VILNIUS UNIVERSITY

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YEAST-GENERATED PARVOVIRAL VIRUS-LIKE PARTICLES AND THEIR USE IN DIAGNOSTICS

Doctoral dissertation

Physical sciences, biochemistry (04 P)

Vilnius, 2017

The work presented in this doctoral dissertation was carried out at the Institute of Biotechnology, Vilnius University in 2010-2014.

Dissertation is defended extramurally.

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Dissertation will be defended in the public session on 31 May, 2017, 13:00, at Vilnius University Life Sciences Centre, R-401 auditorium.

Summary of the dissertation was distributed on 28 April, 2017.

The dissertation can be reviewed at the library of Vilnius University.

CONTENTS

LIST OF ABREVIATIONS

aa	amino acid(s)
AAV	adeno-associated virus
Ac	acetate
AGE	acute gastroenteritis
ARTI	acute respiratory tract illness
BICS	baculovirus-insect cell system
CI	confidence interval
CPV	canine parvovirus
EIA	enzyme
HBoV	human bocavirus
HCV	hepatitis C virus
HP	hairpins
HRP	horseradish peroxidase
IDU	intravenous drug user
IFA	immunofluorescence assay
ITR	inverted terminal repeats
kb	kilobase
LRTI	lower tract respiratory tract illness
mVLP	mosaic virus-like particles
MVM	minute virus of mice
NLS	nuclear localization signal
NP	phosphorylated non-structural protein
NS	non-structural protein
OD	optical density
PARV4	human parvovirus 4
PEG	polyethylene glycol
PLA2	phospholipase type A2
PMSF	phenylmethylsulfonyl fluoride
PPV	porcine parvovirus
RT	room temperature
SDS	sodium dodecylsulphate
VLP	virus-like particle
VP1u	VP1 unique domain
WB	Western blot

INTRODUCTION

Parvoviruses are single-stranded DNA viruses that infect both vertebrates and insects. Having a diameter of only 18-27 nm and a genome of 4-6 kb, they are one of the smallest viruses known. Some animal parvoviruses, like canine and porcine parvoviruses, are highly contagious with mortality reaching up to 91% of untreated cases. Until 2005, parvovirus B19 was known as the only pathogenic human parvovirus, causing *erythema infectiosum* and chronic arthralgia. In the recent decade, using the high throughput sequencing methods, several new human-infecting parvoviruses were discovered. Human bocavirus 1 was detected in paediatric respiratory samples, and its prevalence was confirmed worldwide with high incidence rate already in the early childhood. In 2010, three more human bocavirus genotypes (HBoV2–4) were found in gastrointestinal samples. Human parvovirus 4 was identified in the plasma of an intravenous drug user with the acute viral syndrome, and this pathogen was initially considered to be transmitted by parenteral means and prevalent primarily among high-risk individuals. Data on the clinical significance of these novel human parvoviruses are still sparse. However, HBoV and PARV4 presence in children with encephalitis and encephalopathy show its possible involvement in the pathogenesis of inflammatory neurological diseases. Further studies of these parvoviruses require recombinant viral proteins as tools for both diagnostic and virus structure research purposes.

Animal viruses naturally propagate in mammalian cells, however, the proteins synthesised in such cell cultures for extensive protein production for research and industry is complicated due to cost and yield. Yeast provide a more efficient and cost effective means of producing viral proteins, for use in research and industry. As with bacteria, yeast offers the ease of microbial growth and gene manipulation as well as taking advantage of eukaryote-specific post-translational modification processes. Yeast-generated viral antigens were previously successfully used to develop sensitive diagnostic assays for viruses from families of *Paramyxoviridae* (Warrerner *et al.,* 2010*), Bunyaviridae*

(Petraityte *et al.,* 2008) and other. This PhD thesis focuses on the investigation of yeast *S. cerevisiae* expression system for synthesis of recombinant structural proteins of novel human and economically relevant porcine parvoviruses for virus prevalence studies.

Research aim:

To investigate the synthesis of structural proteins of mammalian parvoviruses in yeast and to test their aptness for use in serological diagnostics.

Research objectives:

1. To synthesise the capsid-forming proteins of novel human parvoviruses HBoV1, 2, 3, 4, PARV4, and porcine parvovirus PPV in yeast *S. cerevisiae*, purify them and investigate their immunogenic properties.
2. Using the recombinant antigens, to develop and validate the serological test systems for detection specific antibodies against human bocaviruses 1-4, human parvovirus 4 and porcine parvovirus in serum samples.
3. To assess the extent of human bocaviruses 1-4, human parvovirus 4 circulation in Lithuania.

The novelty and significance of the project

Rapid detection of viral infection relies on PCR-based viral gene amplification or analysis of host immune response to viral antigens. The latter provide additional information about previous infections and may be developed as point-of-care tests enabling rapid diagnostics at the places where trained personnel or laboratory equipment is unavailable. The surface antigens of novel human parvoviruses (HBoV1-4 and PARV4) generated in yeast in this study were proved to be highly immunogenic and can be successfully employed in serological tests. It opens a platform for a rapid and economically effective expression system for the surface antigens of other parvoviral pathogens to be discovered in the future. In 2013-2016, several new parvoviruses infecting livestock (porcine and bovine hokoviruses, porcine parvovirus 6 (Schirtzinger *et al.*, 2015) and humans (bufavirus and tusavirus (Väisänen *et al.*, 2016)) were

discovered. Thus, the approach used in this study may be applied in the research of these new parvoviruses.

The serological study of the prevalence of human parvovirus 4 and human bocaviruses 1-4 in Lithuania was performed for the first time. A very high prevalence (91.6%) of human bocavirus specific antibodies in the serum samples of Lithuanian patients with respiratory infections revealed high incidence rate of these pathogens in this population. The serological study of PARV4 prevalence among low-risk individuals in Lithuania revealed the highest rate (9.4%) reported in Europe to date. Taking into consideration that the patients had no records of parenteral exposure, this study supports the increasing objection to the prevailing theory that PARV4 is transmitted via the parenteral mode.

To our best knowledge, the recombinant mosaic virus-like particles composed of major and minor structural parvoviral proteins were generated for the first time in yeast expression system. These mVLPs of HBoV1 and PARV4 were evaluated as more sensitive antigens in EIA in comparison to VP2-only VLPs.

Development of an effective viral antigen production systems is also of commercial interest. Porcine parvovirus infections have an important economic impact on swine farming, thus, our system developed to obtain a highly immunogenic PPV VP2 antigen is valuable for use in diagnostics and shows potential as an alternative to currently administrated inactivated vaccines.

Thesis statements:

1. Yeast *S. cerevisiae s*ynthesised major capsid proteins of PARV4, PPV and HBoV1-4 form stable, immunogenic virus-like particles that are suitable for diagnostic immunoassays and elicit the antigen-specific immune response in mice.
2. Coexpression of minor and major structural proteins of HBoV1 and PARV4 in yeast generates stable, highly antigenic mosaic virus-like particles.

3. Novel human parvovirus 4 is circulating in the low-risk Lithuanian patients, suggesting the nonparenteral transmission mode of this virus.
4. Human bocaviruses 1-4 are highly prevalent in Lithuania.
5. Yeast-generated porcine parvovirus VP2 VLPs are suitable antigens for PPV-specific antibody detection in swine serum samples.
6. Major human parvovirus 4 VP2 protein-specific B-cell linear epitopes are located in the EF, HI and C-terminal loops.

1. REVIEW OF LITERATURE

1.1. Viruses of the family *Parvoviridae*

1.1.1. Taxonomy and phylogeny

Parvoviruses, as suggests their name (Latin *parvum* – small), are among the smallest and simplest eukaryotic DNA viruses, which infect a wide range of species, both vertebrates and invertebrates. The family *Parvoviridae* encompasses the viruses with linear single-stranded DNA (group II according to the Baltimore classification) genomes and small unenveloped protein capsids that exhibit T=1 icosahedral symmetry. Fifty-six virus species of this family has been renamed and reassigned into genera by the International Committee on Taxonomy of Viruses in 2014 (Cotmore et al., 2014). In this thesis, the taxonomy down to the genera level is based on this new classification, however, the dual (new binomial as well as widely used former) virus species names are provided in this chapter for cross-reference. The former names will be used in other sections in order to concur with the vast majority of the research literature available to date. Historically this family has been subdivided into two subfamilies (Fig. 1.1), based on the host: *Parvovirinae*, which is defined predominantly by the ability of constituent viruses to infect vertebrate hosts, and *Densovirinae*, which includes viruses that infect arthropods.

The subfamily *Parvovirinae* comprises eight genera:

1. The genus *Dependoparvovirus* includes the adeno-associated viruses (*adeno-associated dependoviruses*) and its members also infect humans. The members of this genus are only able to replicate efficiently when the cells are co-infected with a helper virus (adenovirus, vaccinia virus or herpesvirus) (Berns & Parrish, 2007). Dependoparvoviruses are capable of establishing a latent infection by integrating their genomes into the genome of the host cell (Cheung *et al.*, 1980).
2. The genus *Erythroparvovirus* comprises autonomous parvoviruses that do not require a helper virus and have a pronounced tropism for erythroid progenitor cells. The best-studied member of this genus is the human pathogenic parvovirus B19 (*primate erythroparvovirus 1*) that causes fifth disease (erythema infectiosum).

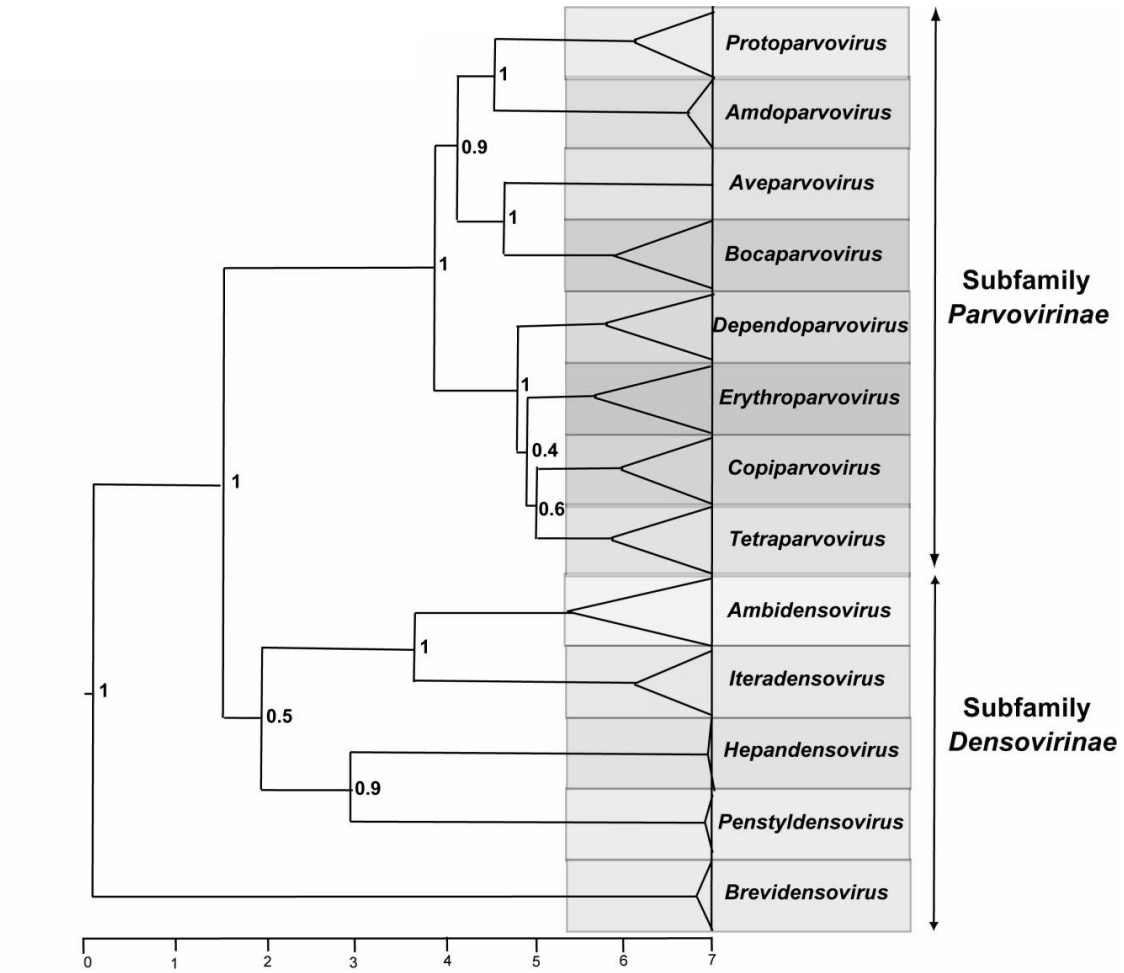


Figure 1.1. The phylogenetic tree of the *Parvoviridae* family viruses (adapted from (Cotmore *et al.*, 2014)). The Bayesian tree is based on the amino acid sequence of the viral replication initiator protein, NS1, and is displayed in the ultrametric format on an arbitrary scale, with posterior probability scores indicated at statistically significant nodes.

3. The genus *Protoparvovirus* contains animal pathogenic viruses, usually causing enteritis and myocarditis in domestic and farm animals. The porcine parvovirus (PPV, u*ngulate protoparvovirus 1*) is the cause of economically important fertility disorders in swine. The feline panleukopenia virus (*carnivore protoparvovirus 1*), namely of the canine parvovirus (CPV) type 2c, infections yield to up to 70% mortality rate in puppies if untreated (for review: (Decaro & Buonavoglia, 2012)). The asymptomatic minute virus of mice (MVM, *rodent protoparvovirus 1*) is a well-studied prototype for parvoviral replication and transcription. The newly discovered bufaviruses (*Primate protoparvovirus 1*) that cause gastroenteric diseases in humans, are assigned to this genus (Phan *et al.*, 2012; Yahiro *et al.*, 2014).

4. The genus *Amdoparvovirus* comprises solely animal pathogenic viruses. A member of this genus, the Aleutian mink disease virus (*Carnivore amdoparvovirus 1*) is considered a serious threat in regions where fur farming is of economic importance. The viruses of this genus lack a cognate phospholipase A_2 (PLA2) domain, which is commonly used by members of the family to penetrate bilayers during cell entry (Li *et al.,* 2011).

5. The viruses of genus *Bocaparvovirus* cause respiratory and gastrointestinal disorder in animals and humans. The genomes of bocaparvoviruses have an additional open reading frame coding for a highly phosphorylated nonstructural protein (NP1) (Sukhu *et al.,* 2013). The genus name was formed after widely spread viruses: *bo*vine parvovirus and *ca*nine minute virus (*ungulate bocaparvovirus 1* and *carnivore bocaparvovirus 1*, respectively). More attention towards the genus was drawn after human bocavirus 1 (*Primate bocaparvovirus 1*) was discovered in respiratory swabs from children with lower respiratory tract infections (Allander *et al.,* 2005) and is reported to be among the four most frequent respiratory viruses (Cebey-López *et al.,* 2015). Three additional genotypes, human bocavirus 3 (also assigned as *Primate bocaparvovirus 1*) and bocaviruses 2 and 4 (both renamed as *Primate bocaparvovirus 2*) had been identified in human stool samples from children with gastrointestinal illness (Arthur *et al.,* 2009; Kapoor *et al.,* 2009).

6. The genus *Copiparvovirus* is comprised of *ungulate copiparvovirus 1* (formerly known as bovine parvovirus 2 and was found to be a common contaminant of commercial bovine serum (Allander *et al.,* 2001) and *ungulate copiparvovirus 2* (porcine parvovirus 4, isolated from porcine lung lavages in USA (Cheung *et al.,* 2010)).

7. The parvovirus infecting poultry has been assigned into genus *Aveparvovirus* with the only species *Galliform aveparvovirus 1* (formerly named as chicken parvovirus). The virus strains in this species are widespread in turkeys and chickens in the United States and Europe, and are highly infectious in young poultry but of uncertain pathology (Kisary, 1985).

8. The genus *Tetraparvovirus* proposed for human parvovirus 4 (*Primate tetraparvovirus 1)*, which has been discovered in human blood (Jones *et al.*, 2005) and in pooled plasma used to manufacture blood products (Fryer *et al.*, 2007c). Symptoms or diseases have not been correlated with the acute infection. Similar viruses have been isolated from several mammals, e.g.: hokoviruses in pigs, wild boars, sheep, cows as well as *Chiropteran tetraparvovirus 1* in bats *Eidolon helvum* in Ghana.

The subfamily *Densovirinae* is divided into five genera: *Ambidensovirus, Brevidensovirus,* and *Iteradensovirus* viruses infect insects, whereas members of *Hepandensovirus* and *Penstyldensovirus* genera replicate in decapods. These parvoviruses may be of special interest due to the hosts of immediate human environment like common (*Culex pipens*) or yellow fever (*Aedes aegypti*) mosquitoes, as well as German cockroach (*Blattella germanica*). *Lepidopteran iteradensovirus 1* (formerly *Bombyx mori* densovirus) is one of the causes of the infectious silkworm flacherie that results in economic losses.

1.1.2. Structure of parvovirus virion

Viral capsids have an icosahedral T=1 structure and a diameter of 18–26 nm. The virions are non-enveloped and composed of proteins VP2 and VP1 in a ratio of 95% and 5%, respectively (Gurda *et al.,* 2010). Dependoparvoviruses contain a third capsid protein (VP3) in differing amounts. The viral genome resides inside the capsid (Fig. 1.2). It is associated through 11 nucleotides with the internal moiety of VP2 proteins (Reed *et al.*, 1988).

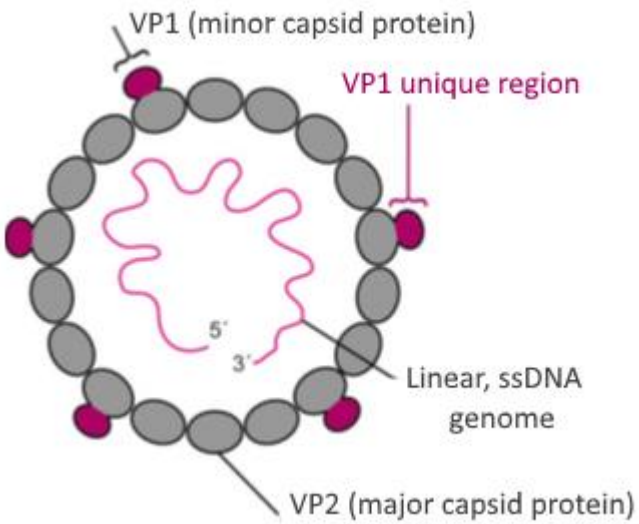


Figure 1.2. Schematic structure of parvovirus particle. Adapted from (Modrow *et al.*, 2013)

1.1.3. Genome structure and organization

Viruses of the *Parvovirinae* subfamily have a single-stranded, linear DNA genome with a length of 4.7 – 5.6 kb (Table 1.1). Most parvoviruses preferentially package genome of negative polarity (complementary to the messenger RNAs (mRNAs) that are synthesised during infection). However, some viruses, like AAV, pack genomes of both polarities in equal amount, or ratio can vary depending on the host cell type (Tijssen *et al.*, 2011). The coding sequence is flanked by short (116–385-base) imperfect hairpins or inverted terminal repeats (ITR), that can form T-shaped hairpin loops. Individually, these telomeres give rise to viral replication origins in replicative form (RF) DNA, and together they contain most of the *cis*-acting information, required for viral DNA replication and packaging. Alternatively, genomes can adopt the panhandle-like (Y-shaped) structures, where ITRs form the double-stranded region (Cotmore & Tattersall, 2014).

The genomic arrangement is similar in all parvoviruses (Fig. 1.3). At the first level of organisation the parvovirus genome is a model of simplicity. All proteins are encoded on one strand: the 3' half of the genome (ORF1) encodes non-structural proteins involved in both viral DNA replication and regulation of gene expression (NS; Rep1-4 in AAV), and the ORF2, located near the 5' terminus, code for structural, coat proteins (VP). However, the organisation is quite complex, because there are 2-4 overlapping NS proteins and 2-3 overlapping

structural proteins in each case. In *Bocaparvovirus* genus, there are additional reading frames that direct the synthesis of NS2 and NP1 proteins using alternative start codons (Chen *et al.*, 2010a). In parvovirus B19, additional small ORFs in the central region and at 5' end code for small non-structural proteins (Ozawa *et al.*, 1987).

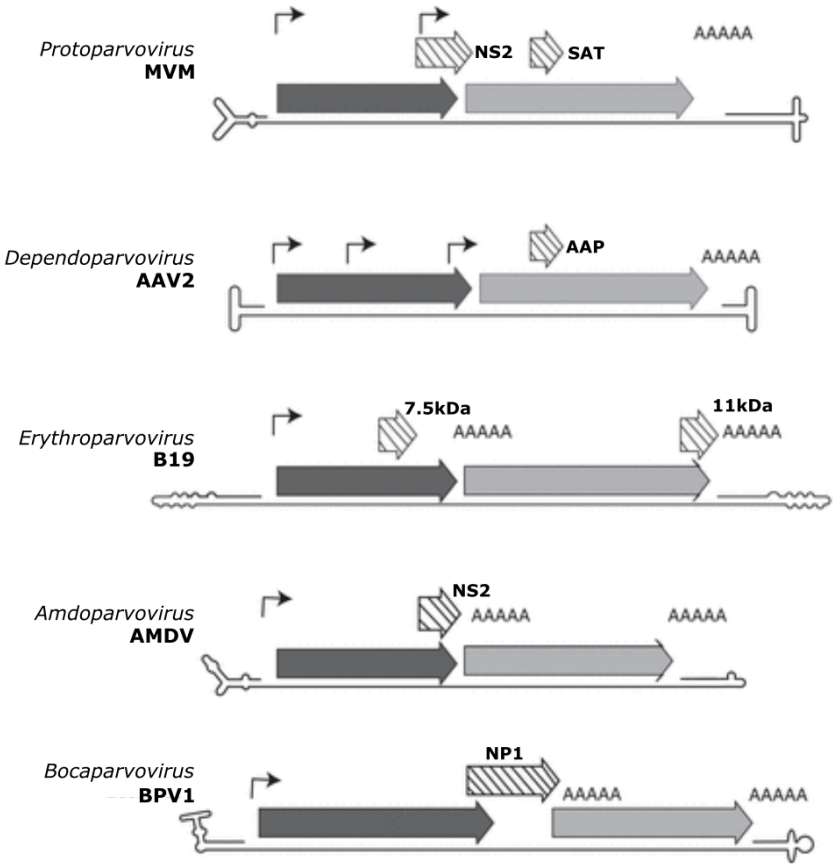


Figure 1.3. Genetic strategies of representative viruses from genera in the subfamily *Parvovirinae*. (adapted from (Cotmore & Tattersall, 2014)). Genomes from the type species of each genus are denoted as a single line terminating in hairpin structures. The hairpins, drawn to represent their predicted structures, are scaled about 20 times with respect to the rest of the genome. Open reading frames are represented by arrowed boxes, shaded dark grey for the major replication initiator protein (NS), light grey for the structural proteins of the capsid, and cross-hatched for sequences unique to the ancillary non-structural proteins. Transcriptional promoters are indicated by solid arrows and polyadenylation sites by the AAAAA sequence block.

There are up to three promoters that organise the transcription from parvovirus genome (Table 1.1). Parvoviruses have the unusual property that both the unspliced and spliced transcripts from a single promoter can serve as mRNAs. In *Erythroparvovirus*, *Amdoparvovirus* and *Bocaparvovirus* genera, all alternatively spliced mRNA species are initiated from a common promoter at the 3' end of the genome, and there are two polyadenylation sites in the genome of

these viruses (in the middle and the 5’ terminus). Protoparvoviruses, tetraparvoviruses and aveparviviruses have a single polyadenylation site and two promoters that regulate transcription of non-structural and structural genes, respectively. Adeno-associated viruses also use a common polyadenylation site at the end of the genome for all mRNA species, but their genome contains three promoters. Two promoters at the 3’ end of the genome (positions 5 and 19) regulate the expression of four non-structural (Rep) proteins. The expression of capsid proteins is under the control of the third promoter (genome position 40) (Laughlin *et al.*, 1979). Hence, despite the similar gene arrangement found in all parvoviruses, gene expression is regulated differentially and specifically by the combination of the different localisation of transcriptional control elements and alternative splicing.

Table 1.1. Characteristics associated with genera in the subfamily *Parvovirinae.* Adapted from ICTV, 2013.

Genus	Type species	Genome size (kb) and sense	Termini	Left (5‘) terminus	Right (3‘) terminus	Promoters (mu)	PLA2 in capsid	Ancilliary proteins	VP proteins
Amdo-parvovirus	*Carnivore amdoparvovirus 1* (Aleutian mink disease virus)	~4.8, "-"	HPs	~116nt	~240nt	1 (mu 4)	no	NS2, SAT	2
Aveparvovirus	*Galliform aveparvovirus 1* (Chicken parvovirus)	~5.3,"+/-"	ITRs, HPs	206nt ITR 39nt HP	206nt ITR 39nt HP	2, (mu 5 and 56)	no	uk	2
Bocaparvovirus	*Ungulate bocaparvovirus 1* (Bovine parvovirus)	~5.5, "-"	HPs	~140- 180nt	~180-200nt	1, (mu ~6)	yes	NP1, (NS2-4)?	2
Copiparvovirus	*Ungulate copiparvovirus 1* (Bovine parvovirus 2)	~5.6, uk	uk	uk	uk	uk	Motifs present	uk	uk
Dependo-parvovirus	*Adeno-associated dependoparvovirus 1 (Adeno-associated virus)*	~4.7 "+/-"	ITRs, HPs	~145nt	~145nt	3, (mu 5,19, and 40); rarely 2 (mu 5 and 40)	yes	Rep 40, AAP	3
Erythro-parvovirus	*Primate erythroparvovirus 1* (Human parvovirus B19)	~5.6 "+/-"	ITRs, HPs	~385nt	~385nt	1, (mu ~6)	yes	11k, 7.5k	2
Protoparvovirus	*Rodent protoparvovirus 1* (Minute virus of mice)	~5.1, "-"	HPs	~120nt	~250nt	2, (mu ~4 and 40)	yes	NS2, SAT	2
Tetraparvovirus	*Primate tetraparvovirus 1* (Human parvovirus 4)	~5.3, uk	uk	uk	uk	2. (mu 6 and 38)	Motifs present	uk	2

Abbreviations: HP, hairpins; ITR, inverted terminal repeats; uk, unknown; mu, map unit; PLA2, phospholipase A_2.

1.1.4. Viral proteins

Non-structural (regulatory) proteins. In parvoviruses, the 70-100 kDa viral replication initiator protein NS1 (or Rep in AAV) is encoded by ORF1. NS1 is essential for viral DNA replication (Cotmore *et al.*, 2007; Han *et al.*, 2013; Niskanen *et al.*, 2010) and packaging of viral DNA into capsid (Cotmore & Tattersall, 2005; King *et al.*, 2001). The protein was shown to play a role in the *trans*-activation of viral and cellular gene expression (Deleu *et al.*, 1999; Moffatt *et al.*, 1996), DNA damage response (Kivovich *et al.*, 2011), cell cycle arrest, apoptosis (Best *et al.*, 2003; Chen *et al.*, 2010b), and the modulation of innate immunity (Hsu *et al.*, 2011). NS1 contains an N-terminal DNA-binding/endonuclease domain, a C-terminal zinc-finger domain, and a central superfamily III (SF3) helicase domain (Tewary *et al.*, 2013). Rep68 of AAV was shown to form multiple oligomeric complexes when interacting with DNA (Zarate-Perez *et al.*, 2013), which are similar to hexameric SF3 helicase complexes.

All parvoviruses encode a few small ancillary proteins which have diverse structures and functions and are variably disposed throughout the genome (Fig. 1.3). Members of the genera *Amdoparvovirus* and *Protoparvovirus* encode essential ancillary NS2 and small alternatively translated (SAT) proteins (Zádori *et al.*, 2005). Recently, four NS proteins were demonstrated to be expressed during infection of polarised human bronchial airway epithelium with HBoV1 (Shen *et al.*, 2015). NS2 molecules of MVM are short-lived and associate *in vivo* with proteins from the cellular 14-3-3 family (Brockhaus *et al.*, 1996), and with the nuclear export factor CRM1 (Bodendorf *et al.*, 1999), which is essential for the efficient export of virions from the nucleus (Eichwald *et al.*, 2002). Bocaparvoviruses encode nuclear phosphoprotein, NP1, which is required for efficient duplex DNA amplification. NP1 is multifunctional, because it also influences RNA processing, promoting read-through of the internal polyadenylation signal and thus allowing capsid protein expression (Sukhu *et al.*, 2013). The induced expression of the HBoV1 NP1 protein in murine cells rescued viral DNA replication in NS2-null MVM infection (Mihaylov *et al.*, 2014). The

small alternatively translated (SAT) protein of protoparvoviruses is expressed late in the infection, from the same mRNA as VP2 (ORF2) (Zádori *et al.*, 2005). Analogously, AAVs encode an assembly-activating protein (AAP) that targets newly synthesised capsid proteins to the nucleolus and promotes capsid assembly via interactions with VP C-terminal peptides (Naumer *et al.*, 2012). Parvovirus B19 (genus *Erythroparvovirus*) encodes two ancillary proteins: an 11-kDa protein that plays a role in virus infectivity, virion production and trafficking, and a 7.5-kDa protein of yet unknown function. (Zhi *et al.*, 2006)

Structural (capsid) proteins. The expression of ORF2 leads at least to 2 structural proteins VP, which share the same 500-560 aa C-terminus. VP1 is the largest, 70-100 kDa protein and is essential for infectivity. Most of the parvoviruses (Table 1.1) have the active phospholipase A_2 (PLA2) enzymatic domain in their VP1 unique N-terminus (VP1u), which enables virions to escape through the endosomal bilayer to the cytoplasm and contains the catalytic site of a PLA2 and a conserved Ca^{2+} binding loop (Zádori *et al.*, 2001). The MVM mutant with a single H42D amino acid substitution in its PLA2 active site severely impaired its enzymatic activity and abrogated its infectivity (Farr *et al.*, 2005). VP1u is either sequestered in mature capsid and is exposed through the 5-fold channel in low pH while in endosomes (AAV2, MVM) (Farr *et al.*, 2005; Girod *et al.*, 2002), or, in the case of parvovirus B19, prepositioned at the virion surface, but masked by a N-terminal peptide extension (Leisi *et al.*, 2013). The PLA2 of B19 is unmasked when VP1 attaches to the cell receptor, the P antigen globoside. Furthermore, after a heat or a low-pH treatment, the native VP1u was irreversibly accessible to the specific VP1u antibody, whereas the natural capsid was not recognised (Ros *et al.*, 2006a). Additionally, a nuclear localisation signal on the VP1 N-terminal residues in the CPV and MVM was detected, aiding the virions to reach the nucleus via ATP-mediated process (Lombardo *et al.*, 2002; Vihinen-Ranta *et al.*, 1997). Porcine parvovirus has more than one activated NLS, including a novel nuclear localisation motif (NLM) (Boisvert *et al.*, 2014) (Fig. 1.4 C). VP1u of B19 was shown to harbour most of the neutralising epitopes (Soderlund *et al.*, 1992).

The parvoviral capsid is composed of various N-terminally truncated VP polypeptides with only a few (up to 10 copies) of VP1. Besides the structural function, VP2 acts as a host cell receptor binding molecule and begins internalisation (see section 1.1.5). A new NLS of the B19 virus was located on the VP2 N-terminus, which could facilitate nuclear transport (Pillet *et al.*, 2003). In the case of MVM, the VP2 N-terminus also acted as a nuclear export signal (NES) (Sánchez-Martínez *et al.*, 2012).

Most of the parvoviruses have two capsid-forming proteins (Table 1.1), but AAV capsids are composed of 3 VPs, the smallest, 61 kDa VP3 being the most abundant (Xie *et al.*, 2002). In the MVM, the VP3 is generated from the cleavage of VP2 at approximately 25 amino acids from the N- terminal and only occurs when the viral genome has completed the capsid assembly and packaging. Although the VP1 has an identical proteolytic site, cleavage does not occur (Weichert *et al.*, 1998).

The assembled capsid exhibits two-, three- and fivefold symmetry elements (Fig. 1.4), which interact with the host cells. The 2-fold symmetry depressions mediate tissue host range via glycan interactions and the 3-fold elevations interact with the receptors. At the 5-fold axes, the capsids possess the cylindrical projections, surrounded by a conserved circular, canyon-like depression of unknown function. Antiparallel β-hairpins from each of the five symmetry-related VPs enclose a narrow central channel that connects the interior of the capsid to its outer surface (Berns & Parrish, 2007; Cotmore & Tattersall, 2007).

Structures of several major parvoviral capsid proteins (VP2) have been resolved to 2.4-3.5 Å resolution (Halder *et al.*, 2013; Kaufmann *et al.*, 2004; Wu & Rossmann, 1993). The B19 VP2 structure contains an eight-stranded, antiparallel β-barrel consisting of two β-sheets in the standard BIDG and CHEF arrangement (Fig. 1.4 A, B). This motif occupies the same position relative to the icosahedral symmetry axes as in other *Parvovirinae* viruses. Although most of the 50 C-terminal residues of B19 VP2 are accessible on the particle surface, the final four amino acids are bundled under the 5-fold axis and extend the 5-fold channel into the capsid interior (Kaufmann *et al.*, 2004). The members of the

Parvovirinae subfamily show high-level VP2 sequence divergence, with percent identities in the range of 22 to 26%. Shared sequence identities are localised to the residues within the conserved β-barrel core and αA helix (Kailasan *et al.*, 2015). All parvoviral VPs share conservative glycine-rich C-terminus, that is positioned between neighbouring VPs and emerges on the particle surface next to the 5-fold cylinder, where the VP N-termini are located (Kaufmann *et al.*, 2008).

In many documented cases the recombinant major capsid protein alone is sufficient to form the intact capsids (Fang *et al.*, 2014; Kailasan *et al.*, 2015; Kaufmann *et al.*, 2004; Ros *et al.*, 2006a; Tuke *et al.*, 2010). Chimeric parvoviral VLPs and other viral capsids carrying heterologous epitopes, inserted in the loops that connect the β-sheets in VP2, hold considerable promise to develop new vaccines. Chimeric recombinant VLPs from human parvovirus B19 (Brown *et al.*, 1994; Miyamura *et al.*, 1994), porcine parvovirus (Sedlik *et al.*, 1997), or canine parvovirus (Rueda *et al.*, 1999), were successfully employed to elicit immune responses specific to the epitopes inserted, and induce protection in animals against infection with the donor virus. Moreover, parvovirus capsids are extremely stable against heat and other denaturing agents.

