Tampere University

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SETTING UP AN ACUTE INFECTION MODEL FOR CVB1 IN MICE

Faculty of Medicine and Health Technology

Master's thesis

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ABSTRACT

Mustonen, Iiris Mariitta: Setting up an acute infection model for CVB1 in mice

Master's Thesis

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Background and aims: Coxsackie B viruses (CVB) under Enterovirus (EV) genus are small yet highly contagious human pathogens. CVB infection can cause severe inflammation in multiple organs, including heart and pancreas. Infection is also linked with diseases such as type 1 diabetes (T1D) and dilated cardiopathy (DCM), among others. The pathogenic mechanisms of CVBs and their role in disease aetiologies are not well known. In addition, there are no treatments or vaccines against CVBs. The aim of this thesis is to set up an animal infection laboratory for Tampere University and to set up an acute infection model for CVB1 in mice. Animal infection model would enable research on CVB infection mechanisms, disease associations and testing for preventive strategies.

Methods: CVB1 was produced in Green Monkey Kidney (GMK) cells and purified by ultracentrifugation. Purified virus was characterized with SDS-PAGE, Western blot, Dynamic Light scattering and BCA total protein quantification. Infectivity of the virus was measured with tissue culture infectious dose 50% ($TCID_{50}$) method. Animal infection laboratory was established, and operating procedures were determined according to risk assessment and mock studies, among other factors. Infection model was established by intraperitoneal inoculation of CVB1 in BALB/c mice. Infection studies were conducted in four separate studies. The model was optimized according to infectious dose, study length, and sex. The infection was determined from the amount of replicative virus in the pancreas, heart, and blood by $TCID_{50}$ method. During the studies, wellbeing of the mice was evaluated with body score indexing, and weights were measured daily.

Results: Induction of systemic CVB1 infection in BALB/c mice was successful. Replicative virus was most consistently found in pancreas but was also detected from blood samples until day 4. Viremia increased during the first 4 days post infection (dpi) in female mice. Viral doses 10^6 and 10^7 $TCID_{50}$ per mouse were able to cause infection in the pancreas most consistently. Replicative virus seemed to be cleared from blood and pancreas around 7 dpi. Female mice had higher viral titres in pancreas 4 dpi compared to male mice. Wellbeing of male mice deteriorated during the infections as indicated by diarrhea and ruffled fur. Also, weights decreased significantly. Female mice, on the other hand, did not show external signs of infection and the weights remained similar. Replicative virus was not found in heart at any points of the studies.

Conclusions: The aims of the thesis were successfully completed. The animal infection laboratory was set up and we were able to induce systemic CVB1 infection in mice. For further optimization of the infection model, we should use the optimized viral doses of 10^6 and 10^7 $TCID_{50}$. For detection of replicative virus in pancreas, the infection should be followed 4 dpi. Moreover, our results support the use of female mice to reduce animal suffering. This infection model could further enable conduction of challenge studies, where safety and efficacy of vaccines can be tested. Moreover, established facility would support infection models with different viruses serotypes or modelling CVB induced diseases.

Keywords: Coxsackie B virus, CVB1, infection model, BSL-2, $TCID_{50}$ method, mouse model, disease association, viral dose optimization

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TIIVISTELMÄ

Mustonen, Iiris Mariitta: Akuutti infektiomalli CVB1 virukselle hiirissä.

Pro Gradu -tutkielma

Tampereen yliopisto

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Tutkimuksen tausta ja tavoitteet: Enteroviruksiin (EV) kuuluvat Coxsackie B virukset (CVBt) ovat pieniä mutta helposti tarttuvia ihmisen patogeenejä. CVB voivat aiheuttaa vakavia tulehduksia useissa elimissä kuten sydämessä ja haimassa. Infektio on myös liitetty vakaviin sairauksiin kuten tyypin 1 diabetekseen (T1D) ja sydänlihassairauteen. Viruksen tapaa aiheuttaa infektioita tai sairauksia ei kuitenkaan tunneta hyvin. Virusta vastaan ei myöskään ole rokotteita tai hoitomuotoja. Tämän pro gradu -tutkielman tavoitteena on pystyttää infektiolaboratorio Tampereen Yliopiston prekliiniselle osastolle, sekä luoda infektiomalli CVB1 virukselle BALB/c hiirissä. Hiiri-infektiomalli mahdollistaisi viruksen infektiomekanismin ja tautiyhteyksien tutkimisen, sekä ennaltaehkäisevien strategioiden testaamisen.

Tutkimuksen menetelmät: CVB1 virus tuotettiin GMK-soluissa ja puhdistettiin ultrasentrifugoimalla. Puhdistettu virus karakterisoitiin SDS-PAGE:n, Western blottauksen, dynaamisen valonsironnan ja BCA konsentraationmittauksen avulla. Viruksen infektiokyky testattiin $TCID_{50}$ -menetelmän avulla. Infektiolaboratorion pystytykseen kuului toimintamenetelmien määrittely muun muassa riskiarvion ja harjoitusinfektioiden pohjalta. Infektiomalli luotiin vatsaonteloon annosteltavalle CVB1 virukselle BALB/c hiirissä. Infektiotutkimukset tehtiin neljässä osassa. Infektiomalli optimoitiin virusannoksen, tutkimuksen pituuden ja hiirten sukupuolen perusteella. Infektio määritettiin $TCID_{50}$ -menetelmällä haimasta, sydämestä ja verestä löytyvän infektiivisen viruksen perusteella. Tutkimusten aikana hiirten hyvinvointia ja painon kehittymistä seurattiin päivittäin.

Tutkimustulokset: CVB1 infektio aiheutettiin BALB/c hiirissä onnistuneesti. Infektiivistä virusta löytyi pääosin haimasta. Viruksen määrä veressä lisääntyi naarashiirillä koko nelipäiväisen tutkimuksen ajan. 10^6 ja 10^7 $TCID_{50}$ virusannokset aiheuttivat infektion haimassa kaikista luotettavimmin. Infektiivinen virus näytti poistuneen haimasta seitsemännen päivän kohdalla infektion aloittamisesta. Virusta havaittiin enemmän naarashiirien haimoissa uroshiiriin verrattuna. Uroshiirten hyvinvointi heikkeni tutkimuksen aikana ripulin ja epäsiistin turkin perusteella. Myös painot laskivat merkittävästi. Naarashiirillä vastaavaa painonlaskua ei ollut ja vointi pysyi vakaana tutkimusten ajan. Infektiivistä virusta ei havaittu lainkaan sydämistä tutkimusten aikana.

Johtopäätökset: Tutkielman tavoitteet saavutettiin onnistuneesti. Infektiolaboratorio pystytettiin ja hiiret saatiin infektoitua CVB1 viruksella. Luodun infektiomallin kehittämistä varten tulisi käyttää 10^6 ja 10^7 $TCID_{50}$ virusannoksia. Jotta infektiivistä virusta havaittaisiin haimassa, tutkimuksen pitäisi kestää neljä päivää infektion aloittamisesta. Tutkimuksen tulokset tukevat myös naarashiirien käyttöä tulevissa tutkimuksissa eläimiin kohdistuvan haitan vähentämiseksi. Luotu infektiomalli voisi mahdollistaa esimerkiksi rokotehaastokokeiden tekemisen, millä varmistettaisiin rokotekandidaattien tehokkuus ja turvallisuus. Lisäksi pystytetty laboratorio mahdollistaisi myös muiden virustyyppien tai viruksen aiheuttamien sairauksien tutkimisen.

Avainsanat: Coxsackievirus B, CVB1, infektiomalli, BSL-2, $TCID_{50}$ menetelmä, hiirimalli, tautiyhteys, virusannoksen optimointi

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

PREFACE

This Master's Thesis was conducted in the Virology and Vaccine Immunology research group in the Faculty of Medicine and Health Technology, Tampere University.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABSL	Animal Biosafety Level
BCU	Bio containment unit
BSL	Biosafety level
CAR	Coxsackie- and Adenovirus Receptor
CVA	Coxsackievirus A
CVB	Coxsackievirus B
DAF	Decay-accelerating factor
DCM	Dilated Cardiomyopathy
dpi	days post infection
ER	Estrogen receptor
EV	Enterovirus
FBS	Fetal bovine serum
GMK	Green Monkey Kidney cell
i.p.	intraperitoneal
i.v.	intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KO	Knockout
MOI	Multiplicity of infection
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFU	Plaque-forming units
PP	Peyer’s patch
PRR	Pattern recognition receptor
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T1D	Type 1 diabetes
TCID50	Tissue culture infectious dose 50%
Th	T-helper cell
TLR	Toll-like receptor
VP	Viral protein

1. INTRODUCTION

Viruses are microscopic infectious agents that need living host cells for replication. Viruses can infect many life forms from bacteria to animals and humans. Enterovirus genus belongs to a large family of Picornaviruses which stands for “small RNA viruses”. Enteroviruses spread through faecal-oral route or respiratory transmission. Enteroviruses can disseminate from their primary infection site to several organs including central nervous system and heart. Among non-polio enteroviruses Coxsackieviruses are one pathologically important species which can be divided into two groups: Coxsackievirus A and B.[1]

Coxsackie B viruses (CVB) infections are mainly mild, but the viruses are also strongly associated with myocarditis, aseptic meningitis, and type 1 diabetes (T1D), for instance. CVBs spread though faecal-oral route and cause more severe conditions with younger individuals[2]. Even though the disease associations are widely known and there are research efforts considering CVBs, there is no comprehensive conclusion about their infection mechanisms and disease aetiologies.

Animal models, and especially mouse models are important for studying infection mechanism within a living organism. Established infection models provide information from viral kinetics to disease association. However, working with living animals require consideration of many factors affecting the infection. In addition to factors considering the virus such as serotype, dosage, and inoculation route, also details of used mice must be considered, including mouse strain, age, and sex[3]. Different infection designs will likely have different outcomes.

Conduction of infection models require specially equipped facilities to ensure safe working environment with an infectious agent[4]. Coxsackieviruses require biosafety level 2 (BSL-2) equipped facilities. Within animal facility this requires for example special biocontainment cages for the mice. In addition, working within BSL-2 require defined operating procedures ensuring safe and optimal working within the facility.

In the conducted study, an infection model was set up for CVB1 in mice. This thesis also included setting up a suitable animal infection laboratory for Tampere University, as there was no suitable area for it yet. Established infection model enables further research of CVB1 infection and provides a platform for other infection application, including disease models and challenge studies.

2. LITERATURE REVIEW

2.1 Coxsackieviruses

2.1.1 Overview of coxsackie B viruses

Coxsackieviruses are classified under enterovirus genus of Picornaviridae family. Coxsackieviruses are further divided into coxsackieviruses A (CVA) and B (CVB). CVBs are under enterovirus B species and further consist of six serotypes (CVB1-6). The serotypes can be further discriminated by virus strains. The most relevant information of classification of picornaviruses can be found from www.picornaviridae.com. Serotype classification was originally based on histopathologic lesions in suckling mice[5]. Currently, the serotypes are discriminated by molecular typing methods based on differences in the viral capsid protein VP1[1].

Structurally CVBs are small nonenveloped viruses with a diameter of 30 nm. Their 7.5 kb single-stranded RNA genome consist of structural and non-structural proteins. Structural proteins involve viral proteins (VP)1-4 proteins responsible for forming an icosahedral capsid having a pseudo T=3 symmetry[6]. Non-structural proteins include viral proteases and proteins required for viral replication and protein synthesis[7].

Coxsackieviruses are the most prevalent non-polio enteroviruses[8]. Just like enteroviruses in general, coxsackieviruses spread through faecal-oral route and by respiratory aerosols[9]. CVBs are highly contagious. Their prevalence peaks during summer or early fall on temperate countries. In warmer countries the prevalence can be constant. In addition, the prevalence varies within serotypes. For example, CVB1 and CVB3 have shown more constant prevalence compared to cyclic outbreaks of CVB2, CVB4 and CVB5[10].

Most of the infections are asymptomatic or subclinical. Infections are most likely to occur during the first years of life. However, the severity of the infection varies. The infections are often more severe in younger individuals and can be even fatal in neonates[1]. The infection can become chronic and cause remarkable harm in different organs[3]. If the infection spreads to secondary organs such as the heart, brain, pancreas, and liver, the infection can lead to severe diseases such as myocarditis, meningitis, pancreatitis, or hepatitis, respectively[11].

Table 1. *CVB serotypes and their disease associations.*

Disease	Serotype	Ref
Myocarditis, dilated cardiomyopathy	CVB1-6	[12,13]
Meningitis	CVB1-6	[13,14]
Encephalitis, meningoencephalitis	CVB1-6	[15]
Pancreatitis	CVB1, CVB3, CVB4	[13,16]
Neonatal systemic illness	CVB1-6	[12,15]
Hand, Foot, and Mouth Disease (HFMD)	CVB1, -2, -4, -5	[12,17]
Sepsis	CVB1, CVB3	[12]
Type 1 diabetes (T1D)	CVB1, CVB4	[12,18–21]

Different serotypes are linked with distinct diseases, as seen in Table 1 showing associations between disease and CVB serotypes. For instance, all serotypes are associated with myocarditis. However, CVB1 is especially linked with type 1 diabetes (T1D), whereas CVB3 with myocarditis and CVB2 with aseptic meningitis[12]. Therefore, even though two serotypes can cause a certain disease, one of them may cause a more severe condition. Thus, there are differences in the severity of the infection, virulence, and tissue tropism between CVB serotypes.

2.1.2 Infection mechanism of CVBs

Coxsackieviruses are mainly human pathogens. Spreading of the virus occurs through faecal-oral route and respiratory secretions. However, airborne transmission is not exclusively reported[8,10]. The primary infection site is therefore in respiratory or gastrointestinal tract. Overall, the infection mechanisms consist of virus invasion, translation, replication, assembly, and finally release. CVBs are mainly lytic viruses meaning the virus is released when infected cells rupture. However, enteroviruses can also be released through a non-lytic mechanism, wherein membrane-bound structures are shed from the cell membrane[1].

Virus invasion happens through receptor mediated endocytosis via specific coxsackie- and adenovirus receptor (CAR)[22–24]. All serotypes have been shown to bind CAR. In general, CAR is found in tight junctions as a cell adhesion molecule. CAR mRNA is expressed in tissues linked with CVB infection including human pancreas, heart, and brain. High expression of CAR has been seen in tight junctions of developing brain and heart[15]. Consistently, mouse models have shown knockout (KO) mouse for CAR do not show virus induced myocarditis and pancreatitis[25].

Baggen et al. discussed whether infection induces expression of CAR, since tissues that do not normally express CAR can be infected by CVBs[1]. CAR works together with a coreceptor called decay-accelerating factor (DAF/CD55), which assist in exposing the CAR receptor to the virus. DAF is an abundant component of cell membrane. CVB1, CVB3 and CVB5 have been shown to bind DAF[1]. Binding of DAF causes a conformational change leading to release of the viral RNA into cytosol. Despite the role of CAR in virus entry, a number of other receptors for cell entry have been identified[1,15].

Translation of the viral genome happens with the help of the host's ribosomes. It is suggested that cysteine proteases within the viral polypeptide start proteolytic processing already during the translation process[18]. Replication of the viral RNA happens in host-derived replication organelles and non-structural proteins of the genome are essential for its formation. Further, structural proteins are assembled into protomers and further pentamers to build the viral capsid. Virions mature after viral the RNA is inserted. Finally mature virions are released into the bloodstream in either lytic or nonlytic way. There isn't an agreement on which enteroviruses that can spread in a nonlytic way, and whether is linked only to specific tissues or cell types. If the infection is not cleared in the primary infection site, virus release into bloodstream enables secondary infection in susceptible tissues[1]. The life cycle of enteroviruses from entry to release is comprehensively illustrated in figure 1.

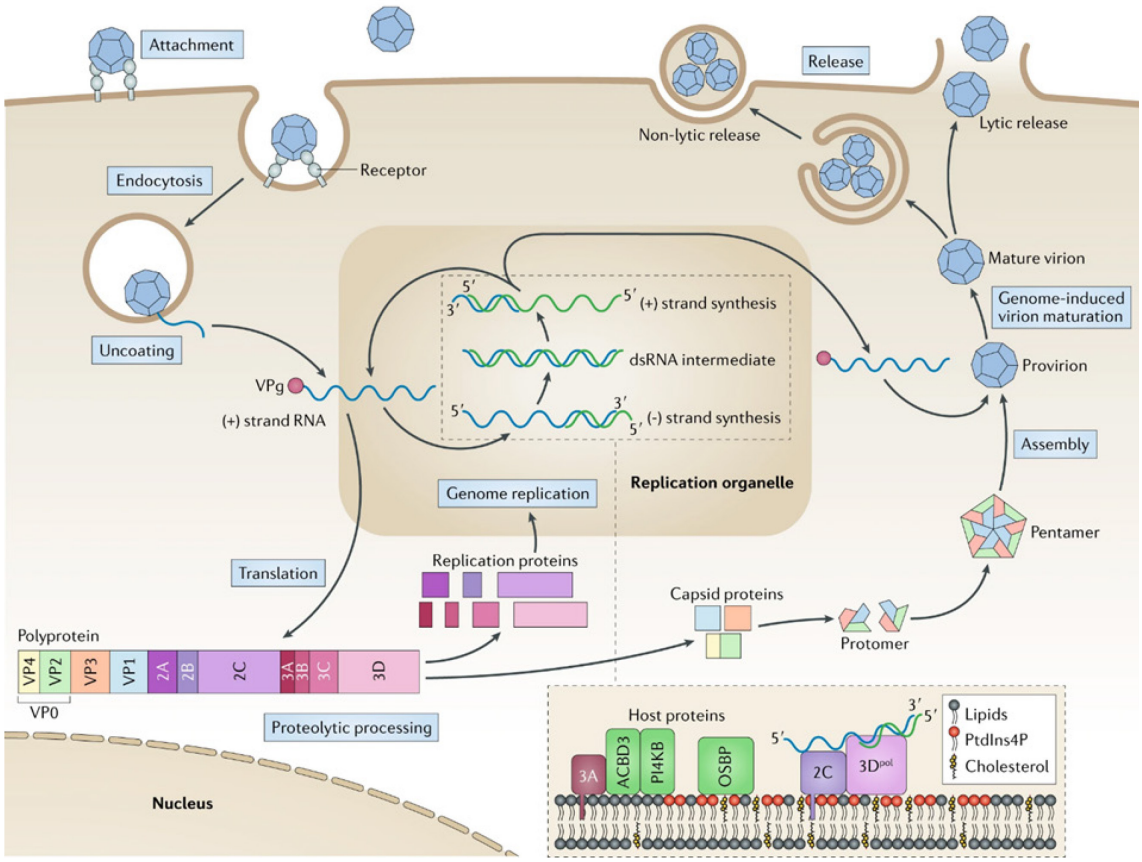

Figure 1. *Overview of enterovirus life cycle. Adapted from Baggen et al.*[1]

2.1.3 Immune response to CVBs

CVBs can elicit a robust immune response. Both parts of the immune response, innate and adaptive are activated upon CVB infection. Freely circulating viruses can be detected by immune cells and infected cells are recognized by presenting viral material on the cell surface. Proper innate immune response is needed for activation of adaptive immunity. Here, basics of immune response against CVBs are discussed. However, immune response to CVBs consists of many other aspects as well.

Innate immunity is activated upon the virus invasion. Innate immunity cells such as NK cells and sensor cells can detect viruses through pattern-recognition-receptors (PRRs) that detect pathogen-associated molecular patterns on the virus surface. Toll-like receptors (TLRs) on the surface of immune cells such as macrophages and dendritic cells are one example of PRRs essential for inducing innate immune response. TLRs activate signalling pathways that will eventually activate type 1 interferons and proinflammatory cytokines. TLR-3, TLR-7 and TLR-8 are shown to activate during virus infections in pancreas and B cells[26]. Interestingly, even though TLR-4 is not generally linked to viral in-

fections, it has been connected with CVB3 and CVB4 infections. TLR-4 induced proinflammatory cytokines have been seen in endomyocardial tissues and pancreas upon enterovirus infection, which may can affect the infection outcome in secondary tissues[27].

