EHDV-2 transcripts from the 10 T7 plasmids clones

To generate the 5` cap analogue of the 10 EHDV-2 T7 transcripts, 1µg of the restriction digested T7 plasmid clones of each segment was used. For efficient generation of the capped transcripts, mMESSAGE mMACHINE T7 Ultra Kit was used. The precipitated RNA of each individual transcript was predicted to have equivalent size to that of the messenger RNA (mRNA) strands corresponding to the EHDV-2 genome segments as visualised on 1% denaturing agarose gel (Fig. 95).

Post transcription, BSR monolayers were transfected with 150 to 250 ng of each EHDV-2 transcript. Transfection process was performed twice, 18 hours apart, as discussed in detail in Materials and Methods. After the second transfection, the transfected monolayers were overlaid with agarose and incubated at 35°C for 72 hours. However, though a clear difference was observed between the transfected and non-transfected BSR cells morphology, no plaques were observed (Fig. 96, A and B), indicating no virus recovery.

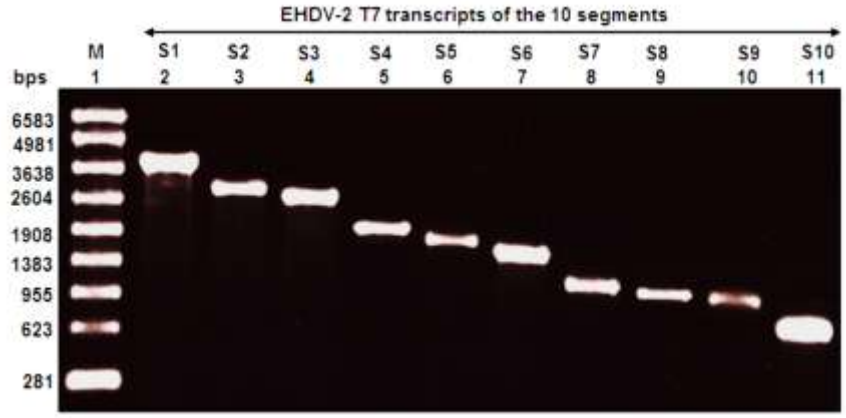

Fig. 95. Denaturing 1% agarose gel electrophoresis of EHDV-2 T7 transcripts generated from digested clones. Lane 1: ssRNA marker (1.0 µg), lane 2: EHDV-2 S1, lane 3: EHDV-2 S2, lane 4: EHDV-2 S3, lane 5: EHDV-2 S4, lane 6: EHDV-2 S5, lane 7: EHDV-2 S6, lane 8: EHDV-2 S7, lane 9: EHDV-2 S8, lane 10: EHDV-2 S9 and lane 11: EHDV-2 S10.

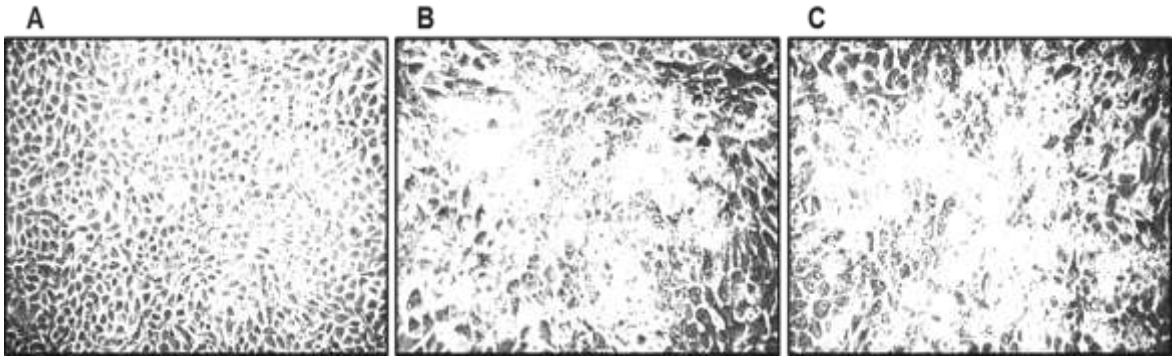

Fig. 96. BSR cells transfection with the entire T7 transcripts of EHDV-2 showing a difference between the treated and untreated cells. (A) Untreated BSR cells. (B) BSR cells transfected with 1.0 µg of EHDV-2 T7 transcripts 72 hpt. (C) BSR cells transfected with 1.5 µg of EHDV-2 T7 transcripts 72 hpt.