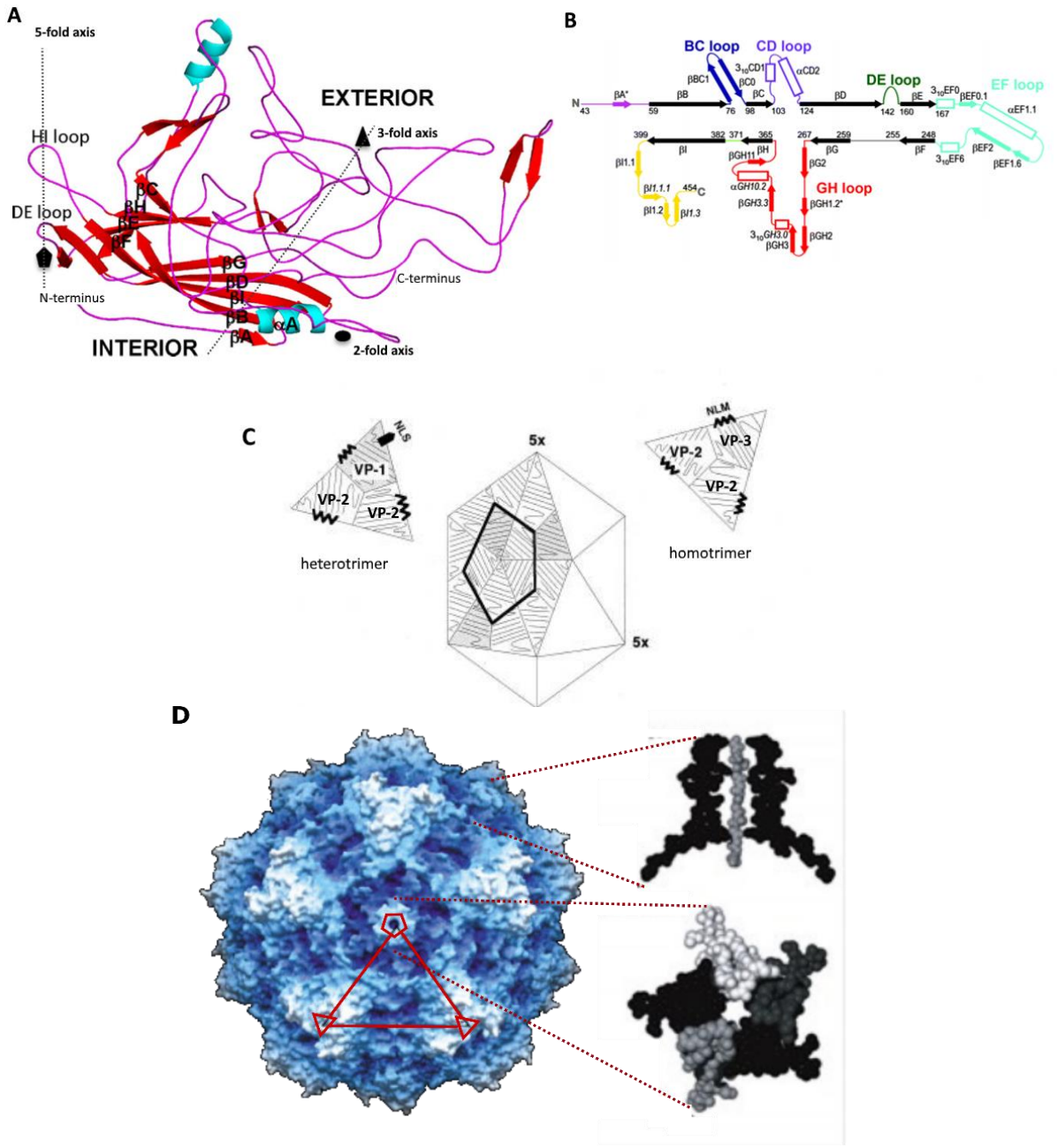


Figure 1.4. The secondary structure of parvovirus VP2 and its assembly to capsid. **A.** Ribbon diagram of BPV VP2 (modified from (Kailasan *et al.*, 2015)). **B.** Schematic BIDG and CHEF arrangement of β-sheets in VP2 of *Bombyx mori* densovirus 1 (adapted from Kaufmann *et al.*, 2011)). **C**. Two types of trimeric capsid precursors (with and without a minor capsid protein) and their position forming an icosahedral capsid (adapted from Lombardo *et al.*, 2000). NLS, nuclear localisation signal; NLM, nuclear localisation motif. **D.** The topology of the parvoviral (MVM) particle. Left—depth-cued, space-filling model of MVM, centred on a fivefold symmetry axis (adapted from Cotmore and Tattersall 2007). Asymmetric unit is indicated by the large triangle, bordered by a fivefold axis, two threefold axes (triangles), and a twofold axis (oval). Upper right—cross-section of the fivefold channel, showing two of the five β-ribbons that comprise the cylinder, and residues 28–37 of VP2 in light grey. Lower right—view down the fivefold cylinder, with the five β-ribbons differentially shaded.

Capsid assembly and genome packaging. Parvoviral capsids are assembled and loaded with DNA within the nucleus of a host cell. In the case of MVM, the transport of trimeric capsid precursors into the nucleus is governed by the nonconventional nuclear localisation motif in VP2 (Lombardo *et al.,* 2000). Two forms of trimer have been characterised, a homotrimer of VP2 and a less abundant heterotrimer that contains one VP1 complexed with two VP2 molecules (Riolobos *et al.,* 2006) (Fig. 1.4 C). Different oligomers, possibly trimers and/or pentamers of the various VP1-3 proteins, are transported into the nucleoli in the AAP-mediated assembly of AAV capsids (Naumer *et al.,* 2012).

The packaging of AAV2 genome is driven by the 3'-to-5' helicase activity of Rep40/52 that is an independently expressed C-terminal domain of the initiator protein. (King *et al.,* 2001). In MVM, DNA is pumped into the capsid through a 5-fold pore (Fig. 1.5) by the helicase activity of a pore-associated oligomeric NS1. Ultimately, ~24-nt DNA sequence and the 5'-associated NS1 molecule remains threaded through the packaging portal and exposed at the virion surface (Cotmore *et al.,* 2010).

MVM and HBoV1 virions were shown to be packaged at early infection stages and rapidly exported from the nucleus to be released from viable cells, allowing rapid infection spreading (Deng *et al.,* 2013; Maroto *et al.,* 2004). Nuclear export of MVM virions is driven by their interaction with NS2 and the nuclear export protein CRM1 and involves internalisation in COPII vesicles in the endoplasmic reticulum (Bär *et al.,* 2013; Engelsma *et al.,* 2008). Further, the MVM capsids are modified by tyrosine phosphorylation in the Golgi complex and delivered via gelsolin-dependent transportation to the plasma membrane (Bär *et al.,* 2008). However, similar early export mechanisms have not been reported for AAV or B19.

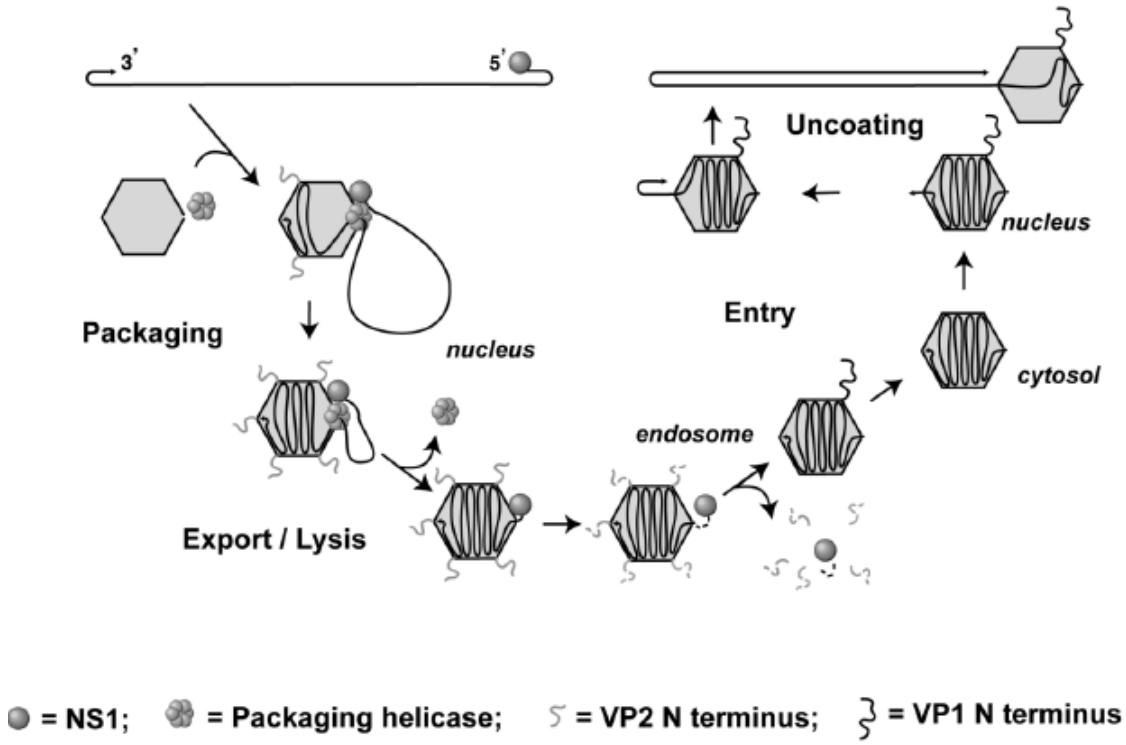


Figure 1.5. The two-portal model of parvovirus virion packaging and uncoating (adapted from Cotmore and Tattersall 2014).

1.1.5. Cell entry

The parvoviral infection starts through capsid-mediated binding to one or more glycosylated receptor molecule on the cell surface (Table 1.2) and is followed by virion uptake into the cell via receptor-mediated endocytosis.

Not all incoming DNA-containing particles follow the same intracellular pathway. It has been shown that a proportion of AAV incoming particles are degraded by the proteasomes, and a large percentage of particles accumulate in a perinuclear region, in the crescent-shaped clusters of vesicles that are focused on one side of the cell nucleus (Ding *et al.*, 2005). Some of the perinuclear particles are slowly transported into the nucleus where it seems that only a few are able to release the DNA, while the rest of particles remain intact (Hauck *et al.*, 2004). The similar tendency was observed for recombinant AAV VLPs, used as viral vectors for gene delivery (Nonnenmacher & Weber, 2012).

Table 1.2. The cellular receptors and confirmed cellular invasion pathways for some members of *Parvovirinae* subfamily. *Adapted from* (Tu *et al.,* 2015).

Genus	Virus	Host	Cellular receptor	Invasion pathway
Amdoparvovirus	Aleutian mink disease virus	Mink	Sialic acid	-
Bocaparvovirus	Bovine parvovirus	Bovine	α2-3 O-linked sialic acid	Clathrin-dependent endocytic pathway
Dependoparvovirus	Adeno-associated virus	Human	Heparan sulphate proteoglycan, sialic acid, aVβ5 integrin	Clathrin-dependent or independent internalisation
	Goose parvovirus	Goose, Muscovy duck	-	-
Erythroparvovirus	Human parvovirus B19	Human	P antigen, α5β1, ku80	Clathrin-dependent endocytic pathway
Protoparvovirus	Canine parvovirus	Dogs, cats	Transferrin receptor	Clathrin-dependent endocytic pathway
	Minute virus of mice	Rodents	α2-3 and α2-8 N-linked sialic acid	Both clathrin- and lipid-raft mediated endocytosis
	Porcine parvovirus	Swine	α2-3 N- and O-linked sialic acid	Macropinocytosis and clathrin-dependent endocytic pathway
	Mink enteritis virus	Mink	Transferrin receptor	-

Infectious entry probably occurs via a late endosomal or lysosomal route. These compartments are rich in proteases and nucleases, that would explain how genomes lose their covalently linked NS1 molecules, and the nucleotides of the ‘‘tether’’ DNA sequence, prior to arrival in the nucleus (Fig. 1.5). CPV remains physically associated with its receptor, TfR, for at least 4 h after internalisation. For MVM, endosomal proteases are known to generate VP3 polypeptides from VP2 molecules following engulfment (Mani *et al.,* 2006; Ros and Kempf, 2004), which is likely important because it both removes the nuclear export signals in the VP2 N-termini (Maroto *et al.,* 2004) and primes the virion for its subsequent conformational transition (Farr *et al.,* 2006). Exactly where the infecting viruses penetrate the endosomal bilayer is uncertain, but CPV infectivity can be blocked by the antibodies against structural epitopes on the VP1u-specific (PLA2) sequences, indicating that the exposure of the VP1u must accompany or precede infectious entry into the cytoplasm (Vihinen-Ranta *et al.,* 2000, 2002).

1.1.6. Replication

Parvoviral genomes are amplified via a single-strand displacement mechanism, called *rolling hairpin replication* (RHR) (Fig. 1.6), that ensures duplication of both termini. The viral replication must occur in mitotic cells because the virus must use the host polymerase to proliferate efficiently. Replication is most likely

mediated by the polymerase δ. It depends on the sliding clamp protein proliferating cell nuclear antigen (PCNA), the single strand binding protein replication protein A (RPA) (Tattersall 1976).

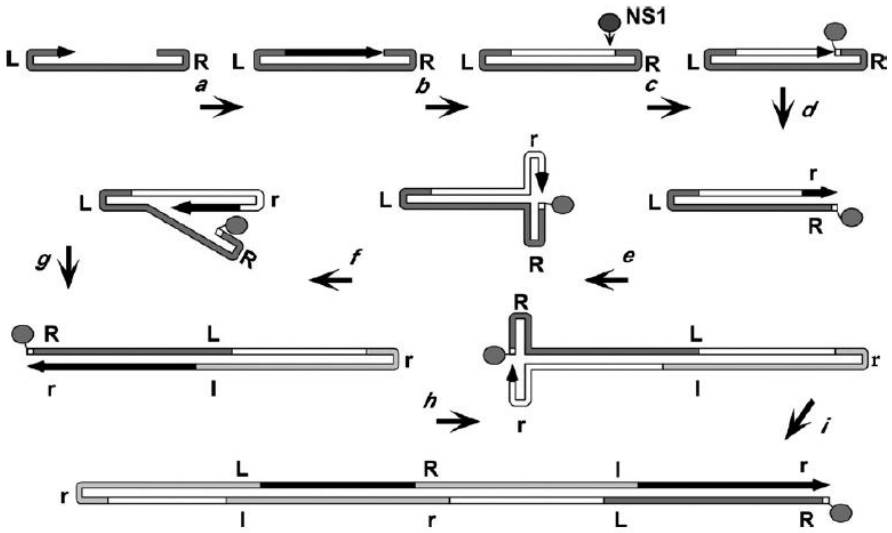


Figure 1.6. Rolling hairpin replication model for parvovirus genome replication. L and l represent flip and flop forms of the left-end telomere; R and r represent these sequence arrangements of the right-end telomere. Newly synthesised DNA is represented by dashed lines, and its 3'end is capped with an arrowhead. Adapted from (Tijssen *et al.,* 2011).

In the initial stage of replication, left (3') telomere of incoming virion DNA folds back on itself, allowing the base-paired 3' nucleotide to prime synthesis of a complementary positive-sense strand and duplex replicating form (RF) DNA is amplified (Fig. 1.6). In MVM, the 3' intact Y-shaped hairpin containing small axial palindromes is essential for the establishment of the initial transcription complexes (Li *et al.,* 2013). These early RF molecules support viral gene expression, including synthesis of NS1, allowing the virus to seize control of cell metabolism rapidly. NS1 is essential for all further steps in the replication pathway as an ATP-powered helicase to resolve terminal hairpin structures of the viral genome (Christensen & Tattersall, 2002; Willwand *et al.,* 2002). Overall, the result of rolling hairpin synthesis is that the coding sequences of the virus are copied twice as often as the termini, and duplex dimeric and tetrameric concatemers are generated.

Unit-length genomes are excised from the duplex intermediates during the terminal resolution. A single-strand nick is introduced into the DNA via a *trans*-esterification reaction that transfers the phosphodiester bond to a tyrosine

hydroxyl in the active site of the NS1 nuclease, leaving it attached covalently to the new 5' nucleotide and liberating a base-paired 3' hydroxyl to prime a new fork. NS1 possess the site- and strand-specific *trans*-esterification activity, characteristic to all rolling circle replication initiator endonucleases, that are used by many small circular replicons. However, NS1 has lost the subsequent joining function to circularise progeny genomes. Instead, this nickase is left covalently attached to the 5' ends (Fig. 1.6) of all viral DNAs via its active-site tyrosine throughout replication, packaging, and virion release (Nüesch *et al.,* 1995).

Replication can take several days, during which the host cell nucleus is structurally rearranged and enlarged (Ihalainen *et al.,* 2009). For productive infection, parvoviruses evoke a broad and variable range of host DNA damage repair responses (Adeyemi *et al.,* 2010; Lou *et al.,* 2012b; Luo *et al.,* 2011), possibly because it provides opportunities for suspending cellular, but not viral, DNA replication. The cell cycle is blocked prior to metaphase, creating an extended S-like phase, leaving cells actively synthesising viral DNA until subsequent cell death results in the release of the progeny virus (Adeyemi & Pintel, 2014; Luo *et al.,* 2013).

1.2. Human bocaviruses (HBoV)

1.2.1. HBoV 1-4 classification

Human bocavirus (HBoV1) was first detected in patients with respiratory illness and has been associated with respiratory tract infections (Allander *et al.,* 2005). Additional three genotypes (HBoV2-4) were identified with a possible connection to gastrointestinal illnesses (Arthur *et al.,* 2009; Kapoor *et al.,* 2009, 2010). Kapoor *et al.,* (2010) suggested splitting of HBoV2 into 2A and 2B genotypes due to a high degree of genetic diversity. Although the bocaviruses have come into the spotlight during the last decade, a retrospective study by Levican *et al.* (2013) has demonstrated the HBoV1-3 DNA detection in the samples collected in the 1980s.

Human bocaviruses belong to the genus *Bocavirus* in the subfamily *Parvovirinae* of the family *Parvoviridae*. According to the latest changes in the taxonomy of

viruses, the genus *Bocavirus* has been proposedly renamed as *Bocaparvovirus*. As viruses that encode NS1 proteins that show over 85% amino acid identity were subgrouped into the same species, Primate bocaparvovirus 1, was created to include HBoV1, HBoV3, and Gorilla bocavirus, and Primate bocaparvovirus 2 to include HBoV2 and HBoV4 (Cotmore *et al.*, 2014).

1.2.2. Pathogenesis of HBoV1

The pathogenetic mechanism for bocavirus infection has been characterised only for HBoV1. A few in vitro models for HBoV1 cultivation have been developed, including the differentiated human airway epithelial (HAE) cells and a reverse genetics system using a plasmid clone of HBoV1 and human embryonic kidney 293 cells (Dijkman *et al.*, 2009; Huang *et al.*, 2012). These models revealed that HBoV1 infection causes disruption of the tight junction barrier (activity associated with the unique region of the VP1 protein), epithelial cell hypertrophy, and the loss of cilia, which are signs of respiratory tract damage. It has been suggested that HBoV1 infection caused damage of the epithelial lining of the respiratory tract may expose to the secondary infections by other microbes and increase the permeability of the epithelia to allergens. Additionally, a lack of cilia may predispose to bronchiolitis (Huang *et al.*, 2012). HBoV1 was shown to enter the epithelial cells from both basolateral and apical surfaces, but the latter pathway being more efficient (Deng *et al.*, 2013).

Despite a significant number of studies worldwide, reporting HBoV1 DNA in children with a variety of respiratory symptoms, mainly wheezing and pneumonia (Table 1.3), even a large meta-analysis of the viral cause of acute lower respiratory tract infections failed to reveal a causal linkage of these conditions to HBoV1 infection (Shi *et al.*, 2015). HBoV1 is frequently co-detected with other viruses mainly in respiratory specimens from children with acute respiratory disease (Christensen *et al.*, 2010; Guido *et al.*, 2011; Moriyama *et al.*, 2010) and is reported to be among the four most frequent respiratory viruses (Brieu *et al.*, 2008; Cebey-López *et al.*, 2015; Pozo *et al.*, 2007). Infections are more prevalent in children under two years of age than in older

children (Sloots *et al.,* 2006; Wang *et al.,* 2010), and in sporadic cases HBoV1 has even been associated with life-threatening respiratory infections in children (Edner *et al.,* 2012; Körner *et al.,* 2011; Pekcan *et al.,* 2014). HBoV1 has also been found in asymptomatic children (Christensen *et al.,* 2010; García-García *et al.,* 2008; Martin *et al.,* 2010). A severe HBoV1 infection requiring intensive care and leading to pneumonia has also been reported in elderly patients (Krakau *et al.,* 2015).

HBoV1 DNA in stool samples has also been reported in several studies (Table 1.3.) with an overall incidence of 0.8-10% of stool samples from children with acute gastroenteritis, but coinfections with other viruses were found in most cases. Although the study by Proença-Modena *et al.,* 2011 showed that diarrhoea was more common in cases with a very high HBoV1 viral load, several case-control studies did not find an association between HBoV1 and gastroenteritis (Jin *et al.,* 2011; Nawaz *et al.,* 2012; Yu *et al.,* 2008). Neske *et al.,* 2007 suggests, that HBoV1 DNA in stools may be swallowed from the respiratory tract. Pozo and colleagues (Pozo *et al.,* 2007) have detected HBoV1 DNA in two of six urine samples of hospitalised LRTI children patients.

HBoV1 DNA has also been detected in the cerebrospinal fluid (CSF) of children with encephalitis without other potential pathogens (Mitui *et al.,* 2012), and a case of invasive HBoV1 infection with hepatitis and skin manifestations has been reported in a child with T-cell deficiency (Kainulainen *et al.,* 2008). HBoV1 DNA has been detected in a cord blood sample from new-born with hydrop, therefore pass through the placenta barrier was suggested (Shao *et al.,* 2015).

Recent studies suggest that HBoV may indirectly contribute to the development of some colorectal and lung cancers, or may play an active role in cancer by interacting with the host genome (Schildgen *et al.,* 2013). Most recently, twenty-four out of 101 colorectal cancer tissue specimens were found to contain HBoV1 DNA (Abdel-Moneim *et al.,* 2016).

1.2.3. Pathogenesis of HBoV2-4

Since the discovery of HBoV2, 3 and 4, several groups have detected HBoV in faecal samples of children with acute gastroenteritis, at a frequency of 0.9-33% (Table 1.4.), and co-detection of other pathogens varies as high as 0.2-100%. HBoV2 has been specifically associated with gastrointestinal symptoms (Arthur *et al.,* 2009; Jin *et al.,* 2011; Nawaz *et al.,* 2012) but similar data for HBoV3 and HBoV4 is still lacking (Chow and Esper, 2009; Ong *et al.,* 2016). Substantially higher prevalence of HBoV genotype 2B than other HBoV species has been detected in children with acute flaccid paralysis (Kapoor *et al.,* 2010). HBoV2 has also been detected in in 0.6-4.3% of respiratory samples of children with ARTI, usually together with other viruses (Han *et al.,* 2009; Koseki *et al.,* 2012). HBoV2 has also been detected in stool samples of children with ARTI (Vicente *et al.,* 2007). HBoV2 DNA, as well as bocavirus-like particles, has been detected in the CSF of children with encephalitis, indicating the crossing of the blood-brain barrier (Mitui *et al.,* 2012). There is also a case report of fatal subacute myocarditis associated with HBoV2 in a 13-month-old child (Brebion *et al.,* 2013).

HBoV3 and 4 detection rates in children with AGE have been lower than those of HBoV2 (Table 1.4). HBoV3-positive cases have been found mostly in young children (Nawaz *et al.,* 2012). HBoV3 and 4 DNA has been detected in respiratory samples from the children with ARTI (0.4% and 0.6% os samples, respectively) (Koseki *et al.,* 2012). However, none of the studies to date has established any aetiological role of HBoV3 or 4 in human pathogenesis.

1.2.4. Epidemiology of HBoV

During the decade after the discovery of HBoV1, a number of epidemiological studies have been conducted. Primarily, cohorts of patients with respiratory tract illnesses (Table 1.3) and gastroenteric symptoms (Table 1.4) are targetted for HBoV DNA in nasopharyngeal or stool samples, respectively. HBoV1 has a global distribution, with prevalences varying considerably from 0.3% to 33.3%

in ARTI patients, mainly in young children aged 6-24 months, when primary infection is mostly possible. Complication of assigning an explicit aetiological role of HBoV1 infections is that in 18-90% of reported cases, HBoV1 DNA is co-detected with other pathogens (for references, Table 1.3). Detection of HBoV1 in the respiratory specimens of immunocompetent adults and the elderly is still rare.

Several studies have demonstrated the detection of HBoV1 DNA (1-17% prevalence) in the respiratory samples of completely asymptomatic individuals and individuals with non-respiratory symptoms (Cheng *et al.,* 2008; Christensen *et al.,* 2010; Fry *et al.,* 2007; García-García *et al.,* 2008; von Linstow *et al.,* 2008; Martin *et al.,* 2009). However, two highest HBoV1 DNA prevalences ever published have been among individuals without respiratory symptoms: 20 of 45 nasal swab specimens (44%) from asymptomatic children who attended daycare in US (Martin *et al.,* 2010) and 43 of 100 nasopharyngeal aspirates of children undergoing elective surgery (mostly ear, nose and throat surgeries) without concomitant respiratory symptoms or fever at admission in Canada (Longtin *et al.,* 2008). Prolonged persistence of bocavirus DNA in nasopharynx up to several months (Blessing *et al.,* 2009) may influence distorted real picture of HBoV prevalence in asymptomatic cases. HBoV1 DNA detection in the stools of adults is rare, and the most comprehensive study involving 2256 stool samples from patients of various ages with AGE found no HBoV1 DNA in adults in contrast to 2% of children aged <4 years (Nawaz *et al.,* 2012). Variations in average detection rates between the seasons have been observed (Proença-Modena *et al.,* 2011), with the highest detection rates mainly found in the cold seasons (Lau *et al.,* 2007; Weissbrich *et al.,* 2006; Yu *et al.,* 2008).

In contrast to HBoV1, HBoV2-4 are considered enteric viruses because they are mainly detected in the stool (Table 1.4) and only rarely in respiratory specimens. In a comprehensive study of over 6000 nasopharyngeal swabs/aspirate samples, HBoV2 DNA has not been detected in any of samples, in comparison to 14% and 3.4% HBoV1 DNA prevalence in samples from Thailand and the UK, respectively (Chieochansin *et al.,* 2009). Later studies in PR China, Korea, Japan

respectively reported HBoV2 in 4%, 2%, and 0.5% respiratory specimens from children with respiratory tract disease (Koseki *et al.*, 2012; Song, 2010; Han *et al.*, 2009).

Based on the number of studies worldwide (Table 1.4), HBoV2 (more specifically, genotype 2B) is the most abundant bocavirus in human stool samples with reported prevalence of 0.4-24% in patients with gastroenteric symptoms. Interestingly, one of the highest percentage (24%) of HBoV2 DNA incidences was reported from unsymptomatic children in Tunisia (Kapoor *et al.*, 2010). HBoV2-positive cases have been found throughout the year without a clear seasonal variation (Xu *et al.*, 2011). HBoV2 and HBoV3 prevalence appear to be relatively high among young children and low in adults, similarly to HBoV1 DNA in respiratory samples (Nawaz *et al.*, 2012). HBoV3 DNA occurs less frequently (0.4-5.2%) in stool than HBoV2 DNA but may exceed the occurrence of HBoV1 DNA, but coinfection is common (50-100%). HBoV4 has occasionally been detected in the stools of children with AGE, detection rates varying 0- 2.0% (Kantola *et al.*, 2010; Kapoor *et al.*, 2010; Nawaz *et al.*, 2012; Tymentsev *et al.*, 2016).

Seroepidemiological studies reveal mainly the previous infections, thus, the absolute majority (up to 99.3%, in non-competition EIA) of adults tested have been reported to be seropositive for HBoV. The summarised data from seroepidemiological studies are provided in Table 1.3. An extensive follow-up study with longitudinally collected serum samples showed that 80% of the children became HBoV1 seropositive before the age of six years, the mean age for seroconversion being 1.9 years (Kantola *et al.*, 2015). Another study in China revealed that 50% of healthy children under 14 years old had HBoV1 seroprevalence and it was 66.9% in adults (Guo *et al.*, 2012). A number of serological studies confirmed HBoV1 being the most prevalent bocavirus in humans, followed by HBoV2, 3 and 4, respectively (Fang *et al.*, 2014; Guo *et al.*, 2012; Kantola *et al.*, 2011, 2015).

1.2.5. HBoV diagnostics

HBoV infection has a variety of signs and symptoms, including rhinitis, pharyngitis, cough, dyspnoea, wheezing, pneumonia, acute otitis media, fever, nausea, vomiting, and diarrhoea. However, most of these symptoms are not bocavirus-specific, hence molecular diagnostic tools are essential in pin-pointing this pathogen. Current diagnosis of HBoV infections mainly relies on PCR assays targeting primarily NS1 gene, but also NP1 or VP1/VP2 gene regions are amplified (see Table 1.3 for references). PCR methods have been used in HBoV diagnostics widely, but frequent HBoV detections in asymptomatic persons (including blood donors) have made the sole use of this method problematic (Bonvicini *et al.,* 2011; Li *et al.,* 2015a). Additionally, bocaviral DNA persists in the blood (Berns & Parish, 2007; Simmons *et al.,* 2011) and has been detected in patients 1–7 months after infection (Brieu *et al.,* 2008; von Linstow *et al.,* 2008). In general, a high HBoV1 DNA load (>10,000 copies/ml) in the nasopharynx and viremia are generally good indicators of acute infection (Söderlund-Venermo *et al.,* 2009).

For EIA, Western blot, and immunofluorescence assay based serological studies, prokaryote-derived recombinant HBoV proteins and insect cell-generated HBoV VP2 VLPs has been employed (see Table 1.5 for reference). IgG-avidity assays have also been developed for HBoV1 (Hedman *et al.,* 2010). Conserved B-cell epitopes of HBoVs have also been tested as diagnostic tools and have shown to be comparable to VLP-based methods (Zhou *et al.,* 2014). As antibodies between different HBoV species were shown to be cross-reactive, and, thus, complicates the serodiagnostics of HBoVs, serological competition assays have been proposed for correct estimation of seroprevalence of different species (Guo *et al.,* 2012; Kantola *et al.,* 2011). Additionally, serological methods are of limited use due to reported original antigenic sin phenomenon (Li *et al.,* 2015b), that may explain the lack of an IgG increase in heterologous secondary infections.

Table 1.3. Summary of HBoV1 DNA prevalence studies in respiratory illness-related cases.
The list of studies was adapted and extended from a 2012 systematic review by Jartti *et al.*, (2011).

Country	Sample collection year(s)	Age (years)	Sample size	Major clinical symptoms reported	HBoV1 pos., n (%)	Diagnostic method, (HBoV1 gene(s) targeted)	Coinfection with other viruses, n (%)	Reference
Sweden	2003-2004	-	540	LTRI	17 (3%)	PCR, (NP1)	3 (18%)	(Allander *et al.*, 2005)
Thailand	2004-2005	-	1178	Pn	39 (3%)	RT-PCR, (NS1 & NP1)	n/r	(Lu *et al.*, 2006)
Canada	2003-2004	<6 6-15 16-50 >50	290 149 444 324	ARTI	8 (2.8%) 3 (2.0%) 6 (1.4%) 1 (0.3%)	PCR, (NP1)	n/r	(Bastien *et al.*, 2006)
S. Korea	2000-2005	<6	515	LRTI	58 (11.3%)	RT-PCR, (NS1)	22 (37.9%)	(Choi *et al.*, 2006)
France	2003-2004	<6	589	ARTI	26 (4.4%)	PCR, RT-PCR, (NP1)	9 (34.6%)	(Foulongne *et al.*, 2006)
Germany	2002-2005	<9	835	ARTI	87 (10.4%)	PCR, (NP1)	34 (39.1%)	(Weissbrich *et al.*, 2006)
Finland	2000-2002	<16	259	acute expiratory wheezing	49 (18.9%)	(NP1)	37 (75.5%)	(Allander *et al.*, 2007)
Thailand	2004-2005	all	1680	Pn or infuenza-like illness	73 (4.3%)	RT-PCR, (NS1 & NP1)	42/53 (79.2%)	(Fry *et al.*, 2007)
Hong Kong	2004-2005	all <18	400 1200	LTRI	20 (5.0%) 83 (6.9%)	PCR, (NP1)	26/79 (32.9%)	(Lau *et al.*, 2007)
Japan	2002-2003 & 2005	median age 21.3 months	318	LTRI	18 (5.7%)	PCR, (NP1)	n/r	(Ma *et al.*, 2006)
Spain	2005-2006	<3	520	RTI	40 (7.7%)	PCR, (NP1 & VP1)	25 (63.5%)	(Vicente *et al.*, 2007)
Germany	2005-2006	3-17 months	389	RTI, hospitalised	11 (2.8%)	PCR	4 (36.4%)	(Völz *et al.*, 2007)
Spain	2004-2006	<14	730	LTRI	115 (15.7%)	nested PCR, (NP1, VP1)	74/123 (60.1%)	(Pozo *et al.*, 2007)
Italy	2004-2005	<15	1332	RTI, hospitalised	99 (7.4%)	RT-PCR, (NS1)	50 (50.5%)	(Esposito *et al.*, 2008)
Thailand	2006-2008	all	386	n/r	55 (14.1%)	nested PCR, (NP1)	n/r	(Chieochansin *et al.*, 2009)
UK	2007-2008	all	6138	n/r	67 (3.4%)	nested PCR, (NP1)	n/r	(Chieochansin *et al.*, 2009)
Norway	2006-2007	children	376	LTRI	45 (12.0%)	RT-PCR, (NP1)	35 (77.7%)	(Christensen *et al.*, 2008)
Hong Kong	2005-2006	1-15	1906	RTI, hospitalised	95 (5.0%)	PCR, (NP1)	18 (19%)	(Margaret *et al.*, 2008)

Spain	2005-2007	<14	710	RTI	99 (13.9%)	nested PCR, (NP1, VP1)	64 (64.6%)	(Calvo *et al.*, 2008)
France	2006-2007	<5	507	RTI	55 (10.8%)	PCR, RT-PCR, (NP1)	22 (40%)	(Brieu *et al.*, 2008)
Jordan	2003-2004	<5	312	ARTI	57 (18.3%)	PCR, (NP1)	41 (71,9%)	(Kaplan *et al.*, 2006)
Italy	2004-2007	children	415	RTI, hospitalised	34 (8.2%)	PCR, (NS1 & NP1)	21 (61.8%)	(Pierangeli *et al.*, 2008)
Canada	2002-2003	adults	126	chronic bronchitis or Pn	1 (0.8%)	PCR, (NS1 & NP1)	0	(Longtin *et al.*, 2008)
Canada	2002-2003	<4	225	RTI, hospitalised	31 (13.8%)	PCR, (NS1 & NP1)	22 (71%)	(Longtin *et al.*, 2008)
France	2004-2005	<15	828	RTI, hospitalised	11 (1.4%)	PCR, (NP1, VP1/2)	n/r	(Dina *et al.*, 2009)
France	2003-2004	<15	448	LTRI, hospitalised	7 (1.6%)	PCR, (NP1, VP1/2)	5 (71.4%)	(Dina *et al.*, 2009)
Australia	2003-2004	<5	96	ARTI	17 (17.7%)	PCR, RT-PCR (NP1, VP1)	4 (23.5%)	(Tozer *et al.*, 2009)
PR China	2006-2007	<10	351	ARTI	16 (4.6%)	PCR, (NP1, VP1/2)	3 (19%)	(Zeng *et al.*, 2010)
Germany	2006-2007	<15	156	RTI	15 (9.6%)	RT-PCR, (NP1)	2 (13.3%)	(Karalar *et al.*, 2010)
PR China	2006-2008	0.5-9	817	RTI with fever	96 (11.8%)	RT-PCR, (NP1, NS1, VP1)	49 (51%)	(Wang *et al.*, 2010)
Germany	2006-2008	<16	404	LTRI	28 (6.9%)	RT-PCR	19 (67.9%)	(Franz *et al.*, 2010)
Spain	2005-2008	<2	318	bronchiolitis	42 (11.4%)	RT-PCR, (NP1, VP1/2)	28 (66.7%)	(Calvo *et al.*, 2010)
Israel	2005-2006	<3	465	acute bronchiolitis	31 (6.7%)	RT-PCR, (NS1)	28 (90.3%)	(Miron *et al.*, 2010)
USA	2006-2008	<2	318	RTI	106 (33.3%)	RT-PCR, (NP1)	76 (71.7%)	(Martin *et al.*, 2010)
Finland	2009-2011	<15	545	ARTI	23 (4.2%)	PCR, (NS1)	n/r	(Paloniemi *et al.*, 2014)
PR China	2006-2013	<	4941	ARTI	82 (1.7%)	PCR, (NS1)	22/49 (44.9%)	(Zhao *et al.*, 2016)
Australia	2004	all	324	ARTI	18 (5.6%)	RT-PCR, (NP1)	10/18 (55.6%)	(Sloots *et al.*, 2006)
Iran	2007-2008	<17	133	asthma and RTI	9 (6.8%)	PCR, (NS1)	n/r	(Nadji *et al.*, 2010)
USA	2005-2006	all	868	RTI	0 (0%)	PCR, (NS1)	n/r	(Chow *et al.*, 2010)
Norway	2007-2009	children	1316	RTI	144 (10.9%)	PCR, (NP1)	108/144 (75%)	(Christensen *et al.*, 2010)
Latvia	2012-2015	<4.2	130	LTRI	42 (32%)	PCR, (NS1)	n/r	(Nora-Krūkle *et al.*, 2016)
Lithuania	2012-2015	<4	56	LTRI	8 (14%)	PCR, (NS1)	n/r	(Nora-Krūkle *et al.*, 2016)

Japan	2005-2011	< 11.3	850	RTI	132 (15.5%)	PCR, (VP1)	56/132 (42.4%)	(Koseki *et al.*, 2012)
Cambodia	2007-2009	all	2773	ARTI	43 (1.5%)	multiplex RT-PCR/PCR, NP1	19/43 (44%)	(Arnott *et al.*, 2013)

Pn, pneumonia; RTI, respiratory (lower tract (L), acute (A)) tract illness; n/r, not reported.

able 1.4. Summary of HBoV DNA prevalence studies in human stool.

