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**Author(s):** Hankaniemi, Minna M.; Stone, Virginia M.; Andrejeff, Tanja; Heinimäki, Suvi; Sioofy-Khojine, Amir-Babak; Marjomäki, Varpu; Hyöty, Heikki; Blazevic, Vesna; Flodström-Tullberg, Malin; Hytönen, Vesa P.; Laitinen, Olli H.

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## Research paper

## Formalin treatment increases the stability and immunogenicity of coxsackievirus B1 VLP vaccine



Minna M. Hankaniemi<sup>a,1</sup>, Virginia M. Stone<sup>a,b,1</sup>, Tanja Andrejef<sup>a</sup>, Suvi Heinimäki<sup>c</sup>, Amir-Babak Sioofy-Khojine<sup>a</sup>, Varpu Marjomäki<sup>d</sup>, Heikki Hyöty<sup>a,e</sup>, Vesna Blazevic<sup>c</sup>, Malin Flodström-Tullberg<sup>a,b</sup>, Vesa P. Hytönen<sup>a,e</sup>, Olli H. Laitinen<sup>a,\*</sup>

<sup>a</sup> Faculty of Medicine and Health Technology, Tampere University, FI-33014, Tampere, Finland

<sup>b</sup> The Center for Infectious Medicine, Karolinska Institutet, Department of Medicine Huddinge, Karolinska University Hospital, Alfred Nobels Allé 8, SE-14152 Stockholm, Sweden

<sup>c</sup> Vaccine Research Center, Faculty of Medicine and Health Technology, Tampere University, Finland

<sup>d</sup> Department of Biological and Environmental Science/Nanoscience Center, University of Jyväskylä, P.O. Box 35, FI-40014, University of Jyväskylä, Finland

<sup>e</sup> Fimlab Laboratories, Pirkanmaa Hospital District, FI-33520 Tampere, Finland

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## ABSTRACT

Type B Coxsackieviruses (CVBs) are a common cause of acute and chronic myocarditis, dilated cardiomyopathy and aseptic meningitis. However, no CVB-vaccines are available for human use. We have previously produced virus-like particles (VLPs) for CVB3 with a baculovirus-insect cell production system. Here we have explored the potential of a VLP-based vaccine targeting CVB1 and describe the production of CVB1-VLPs with a scalable VLP purification method. The developed purification method consisting of tangential flow filtration and ion exchange chromatography is compatible with industrial scale production. CVB1-VLP vaccine was treated with UV-C or formalin to study whether stability and immunogenicity was affected. Untreated, UV treated and formalin treated VLPs remained morphologically intact for 12 months at 4 °C. Formalin treatment increased, whereas UV treatment decreased the thermostability of the VLP-vaccine. High neutralising and total IgG antibody levels, the latter predominantly of a Th2 type (IgG1) phenotype, were detected in female BALB/c mice immunised with non-adjuvanted, untreated CVB1-VLP vaccine. The immunogenicity of the differently treated CVB1-VLPs (non-adjuvanted) were compared in C57BL/6J mice and animals vaccinated with formalin treated CVB1-VLPs mounted the strongest neutralising and, CVB1-specific IgG and IgG1 antibody responses. This study demonstrates that formalin treatment increases the stability and immunogenicity of CVB1-VLP vaccine and may offer a universal tool for the stabilisation of VLPs in the production of more efficient vaccines.

## 1. Introduction

The six Coxsackievirus B serotypes (CVB1-6) are common human pathogens belonging to the family of Enteroviruses (EVs). While the majority of CVB infections cause mild symptoms, the major importance of CVBs in public health is associated with their potential for inducing myocarditis and subsequent cardiomyopathy (Yajima, 2011), aseptic meningitis (Wong et al., 2011) and pancreatitis (Huber and Ramsingh, 2004). CVB1 became the predominant enterovirus in the United States in 2007 among neonates (Centers for Disease Control and Prevention (CDC), 2008) and since then CVB1 epidemics causing life-threatening infections have occurred in neonates (Verma et al., 2009; Wikswo et al.,

2009; Centers for Disease Control and Prevention (CDC), 2008; Chu et al., 2015). Moreover, associations between CVB infections (in particular CVB1) and type 1 diabetes (T1D) have been documented (Laitinen et al., 2014; Sioofy-Khojine et al., 2018).

All enteroviruses have a similar genetic organization with a 7.5 kb single-stranded positive-sense RNA genome that is translated into a single polypeptide containing the P1–P3 regions. The P2 and P3 regions of the polypeptide contain the non-structural proteins, such as viral protease 3CD, that cleave the P1 region into the structural proteins VP0, VP1 and VP3. These structural proteins assemble into the viral capsid, wherein the viral RNA is encapsidated. VP0 is further processed into VP2 and VP4 in a viral RNA-driven autocatalysis reaction yielding the

\* Corresponding author.

E-mail address: [olli.laitinen@tuni.fi](mailto:olli.laitinen@tuni.fi) (O.H. Laitinen).

<sup>1</sup> Contributed equally.

mature virus (Laitinen et al., 2016). The mature capsid exhibits icosahedral symmetry and consists sixty copies of VP1-4 proteins (Muckelbauer et al., 1995).

Aside from the polio (Lien and Heymann, 2013) and EV71 (Chang et al., 2016) vaccines, there are no treatments or vaccines available against enteroviruses. However, clinical development of a vaccine against CVBs has recently commenced (Hyoty et al., 2018). EV-vaccines contain either live-attenuated viruses (oral polio vaccine, OPV) or formalin inactivated viruses (inactivated polio vaccine, EV71-vaccine). However, attenuated and inactivated vaccines face safety issues, such as reversion to the virulent form. OPV has been shown to regain virulence through reversion or recombination, resulting in cases of vaccine-associated paralytic polio (VAPP) and subsequent polio outbreaks (Lukashev, 2010).

Virus-like particles (VLPs) are empty particles assembled from recombinantly expressed viral structural proteins, that lack the viral genome and are thus non-infectious (Mohsen et al., 2017). As such, VLP-based vaccines are not associated with safety concerns related to whole virus vaccines. Various studies have shown that VLPs are promising vaccine candidates against EVs as demonstrated by monovalent VLP-vaccines against CVB3 (Zhang et al., 2012; Koho et al., 2014), EV71 (Salmons et al., 2018), CAV6 (Shen et al., 2016), CAV16 (Liu et al., 2012) and EV-D68 (Dai et al., 2018) or polyvalent VLP-vaccines against EV71, CAV6, CAV10 and CAV16 (Zhang et al., 2018a) studied in preclinical models.