In conclusion, EHDV-2 was successfully recovered from ssRNAs after the efficient *in vitro* synthesis of the ssRNA transcripts from purified EHDV-2 cores. The entire 10 T7 transcripts of EHDV-2 were efficiently generated from the linearised T7 plasmid-derived clones, which, in this study, were all cloned into pUC19 vector with the designed specific primers from a purified clonal population of EHDV-2. Due to the limited time for this study, it was not possible to investigate further the concentrations of T7 transcripts required for the recovery of infectious virus.

6.3. DISCUSSION

The aim of this study was to establish of a plasmid-based RG system for EHDV entirely based on synthetic transcripts, in a manner similar to that of BTV and AHSV. Since EHDV is genetically closely related to BTV, a similar RG system for EHDV could be utilised for the development of a rationally designed attenuated vaccine. EHDV-2 was chosen in particular, since this serotype has been circulating in many countries in the past and more recently, caused outbreaks in both domestic and wild animals (Omori et al., 1969, Campbell and St George, 1986).

The RG system set up for BTV (Boyce et al., 2008, Boyce and Roy, 2007) has been demonstrated to be a novel approach for the generation of a highly successful defective vaccine strain (Roy et al., 2009, Matsuo et al., 2011). The production of such defective virus strains was due to the understanding of BTV virus infection at molecular levels, which permitted to the generation of specific mutations in the enzymatic protein that is essential for the viral replication (Matsuo and Roy, 2009, Kaname et al., 2013).

Applying the RG system, a rapid generation of attenuated vaccines against several emerging serotypes could be achieved. This was previously reported by using a consistent genetic background of the defined attenuated serotype (i.e. BTV-1), and replacing the antigenically genome segments encoding the variable outer capsid proteins VP2 and VP5 with the serotype of highly virulent strain (i.e. BTV-8) (Matsuo et al., 2011).

These vaccines would be as safe as the live attenuated vaccines but retain the ability to produce viral proteins at the infection sites. Moreover, protective immune

responses could be elicited in vaccinated animals but lose the ability to revert to the virulent strain making them safer than the current live vaccines.

Generally, in the members of *Reoviridae* family, the core particles have two functions; the synthesis of ssRNAs in the cytoplasm of infected cells which then act as mRNAs for viral protein formation, and also serve as templates for the production of the new dsRNA genome. Several published literatures confirmed that BTV (Boyce and Roy, 2007) and AHSV (Matsuo et al., 2010) core transcripts were infectious when BSR monolayers were transfected with them. This work investigated the possibility of recovering the infectious EHDV-2 from the transcripts synthesised *in vitro* from cores, without the use of helper virus (Roner and Joklik, 2001).

Several attempts were carried out to increase the yield and purity of EHDV-2 cores. Eventually, following the optimised protocols described for BTV, the EHDV-2 cores were successfully prepared and purified from infected cells, and cores purity was shown to be high by 10% SDS-PAGE gel analysis. In consistent with BTV and AHSV findings (Boyce and Roy, 2007, Matsuo et al., 2010), all five core proteins (VP1, VP3, VP4, VP6 and VP7) were detected in the expected ratios.

For *in vitro* synthesis of EHDV-2 ssRNAs, this study was based on the protocols described for BTV, due to the lack of published reports regarding EHDV. Similarly to *Matsuo et al.* (Matsuo et al., 2010), 60 µM $MnCl_2$ was added to the transcription reaction mixture, which enhances the efficiency of *in vitro* transcription synthesis. A sufficient amount and high purity profile of the 10 transcripts synthesised *in vitro* as observed on denaturing agarose gel electrophoresis, with no indications of early termination or noticeable degradation.