The list of studies was adapted and extended from a 2012 systematic review by Jartti *et al.,* (2011)**.**

Country	Sample collection year(s)	Age (years)	Sample size	Major clinical symptoms reported	HBoV species prevalent (number of cases)	HBoV pos., n (%)	Method, (HBoV gene(s) targeted)	Coinfection, n (%)	Reference
Spain	2005-2006	<3	527	respiratory tract infection	n/s	48 (9.1%)	PCR, NP1 & VP1	28 (58.3%)	(Vicente *et al.*, 2007)
S. Korea	2005-2006	<18	1435	AGE	n/s	30 (2.1%)	PCR, NP1	14/25 (56%)	(Lau *et al.*, 2007)
PR China	2006-2007	<5	1216	AGE	n/s	67 (5.5%)	RT-PCR or PCR, VP1/VP2	57/67 (77.6%)	(Yu *et al.*, 2008)
India	2009-2011	<5	418	AGE	1 (15)>2 (4) >4 (3)>3 (2)	24 (5.7%)	PCR, VP1/VP2	5/24 (21%)	(Lasure & Gopalkrishna, 2016)
Thailand	2011	<5	222	diarrhoea	1 (11) > 2A (3) >3 (2) >4 (1)	17 (7.7%)	PCR, VP1/VP2	10/17 (58.8%)	(Khamrin *et al.*, 2012)
Finland	2009-2011	<15	172	AGE	2 (10) >1 (2)> 3(1)	13 (7.6%)	PCR, NS1	n/r	(Paloniemi *et al.*, 2014)
Albania	2013-2015	<18	142	AGE	1 (12)>2 (1)	13 (9.1%)	PCR, (NP1 & VP1/VP2)	13/13 (100%)	(La Rosa *et al.*, 2015)
PR China	2010-2012	<14	1121	Acute diarrhoea	2 (15)> 1 (9)> 3 (1)	25 (2.2%)	PCR, (NS1)	n/r	(Zhao *et al.*, 2016)
Thailand	2005-2006	4 m - 4 y	225	Acute diarrhoea	n/s	2 (0.9%)	PCR, (NS1 & VP1/VP2)	1/2 (50%)	(Chieochansin *et al.*, 2008)
Hong Kong	2004-2005	<10	1435	AGE	n/s	25 (2.1%)	PCR, (NP1)	9/25 (36%)	(Lau *et al.*, 2007)
PR China	2006-2007	<5	397	diarrhoea	n/s	14 (3.5%)	PCR (VP1/VP2), qPCR (NS1)	9/14 (64%)	(Cheng *et al.*, 2008)
Germany	2007	all	307	vomiting and/or diarrhoea	n/s	14 (4.6%)	qPCR (NP1)	8/14 (57.1%)	(Campe *et al.*, 2008)
Australia	2001	<17	186	AGE	2(22) >1(16) >3(5)	32 (17.2%)	PCR (NS1)	n/r	(Arthur *et al.*, 2009)
Pakistan	archive	all	98	mixed	2 (only targeted)	5 (5.1%)	PCR (NS1)	n/r	(Kapoor *et al.*, 2009)

United Kingdom	archive	all	699	mixed	2 (only targeted)	3 (0.4%)	PCR (NS1)	n/r	(Kapoor *et al.*, 2009)
Japan	2003-2006	<15	877	AGE	n/s	4 (0.5%)	PCR (NS1)	1/4 (25%)	(Nakanishi *et al.*, 2009)
S. Korea	2008-2009	<17	358	AGE	2(13) >1(2)	15 (4.2%)	PCR (NS1)	9/15 (60%)	(Han *et al.*, 2009)
Hungary	2007-2008	<5	61	AGE	1	2 (3.8%)	PCR (VP1/VP2)	0/2 (0%)	(Szomor *et al.*, 2009)
Australia	2002	<6	136	AGE	1	13 (9.6%)	PCR, RT-PCR (NP1, VP1)	3/13 (23%)	(Tozer *et al.*, 2009)
Australia	2006	all	239	AGE	1	5 (2.1%)	PCR, RT-PCR (NP1, VP1)	1/5 (20%)	(Tozer *et al.*, 2009)
Finland	2007	<15	50	AGE	1	4 (8%)	RT-PCR (NP1)	3/4 (75%)	(Räsänen *et al.*, 2010)
Iran	2006-2008	<17	47	AGE	1	6 (12.8%)	PCR, (NS1)	n/r	(Nadji *et al.*, 2010)
Brazil	N/R	all	807	AGE	1 (10) >3 (5)	15 (1.8%)	PCR, (NS1)	2/807 (0.2%)	(Santos *et al.*, 2010)
Brazil	N/R	all	144	AGE	2	30 (20.8%)	PCR, (NS1)	13/30 (43.3%)	(Santos *et al.*, 2010)
USA	2007-2008	all	479	mixed	1(9)> 2(6) > 3(0)	15 (3.1%)	PCR (NS1)	n/r	(Chow *et al.*, 2010)
Germany	2006-2007	<15	64	mixed	1	5 (7.8%)	RT-PCR (NP1)	3/5 (60%)	(Karalar *et al.*, 2010)
Nepal	N/R	adults	96	diarrhoea	2B (4)> 1,2A,3,4 (0)	4 (4.2%)	PCR (VP1/2)	n/r	(Kapoor *et al.*, 2010)
Nigeria	N/R	<15	96	AFP	2B (19)>3(5)>4(2) >1(1),2A(1)	28 (29.1%)	PCR (VP1/2)	n/r	(Kapoor *et al.*, 2010)
Tunisia	N/R	<15	96	AFP	2B (24)>3(3) >4(2),1(2)>2A(1)	32 (33.3%)	PCR (VP1/2)	n/r	(Kapoor *et al.*, 2010)
USA	N/R	adults	87	diarrhoea	2B (4)> 3(1),4(1) >1,2A(0)	6 (6.9%)	PCR (VP1/2)	n/r	(Kapoor *et al.*, 2010)
Finland	N/R	all	250	diarrhoea	2(4)>3(1)>1,4 (0)	5 (2.0%)	RT-PCR (NS1)	3/5 (60%)	(Kantola *et al.*, 2010)
Russia	2010-2012	≤5	5250	diarrhoea	2(35)>1(24) >4(2)>3(1)	62 (1.2%)	PCR (NS1)	28/62 (45%)	(Tymentsev *et al.*, 2016)
Egypt	2013-2015	<2	100	AGE	1	2 (2.0%)	PCR (VP1/2)	1/2 (50%)	(El-Mosallamy *et al.*, 2015)
PR China	2010-2011	<15	553	acute diarrhoea	2	15 (2.7%)	RT-PCR (NS1)	n/r	(Zhao *et al.*, 2012)
UK	N/R	all	2256	AGE	2 (32)>3(10) >4(1); 101 untyped	149 (6.6%)	qPCR (NS1)	88/149 (59.1%)	(Nawaz *et al.*, 2012)

AGE, acute gastroenteritis; AFP, acute flaccid paralysis; n/s, not specified; n/r, not reported

Table 1.5. Summary of HBoV antibody prevalence studies.
The list of studies was adapted and extended from a 2012 systematic review by Jartti *et al.*, (2011)**.**

Country	**Study year**	**Age**	**Clinical symptoms of selected sample**	**Method**	**Antigen, expression system**	**Seropositive (serum), n, (%)**	**PCR+, n (%)**	**Reference**
Japan	1998-2007	0-41 y	n/r	IgG (IFA)	VP1-his, BICS	145/204 (71.1%)	n/r	(Endo *et al.*, 2007)
Germany	n/r	<10 y	non-infectious (HBoV DNA-)	IgG, IgM (EIA)	VP2 VLPs, BICS	IgG: 27/52 (52%) IgM: 0/52 (0%)	0, (0%)	(Lindner *et al.*, 2008)
	n/r	4 mo – 7.5 y	RTI (HBoV DNA+)	IgG, IgM (EIA)	VP2 VLPs, BICS	IgG: 10/24 (42%) IgM: 10/24 (42%)	24, (100%)	
	n/r	19-78 y	none	IgG, IgM (EIA)	VP2 VLPs, BICS	IgG: 280/299 (94%) IgM: 2/299 (1%)	n/r	
PR China	2006	<9 y	LTRI	IgG (EIA)	VP2 VLPs, BICS	50/161 (31%)	5/161, (3%)	(Lin *et al.*, 2008)
Finland	2000-2002	3 mo -15 y	acute expiratory wheezing	IgG, IgM (EIA)	VP2 VLPs (biotinylated), BICS	IgG: 111/258 (43%) IgM: 48/258 (19%)	64/258 (24.8%)	(Söderlund-Venermo *et al.*, 2009)
PR China	2006-2008	6 mo – 9 y	ARTI with fever	IgG, IgM (EIA)	VP2 fragment, *E. coli*	IgG: 40/79 (50.6%) IgM: 44/79 (55.7%)	96/817 (11.8%)	(Wang *et al.*, 2010)
Italy	2001-2002	0.3 - 16.6 y	pneumonia	IgG, μ-capture IgM (EIA)	VP2 VLPs (biotinylated), BICS	IgG: 12/101 (12%) IgM: 11/101 (11%)	n/r	(Don *et al.*, 2010)
Germany	2006-2007	<14.8 y	RTI	IgG (EIA)	VP2 VLPs, BICS	IgG: 95/156 (60.9%)	15/156 (9.6%)	(Karalar *et al.*, 2010)
Jamaica	2009	<18 y	n/r (hospitalized)	IgG (EIA)	VP2 VLPs, BICS	IgG: 220/287 (76.7%)	n/r	(Hustedt *et al.*, 2012)
USA	2003-2004	≤20 y	n/r (hospitalized)	IgG (EIA)	VP2 VLPs, BICS	IgG: 195/270 (72.2%)	n/r	(Kahn *et al.*, 2008)
USA	n/r	adult	healthy, blood donors	IgG (EIA)	VP1-2-3 VLPs, BICS	IgG: 255/404 (63%)	n/r	(Cecchini *et al.*, 2009)
Japan	1993	1-80 y	healthy	IgG, IgM (EIA)	VP2 VLPs, BICS	IgG: HBoV1: 346/372 (93.0%);	n/r	(Fang *et al.*, 2014)

						HBoV2: 261/372 (70.1%); HBoV3: 252/372 (67.7%); HBoV4: 285/372 (76.6%). IgM: HBoV1: 1/372 (0.54%); HBoV3: 1/372 (0.54%).		
PR China	2008	<14 y	healthy	IgG (EIA, competition format)	VP2 VLPs, BICS	IgG: HBoV1: 166 (122[a])/244 (68%); HBoV2: 119 (90[a])/244 (48.8%); HBoV3: 112 (70[a])/244 (49.6%); HBoV4: 55 (2[a])/244 (22.5%).	n/r	(Guo *et al.*, 2012)
PR China	2008	15-70 y	healthy	IgG (EIA, competition format)	VP2 VLPs, BICS	IgG: HBoV1: 141 (95[a])/142 (99.3%); HBoV2: 137 (70[a])/142 (96.5%); HBoV3: 137 (55[a])/142 (96.5%); HBoV4: 73 (2[a])/142 (51.4%).	n/r	(Guo *et al.*, 2012)
PR China	2011	<14 y	healthy	IgG (EIA)	VP2 VLPs, BICS	IgG: HBoV1: 1021/1391 (73.4%); HBOV2: 986/1391 (70.9%).	n/r	(Hao *et al.*, 2015)
Finland	n/r	21-32 y	healthy	IgG (EIA, competition format)	VP2 VLPs, BICS	IgG: HBoV1: 109 (74[a])/115 (95%); HBoV2: 110 (33[a])/115 (96%); HBoV3: 100 (9[a])/115 (87%); HBoV4: 90 (1[a])/115 (78%).	n/r	(Kantola *et al.*, 2011)
Finland	n/r	0.2–15 y	acute expiratory wheezing	IgG (EIA, competition format)	VP2 VLPs, BICS	IgG: HBoV1: 140 (107[a])/252 (59%); HBoV2-4: 65[a]/252 (27%[a]);	HBoV1: 52/487	(Kantola *et al.*, 2011)
Pakistan	n/r	18-20 y	healthy, blood donors	IgG (EIA, competition format)	VP2 VLPs, BICS	IgG: HBoV1: 79 (42[a])/80 (99%); HBoV2-4: 36[a]/80 (45%[a]);	n/r	(Kantola *et al.*, 2011)

BICS, baculovirus-insect cell system; IFA, immunofluorescence assay; RTI, respiratory (lower (L), acute (A)) tract illness; n/r, not reported.
[a] number of seropositive samples after antigen competition.

1.3. Human parvovirus 4 (PARV4)

1.3.1. PARV4 epidemiology and transmission

Human parvovirus 4 (PARV4) was identified in the plasma of an intravenous drug user with the acute viral syndrome in 2005. The isolation of the full-length genome of new parvovirus was achieved through the use of a sequence–independent PCR amplification method. PARV4 was found to display <30% of amino acid sequence similarity to other parvoviruses and it formed a distinct branch upon phylogenetic analysis. It was initially described as 'not closely related to any known parvoviruses' (Jones *et al.,* 2005). However, further work has shown that PARV4 is most similar to the recently discovered bovine and porcine hokoviruses (Lau *et al.,* 2008).

PARV4 isolates have been subclassified into three genotypes. Genotypes 1 and 2 (the latter originally termed PARV5) are predominant in Europe, North America, and Asia (Manning *et al.,* 2007; Yu *et al.,* 2012); genotype 3 is most widespread in Africa (Panning *et al.,* 2010; Simmonds *et al.,* 2008). Due to minimal genetic diversity within each genotype, the recent spread within the past 20–30 years has been suggested. Manning *et al.,* (2007) observed an apparent temporal shift in PARV4 genotypes, with genotype 1 representing the current 'modern' infection and genotype 2 the older strain. In a study by Tuke *et al.,* (2010) the subjects positive for genotype 1 were all born after 1958 and those infected with genotype 2 were born between 1949 and 1956. It is possible that the three genotypes represent separate zoonotic transmissions of PARV4 into human populations, perhaps from chimpanzees and monkey species that harbour the most closely related parvoviruses to PARV4 (Sharp *et al.,* 2010a). Viruses closely related to PARV4 were also identified in porcine and bovine serum samples (Lau *et al.,* 2008).

The epidemiology and the clinical implications of PARV4 in the human population are still under evaluation. There are two focus cohorts in the PARV4 epidemiology studies: one being the immunocompromised patients, usually intravenous drug users and/or infected with other blood-borne viruses, like HIV or hepatitis (B, C) viruses. Another group of interest is the newborns and pregnant

women. Both groups correspond to the initially proposed parenteral mode of PARV4 transmission (Lavoie *et al.,* 2012; Lurcharchaiwong *et al.,* 2008), however, some studies revealed the presence of viral DNA in nasal and faecal samples, mainly in sub-Saharan and Western African countries (Drexler *et al.,* 2012; Panning *et al.,* 2010). The reported prevalence of PARV4 DNA in healthy individuals from different parts of the world varies from 0.1% to 24%. Epidemiologic situations may differ significantly from country to country and are summarised in Table 1.6.

Studies in Sweden and Finland, as well as South Africa, demonstrated a high prevalence (45-78.2%) of antibodies against PARV4 in IDUs, persons co-infected with HIV and HCV, and individuals with hemophilia who were exposed to nonvirally inactivated clotting factors (Lahtinen *et al.,* 2011; Matthews *et al.,* 2015; Sharp *et al.,* 2009). In contrast, PARV4 seroprevalence was 25%–37% in adults in the Democratic Republic of Congo, Cameroon, Burkina Faso who were not infected with HIV and hepatitis C virus (Sharp *et al.,* 2010b). PARV4 DNA was detected in the blood of 8.6% of children 15 or 24 months of age in Ghana (Panning *et al.,* 2010). There was no history of exposure to multiple-use needles or blood transfusion in any of these children. These data suggested alternative modes of PARV4 transmission in African countries. Nonparenteral modes of transmission of PARV4 have also been proposed in South Africa (Sharp *et al.,* 2010c), South-eastern Asia (Benjamin *et al.,* 2011; Lurcharchaiwong *et al.,* 2008; Yang *et al.,* 2011; Yu *et al.,* 2012), and United Kingdom (Maple *et al.,* 2013).

PARV4 DNA has been found to be a common contaminant of plasma pools and can be detected by polymerase chain reaction in 4-8.9% of plasma specimens (Fryer *et al.,* 2006; Schneider *et al.,* 2008). A higher percentage of contaminated blood products was reported from the samples prepared until 2000 and were as high as 16-33% (Fryer *et al.,* 2007a; Schneider *et al.,* 2008; Touinssi *et al.,* 2010). Parvoviruses are potentially resistant to traditional viral inactivation methods employed for plasma-derived products (Fryer *et al.,* 2007b; Jones *et al.,* 2005). Although these risks are lessened by modern virus inactivation methods and by the increasing use of recombinant clotting factors instead of plasma-derived

products. However, transfusion-mediated transmission remains a potential issue in developing countries. The unknown clinical significance of PARV4 infection provides even more concern on the safety of plasma-derived products.

Table 1.6. PARV4 prevalence in different countries.

Country	Sample collection year(s)	Study group	Sample size	Incidence rate,%	Method	Tissue	Reference
High-risk individuals							
France	2003-2007	HIV+	150	7.30	EIA	Serum	Servant-Delmas *et al.*, 2014
Thailand	2000	IDU	88	7.95	PCR	Serum	Lurcharchaiwong *et al.*, 2008
Denmark	1997-2012	HIV+ children	46	8.70	EIA	Serum	Rosenfeldt *et al.*, 2015
France	2003-2007	HCV+	216	20.80	EIA	Serum	Servant-Delmas *et al.*, 2014
PR China	2012	HCV+	153	33	PCR	Serum	Zhang *et al.*, 2012
PR China	2008-2009	HCV+	153	33.3	PCR	Serum	Yu *et al.*, 2012
Finland	2012	HCV+	200	34.5	EIA	Serum	Lahtinen *et al.*, 2011
South Africa	2007	HIV+	170	36.2	EIA	Serum	Sharp *et al.*, 2010
France	1985-1999	HIV+	164	36.40	EIA	Serum	Servant-Delmas *et al.*, 2014
South Africa	2009-2013	HIV+ children	157	37	EIA	Serum	Metthews *et al.*, 2015
Italy	1997-2005	HIV+	35	40	PCR	Bone marrow	Longhi *et al.*, 2007
PR China	2012	HBV+	248	41	PCR	Serum	Zhang *et al.*, 2012
PR China	2008-2009	HBV+	188	41.5	PCR	Serum	Yu *et al.*, 2012
US	1989-1994	Haemophiliacs	195	44	EIA	Serum	Sharp *et al.*, 2012
UK	1992	HCV+	33	45.45	EIA	Serum	Sharp *et al.*, 2009
UK	1992	Haemophiliacs	20	55	EIA	Serum	Sharp *et al.*, 2009
Germany	ND	HCV+	9	66.6	PCR	Liver	Schneider *et al.*, 2008
Finland	2012	HIV+	78	78.2	EIA	Serum	Lahtinen *et al.*, 2011
Thailand	2000	HCV+	88	88.64	PCR	Serum	Lurcharchaiwong *et al.*, 2008
Switzerland	ND	HCV+/HIV+/IDU	94	95	EIA	Serum	Simmons *et al.*, 2012
Low-risk individuals (HIV-, HCV-, non IDU's)							
UK	2005	-	161	0	EIA	Serum	Sharp *et al.*, 2010
France	2008	-	199	0	EIA	Serum	Sharp *et al.*, 2010
Ghana	2012	Children	943	0.53	PCR	Faecal samples	Drexler *et al.*, 2012
Ghana	2012	Children	961	0.83	PCR	Nasal secretions	Drexler *et al.*, 2012
Thailand	2000	-	176	3.95	PCR	Serum	Lurcharchaiwong *et al.*, 2008
South Africa	2009	-	180	4.4	EIA	Serum	Sharp *et al.*, 2010
Ghana	2004-2005	Children	279	8.6	PCR	Serum	Panning *et al.*, 2010

Ghana	2012	Children	361	8.9	PCR	Serum	May *et al.*, 2012
PR China	2012	-	289	16-22	PCR	Serum	Zhang *et al.*, 2012
Cameroon	2007	-	238	24.8	EIA	Serum	Sharp *et al.*, 2010
Congo DR	2007	-	221	35.3	EIA	Serum	Sharp *et al.*, 2010
Burkina Faso	2007	-	167	37.1	EIA	Serum	Sharp *et al.*, 2010

Table 1.7. Clinical symptoms reported in subjects with PARV4 infection. Adapted from Matthews *et al.*, 2014.

Reference	Characteristics and location of subject(s) with PARV4 infection	Method of laboratory detection of PARV4 infection	Presenting clinical symptoms(s)
Benjamin *et al.*, 2011	N=2; children aged 2–3 years with suspected CNS infection; India	PARV4 DNA in CNS.	Encephalitis (fever and generalised convulsions).
Chen *et al.*, 2011	N=6; mother-infant pairs with nonimmune idiopathic hydrops in the foetus; Taiwan.	Infants: 5/6 had PARV4 DNA in plasma. Mothers: 4/6 had PARV4 IgM; 2/6 had PARV4 IgG	Foetal hydrops (≥2 of ascites, pleural/ pericardial effusion, skin oedema, polyhydramnios). Two of six babies died.
Drexler *et al.*, 2012	N=13; Children with respiratory or gastrointestinal symptoms; Ghana	PARV4 DNA in nasal secretions (N=8) or faeces (N=5).	Upper/lower respiratory tract symptoms or gastrointestinal symptoms.
Jones *et al.*, 2005	N=1; homeless male IDU, Hepatitis B-positive, HIV-negative; United States.	PARV4 DNA in serum	Fatigue, arthralgia, neck stiffness, pharyngitis, diarrhoea, vomiting, confusion, night sweats.
Sharp *et al.*, 2012	N=9; haemophilia patients (seven HIV-positive); United States.	PARV4 IgG seroconversion; two had transient positive PARV4 IgM. All were positive for PARV4 DNA in serum (viral titre, 10^3–10^{10} copies/mL)	Rash in three subjects, unexplained hepatitis (but minimal disturbance of LFTs at the time of PARV4 IgG seroconversion)
Simmons *et al.*, 2012	N=193; HIV-positive; Switzerland	PARV4 IgG positive.	Early HIV-related symptoms (CDC-B symptoms).
Vallerini *et al.*, 2008	N=1; patient with Wegener's Granulomatosis on long-term steroid therapy; Italy	PARV4 DNA in serum.	Fever, anaemia (with erythroid hypoplasia on bone marrow biopsy), post-infectious glomerulonephritis, subsequent multiorgan failure.

CNS, central nervous system; CSF, cerebrospinal fluid; IDU, injecting drug user; LFTs, liver function tests.

1.3.2. PARV4 clinical significance and diagnostics

There is currently no definitive clinical syndrome associated with PARV4 infection, neither it was detected for related hokoviruses in animals. PARV4 viraemia appears to be self-limiting and asymptomatic in the majority of cases (Panning *et al.,* 2010). However, a range of possible disease outcomes is described in individuals with evidence of past or current PARV4 infection and are summarised in Table 1.7.

Compared to other parvoviruses that are predominantly transmitted through respiratory or gastrointestinal routes, PARV4 infections show an unusual risk-group association. In a study on three human parvoviruses, B19, bocavirus and PARV4, in HIV-1-infected and non-infected individuals, Manning *et al.,* (2007) revealed that a high proportion (70.8%) of HIV-1-infected individuals harboured PARV4 in lymphoid and bone-marrow tissues, but none had viraemia. In another study, seven of the eight individuals in whom PARV4 was detected in the plasma were co-infected with hepatitis B and C viruses (Tuke *et al.,* 2010). Flowingly, a statistical correlation has been described between PARV4 positivity and early features of AIDS, this association is potentially confounded by the close relationship between PARV4 and both HCV status and individuals with a history of IDU (Simmons *et al.,* 2012). The infected intravenous drug users complained of fatigue, night sweats, pharyngitis, neck stiffness, vomiting, diarrhoea, arthralgias, and confusion (Jones *et al.,* 2005). Markedly, the afore-mentioned studies involved small numbers of patients and establishing cause and effect is limited.

PARV4 was suggested as a cause of encephalitis in two children with high viral load in cerebrospinal fluid and positive IgM and negative IgG titres in serum (Benjamin *et al.,* 2011). A link to PARV4 infection and liver and heart diseases was proposed by Corcioli *et al.,* (2010). In a study from Taiwan, PARV4 DNA was detected in plasma from three mothers and their new-borns with hydrops, pointing to transmission through the placenta (Chen *et al.,* 2011). PARV4 genotype 3 was recently detected in nasal and faecal samples from children in

Ghana (Drexler *et al.*, 2012). However, such findings do not prove that PARV4 was the cause of respiratory or gastrointestinal diseases as co-infections with known pathogens were found together with PARV4.

The reservoir tissue for PARV4 is not exactly known, either in the acute or chronic phases of infection. However, different studies have described the detection of PARV4 DNA from a variety of sites including blood, lymphoid tissue, bone marrow, liver, and central nervous system (Jones *et al.*, 2005; Manning *et al.*, 2007; Sharp *et al.*, 2012). Viral DNA may persist indefinitely, but this archived virus does not necessarily reflect local disease. Conversely, specific pathology does not clearly relate to viral replication in local tissues. For example, anaemia in acute infection was not associated with high viral titres in bone marrow (Vallerini *et al.*, 2008). In humans, a low DNA content of parvovirus in the myocardium is a frequent finding (Kuethe *et al.*, 2009; Lotze *et al.*, 2010; Corcioli *et al.*, 2010), and the persistence of parvovirus B19 infection in this tissue is suggested to be caused by DNA defective interfering particles or inactive replicative DNA intermediates. Another possible explanation may be that this particular tissue provides an immunologically privileged site protected from the immune system (Söderlund-Venermo *et al.*, 1990).

Diagnostic test systems for parvovirus infection are based on the detection of either virus–specific antibodies or viral DNA. The long-lasting IgG antibodies against parvoviruses are preferentially directed against conformational epitopes present in VP2 proteins. Weak or transient VP-2 IgM positivity has also been reported in acute infection (Lowin *et al.*, 2005; Sharp *et al.*, 2012; Söderlund *et al.*, 1995). This means that current diagnostic enzyme immunoassay (EIA) tests for parvoviruses rely on recombinant VP2-based virus-like particles (VLPs) as a specific antigen that is produced preferentially in baculovirus expression systems (Lahtinen *et al.*, 2011; Simmons *et al.*, 2011). Alternatively, T cell response was successfully detected using NS protein derived peptides in HIV and HCV-positive patients (Simmons *et al.*, 2011). To date, there is no commercially available serological test for PARV4 to be used as a golden standard.

PARV4 DNA may be isolated from plasma in acute infection with viral loads up to 10^{10} copies/mL, but generally lower loads are observed ($<10^5$ copies/mL) (Benjamin *et al.*, 2011; Sharp *et al.*, 2012). ORF1 directed qPCR and semi-nested PCR techniques to detect PARV4 DNA up to viral loads of 5-100 copies/mL in plasma, serum or whole blood has been developed (Tuke *et al.*, 2010).

1.4. Porcine parvovirus (PPV)

1.4.1. Epidemiology of PPVs

Porcine parvovirus (PPV) was first isolated in 1965 in Munich, Germany, as a contaminant of a porcine primary cell culture used for the propagation of classical swine fever virus (Mayr and Mahnel. 1964). Soon after, this virus was found to be associated with reproductive disorders in pigs, and has been found to occur worldwide (Cui *et al.*, 2012; Kim & Chae, 2004; Mengeling *et al.*, 1991). With new genetically distinct parvoviruses sequentially discovered in swine, this virus has been designated as "classical" PPV (cPPV) or PPV type 1 (PPV1) (Cadar *et al.*, 2013, Cheung *et al.*, 2010 and Cságola *et al.*, 2012;), and recently named Ungulate protoparvovirus 1.

PPV1 is the major causative agent of a syndrome of reproductive failure in swine. This syndrome is characterised by stillbirth, mummified foetuses, early embryonic and foetal death, delayed return to oestrus, and infertility (abbreviated as *SMEDI*) (Szelei *et al.*, 2006; Truyen & Streck, 2012). Classic PPV has one serotype subdivided into four clinical genotypes (biotypes) according to their pathogenicity. The NADL-8 strain can cause viremia and crosses the placenta to infect foetuses, leading to foetus death (Mengeling & Cutlip, 1975). In contrast, the non-pathogenic NADL-2 strain is currently widely used as an attenuated vaccine and causes only limited viremia without crossing the placental barrier in experimental infections (Paul & Mengeling, 1980). The other two groups are the Kresse and IAF-A83 strains, which are associated with dermatitis and enteric diseases, respectively (Opriessnig *et al.*, 2011). PPV1 is also shown to be an agent able to increase the effects of porcine circovirus type 2 infection in the clinical

course of postweaning multisystemic wasting syndrome (Krakowka *et al.*, 2000), which is a significant disease in global swine production (Segalés *et al.*, 2005). Despite the continuous use of vaccines, several new strains have recently been described, and there is cause for concern because changes in only a few amino acids in the capsid protein can potentially cause marked differences in antigenicity and virulence (see the review of Cadar *et al.*, 2013). Virus H-1 was detected in sera from pigs from Myanmar (Hijisaka *et al.*, 2001) and originally named as PPV2. In 2008, a virus closely related to human parvovirus 4 was found in slaughtered pigs in Hong Kong (Lau *et al.*, 2008). Several sequences with high homology (> 98% DNA identity) are found in DNA databanks under different names, including porcine parvovirus 3, porcine hokovirus and PARV4-like. This virus has already been detected in several countries in domestic pigs (Pan *et al.*, 2013 and Xiao *et al.*, 2012) and also in wild boars (Adlhoch *et al.*, 2010, Cadar *et al.*, 2011 and Streck *et al.*, 2013). Subsequently, a porcine bocavirus (PBoV) was identified in the lymph nodes of pigs with the postweaning multisystemic wasting syndrome (PMWS) (Blomström *et al.*, 2009). In 2010, another parvovirus was found in the lungs of a diseased pig co-infected with porcine circovirus type 2 and was designated as porcine parvovirus 4 (Cheung *et al.*, 2010); the virus was also found in diseased animals in China (Huang *et al.*, 2010). Bayesian phylogeography indicates that Romania (for PPV2 and PPV4) and Croatia (for PPV3) are the most likely ancestral areas from which PPVs have subsequently spread to other European countries and regions (Cadar *et al.*, 2013). Recently, another porcine parvovirus was described in the lungs of pigs in the USA (Xiao *et al.*, 2013). This virus, tentatively designated porcine parvovirus 5, was also observed in the lung of pigs of different ages, but no association with clinical signs was found. Finally, in 2010 and 2013, several samples collected from aborted pig foetuses, piglets, finished pigs and sows were analysed, revealing a novel porcine parvovirus circulating in China. The virus, provisionally named porcine parvovirus 6, possesses a genome of more than 6.1 kb and shows a high dissimilarity in amino acid composition compared with other porcine parvoviruses (Ni *et al.*, 2014).

For PPV1, a virus is considered to have a more conservative genome than other parvoviruses and ssDNA viruses (Lukashov and Goudsmit, 2001, López-Bueno *et al.,* 2006, Duffy *et al.,* 2008). However, high mutation rates (approximately 3–5 × 10^{-4}) in the VP genes were found by Streck *et al.,* (2015), with a more moderate evolution rate for NS genes (approximately 10^{-5}) in the genome of PPV, as well as in viruses affecting wild boars. The analysis of the wild boar population reported that PPV isolated from these animals showed higher genetic diversity than that of domestic pigs (Cadar *et al.,* 2012). The point mutations alter the surface antigenicity and may modify protein assembly as well as the DNA packaging processes, resulting in more efficient replication and, possibly, enhanced virulence. This was observed for PPV1, as three out of six amino acid differences of the non-virulent strain NADL2 to the virulent Kresse strain are located in β-strands (Simpson *et al.,* 2002), closer to the centre of the PPV1 capsid compared to other variable capsid sites.

The fact that the novel porcine parvoviruses have been found in domestic swine as well as wild boars suggests active intraspecies and interspecies genetic flow (Adlhoch *et al.,* 2010, Cadar *et al.,* 2011, Cadar *et al.,* 2013, Streck *et al.,* 2013, Xiao *et al.,* 2013). PPV sequences have already been detected in rats (*Rattus norvegicus*), and productive infection in rats has been described (Cutler *et al.,* 1982). As PPV is able to replicate in immortalised cells of both bovine and human origin, its host range may be broader than commonly thought. A wide variety of porcine cells supports replication of PPV in vitro. Early-passage cells are generally considered to be more susceptible than established cell lines. Foetal porcine kidney cell cultures are commonly used for virus detection and propagation (Mengeling, 1975).

Horizontal transmission of PPV is thought to occur directly by contact among acutely infected and naive pigs, and indirectly by naive pigs ingesting or inhaling virus-laden secretions and excretions. There is no evidence that pigs commonly shed PPV over an extended period of time. It is more likely that the primary virus reservoir is a contaminated environment. By adding naive pigs to a room previously inhabited by acutely infected pigs, it was shown that PPV could

remain infectious outside its host for at least 4 months. Conversely, it was shed for only a few weeks when the same acutely infected pigs were moved to a separate isolation room and put in contact with additional naive pigs (Mengeling and Paul, 1986). Rats were shown to be one of the possible transmission vectors (Cutler *et al.*, 1982). PPV has little, if any, clinical effect on mature boars. However, it has been isolated from scrotal lymph nodes for as long as 7 weeks after acute infection, and from semen. Boars may also serve as non-infected carriers of PPV as they move among infected and non-infected females. Additionally, artificial insemination could lead transfer of PPV into distant piggeries (Althouse & Rossow, 2011; Lucas *et al.*, 1974).

1.4.2. PPV transmission, clinical signs, and pathogenesis

PPV is ubiquitous in swine throughout the world, and in most herds infection is endemic. Typically, at the time of farrowing most gilts and sows are immune and impart a high level of PPV antibody to their offspring via colostrum. This passively acquired antibody persists at progressively lower levels for 4 to 6 months, during which time pigs are relatively refractory to infection (Paul *et al.*, 1980, 1982). As passively acquired antibody wanes pigs become progressively more likely to be infected and acquire active immunity. Active immunity apparently persists for life, perhaps as a result of repeated exposure to the virus. Some gilts escape infection until their first gestation. If they are first exposed to PPV anytime during the first half of gestation, transplacental infection and reproductive failure are likely to result (Mengeling and Cutlip, 1976; Joo *et al.*, 1976). If they are infected anytime during the second half of gestation, the transplacental infection is possible. However, at least most foetuses infected after about 70 days of gestation produce antibody and survive the infection. They may, however, shed PPV during at least the early postnatal period (Mengeling *et al.*, 2000).

PPV is a highly infectious and extremely durable virus. The major consequence of PPV infection in swine is a reproductive failure, characterised by foetal death and mummification (Mengeling *et al.*, 2000; Mengeling & Cutlip, 1976).

Additionally, it plays a role in porcine respiratory disease complex (Kim *et al.*, 2003) and postweaning multisystemic wasting syndrome (Allan *et al.*, 1999; Ellis *et al.*, 2000). When susceptible adult pigs are exposed to PPV at mating or during gestation, the virus readily crosses the placental barrier and infects the embryos or foetuses. PPV is able to spread intrauterinely between foetuses, giving the variable numbers of affected foetuses in the litter (Joo *et al.*, 1976). Mengeling *et al.*, (2000) suggests that PPV reaches the conceptus as a passenger in or on maternal macrophages. Once the virus reaches the embryo or foetus, it finds an environment particularly conducive to replication because of the high mitotic index of most tissues. When transplacental infection of PPV occurs before 35 days of gestation, resorption of some or all foetuses takes place, resulting in a reduction of the litter size or return to service. If an infection occurs between 35 to 70 days of gestation, one or more foetuses may die and mummificate. Infections later than day 70 will remain mainly sub-clinical. The foetuses are immune competent for PPV, and seropositive piglets would be born (Christianson, 1992).

Although acute infection of postnatal, non-pregnant pigs (as well as wild boars) is usually subclinical (Cutlip & Mengeling, 1975), PPV has also been linked to skin lesions in piglets (Kresse *et al.*, 1985, Whitaker *et al.*, 1990, Lager and Mengeling, 1994), interstitial nephritis in slaughter-aged pigs (Drolet *et al.*, 2002), and non-suppurative myocarditis in lactating piglets (Bolt *et al.*, 1997).

1.4.3. PPV diagnostics, vaccines and prevention

Clinical diagnosis of PPV infection is difficult because the main signs of disease are similar to those of other diseases. Accordingly, laboratory confirmation is required for suspected cases. Classical and highly reliable means to diagnose PPV-induced reproductive failure is to examine foetal tissues by immunofluorescence microscopy (Mengeling and Cutlip, 1976). Cryostat-microtome sections of the foetal lung are reacted with fluorescein-labelled PPV antibody. Molecular detection of PPV has been based on virus isolation (VI), latex agglutination (LA), hemagglutination (HA), electron microscopy (EM),

enzyme-linked immunosorbent assay (ELISA or IEA), and polymerase chain reaction (PCR, RT-PCR) (for review: Hohdatsu *et al.*, 1988; Song *et al.*, 2010)). Multiplex PCR systems were developed for simultaneous detection of four viruses involved in reproductive and respiratory failure in pigs (Jiang *et al.*, 2010; Ogawa *et al.*, 2009), with specific primers targeting NS1 or VP genes. A sensitive and time-saving loop-mediated isothermal amplification (LAMP) method has also been shown to be a valid diagnostic tool for PPV (Chen *et al.*, 2009).