We have previously developed a CVB3-VLP vaccine, which induced a robust neutralising antibody response in mice (Koho et al., 2014). In the present study we produced CVB1-VLPs using a new construct design with the goal to enhance the VLP yield. We also optimised the purification process of the VLPs to obtain a production process that would be applicable for industrial scale production. We analysed the stability of the CVB1-VLP vaccines, as previous studies have shown that poor thermal stability of polio-VLPs has restricted their applicability as vaccines (Adeyemi et al., 2017; Fox et al., 2017). Therefore, we studied if UV or formalin treatments could improve the stability or immunogenicity of the CVB1-VLP-vaccines.

According to our knowledge, other EV-VLP immunogenicity studies published to date, with the exception of one polio-VLP study (Fox et al., 2017), have been performed in the presence of adjuvants (Koho et al., 2014; Zhang et al., 2012; Zhang et al., 2018a, Zhang et al., 2018b; Salmons et al., 2018; Shen et al., 2016; Liu et al., 2012; Dai et al., 2018; Wang et al., 2017; Ku et al., 2014). Here, the potential of non-adjuvanted CVB1-VLPs as vaccines was demonstrated by the immunisation of mice. The effect of UV or formalin treatment on vaccine immunogenicity of CVB1-VLPs was also examined. Our results provide insights into the feasibility of non-adjuvanted CVB1-VLPs in the development of vaccines against Coxsackie B viruses.

## 2. Materials and methods

### 2.1. Generation of the VLP-producing baculoviruses

The baculoviral transfer vector pOET5, containing separate cassettes for the CVB1 VP1-4 capsid under control of the polyhedrin promoter and the 3CD-protease under CVM promoter control was ordered from GeneArt (Regensburg, Germany). A CVB1 field isolate (Laitinen et al., 2014) was chosen as template. The recombinant baculovirus was produced according to the FlashBAC baculovirus expression system manual utilising the flashBAC ULTRA baculovirus genome.

### 2.2. Production and purification of the VLPs

CVB1-VLPs were produced in High-Five insect cells. After clarification by centrifugation (9610 × g, 4 °C, 30 min) and filtering through 0.45 µm and 0.2 µm filters, CVB1-VLPs were concentrated from the culture supernatant by Tangential Flow Filtration (TFF), utilising 750

MWCO hollow fiber with an ÄKTA Flux system. The buffer was exchanged to 40 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub>, 40 mM NaCl, 0.1% Tween80 with the same system. Impurities were removed from the preparation using HiTrap Q and SP columns (GE Healthcare), as well as CIMmultus QA and SO<sub>3</sub> ion exchange chromatography (IEX) columns (BIASeparations). Finally, CVB1-VLPs were captured to CIMmultus SO<sub>3</sub>-column and eluted using NaCl gradient (peak with 200 mM NaCl).

### 2.3. Characterisation of the VLPs and vaccine formulation

Purified VLPs were analysed either on Bolt 4–12% Bis-Tris Plus SDS-PAGE gels (Invitrogen) and stained with the Pierce Silver Stain Kit (Thermo Scientific) or on mini-PROTEAN TGX stain-free precast gradient gels (4–20%) (BioRad). The proteins were analysed by Western blotting using in-house produced rabbit anti-CVB1-6 polyclonal antibody and IRDye-labeled secondary antibody. VLP proteins and impurities were assessed by densitometry analysis of SDS-PAGE gels using the ImageJ software (Schindelin et al., 2012). Determination of VLP total protein concentration, dynamic light scattering (DLS) analysis and transmission electron microscopy (TEM) imaging were performed as described (Hankaniemi et al., 2017). The thermal stability of the VLP particles was characterized by a thermofluorometric dye-binding assay using the protein-binding dye SYPRO orange (Invitrogen). Reaction mixtures of 25 µl containing 4.0 µg VLP and 6 x SYPRO orange were prepared in PBS and heated from 25 to 110 °C, with fluorescence reads taken at 1 °C intervals every 30 s within the Biorad quantitative PCR system.

The vaccine was formulated in M199 medium containing 0.1% Tween80.