However, TLR-3 has been shown to be especially important in facilitating innate immune response against CVB3 in mice[28]. TLR-3 KO mice were not able to clear CVB3 infection and showed increased pancreatic injury and stronger inflammation in heart. In contrast, infected wild type mice recovered from infection. Overall, innate immunity aims to trigger antiviral state by blocking viral transcription, suppressing viral translation, and increasing antigen presentation to adaptive immune cells. Type 1 interferons (IFNs) are one important group of cytokines, which are upregulated upon viral infection and responsible for triggering the adaptive immune system[26].

Adaptive immune response is responsible for specific recognition and clearance of the virus. Its role is significant as CVBs can spread from primary infection site and disseminate into other organs. Cell mediated adaptive immunity functions through CD4+ and CD8+ T cells. Simplified, CD8+ cells can destroy infected cells and CD4+ cells orchestrate differentiation of B cells. B cells, in turn, are responsible for humoral response. One study discussed that the role CD4+ T cells is crucial for virus clearance and prevention of persistent cardiac inflammation. CD8+ T cells, on the other hand, would be associated with autoimmune response against cardiac myosin and not clearance of the virus[29]. In contrast, there are studies reporting that CD8+ cells are crucial for alleviating the infection[30]. In conclusion, mutual effect of cell mediated adaptive immunity is crucial in haltering CVB infection.

Humoral adaptive immunity orchestrated by B cells is crucial for creating antibodies and memory cell against CVB infections. Antibody response is necessary against persistent infections. Immunoglobulin M (IgM) and IgG responses have been shown to be mainly against the virus capsid protein VP1[31]. IgM response appears first and later decreases as the immunoglobulin isotype is switched to IgG. Formed IgG response, on the other hand, persists. Also, CD4+ T cells have a role in eliciting proper antibody responses. The balance between cell-mediated and humoral immunity is overall dependent of several cytokines, which drive the response to certain direction. Simplified, T-helper cell (Th)-1 response is pro-inflammatory and induces cell-mediated response, whereas Th2 is anti-inflammatory and leads to humoral response[26].

In conclusion, the immune response against CVBs is dependent on efficient responses of both, innate and adaptive immunity. In this review, only the fundamental aspects of immune responses against CVBs were discussed. It should be noted that the overall

response to eradicate the virus is highly complex. In addition, coxsackieviruses have a high potential to evade from host's immune responses[31], which is a topic which was not described herein.

2.1.4 CVB biosafety level

Studying infective agents such as viruses requires specified facilities to ensure safe work environment. The facility requirements are based on the biosafety level of the pathogen[32]. Coxsackieviruses are classified under Biosafety Level 2, according to the Finnish Ministry of Social Affairs and Health, under Section 40, Subsection 3 of the Occupational Safety and Health Act (738/2002). Pathogens that belong to level 2 can cause diseases to humans and thus be dangerous to workers. However, it is not probable for the virus to spread to the environment. Also, in general, there is an efficient cure or care for the disease.

Multiple aspects are considered in biosafety level laboratories. Some main aspects include risk assessment, laboratory design, personal protective equipment, and decontamination[4]. Evaluating possible risks is a crucial part of creating a safe work environment. Risk assessment includes determination of how pathogen infects, how long workers are predisposed to the pathogen, and what are the quantities of the pathogen[32]. Importantly, aspects of risk assessment are dependent on the workers, and therefore proper training is inevitable. Workers are mainly responsible for careful work, proper cleaning, and continuous validation of the procedures[33]. Overall, personnel must be trained to be competent in following advanced microbiological practices and procedures[4].

Proper designing of the biosafety laboratory, quality of the equipment and proper education further promote safe working. Core requirements for the facility include restricted access, appropriate labelling of biohazard warning, proper equipment such as biosafety cabinets, and appropriate waste management. Personal protective equipment is also one essential factor to be considered. For work in biosafety laboratory, designated laboratory clothing, footwear and gloves are mandatory. Different aspects of working in biosafety laboratory are discussed in Laboratory Biosafety Manual by World Health Organization[4].

Extensive guidelines and strict requirements are essential, as many occupational diseases are reported relating to working with biological agents[33]. Examples of potentially risky occupations include animal-care and laboratory workers. Risks within the work can be related to handling of infected laboratory rats and mice, handing of bodily fluids such as blood and handling of contaminate needles and surgical instruments. Modelling CVB

infections in mice include all these risk factors. Even though CVBs can infect humans, among viral agents enteroviruses were not reported to cause a significant risk for occupational acquisition of the agent[34].

2.2 Murine infection models

Preclinical infection models are used to study infection mechanisms in model animals, such as mice, rats, and rhesus macaque monkeys. These studies give crucial information about the pathophysiological processes caused to the host by the pathogen. Animal models enable research on diagnostics, treatment, and preventive strategies. Even though Coxsackieviruses are primarily human pathogens, they can infect, for instance, certain nonhuman primates and mice[35]. Because CVBs do not naturally infect mice, modelling the infection can be difficult. However, it is shown that CVBs efficiently replicate in suckling mice, causing pathological outcomes in several tissue types. In addition, mice models are claimed to be the best models within murine models for studying CVBs, considering a 3R principle discussed in section 2.4.[3]

There are several similarities in humans and mice when considering CVB infections. Mice have shown to express a murine homolog of the CAR receptor that can bind to different coxsackievirus serotypes[23]. Possibility to study same virus that infects humans in mice through similar receptor validates the experimental results from murine models. However, there are differences on CAR expression between humans and mice. Predominant CAR transcript in humans is reported to be expressed considerably in the heart, testis, prostate, and pancreas. In mice, abundant CAR expression is seen in liver, kidney, lung, and heart[24]. These differences can become crucial when studying specific disease models, as discussed in later sections. Yet, it should be noted that CAR expression alone does not directly cause or drive the infection. In addition, infancy predisposes to severe CVB infection in both humans and mice[1,3]. Consistently, disease models such as myocarditis models have demonstrated that CVB infected human and murine hearts histopathologically resemble each other[29]. These similarities further validate the modelling of CVB infection in mice, as it provides important information about the infection in humans as well.

However, there are certain challenges in operating with mice models. Often, the administration route is not natural, as the virus is for example inoculated into the peritoneum, instead of resembling the ordinary oral route. In addition, the viral doses must be large, containing even hundreds of millions of infective viral units/ml. Furthermore, working with infectious agents can be dangerous and require special facilities and procedures according to the biosafety level of the pathogen. Yet, despite the challenges, preclinical models

give essential information about the virus and infections. Alike, modelling of disease mechanisms and pathology following CVB infection is enabled with mouse models.

Table 2. *Simplified overview of different designs for CVB infection models in mice. In model column AV: antiviral, CS: challenge study, D: diabetes, IM: infection mechanism, M: myocarditis and SF: sex difference. In mouse strain SAM: Swiss Albino mouse and GMO: genetically modified mouse strain. For more detailed information see the reference.*

Model	Mouse strain	Serotype: dose	Unit	Route	Age (weeks)	Ref
AV	BALB/c	CVB5: 10^5	$TCID_{50}$	i.p.	3-4	[36]
CS	BALB/c, SOCS-1-tg	CVB1: 10^6 CVB3: $5x10^4$, 10^6 CVB4: 10^5	PFU	i.p.	10-12	[35]
CS	GMO C57BL/6	CVB1: 10^6 CVB3: 10^5	PFU	i.p.	18+	[37]
CS	NOD	CVB1: 10^6	PFU	i.p.	4-6	[38]
D	NOD	CVB3: $5x10^5$	$TCID_{50}$	i.p.	4+	[39]
D	NOD, SOCS-1-tg,	CVB1: 10^6	PFU	i.p.	4-7	[40]
D	NOD	CVB1: 10^7	PFU	i.p.	10-13	[41]
D	NOD	CVB3: $5x10^5$ CVB4: $5x10^5$	$TCID_{50}$	i.p.	4-8	[42]
IM	SAM	CVB3: $5x10^9$ CVB4: $5x10^9$	$TCID_{50}$	Oral	3-4	[43]
IM	CD1, SAM	CVB3: $5x10^3$-10^9 CVB4: $5x10^7$	$TCID_{50}$	Oral, i.p.	3-4	[44]
IM	C3H/HeJ	CVB1: 10^4	PFU	i.p.	3	[45]
IM	BALB/c	CVB2: $2,5x10^4$	PFU	i.p.	4	[14]
IM	GMO	CVB3: 10^5	PFU	i.p.	6-8	[25]
IM	C57BL/6, GMO	CVB3: 10^6-10^8	$TCID_{50}$	i.p.	5-7	[28]
IM	C57BL	CVB1: 10^4-10^8	PFU	Oral, i.p.	8-14	[46]

IM	BALB/c, C57BL/6…	CVB1: 10^3	$TCID_{50}$	i.p.	<1	[47]
IM	SAM	CVB3: 10^3-10^9	$TCID_{50}$	Oral, i.p.	3-4	[48]
IM	SAM, CD1, SJL, NOD...	CVB4: $2x10^6$	$TCID_{50}$	Oral	3-4	[49]
M	BALB/c,	CVB3: 10^3	PFU	i.p.	6-8	[50]
M	NOD,	CVB3: 10^4	PFU	i.p.	6-8	[51]
M	BALB/c, C57BL/6	CVB3: 10^4	PFU	i.p.	8-13	[52]
M	BALB/c	CVB3: 10^3	$TCID_{50}$	i.p.	6	[53]
M	NMRI, BALB/c,	CVB3: 10^5-10^8	$TCID_{50}$	i.p., i.v.	6	[54]
SF	GMO C57B/6	CVB3: 10^4-10^8	PFU	Oral, i.p.	8-14	[30]
SF	BALB/c	CVB3: 10^4	PFU	i.p.	6	[55]
SF	C57BL/6	CVB3: $5x10^7$	PFU	Oral	10-15	[56]

As seen in Table 2, there are several different mouse strains, infection routes, and infection models used in preclinical studies considering CVB infections. The infection is confirmed with numerous methods varying form immunohistochemical analysis and imaging techniques to Polymerase Chain Reaction (PCR) and viral end-point dilution assays. Reverse transcriptase-PCR (RT-PCR), for instance, can indicate the presence of viral RNA in organ samples. The presence of replicating, infective virus can be detected with end-point dilution assay such as plaque assay and tissue culture infectious dose 50 ($TCID_{50}$) method. The result from plaque assay is reported in Plaque Forming Units (PFU). The infection can also be evaluated according to the wellbeing of the mice. Reported clinical symptoms for the infection include inactivity, emaciation, limb weakness, hair thinning, hunching and even death[47]. It should be noted that infection models are mostly reported with CVB1 and CVB3 serotypes. Even though the effects of CVBs can be similar between serotypes, these studies will not comprehensively represent the coxsackievirus B group.

2.2.1 Designing an infection model

Many aspects affect infection outcomes in murine models, including the genetic background of virus and mice to begin with. Therefore, it is crucial to take in consideration the

aim of the infection study and choose an appropriate design according to it. For example, design for studying infection in heart should be different than for testing the effect of inoculation route. Most important aspects to consider when designing an infection are discussed in this section.

Mouse strain. Different mouse strains can have a distinctive response to infections, and thus it must be carefully considered which strains to use in specific infection models. Inbred mice, such as BALB/c and C57BL/6 tend to be more susceptible for CVBs than outbred mice, like Swiss albino mice[21]. Yin et al tested the susceptibility to infection in different mouse strains when establishing a lethal neonatal model for CVB1[47]. They reported that inbred mice were more sensitive to CVB1 infection, and especially BALB/c strain is ideal for infection model according to their modest individual differences and coherent genetic background. They summarized that the major pathological changes affected pancreases which showed signs of necrosis and inflammatory infiltration. They also concluded that age and viral dosage greatly impact the success of the infection.

Leipner et al. studied differences on CVB3 infection between BALB/c and C57BL/6 mouse strains, as they are reported to have distinct immune responses[52]. They demonstrated only mild differences between the mouse strains when studying outcome of a CVB3 induced myocarditis. Their conclusion reported somewhat faster clearance of the virus from myocardium in C57BL/6 mice, which could be because of a stronger IgM but weaker IgG response when compared to BALB/c.

Interestingly, Precechtelova et al. showed no differences in viral kinetics and persistence between SJL, NOD, CD1 and Swiss albino mouse strains when they studied orally induced CVB4 infection[49]. They studied effects mainly in pancreas in different mouse strains, including outbred (CD1, Swiss albino), inbred (NOD, SJL) and genetically modified (NOD.SCID) strains. The main difference between the strains was mortality only in infected NOD and NOD.SCID mice. However, it is discussed that outbred mice would better represent the natural infection in humans, as they show normal genetic variation[43]. Genetically modified mouse strains can be used for specific study questions. For example, the role of CAR in infection can be studying by creating CAR KO mouse strain[25]. Similarly, mouse strains created to be diabetes-prone can be used in mouse models for diabetes[39], as discussed later. All in all, the mouse strain for specific infection model should be carefully determined.

Sex. There is evidence on differential immunopathogenic response in CVB3 infected male and female mice. Female mice expressed lower levels of virus receptors on cardiac

cells and therefore were only mildly affected by myocarditis compared to severe myocarditis in male BALB/c mice[55]. In addition, the polarity of the immune response differed. Females produced more interleukin 4 (IL-4) which drives towards Th2 phenotype. Male, in contrast, expressed more IFN-γ which drive towards Th1 phenotype. Similarly, female mice have shown differential profile of infiltrating macrophages to myocardium following a CVB3 infection[53]. Distinct myocardial-derived cytokines would drive to macrophage M1 phenotype in male mice and M2 phenotype in females. IL-4 is one inducer of M2 phenotype, which is consistent with results showing Th2 oriented immune response and higher production of IL-4 in females.

Furthermore, young male mice (6-8 weeks) were reported to be more susceptible CVB3 induced myocarditis and showed increased number of CD45+ cells compared to females[50]. However, another study showed protective CD4+ T cell response in female mice[46]. CD4+ T depleted female mice had increased mortality when compared to control group, whereas depletion did not significantly alter morbidity in male mice. One study challenged a common though of CVB3 not inducing CD8+ T cell responses[30]. The results supporting the lack of CD8+ response had been done mostly in male mice. Therefore, they conducted a study using female mice and were able to show that amount of CD8+ increased in female mice but not in males upon CVB3 infection[30]. This response was not dependent on the inoculation route or IFN-γ. Their result indicates that also CD8+ T cells are crucial for protection against CVB3 infection. However, sex affects remarkably on the T cell response against CVBs.

Further, it has been shown that sex associated hormones facilitate CVB3-induced myocarditis susceptibility[57]. High estrogen levels protect from heart damage via Estrogen Receptor Alpha (ER-α) which promotes type I interferons and regulatory T-cell response. In male mice, the infection leads to strong proinflammatory response and higher mortality and myocarditis more readily. The effect of estrogen is underlined as the immune response tilts towards pro-inflammatory response in older females or in younger females in diestrus phase where Estrogen Receptor Beta (ER-β) is predominant. This is align with the observation of testosterone promoting susceptibility to the infection and estrogen treatment of males is protective[55]. Likewise, testosterone has been shown to enhance intestinal CVB3 replication, dissemination to organs such as pancreas, and increasing mortality upon infection[56]. Further supporting the results discussed above, testosterone also affected the immune response by increasing proinflammatory signals and reducing the activation of CD8+ T cells.

Nevertheless, it should be noted that these results are from studies concentrating on CVB3 and largely myocarditis, and therefore may not be fully applicable on other strains

or target organs. However, they do highlight the differential immunopathological response to CVB infection between male and female mice. Therefore, it is underlined that sex must be taken in consideration when designing an infection model.

Infection route. Administration route of the virus alters the infection kinetics. As seen in the Table 2, intraperitoneal (i.p.) inoculation of the virus, where the virus is administered directly into the peritoneum, is the most used route of infection. It is preferred route as i.p. inoculation can effectively cause infection in mice. Another commonly used technique is oral inoculation. In mice, this is commonly performed as oral cavage, where the virus is administered straight to the oesophagus[43,44,48,49]. Infection after oral inoculation tends to be milder and may require higher viral loads to cause detectable infection[43,48]. In addition to the most common administration routes, other inoculation routes have been used as well. One example is intravenous inoculation (i.v.) where the virus in injected into the left jugular vein[54].

Intraperitoneal inoculation is a rather simple method, which may explain its wide usage. However, its fitness for the infection models is questioned as it does not resemble the natural infection route of a gastrointestinal virus. Bopegamage et al. has conducted several studies that systematically compare oral and i.p. route of infection and investigate the differences in viral kinetics, morbidity, and pathology. One study focused on how viral kinetics differ with orally and i.p. inoculated CVB3 and CVB4[43]. They showed acute infection in heart from fibrosis and mild infiltration with both inoculation techniques. However, in pancreas, oral route caused prolonged presence of the infectious virus and absence of histopathological changes. A follow up study used various CVB3 doses with either oral or i.p. inoculation[48]. Results showed that pancreatic lesions were seen only after i.p. inoculation, where the severity of pancreatitis dependent on the viral dose. However, PCR results showed virus in the pancreas following both routes. The authors discussed the role of gut barrier and its importance in immune response to be responsible for the contrasting outcome in the pancreas. They concluded that oral infection route could better resemble the mild course of infection seen in humans, as the intraperitoneal route neglects the effect of gut barrier and its immune response.

Also other experiments have shown similar results of the pathology of pancreas, with no histopathological changes but prolonged presence of viral RNA[49]. However, there are also results that are not dependent on the inoculation route. For example, oral and i.p. inoculation routes did not have an impact CD8+ T cell response in CVB3 infected female C57BL/6 mice[30]. Again, it should be considered that these studies have used different mouse strains and viral loads. Thus, the infection route should be chosen to be suitable for a specific study. For example, oral route could be better suitable for models studying

the natural infection route, whereas intraperitoneal infection could be better for studying the pathology of infected pancreas.

Age. CVB infections are shown to be more severe in younger mice and even lethal for neonates[47]. Therefore, as seen in Table 2 as well, majority of mouse models are done with mice from days to only couple week old. However, studies comprehensively comparing CVB infection according to mice age were not found. However, it seems the needed dose of CVB1 could be smaller the younger the mice are at the induction of infection[35,38,47]. Hence, the older the mice are, the harder it would be to cause the infection. At least it is reported that 1-day old BALB/c mice were most sensitive to the infection when compared to 2-19 days older mice[47]. Thus, mouse infection resembles CVB infection in humans, where most of the infections pass as mild or subclinical without requiring specific care[10].

Aging and maturation of immune system is thought to protect from overwhelming infection[47,48]. Supporting the idea, maternal antibodies have been shown to protect against enterovirus infections[58]. Therefore, undeveloped immune system against a highly prevalent virus would cause higher risk for severe infection in young individuals. Neonatal models are argued to provide highly sensitive, stabile, and feasible model for evaluating preventive strategies such as vaccines[47]. However, use of older mice could potentially resemble the development of chronic diseases better. Therefore, the age should be carefully considered as well, especially when designing an infection model for CVBs.

Viral dose. The optimal viral dose for causing the infection depends greatly on the specific study. All factors discussed above such as the infection route and age affect the needed amount of virus. Also, the virus infectivity is measured with different methods and reported with varying units. For example, PFU titre is approximately the same as 70% of $TCID_{50}$ (https://www.atcc.org/resources/culture-guides/virology-culture-guide, cited 22.4.24). In addition, outcome of these preclinical studies relies greatly on the persons performing the work, success of the inoculation, and the production and purification of the virus. Thus, the optimal viral dose should be determined case by case. For example, Bopegamage et al. has used multiple titres of CVB3 with enormous differences in their studies, starting from $5x10^3$ to $5x10^9$ $TCID_{50}$[44,48]. They reported that a higher viral dose affected primarily pancreas, where the severity of the pancreatitis was concordant with the viral dose. In addition, only higher doses caused mild inflammation in the heart[48]. Stone et al, on the other hand, reported 10^6 PFU to be the optimal dose for intraperitoneal CVB1 infection[40]. In contrast, Yin et al. determined 10^3 $TCID_{50}$ to be the optimal infectious

dose for CVB1 in their neonatal model, according to the time to death and clinical symptoms[47]. This further highlights the effect of study in question and for example the age of the mice to the optimal viral dose.