The recovery of EHDV-2 infectious virus from ssRNA was successfully achieved in BSR cells transfected twice with the core transcripts. This was based on earlier study demonstrated that double-transfection is more effective in the recovery of AHSV serotype 4 than singly transfecting alone (Matsuo et al., 2010). Additionally, the study confirmed that virus recovery was not due to the quantity of ssRNAs, but due to the primary replication (Matsuo et al., 2010). Such findings prevented the need for high concentration of transcripts, such as that used for the recovering of BTV-1 from ssRNAs in double transfection (4-8 µg) (Boyce and Roy, 2007).

Since, no previous reports were published addressing the recovery of EHDV from core transcripts, in this study, the sufficient amount of EHDV-2 ssRNAs needed to recover the infectious virus was determined by transfecting BSR monolayers twice, 18 hours apart, with 1.0 µg or 1.5 µg. The 1.0 µg of ssRNAs were found to be the optimal quantity, as a 100% CPE was observed at 72 (hpt). In contrast, a complete CPE was shown in less than 24 h of the first transfection when 1.5 µg of ssRNAs were used, indicating that this amount of ssRNAs is too much and not suitable for the recovery of EHDV-2 virus. For further confirmation of the obtained data, the experiment was repeated twice followed by BSR cells infection with the supernatant harvested from that transfected with 1.0 µg and 1.5 µg of the ssRNAs. The presence of the infectious virus was detected in BSR cells infected with supernatant harvested from transfected cells with 1.0 µg, while no CPE was observed from that infected with the supernatant harvested from BSR cells transfected with 1.5 µg. Comparing the morphology of the plaques produced from BSR cells transfected with EHDV-2 ssRNAs to that generated by EHDV-2 infection, no recognisable differences in terms of size and appearance were identified. These results confirmed that infectious

EHDV-2 was effectively produced in BSR cells transfected with purified ssRNAs and the utility of the RG system described for BTV to other closely related orbiviruses.

Whilst recovery from core ssRNA transcripts demonstrates the utility of the RG system, in order to effectively design attenuated vaccine strains transcripts must be amenable to manipulation by molecular biology techniques. To such an end, the synthesis of *in vitro* RNA transcripts by T7 RNA polymerase of cDNA clones is required, as shown in earlier studies (Boyce et al., 2008). This RG system has been established using two different approaches; either by mixing the viral transcripts and T7 transcripts, or by utilising complete set of the *in vitro* synthesised T7 transcripts (Matsuo et al., 2010, Boyce et al., 2008). In this work, in order to investigate whether EHDV-2 could be recovered from transcription of cDNA clones of its ten segments, an attempt of recovery from 10 *in vitro* transcribed, T7 transcripts was carried out.

To date, no published reports were found regarding the establishment of an EHDV-2 RG system. Therefore, to design exact T7 primers containing gene specific sequence, the full-length sequences of the 10 segments were cloned and compared with published data. Applying a FLAC2 technique, RT-PCR amplification was undertaken using the purified EHDV-2 dsRNAs. Initially, this technique was developed and optimised for dsRNA templates (Maan et al., 2007), which permitted the amplification of the large and uncharacterised dsRNA virus genome (Shapiro et al., 2005). Amplification and sequencing of cDNAs from dsRNA were earlier reported in several studies, such as the single prime amplification technique (SPAT) initially described by *Lambden et al* (Lambden et al., 1992) and then modified by *Attoui et al* (Attoui et al., 2000). However, mispriming was the significant disadvantage of SPAT in comparison to FLAC2, which was solved by the design of modified C9 anchor primer allowing the synthesis of full-length cDNA by RT-PCR. Prior to cDNA

synthesis, the excess of C9 primer was removed by excising the 10 dsRNA segments from 0.8% agarose gel. This was an essential step to avoid mispriming or any non-specific amplification, permitting the production of full-length single-strand cDNAs in the absence of free primer molecules.