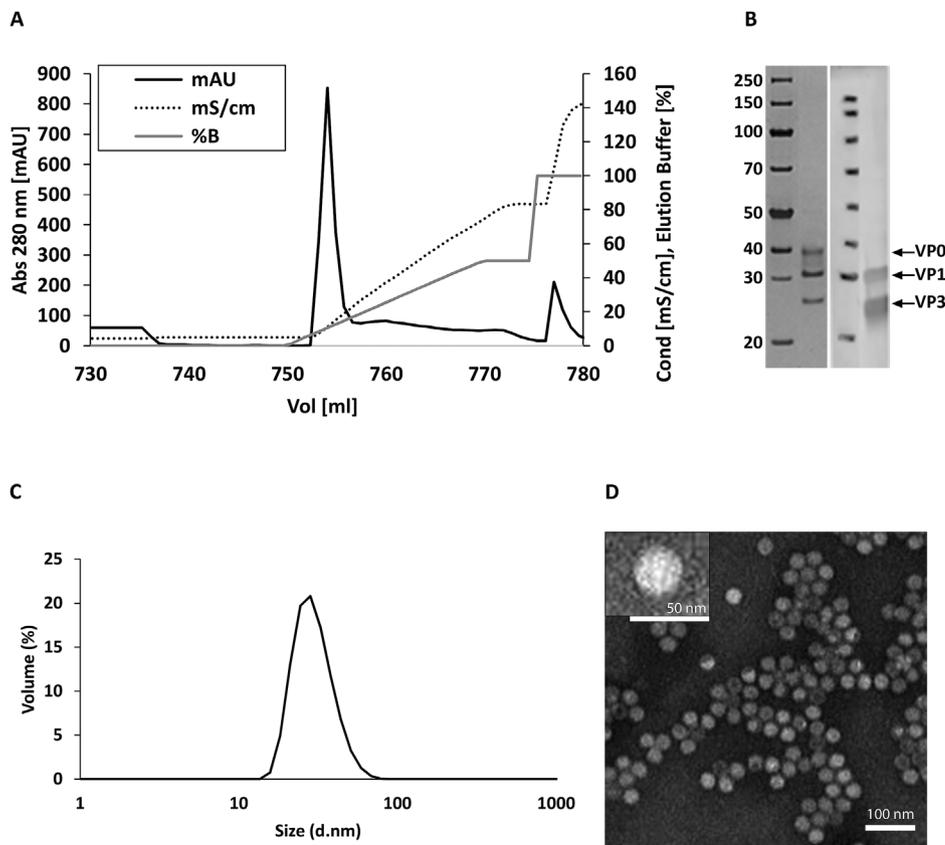
### 2.4. Mouse immunisations

Female BALB/c OlaHsd mice (Envigo, Horst, the Netherlands) were vaccinated twice using 50 µl volume intramuscularly (i.m.) on days 0 and 21 with 0.3, 1 or 10 µg of CVB1-VLP (n = 5) with no adjuvants. Mice receiving sterile PBS served as negative controls (n = 5). Whole blood samples from each mouse were collected at the time of euthanization (day 35) and the serum was separated as described (Tamminen et al., 2012). All experimental procedures with BALB/c mice were conducted in accordance with the regulations of the Finnish National Experiment Board (permission number ESAVI/10800/04.10.07/2016).

C57BL/6 J mice were vaccinated with subcutaneous injections interscapularly (i.s.) on days 0, 21 and 35 with 1 µg of non-adjuvanted formalin treated, UV treated, untreated CVB1-VLP or vaccine buffer (n = 10). All experiments with C57BL/6 J mice were conducted at Karolinska Institutet, Stockholm, Sweden in accordance with the NIH Principles of Laboratory Animal Care and national laws in Sweden and were approved by the local ethics committee (permission number S 48–14).

### 2.5. Neutralisation assays and CVB1-VLP specific ELISA

Mouse sera were tested for CVB1-specific IgG and IgG subtype antibodies by ELISA as previously described (Heinimäki et al., 2018). Briefly, 96-well plates (Corning Inc., Corning, NY) were coated with 50 ng of CVB1-VLPs per well, antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, MO), IgG1 (Invitrogen) or IgG2a (Invitrogen) and SIGMA FAST OPD substrate (Sigma-Aldrich). CVB1 neutralising antibodies were measured as previously described (Laitinen et al., 2014). The dotted line in each graph represents the positive/negative threshold of neutralising capacity (dilution 1:16) in the virus neutralisation assay. According to previous studies, uninfected animals that have not been vaccinated occasionally show a neutralising antibody titer of 1:4 but not beyond this titer (Stone et al., 2018).



**Fig. 1.** Final cation exchange chromatography concentration step and characterisation of the purified CVB1-VLPs with SDS-PAGE, Western blotting, DLS and TEM. (A) In the final purification step, a buffer containing 20 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 20 mM NaCl and 0.1% Tween80 was used as the running buffer, and the same buffer containing 2 M NaCl was used as the elution buffer. After sample loading, unbound proteins were washed out from the column with running buffer and column-bound VLPs eluted at approximately 200 mM NaCl concentration using a linear gradient. (B) SDS-PAGE and Western blot analyses of purified VLPs. The left panel shows the silver-stained SDS-PAGE analysis of VLPs, and in the right panel VP1 and VP3 capsid proteins were detected by Western blot using an in-house produced rabbit anti-CVB1-6 pAb. VLP capsid proteins are indicated with arrows. (C) Dynamic light scattering analysis of the chromatography-purified CVB1-VLP. (D) Transmission Electron Microscopy (TEM) images of the chromatography-purified VLPs. Scale bar 50 nm, 25 000× magnification (boxed region on top), scale bar 100 nm, 15 000× magnification.

## 2.6. Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.02. Neutralising antibody and CVB1-specific IgG, IgG1 and IgG2a titres were analysed with Mann-Whitney *U* test.

## 3. Results

### 3.1. Optimised production and purification of CVB1-VLPs

CVB1-VLPs were produced in High Five insect cells, isolated from the clarified insect cell culture supernatants by TFF and purified with a combination of subsequent anion and cation exchange chromatographic steps. As final purification step, VLPs were eluted from the SO<sub>3</sub> cation exchange chromatography column at a NaCl concentration of 200 mM (Fig. 1a). The yield after purification was approximately 3 mg/L, which is approximately six times higher than the yield of CVB3-VLP in our previous study (Koho et al., 2014). SDS-PAGE analysis and subsequent silver staining (Fig. 1b, left panel) of the purified proteins revealed three prominent proteins of approximately 38 kDa, 31 kDa and 26 kDa corresponding to the CVB1 capsid proteins VP0, VP1 and VP3. The purity confirmed by silver staining was > 95% for the VLP as measured by densitometric analysis of the SDS-PAGE gel. VP1 and VP3 capsid proteins were also detected by Western Blotting using a rabbit anti-CVB1-6 polyclonal antibody (Fig. 1b, right panel). DLS analysis showed that 100% of the particles had an average hydrodynamic diameter of 30.5 (± 0.23) nm and the sample was very monodisperse (polydispersity index, Pdi: 0.065 ± 0.005) indicating that no aggregation or disintegration of VLPs occurred during the production and purification. TEM imaging revealed intact particles with the correct morphology with an average size of 30 nm (Fig. 1d).