In conclusion, the optimal preclinical model depends on the study plan. As seen, the mouse strain, sex and age can remarkably affect the infection, altogether with different inoculation routes and virus serotypes and strains. Therefore, it is important to carefully plan the infection to be able to study infection mechanisms or specific diseases. For example, when establishing disease model for strong viral infection in a specific organ, the model should not resemble one where a mild course of the infection was induced. As the models differ remarkably, it is important to acknowledge these factors when comparing experimental results and effects.

2.2.2 Murine CVB infection in different organs

CVBs can infect multiple organs. In infants, outcome of the infection can even be systematic multiorgan disease[12]. However, organs linked to severe diseases, such as heart to myocarditis and pancreas to T1D are extensively studied. Yet, the importance of other organs such as GI tract to infection mechanisms should not be overlooked. This chapter discusses how the infection affects different organs in mouse models.

CVBs have strong tropism towards pancreas. Several studies confirm the claim by reporting signs of pancreatitis, significant changes in pancreas morphology, and positive results of CVB presence in pancreas[35,40,47]. Virus replication has been detected in pancreas 3 days post infection (dpi) after CVB1 infection[40]. Other studies have detected virus already 24 h after infection[52]. In neonatal model, the amount of replicative CVB1 increased from 2 to 4 dpi[47]. Consistently, CVB4 infected mice showed peak in the amount of replicative virus on days 3 and 5 depending on mouse strain[49]. Replicative virus was cleared out from pancreas approximately within two weeks, according to $TCID_{50}$ method. Viral RNA, however, had been detected in mice pancreases even 49 dpi[49]. In conclusion, the infection spreads within few days to pancreas and viral RNA can persist prolonged times in pancreas, depending on the infection model. Infection can be easily detected from inflamed pancreas showing severe pancreatitis by morphological changes and infiltrates.

Other susceptible organ for CVB infection is the heart. Replicative CVB3 has been detected in the heart as early as 2 dpi, having the highest peak in 4 dpi or 7 dpi depending on the mouse strain[52]. Consistently, in another study virus titres peaked in the heart 5 dpi after CVB4 infection[49]. Replicative virus was cleared out from heart approximately within two weeks[52], whereas viral RNA has been detected in some mice even 105 dpi[49].

In conclusion, the infection is detected from the heart within a week, somewhat later than from pancreas. The viral RNA can be detected for months after infection, whereas the virus is typically cleared within couple of weeks. Infection is detected from the heart with histopathological examinations and infiltrates, for instance.

It is shown that CAR is essential for virus replication, acute inflammation and necrosis in heart and pancreas. Tissue specific CAR knockout mice for pancreas and heart showed remarkably lower viral titres compared to wildtype control mice. CAR deletion in pancreas did not reduce the viral titres in the heart. Similarly, the infection was alike in dual knockout mice and single knockouts, implicating that the infection in heart is not dependent on infection in pancreas.[25]

However, contradictory findings suggest that infection in the heart may depend on the presence of infection in the pancreas. CVB3 can cause stronger and more prevalent CVB3 titres in the heart when compared to pancreas in BALB/c and C57BL/6 mouse strains[52]. Yet, several CVB3 infection models have not caused myocarditis without causing pancreatitis as well[14,16,51,52]. Pinkert et al. have criticized that heart focused murine CVB3 models resemble multiorgan failure seen in infants, rather than myocarditis in human adults when pancreas is also infected. To overcome this bias, they established a model with genetically engineered pancreas attenuated CVB3 variant, enabling establishing of myocarditis without developing pancreatitis and systemic infection. Infection only in the heart was achieved only in i.v. inoculated virus, whereas i.p. inoculation failed to cause inflammation in heart in the absence of inflammation in the pancreas[54].

Most studies focus on pancreas and heart when investigating CVB infections. However, the infection affects several other organs as well. A study made with a neonatal model detected replicative virus with $TCID_{50}$ methods from spleen, lungs, intestine, brain, spinal cord and muscles, 4 dpi at the latest[47]. Similarly, other studies have shown detectable viral titres with plaque assay from liver and spleen from 6 to 8 dpi[25]. Consistently, signs of inflammation after CVB2 infection has been seen in lungs, liver and spleen[14]. CVBs spread through bloodstream, and indeed, replicative CVB1 and CVB4 have been detected in blood samples 3 dpi[35,48]. CVB3 viral titre has been detected in serum from 2 dpi up to 4 dpi, but it has also cleared form serum within 4 dpi depending on mouse strain[52].

As the virus naturally infects through faecal-oral route, the role of small intestine has been further studied. CVB4 has been detected from small intestine, stool samples and spleen within two weeks of oral infection, with highest peaks 5 dpi[49]. Especially Peyer's patches (PP) have been though to function as primary replication site. Bopegamage et al. first demonstrated inflammation and prolonged presence of CVB3 in small intestine

and enlarged PPs after oral inoculation[48]. Their later study focused on viral presence in small intestine and especially in PPs after oral inoculation[44]. They showed no viral markers, such as viral RNA in germinating centres of Peyer's patches after CVB3 and CVB4 infection. Instead, they discovered viral proteins in the follicle-associated epithelium. In addition, interferon alpha (IFN-α) and enlarged germinating centres were detected. Therefore, their results contradict with the hypothesis of PPs being the central place for viral replication in small intestine.

In conclusion, pancreas and heart are highly studied organs in CVB infection models. However, the virus can be detected from multiple organs, including small intestine, spleen, blood, and liver. The infection is typically cleared within 2 weeks of the infection. With specific mouse strains, virus serotypes or techniques also prolonged and persistent infections can be caused in different organs. These models are important for modelling different diseases related to specific organs, such as myocarditis and type 1 diabetes.

2.3 Murine disease models for CVBs

Murine disease models provide a platform for studying CVB associated diseases such as myocarditis and type 1 diabetes. As previously discussed, careful planning is crucial in designing the disease model, ensuring the selection of suitable mouse strain, virus serotype, mice ages, and other applicable factors. Especially when mimicking human diseases in model animals, the similarities between species should be considered. Modelling of a disease which differs considerably between mice and humans would not be reliable. Yet, disease models provide essential information about the disease pathology and possible therapeutics and preventive strategies.

2.3.1 Diabetes model

Type 1 diabetes mellitus (T1D) is a severe autoimmune disease where insulin producing β-cells in the pancreatic islets are destroyed. It is a chronic condition which is caused by multiple factors, including predisposing genetics and several environmental factors. On this day, many studies have suggested that coxsackievirus infection is associated with the onset of T1D. There are several studies including case-control studies and experimental data further validating the relationship[2,19–21,39–42,59]. However, the specific mechanism of how the infection alters the onset of the disease remains unclear. At least some key features, such as peak incidence during infancy, β-cell permissiveness and high prevalence link CVB infection and T1D together[19].

Several mouse models have been established to understand the development of the disease. Especially, non-obese diabetic (NOD) mice are widely used in murine diabetes models. NOD mice will spontaneously develop diabetes in most of the mice around the age of 30 weeks. Diabetes onset is preceded by lymphocyte infiltration to pancreas and formation of anti-insulin autoantibodies from the age of 5 weeks[21]. Also genetically modified strains, such as SOCS1-tg are used[40]. In SOCS1-tg strain, suppressed cytokine signalling prevent forming a response to interferon mediated signals, thus leaving beta cells defenceless against CVB infection. Especially CVB1 and CVB4 are linked with T1D[19–21]. However, also CVB3 is used in murine diabetes models[19,42]. As seen in Table 2, the inoculation route for diabetes models have been intraperitoneal. Viral loads have varied form $5x10^5$ $TCID_{50}$ to 10^7 PFU. The age of mice in the beginning of the studies have varied from 4 to 13 weeks. Diabetes has been determined by blood glucose monitoring[41] and immunohistochemical staining of insulin[39], for instance.

It has been shown that especially CVBs of all enteroviruses have a strong tropism for pancreas in both humans and in animal models[59]. However, there are results implicating that the virus infects exclusively pancreatic islets in humans[60], whereas in mice the infection focuses on exocrine pancreas, sparing the islets[14]. Expression of CAR has been thought to facilitate the expression. However, there has been contradictory results on whether CAR is expressed in murine pancreatic islets cells. In result, CVBs could not cause inflammation in pancreatic islet cells and thus drive toward diabetes because of the lack of the receptor. Yet, in addition to studies reporting lack of expression[60], there are ones showing CAR expression in the islets[39]. There are yet no broad and comprehensive conclusion explaining the variance of CAR expression within pancreas. It is concluded that the susceptibility of mouse islet cells is driven by multiple factors, not only by CAR expression[48]. All in all, these differences and variability of results depending on disease model must be taken into consideration when analysing and concluding the experimental results.

Even though the specific role of CVBs as an etiologic agent of T1D is still unclear, it is many times proven how CVB infection can cause pancreatic insufficiency, pancreatitis and necrosis[45]. These findings have led to wide use of mouse models to study the onset and development of virus-induced type 1 diabetes. There are specific mouse strains and disease model set up that can mimic human diseases. Comprehensive and repeatable disease models enable safety and efficacy testing of therapeutics and the development of preventive strategies for T1D. The association between CVB infection and T1D have led to efforts for creating a diabetes vaccine. There are promising results from mouse challenge studies, where a vaccine has prevented CVB infection without causing the

onset of T1D[40,41]. However, there are several challenges in the development, including the need for further validation of virus serotypes and host responses facilitating the onset of T1D. Nonetheless, in addition to ongoing research, there is already a diabetes vaccine in clinical trials[61]. All in all, more research is needed to be able to produce efficient vaccines candidates that could pass clinical trials. Preclinical models, and especially diabetes mouse models can provide invaluable information about the progression of T1D.

2.3.2 Myocarditis model

Myocarditis means inflammation in the heart muscle. It is a severe condition and common cause of death in western countries[29,50]. Acute inflammation in the heart is called cardiomyopathy, which can either resolve or develop into chronic myocarditis and dilated cardiomyopathy (DCM), eventually leading to congestive heart failure. CVBs have been reported to cause majority of enterovirus-related viral myocarditis[45]. Further, CVB is said to be found in almost one-fourth of patients with viral myocarditis[54]. Specifically, coxsackievirus B3 has been proven to infect and replicate in cardiomyocytes and cardiac fibroblasts[54]. Animal models, especially mouse models have shown to highly resemble the human disease. The same virus serotype can infect both species, humans, and mice. Also, heart-specific autoantibodies are seen in myocarditis patients and mouse models further resembling the similarities[29]. In fact, CVB3 is said to be the most studied pathogen of viral myocarditis[54]. However, it is noteworthy that while CVB1 and CVB5 are also associated with myocarditis injury, merely CVB3 is used for preclinical models[12]

The viral model for myocarditis can be induced with mammalian cell passaged and purified virus, or with heart-passaged model. In latter, the infection is started with substance derived from CVB3 infected hearts, where cardiac myosin is inoculated together with the virus. It is reported that at least BALB/c, A/J, DBA-2 and C57BL/6 are susceptible mouse strains for one or both models[29]. However, it is also reported that C57BL/6 mouse strain would be resistant to DCM after CVB3 induced myocarditis with heart-passaged virus[50]. As a conclusion of individual studies and review articles, CVB3 serotype is mostly used for the virus induced infections. The viral load is typically between 10^3-10^5 $TCID_{50}$ or PFU. The age of the mice for these models has been from 4 to 13 weeks.[29,50,52]

Acute infection in the heart can be characterized by high virus titres in the organ, focal cellular infiltration, and necrosis, for instance. It is demonstrated that inoculation of heart passaged CVB3 leads to an acute infection in heart in 7 to 14 dpi. DCM was seen in BALB/c mice from day 35, but not in C57B/L mice. In addition, male mice were more susceptible to acute and chronic myocarditis compared to females[50]. Similarly, another

study reported acute myocarditis in heart at 7 dpi. both C57BL/6 and BALB/c mice. However, the inflammation was cleared upon three weeks and neither of the strains showed chronic myocarditis[52]. Thus, myocarditis models have provided valuable information about the progression of the infection and development of the disease together with the mechanisms of recovering from the disease.

2.3.3 Other infection model applications

As briefly discussed before, stable infection and disease models enable the testing of different therapeutic agents against the virus or induced diseases. When the outcome of induced infection is well known, the effect of different therapeutic substances can be evaluated. Important applications include vaccine challenge studies and testing antivirals. Vaccine research is particularly important, especially considering the lack of available vaccines for coxsackieviruses, despite their prevalence and role in disease aetiology.

The safety and efficacy of a vaccine candidate must be preclinically validated before clinical trials can be considered. In challenge studies, model animals are first vaccinated against specific pathogen and then predisposed to it. Therefore, these studies give more powerful and essential information about the protective role and effectiveness of the vaccine candidate. Stone et al. have conducted several rather successful challenge studies for vaccines against CVBs, especially CVB1. One of their studies demonstrated how inactivated CVB1 vaccine prevented viraemia in the pancreas of NOD mice 3 dpi, whereas clear viremia was seen in the control mice. Consistently, CVB1 vaccinated SOCS1-tg mice did not develop virus-induced diabetes within 21 dpi, whereas half of the control group mice showed hyperglycaemia[40]. Relatedly, a hexavalent CVB1-6 vaccine produced in our laboratory protected from viraemia in pancreas and heart. They showed that no acute infection was seen in vaccinated and CVB3 infected BALB/c mice. Also, vaccinated SOCS-1-tg mice did not develop diabetes after induced CVB1 or CVB3 infection[35]. They further showed similar result with NOD mice, where our CVB1-6 vaccine could prevent diabetes in another diabetes prone mouse strain as well[41]. The virus challenge was reported to be done with model that is known to cause systemic infections. As seen in the variable parameters for the discussed studies in Table 2, their infection models highlight the importance of specifying the optimal viral dose, mouse age and mouse and virus strains to be able to cause wanted infection and for studying specific conditions.

There is overall emerging amount of vaccine studies against CVBs. In addition to myocarditis and T1D, efficient CVB vaccines would prevent from other severe conditions such as cystic fibrosis[37]. Mone et al. have recently listed and discussed different vaccine

approaches against CVBs, including vaccines containing live-attenuated and inactivated viruses to recombinant subunits and virus-like particles[12].

Several antivirals from distinctive approaches have been tested in murine infection models as well. Reduced virus titres in hearts and pancreases are seen with specific molecules, repurposed drugs, and plant extracts. Antivirals halter the virus' life cycle in several ways. For example, an antiviral called Pleconaril interferes with the virus capsid preventing the attachment to receptor on host cell's membrane. Ribavirin, on the other hand, could hinder the function of viral proteases. Similarly, repurposed drugs have shown alleviate CVB infections. As an example, a statin called Lovastatin can decrease the expression of CAR.[2]

To conclude, established infection models provide crucial information about CVB pathology, progression of infection and disease mechanisms. Such knowledge is essential for testing safety and efficacy of preventive strategies such as vaccines and antivirals. Optimal vaccine for CVBs could protect from a range of adverse outcomes in addition to myocarditis and T1D.

2.4 Ethical aspects of preclinical studies

Preclinical experiments in EU must be done in accordance with the European principles of laboratory animal care (Directive 2010/63/EU). Further, the experiments must be approved by local ethics committees. For example, in Finland, animal experiments are expected to be done under specific Laboratory animal usage permission which ensure the following of guidelines of the Finnish National Experiment Board. These permissions ensure that the experiments are done with minimal suffering of the animals. Responsible animal experiments follow 3 Rs, which stand for Replacement, Reduction and Refinement. Briefly the aim is to replace the use of animals, when possible, use as few animals without reducing reliability of results, and minimize the suffering and maximize the welfare of the experimental animals. The importance methods for following the 3R principle are extensively discussed with guidelines and training materials in (www.nc3rs.org.uk, cited 21.4.24).

Preclinical models rely greatly on persons implementing them. Handling and husbandry affect the mice wellbeing as well as the modelled infection. Often, the reporting of animal experiments is concentrated only on the performed procedures. Information about the husbandry or clinical evaluation of the mice is rarely reported. This is a major drawback when considering the reliability and replicability of preclinical models. As seen in Table

2 and discussed within literature review, the way of inducing CVB infection can remarkably vary even when the goal of the model is the same. Several studies cited in this thesis have not reported the sex of the mice in their studies. As discussed, the sex can remarkably affect the progression of CVB infection. Therefore consistent, altogether more meaningful results and conclusions are hard to achieve. There are clear instructions for high quality and reliable reporting for animal experiments. The ARRIVE guidelines (Animal Research, Reporting of In Vivo Experiments) consist of step-by-step instructions for sufficient reporting of the experiments (https://arriveguidelines.org, cited 20.3.24). The aim of the guidelines is to enhance the reporting of preclinical studies and therefore support reliable and open information.

Preclinical models are essential for studying complex infection mechanisms in animals. There are cell models available for researching coxsackievirus infections and for example drug screening. Cell models can answer to definite questions, such as studying the specific receptors or interactions with the virus in isolated pancreatic tissue. For example, in situ hybridization studies have shown that enteroviruses have islet-cell tropism for human pancreases[60]. The same study demonstrated how receptor involvement in the infection can be studied in cultured islet beta cells. Another study revealed important information about the function of viral proteases in immune evasion and pathogenesis in infected HeLa cells[62]. Therefore, just a couple of examples show the importance of cell models in giving information about the infection mechanisms, testing specific aspects of the infection, and enabling for example drug screening. However, they cannot yet represent a living organism and the extraordinarily complex immune reactions reliably enough to replace animal models. Nonetheless, cell models give crucial information which can be utilized to reduce unnecessary animal testing and should be used as widely as possible.

Organoids can provide further information about infection in more complex environment compared to cell models. For example, enteroids, derived from human intestinal epithelium are used for studying molecular event upon EV infections[63]. As mouse models may not represent the natural infection route, role of the GI tract as primary infection site may be bypassed. Therefore, organoid models can give crucial information about the infection is specific tissues. For example, enteroviral replication was not differential between adult and foetal tissues when studied on organotypic cultures derived from human airway- and intestinal epithelium[64]. In the future, further developed organoid models could lessen the need of animal studies especially according to specific tissue responses.

In conclusion, despite several cell models, animal studies are still necessary for complete understanding of infections and their comprehensive evaluation. There are specific laws

and regulations for animal experiments in countries and for example for EU. These regulations and permissions ensure that the animal experiments are reasoned and done in competent facilities. However, a large part of experiments and animal welfare is dependent on the researchers. One way to show responsibility is to comprehensively report the animal experiments together with specs about the animal husbandry and welfare. Sufficient reporting drives towards more ethical, reproducible, and significant results considering animal experiments overall.

3. MAIN OBJECTIVES

This study has two main objectives:

1. To set up operating procedures for the new animal infection laboratory in Tampere University
2. To set up an acute CVB1 infection model for BALB/c mice via intraperitoneal administration route.

Before carrying out this thesis work, there has not been a dedicated laboratory for murine infection studies at Tampere University. As our research group focuses on vaccine development against different viruses, it is crucial to understand the molecular mechanisms behind the progression of virus infections. Establishing and infection model enables studying the mechanisms behind viral infections and allows the evaluation of the safety and efficacy of the vaccine candidates against infective virus. Once established, the same facility can be used for other viruses and applications as well, such as various administration routes and modelling infectious diseases such as myocarditis.