Immunogenic major capsid protein VP2 of PPV has been synthesised in several expression systems including bacteria (Qi & Cui, 2009; Xu & Li, 2007). PPV VP2 protein expressed using the baculovirus expression vector system was shown to assemble into virus-like particles (VLPs) similar in size and morphology to the original virions. Such VLPs were shown to induce antibodies in immunised pigs (Martínez *et al.*, 1992) and guinea pigs (Antonis *et al.*, 2006). VLPs generated in baculovirus system exhibit positive immunoreactivity for PPV and are used in most commercial EIA tests (Maranga *et al.*, 2003). Most recently, immunogenic PPV VP2 protein was synthesised in yeast *Pichia pastoris* (Guo *et al.*, 2014). PPV VP2-derived VLPs have been shown to be effective epitope carriers to elicit a strong immune response in mice (Sedlik *et al.*, 1999; Pan *et al.*, 2008).

PPV-induced reproductive failure can be prevented by making sure that all females have developed an active immunity before they conceive for the first time (Mengeling & Cutlip, 1976). Because infection is endemic in most herds, immunity is often the result of natural exposure. However, to ensure immunity, it is a common practice to vaccinate gilts once or twice before conception and at least once annually thereafter (Mengeling *et al.*, 1979).

There are several types of vaccines against PPV including attenuated vaccine, inactivated vaccine, subunit vaccine, and DNA vaccine, but they are likely to be less immunogenic (Chen *et al.*, 2010c; Lima *et al.*, 2004). The live attenuated vaccine has been using for several years in some countries, but it is expensive, causes side effects, and may revert to a pathogenic strain, so inactivated vaccines are the most common type of PPV vaccines used in animals (Wrathall *et al.*,

1984). However, an inactivated whole-virus PPV vaccine protects pigs against the disease, but was demonstrated to fail in prevention of virus shedding even after homologous virus challenge (Foerster *et al.,* 2016). Porcine transfer factor (TF) could be used as adjuvant of inactivated PPV oil emulsion vaccine to enhancing immune response (Wang *et al.,* 2012). Several subunit vaccines has been developed (Alvarez *et al.,* 1996; Martínez *et al.,* 1992), and it has been reported recently that a single immunization with as low as 0.7 µg of baculovirus produced PPV VLPs yielded complete protection in targeted animals against infectious PPV strains (Antonis *et al.,* 2006).

1.5 Parvoviral capsid protein synthesis in various expression systems

Parvoviral VLPs, composed of the major capsid protein, have been increasingly used in diagnostics and vaccine research, but also as epitope carriers and gene therapy experiments. Chimeric PPV-VLPs carrying heterologous epitopes, when injected intraperitoneally into mice, activate strong CD4+ and CD8+ T-cell responses specific for the foreign epitopes, and these responses are mediated by dendritic cells and influenced by the flanking sequences (Morón *et al.,* 2002; Rueda *et al.,* 2004). Similarly, epitope-carries were developed by modification of B19 (Brown *et al.,* 1994) and canine parvovirus (Gilbert *et al.,* 2004) VP2 proteins. A high airway tropism of HBoV1 virions was successfully exploited by developing a chimeric HBoV1-AAV2 viral vector that specifically and efficiently infects human-polarized airway epithelia from the apical membrane and is able to correct CFTR-dependent chloride transport in cystic fibrosis case (Yan *et al.,* 2013).

Porcine parvovirus VP2-VLPs are the premiere choice for subunit vaccine. Therefore large scale antigen expression system is of economic value. However, to date commercially available vaccines are developed using BICS expression system (Martínez *et al.,* 1992) or by the chemical inactivation of isolated virus particles grown in primary cell cultures of porcine origin (Casal, 1999). Alternatively, PPV VP2 VLPs were generated at moderate yield in the leaves of low alkaloid transgenic tobacco (Rymerson *et al.,* 2003).

Recombinant HBoV structural proteins for diagnostic purposes were synthesised in *Escherichia coli* (Kantola *et al.,* 2008) and were generated in a baculovirus system as VP2-VLPs (Kahn *et al.,* 2008; Kantola *et al.,* 2011). Recently, Fang *et al.,* (2014) reported mosaic VP1-VP2 VLPs of HBoV1 generated by co-expression in insect cells. By introducing additional Kozak sequence in the optimised HBoV1 VP1 coding sequence, Cecchini *et al.,* (2009) managed to produce in BICS system mosaic VLPs composed of three proteins: VP1, VP2, and intermediate size (68 kDa) VP3. However, this study was the only case to report the third protein in bocaviral capsids. In mammalian cells, HBoV1 production as infectious virions has been reported in well-differentiated human airway epithelia (HAE) (Dijkman *et al.,* 2009) and human embryonic kidney 293 cells (Huang *et al.,* 2012). No mammalian cell cultures were reported to propagate HBoV2-4 or PARV4 virions in vitro to date successfully. VLPs of these viruses for all studies to date have been produced in the insect cell system (for references, see Tables 1.5 and 1.6).

Yeast expression systems for production of a few parvoviral VLPs have recently been reported. Immunogenic VP2 virus-like particles of porcine parvovirus (Guo *et al.,* 2014) were generated in *Pichia pastoris,* and parvovirus B19 VP2 VLPs were produced in *S. cerevisiae* (Lowin *et al.,* 2005).

To conclude, a baculovirus-insect cell expression system to date was a dominating platform for the production of non-infectious parvoviral VLPs for structural studies and diagnostics. The VP2 structural proteins of many parvoviruses, including canine parvovirus (Saliki *et al.,* 1992), human parvovirus B19 (Söderlund *et al.,* 1995) and goose parvovirus (Ju *et al.,* 2011) have been produced in this system.

1.6. Viral protein synthesis in yeast

Synthesis of recombinant viral proteins in heterologous systems is essential for the study of their structure and properties, diagnostics of viral diseases and development of vaccines. The structural similarity to the native viral protein and post-translational modifications are essential for the above-mentioned purposes.

A number of viral structural proteins possess the intrinsic capacity to self-assemble into virus-like particles (for review, Zeltins, 2013) that resemble native virions in size, shape and antigenicity, and thus are useful as antigens for immunoassays, vaccination or structural studies. The interest in new VLPs was mostly inspired by the successful development of commercial vaccines based on hepatitis B virus (HBV) surface antigen and the human papillomavirus (HPV) capsid protein L1 (Frazer, 2004; McAleer *et al.*, 1984; Pumpens *et al.*, 2008). A number of VLP-forming viral proteins are able to accommodate inserted additional sequences and expose them on the VLP surface (for review, Palucha *et al.*, 2005), thus evoking an immune response against such heterologous epitopes.

Human viruses naturally propagate in mammalian cells. However, the use of such cell cultures for extensive protein production for research and industry is complicated due to cost and yield. Alternatively, the microorganisms (prokaryotic as well as eukaryotic) are advantageous hosts for the production of proteins because of high growth rates and, commonly, ease of genetic manipulation. Yeasts offer the ease of microbial growth and gene manipulation found in bacteria as well as the eukaryotic environment and ability to perform many eukaryote-specific post-translational modifications, such as proteolytic processing, folding, disulphide bridge formation, and glycosylation (Eckart & Bussineau, 1996; Hou *et al.*, 2012). Yeast cells are particularly advantageous as hosts for biopharmaceutical production in that they are generally recognised as safe (GRAS) organisms by the US Food and Drug Administration. Bacteria lack these capabilities and often produce eukaryotic proteins that are misfolded, insoluble, or inactive. Therefore any use of such proteins for the treatment of humans is also excluded unless they can be correctly refolded in vitro. The baculovirus expression system was found to be highly effective for VLP production of various viruses (for review, (Liu *et al.*, 2013), but it is prone to contamination, costs are high and skilled hands are needed to get satisfactory results.

The use of yeast, primarily *Saccharomyces cerevisiae,* for recombinant protein production started in the 1980s (Hitzeman *et al.*, 1981), but other species have been employed since then, to name: (methylotrophic yeasts) *Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Candida boidinii,* and (nonmethylotrophic yeasts) *Kluyveromyces lactis, Yarrowia lipolytica, Pichia stipitis, Zygosaccharomyces rouxii, Z. bailii, Schwanniomyces (Debaryomyces) occidentalis,* (for review, Mattanovich *et al.*, 2012). However, there are some disadvantages to using yeasts for expression of some heterologous proteins, mostly related to their inability to perform certain complex post-translational modifications, such as prolyl hydroxylation and amidation, as well as some types of phosphorylation and glycosylation (Cereghino & Cregg, 1999; Cregg & Higgins, 1995). Additionally, the proteins produced by *S. cerevisiae* may be hyperglycosylated (high-mannose type N-glycosylation), and retention of the products within the periplasmic space is frequently observed (Reiser *et al.*, 1990; Wildt & Gerngross, 2005).

The heterologous gene can be introduced into the yeast host cells by means of an integrative plasmid as well as by autonomous or episomal circular plasmids. The vectors used are often hybrids between yeast-derived and bacterial sequences. The bacterial fraction of these vectors bears an origin of replication for the chosen bacteria (essentially, *E. coli*) and selection markers (e.g., antibiotic resistance). The yeast-origin part comprises transcription regulation elements and sequences responsible for selection, replication and distribution (or integration). For the autonomous plasmids, replication is governed by the autonomous replicating sequences (ARS), while the episomal plasmids are based on endogenous circular yeast plasmids like the 2µ plasmid of the yeast *S. cerevisiae,* and are generally maintained with a high copy number per cell (from 1–10 up to 100 copies per cell). Episomal plasmids may be unstable and lost when cells are grown in unselective conditions.

Efficient expression of the heterologous protein is ensured by strong promoters. In the case of *S. cerevisiae,* constitutive (*GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *PGK,* phosphoglycerate kinase; *TP1,* triose phosphate

isomerase; *ENO*, enolase; *α-MP*, α-mating factor; *PYK1*, pyruvate kinase), galactose-induced (upstream activating sequences of the GAL1-10 and GAL7 promoters), and ethanol-induced (*ADH1*, alcohol dehydrogenase) promoters are used. Inducible promoters enable regulation of the recombinant protein expression timing; as overexpressed viral proteins may influence yeast cell viability or be toxic. Recombinant proteins are either targeted to the cytoplasm or secreted, best into the culture medium. A cytoplasmic expression often leads to very high expression levels. However, some authors have described the deposition of recombinant proteins as insoluble aggregates (inclusion bodies) in the cytoplasm of yeasts (for review, Mattanovich *et al.*, 2012). Alternatively, proteins can be tagged to be secreted essentially by adding an appropriate secretion signal sequence, e.g., *S. cerevisiae* α mating factor (α-MF) signal. Yeast-generated viral proteins were demonstrated to be highly immunogenic and structurally resembling the native virions and suitable for serological assays as single proteins (Petraityte *et al.*, 2008; Razanskiene *et al.*, 2004) or as VLPs (Petraitytė-Burneikienė *et al.*, 2011; Slibinskas *et al.*, 2004).

2. Materials and methods

2.1. Materials

2.1.1. Reagents, enzymes and kits

Table 2.1. Reagents and enzymes used in this study and their producers.

Producer	Reagent, enzyme, kit
ThermoFisher Scientific Baltics (Lithuania)	DNA modifying enzymes (restriction endonucleases, DNA polymerases (*Taq* High Fidelity PCR Enzyme Mix), FastAP alkaline phosphatase, T4 DNA ligase, T4 Polynucleotide Kinase) and corresponding enzymatic reaction buffers; dNTP mixture, DNA and protein mass standards, IPTG, 50xTAE buffer, Phusion Site-Directed Mutagenesis Kit, GeneJET Gel Extraction and GeneJET Miniprep Plasmid Kit, agarose, GeneJET Viral DNA/RNA Purification Kit
Qiagen (Germany)	Ni-NTA Agarose, QIAamp UltraSens Virus Kit
Sigma Aldrich (Germany)	CsCl, EDTA, PEG, Tris, Tween-20, $MgSO_4$, $CaCl_2$, lithium acetate, glucose, agar, formaldehyde (37% solution), 2-mercaptoethanol, 3,3',5,5'-tetramethylbenzidine, ethidium bromide, 4-chloro-1-naphthol, xylolcyanol FF
Amresco (USA)	Sodium chloride, glycine, sodium phosphates (monobasic and dibasic), LB broth, yeast extract, bovine serum albumin
Fluka (Switzerland)	Hydrogen peroxide, PMSF, glycerol, glucose
Carl Roth GmbH (Germany)	Ammonium acetate, yeast extract, peptone, skim milk powder, ampicillin, protease inhibitor cocktail, Tris, 10xRoti Block™ blocking solution, chloroform, phenol
Serva (Germany)	Sodium azide, SDS, PEG (4000), acrylamide, N,N′-Methylenebis(acrylamide), ammonium persulphate
Applichem (Germany)	Tris, L-arginine, calcium chloride, caesium chloride, SDS, magnesium chloride, galactose
Merk (Germany)	Agar, bromophenol blue, isopropanol, calcium chloride, methanol, triton X-100
Reachim (Russia)	Potassium chloride, glucse, galactose, potassium phosphate monobasic, sodium acetate, sodium carbonate, sodium bicarbonate, uranyl acetate
Standard (Poland)	Sodium hydroxide, acetic acid, sulphuric acid
Vilniaus degtinė (Lithuania)	Ethanol

2.1.2. Oligonucleotides and peptides

Table 2.2. Oligonucleotides and peptides used in this study. Oligonucleotides were purchased from IDT (Munich, Germany) or MWG-Biotech AG (Ebersberg, Germany).

Oligonucleotides used for cloning and colony screening:		
Oligonucleotide	Sequence (5' to 3')	Aditional information
PPV-vp2-F	TCTACTAGTACAATGAGTGAAAATGTGGAACAA	Amplification of PPV VP2
PPV-vp2-R	GAGACTAGTCTAGTATAATTTTCTTGGTATAAGT	
P4_FW2	GCACCTAGGACAATGTAGTAGTTGGCAATGC	Amplification of PARV4 VP2
P4_RV	CGACCTAGGTTATAGCAAATGAGAATAATTTCGCG	
hP4_FW1	GCACCTAGGACAATGTCTGCTGCTGATGC	Amplification of PARV4 VP1
HBoV1_VP2F	GGATCTAGAACAATGTCTGACACTGACATTCAAGAC	Amplification of HBoV1 VP2
HBoV1_VP2R	CTTTCTAGATTACAACACTTTATTGATGTTTGTTTTTAC	
BocDir1	GCATCTAGAACAATGCCTCCAATTAAGAGACAGCCT	Amplification of HBoV1 VP1
N101Y_F	ACTTCAACTTCTACCAATACTCTTCAC	Introducing N101Y mutation in HBoV3 VP2
N101Y_R	ATGACCATGGAGTAGAAACACATC	
PYK5	TATTCATTCTTTTTCATCCTTTGG	Colony screening (primers are specific to sequences in pFX7 and pFGG3 plasmids.
PGK3	TCCTTACCTTCCAATAATTCCAAAC	
GAL7P	ATTATGCAGAGCATCAACATG	
GAL7T	GTCTTTGTAGATAATGAATCTG	
T7 TER	GCTAGTTATTGCTCAGCGG	Colony screening (pET43(a)+ constructs
T7 PRO	TAATACGACTCACTATAGG	

Oligonucleotides used for PARV4 VP2 epitope mapping:	
Oligonucleotide	Sequence (5' to 3')
121-135F	AATTCGTTTCTGATTCTGCAAGTGGTGGGATTACTGTATTTGCTGATGATG
121-135R	TCGACATCATCAGCAAATACAGTAATCCCACCACTTGCAGAATCAGAAACG
128-142F	AATTCGGGATTACTGTATTTGCTGATGATAGCTATGACTATCCATATGTAG
128-142R	TCGACTACATATGGATAGTCATAGCTATCATCAGCAAATACAGTAATCCCG
136-150F	AATTCGGGATTACTGTATTTGCTGATGATAGCTATGACTATCCATATGTAG
136-150R	TCGACTACATATGGATAGTCATAGCTATCATCAGCAAATACAGTAATCCCG
144-158F	AATTCGGTCATAATCAAGATACATTACCAGGTCATTTACCAGGAGAAAATG
144-158R	TCGACATTTTCTCCTGGTAAATGACCTGGTAATGTATCTTGATTATGACCG
248-262F	AATTCAATGATGGGACTGCCATTTGGAAACGCCCTGAAGGCATGGATGTTG
248-262R	TCGACAACATCCATGCCTTCAGGGCGTTTCCAAATGGCAGTCCCATCATTG
256-270F	AATTCCGCCCTGAAGGCATGGATGTTGGCAGACTCCCATTAAATTATGTTG
256-270R	TCGACAACATAATTTAATGGGAGTCTGCCAACATCCATGCCTTCAGGGCGG
264-278F	AATTCAGACTCCCATTAAATTATGTTCCAGGGCCAGCTCTAATGATGCCAG
264-278R	TCGACTGGCATCATTAGAGCTGGCCCTGGAACATAATTTAATGGGAGTCTG
hp4 VP2 N120	GAAAGATCTGTTTCTGATTCTGCA
hp4 VP2 N150	GAAAGATCTCCAGGTCATTTACCA
hp4 VP2 N180	GAAAGATCTGCTATTAGTGATCAT
hp4 VP2 N210	GAAAGATCTGAGTTTCCAGATGAC
hp4 VP2 N240	GAAAGATCTGCTATTATGACAGGT

Table 2.2 continued

Oligonucleotide	Sequence (5’ to 3’)
hp4 VP2 N270	GAAAGATCTCCAGGGCCAGCTCTA
hp4 VP2 N300	GAAAGATCTGACAGGTATAGTGTA
hp4 VP2 N330	GAAAGATCTAATTATTTAGGAGGT
hp4 VP2 N360	GAAAGATCTCCATCCAGAGTTGTT
hp4 VP2 N390	GAAAGATCTGGTGGTACTGATACA
hp4 VP2 N420	GAAAGATCTTCATATGATTGCCAA
hp4 VP2 C165	CGAAGATCTTTACCCATACTGAGGCAA
hp4 VP2 C195	CGAAGATCTTTAATGGTGCTCTAAAAA
hp4 VP2 C225	CGAAGATCTTTAGTTGGGAGTTGACAA
hp4 VP2 C255	CGAAGATCTTTATTTCCAAATGGCAGT
hp4 VP2 C285	CGAAGATCTTTAGTTTCTAATTTGGGT
hp4 VP2 C315	CGAAGATCTTTAGACAGACCATGGTTG
hp4 VP2 C345	CGAAGATCTTTACTCTTCATGCTTTCT
hp4 VP2 C375	CGAAGATCTTTAATGAGGAGCTGCTAA
hp4 VP2 C405	CGAAGATCTTTAAGGTTCCTGATATAA
hp4 VP2 C435	CGAAGATCTTTAACATTCTGTATTAGG
hp4 VP2 C465	CGAAGATCTTTAGCCTGGTTGTGACCT
hp4 VP2 N130	GAACCTAGGACAATGACTGTATTTGCT
hp4 VP2 N320	GAACCTAGGACAATGCTAGCAAACAAA
hp4 VP2 C420	CGACCTAGGTTATAAAGGATTTGG
hp4 VP2 C490	CGACCTAGGTTAACTATAATGCAA
hp4 VP2 C525	CGACCTAGGTTAGGCAAGGGTAAA
hp4 VP2 C552	CGACCTAGGTTATAGCAAATGAGAATAATTTCGCG
492-506F	AATTCCAGTTTTTAGTTAAGCGCCGCAAGAGATCTCGCCGCCATAATCCCG
492-506R	TCGACGGGATTATGGCGGCGAGATCTCTTGCGGCGCTTAACTAAAAACTGG
507-521F	AATTCGAGAAACCTGCTCCTTTCCCGACAACAGATTCGGGACGTATGCCTG
507-521R	TCGACAGGCATACGTCCCGAATCTGTTGTCGGGAAAGGAGCAGGTTTCTCG

Peptides used for *PARV4 VP2 epitope mapping (Pepscan Presto BV, The Netherlands):*	
Peptide	*Sequence (N- to C-terminus)*
hp4A-1	GHNQDTLPGH
hp4A-2	NQDTLPGHLP
hp4A-3	DTLPGHLPGE
hp4A-4	LPGHLPGEN
hp4A-5	GHNQDTLP
hp4B-1	NDGTAIWKRP
hp4B-2	GTAIWKRPEG
hp4B-3	AIWKRPEGMD
hp4B-5	WKRPEGMDV
hp4B-5	NDGTAIWK

2.1.3. Plasmids

pFGG3, pFX7, pFX7-His6N - yeast expression vectors (Razanskiene *et al.*, 2004; Sasnauskas *et al.,* 1999)

pET-43.1a(+) – bacterial expression vector for the expression of fusion proteins with soluble poli-tagged NusA protein (Novagen, Madison, WI, USA).

Chemically synthesised HBoV1 VP1, HBoV2-4 VP2 and PARV4 VP1 genes were obtained cloned into pUC57 vector (GenScript, Piscataway, NJ, USA).

2.1.4. Strains

All cloning procedures were performed in *E. coli* DH5αF′ (F^-*gyr*A96(NaI^r) *rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17($r_k^-m_k^+$) *gln*V44 *deo*R Δ(*lac*ZYA-*arg*F) U169 [φ80d*lac*Z ΔM15] (ThermoFisher Scientific Baltics) or GM119 (F^- *dam*-3 *dcm*-6 *lac*Y1 *gal*K2 *gal*T22 *ton*A31 *tsx*-78 *sup*E44 *mtl*-1(*thi*-1)) (ATCC™ 53339) cells. For protein synthesis in *E. coli,* strain BL21(DE3) *(*F^- *omp*T *gal dcm lon hsd*SB (rB- mB-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]) was used.

Saccharomyces cerevisiae strains AH22-214 *(MATa leu2-3 leu2-112 his4-519 can1 [KIL-o])*(ATCC™ 38626), 214Δ*pep4 (a ura3 leu2 his3* Δ*pep4)*, FH4 (a/α) (ATCC™ 42368) and gcn2 (ATCC™4033642) were used for heterologous protein expression.

2.1.5. Serum samples

Serum samples from 170 patients (mean age, 20.5 years; range, 1 month–66 years; gender, 92 male and 78 female) with acute respiratory disease were obtained from Vilnius University, Faculty of Medicine, Clinic of Children Diseases (Lithuania) in 2008–2011. The clinical diagnosis of respiratory tract infections was confirmed at the sera collection day by the presence of typical symptoms of an acute respiratory viral infection, including sneezing, nasal obstruction and discharge, sore throat, cough and moderate fever. A collection of specimens and clinical data was approved by the Lithuanian Bioethics Committee (No. 53, 2008-09-03). Human serum samples were provided by Dr. R. Ėmužytė and Dr. R. Firantienė (Vilnius University, Faculty of Medicine).

One hundred and eighty-three swine serum samples from farms in Lithuania (n=160), Romania (n=14) and Ukraine (n=13) were collected in years 2008-2010 and kindly provided for this study by Dr. V. Sereika and Dr. R. Lelešius from Veterinary Academy, Lithuanian University of Health Sciences. Samples were stored at -70°C prior to testing.

2.1.6. Antibodies, diagnostic kits

Table 2.3. Antibodies and immunodiagnostic kits

Antibody	Producer
Goat anti-human IgM, HRP conjugate	ThermoFisher, Carlsbad, CA, USA
Rabbit anti-human IgG, HRP conjugate	DakoCytomation, Denmark
Rabbit anti-pig IgG, HRP conjugate	Sigma-Aldrich Biosciences, Seattle, USA
Goat anti-mouse IgG, HRP conjugate	BioRad, USA
INGEZIM PPV compact kit	Ingenasa, Madrid, Spain
PPV KY-11 infected swine testicle cells on slides	VMRD, Inc., Pullman, WA, USA

2.1.7. Molecular mass standards

All DNA and protein molecular mass standards were purchased from Thermofisher Scientific Baltics. For DNA electrophoresis, GeneRuler™ DNA Ladder Mix (#SM0333) was used. For SDS-PAGE and WB, Unstained Protein Molecular Weight Ladder (#SM0431) or PageRuler™ Prestained Protein Ladder (#SM0671) were used.

2.1.8. Solutions and growth media

Table 2.4. Solutions for DNA purification and electrophoresis

Solution	Composition	Remarks
NaOH-SDS	1% NDS, 0.2 M NaOH	
NaAc (pH = 4.8)	3 M NaAc, pH = 4.8	
NaAc (pH = 5.2)	3 M NaAc, pH = 5.2	
NH4Ac	7.5 M NH4Ac	
RNAse A solution	10 mg/mL bovine pancreas RNAse A	RNAse is solubilized by boiling the solution 10 minutes, and stored at -20°C.
Phenol-chloroform sol.	Saturated 50 mM Tris-HCl solution in 1:1 (v/v) phenol:chloroform micture, pH = 7.5-8	
TAE 1x	0.04 M Tris Acetate, 0.2 mM EDTA, pH=8.3	Diluted to working solution from 50x stock
Agarose (gel)	0.8 – 2% agarose in TAE 1x	Supplemented with 0.05 µg/mL ethidium bromide
Ethidium bromide	10 mg/mL	Handling with caution (nitrile gloves)
DNA loading dye	0.04% bromophenol blue, 0.04% xylolcyanol FF, 30% glycerol	Used as 6x

Table 2.5. Solutions for yeast and bacterial transformation

Solution	Composition	Remarks
$CaCl_2$	100 mM $CaCl_2$	Sterile
$CaCl_2$-glycerol	4:1 mixture of glycerol and 100 mM $CaCl_2$	Sterile, ice-cold
TE	10 mM Tris-HCl (pH 7.6), 1 mM EDTA	Sterile
TE-LiAc	0.1 M lithium acetate in TE	Sterile
TE-PEG	50% (w/v) PEG 4000 solution in TE	Sterile

Table 2.6. Growth media composition

Medium	Composition	Remarks
YEPD	1% yeast extract, 2% peptone, 2% glucose, solution in water	Autoclaved (0.5 atm, 30 min)
YEPD agar	1% yeast extract, 2% peptone, 2% glucose, 2% agar, solution in water	Autoclaved (0.5 atm, 30 min)
YEPG	1% yeast extract, 2% peptone, 3% galactose, solution in water	Autoclaved (0.5 atm, 30 min), prepared as 2x
Formaldehyde	37% formaldehyde solution	Yeast transfpormant are plated onto YEPD-agar with 0.3 μL/mL, replated onto 0,6 μL/mL 37% formaldehyde. For cultivation in liquid medium, 0.3 μL/mL concentration was used
LB	1% peptone, 0,5% yeast extract, 0,5% NaCl, solution in water	Autoclaved (1 atm, 40 min)
LB agar	1% peptone, 0,5% yeast extract, 0,5% NaCl, 2% agar solution in water	Autoclaved (1 atm, 40 min)
ampicillin	50 mg/mL solution in ethanol	Store at -20°C. 50 μg/mL concentration in LB agar was used for selection of *E. coli* transformants
IPTG	100mM (100x) IPTG solution in water	Stored at -20°C

Table 2.7. Solutions for purification of proteins produced in yeast and *E. coli*

Solution	Composition	Remarks
PMSF 10x	20 mM phenylmethylsulfonyl fluoride in isopropanol	Stored at -20°C
0.2 M EDTA	0.2 M EDTA solution in water	Sterilised, used as a stock solution
5x PBS	250 mM NaH2PO4 (pH 7,5), 0.5 M NaCl, solution in water	Sterilised, pH adjusted, used as a stock solution

Table 2.7 continued:

Solution	Composition	Remarks
500 mM sodium citrate	500 mM sodium citrate in water, pH 3.2	Sterilised, pH adjusted, used as a stock solution
500 mM sodium acetate	500 mM sodium acetate in water, pH 4.7	Sterilised, pH adjusted, used as a stock solution
500 mM Tris	500 mM Tris, pH 8,7	Sterilised, pH adjusted, used as a stock solution
500 mM NaHCO3	500 mM $NaHCO_3$, pH 9,6	Sterilised by filtration through 0.2 μm filter, pH

		adjusted, used as a stock solution
Sucrose solution	30% (w/v) sucrose solution in lysis buffer	
CsCl solution	0.40 g/mL CsCl solution in PBS	
Buffer A	6 M guanidine HCl, 100 mM NaH_2PO_4, 10 mM Tris-HCl, solution in water, pH 8.0	
Buffer B	8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl solution in water	pH 8.0
Buffer C		pH 6.3
Buffer D		pH 5.9
Buffer E		pH 4.5

Table 2.8. Solutions for protein electrophoresis

Solution	Composition
Acrylamide/bisacrylamide solution	40% (w/v) acrylamide, 0.8% N,N'-metilenbis(acrylamide) solution in water
4x stacking gel buffer	0.5 M Tris-HCl solution in water, pH = 6.8
4x resolving gel buffer	1.5 M Tris-HCl solution in water, pH = 8.8
Ammonium persulphate	10% $(NH_4)_2S_2O_8$ solution in water
SDS solution	10% Sodium dodecyl sulphate, solution in water
Stacking gel	3.2% acrylamide/bisacrylamide solution, 1.25 M Tris-HCl (pH = 6.8), 0.1% ammonium persulphate, 0.1% SDS, 0.001% TEMED
Resolving gel (12%)	12% acrylamide/bisacrylamide solution, 0.375 M Tris-HCl (pH = 8.8), 0.1% NDS, 0.1% ammonium persulphate, 0.001% TEMED.
2xSDS-PAGE Loading Dye	0.5 M Tris-HCl (pH = 6.8), 20% (w/v) glycerol, 4% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol
Tris-Gly-SDS electrophoresis buffer	25 mM Tris, 0.2 M glycine, 0.1% SDS solution in water, pH = 8.3
Coomassie Brilliant Blue solution	50% (v/v) ethanol, 0.25% (v/v) Coomassie Brilliant Blue R-250, 10% (v/v) acetic acid
Destaining solution	5% acetic acid solution in water

Table 2.9. Solutions for Western blotting and enzyme immunoassay

Solution	Composition
PTB	25 mM Tris, 150 mM glycine, 10%(v/v) ethanol solution in water
TBS buffer	20 mM Tris, 0.5 M NaCl solution in water (pH = 7.5)
PBS-T	1x PBS supplemented with 0,1% (v/v) Tween-20
Blocking solution	1x Roti-Block solution (in water)
Detection solution	6 mg 4-chloro-1-naphthol (dissolved in 2 mL of methanol), 5 µL 30% H_2O_2, 10 mL TBS (mixture is prepared prior detection.
Coating buffer	66.67 mM $NaHCO_3$, 30 mM Na_2CO_3, solution in water, pH 9.6
1 M H_2SO_4	1 M H_2SO_4 solution in water

2.19. Equipment and software

Table 2.10. List of equipment used in this study

Apparatus	Manufacturer

SE260 MSII vertical electrophoresis apparatus	Hoefer, Holliston, MA, USA
Bench-top centrifuge 5415 R	Eppendorf, Germany
Bench-top centrifuge 5810R	Eppendorf, Germany
Beckman J-6B centrifuge	Beckman (USA)
Beckman K80, K90 ultracentrifuges	Beckman (USA)
Eppendorf Mastercycler AG 22331 thermal cycler	Eppendorf, Germany
Nanodrop2000 spectrophotometer	Thermo Scientific (USA)
Herolab E.A.S.Y 442 K gel documentation system with UV transilluminator	Herolab (Germany)
TDB-120 Dry block thermostat	Biosan (Latvia)
Haake DC10 water bath	Thermo Scientific (USA)
Vortex Genie-2	Scientific Industries (USA)
MiniRocker MR-1 horizontal shaker	Biosan (Latvija)
Innova 44 incubator shaker	New Brunswick (USA)
LabQuake orbital rotator	Biometra (Goettingen, Germany)
Fastblot Semi-Dry Electrophoretic Transfer Apparatus	Biometra (Goettingen, Germany)
infinite M200 spectrophotometer	TECAN (Switzerland)
Power Pack P25 T	Biometra (Goettingen, Germany)
BABCOCK-BSH Laminar flow cabinet	Babcock (Germany)
Labconco freeze dryer	Labconco (USA)
Morgagni 268 (D) electron microscope	FEI (USA)

Table 2.11. List of software used in this study

Software	**Application**
Expasy	Online tool for protein molecular mass and pI calculations
ImageJ 1.50b	Evaluation of band intensity in Western blot and SDS-PAGE
Tecan i-control 1.5	TEKAN infinite M200 spectrophotometer operation and data accession.
Nanodrop 2000/2000c	Data collection with spectrophotometer Nanodrop 2000
Unipro UGENE v1.21	Sequence (DNA and protein) data manipulation tool
MS Excel 2013 Analysis ToolPak	Statistical analysis

2.3. Methods

2.3.1. DNA amplification by PCR

Reaction mixtures for DNA amplification by polymerase chain reaction were made following the user guides published by the DNA polymerase producer (ThermoFisher Scientific Baltics). VP1/2 genes were amplified using High Fidelity PCR Enzyme Mix with at least 30 cycles of amplification. For routine PCRs (colony screening) *Taq* DNA polymerase was used. Amplification reactions were performed in "Eppendorf Mastercycler" thermal cycler. Primer annealing temperatures were calculated with on-line tools (ThermoFisher Tm Calculator).

2.3.2. Site-directed mutagenesis

Point-mutations in DNA sequence was introduced using Phusion Site-Directed Mutagenesis Kit and following manufacturer's recommendation. Primers for this reaction were phosphorylated by T4 Polynucleotide Kinase as described in the user manual.

2.3.3. DNA electrophoresis

DNA electrophoresis was performed in 0.6-1.2% agarose gels prepared in TAE buffer (supplemented with ethidium bromide (10 ng/mL)) and using horizontal electrophoresis system at a voltage of 10 V/cm. DNA fragments visualised with UV transilluminator (Herolab E.A.S.Y 442 K).

2.3.4. DNA fragment purification from agarose gel or PCR reaction mixture

A targeted DNA fragment after electrophoresis was cut out with a piece of agarose from a gel. Further DNA extraction from an agarose gel or from a PCR mixture was performed using GeneJET™ Gel Extraction Kit (ThermoFisher Scientific Baltics) according to the provided user instructions.

2.3.5. Plasmid purification from *E. coli* (for sequencing and restriction analysis)

A tube with 4 mL LB medium supplemented with ampicillin (50 µg/mL) is inoculated with *E. coli* culture and incubated overnight with agitation at 37^{o}C. Cells are harvested by spinning at 3000 rpm for 10 min (table-top centrifuge Eppendorf 5810R). The further DNA extraction was performed with GeneJET™ Plasmid Miniprep Kit (ThermoFisher Scientific Baltics) following the user manual instruction.

2.3.6. Construction and analysis of expression plasmids (hydrolysis, dephosphorylation and ligation)

All DNA manipulations (digestion with restriction endonucleases, dephosphorylation of 5'-ends, DNA ligation) were performed according to

standard procedures (Green and Sambrook, 2012). Enzymes, molecular mass standards and kits for DNA manipulations were purchased from ThermoFisher Scientific Baltics.

2.3.7. Preparation of competent *E. coli* cells and their transformation

The overnight culture (5 mL) of *E. coli* strain was transferred into 500 mL medium in 2 L flask for further growth until OD (600 nm) reached 0.6-0.7. Cells were pelleted (10 min 3000 rpm 4°C in Eppendorf 5810R centrifuge) and washed with 25 mL ice-cold 100 mM $CaCl_2$ solution. After additional pelleting, cells are stored in 25 mL 100 mM $CaCl_2$ at 0-4°C overnight. After incubation, pelleted cells are resuspended in 5 mL $CaCl_2$-glycerol solution, aliquoted and frozen for storage at -70°C freezers.

Prior the transformation, 0.1 mL of competent cells were thawed on ice for 5-10 min, mixed with 5-10 µL of ligation mixture (concentration at least 100 ng/mL of DNA). After the incubation on ice for 15-30 min, cells are subjected to thermal shock (42°C for 90 s), and the mixture is diluted with 1 mL of room temperature LB medium. After 30-40 min incubation in 37°C with agitation, cells are pelleted and plated onto the selective medium.

2.3.8. Preparation of competent yeast *Saccharomyces cerevisiae* cells

A liquid overnight culture of *S. cerevisiae* strain is diluted 10-20 times into a new sterile YEPD medium and incubated with agitation at 30°C till OD (600 nm) reaches 0.5-0.8. Cells are pelleted (2 min 3000 rpm at RT; Eppendorf 5810R centrifuge) and washed with sterile water. After resuspension in 300 µL TE-LiAc solution, cells are incubated at 30°C for 1 h. After incubation, cells are resuspended in the fresh aliquot of 300 µL TE-LiAc solution. In a new sterile tube, 100 µL of cell suspension is mixed with 10 µL 96% ethanol and 0,1-1 µg of plasmid. After the 30 min incubation at RT, 240 µL 50% of TE-PEG is added, and the suspended mixture is transferred for 1 h incubation at 30°C. The competent cells are then subjected to thermal shock for 20-25 min in a 42°C water

bath, and subsequently, cells are washed twice with fresh YEPD medium. After overnight incubation at 30°C, pelleted cells are plated onto YEPD agar medium supplemented with 5 mM formaldehyde.