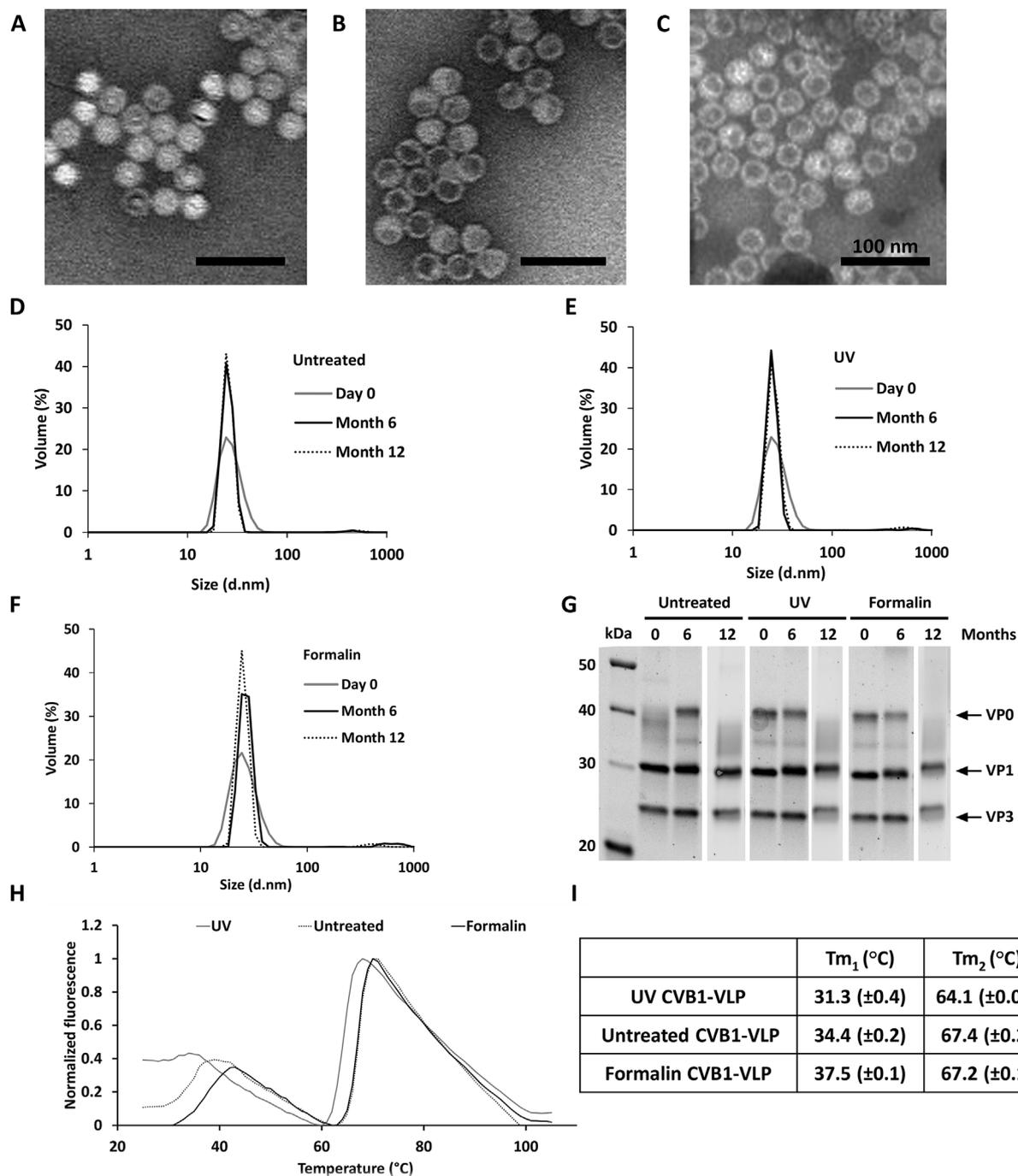
### 3.2. CVB1-VLPs remain stable for 12 months at 4 °C

An optimal vaccine-candidate is immunogenic and stable during storage. Chemical crosslinking and genetically engineered disulphides have been used to improve the stability of viruses and VLPs (Frietze et al., 2016; Rohovie et al., 2017; Adeyemi et al., 2017; Fox et al., 2017). We treated the purified VLP with UV and formalin to study their effects on VLPs stability and immunogenicity. According to TEM, treatment by UV or formaldehyde did not affect the morphology of the VLPs (Fig. 2a–c). An important indicator of the vaccine stability is particle size, as VLPs might dissociate into subunits or aggregate during storage, causing changes in particle size and polydispersity index (Pdi), that both can be monitored by DLS. Here, differently formulated CVB1-VLP vaccines were stored at 4 °C in M199-Tween80 buffer for twelve months and analysed by DLS on day 0 as well as after six and twelve months (Fig. 2d–f). After the twelve months storage period, light scattering intensity and Pdi of untreated, formalin or UV treated VLPs were found to increase slightly, while the mean particle sizes did not change (Table 1). These findings indicate that a minor part of the VLP samples may have been aggregated during the storage, but in general the VLPs were stable over 12-month storage.

The concentration of the untreated CVB1-VLP vaccine was determined using a BCA assay. Concentrations of the differently formulated vaccines from day 0 and after six and twelve months storage at 4 °C were analysed by densitometric analysis, according to which the concentration of the vaccines did not change during the twelve months storage period (data not shown). Additionally, the three CVB1 capsid proteins VP0, VP1 and VP3 were detected by total protein staining of the vaccines in a SDS-PAGE gel (in some of the samples the VP0 protein band was blurry in appearance) (Fig. 2g, Supplementary Fig. 1).

### 3.3. Formalin treatment stabilises CVB1-VLP at elevated temperatures

Previous studies have indicated EV-VLPs to have a lower thermal



**Fig. 2.** The stability profiles of untreated, UV and formalin treated CVB1-VLP vaccines. Transmission Electron Micrographs of chromatography purified (A) untreated, (B) UV treated and (C) formalin treated CVB1-VLP vaccines. Scale bars 100 nm. 25 000× magnification. VLPs formulated in the vaccine buffer (M199–0.1% Tween80) stored at 4 °C for zero, six and twelve months were analysed by dynamic light scattering (DLS) for their size and volume distributions: (D) untreated CVB1-VLP, (E) UV treated CVB1-VLP and (F) formalin treated CVB1-VLP. (G) SDS-PAGE analysis of the CVB1-VLP vaccines from day zero and after storing at 4 °C for six and twelve months. 2 µg of each vaccine were loaded per well. (H) Thermal stability profile of UV treated, untreated and formalin treated CVB1-VLP with Differential Scanning Fluorimetry. SYPRO Orange, a fluorescence dye binding to hydrophobic amino acid residues was used to analyse the unfolding or denaturation of the capsid proteins and, thus the conformational stability of the VLPs. Fluorescence intensity of the dye in the presence of VLPs was plotted as a function of the temperature, and melting temperatures (T<sub>m</sub>) of the VLPs were derived from the inflection points of the transition curve using the Boltzmann equation (Niesen et al., 2007). (I) T<sub>m</sub> values of different VLP preparations determined from fluorogram. The data is shown as an average of three independent measurements ± Standard Deviation.