The chosen virus strain for our infection model is CVB1-10796, since it has been shown to infect mice[38,40]. Infections will be started with BALB/c mice, as this strain has been shown to be susceptible for CVB infections[3,14,35,47,50,52,53,55]. Our objective is to establish an acute infection model using a high viral dose. We hypothesize that the infection is detectable from target organs such as pancreas, heart, and blood within a week from infection induction. To confirm infection, titres of replicative virus will be measured from these organs. Mice will be age-matched to be 13-week-old in the beginning of the infection as the mice undergoing vaccine immunogenicity studies are usually 13 weeks old. Previous studies have shown that the CVB1 infection is successful in mice with similar age[35,41].

Briefly, the infection model is optimized according to the infectious dose, length of the study, and sex. According to the literature, we hypothesize that the infectious dose must be relatively high for inducing the infection. Therefore, 4 different doses from 10^4 to 10^7 $TCID_{50}$ will be tested. This study is based on a hypothesis that the amount of replicative virus in organs and blood is expected to correlate with the infectious dose, and the infection should be most clearly seen in the pancreas[47]. According to the literature, male mice should be more susceptible for the infection, especially in the heart[54]. We expect the viral dose in blood to be highest around 3 dpi[35,48,52], and replicative virus to be found in pancreas and heart 4 dpi[47,49]. We will also measure the viral titres in organs 7 dpi.

4. MATERIALS AND METHODS

4.1 Work procedures and risk assessment for ABSL-2 laboratory

Operating procedures for the new animal biosafety level 2 (ABSL-2) laboratory were established before the infection studies. Detailed written procedures are available in the Appendix A: Operating Procedures for ABSL-2 laboratory. Before starting infections, possible risks in this specific project had to be identified and a risk assessment was performed. To determine and lessen the risks, infection studies were started with mock studies. These mock studies simulated all steps of infection studies except no virus was used at any point. The infections were started only after confirming the competence of workers and the effectiveness of the workflow.

There are different ways to prepare for possible risks. To minimize the risks of virus exposure, all procedures done with the virus or infected mice were conducted under specified laminar flow hood designed for BSL-2 work (biological safety cabinet) to maintain a controlled environment. The biocontainment unit (BCU)-cages, where the mice were housed, were only opened inside the laminar after the mice were infected. All required equipment such as pipettes, scalpels, scale, and inhalation anaesthesia were available inside the laminar. The person working in the laminar used an extra pair of gloves that were discarded inside the laminar once contaminated or finishing the work.

In case of virus exposure, the area and items were decontaminated with ProChlor (Contec®). ProChlor is a strong commercial sporicide which is effective against viruses and many other pathogens. To minimize the risk of accidental cuts or needle pokes, workers wore cut-resistant gloves beneath two sets of laboratory gloves. A sharp waste box was easily accessible inside the laminar and sharp objects were put away immediately after procedures. To prevent mouse stress, bites and needle pokes, the mice were under inhalation anaesthesia during procedures where sharp tools were used. When possible, weighting and transferring mice was done with a tube to prevent mice form biting or fleeing.

4.2 CVB1 production, purification, and characterization

The infection studies were conducted with a CVB1-10796 strain isolated from Argentina[65]. The virus was propagated in green monkey kidney (GMK)-cells. Briefly, cells were

infected with multiplicity of infection (MOI) 10-20 and virus was harvested from supernatant 2 days post infection as follows: 0.1% Tween80 was added in the supernatant, cell debris was pelleted and discarded and virus was concentrated by pelleting through 30% sucrose cushion in phosphate-buffered saline (PBS)-0.1% Tween80 using ultracentrifugation (32 k rpm, 16 h at 4°C). The pellets were dissolved in PBS-0.1% Tween80. The virus stocks were stored in -80°C until further usage.

The quality of virus purification was characterized by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) after ultracentrifugation. The samples were first denatured and charged by adding SDS-PAGE sample loading buffer (containing β-mercaptoethanol). The samples were then boiled 10 min in +100°C and loaded to gel with PageRuler Broad Range, unstained Protein Ladder (Thermo Scientific). Used gel was Criterion TGX Stain-Free Precast gel (26 wells, Bio-Rad). The electrophoresis was carried out with 100 V for 1 h 13 min and then 250 V until the stained front line reached the bottom of the gel. Gels were UV activated for 5 min and the UV-induced fluorescence was imaged with Chemi-Doc XRS+ (Bio-Rad). The virus and impurities were evaluated by densitometry analysis of SDS-PAGE gels using ImageLab software.

In addition, CVB1 VP1 and VP3 structural proteins were detected with Western Blotting using in-house produced anti-CVB1-6 rabbit polyclonal antiserum[66]. The virus proteins from SDS-page gel were blotted onto nitrocellulose membrane with the semi dry, Trans-Blot Turbo Blotting System (Bio-Rad) according to the user manual. Electroblotting was carried out by using a pre-programmed Midi-run protocol (25 V, 2.5 A, 7 min). After blotting, non-specific binding of antibody was blocked by incubating the membrane in EveryBlot blocking Buffer (BioRad) and in in-house produced Rabbit anti-CVB1-6 polyclonal antibody was used in dilution 1:1000 to detect the VP3 and VP1 capsid proteins. IRDye Goat anti-Rabbit IgG (LI-COR) in dilution 1:40000 was used as the secondary antibody. Primary antibody was incubated on a table shaker o/n in 4°C, apart from Rabbit anti-CVB1-6, which was incubated at RT for 1.5 h. In between of antibodies, the membrane was washed for 3 times 10 min with 1 x TBS + 0.05 % Tween20. Imaging was carried out with Odyssey CLx Imaging System, (LI-COR). Before imaging the membrane TBS-Tween was replaced with only 1 x TBS to remove Tween which can interfere the imaging process.

Total protein concentration of the purified virus preparation was measured with Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) following the microplate procedure and colorimetric detection and quantitation of virus concentration. The absorbance (562nm) was measured by EnVision Multilabel Plate Reader (PerkinElmer) spectropho-

tometer and the total protein concentrations were determined based on the BSA standard curve. The purity of the virus was analyzed by densitometric analysis from the stain-free stained SDS-PAGE gel using the ImageJ software[67].

The hydrodynamic particle size of the purified CVB1 was determined with dynamic light scattering (DLS) analysis using Zetasizer Nano ZS (Malvern Panalytical) system. DLS was equipped with HeNe gas laser with wavelength of 633 nm. DLS measures Brownian motion and relates this to the size of the particles. For determining the particle sizes accurately, predefined standard operating procedures (SOPs) considering the temperature and viscosity of the sample were followed. The measurements were done at room temperature (25°C) and the hydrodynamic diameters were determined from the average size of three to four measurements, each measurement containing 15 readings.

4.3 Determining the virus infectivity

Tissue Culture Infectious Dose 50 ($TCID_{50}$) assay was used for determining the virus infectivity in both infection samples and purified virus. The assay measures the dilution of virus that is needed to infect 50 % of certain cell culture. The titre is defined as number of viral units/unit volume[68]. This assay is based on the original protocol by Reed and Muench[69]. In this protocol, GMK cells are cultured as monolayers in cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM; Gibco™) containing 5 % Fetal Bovine Serum (FBS) (Sigma).

For the assay, 50 µl of 20 mM HEPES buffer (Sigma) in Hanks balanced salt solution (Sigma) with 0.6% FBS is pipetted on Nunclon™ Delta Surface 96 well plates (Thermo Scientific). 10 µl of the sample is serially diluted 1/6 by moving 10 µl to the next well within the row. The samples are left to incubate for 1 h in 37°C. 150 µl of cell suspension containing 16000 cells is added on top of the sample dilutions and left to incubate for 46 h. Finally, 20 µl in each well is replaced with alamarBlue (Invitrogen) and incubated for 2 h in 37°C. The samples are transferred to SW AlphaPlate™ 384 well plate (PerkinElmer) and read with PerkinElmer wallac EnVision 2104 Multilabel Reader which measures the viability of the cells according to the fluorescent signal in the wells.

$TCID_{50}$ calculation is based on Karber's formula,

$$TCID_{50} = (dilution\ in\ the\ first\ row) \times (dilution\ rate)^{(\Sigma - 0.5)},$$

where $\sum$ = the total sum of the number of wells at each dilution showing cytopathic effects divided by a number of total wells at each dilution. The final values are obtained with a

python script which takes in consideration the original and sample dilutions. Within this thesis, all samples were analysed only once.

4.4 CVB1 infection studies

The mice strain used in all infection studies was BALB/cJRj (n = 54) obtained from JanvierLabs, France. All mice were age matched to be 13 weeks in the beginning of the infection studies. Extended health monitoring was performed throughout the studies, according to body score indexing. Monitoring included observing the posture, movement, reaction to handling, fur, skin, eyes, breathing and faeces. If body score index was higher than 3, mice were euthanized. Weights of the mice were followed daily at least onwards 3 dpi. If the body weight decreased over 15% from the weight in the beginning of the study, MediGel® Hazelnut (ClearH_2O) dietary supplement was given to the cage. If the weight did not recover within 48 h, the mouse was euthanized.

Upon arriving to the facility, the mice had an acclimatization period for at least a week in the standard preclinical facility. Mice were housed in randomly assigned groups of three mice in individually ventilated cages. In the beginning of infection studies, mice were transferred to infection laboratory in BCU-cages having negative pressure and individual air exchange. Material from old cage including nesting material and plastic tubes were moved from old cage to the BCU-cages. No mice were single-housed and maximum of 3 mice were kept in the same ventilated cage. BCU-cages included bedding, nesting material, cardboard shelter, plastic, cardboard tubes, and a gnawing stick. Laboratory food and water was provided *ad libitum*. The temperature and humidity of the cages were followed daily during the studies. The automatic light cycle in the room followed a 12-hour cycle, alternating between 12 hours of light and 12 hours of darkness.

Animals were randomly allocated to treatment groups. Researchers were not blinded to treatment groups during the experiments. However, $TCID_{50}$ analyses were performed by a worker who was unaware of treatment groups. Standardization of experimental procedures was achieved by maintaining consistent protocols, timings, and arrangements of animal cages throughout the study duration. As this was a pilot study, no power calculation was used to determine the sample size. To minimize the use of animals while achieving preliminary data on infection studies, 3 mice were used per study group.

All blood samples, ear markings and i.p. injections were done while mice were under inhalation anaesthesia by isoflurane (Attane vet, Vet Medic Animal Health). In-between-study blood samples were collected from lateral tail vein 1:1 to PBS + 12 mM EDTA. The mice were terminated with an overdose of injectable anaesthesia (Ketaminol vet

75mg/kg and Medetomidin 1mg/kg in Aqua Sterilisata Freseniuss Kabi) under inhalation anaesthesia either 4 dpi or 7 dpi. Right after the reflexes could not be detected, termination blood sample was collected via heart puncture and diluted 1:1 to PBS-EDTA. Other termination samples included pancreas and heart, and all of the sampes were used for $TCID_{50}$ analysis.

Organs were collected in Bertin Technologies Precellys Lysing Kit (Tissue homogenizing CKMix) tubes, weighted, and 1ml of 4°C Dulbecco's Modified Eagle Medium (DMEM; Gibco™) cell media was added to the tubes before homogenization. The organ samples were homogenized with Precellys 24 by Bertin Technologies with program of 6500 rpm 2 x 30 seconds. All samples (blood and homogenized organ samples) were freeze-thawn at least 3 times to release the virus in the samples. Blood samples were further diluted 1:5 before starting the $TCID_{50}$ analysis. Organ and blood samples were stored in -80°C as soon as possible after collection and preparation.

Laboratory animal usage permission (Regional State Administrative Agency; decision number ESAVI/1408/2021) covered all mice experiments done for this thesis.

4.4.1 Mock study

Mock-study without infective virus was conducted to ensure the competence of personnel working in the BSL-2 laboratory and to assess the efficacy of work procedures determined in section 4.1. Two mock studies included altogether 6 female mice, where 3 mice (n = 3) were used per study. The procedures were similar with other studies, except no virus was present at any point of these studies. Virus injection was mimicked with 150 µl i.p. injection of PBS. Animals were weighted daily, and blood samples were collected on days 0 and 4. Upon termination, blood, pancreas, and hearts were collected to practice the procedures as well as sample handling before the actual infection studies.

4.4.2 Study 1: optimal virus dosage

The primary objective of this study was to determine the optimal viral dose to induce an acute CVB1 infection. The study lasted 5 days including the infection day. The study included altogether 12 female mice that were randomly divided into 4 groups of 3 mice (n = 12). The mice were housed within groups in BCU cages. Each group received a distinct viral dosage: $1.0x10^4$, $1.0x10^5$, $1.0x10^6$ and $1.0x10^7$ $TCID_{50}$ per mouse. Virus preparations were diluted to a final volume of 150µl in PBS and injected i.p. on day 0. Blood samples were collected at 0 dpi, 2dpi and 3dpi to monitor viral kinetics. Mice were sacrificed and termination samples were collected at 4 dpi for further analysis. The experimental design and procedures for study 1 are summarized in figure 2.

Infection study 1

N	Group	Virus dose ($TCID_{50}$)
3	1	10^4
3	2	10^5
3	3	10^6
3	4	10^7

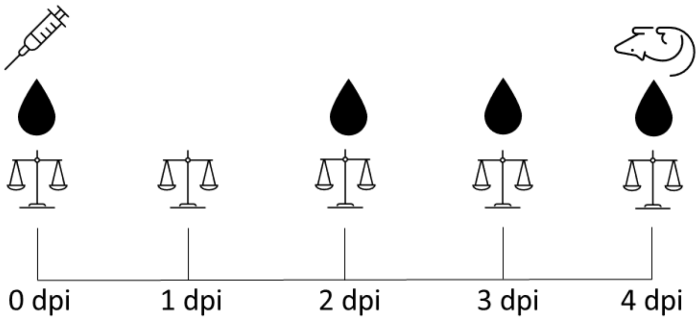

Figure 2. *Experimental design and procedures for study 1. Group division and viral doses are presented in a table. The experimental timeline with procedures is depicted in the right side. Infection was induced 0 dpi. Blood samples were taken 0, 2, 3, and 4 dpi. Weights were measured every day. Mice were terminated 4 dpi.*

4.4.3 Study 2: reproducibility

The aim of this study was to confirm the reproducibility of the results obtained in study 1. Therefore, the experimental design was the same as in study 1, lasting for 5 days including the infection day. The study included altogether 6 female mice that were randomly put in 2 groups of 3 mice (n = 6). The mice were housed within groups in BCU cages. Two different viral doses that were able to induce infection according to the previous study were used: $1.0x10^6$ and $1.0x10^7$ $TCID_{50}$ per mouse. Virus was diluted in the final volume of 150µl PBS and injected intraperitoneally on the day 0. Blood samples were taken at 0 dpi and 2 dpi. Mice were sacrificed and termination samples were collected at 4 dpi. The experimental design and procedures for study 2 are summarized in figure 3.

Infection study 2

N	Group	Virus dose ($TCID_{50}$)
3	1	10^6
3	2	10^7

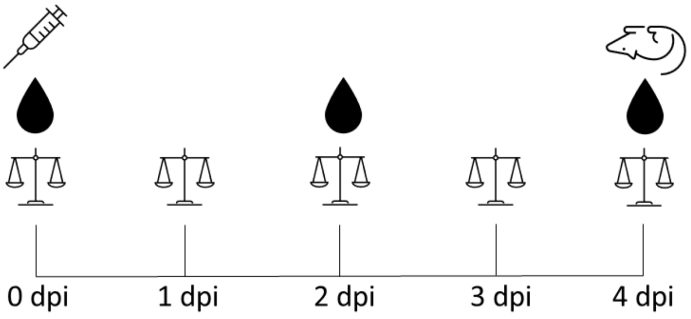

Figure 3. *Experimental design and procedures for study 2. Group division and viral doses are presented in a table. The experimental timeline with procedures is depicted in the right side. Infection was induced 0 dpi. Blood samples were taken 0, 2, and 4 dpi. Weights were measured every day. Mice were terminated 4 dpi.*

4.4.4 Study 3: optimal study length

This study aimed to find the optimal study length and to evaluate the impact of an extended study duration. The experimental timeline was extended to 8 days including the infection day. The study included altogether 6 female mice that were randomly divided into 2 groups of 3 mice (n = 6). The same viral doses were used as in study 2: $1.0x10^6$ and $1.0x10^7$ $TCID_{50}$ per mouse. Virus was diluted in the final volume of 150µl PBS and injected intraperitoneally on the day 0. Blood samples were taken at 0 dpi, 3dpi and 5dpi. Mice were sacrificed and termination samples were collected at 7 dpi. The experimental design and procedures for study 3 are summarized in figure 4.

Infection study 3

N	Group	Virus dose ($TCID_{50}$)
3	1	10^6
3	2	10^7

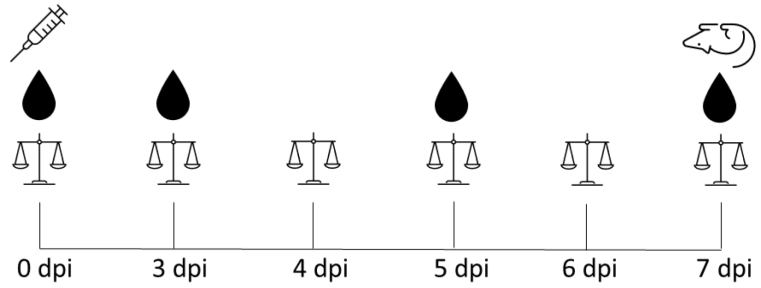

Figure 4. *Experimental design and procedures for study 3. Group division and viral doses are presented in a table. The experimental timeline with procedures is depicted in the right side. Infection was induced 0 dpi. Blood samples were taken 0, 3, 5 and 7 dpi. Weights were measured at 0 and 3-7 dpi. Mice were terminated 7 dpi.*

4.4.5 Study 4: sex differences

This study aimed to replicate previous studies using male mice to determine potential sex-based differences in infection outcomes. The study lasted from 5 to 8 days including the infection day. The study included altogether 24 mice that were randomly put in 6 groups of 3 or 6 mice (n = 24). The mice were housed in groups of 3 mice in BCU cages. The experimental setup and viral doses mimicked the previous studies. Four different viral titres were used: $1.0x10^4$, $1.0x10^5$, $1.0x10^6$ and $1.0x10^7$ $TCID_{50}$ per mouse. Virus was diluted in the final volume of 150µl PBS and injected intraperitoneally on the day 0. For groups 1-4, blood samples were taken at 0 dpi and 3dpi. Mice were sacrificed and termination samples were collected at 4 dpi. For groups 5 and 6, blood samples were taken at 0 dpi, 3dpi, and 5dpi. Mice were sacrificed and termination samples were collected at 7 dpi. The experimental design and procedures for study 4 are summarized in figure 5.

Infection study 4

N	Group	Virus dose ($TCID_{50}$)
3	1	10^4
3	2	10^5
6	3	10^6
6	4	10^7
3	5	10^6
3	6	10^7

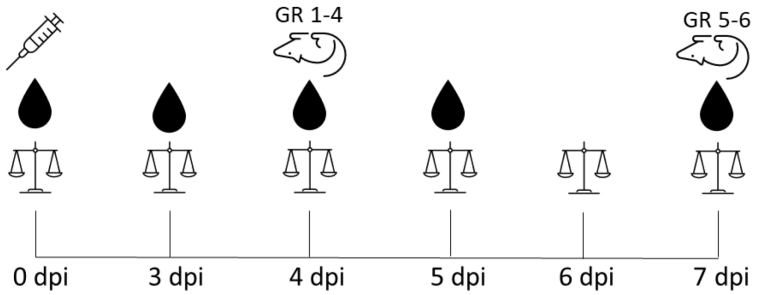

Figure 5. *Experimental design and procedures for study 4. Group division and viral doses are presented in a table. The experimental timeline with procedures is depicted in the right side. Infection was induced 0 dpi. For groups 1-4, blood samples were taken 0, 3, and 4 dpi. Weights were measured at 0, 3 and 4 dpi. Mice were terminated 4 dpi. For groups 5-6, blood samples were taken 0, 3, 5 and 7 dpi. Weights were measured at 0 and 3-7 dpi. Mice were terminated 7 dpi.*

4.5 Statistical analysis

All statistical analyses were done with GraphPad Prism 10 software. All analyses were done with non-parametric test, according to normality tests. Results from $TCID_{50}$ analysis are shown in scatterplot with median and 95% confidence interval. Virus titres in blood and pancreases are shown in log10. Differences in titres between viral doses were tested with Kruskal-Wallis test. Comparison between studies or dpi within one viral dose were tested with Mann-Whitney test. Weights are shown in boxplot and whiskers displaying all individual data points. Percentage body weight change between sexes was compared with Mann-Whitney U test. Weight change within one viral dose was compared by Kruskal-Wallis between day 0 and later time points. Significance levels are marked as *P < 0.05, **P < 0.01, ***P < 0.001.