2.3.9. Induction of recombinant protein synthesis in yeast *Saccharomyces cerevisiae*

S. cerevisiae transformants were grown in liquid YEPD medium supplemented with 5 mM formaldehyde for 24 h (OD (600 nm) reaching 11-13) with agitation. Induction of recombinant protein synthesis was performed by adding equal volume induction medium (2xYEPG) resulting in final 3% concentration of galactose in the medium. Culture is further incubated at 30°C in an orbital incubator with 220 rpm agitation for 16-24 h until required expression level is reached.

2.3.10. Preparation of yeast cell lysate

Induced yeast cells are harvested by 10 min 3000 rpm centrifugation (Beckman J2-21 centrifuge, rotors JA-20 or JA-14). After washing the biomass with a small volume of water, cells are resuspended in the equal volume of lysis buffer (PBS, supplemented with 2 mM EDTA and 2 mM PMSF) and another equal volume of glass beads (d=212-300 μm) is added to the mixture. The yeast cells were disrupted through vortexing with glass beads (d=212–300 μm) in the lysis buffer (ten bursts for 30 s), and the lysate was clarified by centrifuging at 1300×g for 5 min at 4°C. The lysis buffers tested for PARV4 VP2 purification and stability are specified in section 3.1.2, table 3.2.

2.3.11. Induction of recombinant protein synthesis in *E. coli*

E. coli strain BL21(DE3) cells transformed with pET43.1(a)+ plasmid with an insert are cultivated at 37°C overnight in liquid LB medium with agitation. This overnight culture is then diluted ten-fold with LB medium and incubated till OD (600 nm) reaches 0.6. The synthesis of recombinant protein is then induced by

adding IPTG to the medium till final 1 mM concentration. After 3 h, cells are pelleted.

2.3.12. Preparation of *E. coli* cell lysate for protein electrophoresis

E. coli cells are harvested by centrifugation and suspended in 1% SDS by vortexing, and the mixture is then heated at 100°C for 10 min.

2.3.13. Recombinant protein purification from yeast by ultracentrifugation

The clarified yeast cell lysate (section 2.3.10) was transferred onto the 30% (w/v) sucrose solution in the lysis buffer and spun for 3 h at 140,000×g (Beckman Coulter Optima L-90 K ultracentrifuge, rotor type 70Ti). The resulting pellet was resuspended in the lysis buffer, mixed with 0.40 g/mL CsCl solution in PBS and centrifuged at 140,000×g for 20 h. One millilitre fractions of the formed CsCl gradient were collected and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Positive fractions were pooled and rebanded in a CsCl gradient (1.31 g/cm^3) by ultracentrifugation for additional 20 h. The isolated VLPs were dialysed against PBS and lyophilized or mixed with 50% glycerol for longer storage.

2.3.14. Purification of recombinant proteins with a hexahistidine tag (denaturating conditions)

Ni-NTA sorbent is equilibrated with buffer A. Clarified cell lysate (in buffer A) is mixed with the sorbent, and the mixture is incubated for 1 h on ice with modest agitation (assuring the mixing). After incubation, the mixture is transferred into the flow-through cartridge. The sorbent is washed with 10-20 mL portions of buffers B, C, and, subsequently, D. His-tagged recombinant protein is eluted with 250 μL portions of buffer E those are collected separately. Protein presence in

the fractions is evaluated by quantitative Bradford assay and qualitative SDS-PAGE analysis.

2.3.15. Polyacrylamide protein electrophoresis

The protein containing samples were mixed with 2xSDS-PAGE Loading Dye and boiled at 100^{o}C for 10 min. The prepared samples were separated in 10-12% SDS-PAGE gel in Tris-Gly-SDS electrophoresis buffer, using Hoefer SE260 MSII vertical electrophoresis apparatus in 60 mA current and 200 V voltage. Proteins were visualised by staining with Coomassie Brilliant Blue solution and destained by washing in 5% acetic acid.

2.3.16. Western blotting

Protein samples were fractionated by SDS-PAGE in 12% mini-gels and then electrotransferred onto Immobilon P membrane (Millipore, Bedford, MA, USA) in PTB buffer as described in Green and Sambrook (2012). The membranes were blocked with 1× Roti-Block (Carl Roth GmbH & Co.) for 1 h. The blocking solution was removed, the membrane was washed with PBS-T and incubated for 1 h at RT with antibodies diluted in PBS-T containing 2% milk powder. After washing with PBS-T, the membranes were incubated for 1 h at RT with HRP-labelled secondary antibodies (conjugated with HRP) diluted 1:4000 in PBS-T containing 2% milk powder. The blots were stained with detection solution.

2.3.17. Protein concentration (Bradford) assay

The purified protein concentration was determined using a Bradford protein assay reagent Roti-Nanoquant (Carl Roth GmbH, Germany) following the producer manual. Bovine serum albumin (BSA) was used as a standard protein (0.5 mg/mL).

2.3.18. Electron microscopy

After purification by CsCl ultracentrifugation, suspension of the recombinant protein was placed on 400-mesh carbon-coated copper grids (Agar Scientific, Stansted, UK). The protein samples were stained with 2% aqueous uranyl acetate solution and examined with a Morgagni-268 electron microscope (FEI, Eindhoven, the Netherlands).

2.3.19. Indirect enzyme immunoassay

Indirect EIA was used for virus-specific IgG and IgM detection in human and swine sera. Ninety-six-well EIA plates (Nerbe Plus GmbH, Winsen/Luhe, Germany) were coated with 100 – 400 ng per well of recombinant protein in 100 μL of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed four times with PBS-T, then blocked by the addition of 300 μL of blocking 1× Roti-Block buffer per well and incubation at RT for 1 h. After blocking, the plates were washed three times with PBS-T and 100 μL aliquots of serum specimens diluted 1: 200 in PBS-T buffer with 1% BSA were added to the wells. Plates were incubated for 1 h at 37°C with shaking and washed four times with PBS-T. 100 μL aliquots of either rabbit anti-human IgG or goat anti-human IgM conjugated to horseradish peroxidase, diluted 1: 16,000 (v/v) in blocking buffer, were added to each well, and the plates were incubated for 1 h at 37°C with shaking. For PPV-specific antibody detection in swine sera, rabbit anti-pig IgG diluted 1:30 000 in PBS-T, containing 1% BSA, was used. After washing four times with PBST, 100 μL of TMB substrate was added to each well, and the enzyme reaction was stopped with an equal volume of 1 M H_2SO_4 following a 5-min incubation at RT. The OD at 450 and 620 nm was determined for each sample using an EIA plate reader (TECAN infinite M200, Switzerland).

2.3.20. Capture enzyme immunoassay

Capture EIA was used to evaluate antigenicity of PARV4 mVPLs. Ninety-six-well EIA plates were coated with PARV4 VP2 specific Mab 12E8, diluted 1:1000 in 100 µL of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed four times with PBS-T, then blocked by the addition of 300 µL of blocking 1× Roti-Block buffer per well and incubation at RT for 1 h. After blocking, the plates were washed three times with PBS-T, and 500 ng of PARV4 mVLPs or VP2 VLPs was added in 100 µL in PBS-T, containing 1% BSA. After 1 h incubation at RT with agitation, triple washing with PBS-T was performed. Further steps (incubation with human sera and HRP conjugated anti-human IgG antibodies) were performed as described above (section 2.3.19).

2.3.21. Competitive enzyme immunoassay

For HBoV1-4 VP2 competitive IEA, the serum specimens were incubated with 15 mg/mL of each of competitive antigen at 37°C for 90 min, prior adsorbed antigens were applied. Other procedures were identical to those described in section 2.3.19.

2.3.22. Competitive enzyme immunoassay with peptides

PARV4 VP2 fragments 144-158 or 248-262 fused with bacterial NusA protein and purified as outlined in section 2.3.14 were coated in 96-well plates in 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) overnight. Lyophilized peptides were solubilized according to the manufacturers' instructions. 10 µg of the peptide was incubated in 400 µL MAb solution (hybridoma growth medium, diluted 500 times in PBS-T) at 37°C with agitation. After 1 h incubation, the solution was applied to the wells with the adsorbed antigen (PARV4 VP2 fragments). Other procedures were identical to those described in section 2.3.19.

2.3.23. Statistical analysis

The seroprevalence of HBoV and PARV4 were evaluated using the χ^2 test. The p-value less than or equal to 0.05 was considered significant unless otherwise indicated. The effect size is indicated as odds ratio (OR) with 95% confidence interval (CI). Wilcoxon signed-rank test was calculated to evaluate differences in mVPL epitope exposure. Calculations were performed using MS Excel 2013 Analysis ToolPak.

3. RESULTS AND DISCUSSION

Diagnostic test systems for parvovirus infection are based on the detection of either virus-specific antibodies or viral DNA. VP2 protein is a major immunogen of most parvoviruses (Kamstrup *et al.,* 1998; López de Turiso *et al.,* 1991) and the long-lasting antibodies against parvoviruses are preferentially directed against conformational epitopes present in VP2 proteins (Lowin *et al.,* 2005; Söderlund *et al.,* 1995). This means that current diagnostic enzyme immunoassay tests for parvoviruses rely on recombinant VP2-based virus-like particles as a specific antigen that is produced preferentially in baculovirus expression systems (Lahtinen *et al.,* 2011; Sharp *et al.,* 2009). Moreover, VP2 protein is the major agent for developing vaccines (Lo-Man *et al.,* 1998).

Thus, the main aim of this study was to test yeast *S. cerevisiae* expression system's ability to produce structural proteins (VP) of parvoviruses as virus-like particles and to investigate the aptness of these proteins for serodiagnostics. In this chapter, the results are presented as follows: section 3.1 provides the results on recombinant parvoviral protein synthesis in yeast, their purification and structural properties, whereas section 3.2 presents the results on the antigenic properties of these proteins and their application in serodiagnostics. The work has been done in the Department of Eukaryote Gene Engineering of the Institute of Biotechnology, Vilnius University in close collaboration with the department of Cell Biology and Immunology (head Dr. A. Žvirbliene) and with a contribution from the Clinic of Children's Diseases, Faculty of Medicine, Vilnius University and Institute of Microbiology and Virology, Veterinary Faculty of Veterinary Academy, Lithuanian University of Health Sciences.

3.1. Synthesis of parvoviral capsid proteins in yeast *S. cerevisiae*

3.1.1. Construction of parvoviral protein expression vectors

For the synthesis of parvoviral capsid proteins in yeast *S. cerevisiae*, two heterologous protein expression vectors were employed. Corresponding VP1 or VP2 genes were subcloned into *Xba*I-digested vector pFX7 under the control of a hybrid GAL10-PYK1 promoter (Sasnauskas *et al.,* 1999). Yeast expression vector pFGG3 that had an additional cassette under galactose-inducible GAL7

promoter (Slibinskas *et al.*, 2004) was used to co-express two viral proteins and generate mosaic VLPs of PARV4 and HBoV1 (section 3.1.6.). Both types of expression vectors harboured 2 µm plasmid fragment and *ColE1 ORI* sequence, ensuring replication in yeast *S. cerevisiae* and *E. coli*, respectively. For transformant selection, *bla* gene coding for β-lactamase (resistance to ampicillin) and formaldehyde dehydrogenase gene *FDH1* from *Candida maltosa* were exploited (Sasnauskas *et al.*, 1992). Figure 3.2 schematically illustrates the structure of pFX7 and pFGG3 vectors and location of cloned parvoviral VP proteins.

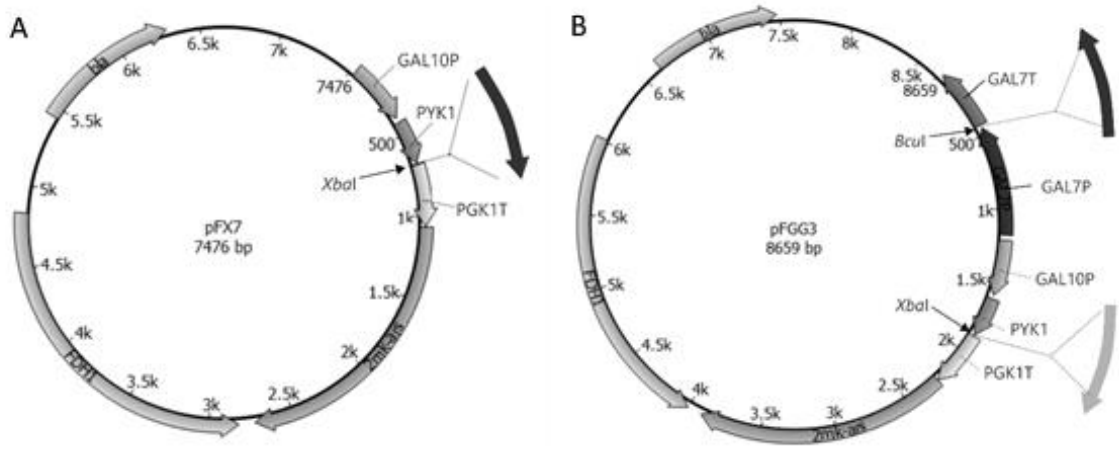


Figure 3.1. (**A**). Expression vector pFX7. (**B**). Expression vector pFGG3. 2 µm–1.74 kb fragment of yeast 2µm plasmid; FDH1—*FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde; pIC19H—bacterial plasmid; GAL7T—*S. cerevisiae GAL7* gene transcription terminator (381 bp); GAL7P—*S. cerevisiae GAL7* gene promoter (−1to −716 nt); GAL10P—*GAL10* gene UAS sequence (−511 to −138 nt); PYK1—*S. cerevisiae PYK1* gene promoter (−271 to −4 nt); PGK1—fragment of *S. cerevisiae PGK1* gene transcription terminator sequence (387 bp). Parvoviral genes were subcloned downstream the corresponding promoters by linearization of vectors using *Xba*I or *Bcu*I restriction endonucleases at the indicated recognition sites. The required direction of heterologous genes for successful expression is indicated by the arrow.

Porcine parvovirus (strain NADL2) VP2 DNA was obtained from Dr. V. Sereika (Lithuanian University of Health Sciences) as pathological specimens of the infected porcine kidney. Viral nucleic acids were isolated from the kidney homogenate using 'QIAamp Ultrasens virus kit'. The isolate was subsequently employed for amplification of the coding sequence of PPV VP2 gene by PCR. The resulting reaction mixture was analysed in agarose gel electrophoresis

revealing ~1700 bp fragment (data not shown). The sequence of isolated PCR product was analysed in DNA Sequencing Center (VU IBT), and results were compared to those in GenBank using the Basic Local Alignment Search Tool (BLAST). The amplified fragment proved to be identical to VP2 gene from porcine parvovirus strain NADL-2 (GenBank accession number NC001718). The primers used for amplification (PPV-vp2-F and PPV-vp2-R, Table 2.2) were designed to incorporate *Bcu*I sites for cloning into *Xba*I-digested vector pFX7.

Human parvovirus 4 VP1 gene was chemically synthesised by Genscript (Piscataway, NJ, USA) according to the published sequence (GenBank accession number EU546204), and received in the pUC57 plasmid. The sequence was designed with additional *Xma*JI sites at both ends of the VP1 gene for its excision from the plasmid and subsequent cloning into yeast expression vectors. PARV4 VP2 gene was amplified from the aforementioned chemically synthesised PARV4 VP1 in pUC57 matrix using P4_FW2 and P4_RV primers. The PCR product (~1660 bp, data not shown) was hydrolysed with restriction endonuclease *XmaJ*I and subcloned into pFX7 and pFGG3 vectors.

HBoV1 VP1 and HBoV2–4 VP2 genes were chemically synthesised by GenScript according to the sequences published in GenBank (NC_007455, GU048664, FJ948861 and FJ973561, respectively). Additionally, after insufficient expression protein levels were observed (section 3.1.3), VP2 sequences of HBoV2 and HBoV3, optimised by GenScript OptimumGene™ algorithm for expression in yeast, were purchased. Optimised sequences were published in GenBank with the accession numbers KU212373 (HBoV2opt) and KU212374 (HBoV3opt). All genes with *Xba*I restriction sites designed in both ends were obtained in pUC57 plasmids. HBoV1 VP2 gene was amplified by PCR from VP1 sequence using specific primers HBoV1_VP2F and HBoV1_VP2R that introduced *Xba*I sites at both ends of ~1640 bp PCR product.

All the expression vectors for parvoviral protein synthesis in yeast generated in this work are listed in Tables 3.1 and 3.2. After cloning, the sequences of generated expression vectors were verified by sequencing in DNA Sequencing Center (VU IBT).

Table 3.1. Expression vectors created in this study for parvoviral protein synthesis in yeast. Direction and position of cloned genes are generalised in Fig. 3.1.

pFX7-based vectors	**Gene cloned under GAL10-PYK1 promoter and its accession number at GenBank**	**Vector size, bp**
pFX7-PPV_VP2	PPV VP2 (NC001718, 2035...4833 nt)	10278
pFX7-PARV4_VP2	PARV4 VP2 (EU546204, 3412…5073 nt)	9141
pFX7-PARV4_VP1	PARV4 VP1 (EU546204, 2329…5073 nt)	10224
pFX7-HBoV1_VP1	HBoV1 VP1 (NC_007455, 3056...5071 nt)	9495
pFX7-HBoV1_VP2	HBoV1 VP2 (NC_007455, 3443..5071 nt)	9108
pFX7-HBoV2_VP2	HBoV2 VP2 (GU048664, 3426...5042 nt)	9096
pFX7-HBoV2opt_VP2	HBoV2 VP2, optimised for yeast expression system (KU212373, 1…1617 nt)	9096
pFX7-HBoV3_VP2	HBoV3 VP2 (FJ948861, 3420..5039 nt)	9099
pFX7-HBoV3opt_VP2	HBoV3 VP2, optimised for yeast expression system (KU212374, 1…1620 nt)	9099
pFX7-HBoV4_VP2	HBoV4 VP2 (FJ973561, 3331..4956 nt)	9105
pFGG3-based vectors	**Gene cloned under corresponding promoters**	**Vector size, bp**
pFGG3-HBoV1_VP1$_{GAL7}$+VP2 $_{GAL10}$	GAL7 – HBoV1 VP1 and GAL10-PYK1 - HBoV1 VP2	12296
pFGG3-HBoV1_VP2$_{GAL7}$+VP1 $_{GAL10}$	GAL7 – HBoV1 VP2 and GAL10-PYK1 - HBoV1 VP1	12296
pFGG3-PARV4_VP1$_{GAL7}$+VP2 $_{GAL10}$	GAL7 – PARV4 VP1 and GAL10-PYK1 – PARV4 VP2	13049
pFGG3-PARV4_VP2$_{GAL7}$+VP1 $_{GAL10}$	GAL7 – PARV4 VP2 and GAL10-PYK1 – PARV4 VP1	13049

3.1.2. PARV4 VP2 expression in yeast

Expression vector pFX7-PARV4_VP2 was transformed into yeast *Saccharomyces cerevisiae* AH22-214 strain that earlier had proved to successfully generate immunogenic nucleocapsids of *Paramyxoviridea* family viruses (Petraityte *et al.,* 2009; Slibinskas *et al.,* 2004) or a number of polyomavirus VLPs (Sasnauskas *et al.,* 2002). After induction of heterologous protein expression (as described in section 2.3.10), SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring the above-mentioned expression vectors revealed the presence of an additional protein band. This band was approximately 62 kDa in yeast transformed with pFX-PARV4_VP2 (Fig. 3.2, lane 2). Theoretically calculated molecular mass of unmodified PARV4 VP2 protein should be 61.6 kDa. No additional band of the equal molecular size was observed in crude lysates of *S. cerevisiae* harbouring pFX7 vector (Fig. 3.2, lane 1). After centrifugation of lysates through 30% sucrose cushion, the pellets harbouring recombinant proteins were subjected to CsCl-gradient centrifugation. CsCl

gradients revealed recombinant PARV4 VP2 protein in fractions with buoyant density of 1.28–1.29 g/mL (Fig. 3.2 B, lane 3).

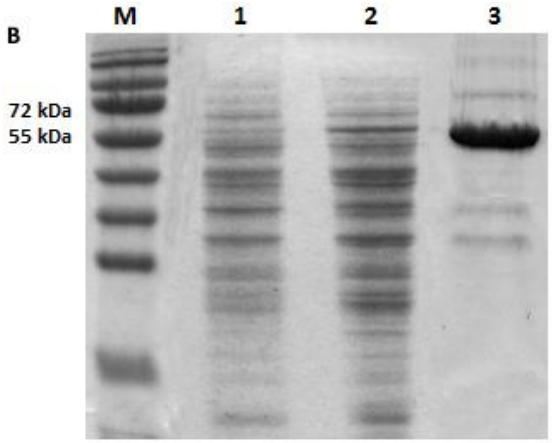


Fig. 3.2. PARV4 VP2 synthesis in yeast. SDS-PAGE analysis of yeast lysates and expressed PARV4 VP2 protein after ultracentrifugation in CsCl. Five µg of purified VP2 protein (lane 3) and 20 µg of yeast lysates (lanes 1 and 2) were separated in a 12% SDS-PAGE and stained with Coomassie brilliant blue. M = PageRuler Prestained Protein Ladder (ThermoFisher Scientific Baltics). Lane 1: *S. cerevisiae* pFX7 lysate; Lane 2: *S. cerevisiae* pFX7-PARV4_VP2 lysate; Lane 3: CsCl purified PARV4 VP2.

Formation of VLPs by PARV4 VP2 protein was confirmed by negative staining electron microscopy. Typical icosahedral structures of parvoviruses with a diameter of approximately 25-28 nm were observed indicating that PARV4 VP2 had self-assembled to VLPs (Fig. 3.3). To confirm the sequence identity of the full-length recombinant PARV4 VP2 protein, MS analysis of purified recombinant protein was performed at Proteomics Centre (VU BChI) (data not shown). In several preparative procedures, the yield of purified recombinant PARV4 VP2 protein was found to be ~6.0±1.1 mg/L of induced yeast culture (data not shown).

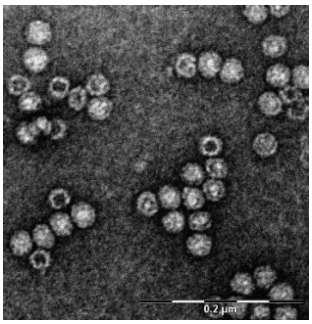


Fig. 3.3. Electron micrograph of recombinant PARV4 VP2 VLPs generated in yeast. Scale bar 200 nm.

After CsCl gradient purification, the protein was dialysed against PBS containing 10 mM EDTA and stored at −20°C in PBS containing 10 mM EDTA and 40% glycerol. Lyophilisation of PARV4 VP2 and subsequent storage at −20°C for three months resulted in the appearance of ~30 kDa partial degradation product (Fig. 3.4) that was less abundant in the glycerol lot. Further protein stability study was conducted to determine the stability of PARV4 VP2 VLPs at different pH conditions. Purified VLPs were dialysed against buffers of pH 3.2 – 9.6 (Table 3.2) at 4°C overnight and subsequently stored in a corresponding buffer for 48 hrs at 4°C. The SDS-PAGE analysis was performed to evaluate the appearance of <65 kDa bands that would suggest that the degradation had occurred. Additionally, electron microscopy analysis was carried out to inspect VLP stability. Results summarised in Table 3.2 show that PARV4 VP2 VLPs are stable in the buffers of pH 7.6-9.3. VLPs were observed to remain intact in PBS supplemented with detergents such as 0.5% of TritonX or 1% Tween-20, as well as chelating agents EGTA or EDTA (10 mM). However, the solubility of purified PARV4 VP2 VLPs was the best in pH 8.7 buffer (50 mM Tris, 2 mM EDTA).

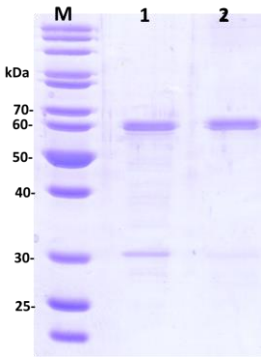


Fig. 3.4 Stability of PARV4 VP2 protein. SDS-PAGE analysis of lyophilized and resuspended in PBS (lane 1) and stored in protein sample in 50% glycerol for 3 months (-20°C) (lane 2).

Table 3.2. Assay of PARV4 VP4 VLPs stability at different pH

Composition of buffers	pH	Degradation products	Uniformity of VLPs
50 mM sodium citrate, 2mM EDTA	3,2	multiple (<35 kDa), >60%	+/-
50 mM sodium acetate, 2mM EDTA	4,7	minor (~30 kDa), <5%	+
PBS, 2mM EDTA	7,6	-	+
50 mM Tris, 2 mM EDTA	8,7	-	+
50 mM $NaHCO_3$, 2mM EDTA, 200mM NaCl	9,6	-	+

Results show that the yeast system employed in this study is suitable for the efficient expression of PARV4 VP2 protein. Moreover, PARV4 VP2 protein produced in *S. cerevisiae* has the capacity to form VLPs in the absence of VP1 protein. VLPs of PARV4 VP2 remain stable in neutral or basic pH (7.6-9.3) and do not need divalent ions for the assembly. Storage in 50 mM Tris, pH 8.7 containing 2 mM EDTA and 40% glycerol is recommended for a greater stability of PARV4 VP2 VLPs.

3.1.3. Synthesis of VP2 proteins of HBoV1-4 in yeast

Yeast expression vectors pFX7, containing HBoV1-4 VP2 gene sequences inserted downstream the galactose-inducible GAL10-PYK1 promoter (Table 3.1) were transformed into *S. cerevisiae* AH22-214 strain. Expression efficiency of recombinant HBoV1-4 VP2 proteins in yeast was analysed by SDS-PAGE. Crude lysates of yeast cells harbouring vector pFX7 with unmodified HBoV1 and HBoV4 VP2 sequences revealed additional bands of 62 kDa which corresponds to the calculated VP2 molecular mass (Fig. 3.5). Similar pronounced bands were absent in protein profiles of yeast cells harbouring the unmodified genes of VP2

of HBoV2 and HBoV3 in pFX7. However, an efficient synthesis of these two structural proteins (Fig. 3.5, A and C) was obtained using gene sequences optimised for their expression in yeast (designated as HBoV2opt and HBoV3opt further in the text). Optimisation was done primarily by improving the codon usage. The codon adaptation index (CAI) (Sharp and Li, 1987) for HBoV2 and HBoV3 VP2 genes were enhanced from 0.75 and 0.76, respectively, to 0.85 for both genes. In yeast, codon optimisation does not always lead to enhanced expression compared to a wild-type, unmodified sequence (for review, Lanza *et al.*, 2014). However, substantial differences in expression levels of highly related (homology among HBoV 1-4 VP2 proteins is >80%) heterologous proteins in yeast were observed in this study.

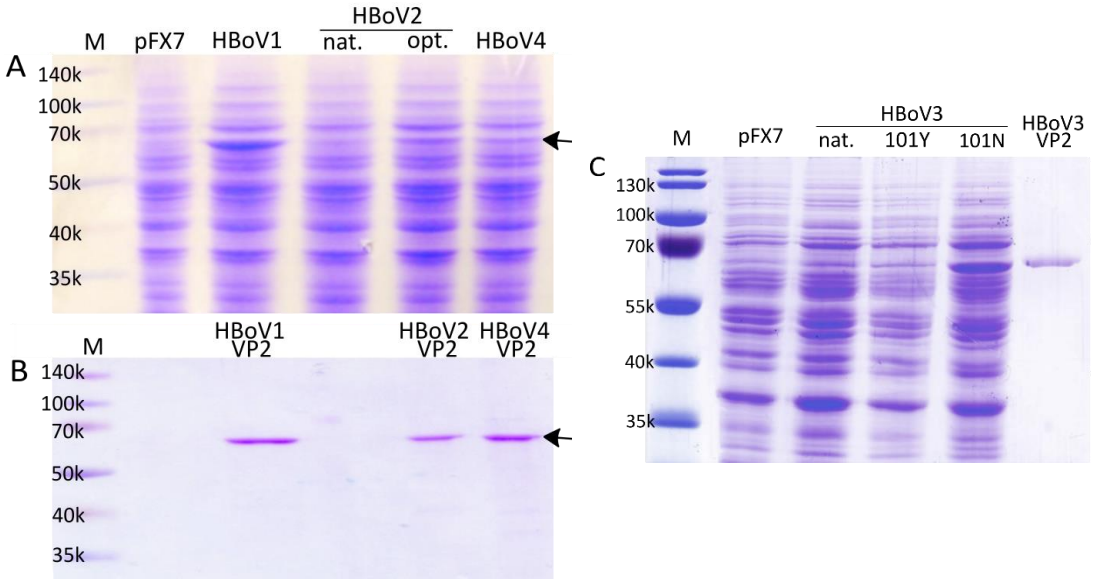


Fig. 3.5 SDS-PAGE analysis of *S. cerevisiae* cell lysates and purified HBoV1-4 VP2 proteins. (**A**) Lysates (20 µg) of *S. cerevisiae* harbouring plasmids pFX7 (lane 2) and pFX7 containing VP2 genes of HBoV1, HBoV2 (native and optimised sequences), and HBoV4, respectively (lanes 3-6). (**B**) CsCl-gradient purified HBoV1, 2 and 4 VP2 proteins (3 µg of each). The molecular mass of VP2 proteins is approximately 62 kDa (indicated by arrows). Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific Baltics) was used as molecular mass standard in lanes M. (**C**) Lysates (20 µg) of *S. cerevisiae* harbouring plasmids pFX7 and pFX7-HBoV3_VP2 (lanes 3-5: unmodified native sequence and two genes, optimized for yeast expression system, coding for tyrosine and asparagine in position 101, respectively) as well as CsCl-gradient purified HBoV3 VP2 protein (lane 6) were separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant blue. PageRuler Prestained Protein Ladder (ThermoFisher Scientific Baltics) was used as a molecular mass standard in lane M.

After centrifugation of yeast lysates through 30% sucrose cushion, the pellets harbouring recombinant proteins were subjected to CsCl-gradient centrifugation. SDS-PAGE analysis of CsCl gradient fractions revealed single bands of recombinant 62 kDa proteins in fractions with a buoyant density ranging 1.30–1.32 g/ml (Fig. 3.5, B and C).

Analysing the published HBoV VP2 gene sequences for possible regional differences, tyrosine instead of arginine at position 101 of the only entry from HBoV3 European sample (Genbank accession number ADD39268, (Chieochansin *et al.*, 2010)) was discovered (Fig. 3.6, A). Sequence alignment with more distant parvoviruses, a conserved motif FdF**N**qysshFS**P** has been detected in parvoviral major capsid proteins (Fig. 3.6, B), suggesting that conservative arginine at this position may play an important role in structural stabilisation. An intrinsic property of parvovirus major structural (VP2) proteins to self-assemble into VLPs is partially determined by a highly conservative eight-stranded, antiparallel β-barrel, which forms most of the intersubunit contacts (Kaufmann *et al.*, 2004). Analysing the structures of related parvoviruses, namely B19 VP2 (PDB accession number 1s58, (Kaufmann *et al.*, 2004)) and canine parvovirus VP2 (PDB accession number 2cas, (Wu & Rossmann, 1993)), revealed that 101N in HBoV3 is adjacent to a conservative α-helix, and larger side chain at this position (for example, that of tyrosine) would sterically interfere with β-barrel that is a core of parvoviral capsid structure (Fig. 3.7). However, parvoviral VLPs have been demonstrated to tolerate sequence substitutions and insertions in surface loops which are an effective foreign epitope display for immunisation (Pan *et al.*, 2013). Our finding may be important for further structural studies of parvovirus-derived antigens and the assembly of parvoviral particles.

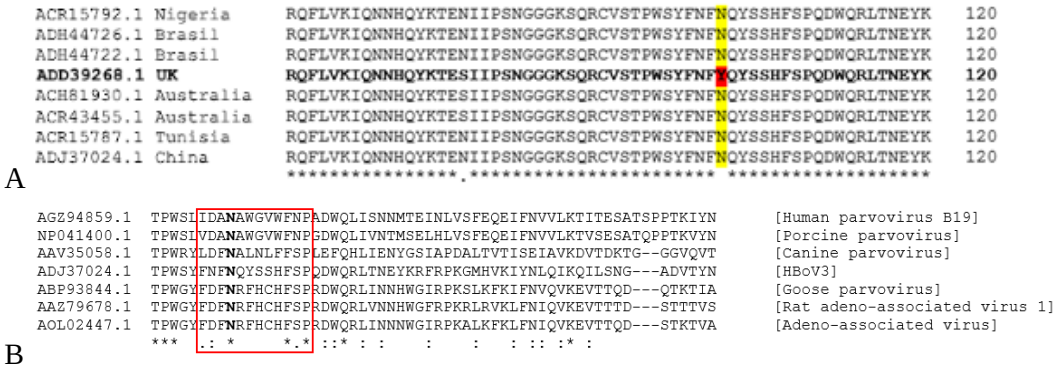


Fig. 3.6. Sequence alignment of the published gene sequences (regions from 60 aa to 120 aa) of the major capsid proteins of some parvoviruses. (**A**) Alignment of HBoV3 VP2 gene sequences. Sequence numbers in Genbank and countries of origin are indicated on the left. (**B**) Alignment of the major capsid protein (VP2 or VP3, for AAV) of various distant parvoviruses. Conservative arginine is indicated in bold in the boxed area. Alignments were performed with Clustal (v.1.2.4) (Sievers *et al.*, 2011)

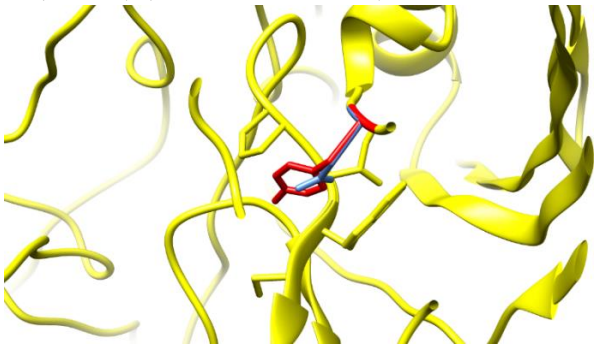


Fig. 3.7 The model of HBoV3 capsid structure. The side-chain of the amino acid at the position 101 in HBoV3 VP2 protein sequence is displayed as red (tyrosine) or blue (arginine). The model was constructed with a SWISS-MODEL tool (Bienert *et al.*, 2017).

The influence of this substitution on protein properties was tested by introducing a N101Y point mutation into HBoV3 VP2 protein via site-directed mutagenesis (Methods section 2.3.2.). The mutation was introduced directly into vector pFX7-HBoV3opt_VP2 using primers N101Y_F and N101Y_R, and newly generated vector pFX7-HBoV3opt-N101Y_VP2 was transformed into *S. cerevisiae AH22-214* strain and further protein expression analysis procedures were performed as described earlier in this chapter. As presented in Fig. 3.5 C, lane 101Y, the expression level of HBoV3 VP2 with tyrosine at position 101 was lower as compared to that of HBoV3 VP2 with asparagine. Furthermore, analysis of CsCl fractions of both purified VP2 proteins by electron microscopy revealed that HBoV3 VP2 mutant N101Y was assembled into irregularly shaped and clustered

particles (Fig. 3.8), unusual to native parvovirus virions (Brieu *et al.*, 2008). In contrast, recombinant VP2 of HBoV1, HBoV 2, HBoV 4, as well as an unmodified VP2 of HBoV3 (101N) formed icosahedral structures with a diameter of 22-25 nm (Fig. 3.8). For further studies (sections 3.2.4-5), VLPs of HBoV3 VP2 (101N) was used.

The yields of the purified VLPs were 8.0±0.3, 4.1±0.3, 10.9±0.5, and 6.7±0.2 mg per litre of induced yeast culture for VP2 of HBoV1, HBoV2, HBoV3 (101N) and HBoV4, respectively, and were similar to the yield of the previously generated B19 VLPs in yeast (Lowin *et al.*, 2005). Concerning the impact of codon usage optimisation, protein yields after gene sequence modification increased substantially. Nonetheless, in the case of HBoV2 VP2 protein, expression levels did not reach those of HBoV1 or HBoV4 VP2 from unmodified genes. The sequence identities of the purified recombinant HBoV1-4 VP2 proteins were confirmed by MS analysis at the Proteomics Centre (VU BChI) (data not shown).