stability compared to native viruses (Koho et al., 2014; Adeyemi et al., 2017; Fox et al., 2017), which may be due to a lack of capsid-genome interactions and also due to the absence of VP0 cleavage. Differential Scanning Fluorimetry (DSF) was used to analyse the thermal stability of the differently formulated CVB1-VLP vaccines. Two SYPRO orange

peaks were detected, with the first (T<sub>m1</sub>) being assigned to capsid expansion (Adeyemi et al., 2017) and exposure of hydrophobic residues, whereas the second (T<sub>m2</sub>) was assigned to denaturation of the VLPs (Adeyemi et al., 2017) (Fig. 2h). UV treatment lowered the final denaturation temperature by three centigrade, while formalin treatment

**Table 1**  
DLS analysis of CVB1-VLPs stored at 4 °C.

	Day 0				Month 6			
	d (nm)	Vol (%)	Pdi	SI	d (nm)	Vol (%)	Pdi	SI
CVB1-VLP	27.3 ( ± 0.53)	100 ( ± 0.0)	0.13 ( ± 0.04)	7962 ( ± 90)	25.4 ( ± 1.17)	98.8 ( ± 0.47)	0.75 ( ± 0.01)	18603 ( ± 1303)
UV CVB1-VLP	26.7 ( ± 0.44)	99.9 ( ± 0.12)	0.15 ( ± 0.02)	7549 ( ± 70)	25.0 ( ± 0.98)	98.43 ( ± 1.33)	0.86 ( ± 0.09)	19829 ( ± 1566)
Formalin CVB1-VLP	26.1 ( ± 1.0)	100 ( ± 0.0)	0.13 ( ± 0.01)	7078 ( ± 97)	26.6 ( ± 0.64)	96.5 ( ± 1.5)	0.82 ( ± 0.11)	23757 ( ± 1912)
	Month 12							
	d (nm)	Vol (%)	Pdi	SI				
CVB1-VLP	25.4 ( ± 0.53)		98.4 ( ± 0.40)		0.85 ( ± 0.13)		16343 ( ± 1870)	
UV CVB1-VLP	25.7 ( ± 0.81)		97.3 ( ± 1.0)		1.00 ( ± 0.01)		19438 ( ± 588)	
Formalin CVB1-VLP	24.8 ( ± 0.20)		97.8 ( ± 0.58)		0.81 ( ± 0.02)		20524 ( ± 1466)	

did not affect the final denaturation temperature (Fig. 2i). These results demonstrate, that formalin treatment stabilises the VLP, whereas UV treatment destabilises it.

### 3.4. Non-adjuvanted CVB1-VLP vaccines induce robust neutralising antibody responses in BALB/c and C57BL/6 J mice

To study the immunogenicity of the CVB1-VLPs as vaccines, we first performed a dose response study with the untreated CVB1-VLP in BALB/c mice. Animals were immunised twice with 0.3 µg, 1 µg or 10 µg non-adjuvanted CVB1-VLP i.m. (day 0 prime immunisation and day 21 boost immunisation) (Fig. 3a). Sera taken 35 days after the prime immunisation were evaluated for their CVB1 neutralising ability *in vitro*. All immunised mice generated neutralising antibodies by day 35 (Fig. 3b). There were no statistically significant differences in the responses between the different doses, although the variation within each group decreased with the increasing dose (Fig. 3b). Geometric mean titres (GMTs) of the neutralising antibodies were 256, 1351 and 446 for mice injected with 0.3, 1 and 10 µg CVB1-VLP, respectively.

The immunogenicity of the differently formulated CVB1-VLP vaccines were tested in C57BL/6 J mice by injecting three 1 µg doses of non-adjuvanted vaccines i.s. to groups of ten mice (Fig. 3c). After the prime (day 21), boost (day 35) and second boost (day 42) immunisations, neutralising antibody GMTs were 2, 11 and 97 for the mice immunised with untreated CVB1-VLP, 2, 11 and 84 for the mice immunised with UV treated CVB1-VLP and 18, 256 and 1351 for the mice immunised with formalin treated CVB1-VLP. On day 49, the neutralising antibody response had waned for all the CVB1-VLP groups, being 42 (untreated), 21 (UV treated) and 588 (formalin treated). At all time points, the group vaccinated with formalin treated CVB1-VLP mounted significantly stronger neutralising antibody responses than the groups vaccinated with untreated CVB1-VLP or UV treated CVB1-VLP (Fig. 3d).

### 3.5. CVB1-VLP vaccine-induced serum IgG antibodies and the profile of IgG1 and IgG2a antibody responses

In the first vaccination experiment, a dose-dependent CVB1-specific IgG antibody response was observed in BALB/c mice immunised twice with 0.3, 1 or 10 µg of untreated CVB1-VLP and the IgG-levels were significantly higher in the group immunised with 1 µg and 10 µg CVB1-VLP compared to the groups immunised with 0.3 µg (Fig. 4a). On day 35 the GMTs were 16890, 135118 and 178289 for the mice immunised with 0.3, 1 or 10 µg of untreated CVB1-VLP, respectively.

The dichotomy of IgG1 and IgG2a immunoglobulin subtypes as markers for Th2 and Th1 type responses was investigated. A strong dose-dependent Th2 type (IgG1) response was detected for the CVB1-VLP immunised mice (Fig. 4b), GMTs being 2786, 135118 and 310419 for IgG1 in the groups immunised with 0.3, 1 or 10 µg of untreated