5. RESULTS

5.1 Establishment of the infection laboratory

Setting up the facility was started when the laboratory room within the preclinical facility of Tampere University was not yet fully equipped for ABSL-2 work. Upon the arrival of equipment, the layout had to be determined for optimal and easy workflow. In addition, waste management, decontamination and cleaning of used tools and overall procedures during infection studies were discussed and planned together with the personnel of the preclinical facility. The layout of the laboratory space in the beginning of the infection studies is illustrated in figure 6. Detailed operating procedures specific to the infection laboratory are provided in the Appendix A: Operating Procedures for ABSL-2 laboratory. In this section, the basic principles of working in this facility are discussed.

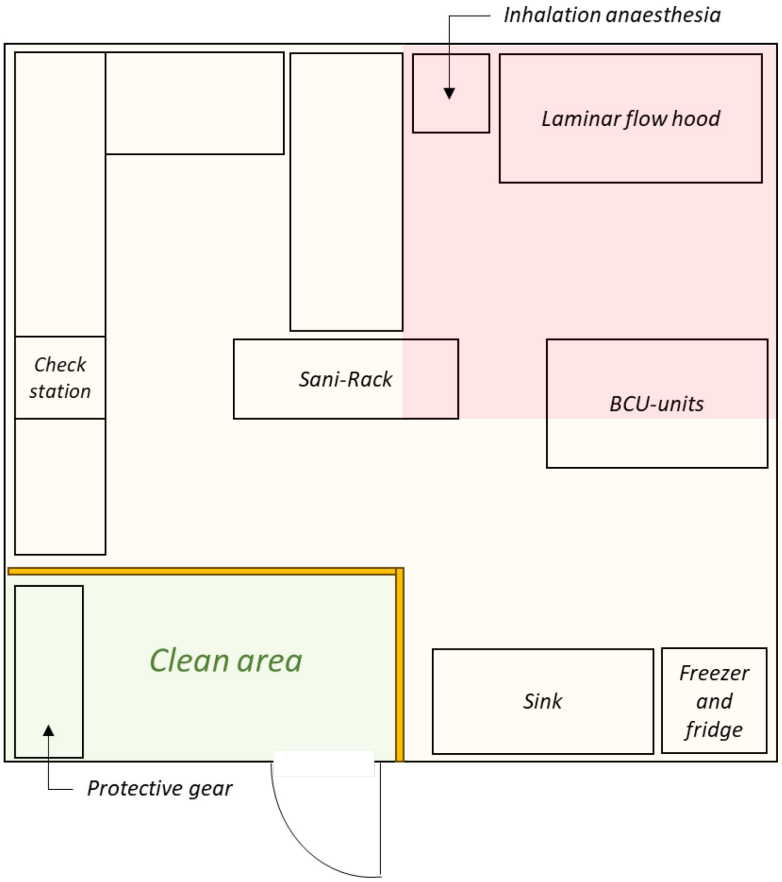

Figure 6. *Layout of the newly established infection laboratory. The layout is not drawn to scale. Different background hues are used to implicate the cleanliness level withing the room, with green representing the clean area and red indicating the dirtiest area where infective virus is handled. Unmarked boxes implicate tables and storage areas.*

Risk assessment was done prior and maintained during the infection studies. Procedure that was identified to have the highest risk was the administration of the virus. Administration procedure includes filling up needles with a high amount of infective virus and administrating the virus into the mice. Therefore, during infection procedures only an appropriate number of needles were loaded with the virus to reduce the risk of sharp objects. The needles were sterilized with ProChlor, and gloves were changed before continuing the protocol. Other procedures posing a higher risk included working with sharp instruments such as scalpels and needles. In addition, invasive procedures increase the risk of animal bites. Thus, those procedures were performed while mice were under inhalation anaesthesia.

The laboratory was organized to segregate clean areas from potentially infective areas. The clean area of the laboratory was separated from the potentially infective areas with a tape, indicated with green hue in figure 6. Personnel entering the infective areas must use personal protective clothing including overalls, long gloves and specific shoes that are put on within the clean area. Access to the dirty side is prohibited without appropriate protective gear. All other areas inside the lab are potentially virus-contaminated and thus are considered “dirty”, indicated with yellow hue in figure 6. All virus-related work is conducted inside the laminar flow hood. The mice cages are transferred between the laminar flow hood and the BCU-2 cage system. Therefore, the areas surrounding the laminar flow hood and BCU-2 cage system are considered the most contaminated, indicated with red hue in figure 6.

Specific equipment inside a BSL-2 infection laboratory include the BCU-2 cage system, Sani-Rack and Check station for BCU-cages. Mice are housed within the BCU-cages which have separate air exchange and negative pressure. The cages are held inside the BCU-2 cage system which facilitates the separate air exchange. Condensation and functioning of the BCU-cages are ensured with Check station, which tests the ventilation within the cage. Finally, after each infection study, the BCU-cages that have housed infected mice are autoclaved to ensure decontamination. Additional equipment includes scale and inhalation anesthesia. Scale, anesthesia induction box and mask for maintaining the anesthesia are inside the laminar flow hood whilst the active carbon filter and gas meters stay outside of the laminar.

The work within the facility is conducted in pairs, comprising a "dirty worker" responsible for tasks involving animals and infective viruses, and a "clean worker" assisting with cage transfers, tool handling, and anesthesia monitoring. Clear communication and good planning of each task is essential for safe pairwise working within the facility. Therefore, mock studies were conducted to identify potential risks and optimize procedures before starting

the infection studies in the new facility. This made sure that the work with infective agents can be started in a safe and optimized way.

5.2 CVB1 production, purification, and characterization

Purity of the CVB1-CDC7 virus strain was analysed with SDS-PAGE total protein staining and densitometric analysis (figure 7A), whereas the identity of the purified virus was confirmed by staining the VP1 and VP3 structural proteins of the virus from Western blot using a rabbit anti-CVB1-6 polyclonal antibody (figure 7B). The hydrodynamic diameter of the purified virus particles and the proportions of the virus specific particle populations in the virus preparations were determined with Dynamic Light Scattering (DLS) analysis (figure 7C). The average hydrodynamic diameter of the purified CVB1 virus was 26.2 (±2.6) nm and 92.5 (±2.1) % of the virus preparation contained this particle population. The total protein concentration of the purified virus was measured with BCA total protein staining method and the purity of the virus was analyzed by densitometric analysis from the SDS-PAGE gel and was found to be ~79%. Therefore, the concentration of the purified virus in the preparation was determined to be 0.3 mg/ml.

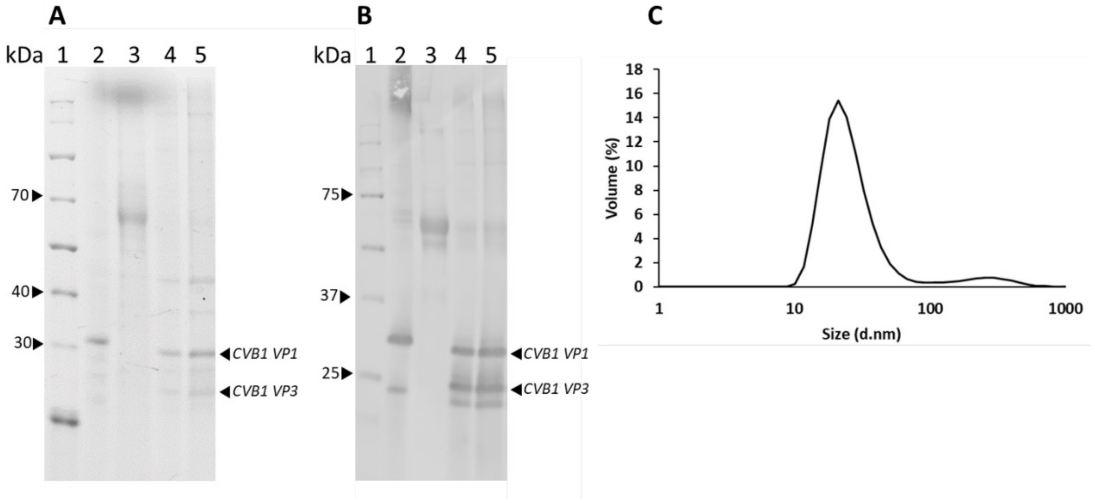

Figure 7. *Characterization of CVB1 after ultracentrifugation. A) SDS-PAGE analysis with stainfree total protein staining. B) Western blot stained with a polyclonal CVB1-6 rabbit antibody detecting VP1 and VP3 capsid proteins. 1. Molecular weight standard, 2. CVB3 VLP 2 µg, 3. Virus supernatant after centrifugation 15 µl, 4. Purified CVB1 1.5 µg, 5. Purified CVB1 3 µg. C) Dynamic light scattering analysis of the purified CVB1.*

5.3 CVB1 infection studies

The average body weights of mice at the start of infection studies were 22.8 $\pm$ 0.4 g for females and 25.8 $\pm$ 0.3 g for males (mean $\pm$ SEM). Throughout the studies, no mortality

was observed and there was no need for premature euthanasia. The following results sections compare weights and viral titres in the blood and pancreas homogenate samples, determined with $TCID_{50}$ method. Additionally, all hearts were collected upon termination and analysed similarly. However, replicative virus was not detected form heart samples in any of the studies. Even though intended, in-between-study blood samples could not be analysed from studies 3 and 4 during the experimental part of this thesis.

5.3.1 Study 1: determination of optimal infection dosage

In this study, the aim was to cause systemic infection in mice (detectable with $TCID_{50}$ assay). To determine optimal viral dose, four different doses were tested. Each viral dose was administered to a group of three mice. The infection was most consistently detected from pancreases of mice infected with the highest viral doses (10^6 and 10^7 $TCID_{50}$) (figure 8). Based on $TCID_{50}$ results, 2/3 and 1/3 mice per group got infected for lower doses. Therefore, we concluded that the reproducibility of the infection is not optimal when the mice are infected with 10^4 or 10^5 $TCID_{50}$ units per mouse.

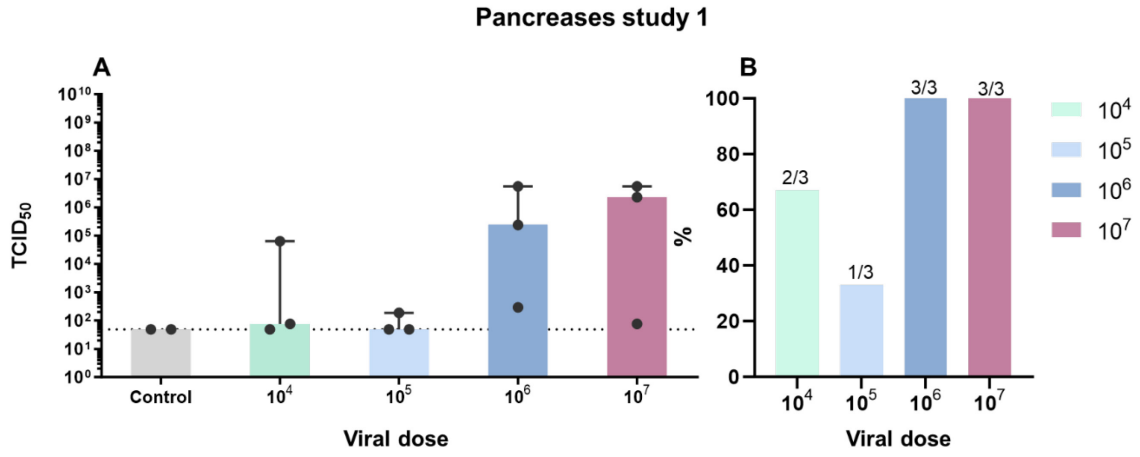

Figure 8. *Viral titres of pancreases form study 1. Three female BALB/c mice per group were intraperitoneally inoculated with four different viral doses ($TCID_{50}$) as indicated with different colours in the figure. The viral titres were analysed from pancreases 4 dpi. A) Virus titres for each mouse are shown individually. Bars represent median + 95% CI. The dotted line shows the limit of detection for the assay. Kruskal-Wallis test did not show significance between any of the groups. B) Percentage of pancreases positive for replicative virus within different viral doses.*

The viral titres from blood samples indicate that the amount of replicative virus in blood increases up to 4 dpi (figure 9). In all groups, the viral titres have increased above the threshold 4 dpi at the latest. Interestingly, lower viral doses seem to induce stronger viremia in the blood when compared to higher doses, but this observation is a trend and

not statistically significant result. Until 3 dpi, the viral titres are similar in all groups. However, upon 4 dpi titres in blood increase more with lower doses compared to higher doses. This could implicate that with higher doses the virus disseminates to pancreas more efficiently and replicates there, whereas with lower doses the virus does not disseminate in the pancreas so efficiently and remains mostly in blood. Our aim was to cause infection where the virus disseminates in organs as well, rather than replicating only in blood. Thus, based on the consistency of replicative virus in pancreas, we chose to continue with only higher doses (10^6 and 10^7 $TCID_{50}$) in subsequent studies.

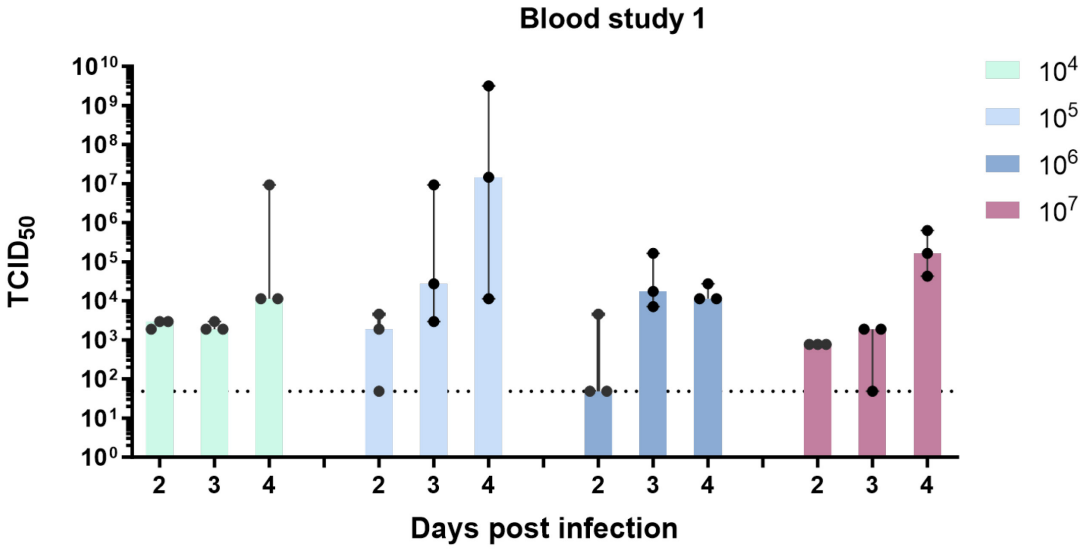

Figure 9. *Viral titres in blood from study 1. Three female BALB/c mice per group were intraperitoneally inoculated with four different viral doses ($TCID_{50}$) indicated with different colours in the figure. The viral titres were analysed from blood 2, 3 and 4 dpi. Virus titres for each mouse are shown individually. Bars represent median + 95% CI. The dotted line shows the limit of detection for the assay. Kruskal-Wallis test did not show significance between any of the groups.*

There were no external signs of infection observed in any of the mice, according to body condition scoring. The weights of the mice did not change considerably during the study (figure 10). However, there appears to be a potential trend for weight decrease after 3 dpi. Nonetheless, due to small sample size, drawing definitive conclusion is challenging. Overall, there were no clear signs of the mice suffering from infection. However, pancreases were observed to be watery upon termination, which implicates infection in the pancreas. Watery pancreases were noted in all the groups, independent of the viral dose, excluding mice that were administrated with buffer. External examination of hearts did not show difference to the hearts of uninfected mice indicating that replicative did not

reach the hearts of the mice. The primary outcome of study 1 was that CVB1 infection in BALB/c mice with i.p. inoculation was successfully induced.

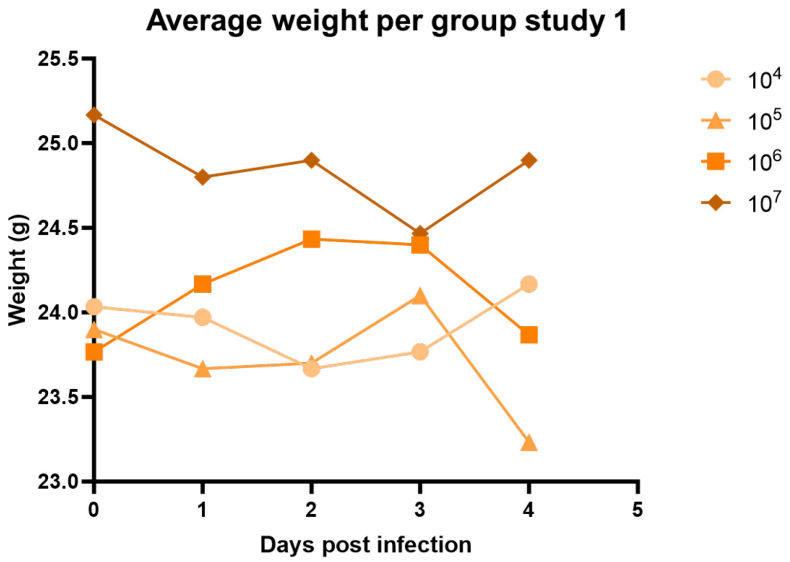

Figure 10. *Average weights of three mice within viral dose group throughout the study 1.*

5.3.2 Study 2: reproducibility of the infection

This study aimed to validate the consistency and reliability of the findings from study 1 by replicating experimental conditions and procedures with the two highest viral doses. Therefore, study 2 was carried out the same way as the study 1, with the exception that only higher viral doses (10^6 and 10^7 $TCID_{50}$) were used. Each viral group consisted of three mice. The viral titres in the pancreas were similar between the studies (figure 11A). However, despite the use of higher doses, one pancreas in each viral dose groups gave a negative result. Viral titres in blood were similar between the two studies at 2 dpi. However, more variance was observed at 4 dpi, where mice form study 1 show stronger viremia (figure 11B). Yet, there were no significant differences observed between the studies for each viral dose and time point. Therefore, it can be concluded that the i.p. infection with the higher viral doses is reproducible.