A notable variation in the efficiency of the production and amplification of EHDV-2 cDNAs of some segments was identified. For instance, it was difficult to amplify EHDV-2 (S1, S2 and S3) due to their large size. Furthermore, it was difficult to separate some segments due to their close migration pattern on o.8% agarose gel, such as; S2 and S3 or S4 and S5 or S7, S8 and S9. To overcome this problem, lower percentage of agarose gel was used (0.7% and 0.6%), and electrophoresised for longer time that permitted a suitable separation of the targeted segments. However, as this method failed to separate three small segments, a pool of them were used for RT-PCR. Consistently, the possibility of preparing cDNA of BTV segment 2 and 3 from a combination containing both segments, was earlier confirmed (Maan et al., 2007). In this work, the yield from S1, S2 and S3 was found to be low which was improved by using high annealing temperature of 60°C – 62°C for each of the large three segments. Ultimately, all of the 10 genomic RNAs were effectively converted into cDNA and amplified with no non-specific products.

To determine the full-length sequence of the 10 amplified EHDV-2 genome, each segment was successfully cloned into pUC19. This was not a straightforward approach, particularly, for S1 and S2 due to their large size. Additionally, since the resulted PCR amplification of S7, S8 and S9 were from the same pool, several colonies were tested by PCR using M13 (F and R) primers, to determine the presence of each segment. In due course, successful cloning of the 10 segments into pUC19 was achieved; and confirmed by sequencing.

To investigate the possibility of recovering EHDV-2 infectious virus from a complete set of T7 transcripts, the T7 plasmid clones were efficiently constructed. The T7 primers for the 10 EHDV-2 segments were designed as described previously by *Boyce et al* (Boyce et al., 2008). Capped T7 transcripts were successfully synthesised from the linearised T7 plasmid clones. The transcripts specificity was analysed by 1% denaturing agarose gel electrophoresis, which showed exactly the same estimated sizes of mRNA strands corresponding to that of EHDV-2 genome segments.

To recover progeny virus with genome segments generating from the 10 T7 transcripts, BSR cell monolayers were transfected twice with 0.15 µg to 0.25 µg of each segment. Following the second transfection, no plaques were observed at 72 (hpt), but a noticeable difference in cell morphology was documented in the transfected BSR monolayers with both amounts of T7 transcripts in comparison to the uninfected cells. Such findings indicated that certain mutations including deletions and stop codons along the full-length sequence of specific segment/s might be exist. Unfortunately, it was not possible to do further investigations due to time limitations.

In conclusion, an approach was presented in this study to establish RG system for EHDV-2, which was not reported earlier. The successful recovery of EHDV-2 infectious virus, entirely from cores transcripts, was achieved using sufficient amount of ssRNAs. Furthermore, the full-length sequence of each of the 10 dsRNA segments was generated and compared with published data. Successful synthesis of the 10 T7 transcripts of EHDV-2 was achieved from the T7 plasmid-derived clones. However, the recovery of infectious EHDV-2 from the complete set of 10 T7 transcripts was not possible.

Chapter 7

Overall discussion and conclusions

7.1. Overall discussion and conclusions

7.1.1. The development of VLPs and heterologous VLPs of EHDV serotypes

Historically, EHDV was considered a disease of deer particularly in North America (Shope et al., 1960). However, this premise has changed following the outbreaks occurred in the USA and European boarding countries, wherein agricultural cattle were the main host (Breard et al., 2004). This was due the emerging pathogenic strains from reassortant viruses of EHDV-2, EHDV-6 and EHDV-7, which were more fatal for domestic animals (Omori et al., 1969, Campbell and St George, 1986, Yadin et al., 2008, Temizel et al., 2009). Genetic characterisation of EHDV-6 highlighted this issue indicating that the outer capsid structural proteins VP2 and VP5 were those of EHDV-6, while the remaining structural and non-structural proteins were derived from EHDV-2 (Allison et al., 2010).