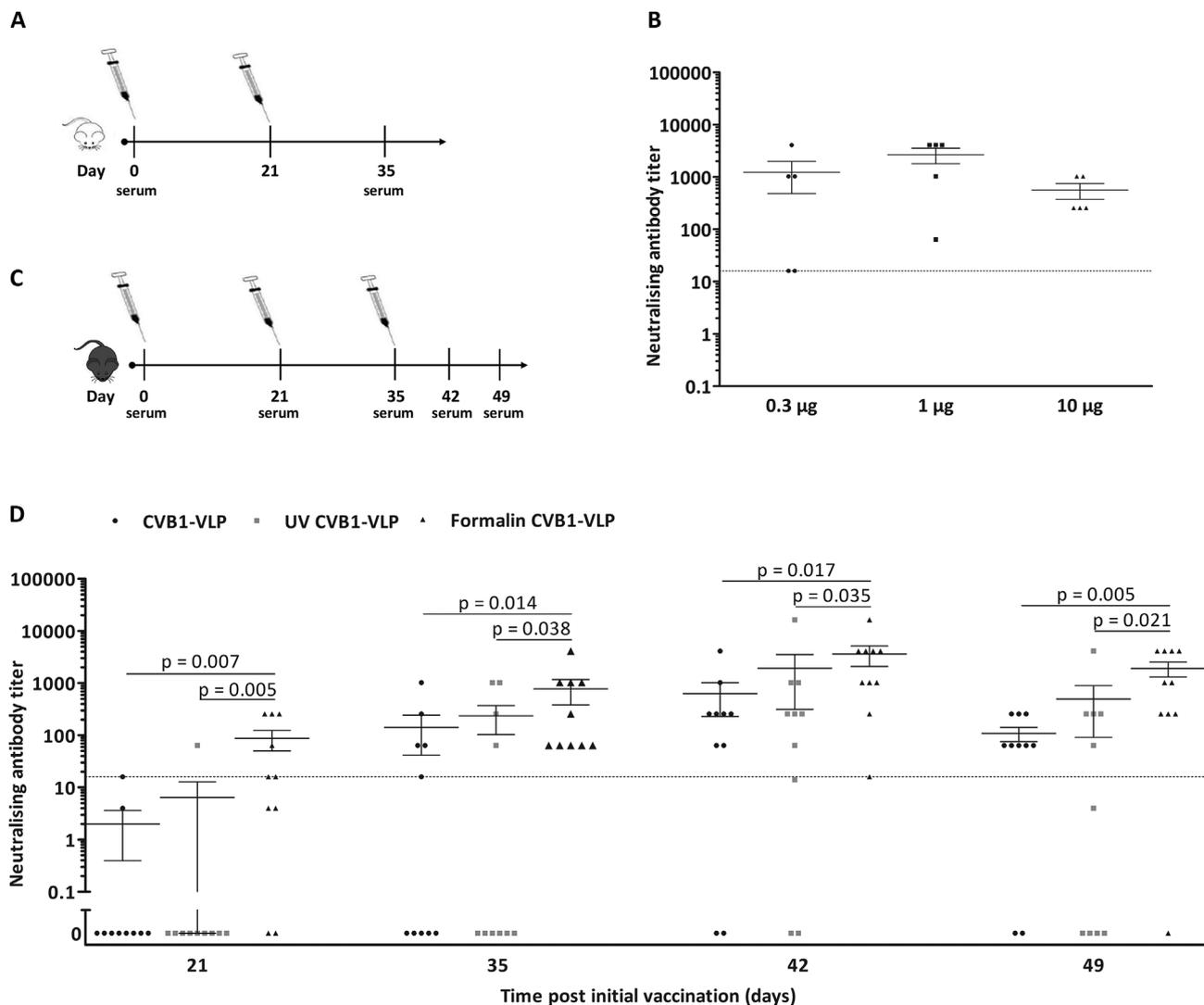
CVB1-VLP. Further, a much weaker CVB1-specific dose-dependent Th1-type (IgG2a) response was detected, the GMTs being 800, 2111 and 29407 in the groups immunised with 0.3, 1 or 10 µg CVB1-VLP. However, the IgG2a-response was significantly higher in the group immunised with 10 µg compared to 0.3 µg CVB1-VLP (Fig. 4b). Therefore, the results show an induction of both Th1 and Th2 type responses in BALB/c mice, with IgG1 antibodies being predominant.

In the second vaccination experiment C57BL/6 J mice immunised with untreated, UV or formalin treated CVB1-VLP were positive for CVB1-specific IgG on day 49 after the prime immunisation, GMTs being 2111, 1600 and 22286, respectively. The strongest IgG response was detected in mice immunised with formalin treated CVB1-VLP, and the response was significantly higher compared to the group immunised with untreated CVB1-VLP or UV treated CVB1-VLP (Fig. 4c). Untreated, UV and formalin treated CVB1-VLP all induced CVB1-specific Th2 type (IgG1) responses in C57BL/6 J mice, with the response being significantly higher in the formalin treated CVB1-VLP group than in the UV treated CVB1-VLP group (Fig. 4d). The IgG1 GMTs were 1838, 1213 and 29407 for the mice immunised with untreated, UV or formalin treated CVB1-VLP. The Th1 type (IgG2a) response was negative for all groups (Fig. 4d). No CVB1-VLP specific IgG, IgG1 or IgG2a antibodies were detected in the sera of BALB/c or C57BL/6 J control mice.

## 4. Discussion

CVB1-6 are known as the most common viral cause of human heart infections (Gaaloul et al., 2014). CVBs share many characteristics with other EVs such as polioviruses, and their manifestations vary from mild or subclinical to severe and even fatal outcomes (Verma et al., 2009; Wikswa et al., 2009; Centers for Disease Control and Prevention (CDC), 2008; Chu et al., 2015). The clinical course of CVB1-caused diseases can vary from cardiac and pancreatic acinar damage to heart failure and pancreatic deficiency (Benjamin et al., 2018). Currently there are no treatments or vaccines available against CVBs. In the present study, our objective was to develop an efficient production and purification method for CVB1-VLP and to study its stability and immunogenicity in two mouse strains.