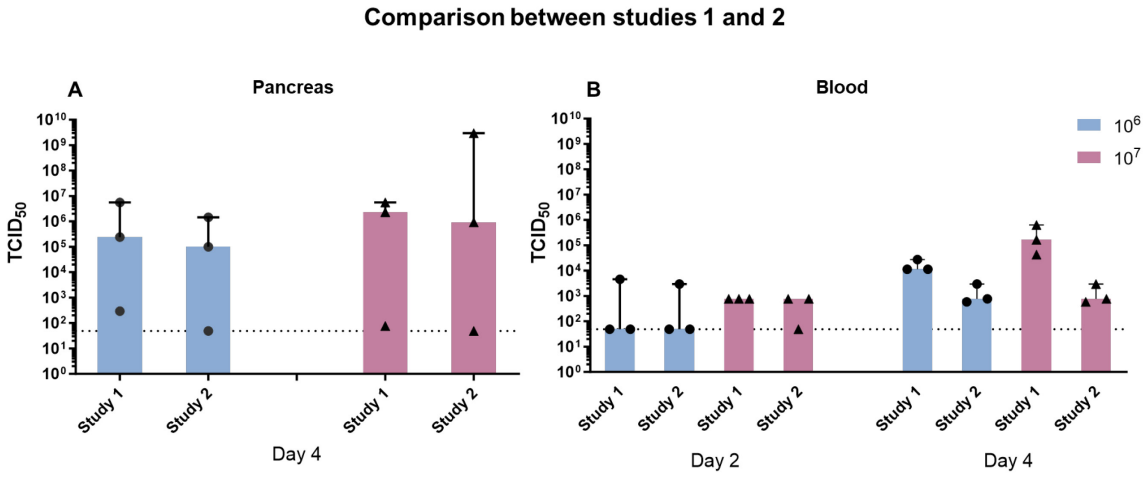

Figure 11. *Comparison between first and second study according to viral titres in pancreas and blood. In study 2, three female BALB/c mice per group were intraperitoneally inoculated with two previously determined doses ($TCID_{50}$) as indicated with different colours and shapes in the figure. Virus titres for each mouse are shown individually. Bars represent median + 95% CI. The dotted line shows the limit of detection for the assay. A) The viral titres were analysed from pancreases 4 dpi. Mann-Whitney test did not show significance between the studies within the same viral dose. B) The viral titres were analysed from blood 2 and 4 dpi. Mann-Whitney test did not show significance between the studies within the same viral dose and day.*

Similarly with study 1, no external signs of infection were seen in any of the mice. Weights of the mice did not vary notably (figure 12). The pancreases were similarly watery as in study 1. Overall, the wellbeing of the mice remained normal throughout the study.

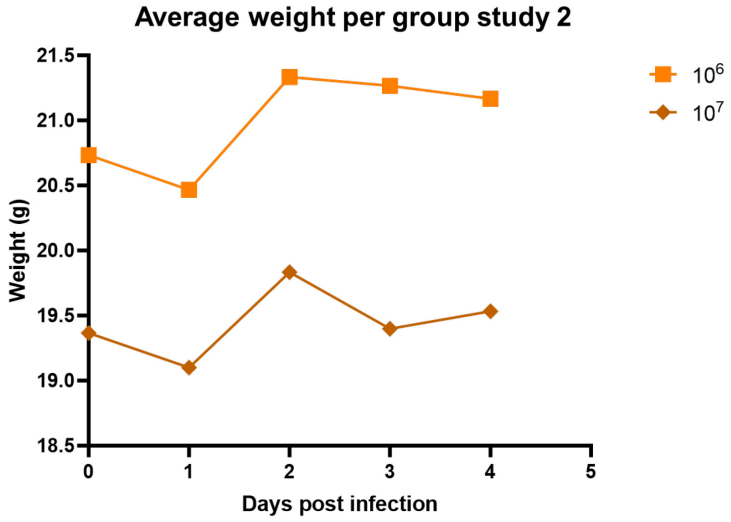

Figure 12. *Average weights of three mice within viral dose groups throughout the study 2.*

5.3.3 Study 3: impact of a prolonged infection

The aim of study 3 was to see how the duration of infection affects the viral titres. The study was carried out in the same way as study 2, except the infection was followed 7 dpi instead of 4 dpi. Again, each viral dose group contained three mice. Surprisingly, there were no positive results from pancreases or termination blood samples 7 dpi with $TCID_{50}$ analysis (figure 13A). Unfortunately, because of small sample size, we were not able to analyse the viral titres from blood samples 3 and 5 dpi and therefore could not observe if virus was present in the blood at those time points.

Similar to studies 1 and 2, there were no external signs of infection in the mice. Interestingly, the weights notably decreased from 5 to 6 dpi and then recovered 7 dpi (figure 13B). This indicates that based on the better appetite of the mice, the virus may have resolved from the mice by 7 dpi. In addition to changes in weight, the pancreases were watery in both groups. In the group of highest viral dose, pancreases were even remarkably watery further confirming that by visual inspection of the pancreases, the mice got infected.

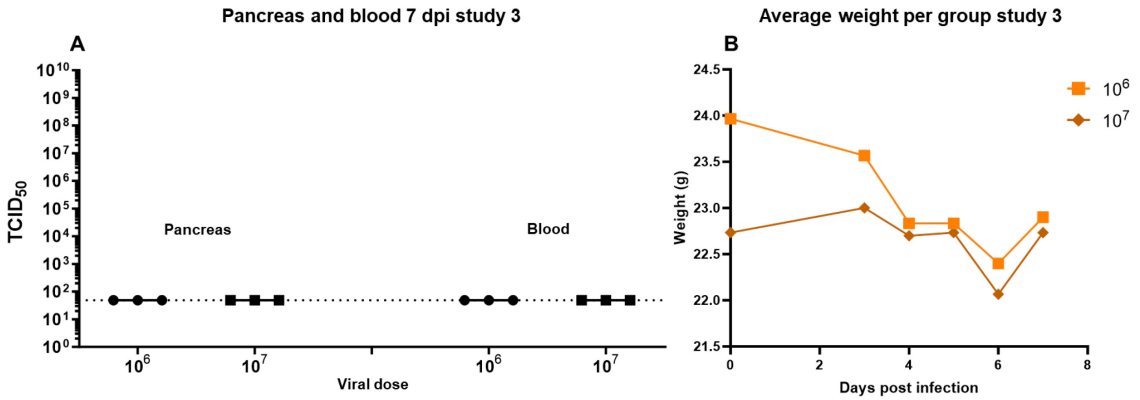

Figure 13. *A) Viral titres in pancreas and blood 7 dpi. Three female BALB/c mice per group were intraperitoneally inoculated with two previously determined doses ($TCID_{50}$) as indicated in the x-axis. Virus titres for each mouse are shown individually. Bars represent median + 95% CI. The dotted line shows the limit of detection for the assay. B) Average weights of three mice within viral dose groups throughout the study 3.*

In conclusion, we were not able to comprehensively show how the longer duration of study period affects the infection. Negative results of termination blood and pancreas samples could imply an unsuccessful infection. However, the decrease of weight and watery pancreases would imply that infection was reproducible. It will be crucial to analyse the in-between-study samples to confirm the presence of replicative virus, as studies 1 and 2 showed replicative virus in blood until 4 dpi.

5.3.4 Study 4: impact of sex

The aim of study 4 was to determine potential sex-based differences in infection outcomes. This study was a replicate of studies 1, 2 and 3 but with male mice. Same number of mice per viral group and study lengths were used. Viral titres in the pancreas show that the infection was successfully induced in male mice as well (figure 14). However, the prevalence of replicative virus was not complete in pancreases 4 dpi. Consistently, none of the termination blood samples from 4 or 7 dpi gave positive results for replicative virus. Interestingly, the percentage of positive pancreases in $TCID_{50}$ analysis decrease from 4 dpi to 7 dpi (figure 15). This result would implicate that the virus is cleared from the pancreas around 7 dpi in male mice. Female mice showed a trend of higher viral titres in pancreas 4 dpi than males (figure 16). However, these were not statistically significant differences.

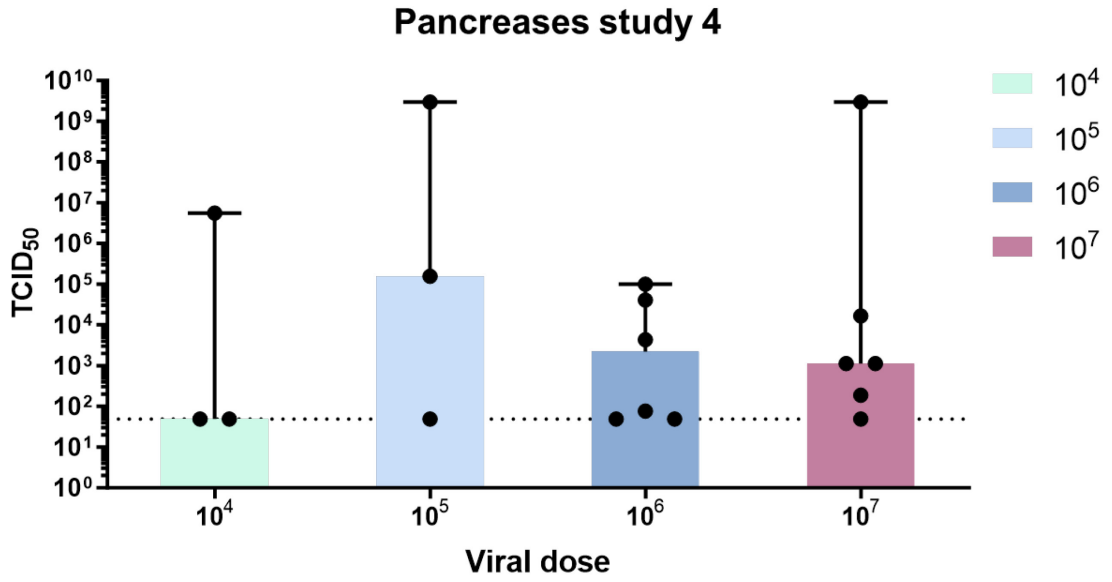

Figure 14. *Viral titres in pancreases 4 dpi from study 4. Male BALB/c mice were intraperitoneally inoculated with four different viral doses ($TCID_{50}$) as indicated with different colours in the figure. There were 3 mice for lower viral doses (10^4 and 10^5) and 6 mice for higher doses (10^6 and 10^7). The viral titres were analysed from pancreases 4 dpi. Virus titres for each mouse are shown individually. Bars represent median + 95% CI. The dotted line shows the limit of detection for the assay. Kruskal-Wallis test did not show significant differences between any of the groups.*

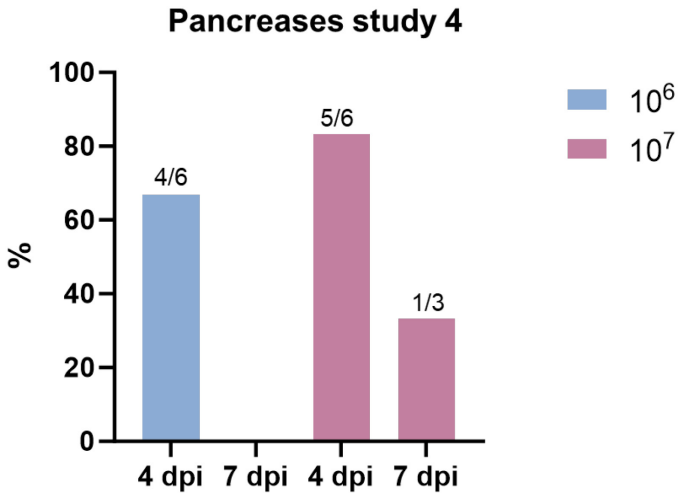

Figure 15. *Percentage of pancreases positive for replicative virus within the two viral doses and time points in study 4. The viral titres were analysed from pancreases either 4 dpi or 7 dpi. There were 6 mice for 4 dpi samples, and 3 mice for 7 dpi samples in both viral groups. Results within the same viral dose are compared and placed next to each other.*

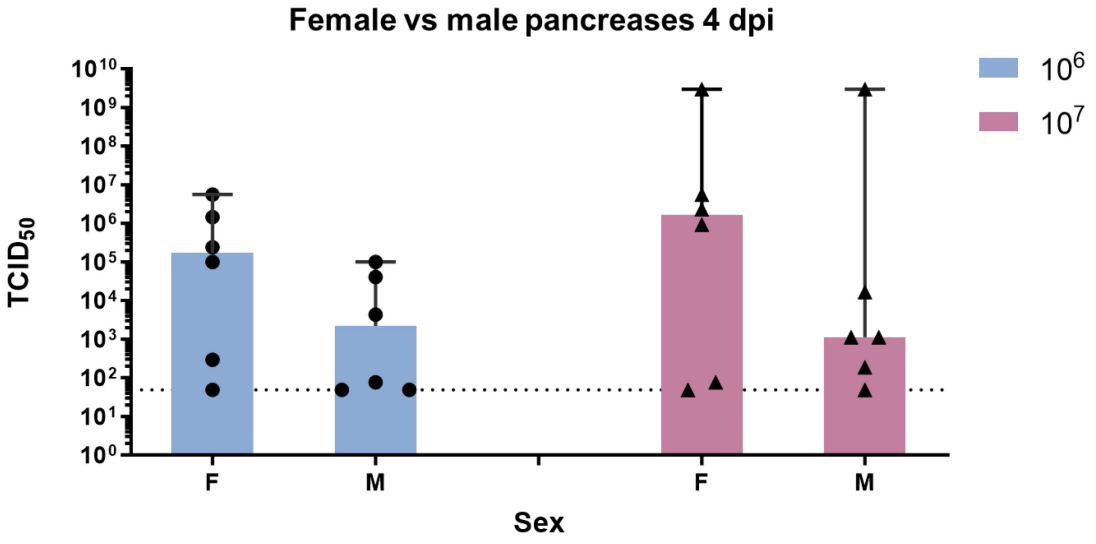

Figure 16. *Viral titres in pancreases are compared between male and female mice. F = Female, M = Male. The viral titres were analysed from pancreases 4 dpi. Titres are compared within viral doses of 10^6 and 10^7 $TCID_{50}$. There were 6 mice in each group. Female data points are from studies 1-3. Virus titres for each mouse are shown individually. Bars represent median + 95% CI. The dotted line shows the limit of detection for the assay. Mann-Whitney test did not show significant differences between sexes within the same viral dose.*

The wellbeing of mice was notably worse in male mice compared to female mice. Especially on 3 dpi, male mice got scoring 1 according to body score indexing from diarrhea and ruffled fur in groups of higher viral doses (10^6 or 10^7 $TCID_{50}$). However, the condition did not decrease after 3 dpi. Diarrhea was absent on 4 dpi, but furs were still ruffled. The groups that were terminated 7 dpi showed improved condition from 5 dpi to the end of the study. We did not need to report any notes on the wellbeing of mice with lower viral doses. We detected that male mice were fighting in several cages during study 4 which affected to the lowered body scoring index as well.

Notable weight decrease was also seen in male mice. Weight decreased up to 4 dpi in all groups (figure 17A). Even significant decrease in weight was seen in groups that received the higher doses of virus, when compared to weights of day 0 (figure 17B). It should be noted that within those groups 6/9 of mice were terminated at 4 dpi, and there are only weights of 3 mice in later time points. Weight of one mouse decreased over the limit of 15% of body weight at 5 dpi. That mouse had received the highest dose of virus. Therefore, all mice in the longer study design received dietary supplement on 5 and 6 dpi. The dietary supplements were eaten only in group of 10^6 viral dose, but it was not consumed in the 10^7 viral dose group.

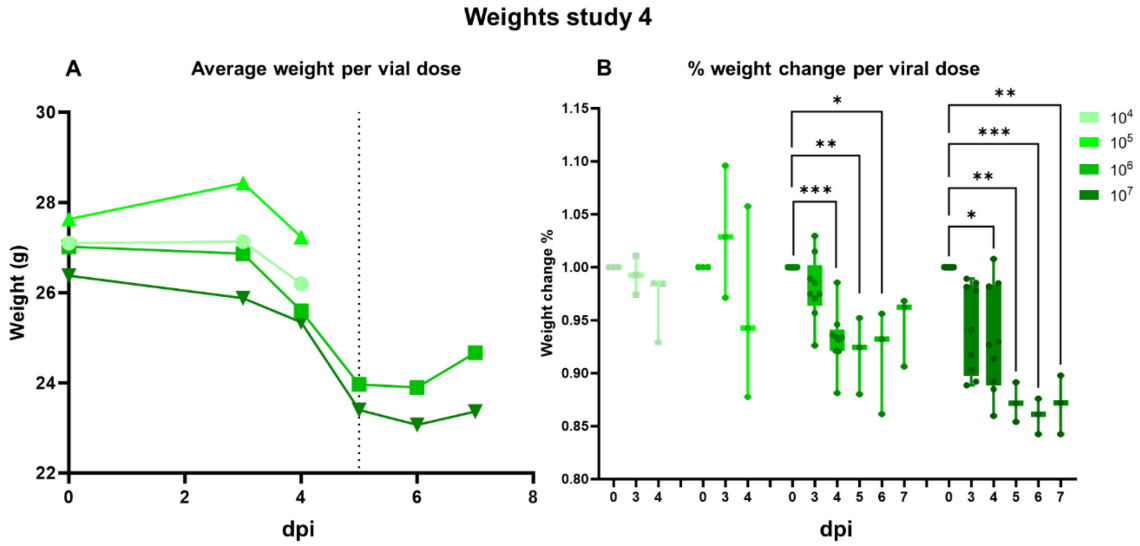

Figure 17. *Weights of male mice throughout study 4. A) Average weight per viral dose group. Viral dose groups of 10^4 and 10^5 include 3 mice per group. In groups 10^6 and 10^7, there are 9 mice until 4 dpi, and 3 mice for later time points. Dotted line at 5 dpi marks day when dietary supplement was provided in all cages. B) Percentual weight change compared to the weight on day 0. Each viral dose groups are represented in different shades of green in separate bundles. The weights are plotted as boxplots & whiskers, showing weights of individual mice. Kruskal-Wallis test showed statistical significance within viral groups when weight in later time points were compared to weight at day 0.*

There is significant difference in percentual weight change when female and male mice are compared. The difference is seen in groups of higher viral dose (figure 18C and 18D), whereas smaller viral doses do not show clear differences (figure 18A and 18B). It should be noted that number of mice is sufficient for significance calculation only in higher doses, until 4 dpi. Therefore, there is also numerical data supporting the difference between wellbeing on female and male mice. Whereas female mice showed no external implication of infection, male mice showed signs of infection during body score indexing and providing dietary supplement was required only for male mice.