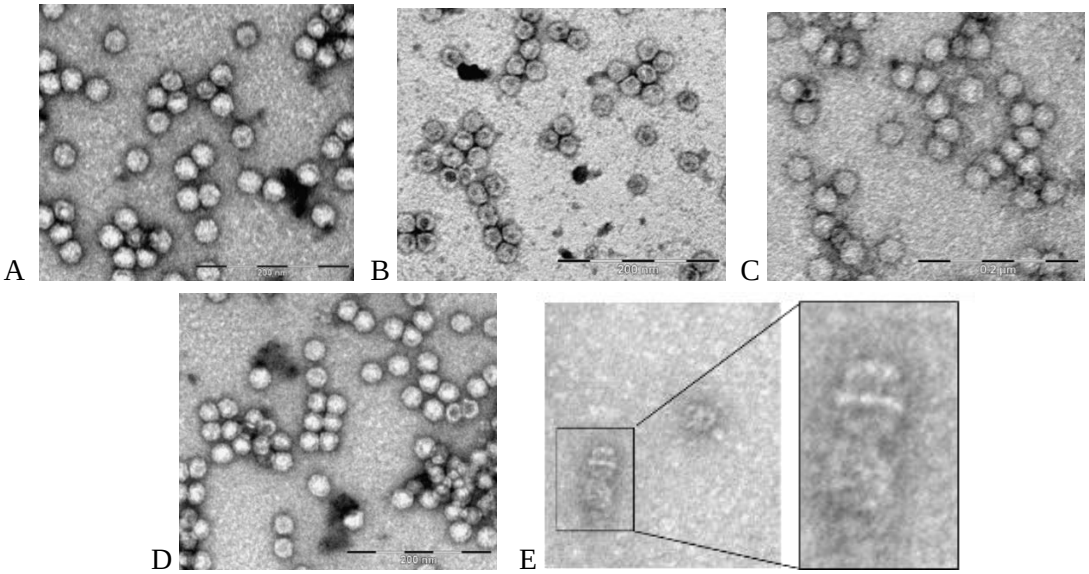


Fig. 3.8 Electron micrographs of yeast-generated recombinant VP2 VLPs of HBoV1 (**A**), HBoV2 (**B**), HBoV3 (**C**), and HBoV4 (**D**) in CsCl fractions (scale bar = 200nm). (**E**) Electron micrograph of structures formed by HBoV3 VP2 (101Y), magnified 110 000 times (left).

3.1.4. Synthesis of porcine parvovirus VP2 in yeast.

For the investigation of porcine parvovirus VP2 synthesis, a yeast expression vector pFX7-PPV_VP2 was transformed into *S. cerevisiae* AH22-214 strain. An SDS-PAGE analysis of the lysate of induced yeast biomass revealed a major protein band of approximately 64 kDa (Fig. 3.9, lane 2). No additional protein bands were observed in crude lysates of *S. cerevisiae* harbouring empty yeast vector pFX7 (Fig. 3.9, lane 1). After centrifugation of lysates through 30% sucrose cushion, the pellets harbouring recombinant proteins were subjected to CsCl-gradient centrifugation. CsCl gradients revealed recombinant PPV VP2 protein (Fig. 3.9, lane 3) in fractions with a buoyant density of 1.28-1.31 g/mL.

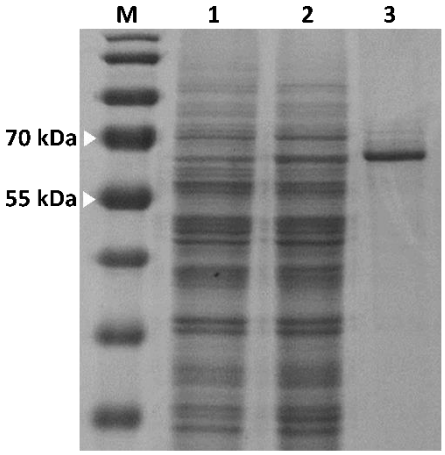


Fig. 3.9 Analysis of *S. cerevisiae* cell lysates and purified PPV VP2 protein by SDS-PAGE. Lysates of *S. cerevisiae* harbouring plasmids pFX7 (lane 1, 20 µg) and pFX7-PPV_VP2 (lane 2, 20 µg) as well as CsCl-gradient purified PPV VP2 protein (lane 3, 4 µg) were separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant blue. PageRuler Prestained Protein Ladder was used as molecular mass standard in lane M

In several preparative procedures, the yield of purified recombinant PPV VP2 protein was found to be 8.2-9.0 mg/L of induced yeast culture. There was no significant yield difference using fresh or frozen biomass. Formation of VLPs by PPV VP2 protein was confirmed by negative staining electron microscopy. Typical icosahedral structures of parvoviruses with a diameter of approximately 25-30 nm were observed indicating that PPV VP2 protein is self-assembled to VLPs (Fig. 3.10 A). VLPs of PPV produced in *S. cerevisiae* expression system were similar to those previously generated in insect cells (Rueda *et al.*, 2000) or native PPV particles (Molitor *et al.*, 1983). To meet the need for stable

recombinant VLPs of PPV, several expression systems were tested as an alternative to baculovirus expression system that is a primary source of the antigen for the market (Maranga *et al.,* 2003). *E. coli* (Qi & Cui, 2009), *Lactobacillus casei* (Xu & Li, 2007) and recently yeast *Pichia pastoris* (Guo *et al.*, 2014) were reported to have been successfully used for producing PPV VP2 protein, but VLP formation in these expression systems has not been confirmed. To our knowledge, our study provides the first evidence of stable recombinant PPV VP2 VLPs produced not in baculovirus expression system.

After CsCl gradient purification, the recombinant VP2 protein was dialysed against PBS and stored at −20 ◦C in PBS containing 50% glycerol or lyophilized and kept at −20 ◦C for prolonged time periods. There were no additional bands of lower molecular mass observed in the SDS-PAGE analysis of samples of both storing conditions after 3 and 12 months (data not shown), that indicates the stability of PPV VP2 protein. The recombinant PPV VP2-derived VLPs were found to be intact when lyophilized and stored at -20 ◦C longer than a year and VLPs remained integral when resolubilized in PBS as no pentamers or disrupted particles were observed by electron microscopy (Fig. 3.10 B). Moreover, the EIA results using freshly prepared and resolubilized after lyophilisation PPV VP2 antigen were fully concordant (data not shown). The stability of VLPs is crucial to ensure their successful transportation and possible application in the point-of-care tests.

Tests of PPV VP2 protein stability in different buffers were performed analogously as described in section 3.1.2. and Table 3.2. Similarly to PARV4 VP2, yeast-generated PPV VP2 remained stable in pH 4.7 to 9.6 and storage in citrate buffer of pH 3.2 caused partial degradation (data not shown).

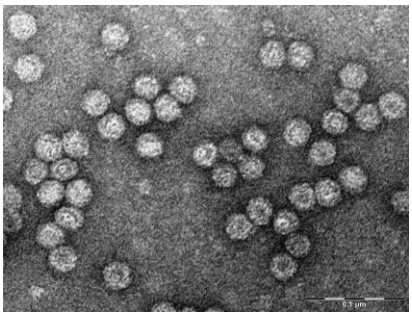
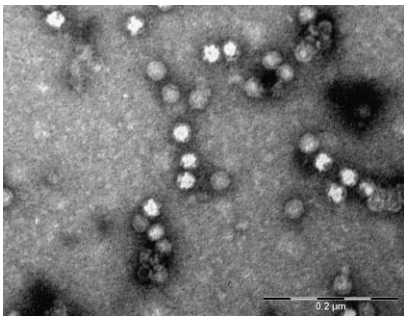
A  B 

Fig. 3.10 Electron micrograph of recombinant PPV VP2 VLPs in CsCl fraction (**A**, scale bar = 100 nm) and VLPs resolubilized after lyophilisation (**B**, scale bar = 200 nm).

3.1.5. Synthesis of HBoV1 and PARV4 minor structural protein (VP1) in yeast

Although VP1 of parvoviruses is a minor structural protein (Gurda *et al.,* 2010), studies reported a cluster of neutralising epitopes within the parvoviral VP1 sequence (Saikawa *et al.*, 1993; Söderlund *et al.*, 1995), suggesting the importance of VP1 protein for serological studies. VLPs, composed of VP1 protein, would potentially retain antigenicity of VP2-VLPs because of the overlapping primary sequences of VP2 and VP1, but also present additional epitopes harbouring in VP1u region. This possibility was tested using two parvoviral genes, namely PARV4 VP1 and HBoV1 VP1, cloned into pFX7 yeast expression vector (Table 3.1.).

After induction of heterologous protein expression (as described in section 2.3.9), SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring vectors pFX7-PARV4_VP1 or pFX7-HBoV1_VP1 revealed the presence of an additional protein band. This band was approximately 105 kDa in the lysates of yeast transformed with pFX-PARV4_VP1 (Fig. 3.11 A, lane 1) and approximately 75 kDa in those transformed with pFX-HBoV1_VP1 (Fig. 3.11 B, lane 2). Theoretically calculated molecular masses of these unmodified proteins should be 101.2 and 75.1 kDa, respectively. No additional band of the equal molecular size was observed in crude lysates of *S. cerevisiae* harbouring pFX7 vector (Fig. 3.11). The expression levels of PARV4 VP1 and HBoV1 VP1 proteins in transformed *S. cerevisiae* AH22-214 yeast were very low, and its purification through sucrose and CsCl gradients was not successful due to minute yields.

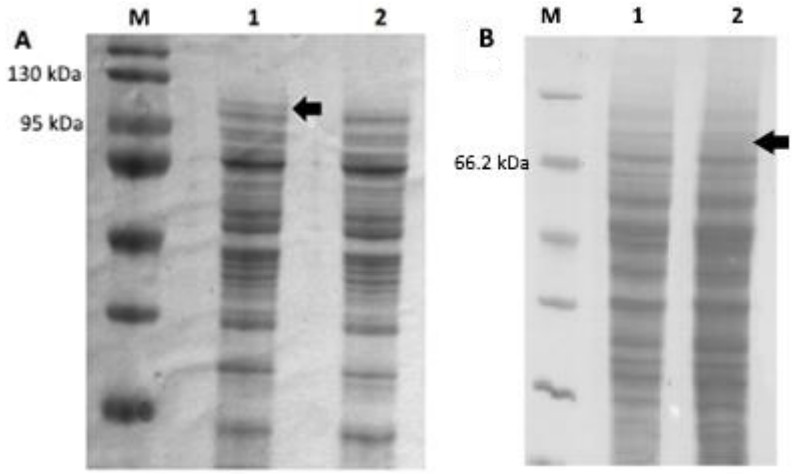


Fig. 3.11. SDS-PAGE analysis of yeast lysates. (**A**) PARV4 VP1 synthesis in yeast. An additional band of approximately 105 kDa in *S. cerevisiae* pFX-PARV4_VP1 lysate (lane 1) is indicated by an arrow. Lane 2: *S. cerevisiae* pFX7 lysate. M = PageRuler Prestained Protein Ladder. (**B**) HBoV1 VP1 synthesis in yeast. Lane 1: *S. cerevisiae* pFX7 lysate. Lane 2: *S. cerevisiae* pFX-HBoV1_VP1 lysate, the 75 kDa band is indicated by an arrow. M = Unstained Protein MW Marker.

For more efficient expression levels of PARV4 and HBoV1 VP1 proteins, additional yeast *S. cerevisiae* strains, namely gcn2, FH4 and AH22-214Δpep4 were tested. The latter strain possesses a mutation in *PEP4* gene coding vacuolar peptidase A (YprA, EC3.4.23.25) that plays a role in the activation of other peptidases (Ammerer *et al.*, 1986) and thus plausibly limiting the accumulation of recombinant proteins. However, none of the tested strains showed enhanced expression level of PARV4 VP1 protein. It suggested that VP1 protein of PARV4 produced in yeast either does not form VLPs or its self-assembly to VLPs is non-efficient, thus the accumulating protein obturates the overall viability of the cell that is observed in up to 26% slower culture growth in comparison to control transformants harbouring pFX7 vector only. Substantially lower expression level to that of VP2 protein as well as failure to form VLPs were previously demonstrated with parvovirus B19 VP1 expressed in insect cells (Michel *et al.*, 2008).

For HBoV1, crude cell lysates of AH22-214Δpep4 strain, harbouring the pFX7-HBoV1_VP1 expression vector, had more prominent ~75 kDa band in SDS-PAGE (Fig. 3.12) indicating enhanced expression. However, a 75 kDa band was

missing after ultracentrifugation through a sucrose cushion, suggesting that this protein or VLPs are unstable in standard preparatory conditions. The change of lysis buffer pH to 8.3 using alternative lysis buffer (50 mM Tris, supplemented with 2 mM EDTA and peptidase inhibitor cocktail (Sigma)), enhanced the stability of the HBoV1 VP1 protein. The overall yield of HBoV1 VP1 was as low as 0.77±0.12 mg/L of induced medium and no virus-like particles were observed by electron microscopy. Therefore, the results show that contrarily from stable VP2-VLPs, yeast expression system is not an efficient producer of parvoviral VP1 proteins from unmodified gene sequences.

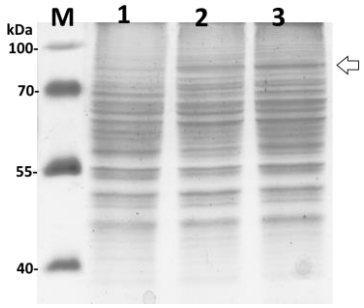


Fig. 3.12 Analysis of HBoV1 VP1 expression in different yeast *S. cerevisiae* strains. SDS-PAGE analysis of 20 µg samples of the lysates of yeast strains harbouring plasmid pFX7-HBoV1_VP1: AH22 (lane 2), AH22-214Δpep4 (lane 3). Lane 1: *S. cerevisiae* AH22 pFX7 lysate. M: PageRuler™ Prestained 10-180kDa Protein Ladder, (ThermoFisher Scientific Baltics).

In native parvovirus virions, the VP1u domain of minor structural protein exhibits phospholipase A_2 (PLA2) activity (Qu *et al.*, 2008). *S. cerevisiae* cells were shown to be sensitive to overexpressed heterologous phospholipases (Sato *et al.*, 2004). VP1u of PARV4 is the N'-terminal 362 amino acids sequence with PLA2-like domain spanning at 216-260 aa position (Lou *et al.*, 2012a). Aiming to test the influence of plausibly active PLA2-activity of recombinant proteins, coding sequences of four N'-truncated variants of PARV4 VP1 protein (Fig. 3.13 A) were amplified by PCR and subcloned into *Xba*I-digested pFX7 vector. Resulting four expression vectors were transformed into *S. cerevisiae* AH22-214 strain and recombinant protein synthesis was induced as described in Methods section 2.3.9. Crude yeast cell lysate samples were diluted to 10.0 mg/mL of total protein and analysed with SDS-PAGE (not shown) and WB (Fig. 3.13 B). Expression levels of N'-termini truncated variants were estimated by comparison of the intensity of

bands in 105-65 kDa region. As seen in Fig. 3.13 B, removing up to 52 aa from N'-terminus of VP1 protein did not enhance the expression levels, whereas removal of 272 aa resulted in substantial increase in recombinant protein synthesis up to a level comparable to that of VP2. Taking into the consideration that PLA2-like motif is located 216-260 aa from N'-terminus, both truncations, affecting this region (-254 and -272) resulted in increased VP1 variant synthesis, suggesting that removal of this motif improves the expression levels. However, Lou *et al.,* (2012a) demonstrated that *E. coli* synthesised VP1u lacks PLA2-like activity *in vitro,* that was observed in B19 VP1u (Dorsch *et al.*, 2014) and HBoV1 VP1u (Chiu *et al.,* 2014; Lou *et al.*, 2012a). We were unable to test the PLA2-like activity of the yeast-generated VP1 due to very low expression level, but data does not omit the possibility of PARV4 VP1 phospholipase activity *in vivo* and, thus, toxic action onto producer cells.

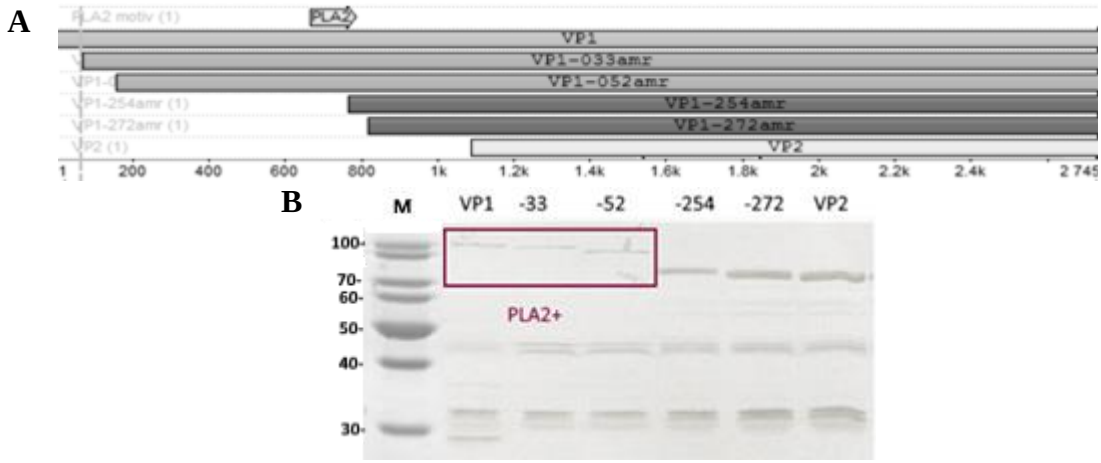


Fig. 3.13 Analysis of the influence of PLA2-like motif for the expression level of PARV4 VP1-VP2 proteins. (**A**) Schematic representation of constructed truncated PARV4 VP1 protein variants. Numbers under the ribbons represent the nucleotide position of PARV4 VP1 gene. The position of the PLA2-like motif is indicated in the first row. (**B**) WB of yeast cell lysates harbouring full and truncated variants of PARV4 VP1. Numbers above the picture show the number of amino acids removed from N-terminus of VP1. Boxed bands represent proteins that possess putative PLA2 domain (216-260 aa in PARV4 VP1). WB was performed using polyclonal mouse Ab against PARV4 VP2.

Analysis of the codon usage and CG content of VP1u region (Fig. 3.14) reveals that native sequence of PARV4 VP1, especially 400-1100 nt region, is abundant in rear yeast codons and have the highest GC content (40-65%) in the gene. These gene sequence features may be limiting factors of VP1 expression, as so-called

"translational ramp", a sequence of less used codons in N'-terminus of heterologous genes, was shown to reduce recombinant protein yields (Shah *et al.*, 2013).

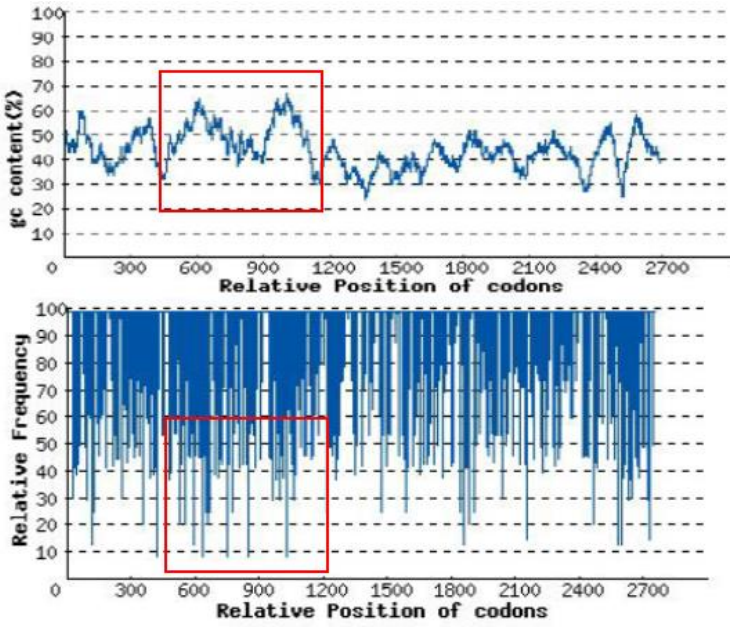


Fig. 3.14. CG content and codon usage (as the relative frequency of the codon usage in yeast *S. cerevisiae*) of PARV4 VP1. The region of high CG content and rare yeast codons (400-1100 nt) is boxed.

To test the structural ability to form VLPs, PARV4 VP1 truncated variant expressing yeast cell lysates were subjected to ultrafugation through a sucrose cushion and, subsequently, in CsCl gradient. Based on the SDS-PAGE analysis, fractions that contained PARV4 VP1 truncated variant proteins were analysed by electron microscopy. However, none of the four PARV4 VP1 protein truncated variants (-33, -52, -254, -272 aa) were observed to be assembled as VLPs (data not shown), showing that as few as 90 aa attached to VP2 protein at the N'-terminus (VP1 truncated variant -272) interfere with VLP formation, possibly due to steric hindrance. Ros *et al.*, (2006a) suggest that B19 VP1u domain is located internally near 5-fold axial channel (Fig. 1.4), and is exposed onto virion surface after thermal or pH stress. The stoichiometry of VP1:VP2 in native virions is 1:5 to 1:20 (Gurda *et al.*, 2010), suggesting that only one VP1u may be located near the 5-fold channel. VP1u of PARV4 is longer (362 aa) than that of other parvoviruses (200-250 aa), this raises doubts about possibility of such

exposing transition. Additionally, removal of 37 aa from PARV4 VP2 N'-terminus disrupts the ability of VLP forming in yeast (data not shown).

A similar approach was chosen to test the influence of PLA2-like motif (21-63 aa) in expression levels of HBoV1 VP1 in yeast. Three genes coding N'-truncated VP1 variants (-20, -40 and -90 aa) were amplified by PCR with specific primers (Table 2.1.2.) and subcloned into *Xba*I-digested pFX7 vector. HBoV1 VP1 truncated variant -90 lacks the PLA2-motif. Similarly to the results observed with PARV4 VP1, expression of truncated HBoV1 VP1 protein variant, lacking the PLA2 motif substantially increased the fraction of this protein in the yeast cell lysate (Fig. 3.15). Differently from PARV4, the PLA2-like activity of HBoV1 VP1u was demonstrated *in vitro* (Chiu *et al.*, 2014) and affects the membrane permeability of human airway epithelial cells. Previous study show that H41A or D42N point mutations in HBoV1 VP1 fully inhibit PLA2 activity *in vitro* (Qu *et al.*, 2008). Point mutation H41A was introduced into the native sequence of HBoV1 VP1 by site-directed mutagenesis (Methods section 2.3.2.) with primers hBo1_H41A_F and hBo1_R (Table 2.1.2). Nucleotide changes were confirmed by sequencing. However, analysing crude lysates of yeast cells harbouring altered VP1 gene in SDS-PAGE, no substantial change in VP1 expression level was observed (data not shown). This result support that the limitation of parvoviral VP1 synthesis in yeast may be primarily influenced by the unsuitable codon usage rather than the enzymatic action of PLA2 in the producer cell.

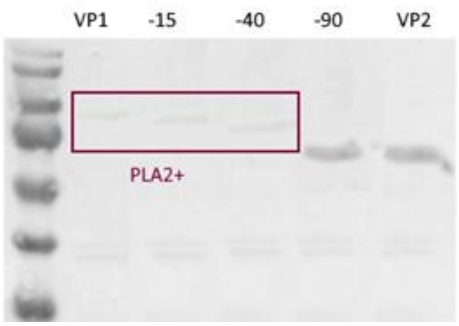


Fig. 3.15 Analysis of the influence of PLA2-like motif for the expression level of HBoV1 VP1-VP2 proteins in WB of yeast cell lysates harbouring full and truncated variants of HBoV1 VP1. Numbers above the picture indicates the number of amino acids removed from N-terminus of VP1. Boxed bands represent proteins that possess putative PLA2 domain (21-63 aa in HBoV1 VP1). WB was performed using MAb 19G7 against HBoV1-4 VP2 (section 3.2.3, table 3.8). PageRuler Prestained Protein Ladder (ThermoFisher Scientific Baltics) was used as a molecular mass standard in the first lane.

3.1.6. Generation of mosaic HBoV1 and PARV4 VP1-VP2 particles (mVLPs)

Due to the unsuccessful expression of HBoV1 and PARV4 VP1 proteins in yeast, its co-expression with VP2 protein was tried. A similar approach for human bocavirus capsid proteins was reported to be successful in insect cells (Fang *et al.*, 2014) and for parvovirus B19 in yeast (Chandramouli *et al.*, 2013). Yeast expression vector pFGG3 was used for a simultaneous co-expression of VP1 and VP2 proteins (section 3.1.1). Two constructs that differ only in swapped VP1 and VP2 positions under their respective promoters were created for each HBoV1 and PARV4 (Table 3.1) and transformed into yeast *S. cerevisiae* AH22.

Samples of yeast cultures harbouring HBoV1 VP1/VP2 constructs were taken and analysed 6 to 48 h after induction with galactose and analysed in SDS-PAGE and WB (Fig. 3.16). Two bands of both VP1 and VP2 proteins are observed in WB starting 6 hours after induction, and their intensity increases until 48 h after induction. Protein purification procedures were performed identically to those for VP2-formed VLPs described in section 2.3.13. As determined by SDS-PAGE, the VP1:VP2 ratio in CsCl gradient fractions of 1.31-1.33 g/mL density is approximately 1 to 15-20 which corresponds to other earlier observations for native parvovirus virions (Cotmore *et al.*, 1986). This ratio was similar in VLPs obtained from both pFGG3 constructs, regardless the observations that VP1 is

better expressed under the regulation of GAL7 promoter (Fig. 3.16 A). Recombinant VLPs of a very similar size and structure as native HBoV1 virions were observed by electron microscopy when analysing the fractions of HBoV1 VP1-VP2 proteins isolated in CsCl gradient (Fig. 3.18 A).

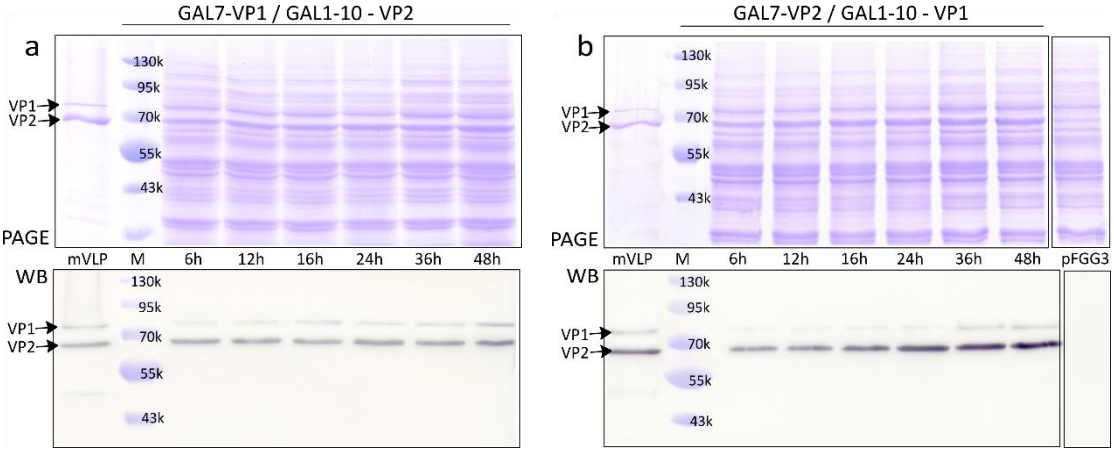


Fig. 3.16 Co-expression of HBoV1-VP1 and VP2 in *S. cerevisiae*. Time course samples of yeast cultures harbouring expression plasmid pFGG3_(HBoV1)_GAL7-VP1_GAL1-10-VP2 (**A**) or pFGG3_(HBoV1)_GAL7-VP2_GAL1-10-VP1 (**B**) were analysed by PAGE and Western Blot. First lanes (mVLP) – CsCl-gradient purified mosaic HBoV1 VP1-VP2 mVPLs, lanes M – PageRuler Prestained Protein Ladder (ThermoFisher Scientific Baltics). Samples of cultures were taken and analysed 6 to 48 hours after induction with galactose. Lanes pFGG3 – lysate of yeast culture harbouring pFGG3 plasmid, 16 hrs after induction. Positions of VP1 and VP2 protein bands are indicated by arrows.

Similarly, two protein bands (~105 and ~62 kDa) were observed in the SDS-PAGE of crude cell lysate of yeast cells harbouring pFGG3_(PARV4)_GAL7-VP1_GAL1-10-VP2 vector (Fig. 3.17) that were absent in a control sample of yeast harbouring pFGG3 vector only. VLP purification procedures identical to the above mentioned were performed, except buffers were changed to 50mM Tris (pH 8.7), supplemented with 2 mM EDTA for better stability of PARV4 VP proteins, as described in section 3.1.2. Analysis of ~1.32 g/mL CsCl fraction in SDS-PAGE, revealed that both above-mentioned protein bands retained in the same fraction (Fig. 3.17, lane 4). Thus, results suggest that this fraction is composed of mosaic VLPs, not of two different type particles (VP2-VLPs and VP1-VLPs). The typical icosahedral particles characteristic to parvoviruses were observed by electron microscopy (Fig. 3.18 B).

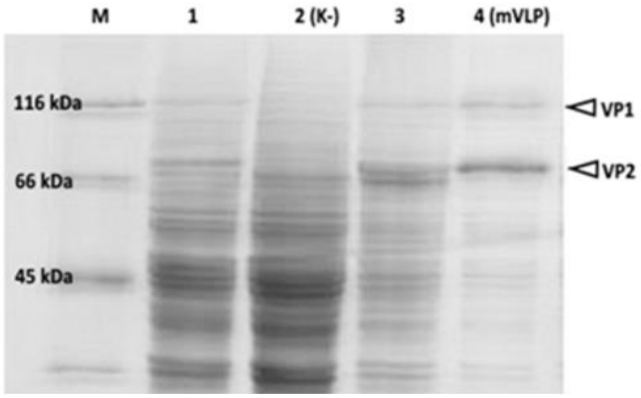


Fig. 3.17 Co-expression of PARV4 VP1 and VP2 in *S. cerevisiae*. PAGE analysis of cell lysates harbouring expression plasmid pFGG3_(PARV4)_ GAL7-VP1_GAL1-10-VP2 (lane 1) and pFGG3 (lane 2). Lane 3 – lysate after centrifugation through a sucrose cushion, lane 4 – CsCl fraction with two bands of mVLP composing proteins. M = Unstained Protein MW Marker, (ThermoFisher Scientific Baltics).

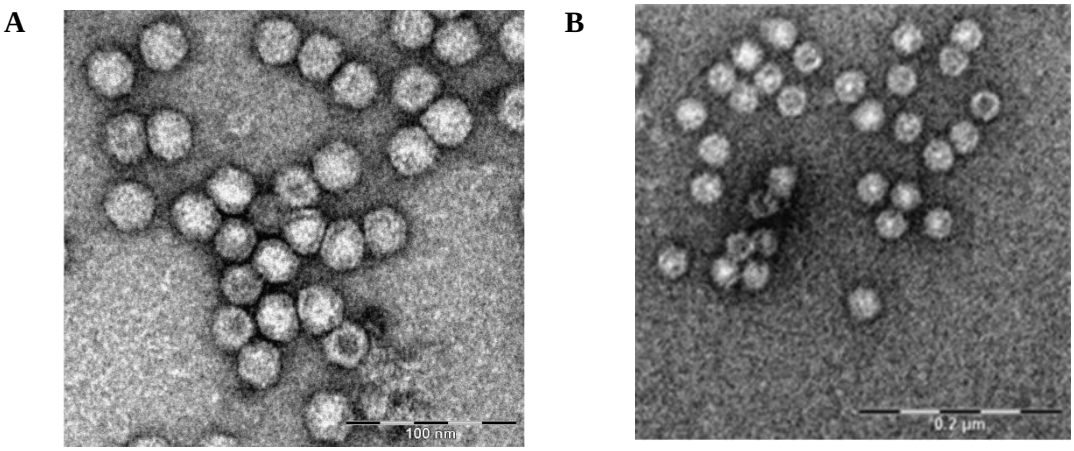


Fig. 3.18 Electron micrograph of recombinant HBoV1 VP1–VP2 mVLPs (**A**) (scale bar = 100 nm) and PARV4 VP1-VP2 mVLPs (**B**) (scale bar = 200 nm) in CsCl fractions

An approach to express a full-length HBoV1 and PARV4 VP1 proteins alone in yeast was not successful (section 3.1.5.). The comparatively better yield of HBoV1 VP1 was obtained when both structural proteins (VP1 and VP2) were co-expressed, suggesting that substantial fraction of the synthesised VP1 is incorporated into soluble VLPs and hence its toxicity for yeast cells is reduced. However, as shown in Fig. 3.16, GAL7 promoter slightly enhanced HBoV1 VP1 expression in yeast, although the fraction of the minor structural protein within a formed recombinant mosaic VLPs remained similar. Fifteen-to-one VP2:VP1 ratio in yeast-generated HBoV1 mVLPs is similar to that in mVLPs produced using the baculovirus system (Fang *et al.*, 2014) and resembles the structure of native parvoviral virion (Ozawa and Young, 1987). However, for parvovirus

B19, manipulation of the multiplicity of infection of the recombinant insect virus led to production of stable mosaic VLPs, eliciting high levels of virus neutralizing activity, that contained up to 41% of VP1 protein (Bansal *et al.,* 1993), although HBoV VP1u domain is 98 aa shorter than that of B19. Generation of HBoV mVLPs in baculovirus expression system from a single reading frame revealed that the third intermediate size (68 kDa) protein was incorporated in the mVLPs (Cecchini *et al.,* 2009). Electron micrograph (Fig. 3.18 B) of PARV4 mVLPs reveal icosahedral particles of 25-28 nm in diameter, that is similar to that of VP2-VLPs. This observation suggests that bulky VP1u domain of PARV4 may be hidden within the particle as it was shown in recombinant B19 mVLPs (Ros *et al.,* 2006b). It, however, opens discussion how this domain, responsible for escaping from the late endosome in other parvoviruses, is displayed onto the surface of the particle without disruption of the structure. However, taking into consideration unusually large PARV4 VP1u domain (362 aa vs. 100-230 aa in other parvoviruses) and data that PARV4 VP1u lacks PLA2 activity *in vitro* (Lou *et al.,* 2012a), this domain may play a different role in PARV4 infection and may not be participating in virus entry.

Purified mVLPs of HBoV1 and PARV4 were further used in antigenicity study (section 3.2.8). Proteins were stored at −20 ◦C in PBS containing 2 mM EDTA and 40% glycerol (HBoV1 mVPLs) or 50 mM Tris (pH 8.7), supplemented with 2 mM EDTA and 40% glycerol (PARV4 mVLPs). No significant degradation signs were observed in SDS-PAGE after 3 months.

In summary, *S. cerevisiae* expression system allowed us to generate and purify stable virus-like particles composed of major capsid protein VP2 of newly discovered human parvoviruses HBoV1-4 and PARV4, as well as economically significant porcine parvovirus (strain NADL-2). Attempts to synthesise the minor structural protein, VP1, in yeast were unsuccessful, despite the elimination of putative PLA2-motiff of HBoV1 and PARV4 VP1. However, VP1 of HBoV1 and PARV4 were successfully incorporated into stable mosaic VLPs when co-expressed with VP2 proteins in yeast cells.

3.2. Antigenic properties of recombinant parvovirus VP proteins synthesised in yeast.

3.2.1. The reactivity of recombinant PARV4 VP2 VLPs with serum antibodies from low-risk group individuals

Recombinant PARV4 VP2 VLPs were employed to develop EIA tests for the detection of PARV4 specific IgM and IgG antibodies in human serum samples. Since commercial serologic test systems for PARV4 infection are still not available, it was not possible to identify anti-PARV4 positive and negative sera. The PCR analysis of serum specimens with earlier published specific primers (Fryer, 2006) was unable to confirm PARV4 infection because PARV4 DNA was not detectable in the sera (data not shown). It meant that the cut-off values for the serologic assay were set conservatively as the mean absorbance +3 standard deviations and used to assign samples as anti-PARV4 positive or negative (Sharp *et al.,* 2009). The cut-off values for the positive sera were determined using the mean OD value of the negative controls (n=144) plus 3 standard deviations ($\bar{x}$ +3SD). Sera with an OD between $\bar{x}$+2SD and $\bar{x}$+3SD were regarded as equivocal (Jacobson, 1998). In this study, the cut-off OD values for positive sera were 0.411 and 0.597 for IgG and IgM, respectively. Equivocal sera for IgG had OD values between 0.290 and 0.411 while for IgM, the ODs were between 0.415 and 0.597. Human serum samples (section 2.1.5.) were analysed in optimised indirect immunoassay (section 2.3.19). Briefly, the yeast-generated PARV4 VP2 antigen was absorbed in 96-wells plate during the overnight incubation. Subsequently, human serum samples were added, and viral antigen-recognising human IgG and IgM antibodies were detected with secondary HRP-conjugated antibodies.

Analysis of the 170 serum specimens isolated from patients with acute respiratory disease (Table 3.3) was performed. Results revealed that 5.8% (n=10) sera were IgG positive and 7% (n=12) sera were IgM positive. PARV4 seroprevalence may be grouped into three groups: 4 patients with serological evidence of past infection (IgM-, IgG+), 6 patients with possible current infection (IgM+, IgG- or equivocal), and 6 patients with ongoing or recent infection (IgM+, IgG+). Among

16 seropositive individuals, 7 were children between the ages of 3 and 11. Results of the VP2-based EIA test are presented in Table 3.4.

Table 3.3. Demographic characteristics of PARV4 study subjects.