To control the invasion of EHD, vaccination is the optimal solution. The necessity for the development of an effective vaccine has economically increased with recent overlapping outbreaks. However, no vaccine is currently available against EHDV apart from the live-attenuated vaccine utilised in Japan for EHDV-2 (IBAV) (Aradaib et al., 2009). Therefore, the production of VLPs as vaccine candidates for EHDV has been considered a promising approach. Several reports have shown that VLPs are highly immunogenic, effective and a safe subunit vaccine, for example the HPV vaccine (Koutsky et al., 2002, Harper et al., 2004, Harper et al., 2006), as well as DIVA compliant. This is because although VLPs mimic the structure of authentic native virions, they lack the viral genome and therefore do not express non-structural proteins.

High level expression and correct folding of the target recombinant proteins are the key elements in the production of the VLPs. In this study, a baculovirus expression vector system was utilized due to its versatility and its ability to meet the described criteria of high level expression of correctly folded proteins for large proteins >100 kDa in size. This was exemplified by validation experiments of single expression of EHDV VLP protein constituents. No earlier reports were available regarding the single synthesis of the outer and inner capsid proteins of EHDV-1, EHDV-2 and EHDV-7 except for EHDV-1 VP3 (Le Blois et al., 1991). Therefore, as an initial step for synthesising VLPs and heterologous VLPs of EHDV-1, -2 and 7 serotypes, it was necessary to individually express the structural proteins VP2, VP5, VP7 and VP3, of the three serotypes.

In this study, the utility of baculovirus expression system had confirmed its suitability for the generation of EHDV VLPs. Baculovirus system allowed expression of correctly folded large capsid proteins, in particular, VP2 and VP3 of EHDV-1, -2 and 7. Additionally, the expressed recombinant protein VP2 of both serotypes; EHDV-1 and EHDV-2 was shown to be immunogenic, and antibodies raised against the targeted two proteins were highly specific. Achieving such results would not be possible in other expression systems. For instance, post-translational modifications and correctly folded recombinant protein do not occur in bacterial or yeast expression systems (Baneyx, 1999, Mattanovich et al., 2012). However, when mammalian cells can provide these, they are expensive for large scale production (Wurm, 2004).

Earlier literature reported the use of baculovirus multiple gene expression system for simultaneous expression of the structural recombinant proteins within the same insect cell (Noad et al., 2009). Therefore, the possibility of generating heterologous

protein complexes using this system is potentially high. Here, this approach was utilised to generate VLP of EHDV-1, which was reported to cause highly fatal disease in wild-tailed deer, in which morbidity and mortality rates were of concern of wildlife managers. Prior to the formation of EHDV-1 VLPs, the recombinant Bacmid$_{1629}$:EHDV1.S7.S3 was successfully generated by inserting EHDV-1 (S7 and S3) at *egt* and *39k* loci into baculovirus genome. The EHDV-1 CLPs were effectively produced in *Sf*21 insect monolayers and a high level of synthesised recombinant proteins in accurate molecular ratios was detected by 10% SDS-PAGE. Using EM, the assembly of the inner capsid proteins VP7 and VP3 of EHDV-1 into CLPs was confirmed, indicating correct interactions when compared with EHDV-2 purified core particles. Successfully, specific antibodies against EHDV-1 CLPs were raised in Rabbits and determined to be highly specific when examined by western blotting.