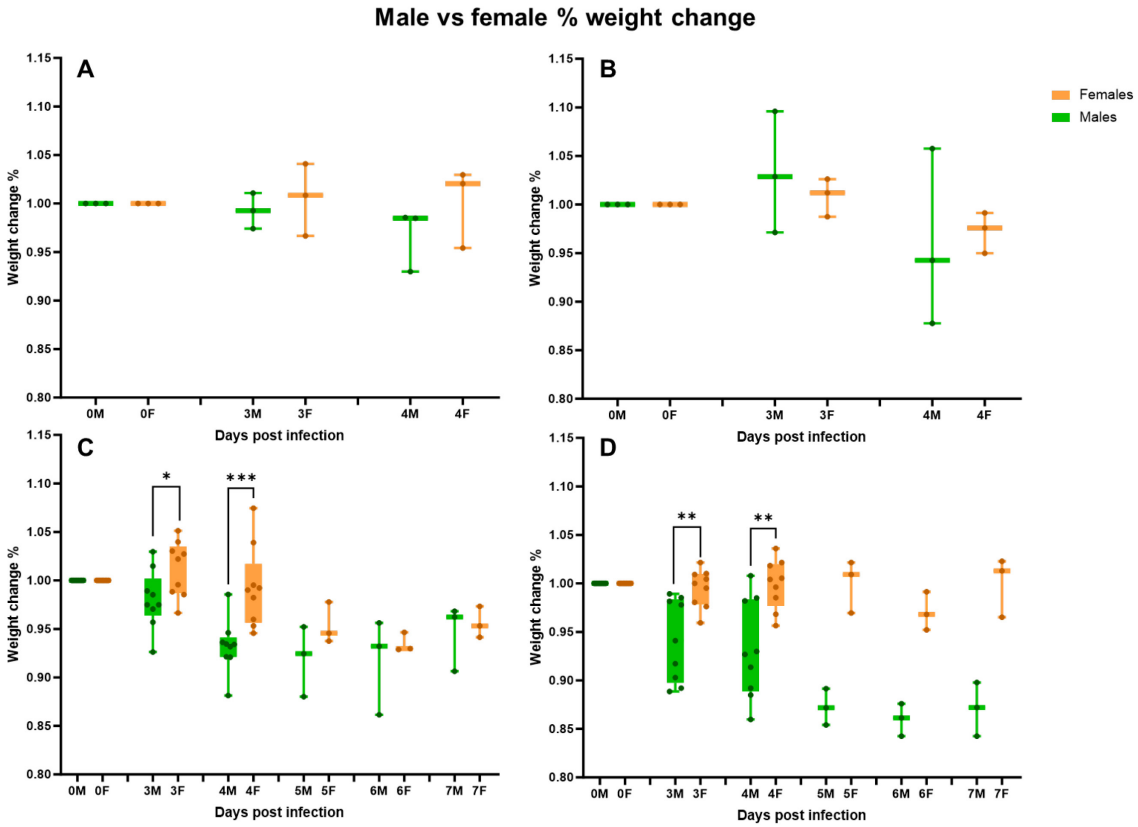

Figure 18. *Comparison of percentual weight change between female and male mice. The weight change is compared against weight on day 0. Weights are plotted as boxplots & whiskers, showing weights of individual mice. F= Female, M = Male. A) Weight change with viral dose of 10^4, comparing three mice per sex. B) Weight change with viral dose of 10^5, comparing three mice per sex C) Weight change with viral dose of 10^6., comparing 9 mice per sex until 4 dpi, and 3 mice per sex for later time points. D) Weight change with viral dose of 10^7, comparing 9 mice per sex until 4 dpi, and 3 mice per sex for later time points. Mann-Whitney test showed significant difference between sexes in C) and D) when sexes were compared within viral doses and time-points.*

6. DISCUSSION

6.1 Establishment of infection laboratory

Animal infection laboratory was established prior to infection studies. As there were no operating procedures for the laboratory, the mock-studies were crucial for figuring out the effective and safe way of working. Previous experience on mice would be preferable before starting work in infection laboratory. For instance, change in wellbeing upon infection is hard to evaluate if the worker is not familiar with normal appearance and behaviour of the mice. Similarly, examining of the organs is difficult. In addition, as all the procedures with mice are done under laminar flow hood, previous experience on working with mice is important. The visibility is limited behind the laminar glass and the movement is restricted within the laminar. Hesitation during the procedures increases the risk of needle pokes, mouse bites and other mistakes. All in all, after the determination of operating procedures and risk assessment the conduction of infection studies were rather simple.

6.2 CVB1 infection studies

We successfully demonstrated that 13-week-old female and male BALB/c mice can be intraperitoneally infected with CVB1, and replicative virus can be detected from pancreas and blood with $TCID_{50}$ method. As hypothesized, inoculation with the highest quantities of CVB1 resulted in detecting the virus consistently from pancreas. Viral titres in pancreas were high on 4 dpi, which falls in line with our hypothesis and literature where the peak of pancreatic titres is reported to be around 4 dpi[40,49].

Severe body condition scores were not given to any animal. Body condition scores were given on diarrhea and ruffled fur. No mortality nor need for euthanasia was seen during our studies. However, studies conducted elsewhere have used mortality and morbidity as a measure of the infection[30,49,56]. Some studies link mortality and morbidity particularly to i.p. infection[48]. Therefore, it was interesting to observe that no physical signs of infection could be detected in our studies with female mice. Yet, it should be noted that several methods and units ($TCID_{50}$, PFU) are used to quantify the amount of virus in studies performed in different laboratories. PFU is approximately 70% of $TCID_{50}$ result, and thus can be calculated to match $TCID_{50}$ titres. Regardless, as the virus infectivity measurement usually involves different cell lines in different passages, there are differences between the final amount of virus used in different mouse infection experiments between

different laboratories. Therefore, these differences can affect the infection level remarkably, even when the doses seem similar.

Interestingly, we were not able to detect replicative CVB1 in the heart in any of the studies, even with longer study period. One explanation can be that the used virus serotype cannot enter heart cells. However, as previous studies have reported, pancreas is an important target organ for CVB-infection[47,59], whereas infection of mouse heart requires specific CVB3 for successful infection[3,54]. However, titre of replicative CVB1 virus in the heart may have peaked later than in the pancreas in the current study, if the mice would have been followed longer[29]. Therefore, to detect virus from heart samples, the samples could be collected later than 4 dpi. Also, hearts of male mice have shown to be more susceptible for CVB3 infection than the hearts of females[57]. Thus, also CVB1 could be more readily detected from male mice. Yet, we did not get positive results from hearts in male or female mice on 4 dpi or 7 dpi. Therefore, CVB1 can be hard to detect from heart with this infection design. For studying CVB1 infection in the heart, a heart adapted version of CVB1 could be used. At least, there are studies using recombinantly modified CVB3 strain which suppressed in pancreas, to show the infection explicitly in the heart[54]. Additionally, CVB3 can be heart-passaged in mice to obtain more heart-adapted version of the virus[50,70]. Therefore, for studying CVB infection in heart overall, CVB3 would be a more competent serotype.

6.2.1 Optimal dosage and reproducibility

The results of the first study showed that viral doses of 10^6 and 10^7 $TCID_{50}$ can consistently cause an infection. The chosen viral doses were consistent with our hypothesis of needing relatively high viral doses. With higher doses all pancreases showed replicative virus. Continuing with only the higher doses was further justified, as all pancreases did not show replicative virus in study 2 or study 4, even with the higher viral doses. Similar viral doses have been used in other infection models using CVB1 and mice that are at least 10-week-old[35,37,41]. However, to determine whether 10^6 or 10^7 would be optimal, we would need larger number of mice to point out statistically significant differences between the viral doses, if there are any.

Amount of replicative virus in blood within all groups blood seemed to increase up to 4 dpi, according to studies 1 and 2. However, with lower doses the titres were higher in blood but not in pancreas 4 dpi. This could implicate that with lower viral doses the virus is not able to disseminate into pancreas, causing the virus to replicate in the blood. Replicative virus was not detected from blood 7 dpi. Likewise, another study has shown absence of replicative CVB3 in blood 7 dpi in BALB/c mice[52]. Therefore, the virus may be

completely cleared and/or disseminated to organs around 7 dpi. All in all, these results are consistent with our hypothesis and literature. To conclude, within our model, to induce an infection in pancreas, the viral dose should be relatively high for the virus to disseminate into pancreas rather than replicating only in blood.

6.2.2 Study length and sex-based differences

$TCID_{50}$ methods showed no positive results for termination blood or pancreas in study 3. The study was done similarly as the previous studies 1 and 2, where the reproducibility was already shown. Exception was that the infection was followed up to 7 dpi instead of 4 dpi. Negative results for viral replication could imply that the infection was not high level enough to exceed the detection limit of our $TCID_{50}$ analysis. However, when considering the weight change of mice in study 3, the weights started to decrease from 3 dpi. The wellbeing was reported to be similar with the previous studies. Consistently, the pancreases were reported to be even remarkably watery. Thus, the visual inspection and external examination of mice and pancreases could imply that infection could be reproducibly induced.

The results from study 3 are supported by the results obtained from study 4, where length of the infection was studied in male mice. Percent of pancreases showing positive results in $TCID_{50}$ on the 4 dpi and 7 dpi were compared. A clear decrease of the number of samples that were positive for replicative virus was seen. On 4 dpi, most of the pancreases were positive whereas only one pancreas of both viral dose groups was positive on 7 dpi. This could imply that the virus is cleared from the pancreas by 7 dpi. Unfortunately, we were not able to analyse viral titres from blood samples from 3 dpi and 5 dpi in study 3 to confirm the viral kinetics in case of female mice. Therefore, these results would imply that probably there was an ongoing infection in mice in the study 3 as well, but replicative virus had been cleared form pancreas and termination blood by 7 dpi and we were not able to detect the viral load in the right time. This is supported by literature, where replicative virus is not detected anymore in blood 7 dpi[52]. To ensure the virus presence in earlier timepoint and its clearance around 7 dpi, a new study should be done where the viral kinetics would be studied in more detail.

In addition, there were no results of replicative virus in blood 4 dpi within male mice, whereas all blood samples gave positive result 4 dpi with female mice. Since none of the blood samples of male mice had detectable levels of replicative virus, perhaps the dissemination kinetics slightly differ between female and male mice. Since the male mice pancreases were positive for replicative virus, the virus could disseminate faster to the organs than in females.

Male mice showed notable decrease in wellbeing in study 4, when compared to studies with female mice. Only male mice received score 1 according to body condition score from diarrhea and ruffled fur. In addition, the weight graphs show the progression of infection. Weights of the male mice decreased significantly in groups that had the highest viral doses (10^6 and 10^7). Alike, the results showed significant difference between the weight changes of male and female mice with highest doses starting from 3 dpi. The largest decrease was seen in male mice 5 dpi, when also dietary supplement was given. According to weight graphs, the dietary supplement was able to increase weights. However, even though weight decrease like this was not seen in female mice, the weights in study 3 showed increase after 6 dpi, without dietary supplement. This could further support the idea that the virus is cleared, and infection is alleviated around 7 dpi, whereas the infection is strongest around 4 dpi.

Interestingly, despite the worse condition of male mice, female mice showed higher titres of replicative virus in pancreas. However, the increased amount of replicative virus in female vs male pancreas was not statistically significant. Nevertheless, male mice seem to have stronger symptoms of the infection, even though the viral doses were the same between sexes. The hypothesis of the infection being stronger in male mice, and especially in heart was not validated. Infection only deceased the wellbeing of male mice, but not induce high viral titres. Also, replicative virus was not seen in any of the hearts, including the hearts of male mice.

6.3 Future prospects

6.3.1 Improving the CVB1 infection model

For this pilot study, the number of mice was chosen to be low in compliance with the principles of 3R. The pilot study provided valuable information on how the infection studies should be conducted and what aspects need to be considered. Therefore, these results were not expected to show statistical significance, but rather validate the procedures and methods that will be used to conduct future studies.

To further validate the findings according to the study length or sex differences, for example, those aspects should be compared within one study. We have seen with our collaborators that sometimes an infection study can simply fail because the amount of virus needed to induce reproducible infection needs to be considerably high. Therefore, to get more reliable data, the differences in virus infection level should only be compared within independent mouse infection experiments.

The results of this thesis have provided valuable information of how the infection develops and what can be expected in future studies. For example, to minimize animal suffering, we should use female mice for studies within this model. When detecting the virus from pancreas, the study should not last over 4 dpi. The virus titres can vary remarkably even between the same viral dose from absence of virus to virus with very high titres. Also, viremia can be considerably different within one viral dose. It was also clear that this infection model is not suitable for studying CVB infection in the heart. For a heart model another virus serotype of genetically modified or passaged CVB1 should be used.

The analysis methods could be further improved. Especially, optimization of the $TCID_{50}$ should be continued. One aim would be downscaling the assay. For example, the volume of in between study blood samples is small (30µl) and with current method is sufficient for only one assay. With smaller volume per sample, also the in between study samples could be analysed multiple times. In addition, for reliable results, the $TCID_{50}$ analysis should be done twice within separate assays, where result would be average of those two analyses. Therefore, also the results of this thesis should be analysed again for more consistent results. Similarly, the samples from this thesis can be utilized for optimization of the method.

Other methods along $TCID_{50}$ could also be used to further research progression of the infection. As discussed, $TCID_{50}$ method detects replicative virus which can infect new cells. However, PCR or RT-PCR could detect where viral RNA or proteins are present[58]. It could be, for example, used for confirming infection in heart even when replicative virus is not found. Also, histopathological analyses would provide important information about inflammation in different organs and is widely used in infection studies overall[14,39,43,49,53]. For example, the severity of inflammation can be evaluated and compared according to the histopathological samples[49]. Immunohistochemical analyses are also used to detect for example VP1 protein or CAR from organ samples[39,48]. Additional methods would enable deeper understanding of the infection mechanism and increase the reliability of the results.

6.3.2 Possible applications of the infection model

Further optimization of this infection model would enable conduction of challenge studies for determining the safety and efficacy of vaccines. We have previous experience and data of female BALB/c mice vaccinated against CVB1[35,66]. The experience and results on females would further support the use of female mice, along with better condition upon infection and the lack of fighting in prolonged housing. However, utilizing more sensitive methods (e.g. RT-PCR[53,58], histology[39,56]) for quantifying the amount of virus

from infected mice, would give more reliable results of the progression of the infection as well as the safety and efficacy of the developed vaccines. With established analyses, the effect of a vaccine could be studied in more detail. For example, with $TCID_{50}$, RT-PCR and histology we could analyse whether a vaccine can reduce the amount of replicative virus, block viral entry to pancreas or alleviate the inflammation in organs, respectively.

The age of 13-weeks for this experimental design was chosen with challenge studies in mind. This age would enable administration of several doses of vaccine before induction of the infection. However, as CVB infections tend to be more severe in younger individual, age differences could be tested as well. Usually, younger mice around 4-week-old are used in models studying the infection mechanism[14,43,48,49]. The age could be tested in our model too, which would enable choosing the optimal age for different applications. Similarly, the length of the infection study could be prolonged from days to weeks, to either study the progression of infection or to model persistent infections.

Additionally, the established facility enables infection studies with different designs. For example, different inoculation routes such as oral cavage could be performed within the facility. In addition, other virus serotypes could be used. However, new designs would require new testing of optimal viral dose, study length, sex, and mouse age. As an example, CVB3 could be used for an acute myocarditis model. According to the literature and table 2 the viral dose could be lower with CVB3. Male mice would be more susceptible for infection, especially in heart. Also, the length of the study should be optimized as replicative virus has been seen later in the heart compared to pancreas.

7. CONCLUSIONS

The aims of the thesis were to establish preclinical infection laboratory for Tampere University and to set up an infection model for CVB1 in mice. In conclusion, both of the aims were completed. The facility was established with functional layout providing safe working environment. Optimal workflow and practices were evaluated in mock-studies and operating procedures for the facility were determined.

Also, the infection model was successfully established. Design of our infection model was intraperitoneal inoculation of CVB1 in male and female BALB/c mice. The infection was confirmed from titres of replicative virus in blood and organs detected with $TCID_{50}$ method. Weights and wellbeing of the mice were checked daily according to body score indexing.

Our infection model was optimized according to viral dose, study length and sex. The most optimal viral doses were 10^6 and 10^7 $TCID_{50}$, according to virus titres in pancreas and blood. Replicative virus was most consistently found from pancreas. With optimal doses, viral replication occurred mainly in pancreas instead of blood 4 dpi. Replicative virus was not found in majority of samples 7 dpi, which implicates that the infection is cleared by 7 dpi. Female mice showed higher viral doses in pancreas and blood 4 dpi compared to male mice, but the difference was deemed insignificant. Only male mice showed significant weight loss and got lowered points during body score indexing.

In conclusion, the aims of the thesis were completed successfully. This thesis work and the results provide a good starting point for future infection studies and applications. In further optimization, our results support the use of female mice. The viral titres were somewhat higher in females, whereas their condition was considerably better than in males. Use of females would avoid the suffering of mice used in the following studies. Also, following the infection until 4 dpi is suitable for causing an infection in the pancreas as viral titres have not yet decreased by that timepoint. Our results implicated that the viral titres in blood increase at least up to 4 dpi. The results obtained from this study, provide valuable insights into infection modelling, disease mechanism studies as well as studying the safety and efficacy of vaccines against infective virus.

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APPENDIX A: OPERATING PROCEDURES FOR ABSL-2 LABORATORY.

Operating procedures for E-124 Infection laboratory.

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Checked by: Minna Hankaniemi

Drafted 6.10.2023 and checked 15.10.2023.

1. PURPOSE

This manual describes the general procedures to be followed when working in the E-124 infectious laboratory of the preclinical facility in the Arvo building.

2. DEFINITION

These premises are authorized for handling biological agents classified up to Biosafety Level 2 (BSL-2). The laboratory conducts infectious work with different strains of mice and utilizes wild-type Coxsackie B viruses within the facility (BSL-2 classification). BSL-2 level containment procedures are also followed in this space.

The infectious laboratory entrance features a biological hazard sign and an electronic access control device, allowing entry only to authorized personnel. Authorization is granted after individuals have received training on the operations of the infectious laboratory. Separate training is required for conducting procedures with mice.

Acceas to the infectious laboratory is strictly limited to authorized personnel, and the doors must always remain closed. In addition to this manual, the general guidelines for Research Laboratories are also followed.

3. RESPONSIBILITIES

Every individual working in the infectious laboratory is responsible for following this operating procedure. The names and contact information of responsible personnel can be found on the infectious laboratory entrance.

4. WORK LIMITATIONS

There are cases in which an employee may not be able to work in the infectious laboratory due to personal reasons. If unsure, consult the laboratory supervisors or seek additional guidance from occupational health regarding pathogens currently under investigation.

Pregnant individuals can work in the infectious laboratory as the viruses handled in the laboratory (as of September 2023) are not harmful to pregnant individuals and do not transfer to the fetus. It is recommended that pregnant individuals have neutralizing antibody levels in their serum measured before performing infectious work involving Coxsackie viruses. Additionally, it is advised that pregnant individuals act only as assistants during procedures. Isoflurane anesthesia is used in the facility, so pregnant employees

should decide whether they want to work in the lab during procedures based on their discretion.

Individuals with immunodeficiencies are advised to use additional protective equipment: FFP2 masks, safety goggles, and cut-resistant gloves. Immunodeficiency makes individuals more susceptible to various infections. Factors contributing to immunodeficiency include virus infections (e.g., HIV), chronic inflammatory diseases, and cancer. Immunodeficiency can also result from cancer treatments (e.g., radiation, chemotherapy, and glucocorticoid therapy). Medications (e.g., opioids) may also cause immunodeficiency. If uncertain about hazards related to one's own health, contact your supervisor, physician, occupational health services (Terveystalo), or the Head of the Preclinical Department.

5. PREMISES

At the entrance to the facility, an area is marked with tape, distinguishing between the clean and potentially infectious sides of the laboratory. The side of the tape facing the door is clean and is used for changing into protective clothing.

The laboratory is equipped with a laminar flow cabinet for BSL-2 work, which includes a balance, heater, inhalation anesthesia induction box, and inhalation anesthesia maintenance mask. Additionally, outside the laminar flow cabinet, there is an active carbon filter for inhalation anesthesia, as well as vaporizers and gas meters. The room also contains a closed-loop animal cage rack system (BCU-2 system) for infected mice.

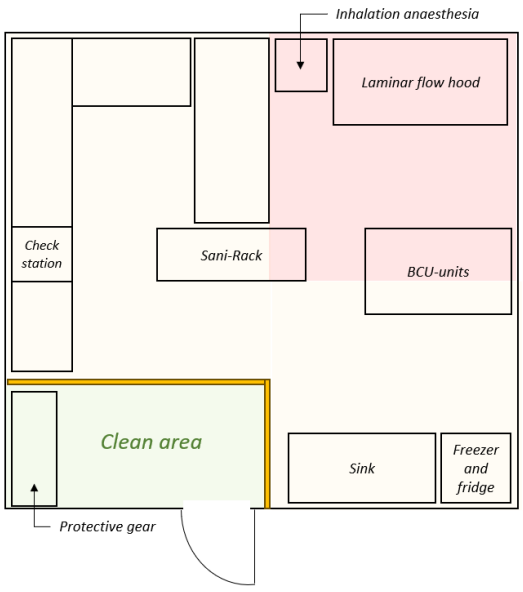


Figure 1. Floor plan of the mouse infection laboratory space E-124.

6. DRESSING AND PROTECTION

Access to the infectious laboratory is through the designated clean area, where individuals put on a protective, disposable coveralls specific to the infectious laboratory, as well as laboratory-specific footwear/foot covers. Protective suits are on the left side upon entry, with footwear stored underneath. Personal indoor shoes are left on the floor to the left of

the footwear rack. If desired, personal clothing can be hung on the coat rack to the left of the lab coats.