	Number of samples	**Mean age (range)** (years)	**Number of EIA-positive samples**
Female	78	21.1 (0.5 – 63)	2
Male	92	20.0 (0.5 – 60)	14
Total	170	20.5 (0.5 – 63)	16

To confirm the results obtained by EIA, an immunoblot analysis of all PARV4 VP2-specific IgM- and IgG-positive sera was carried out. Ten PARV4 IgG-positive and 12 PARV4 IgM-positive serum specimens were analysed. Through immunoblotting, 4 of the ten analysed EIA IgG-positive serum specimens showed a specific positive IgG reactivity with PARV4 VP2 protein while 3 of the 12 analysed EIA IgM-positive serum specimens showed VP2-specific positive IgM reactivity (Fig. 3.19, Table 3.4.). Two of all the analysed sera were positive for both IgG and IgM antibodies specific to PARV4 VP2 protein. The results of both EIA and immunoblot analysis for the human serum specimens are summarised in Table 3.4.

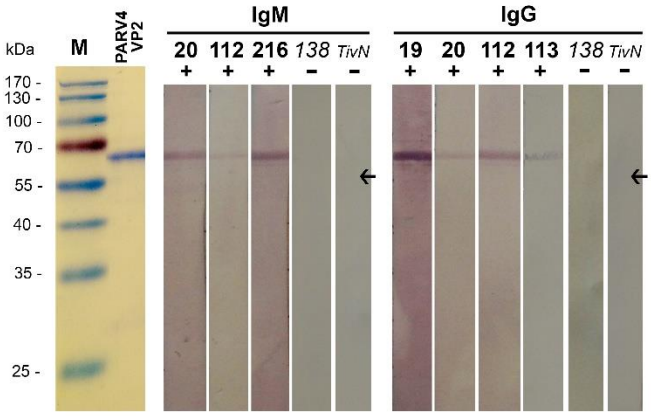


Fig. 3.19 Immunoblot analysis of anti-PARV4-positive human sera. Left: stained SDS-PAGE gel (M – PageRuler Prestained Protein Marker (ThermoFisher Scientific Baltics). Right: Western blot strips of positive sera indicated by the identification number and secondary antibody used. Strips with serum No. 118 were provided to act as a negative control. Negative control antigen Tioman NC (~ 58 kDa) showed no reactivity in Western blotting when used with positive serum No. 20 (arrows indicate the position of blotted antigen).

Table 3.4. PARV4 enzyme immunoassay and immunoblot results. Only values that are interpreted as seropositive are shown in the table.

Serum No	EIA, OD		Immunoblot		Age (years)	Gender
	IgM	***IgG***	***IgM***	***IgG***		
19	0.618	**0.654**		+	25	M
20	**1.014**	**0.687**	+	+	46	M
23	0.619				47	M
31		0.494			23	M
43	0.786				26	M
84		0.445			15	M
85		0.455			16	M
86	0.603				5	F
112	**1.213**	**0.728**	+	+	6	M
113	0.636	**0.453**		+	10	M
116	0.803				10	M
117	0.742				14	M
127	0.628				3	M
138	0.639	0.435			9	F
209		0.419			19	M
216	**0.790**	0.498	+		11	M

Abbreviations: M, male; F, female.

Immunoblot analysis of the same sera confirmed the presence of PARV4 VP2-specific IgM in 4 serum specimens and the presence of PARV4 VP2-specific IgG in 3 serum samples. Two of the immunoblot-positive sera contained both IgM and IgG antibodies specific to PARV4 VP2 protein. Chen *et al.,* (2015) suggests, that IgM antibodies against PARV4 can remain detectable for 12 months or longer, and the high IgM-positive rates are explainable by a prolonged IgM response, but PARV4 DNA might not have been detected in IgM-positive blood samples without conducting PCR replicates. Another study showed the detectable levels of anti-PARV4 IgM after 21 months (Yang *et al.,* 2011).

The results obtained by EIA and Western blotting might be different due to the differences in antigen structures. It is because in EIA native antigens were immobilised onto microtiter plates whereas in the Western blot assays antigens that had been denatured with SDS were used. As the majority of serum antibodies induced by a natural parvoviral; infection target conformational epitopes (Söderlund *et al.,* 1995), only a part of serum antibodies would recognise the

SDS-denatured viral antigens. This would result in a reduction in the sensitivity of the Western blotting assay in comparison to EIA, observed in this study. However, a recent longitudinal study by Chen *et al.,* (2015) demonstrated, that in contrast to the B19 virus, the antibodies against the linear epitopes of PARV4 VP2 did not disappear over time.

Our study is the first attempt to evaluate the seropositivity for PARV4 in low-risk Lithuanian patients. Previous studies indicate that the epidemiologic situation with regards to PARV4 infections may differ greatly from country to country (for reference, see Tables 1.6 and 1.7). These dissimilarities could be related to geographical or social differences, however, no major pattern of virus spread among low-risk cohort has been characterised. We have demonstrated that the seroprevalence of PARV4-specific IgG and IgM antibodies (5.8% and 7%, respectively) in a group of patients with acute respiratory disease was higher when compared to the results of low-risk patient's cohorts obtained from other European countries (UK, France, Finland, (Table 1.6)). However, incidence rate in Sub-Saharan and Central Africa was detected as high as 37% (Sharp *et al.,* 2010). It is important to note that among 16 seropositive individuals, 7 were children between the ages of 3 and 11. These results raised doubts in the proposed theory that PARV4 virus uses a parenteral transmission path which was postulated after investigations of intravenous drug users and HIV-infected individuals (Sharp *et al.,* 2009). In our study, all analysed patients were low-risk individuals for PARV4 infection as they had no medical records of parenteral exposure. The absence of parenteral risks raises the possibility of alternative routes of transmission that might affect the general non-parenterally exposed population. It would explain why more cases of PARV4 infected children have been recently reported in Ghana. Proving non-parenteral transmission would suggest the more widespread distribution of PARV4 in humans than previously expected and occurrence of virus in additional population subsets (Panning *et al.,* 2010).

In summary, the results of the analysis of human serum specimens by VP2-based immunoassays confirmed that yeast-derived VLPs represent a useful diagnostic tool for studying the seroprevalence of PARV4 infection.

3.2.2. Localisation of B-cell linear epitopes of PARV4 VP2

Localisation of epitopes that are recognised by the immune system is a valuable tool to model multivalent vaccines, to evaluate the antigenicity of the protein as well as to give structural insights. A collection of 28 monoclonal antibodies (Table 3.7) was generated by a subcutaneous injection of yeast-generated PARV4 VP2 VLPs in mice, as described by Köhler and Milstein (1975). This work was done by the Department of Cell Biology and Immunology (head Dr. A. Žvirbliene) by Dr. I. Kučinskaite-Kodzė. Only one of the 28 PARV4 VP2 recognising MAbs (16E12) target the conformational epitope, as no reaction with SDS-denatured antigen was observed in WB (data not shown). Thus, epitope mapping was performed in a three-stage process, targeting linear epitopes of the remaining 27 Mabs.

Firstly, PARV4 VP2 sequence was submitted for analysis to BepiPred B cell epitope prediction on-line tool (Larsen *et al.*, 2006) and resulting 7 locations of proposed high antigenicity (Fig. 3.20, A) were targeted by constructing the truncated PARV4 VP2 variants. Seven sequences of different length, spanning 37-552, 61-552, 242-552, 320-552, 37-490, 61-420, and 129-525 aa of PARV4 VP2 protein were amplified by PCR using specific primers listed in Table 2.2. The amplified fragments were framed with *XmaJ*I recognition sites introduced by the primers. Thus, *XmaJ*I digested PCR products were cloned into *Xba*I hydrolyzed yeast expression vector pFX7-NHis (Razanskiene *et al.*, 2004). The resulting plasmids code the PARV4 VP2 fragments, fused with six histidine tag at the N'-terminus under the galactose-inducible GAL10-PYK1 promoter. After transformation into *S. cerevisiae* gcn2 strain and induction of recombinant protein synthesis, seven histidine-tagged proteins were purified by Ni^{2+}-affinity chromatography under denaturating conditions (Fig. 3.21). The purified PARV4 VP2 fragments were employed in WB to determine the reaction with each of 27 MAbs. Results of the testing are summarised in Table 3.5.

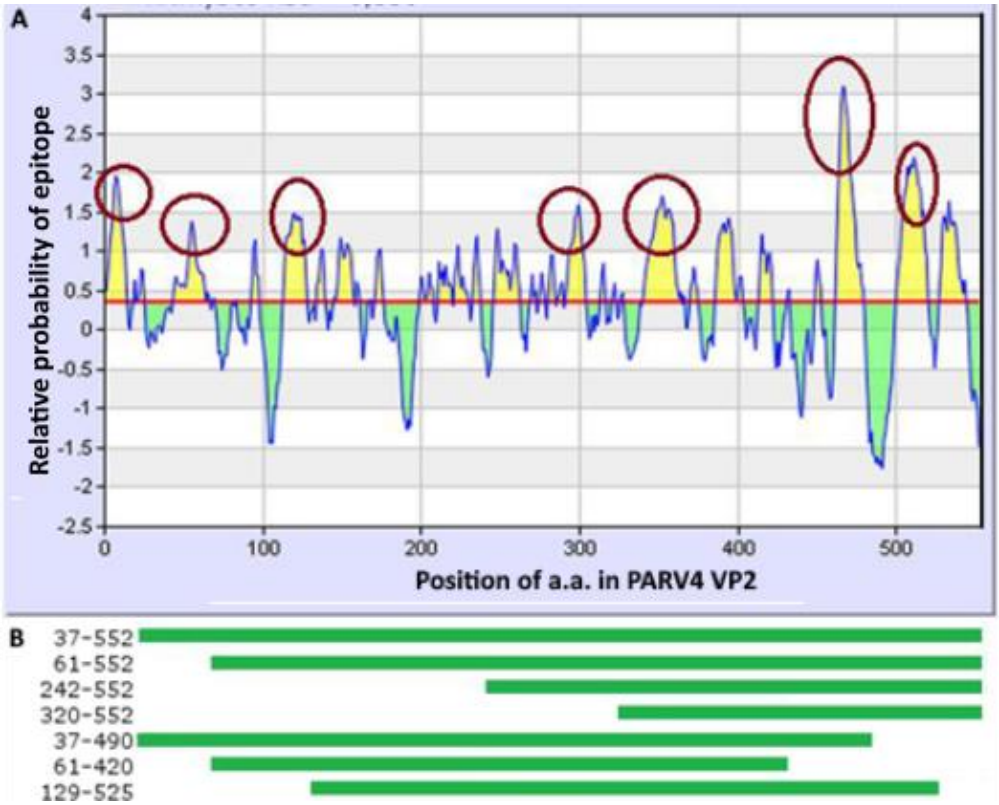


Fig. 3.20. PARV4 VP2 epitope localisation strategy. (**A**) PARV4 VP4 epitope location prediction with BepiPred on-line tool. Protein sequence locations with highest predicted antigenicity is indicated with peaks of the relative probability curve. (**B**) Schematic representation of PARV4 VP2 fragments constructed for epitope localisation. The numbers on the left indicate the amino acids included in the corresponding fragment.

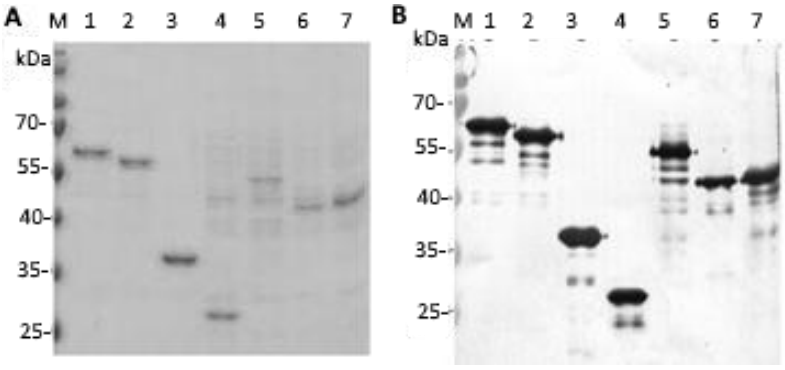


Fig. 3.21. Purification of PARV4 VP2 fragments and their reactivity with polyclonal antibodies. (A) SDS-PAGE of Ni^{2+}-affinity purified PARV4 VP2 fragments. (B) WB of purified PARV4 VP2 fragments with polyclonal mouse anti-PARV4 VP2 antibodies. PARV4 VP2 fragments are indicated with numbers above the corresponding lanes: 1: 37-552 aa, 2: 61-552 aa, 3: 242-552 aa, 4: 320-552 aa, 5: 37-490 aa, 6: 61-420 aa, 7: 129-525 aa, M: PegeRuler Prestained Protein Marker (ThermoFisher Scientific Baltics).

Table 3.5. Reactivity of 27 MAbs raised against PARV4 VP2 with seven truncated variants of the antigen in WB.

MAb	MAb reactivity with PARV4 VP2 truncated variants:							Predicted epitope location (aa)
	37-552	61-552	242-552	320-552	37-490	61-420	129-525	
7F8, 13H11	+	+	+	+	+	+	+	320-420
12F8, 8G8, 1G4, 6E6, 7C5, 11F8, 16H9, 19H9, 3D10, 11H3, 12D6	+	+	-	-	+	+	+	129-242
4A7, 7C7, 8A2, 10C9, 10D1, 12D1, 12F10, 15G7	+	+	+	+	-	-	+	490-525
18E3, 17G2, 17G9, 2G8, 5A6, 12E8	+	+	+	-	+	+	+	242-320

+, positive reaction; -, negative reaction

According to the results, four locations of the epitopes were determined. Eleven MAbs recognise 129-242 aa region, 6 MAbs bind epitopes located in 242-320 aa region, and 7 MAbs interacted with a sequence located in the C'-terminus (490-525 aa) of PARV4 VP2. Additionally, two MAbs recognised 320-420 aa region. No possible epitope locations were determined in neither the N'-terminus (1-129 aa), nor in 420-490 and 525-552 aa regions, resulting the cluster of recognition sequences in 129-420 aa region (19 MAbs). Therefore, this region was targeted in further fragment comminution.

Further, eleven 45 aa PARV4 VP2 fragments of 120-460 aa region (Fig. 3.22, A) were generated by PCR, introducing the *Bgl*II recognition sites at the ends of the amplified DNA sequences. *Bgl*II digestion products were cloned into pET-43.1a(+) (Novagen, Darmstadt, Germany) bacterial expression vector to produce protein fragments fused with Nus-Tag™. This 54,8 kDa protein is soluble and efficiently produced in *E. coli,* allowing high yields of histidine-tagged chimeric proteins (Fig. 3.22, B).

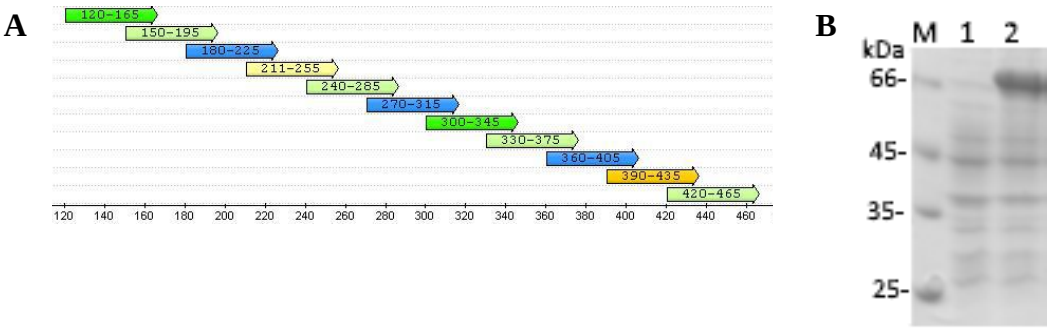


Fig. 3.22 Synthesis of 45 aa PARV4 VP2 fragments for epitope localisation. (**A**) Schematic representation of 11 PARV4 VP2 fragments used to generate NusA-fused proteins. Numbers indicate the amino acids included in the corresponding fragment. (**B**) SDS-PAGE of crude lysates of *E. coli* harbouring pET43.1a(+)-PARV4_VP2(120-165) plasmid before (lane 1) and 3 h after induction (lane 2). M = Unstained Protein MW Marker, (ThermoFisher Scientific Baltics).

The interaction between MAb and the NusA-fused fragment of PARV4 VP2 was tested in 96-well plates by indirect EIA format. The wells were initially covered with the NusA-fused chimeric proteins purified by Ni^{2+}-affinity chromatography under denaturating conditions to expose the inserted PARV4 VP2 sequence. Then, MAbs were added, and the interaction with the chimeric NusA protein was visualised by the colour reaction (Abs(495nm)>1) after HRP conjugated secondary goat anti-mouse IgG antibodies (Biorad, USA) had been added. Two main epitope clusters were identified at this stage: 120 - 165 aa (9 MAbs) and 240 – 285 aa (6 MAbs). Out of the remaining four MAbs, two (12F8 and 8G8) target 150-195 aa, 13H11 - 330-375 aa, and 7F8 - 390-435 aa regions.

The overlapping 15 aa fragments of three PARV4 VP2 regions (120-158 aa (4 fragments), 248–278 aa (3 fragments), and 492-525 aa (4 fragments) (Table 3.6) were generated by hybridization of two complementary primers. Also, the protruding ends, complementary to *Eco*RI and *Sal*I digested pET-43.1a(+) protruding ends were formed after hybridization of these primers. This ensured that only one insert with the desired direction is required for recircularization of the linearized vector, and thus, successful cloning.

As described earlier, the resulting ten pET-43.1a(+) constructs were employed in chimeric NusA protein production, those, after the Ni^{2+}-affinity chromatography purification, were used in EIA for detecting the interaction with the MAbs. The results of this stage are summarised in Table 3.6 and show that there are three

main B-cell linear epitope locations in PARV4 VP2: 144-158 aa (GHNQDTLPGHLPGEN, binds 9 MAbs), 248-262 aa (NDGTAVWKRPEGMDV, binds 6 MAbs), and 507-513 (EKPAPFP, binds 8 MAbs). The latter epitope was narrowed up to 7 aa, as both overlapping fragments (NusA proteins with inserts of 504-513 or 507-521 aa of PARV4 VP2) show reaction of similar magnitude, meaning that amino acids, shared by both fragments (507-513 aa) are responsible for the interaction.

Table 3.6 Summary of NusA-fused 15 aa PARV4 VP2 protein fragments with MAbs.

MAb	**MAb reactivity with PARV4 VP2 fragments (aa), fused with NusA:**				**Predicted epitope location (aa)**
	121-135	128-142	136-150	144-158	
1G4, 6E6, 7C5, 11F8, 16H9, 19H9, 3D10, 11H3, 12D6	-	-	-	+	**144-158**
	492-506	504-513	507-521	516-525	
4A7, 7C7, 8A2, 10C9, 10D1, 12D1, 12F10, 15G7	-	+	+	-	**507-513**
	248-262	257-270	264-278		
18E3, 17G2, 17G9, 2G8, 5A6, 12E8	+	-	-		**248-262**

+, positive reaction (OD_{450}>1); -, negative reaction

Ten overlapping synthetic peptides were ordered to pin-point the epitopes located in 144-158 aa and 248-262 aa regions, five for each of two regions (Fig. 3.23). The peptides were not tagged with any of the anchors for effective primary absorption onto the surface of 96-well surface, therefore the competition EIA format was used. Firstly, the purified NusA proteins with inserts of 144-158 of 248-262 aa of PARV4 VP2 were absorbed in the wells overnight. Then, the MAbs were incubated with 500 ng of each of the peptide, and the resulting mixture was applied to the wells. If there were no interaction of MAb with the peptide, it would bind the absorbed chimeric NusA construct. The MAbs which would fully have been inhibited by the peptides in the pre-incubation mixture, would be washed out and no reaction with the HRP conjugated secondary goat anti-mouse IgG antibodies would be observed. The results of this assay are

summarised in Fig 3.23, showing the full MAb inhibition with NQDTLPGHLP and NDGTAIWKRP peptides.

	12D6	19H9	1G4	16H9	3D10	11F1	11H3	6E6	7C5	12E8	5A6	No MAb
GHNQDTLPGH	1,7897	1,8179	1,8014	1,5495	1,5539	1,7307	1,7758	1,4875	1,5915	0,019	0,022	0,0148
NQDTLPGHLP	0,022	0,0282	0,0233	0,0186	0,0181	0,017	0,0227	0,0232	0,0217	0,0153	0,0144	0,0189
DTLPGHLPGE	0,8121	1,0539	0,9555	0,6535	0,5368	0,7134	0,8146	0,8738	0,6704	0,015	0,0141	0,014
LPGHLPGEN	1,6788	1,6306	1,4655	1,1473	1,3218	1,4065	1,5992	1,5091	1,4589	0,0183	0,0135	0,0148
GHNQDTLP	1,8963	1,4785	1,5688	1,3498	1,5509	1,4093	1,5089	1,2912	1,3955	0,017	0,0164	0,0114
No peptide	1,1959	1,0847	1,0175	0,8574	1,0337	0,8205	0,8013	0,9371	0,7672	0,0267	0,0168	0,0232

	12E8	5A6	18E3	17G2	17G9	2G8	4A7	10C9	7C7	12D6	19H9	No MAb
NDGTAIWKRP	0,042	0,0722	0,0395	0,0408	0,0711	0,041	0,0209	0,0248	0,0232	0,0208	0,0213	0,0178
GTAIWKRPEG	0,3821	0,2882	0,6396	0,5453	0,3947	0,5056	0,018	0,0209	0,0197	0,0194	0,0174	0,0174
AIWKRPEGMD	0,2122	0,2343	0,7636	0,7236	0,4152	0,7771	0,0174	0,0165	0,0172	0,0163	0,0152	0,0154
WKRPEGMDV	1,1304	1,0815	0,9935	0,9526	1,2146	0,9376	0,0172	0,0149	0,0168	0,0174	0,0155	0,0147
NDGTAIWK	1,1828	1,0111	0,9463	0,8518	1,1352	0,7811	0,0147	0,0159	0,0161	0,0142	0,014	0,0131
No peptide	0,8203	0,6898	0,6836	0,6271	0,7706	0,6421	0,0139	0,0153	0,0178	0,0165	0,0167	0,0172

Fig. 3.23. Results of competition EIA with synthetic peptides. Sequences of the peptides are indicated on the left. A positive reaction is indicated by OD(450)>0.5 and illustrates that the peptide did not bind the corresponding MAb and thus, the MAb could bind the adsorbed antigen.

The full list of all 28 MAbs against PARV4 VP2 is provided in Table 3.7. Interestingly, all nine 146-155 aa epitope recognising MAbs are of IgG2a isotype, whereas other epitopes resulted in a mixture of IgG2a and IgG1 isotypes. Stimulation of IgG2a antibodies has been associated with increased efficacy of influenza vaccination (Arulanandam *et al.*, 1999; Huber *et al.*, 2001; John & Crispe, 2004). Additionally, monoclonal antibodies of the IgG2a isotype are more efficient at clearing influenza (Gerhard *et al.*, 1997; Mozdzanowska *et al.*, 1997), Ebola (Wilson *et al.*, 2000), and yellow fever (Schlesinger & Chapman, 1995) virus infections than monoclonal antibodies of the IgG1 isotype displaying similar antigenic specificities.

Table 3.7. Properties of monoclonal antibodies raised against PARV4 VP2 antigens.

Clone	Isotype*	Reactivity in WB*	Epitope location (aa), or epitope sequence
7F8	IgG2a	+	390-420
13H11	IgG1	+	330-375
12F8	IgG2a	+	150-195
8G8	IgG2a	+	150-195
4A7	IgG2a	+	507-513: EKPAPFP
7C7	IgG1	+	507-513: EKPAPFP
8A2	IgG2a	+	507-513: EKPAPFP
10C9	IgG2a	+	507-513: EKPAPFP
10D1	IgG2a	+	507-513: EKPAPFP
12D1	IgG1	+	507-513: EKPAPFP
12F10	IgG2a	+	507-513: EKPAPFP
15G7	IgG1	+	507-513: EKPAPFP
18E3	IgG1	+	248-257: NDGTAIWKRP
17G2	IgG1	+	248-257: NDGTAIWKRP
17G9	IgG2a	+	248-257: NDGTAIWKRP
2G8	IgG1	+	248-257: NDGTAIWKRP
5A6	IgG2a	+	248-257: NDGTAIWKRP
12E8	IgG2a	+	248-257: NDGTAIWKRP
1G4	IgG2a	+	146-155: NQDTLPGHLP
6E6	IgG2a	+	146-155: NQDTLPGHLP
7C5	IgG2a	+	146-155: NQDTLPGHLP
11F8	IgG2a	+	146-155: NQDTLPGHLP
16H9	IgG2a	+	146-155: NQDTLPGHLP
19H9	IgG2a	+	146-155: NQDTLPGHLP
3D10	IgG2a	+	146-155: NQDTLPGHLP
11H3	IgG2a	+	146-155: NQDTLPGHLP
12D6	IgG2a	+	146-155: NQDTLPGHLP
16E12	IgG2a	-	*conformational epitope*

** Isotyping and WB were performed by the Department of Cell Biology and Immunology (I. Kučinskaite-Kodze).*

The location of three localised epitopes, namely NQDTLPGHLP, NDGTAIWKRP and EKPAPFP, were compared with those in human parvovirus B19 and HBoV1 VP2 proteins (Tolfvenstam *et al.*, 2000; Zhou *et al.*, 2014) (Fig. 3.24 A). The epitopes pin-pointed in this study are localised in regions that coincide with those in B19 and HBoV1, showing the conservative antigenic parts of the parvoviral capsid. Based on the alignment onto the resolved structure of

B19 VP2 (PDB accession number 1S58, (Kaufmann *et al.*, 2004)), the localised epitopes of PARV4 VP2 are localised in tree surface accessible loops: EF and HI that connect conservative β-strands and C-terminal loop (Fig. 3.24 B and 1.4 B). The amino acid changes in these flexible loops are likely to cause minute influence on the overall structure of VLP, thus, the pin-pointed epitope locations further may be tested for the ability to carry the heterologous epitopes using chimeric PARV4 VP2 VLPs as epitope carrier.

A

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PARV4_VP2 143  LGHNQDTLPGHLPGENYVLPQYGYITRGREIDQQ--NSI-----VAISDHKTELFFLEHH
B19_VP2   163  LGQGQDTLAPELPIWVYFPPQYAYLSVGDVNTQGITGDS-----KKLASEESAFYVLEHS
BoVI_VP2  169  SHPWDEDVMPDLPYKTWKLFQYGYIPIENELADLDGNAAGGNATEKALLYQMPFFLLENS
                   :: :  .**   :   **.*:       :                 :  ::.**.

PARV4_VP2 196  DAECLGTGDHWSHHYEFPDDLPWRKLSTPNQTLYARHNPIPSSRLAIMTGVDNDGTAVWK
B19_VP2   218  SFQLLGTGGSASMSYKFPAVPP-ENLEGCSQHFYEMYNPLYGSRLGVPDTLGGDP--KFR
BoVI_VP2  229  DHQVLRTGESTEFTFNFD--CEWVNNERAYIPPGLMFNPKVPTRRVQYIRQNGSTA---A
               . : * **   .  ::*       : .         .**   :*        .

PARV4_VP2 256  RPEGMDVGRLPLNYVPGPALMMPTDTQIRNTTFRDP-VAIGNPATSDRYSVAPLVHQPWS
B19_VP2   275  SLTHEDHAIQPQNFMPRPLVNSVSTNEGDSSNTGAGKALTGLRKGTSQNTRISLRPGPVS
BoVI_VP2  284  STGRIQPYSKPTSWMTGPGLLSAQRVGPQSSDT-APFMVCTNPEGTHINTGAAGFGSGFD
                     :    * .::  * :         .:              :   :         .

PARV4_VP2 315  VRTEEWLANKTDYSVHNYLGGVAYTRRKHEESYD----KHEEDRDG-RVSNPSRV-VQID
B19_VP2   335  QPYHHWDTD-------KYVTGINAISHG-QTTYGNAEDKEYQQGVG-RFPNEKEQLKQLQ
BoVI_VP2  343  PPSGCLAPTNLEYKLQWYQTP-----------EG-------TGNNGNIIANPSLSM--LR
                            *                          *  . * .     :

PARV4_VP2 369  GDLAAPHVGHTFFVPGHTRVTSGGTDTVYSPKLYQEPVFPLFPGAVWNPNPLSYDCQIWT
B19_VP2   386  GL-----NMHTYFPN-------KGT-----QQYTDQIERPLMVGSVWNRRALHYESQLWS
BoVI_VP2  383  D-----------------QLLYKGNQTT----YNLVGDIWMFPNQVWDRFPITRENPIWC
                                     *.                ::   **:   :  :  :*

PARV4_VP2 429  KIPNTECHFFAQYPLLGGWGVMTPPPMIFVKLRSQPGPPSPGAHTVPQSNLNQYAIFHLH
B19_VP2   429  KIPNLDDSFKTQFAALGGWGLHQPPPQIFLKILPQSGPIG-GIKSMGITTLVQYAVGIMT
BoVI_VP2  422  KKPRADKHTIM-DPFDGSIAMDHPPGTIFIKMAKIPVPTATNA----DSYLNIYCTGQVS
               * *. :          *. .:  **  **:*:     * .        : *  *.   :

PARV4_VP2 489  YSMQFLVKRRKRSRRHNPEKPAPFPTTD-SGRMPFTLANSLQ----DPSTPVYEVPSDQW
B19_VP2   488  VTMTIKLGPRKATGRWNP-QPGVYPPHA-AGHLPYGLYDPTATDAKQHHRHGYEKPEELW
BoVI_VP2  477  CEIVWEVE-RYATKNWRPERRHTALGMSLGGESNYTPTYH-----VD-PTGAYIQPTSYD
                 :   :  *  : . .* :          .*.  :           :     *  * .
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B

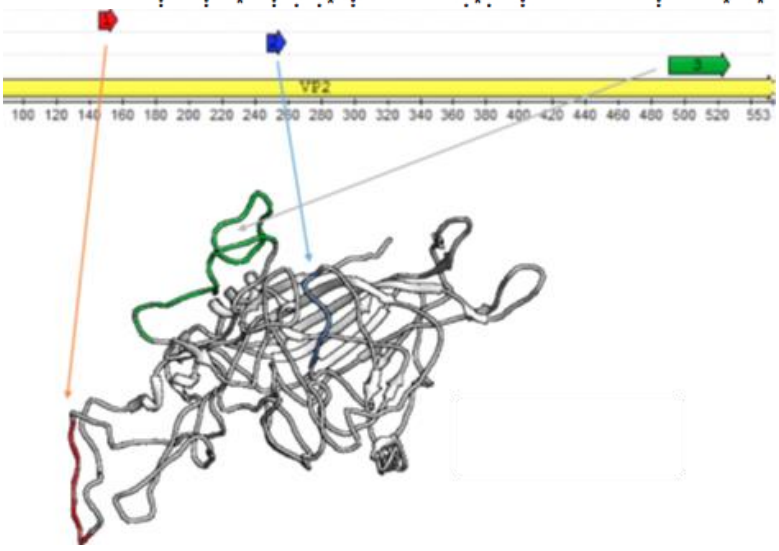

Fig. 3.24 PARV4 VP2 epitopes determined in this study. (**A**) Partial alignment of PARV4, HBoV1 and B19 VP2 sequences. The determined epitopes are underlined in black (PARV4 VP2, this study), purple (B19 VP2), and cyan (HBoV1 VP2). Alignment was performed with Clustal O (Sievers *et al.*, 2011) (**B**) Three major PARV4 VP2 epitope locations determined in this study indicated in the VP2 structural model of parvovirus B19 (PDB accession number 1S58). The picture was produced using Chimera v1.10.1 software.

3.2.3. Antigenic properties of HBoV1-4 VP2 proteins

Purified HBoV1-4 VP2 VLPs (section 3.1.3.) were used to immunise 8-week-old female BALB/c mice to produce polyclonal antisera and monoclonal antibodies using hybridoma technology. This work was done by the Department of Cell Biology and Immunology (head Dr A. Žvirblienė) following the protocols essentially as described by Köhler and Milstein (1975).

Polyclonal mouse antisera produced against recombinant purified VP2 VLPs of single HBoV species were tested for the cross-reactivity in EIA with VP2 proteins of HBoV1–4 and other parvoviruses by limiting dilution analysis (Fig. 3.25). Serum specimens from the pre-immune mice were used as a negative control. As expected, all antisera of the immunised mice strongly reacted with the respective homologous antigens. However, all four antisera showed 10-20-fold lower reactivity with heterologous HBoV VP2 proteins. The HBoV VP2-specific mouse antisera showed a modest reactivity with yeast expressed PARV4 and B19 VLPs, whereas yeast-expressed porcine parvovirus VP2 VLPs displayed low, but detectable cross-reaction (Fig. 3.25). This modest cross-reactivity suggests that distant parvoviruses may share common conformational epitopes. No significant cross-reactivity was observed with unrelated yeast-expressed Tioman virus N protein VLPs (Petraityte *et al.*, 2009) used as a negative control. The immunogenicity and antigenicity analysis of recombinant VLPs showed that HBoV1-4 VP2 VLPs induce high titres of IgG antibodies in mice.

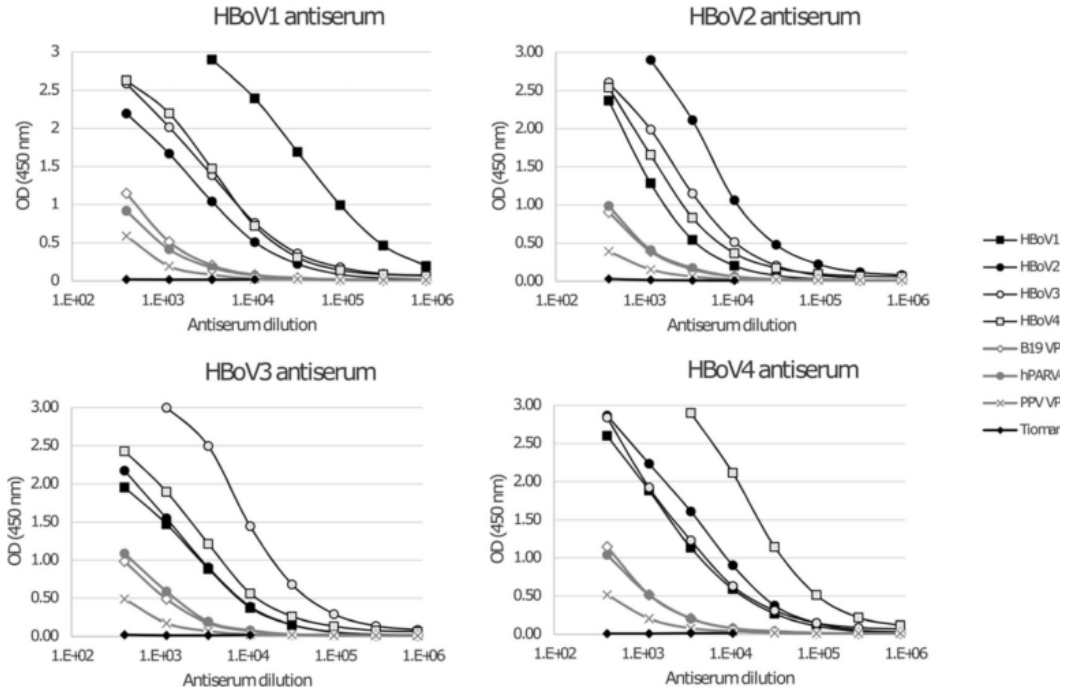

Fig. 3.25. Reactivities of human bocavirus (HBoV) 1–4 mice antisera with parvoviral (HBoV1–4, B19, human parvovirus 4, porcine parvovirus) VP2 virus-like particles. The serum samples were studied in serial dilutions indicated on the x-axis. VLPs of yeast-generated Tioman virus N protein were used as a negative control for unspecific binding.

A collection of 36 monoclonal antibodies was developed that recognise single species of HBoV VP2 antigen or cross-react with other HBoV VP2 proteins. The properties of the MAbs are summarised in Table 3.8. None of the MAbs reacted with yeast-generated PPV, PARV4, B19 VP2 antigens, showing great specificity for HBoV VLPs. A clear majority (28/36) of MAbs were recognising conformational epitopes as no reaction with the antigens was visible in WB or EIA when antigens were coated in the wells after c in 6 M urea. Linear epitope recognising MAbs were created after immunisation with HBoV2 VP2 (5 MAbs of 6) and HBoV4 VP2 (3 MAbs of 24), all of them are cross-reactive: 2 MAbs (4A7 and 7C8) recognise HBoV1, 2 and 3, whereas the remaining 6 MAbs are reactive to all four antigens. This level of cross-reactivity can be easily explained by the high sequence similarity among HBoV1-4 VP2 proteins which is 77.7-90.9% (Fig. 3.26). The primary sequence identity among HBoV2, 3 and 4 is even higher, 88.7-90.9%. Five MAbs were developed that recognise the universal

HBoV conformational epitopes as they recognise all four HBoV antigens in EIA but not in WB. Specific non-cross-reactive MAbs for HBoV1 (3 MAbs), HBoV3 (2 MAbs) and HBoV4 (15 MAbs) VP2 were developed. Thus, this collection of MAbs offers sensitive and versatile tools for both species-specific and broad (reacting with all 4 HBoV species) antigen recognition. Moreover, a significant number of MAbs raised against the yeast-generated HBoV1-4 VP2 VLPs demonstrates high antigenicity of these proteins. Also, since most of the MAbs of this collection recognise the conformational epitopes possessed in the intact VLPs, yeast-generated HBoV VP2 VLPs are stable when mixed with adjuvants and administered intravenously.