Commercially available VLP-vaccines, such as HPV vaccine Cervarix® are based on highly purified VLPs produced in a baculovirus-insect cell production system (Artemchuk et al., 2019). Previously, we have produced a CVB3-VLP vaccine in insect cells and purified it with ion exchange chromatography (Koho et al., 2014). In the present study we aimed to enhance the VLP yield by utilising a similar expression construct design as for the previously produced EV71-VLP (Lin et al., 2015) and a baculoviral (flashBAC) genome containing deletions in the non-essential protease genes (Hitchman et al., 2010). These changes reduce the toxic effects of enteroviral (Laitinen et al., 2016) and baculoviral proteases (Hitchman et al., 2010) on the production cell line. The 3CD protease was produced in low levels by utilising CMV

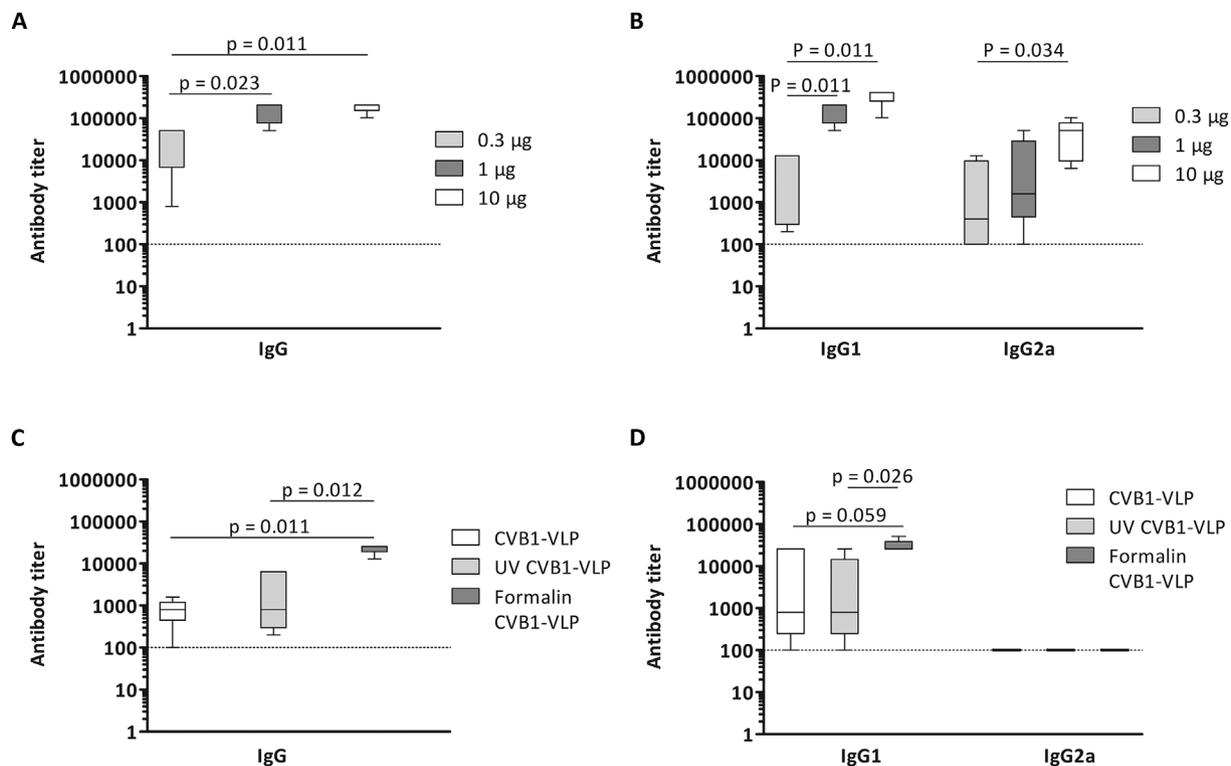


**Fig. 3.** CVB1-VLP vaccines induce neutralising antibody responses in BALB/c and C57BL/6J mice. (A) Schematic showing the experimental timeline of the CVB1-VLP vaccinations in BALB/c mice. The immunogenicity of the CVB1-VLP vaccine was tested by twice injecting 0.3, 1 or 10 µg non-adjuvanted vaccine i.m. to five mice. (B) CVB1 neutralising titre in the sera of BALB/c mice ( $n = 5$ ) immunised with CVB1-VLP vaccine in samples taken 35 days after the prime vaccination. (C) Schematic showing the experimental timeline of the vaccinations in C57BL/6 J mice. The immunogenicity of the differently formulated CVB1-VLP vaccines was tested in C57BL/6 J mice by a three injections of 1 µg non-adjuvanted vaccines i.s. (D) CVB1 neutralising antibody titres induced by differently formulated vaccines at the indicated time points ( $n = 10$ ). Mean neutralising antibody titres are indicated by the line  $\pm$  SEM. Probability values of the differences between the differently formulated vaccines for each time point were determined by Mann-Whitney  $U$  test. The dotted line represents the positive/negative threshold of neutralising capacity (dilution 1:16) in the virus neutralisation assay.

promoter, that drives weak expression in insect cells (Lin et al., 2015). In the CVB1-VLP concentration step we replaced the initial PEG precipitation step causing a loss of viral particles (Hankaniemi et al., 2017) with TFF, and further purification was performed with ion exchange chromatography. Both methods are suitable for an industrial scale production, which is a prerequisite required for the translation of experimental vaccines to clinical trials.

The stability of vaccines has a major impact on the success of an immunisation program, and to assure vaccine quality their stability needs to be evaluated. Immunogenic, but unstable poliovirus-like particles have been produced previously (Urakawa et al., 1989; Rombaut and Jore, 1997). To combat the instability and to improve the immunogenicity of these VLPs, chemical crosslinking and genetically engineered disulphides have been utilised and these particles have been shown to be extremely stable, generating high levels of neutralising antibodies in animal models (Frietze et al., 2016; Rohovie et al., 2017; Adeyemi et al., 2017; Fox et al., 2017). UV irradiation and formalin treatments have also been used in the inactivation of poliovirus for

vaccine development (Tano et al., 2007; Furuya et al., 2010). The effect of formalin treatment on each poliovirus epitope varied, and loss of viral antigenicity was observed in UV inactivated poliovirus that showed antigenic and morphological changes (Tano et al., 2007). Similarly, a UV inactivated EV71 vaccine induced a weaker immune response than a formalin inactivated EV71 vaccine (Chang et al., 2012) and similar effects were observed in the case of CVB1 vaccine (Hankaniemi et al., 2019). Formalin cross-links the functional groups of amino acids, forming non-reversible methylene bridges (Ramos-Vara, 2005), whereas UV causes the formation of thymine dimers (Battigelli et al., 1993). In a previous study we observed 12-day formalin treatment to cause CVB1 virus capsid disintegration (Hankaniemi et al., 2017) and this was also observed with intense UV-C irradiation (unpublished results). In the current study, we studied the effects of UV and formalin on the stability and immunogenicity of CVB1-VLPs and found formalin treatment to improve the thermal stability of the capsid and to enhance the immunogenicity in C57BL/6 J mice, whereas UV treatment decreased the thermal stability. Therefore, formalin treatment of other