Based on the above findings, the possibility of generating EHDV-1 VLPs composed of the three capsid proteins VP3, VP7 and VP5 was investigated in *Sf*21 insect cells. This aim was addressed by efficacious construction of the recombinant pRN296-EHDV1.VP5 followed by a successful integration in to Bacmid$_{1629}$:EHDV1.S5 at *odv-e56* locus. The expression of each protein was determined by SDS-PAGE after the successful formation of the recombinant baculovirus Bacmid$_{1629}$:EHDV1.S5.S7.S3 in *Sf*21 insect cells. Since the above produced VLP was lacking the most variable protein VP2 forming the outermost layer, the construct pAcYM1-EHDV1.VP2 was utilised to recover the recombinant baculovirus Bacmid$_{1629}$:EHDV1.S5.S7.S3 by cotransfection of the insect monolayers. After several rounds of amplification, the purified infected cell lysate showed optimal ratios of the expressed EHDV-1 four recombinant proteins; VP2, VP5, VP7 and VP3 by 10% SDS-PAGE gel. When the

assembly of these proteins into VLPs were examined by EM, micrographs displayed similar morphological structure to the control double-capsid BTV particles.

In order to initially assess the immunogenicity and vaccine potential of the produced EHDV-1 VLPs a neutralisation assay was performed. VLPs were prepared, purified and polyclonal antisera were raised in Rabbits. The authenticity of the developed antisera was tested by western blotting. As a result, significant interactions were detected for each of the four recombinant proteins; VP2, VP5, VP7 and VP3 of EHDV-1. Successfully, EHDV-1 virus was neutralised at 1:64 dilution in cell culture using the antisera produced against EHDV-1 VLPs. In addition, cross neutralising was detected against EHDV-2 and EHDV-7 serotypes at low level. These data are consistent with that of orbiviruses demonstrating that VP2 proteins dictate the host antigenic response but still share some cross neutralising epitopes, due to the presence of conserved regions along VP2 full-length sequence of different serotypes.

The successful construction of EHDV-1 VLPs was encouraging to test the probability of rapid development of the heterologous VLPs of EHDV-1 and EHDV-2 serotypes. This was performed by exchanging the outer capsid proteins (VP2 and VP5) of EHDV-2 onto the conserved CLPs (VP3 and VP7) proteins of EHDV-1, given the neutralisation data this VLP should elicit a neutralising immune response to EHDV-2 VP2. Due to time constraints this possibility was initially tested utilising coinfection of cells with two viruses, the CLPs virus of EHDV-1 and a newly constructed (VP2 and VP5) virus of EHDV-2. In addition, a triple infection was tested with the CLP virus of EHDV-1 and two viruses each singly expressing VP2 and VP5 of EHDV-2, respectively. The triple infection yielded no expression of the outer capsid layer EHDV-2 VP2 and very low expression of the inner shell protein EHDV-2 VP5, while

both of EHDV-1 (VP3 and VP7) were expressed at high level. However, the dual expression of each protein was observed as an intense protein band to the estimated sizes of EHDV-2 [VP2 (114 kDa) and VP5 (59 kDa)] and EHDV-1 [VP3 (103 kDa) and VP7 (39 kDa)]. Further confirmation of the recombinant EHDV-2 VP2 was identified on the nitrocellulose membrane using the earlier developed antibodies against this protein. These results were consistent with the published findings showing that multiple infections (triple and quadruple) of the insect cells are not suitable for the generation of all targeted proteins, since not every cell in the cell culture will be infected simultaneously at the same ratio (Belyaev and Roy, 1993).

An attempt was carried out to generate EHDV-7 heterologous VLP using EHDV-1 CLPs as background. The recombinant pAcYM1-EHDV7.S5 was efficiently constructed and integrated at *odv-e56* locus into baculovirus genome. This work was not taken further due to time restrictions.

7.1.2. The Recovery of EHDV-2 from RNAs transcripts

In conjunction to VLP technology the availability of RG system allows an alternative approach to develop rationally attenuated vaccines, in this work an attempt was made to do so by this methodology for the EHDV-2 Alberta/Canada strain. Prior to the establishment of a plasmid-based RG, similar to BTV (Boyce et al., 2008), the utility of recovering infectious EHDV-2 virus from core transcripts was tested. To this end EHDV-2 cores were prepared from virus culture, and utilised for *in vitro* transcription. Ten core transcripts were efficiently synthesised the correct molar ratio as detected on denaturing agarose gel electrophoresis.