After passing through the air shower into the animal facility, one dresses normally in protective clothing for animal rooms (mask, gloves, protective headgear), and additionally, cotton gloves/cut-resistant gloves must be worn underneath nitrile gloves. Upon entering the laboratory, long gloves are worn over the gloves already in place. Gloves are put over the sleeves so that no bare skin is exposed, and if necessary, they are taped to the protective cover all.

When leaving the laboratory, the long gloves are left in the laboratory in the autoclaved waste container. The entry door opening mechanism is already a 'clean area', so it is not touched by dirty gloves. The infectious disease laboratory coverall and shoes are left inside the taped area.

7. CLOSED-LOOP BCU-2 CAGE SYSTEM

Infected animals housed in a specialized closed-loop cage system called the Biocontainment Unit 2 (BCU-2). The cages are individually ventilated and maintained at negative pressure. The rack can accommodate a total of 48 cages, with at least one being a BioSENSE cage (this cage measures and detects any deviations in air circulation). Cages in long-term storage should be kept open to prevent damage to the cage seals.

7.1. ANIMAL CAGES

To remove a cage from the rack, press the button on the left side of the cage. When returning the cage to its position, it should snap into place with the yellow marker hidden. If the marker is visible, the cage's ventilation is not functioning correctly, and the cage should be pushed into position more securely (see Figure 2).

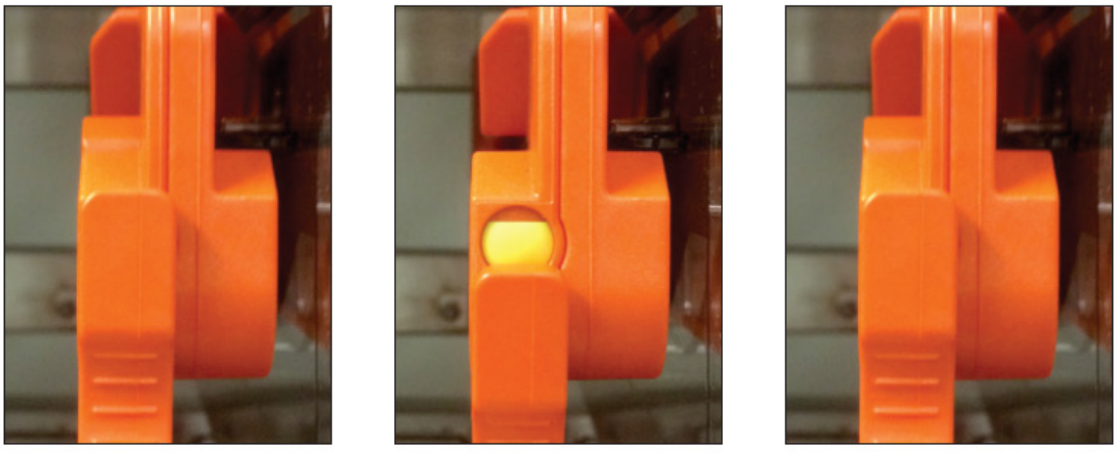


Figure 2. Docking cages into the BCU-2 cage system.

It is crucial to install the cage lid in the correct orientation, as incorrect placement will disrupt the cage's airflow (see Figure 3).

Figure 3. BCU-2 cage assembly.

7.2. BIO-SENSE CAGE

The Bio-SENSE cage must always be securely attached to the BCU-2 system, otherwise, the air circulation in the cage system will not function correctly (see Figure 4). Animals can be kept in the Bio-SENSE cage as usual, but when removing the cage, another Bio-SENSE cage must always be attached to the cage system. In principle, the Bio-SENSE cage is kept empty if it is not necessary to use it to house animals.

Figure 4. Attach the Bio-SENSE recoil hose to the Blower port and the Bio-SENSE cage port.

7.3. CAGE INSPECTION STATION

The cage inspection station is used to inspect the airflow inside the cage (see Figure 5).

1. **Main Power Switch –** Toggles power to the unit either on or off.

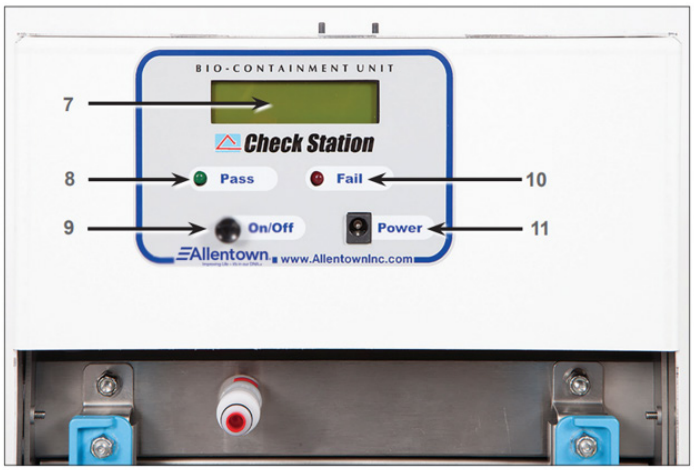
2. **Power Cord Input –** The female end of the supplied power cord is plugged in here, the male end is plugged into a standard outlet.

3. **Supply / Exhaust Ports –** Top port measures the exhaust flow from your test cage. Bottom port supplies the appropriate amount of air necessary to test your cage's seal.

4. **Body –** Body has rubber feet on bottom used to properly secure the unit during operation.

5. **Cage Lock –** This is used to lock the cage in place during the test. It swivels between locked and open positions.

6. **Cage Runner –** Used to properly secure the cage into the unit.



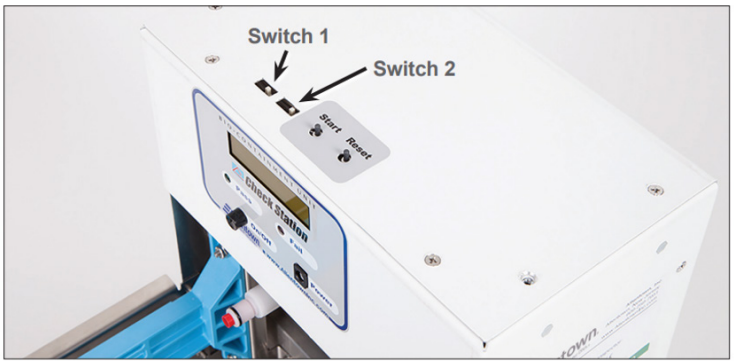
7. **Display –** Information such as whether the unit is ready and test results will be displayed on this screen.

8. **Pass Light –** This green light will illuminate if the cage passes the test.

9. **On / Off Switch –** Used to turn the front display on or off.

10. **Fail Light –** A cage failing the test will cause this red light to illuminate.

11. **Power Port –** Disabled for this application.



12. **Switch 1 –** Switch included for future expansion, not currently usable.

13. **Switch 2 –** Switch included for future expansion, not currently usable.

14. **Start Switch –** Used to begin the test on the cage.

15. **Reset Switch –** Resets the unit, so the next test can begin.

Figure 5. Features and interface of cage inspection station.

The station should only be moved when it is without a cage; otherwise, the sensors on the station may be damaged. The station should not be placed near water sources, and it should not be handled with wet gloves while it is powered. Also, do not touch the metal end of the station (see Figure 6).

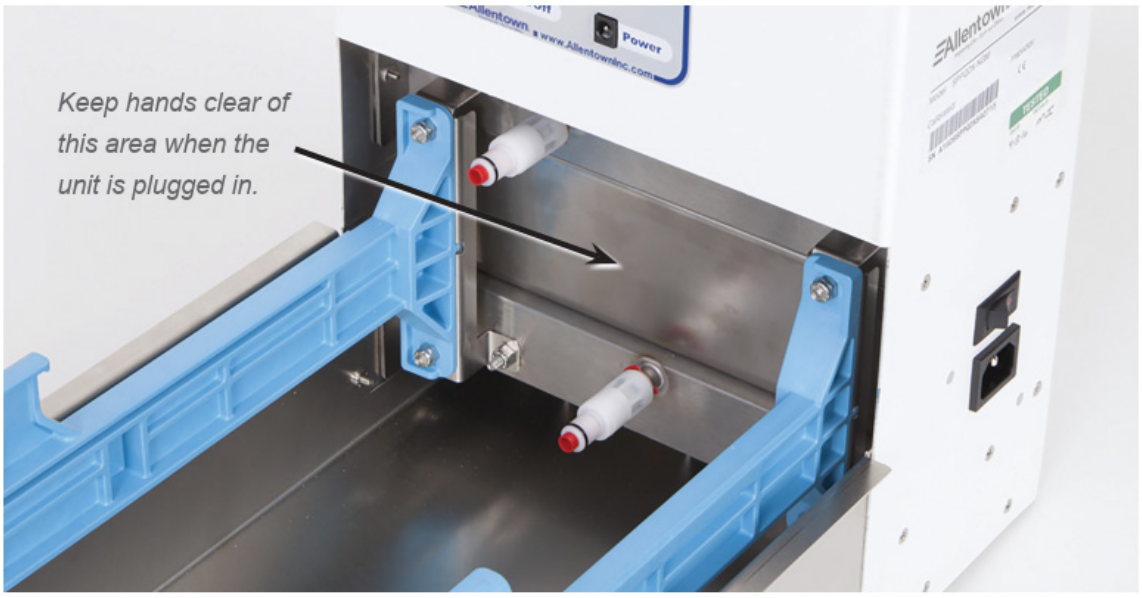


Figure 6. Safety precautions of Fitting Check Station.

The airflow in the cage should be checked every time the cage lid is opened. Docking the cage to the station follows the same principle as docking it to the cage system.

1. Turn on the station
2. Press the "Start" button on the upper panel.
3. If the green light is illuminated next to the "Pass" mark, the cage is ready to be docked to the BCU-2 cage system.
4. If the red light is illuminated next to the "Fail" mark, the cage's airflow is not functioning correctly. Remove the cage from the station, transfer it to the laminar flow cabinet, and check in the laminar flow cabinet that the cage lid is securely closed and that the black filter inside the cage is dry.
5. Try again until the cage passes the airflow testing.

8. WORKING IN THE LABORATORY

All infectious work must be carried out with special care.

The use of needles, glass Pasteur pipettes, scalpels, or other sharp objects requires special caution. When using the aforementioned tools, it is recommended to wear gloves that protect against cuts.

8.1. DOCUMENTATION

Each worker maintains their own laboratory diary according to the group's instructions. There is an updated list on the laboratory wall where individuals working in the facility record their names and phone numbers.

8.2. PERFORMING PROCEDURES

When conducting procedures, work is primarily done in pairs in the room. However, animal welfare checks and weighing can be performed individually.

When working with infections in the facility, work should only be done in pairs so that one member of the pair does not handle the infectious virus; their task is to assist the other member handling the infectious virus. The work pair has a clear division of tasks and work order, which is always planned in advance, and the pair respects the clean and infectious side boundaries set within the facility.

While working, it should be noted that the air in the cages will run out in approximately 20 minutes unless the cage lid is either opened in the laminar flow cabinet or the cage is properly attached to the BCU-2 cage system.

8.3. CLEANING WORKSPACES

- Contaminated surfaces or objects should be initially cleaned primarily with ProChlor, which can be used without cleaning the product away (such as with Virkon). This solution/spay should be used to clean the laminar flow cabinet and any other potentially contaminated items (pipettes, tube racks, etc.), as well as the tabletop surfaces used for virus work.
- Larger spills should be wiped up with absorbent paper. Spill areas are treated with Virkon/ProChlor for at least 10 minutes, and the absorbent papers are placed in autoclave waste.
- Empty containers that have held or been in contact with viruses are treated with 1% Virkon and placed in the sink for washing.
- After working, the UV light in the laminar flow cabinet is turned on for 40 minutes.

8.4. STORAGE OF BSL-2 SAMPLES

- All tubes or containers containing viruses must be stored on designated shelves in the freezer or refrigerator.
- To prevent contamination, samples containing viruses must not be placed on shelves intended for clean materials; the shelves should be clearly labeled.
- All tubes and bottles stored in the refrigerator or freezer must be clearly labeled with their contents, date, and the initials of the responsible person for the sample. Due to limited storage space, all unnecessary samples and bottles should be disposed of in accordance with appropriate guidelines as quickly as possible.

8.5. OTHER GUIDELINES TO AVOID CONTAMINATION

- The laminar flow cabinet in the infectious laboratory is suitable for handling viruses and infected animals, but when working, care must be taken to ensure that work is done deep enough inside the laminar flow cabinet (not on the front grille).
- Nothing in contact with viruses should be removed from the infectious laboratory without being inactivated with ProChlor or 1% Virkon.
- Viral samples are transported out of the laboratory only in a sealed transport container. Transport containers are lidded plastic boxes located in the laboratory's

entrance area. Carts should not be brought into the infectious laboratory (nor should infectious laboratory carts be taken out of the laboratory). The outer surfaces of transport containers must be kept free from viruses. Anything that may have come into contact with viruses should be thoroughly wiped with ProChlor or 1% Virkon and 70% ethanol.

9. WASTE MANAGEMENT

In the infectious disease laboratory, all waste is treated as special waste and collected separately for autoclaving.

Solid loose waste (sawdust, gloves, etc.) is collected in double autoclave bags in a special waste container. Pipette tips can be collected in plastic bags, which, when filled, are placed in the special waste container. No lids or caps should be tightly closed, as the containers may explode during autoclaving or the sterilization result may deteriorate. When the waste container is full, the inner autoclave bag is folded closed inside the outer one. The outer bag is closed loosely with CLEAN GLOVES and loosely taped, and the date and initials are marked on it. The transfer of waste to the autoclave is always arranged separately with the animal caretakers. Two new autoclave bags are placed inside the special waste container.

Cage materials are autoclaved using the SANIRACK system (see section "Sani-Rack Usage"), after which they can be disposed of like normal cage material waste.

Sharp waste is collected inside the laminar flow hood into an empty disposable plastic container. When the container is full, it is closed by loosely taping the lid over the opening and disposed of with solid special waste.

Liquid waste is inactivated with Virkon so that the final concentration of Virkon is approximately 1%, and it is allowed to act for at least 10 minutes before being emptied into the sewer with a large amount of water flush.

Carcasses are collected in closed plastic bags in the freezer until they are sent to Lassila & Tikanoja in identifiable biological waste barrels (200L).

10. SANI-RACK USAGE

Animal cages are sterilized with their cage materials before going for washing. In the infectious disease laboratory, care is taken to ensure that a HEPA filter is placed in the Sani-Rack for those cage locations where a sterilizable cage will be placed (see Figure 7). Attach autoclavable cages to the Sani-Rack system, and arrangements are made with the animal caretakers for moving the rack to the autoclave. After each autoclave cycle, one prong is removed from the filter ribbon disk (see Figure 8).

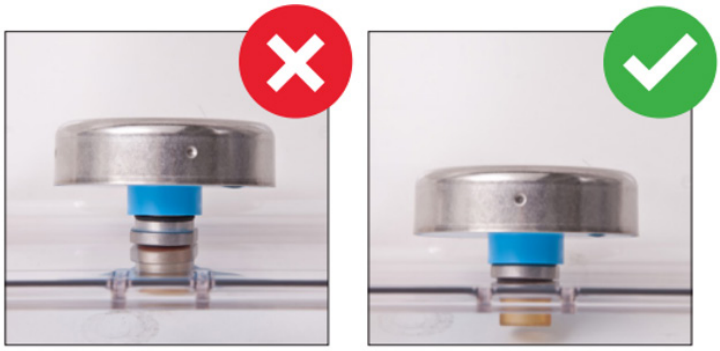


Figure 7. Attaching the Sani-Rack HEPA filter to the Sani-Rack autoclaving system.

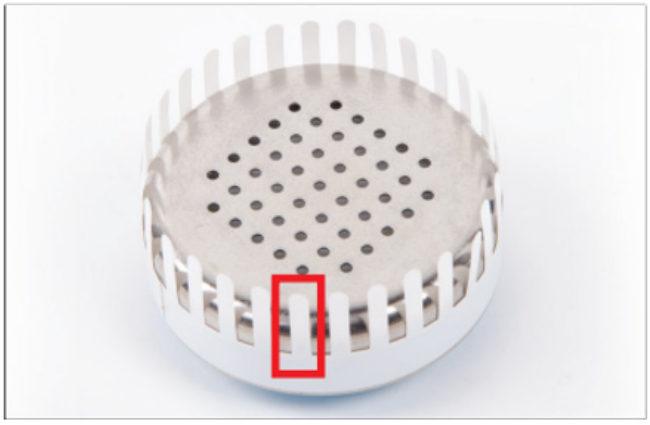


Figure 8. To facilitate counting autoclave cycles for the Sani-Rack HEPA filter, there is a ribbon disk on the filter, from which one strip is always removed at the end of sterilization (indicated with a red box).

11. DAILY ANIMAL INSPECTION

Animal welfare is checked daily, ensuring that there is an adequate supply of water and food. Feed is added as needed. The condition of the animals can be assessed from outside the cage, but if necessary, the cage is opened in the laminar flow hood. Any observed welfare issues are marked on the cage with tape and recorded in the animal information system. In serious cases, contact is made with the researcher or the department veterinarian. A picture of the animal can also be taken and sent for assessment. Even if the researcher cannot be reached, a severely ill animal should be euthanized at the discretion of the personnel. Both deceased and euthanized animals are placed in a bag and stored in the processing room freezer with sufficient identifying information, and the corresponding researcher is notified.

12. ACTION IN CASE OF EMERGENCY

The viruses handled in the infectious disease laboratory are capable of infecting human cells, so there is a risk of emergencies.

Skin contact with material containing viruses is avoided in all situations because there is a risk of infection if the skin is broken (see dressing and protection instructions). Likewise, mucosal contact poses an infection risk, so caution should be exercised when handling solutions containing viruses to avoid splashes (eyes, mouth). Viral entry into a

wound poses a significant infection risk.

Prevention of accidents
Viral work should always be conducted in a laminar flow hood. Only hands may be brought into the biosafety cabinet, and the hands must be completely protected so that no bare skin is visible. Most of the equipment used in viral work is disposable, and virus waste is handled separately. When handling sharp objects in the biosafety cabinet, special care must be taken, and the workflow must be clear. Systematic operation reduces the risk of skin contact for the worker.

Virus tubes should never be opened anywhere other than in the laminar flow hood. The primary safety measure is to avoid skin contact with viruses or virus-contaminated areas. Persons cleaning a contaminated area must always wear appropriate protection (protective jacket, gloves, etc.).

The following measures should be followed if a person has been exposed to virus-containing fluid. Briefly:

Stop exposure
Clean exposed body parts with soap and plenty of water rinse
Remove contaminated materials
If necessary, call for help by phone

1. The exposed person should avoid spreading contamination, so stay at the accident site and immediately remove direct exposure (e.g., remove contaminated clothes in direct skin contact).
2. The skin area exposed to the virus should be immediately cleaned with plenty of water
    a) If there is a wound, it should be allowed to bleed first and then washed with water and soap
    b) Skin contact without a wound is washed with soap and water
    c) In case of mucosal exposure (eyes or nose), the area is washed with water
3. After virus contamination has been washed from the exposed skin area, dispose of all contaminated materials (e.g., clothes) as special waste. Clean contaminated equipment with ProChlor or 1% Virkon.
4. The infectious disease laboratory supervisors and Brian Mphande, the head of the preclinical department, must be informed of the accident.
5. The employee must report the accident to their immediate supervisor.
6. The person exposed to the virus should go to the emergency room (ACUTA) for proper treatment.
7. A report on the accident must be sent to the laboratory manager and the occupational safety representative, Merja Peltomaa (phone +358504689581). The report must include detailed information about the accident, exposure, and actions taken after the accident. The same report must be copied and sent to the person in charge of viral work.
8. After a case analysis, consideration will be given to implementing new procedures to prevent similar accidents in the future. The responsible personnel in the infectious disease laboratory will inform all personnel involved in viral work about the new procedures.