**Fig. 4.** CVB1-VLP specific serum IgG, IgG1 and IgG2a antibody ELISA responses in BALB/c and C57BL/6J mice. (A and B) Level of anti-CVB1 (A) IgG, (B) IgG1 and IgG2a in the sera of female BALB/c mice vaccinated with different CVB1-VLP doses from day 35. (C and D) Level of anti-CVB1 (C) IgG, (D) IgG1 and IgG2a antibodies in the sera of C57BL/6 J mice vaccinated with untreated, UV treated or formalin treated CVB1-VLPs from day 49. The box plots show the antibody titres, expressed as the highest sample dilution giving a positive reading ( $n = 5$ ). The box is defined by the interquartile range (the 25th and 75th percentiles of the distribution), the horizontal line within the box represents the median (50th percentile) and vertical lines extend to the most extreme observation. The positivity cut-off OD value was calculated as follows: control mice mean OD + 3 x SD. An arbitrary titre of 100 (a half of the serum titre 200) was assigned as the positive/negative threshold (indicated by horizontal line).

VLPs might provide a simple method to produce more stable and immunogenic entero- and other viral VLP vaccines. We hypothesise that the virus epitopes inducing the neutralising antibodies and resulting in protective immunity against CVB1 might be better preserved when the VLP is treated with formalin. Alternatively, it is possible that the capsid proteins chemically modified by formalin act like adjuvants, triggering a stronger immune response.

The efficacy of most licenced VLP-vaccines has been optimised by including adjuvants in the vaccine formulas (Cimica and Galarza, 2017). To the best of our knowledge, all immunogenicity studies with EV-VLPs have also included adjuvants (Zhang et al., 2012, Zhang et al., 2018a, Zhang et al., 2018b; Dai et al., 2018; Salmons et al., 2018; Shen et al., 2016; Liu et al., 2012; Koho et al., 2014; Wang et al., 2017; Ku et al., 2014). Adjuvants are immunomodulatory substances that improve the quality, magnitude and duration of the immune response. Generally, it is thought that vaccines composed of VLPs may not sufficiently trigger the innate immunity response to the level required for optimal stimulation of the adaptive immune system (Cimica and Galarza, 2017). However, VLP structures present a repetitive arrangement of antigens that promote B cell activation and can activate a strong humoral immune response, as well as enhanced T-cell stimulation and cellular immunity (Braun et al., 2012). Therefore, we explored the immunogenicity potential of CVB1-VLP vaccines without adjuvant in mice.

CVB infections induce rapid and strong neutralising antibody responses in humans (Torfason et al., 1987) and mice (Stone et al., 2018; Svedin et al., 2017) and the protective efficacy of vaccines correlates well with the magnitude of the antibody response (Stone et al., 2018; Koho et al., 2014; Hankaniemi et al., 2017; Hankaniemi et al., 2019). Here, high neutralising and total IgG antibody levels were detected in

female BALB/c mice in a dose-response study using untreated CVB1-VLP vaccines, and the IgG-response was significantly higher with increased vaccine dose. The magnitude of the IgG antibody response and the preferential patterns of antibody class switching are influenced by the number of immunisations and through the route by which the vaccine is administered (Vidarsson et al., 2014). Previously it has been shown that the immune responses of BALB/c and C57BL/6 J mice to CVB3 infection differ (Leipner et al., 2004). C57BL/6 J mice demonstrated a stronger IgG response than BALB/c mice, and virus elimination from the myocardium of C57BL/6 J mice was also more efficient, with the virus-specific IgG contributing significantly to the rate of CVB3 clearance (Leipner et al., 2004). Most currently licensed vaccines, including the inactivated poliovirus vaccine in the market are administered either by intramuscular or subcutaneous needle injections (Zhang et al., 2015). In the current study we wanted to test whether both routes of administration provide immunity in two mouse strains differing radically in their Th1/Th2 responses. Therefore, BALB/c mice were immunised twice i.m., whereas C57BL/6 J mice were immunised three times i.s. and both immunisation routes were found to be effective in mice. However, it is difficult to compare the antibody responses between the mouse strains because of the differences in the experimental set-up. BALB/c mice did produce CVB1-specific dose-dependent IgG1 antibodies rather than IgG2a antibodies, demonstrating a skew towards a Th2-type immune response. Also, gender-specific differences have been previously observed in the CVB3-specific antibody response in BALB/c mice (Huber and Pfaeffle, 1994). CVB3-infected female BALB/c mice generate more IgG1 isotype than IgG2a, whereas in male mice IgG2a is more prevalent (Huber and Pfaeffle, 1994). Consistent with these findings, also we detected a predominantly Th2 type (IgG1) phenotypic response in the CVB1-VLP vaccinated female BALB/c mice.

In humans it has been shown that the EV71-neutralising activity is mainly mediated by the IgG1 isotype and only to a lesser extent by the IgG2 isotype (Cao et al., 2013). Fittingly, an EV71-VLP vaccine has been shown to induce high levels of IgG1 (Cao et al., 2015).

When the immunogenicity of differently treated CVB1-VLPs were compared in C57BL/6 J mice, we found that formalin treated CVB1-VLP induced the strongest neutralising antibody and CVB1-specific IgG response. Similarly, formalin treated CVB1-VLP induced significantly higher Th2 type responses in C57BL/6 J mice than untreated and UV treated CVB1-VLPs, whereas Th1 type (IgG2a) responses were negative for all CVB1-VLP immunised C57BL/6 J mice.

## 5. Conclusion

We have developed improved protocols for the production and purification of CVB VLP-vaccines. Immunisations with non-adjuvanted CVB1-VLPs resulted in high titres of neutralising antibodies and total IgG antibodies, and a stronger Th2 type (IgG1) rather than Th1 type (IgG2a) response in mice. Non-adjuvanted CVB1-VLP vaccines are highly immunogenic and formalin treatment improves their stability and immunogenicity. Future studies examining the cross-reactivity and longevity of CVB-VLP elicited immunity should be conducted.

## Declaration of interest

HH owns stocks and is the chairman of the board of Vactech Ltd, which develops vaccines against picornaviruses. HH and MFT serve on the scientific advisory board of Provention Bio Inc., which is developing an enterovirus vaccine. The other authors have no conflict of interest to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.104595>.

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