Using micrograms (1.0 µg) of EHDV-2 ssRNAs, their infectivity was determined in BSR cells showing 100% CPE three days after double transfection. Further, the replication of EHDV-2 genome from transfected cells was confirmed by

electrophoresis on agarose gel showing similar dsRNAs segmented genome profile to that extracted from infected cells. Moreover, a complete CPE was clearly observed in the BSR monolayers incubated with the transfected culture medium. The morphology of cells infected by recovered virus was similar to that noticed in BSR cells infected with EHDV-2. It is noteworthy that this is the first report indicating the successful recovery of infectious EHDV-2 from the core transcripts.

For vaccine production, it is necessary to generate virus entirely from T7 transcripts, to allow understanding the infection of the virus on the molecular level, permitting viral genome manipulation, which can be exploited in many research domains. To address this aim, the 10 RNA segments were amplified, cloned into pUC19 and then sequence validated and from which T7 transcription vectors were constructed. T7 Transcripts of each segment were synthesised *in vitro* with the same expected sizes of mRNAs without any signs of degradation as visualised on denaturing agarose gel. Utilising the successful protocol of previous reports for BTV, BSR cells were transfected twice in an attempt to recover infectious EHDV-2 virus (Matsuo et al., 2010, Matsuo and Roy, 2009). After 72 hours of incubation, no CPE was documented in the transfected cells although morphological differences were visualised comparing to the uninfected cells. To confirm the above findings, the experiment was three times repeated indicating that specific mutations might be presented along the full-length sequence in specific segment/s of the viral genome. This problem could be addressed by sequencing full-length of EHDV-2 10 segments and comparing the results with published data. As well as, an attempt to recover the infectious virus using different concentrations of the entire 10 T7 transcripts should be taken into consideration.

7.1.3. Conclusions

Based on the use of baculovirus as multiple gene expression system, the application of this sytem to produce immunogens for EHDV was addressed in this work. Successful generation of stable EHDV-1 CLPs, EHDV-1 VLPs was achieved which allow for the production of heterologous VLPs of EHDV-2 using EHDV-1 CLPs as foundation. The assembly of the recombinant expressed proteins were examined and confirmed by EM to be similar to EHDV core and virion structural particles. The specificity of the raised antisera was tested for VP2 of EHDV-1 and EHDV-2, EHDV-1 CLPs and EHDV-1 VLPs. Furthermore, the biological activity for EHDV-1 VLPs antisera was successfully proved by neutralisation assay against EHDV-1, and cross-neutralising activities were detected for EHDV-2 and EHDV-6 serotypes. Overall, this work lays the pathway to produce VLP and heterologous VLP vaccine for EHDV serotypes to allow emergent disease control.

Alternative approach to develop EHDV-2 vaccine using RG system was performed. In order to validate the RG principles of BTV for EHDV, the successful recovery of the infectious virus EHDV-2 from the core transcripts was achieved. To recover EHDV-2 entirely from the 10 T7 transcripts, the T7 plasmid clones were constructed for the 10 segments and transcribed *in vitro*. However, unfortunately, it was not possible to recover EHDV-2 entirely from the 10 T7 transcripts indicating that studies focusing on detecting full-length sequence of each of the 10 segments should be undertaken. Then, optimising the specific concentration of the 10 T7 transcripts are needed for the recovery of the infectious virus.

7.2. Limitations and future work

This thesis had demonstrated the potential of efficiently developing EHDV-1 VLP and heterologous VLPs of EHDV-2 using EHDV-1 CLPs as scaffold. In addition, alternative approach to generate a rationally designed vaccine for EHDV-2 using RG system was assessed. However, the opportunities for extending the scope of this project, were hampered by time constrains. Nevertheless, the reagents generated in this study will be valuable for two different type of vaccine development in future and also will provide tools for basic understanding of EHDV replication and infection.

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