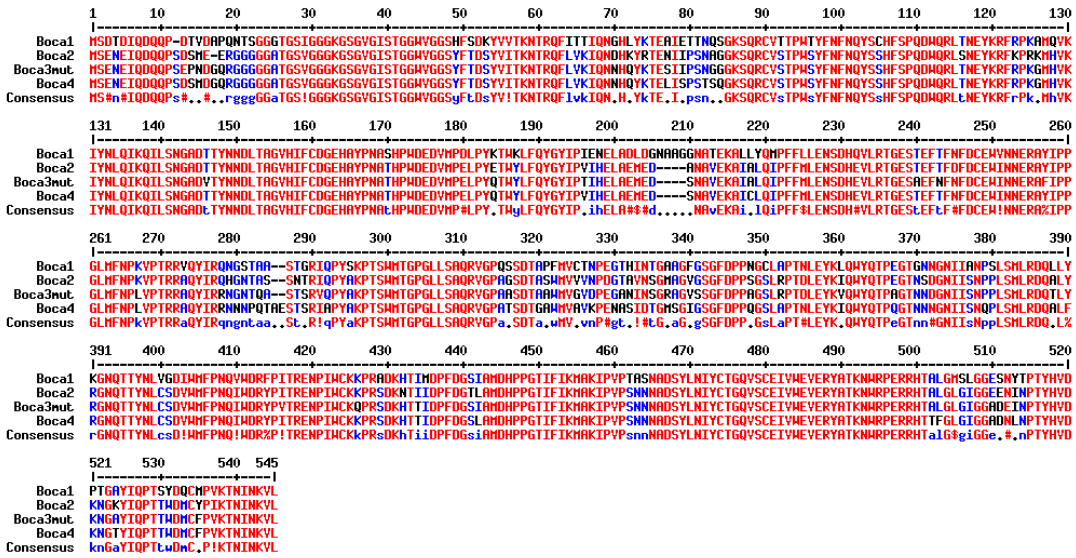

Fig. 3.26 The alignment of HBoV1-4 VP2 sequences.

Table 3.8. Properties of monoclonal antibodies raised against HBoV1-4 VP2 antigens.

Antigen	Clone	Isotype*	Reactivity with HBoV VP2 antigens in EIA:				Reactivity in WB*
			HBoV1	HBoV2	HBoV3	HBoV4	
HBoV1 VP2	**4C2**	IgG1	+	-	-	-	-
HBoV1 VP2	**12C1**	IgG1	+	-	-	-	-
HBoV1 VP2	**9G12**	IgG1	+	-	-	-	-
HBoV1 VP2	**15C6**	IgG1	+	+	-	+	-
HBoV2 VP2	**4B11**	IgG1	+	+	-	+	-
HBoV2 VP2	**4A7**	IgG1	-	+	+	+	+
HBoV2 VP2	**7C8**	IgG2b	-	+	+	+	+
HBoV2 VP2	**5A10**	IgG1	+	+	+	+	+
HBoV2 VP2	**18D11**	IgG1	+	+	+	+	+
HBoV2 VP2	**19C6**	IgG1	+	+	+	+	+
HBoV3 VP2	**9D6**	IgG2b	-	-	+	-	-
HBoV3 VP2	**10G7**	IgG1	-	-	+	-	-
HBoV4 VP2	**2B5**	IgG1	-	-	-	+	-
HBoV4 VP2	**3F12**	IgG1	-	-	-	+	-
HBoV4 VP2	**5B6**	IgG1	-	-	-	+	-
HBoV4 VP2	**7D5**	IgG1	-	-	-	+	-
HBoV4 VP2	**9G11**	IgG1	-	-	-	+	-
HBoV4 VP2	**9F5**	IgG1	-	-	-	+	-
HBoV4 VP2	**13D4**	IgG1	-	-	-	+	-
HBoV4 VP2	**13G6**	IgG1	-	-	-	+	-
HBoV4 VP2	**16D9**	IgG1	-	-	-	+	-
HBoV4 VP2	**17D7**	IgG1	-	-	-	+	-
HBoV4 VP2	**3C4**	IgG1	-	-	-	+	-
HBoV4 VP2	**5E9**	IgG2a	-	-	-	+	-
HBoV4 VP2	**12F7**	IgG2a	-	-	-	+	-
HBoV4 VP2	**18D6**	IgG2b	-	-	-	+	-
HBoV4 VP2	**24C7**	IgG3	-	-	-	+	-
HBoV4 VP2	**20B10**	IgG1	+	-	+	+	-
HBoV4 VP2	**17G11**	IgG2a	+	+	+	+	-
HBoV4 VP2	**25F6**	IgG2a	+	+	+	+	-
HBoV4 VP2	**13H4**	IgG2b	+	+	+	+	-
HBoV4 VP2	**20D10**	IgG2b	+	+	+	+	-
HBoV4 VP2	**4D4**	IgG2a	+	+	+	+	-
HBoV4 VP2	**11F6**	IgG1	+	+	+	+	+
HBoV4 VP2	**13D12**	IgG1	+	+	+	+	+
HBoV4 VP2	**19G7**	IgG1	+	+	+	+	+

** Isotyping and WB were performed by the Department of Cell Biology and Immunology (R. Lasickienė).*

3.2.4. The reactivity of recombinant HBoV VLPs with human serum antibodies

Recombinant HBoV1-4 VP2 VLPs were employed to detect virus-specific antibodies in human serum specimens. Due to high cross-reactivity between HBoV antigens reported earlier (Kantola *et al.*, 2011; Guo *et al.*, 2012; Hao *et al.*, 2015), competition indirect IgG EIA format (c-EIA) was chosen to evaluate the prevalence of bocavirus infection.

Firstly, the optimal concentration of VLPs required for an exhaustive antibody competition was determined. Different concentrations of homologous VLPs were incubated with human serum specimens (n=5) that showed OD>2.5 with all 4 recombinant HBoV VP2 proteins in an indirect IEA (Fig. 3.27). Ninety-five percent incubation was obtained when total concentration of 20 µg/mL of antigen (HBoV1 VP2 alone or a mixture of HBoV2, 3 and 4 VP2 in equal parts) was used. This concentration of total antigen was used in further c-EIA with human sera.

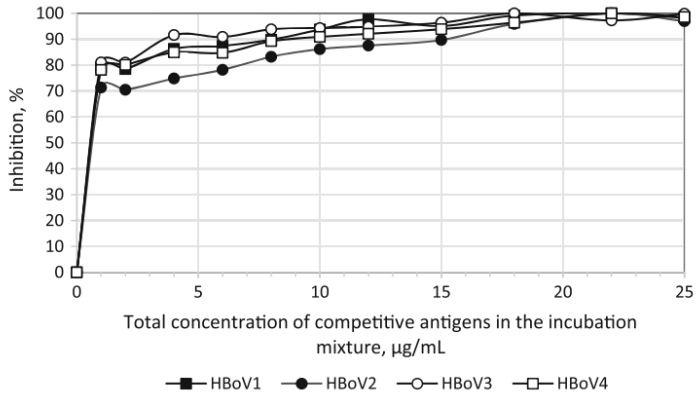

Fig. 3.27 Elimination of cross-reactive IgG from human sera. The y-axis represents the percentage of OD value that is lost due to pre-incubation of the HBoV1 IgG-positive serum sample with indicated concentrations of heterologous antigen (VP2 of HBoV2, 3 or 4). Incubation with HBoV1 VP2 VLPs was used to eliminate the cross-reactive antibodies in HBoV2, 3 or 4 IgG-positive serum samples. Data from five seropositive (OD> 2.5 in EIA) sera are represented as a mean.

All serum specimens were simultaneously tested in an indirect EIA without competition. The cut-off values for the serologic assay were established as described earlier in section 3.2.1. In this study, the cut-off OD values for HBoV-positive serum specimens were set as 0.443, and serum specimens with OD values less than 0.368 were regarded as negative. These cut-off values are similar (0.421-0.463 for positive samples) with the ones that would be deducted from the inflexion point method, proposed by Kantola *et al.*, (2011) (Fig. 3.28). To investigate the HBoV2-4 seroprevalence, competition with only HBoV1 VP2 VLPs was performed in a well with an immobilised single antigen (VP2 VLPs of either HBoV2 or HBoV 3, or HBoV 4). Spearman's rank correlation between the observed results was high (0.834, 0.836, and 0.845, respectively), therefore serum specimen was considered HBoV2-4 positive when at least one antigen (HBoV2, HBoV3 or HBoV4 VP2 VLPs) showed OD>0.443, and HBoV2-4 negative if all three antigens were non-reactive (OD<0.368). All remaining sera were regarded as equivocal.

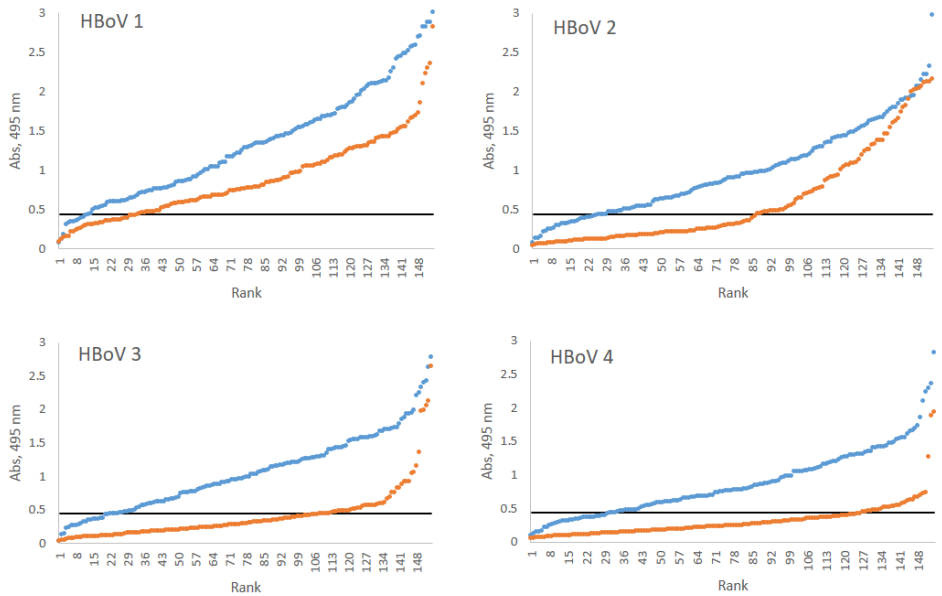

Fig 3.28. OD-value rank graphs of 154 human sera in EIA with HBoV1-4 VP2 antigens. OD (450 nm) values are plotted accordingly to their rank in the sample in the increasing order. Each graph consists of two data sets: without competition (upper, cyan curve) and data points obtained after incubation with the mixture of HBoV2-4 VP2 (for HBoV1 graph) or after incubation with HBoV1 VP2 (for HBoV2, 3, and 4 graphs). The horizontal line indicates the cut-off value (0.443) used in this study.

Serum samples from 154 patients (mean age, 25 years; range, 1 month–66 years; gender, 79 male and 75 female) with acute respiratory disease were obtained from Vilnius University, Faculty of Medicine, Clinic of Children Diseases (Lithuania) in 2008–2011 with collaboration with Dr R. Ėmužytė and Dr R. Firantienė. The clinical diagnosis of respiratory tract infections was confirmed at the sera collection day by the presence of typical symptoms of an acute respiratory viral infection, including sneezing, nasal obstruction and discharge, sore throat, cough and moderate fever.

The seroprevalence of HBoV1-specific IgG was 44.2% after competition with HBoV2-4 antigens and 91.6% without competition (Table 3.9). After removing cross-reactive antibodies with competing antigens, the overall seroprevalence of HBoV1 (44.2%) was the lowest reported to date (53% in Pakistani adults and 64% in Finnish adults (Kantola *et al.,* 2011); 64.4 – 69.2% in China (Hao *et al.,* 2015); 76.7% in Jamaica (Hustedt *et al.*, 2012). However, the overall fraction of human antisera having IgG that recognises HBoV1 remains very high and similar to previous studies (91.6% in this study; 93 – 100% in previously mentioned studies (Table 1.5)). Differences between these results may be explained by different sample sizes, age structure and regional variances, as EIA results reflect the exposure rate of accumulated previous infections.

The combined HBoV2–4 IgG seroprevalence after competition with HBoV1 VP2 was 35.7%. Without competition, HBoV2, HBoV3 and HBoV4 seroprevalences were 81.2%, 85.7%, and 77.9%, respectively. It is lower than that of HBoV1, supporting earlier studies that HBoV1, HBoV2, HBoV3, and HBoV4 are encountered in respective decreasing order (Kantola *et al.*, 2011).

Table 3.9. The Seroprevalence of HBoV1-4-specific IgG Among Lithuanian Patients with Confirmed Respiratory Tract Infections

		Indirect EIA								Competition EIA					
	N	HBoV1 IgG pos.		HBoV2 IgG pos.		HBoV3 IgG pos.		HBoV4 IgG pos.		HBoV1 IgG pos.		HBoV2-4 IgG pos.		*HBoV1-4 IgG neg.*	
All children	**51**	**42**	**82,4%**	**38**	**74,5%**	**42**	**82,4%**	**38**	**74,5%**	**22**	**43,1%**	**17**	**33,3%**	**17**	**33,3%**
<2 y	13	8	**61,5%**	8	61,5%	9	69,2%	8	61,5%	2	15,4%	3	23,1%	8	**61,5%**
2-14 y	38	34	**89,5%**	30	78,9%	33	86,8%	30	78,9%	20	52,6%	14	36,8%	9	**23,7%**
All adults	**103**	**99**	**96,1%**	**87**	**84,5%**	**90**	**87,4%**	**82**	**79,6%**	**46**	**44,7%**	**38**	**36,9%**	**32**	**31,1%**
15-30 y	44	42	**95,5%**	39	88,6%	40	90,9%	37	84,1%	18	40,9%	22	50,0%	8	**18,2%**
30-55 y	47	46	**97,9%**	39	83,0%	42	89,4%	38	80,9%	23	48,9%	13	27,7%	19	**40,4%**
>55 y	12	11	**91,7%**	9	75,0%	8	66,7%	7	58,3%	5	41,7%	3	25,0%	5	**41,7%**
All sera	**154**	**141**	**91,6%**	**125**	**81,2%**	**132**	**85,7%**	**120**	**77,9%**	**68**	**44,2%**	**55**	**35,7%**	**49**	**31,8%**

Analysing the differences in HBoV1-4 seroprevalence between males (n=79) and females (n=75), no significant differences were found for HBoV1 (χ^2=2.146, p≤0.001, OR=0.96, 95% CI=0.51-1.82). However, the statistically higher seroprevalence of HBoV2-4 was detected among males (37 serum specimens HBoV2-4 IgG positive, 46.8%) than in females (18 serum specimens HBoV2-4 IgG positive, 24.0%; p=0.012, OR=2.85, 95% CI=1.41-5.79), similarly as reported by Kantola *et al.,* 2011. Differences between the groups of children (younger than 15 years) and adult patients (aged 15-66) were not statistically significant (HBoV1: χ^2=1.083, p≤0.001, OR=0.96, 95% CI=0.50-2.10; HBoV2-4: χ^2= 0.275, p≤0.001, OR=0.51, 95% CI=0.22-1.17). Higher fraction of seronegative sera for any HBoV among children younger than two years (61.5% as compared to 31.1% in adults) cannot be confirmed due to the limited size of the sample; however, similar tendency was observed in other related studies (Karalar *et al.,* 2010; Kantola *et al.*, 2011; Guo *et al.*, 2012; Hao *et al.*, 2015) and may be explained as bocavirus encounter happening in early childhood. Additionally, all seronegative samples in children younger than two years old had lower OD (for HBoV1, p=1.42×10^{-4}; for HBoV2-4, p=1.22×10^{-4}) than the seronegative serum specimens in the groups of adult or children above 2 years old.

The seroprevalence of HBoV1-4 in various age groups of the studied Lithuanian patients with respiratory tract infections shows similar tendencies to those

reported in other countries. In this study, seropositive sera fraction peaked in samples from 2-14 years old children and remained high (>75% in non-competition IEA) in adult patients. Relatively lower prevalence is observed in elderly patients (>55 years) suggesting a waning of bocavirus specific IgG, and thus the possibility of repeated infection in senescence. Interestingly, 24 serum specimens (15.6%) that displayed high reactivity with all 4 HBoV VP2 proteins in an indirect IEA were interpreted as seronegative after competition with other HBoV VP2 antigens. This effect, also reported by (Kantola *et al.,* 2011), may be a result of a summative exposure to other parvoviruses or antibody waning.

In summary, yeast-generated HBoV1-4 VP2 VLPs are antigenic, and employment of these antigens in human bocavirus seroprevalence study of Lithuanian population revealed high incidence rate of previous bocaviral infection, similarly to other studies worldwide.

3.2.5. Antigenic properties of yeast expressed porcine parvovirus VP2 VLPs

PPV VP2 protein-derived VLPs generated in *S. cerevisiae* were used to develop an indirect EIA for the detection PPV-specific IgG antibodies in swine serum specimens. One hundred and eighty-three swine serum samples from farms in Lithuania (n=160), Romania (n=14) and Ukraine (n=13) were collected in years 2008-2010 were provided by Dr. V. Sereika and Dr. R. Lelešius from Veterinary Academy, Lithuanian University of Health Sciences. To test the antigenic properties of yeast-derived VLPs, all 187 serum samples were tested using INGEZIM PPV Compac test as a gold standard and further retested with the newly developed Indirect IgG PPV EIA.

Both assays were performed in parallel for every serum sample to determine the sensitivity and specificity of the new Indirect IgG PPV EIA. The cut-off value for the new assay was calculated as the mean OD value of the 39 negative sera (identified with the commercial kit) plus 2 standard deviations ($\bar{x}$+2SD) resulting in 95% confidence. The mean OD value and SD were 0.150 and 0.090, respectively. Therefore, sera with OD values above 0.330 were

considered positive (n=129), and those with OD value below this cut-off were assessed as negative (n=58) in the newly developed Indirect IgG PPV EIA.

Thirty-eight out of the 39 sera tested as negative with a commercial kit were assessed as negative by the indirect IgG PPV EIA. Nine out of 137 positive and all 11 doubtful serum samples by INGEZIM assay showed the OD value below the cut-off in the Indirect IgG PPV EIA and were considered as negative. Thus, the calculated specificity and sensitivity for the new indirect IgG PPV EIA were 97.4% (38/39) and 93.4% (128/137), respectively. All nine false-negative samples of the new assay were weak positive in INGEZIM kit showing blocking percentage (BP) in the 33-45% range. All samples above BP equal to 30% were considered positive in this commercial kit. The only false-positive sample in the Indirect IgG PPV EIA showed OD=0.354 that is just above the cut-off OD of 0.330.

Table 3.10 Summary of the concordance of results obtained with the newly developed Indirect IgG EIA and with the commercial INGEZIM test.

EIA test with recombinant antigen		**INGEZIM PPV Compac**			
		Positive	Negative	Doubtful	Total
Indirect IgG EIA test	Positive	128	1	0	129
	Negative	9	38	11	58
	Total	137	39	11	187

To obtain a more precise estimation of the sensitivity and specificity of the new assay, additional evaluation with more serum samples and alternative assays must be done in the future. Alternatively, the precision of the test can be improved using other formats of EIA. In summary, results of the current study are promising to the use of PPV VP2 antigen synthesised in yeast *S. cerevisiae* in diagnostic kits.

3.2.6. Generation of monoclonal antibodies against PPV and their characterization

Purified recombinant PPV VP2 protein was used to immunise mice and generate PPV VP2-specific MAbs. This work was done by the Department of Cell Biology and Immunology (head Dr. A. Žvirbliene) following the protocols essentially as described by Köhler and Milstein (1975).

After screening and cloning of positive hybridoma clones, nine stable hybridoma cell lines producing IgG antibodies were derived. Six MAbs produced by hybridoma clones were of IgG1 subtype, and the remaining three were found to be of IgG2a subtype. All MAbs reacted specifically with recombinant PPV VP2 protein in EIA and did not react with other yeast-expressed proteins used as a negative control (Table 3.11). The MAbs 4F11, 16G11, 25C5, 6D1, and 10A7 recognised SDS-denaturated PPV VP2 protein in Western blotting assay. The remaining MAbs did not react with the antigen in WB, suggesting that these MAbs (clones 1F8, 16A1, 22G2, and 23A7) recognise only conformation-dependent epitopes.

Table 3.11. PPV VP2 specific MAb isotypes and specificity

MAb clone	**MAb isotype**	**Epitope type recognised***	**Indirect EIA results using yeast *S. cerevisia* generated antigens****		
			Porcine parvovirus VP2	*Hantaan (Fojnica) nucleocapsid (N) protein*	*Tioman nucleocapsid (N) protein*
1F8	IgG1	conformational	+	-	-
4F11	IgG1	linear	+	-	-
6D1	IgG1	linear	+	-	-
10A7	IgG1	linear	+	-	-
16A1	IgG2a	conformational	+	-	-
16G11	IgG1	linear	+	-	-
22G2	IgG2a	conformational	+	-	-
23A7	IgG2a	conformational	+	-	-
25C5	IgG1	linear	+	-	-

* epitope type was deduced by reactivity in WB, where positive reaction indicated the linea nature of epitope.
** +, OD in EIA ≥ 1.0; -, No reactivity
Isotyping and WB were performed by the Department of Cell Biology and Immunology (R Lasickienė).

The specificities of the MAbs were further analysed by IFA to verify the ability of the MAbs to recognise native virion. For this purpose, commercial porcine parvovirus control slides containing virus-infected and non-infected fixed cells were used. None of the MAbs reacted with non-infected cells, which confirms the specificity of the assay (Fig. 3.29, negative control). Both groups of MAbs recognising linear or conformational epitopes reacted with infected cells, however only the latter ones produced images with fine nucleus-shaped patterns.

In contrast, the MAbs recognising linear epitopes produced signal outside the nuclei but in lesser intensity (Fig. 3.29). This difference could be explained that PPV VLPs finish their assembly in the nucleus forming conformational epitopes. Taking into consideration trimer translocation model for other parvoviruses (Riolobos *et al.*, 2006), conformational epitopes might be available only in intact capsid but not in trimers or pentamers formed by VP2 protein. This possibility emphasises the importance of properly assembled VLPs to elicit strong immune responses when using recombinant antigens as potential vaccines. Further epitope mapping needs to be done to answer if linear epitopes remain accessible on the intact VLP surface or are hidden within the structure. However, our generated MAbs represent an attractive tool for studying intracellular PPV infection and capsid formation process.

The PPV VP2-derived VLPs generated in *S. cerevisiae* have not yet been tested for possible use as a vaccine in pigs, however, considering results on the antigenic structure and the immunogenicity in mice described in this study, this is an attractive alternative to currently used recombinant PPV vaccines. In previous studies, PPV VP2-derived VLPs have been shown to be effective epitope carriers to elicit a strong immune response in mice (Pan *et al.*, 2008; Sedlik *et al.*, 1999). Furthermore, yeast expression system does not require additional contaminant elimination procedure as described for baculovirus expression system (Rueda *et al.*, 2000) for such recombinant subunit vaccine preparation. Therefore, PPV VP2-derived VLPs generated in yeast *S. cerevisiae* is a promising platform for new PPV vaccine development.

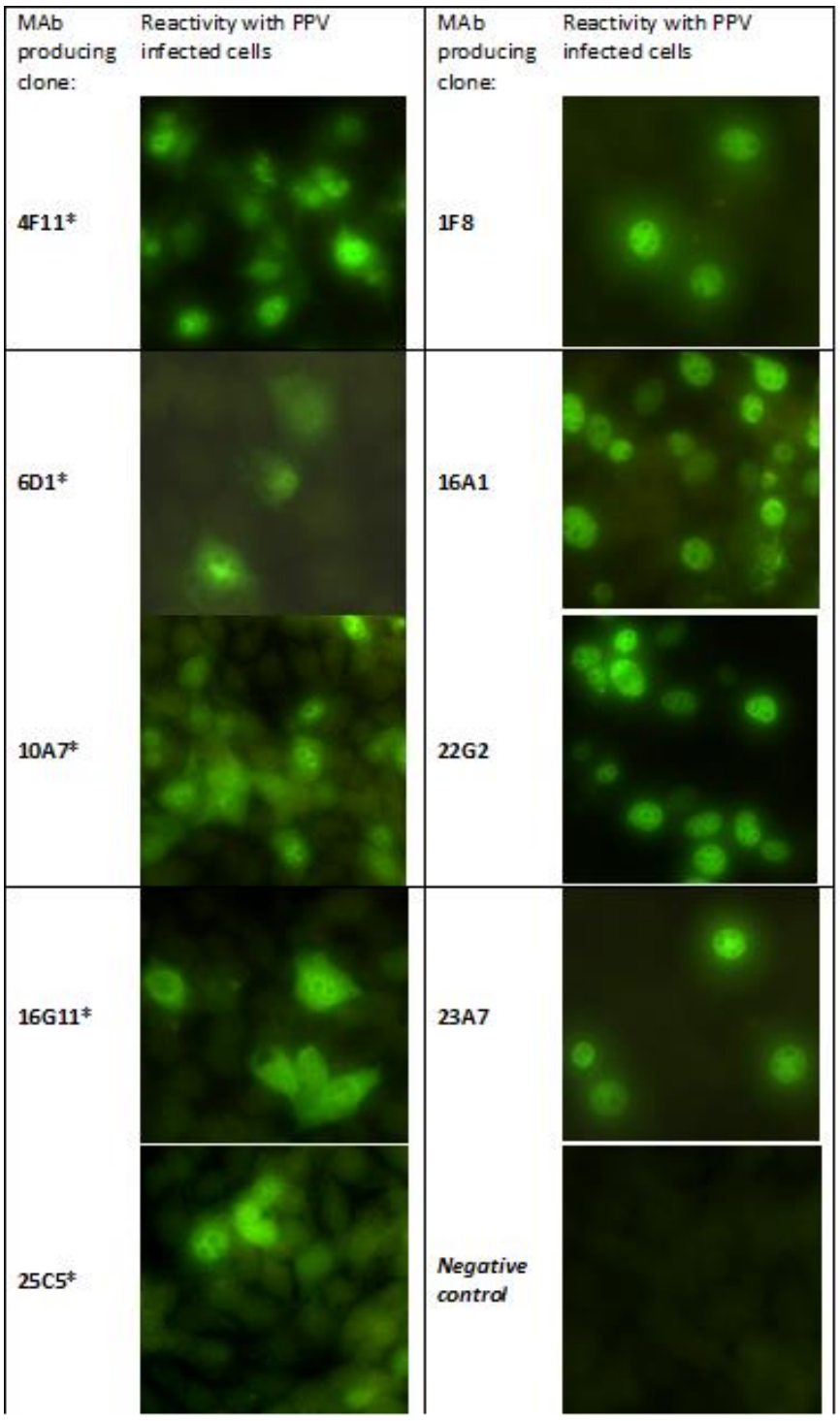

Fig. 3.29 Fluorescence microphotographs showing the reactivity of 9 MAbs raised against yeast-derived PPV VP2 protein with PPV-infected cells on commercial slides (VMRD, Inc.). The codes of the MAbs are indicated on the left side of each picture. The MAbs recognising linear PPV VP2 epitopes are indicated with an asterisk. As a negative control, negative control serum included in the kit is used.

3.2.7. Antigenic properties of mosaic VP1-VP2 parvoviral VLPs

The unique region of the VP1 (VP1u) of parvovirus B19 elicits a dominant immune response, and it has been shown to harbour neutralising epitopes (Anderson *et al.,* 1995; Saikawa *et al.,* 1993). However, B19 VP1u domain was shown to be internalised within the recombinant VLP and is inaccessible to antibodies. Irreversible externalisation of VP1u domain was achieved by acidic treatment or heating of VLPs (Ros *et al.,* 2006a). The efficiency of both strategies was tested with HBoV1 VP1-VP2 mVLPs in EIA. To evaluate VP1u externalisation, recombinant mosaic VLPs (mVLPs), subjected to either heat (60°C) or low pH (pH 5.0), as well as untreated HBoV1 mVLPs and VP2 VLPs, were absorbed in the wells. Twenty-six HBoV1 IgG-positive (HBoV2-4 IgG-negative) human serum specimens were used to test changes in epitope exposure on the surface of HBoV1 mVLPs. Untreated HBoV1 mVLPs were found to be more reactive with human serum specimens than HBoV1 VP2 VLPs (p=1.11×10^{-5}), suggesting the availability of additional antibody epitopes in VP1-VP2 mVLPs. Thermal treatment has not resulted in significant (p=0.204) changes in EIA results as compared to the untreated mVLP, and incubation in low pH resulted in overall lower (p=2.55×10^{-4}) OD values of the samples compared to the untreated mVLPs (Fig. 3.30). No additional bands were observed in SDS-PAGE or WB when analysing treated mVLPs (data not shown), suggesting that incubation in low pH may induce conformational changes, but no proteolytic disintegration which could lead to a decreased OD. Assuming, that additionally exposed epitopes would result in elevated OD, the obtained results suggest that recombinant HBoV1 VP1-VP2 mVLPs are thermally stable, however, structural changes might be induced by incubation in pH 5.0 that results in lower OD values in EIA.

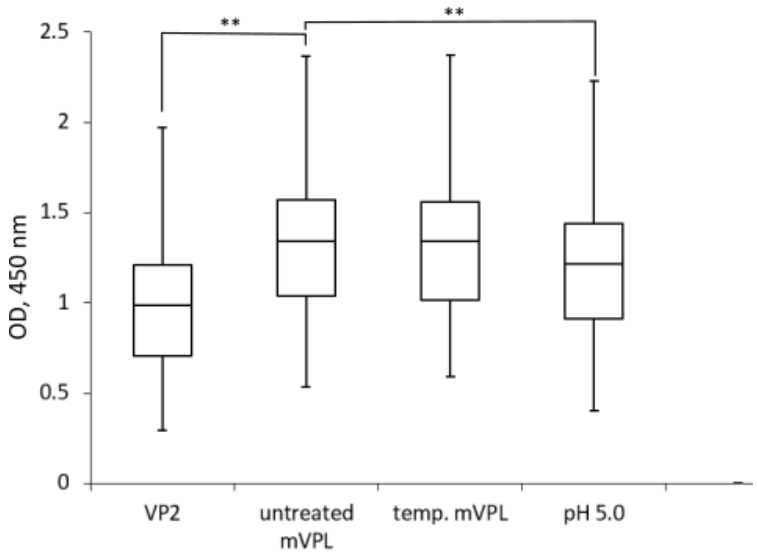

Fig. 3.30 Optical density values of 26 HBoV1-positive samples using HBoV1 VP2 VLPs or VP1-VP2 mVLPs (untreated, heat and low pH treated). ***, significant mean OD change, $p<0.001$

To evaluate the potential of the HBoV1-derived VP1-VP2 mVLPs in serodiagnostics, EIA of human serum specimens previously tested on HBoV1 VP2 VLPs (section 3.24) was performed. No changes in OD ($p<0.001$) were observed comparing HBoV1 VP1-VP2 mVLPs to HBoV1 VP2 VLPs using seronegative (n=24) serum specimens. However, both equivocal serum samples (OD=0.409 and 0.392) after a repeated indirect competition IEA using HBoV1 mVLPs instead of VP2, were interpreted as the newly identified positives due to OD>0.443. Therefore, the higher reactivity of HBoV1 VP1-VP2 mVLPs with HBoV1 IgG positive serum specimens suggests that mVLPs may represent an effective recombinant antigen for more sensitive diagnostic systems.

Similarly, antigenic properties of PARV4 mVLPs were estimated. For this purpose, IgG capture EIA with MAb 12E8 (section 3.2.2) absorbed in the wells was employed. All ten PARV4 IgG-positive sera (OD>0.411) from a previous study (section 3.2.1) were retested using both VP2 VLPs and mVLPs of PARV4. For comparison, 86 IgG-negative human sera (OD<0.29) were also retested. Results are summarised in Table 3.11.

Table 3.11. Results of IgG capture EIA using PARV4 VP2 VLPs and mVLPs captured by the absorbed MAb 12E8. Mean values of three repetitions with sera (positive, OD>0.411, and negative, OD<0.29) are provided.

IgG positive serum	OD (450 nm), capture EIA	
	VP2-VLPs	**mVLPs**
19	**0.654**±0.023	**0.908**±0.036
20	**0.687**±0.045	**0.871**±0.089
31	**0.494**±0.022	**0.501**±0.012
84	**0.445**±0.049	**0.564**±0.067
85	**0.455**±0.069	**0.649**±0.044
112	**0.728**±0.056	**1.018**±0.105
113	**0.453**±0.034	**0.489**±0.069
138	**0.435**±0.078	**0.579**±0.022
209	**0.419**±0.023	**0.724**±0.045
216	**0.498**±0.024	**0.818**±0.028
Average OD of negative sera (n=86)	**0.176**±0.046	**0.179**±0.069

All ten Ig-G positive sera were more reactive with mVLPs than VP2-VLPs in the analogous assay. The mean OD of negative sera did not change significantly. The enhanced positive signal obtained by using mVLPs instead of VP2-only VLPs can be similarly explained by the introduction of additional epitopes, recognised by IgG in human sera. However, due to a limited number of PARV4-positive sera in our disposition, this finding needs additional validation.

CONCLUSIONS

1. Recombinant yeast *S. cerevisiae* synthesises major capsid proteins of PARV4, PPV and HBoV1-4 as the stable and immunogenic virus-like particles, suitable for the diagnostic immunoassays and eliciting a specific immune response in mice. Effective synthesis of HBoV2 and HBoV3 VP2 in yeast *S. cerevisiae* requires codon usage-optimised gene sequences.
2. Synthesis of human parvovirus 4 and human bocavirus 1 minor capsid protein VP1 is ineffective in yeast, however, the co-expression with VP2 leads to the generation of stable mosaic virus-like particles. Such parvoviral mVLPs may be used as antigens in immunoassays for more sensitive diagnostic tests.
3. Sixteen samples (9.4%) from low-risk Lithuanian patients were diagnosed as seropositive for PARV4 using yeast-generated VLPs, suggesting the nonparenteral transmission mode of this virus.
4. VP2 antigens of human bocaviruses 1-4 are cross-reactive. After depletion of cross-reactive antibodies in serum samples from Lithuanian patients with diagnosed acute respiratory disease, the actual seroprevalence of HBoV1 was 44.2% and the seroprevalence of HBoV2–4 was 35.7%.
5. Yeast-generated porcine parvovirus VP2 VLPs are suitable for PPV-specific antibody detection in swine serum samples with high sensitivity and specificity.
6. Major linear B-cell epitopes of parvovirus 4 VP2 are conservatively located in the EF, HI and C-terminal loops.

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Petraitytė-Burneikienė R, **Tamošiūnas PL**, Lasickienė R, Firantienė R, Ėmužytė R, Žvirblienė A, Sasnauskas K. Production in yeast of human bocavirus 1-4 VP2 virus-like particles and generation of VP2-specific monoclonal antibodies as novel tools for bocavirus serology. 6th Congress of European Microbiologists. 2015 Jun 7-11, Maasticht, The Netherlands. (Poster presentation)

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ACKNOWLEDGEMENTS

I would like to express my special appreciation and gratefulness to my supervisor Professor Dr. habil. Kęstutis Sasnauskas for all the guidance, discussions, and encouragement when it was needed. I am heartily grateful to all the staff of the Department of Eukaryote Gene Engineering of Vilnius University Life Sciences Centre Institute of Biotechnology for all the productive collaboration and support, especially Dr. Rasa Petraitytė-Burneikienė, who was the most patient teacher of all the methods and the principal consultant in all the doubtful moments. I'm thankful to all the students, namely, Karolis Simutis, Artiomas Akatov, and Kornelija Marcinkevičiūtė, for a possibility to grow as a tutor.

For a productive collaboration in immunological aspects of this research, I am grateful to Rita Lasickienė, Dr. Indrė Kučinskaitė-Kodzė and Prof. Dr. Aurelija Žvirblienė from the Dept. of Immunology and Cell Biology. For all the collected serum samples that enabled to validate the developed diagnostic systems, I am thankful to Dr. Regina Ėmužytė and Dr. Regina Firantienė (Vilnius University, Faculty of Medicine), as well as Dr. Vilimas Sereika and Dr. Raimundas Lelešius (Veterinary Academy, Lithuanian University of Health Sciences).

The financial support from the Lithuanian Science Council, Grant no. MIP-060/2011, was essential to conduct the experiments of this research.

From the bottom of my heart, I am thankful to my lifetime teachers: my parents and sister, who stimulated in every step not to give up and reach for more.

And I thank to all the most faithful comrades of mine, especially Mindaugas.