DETECTION OF ENTEROVIRUS D68 INFECTION AMONG MALAYSIAN POPULATION USING A PRELIMINARY LUCIFERASE-BASED SERONEUTRALIZATION TEST

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DETECTION OF ENTEROVIRUS D68 INFECTION AMONG MALAYSIAN POPULATION USING A PRELIMINARY LUCIFERASE-BASED SERONEUTRALIZATION TEST

ABSTRACT

Enterovirus D68 (EV-D68) is a respiratory tract pathogen which causes a range of mild to severe respiratory symptoms and in rare cases, neurological symptoms such as acute flaccid myelitis. In 2014, major outbreaks of EV-D68 occurred in several countries such as the United States and Canada, demonstrating that EV-D68 is an emerging pathogen. Therefore, studying the prevalence of EV-D68 infection in the Malaysian population is important to predict future outbreaks. In this study, a recombinant EV-D68 virus expressing a NanoLuc luciferase reporter gene (EV-D68_Nluc) was constructed using restriction enzyme-based cloning. This reporter-expressing infectious clone was used to develop a preliminary luciferase-based seroneutralization test which yields results 3 days faster than the conventional test. Subsequently, the luciferase-based test was used to assess the seroprevalence of EV-D68 neutralising antibodies (NAbs) in Malaysia using serum samples from three age groups, children (1 to 12 years old), adults (13 to 49 years old) and elderly (50 years old and above) collected in 2013, 2014 and 2015. We hypothesised that EV-D68 NAb seroprevalence would increase after 2014 in all age groups, but found a significant increase only in adults. Furthermore, we also hypothesised that EV-D68 NAb seroprevalence would increase with age. This was supported by our finding that children had significantly lower seroprevalence rates compared to adults and elderly. In addition, within the children group, seroprevalence rates increased with age: 32%, 59% and 77% in 1-3, 4-6 and 7-12 years old children, respectively. Hence, children should be prioritised in future vaccination programmes.

Keywords: Enterovirus D68, NanoLuc luciferase, infectious clone, seroepidemiology

PENGESANAN JANGKITAN ENTEROVIRUS D68 DALAM KALANGAN PENDUDUK MALAYSIA MENGGUNAKAN UJIAN AWAL PENEUTRALAN BERASASKAN LUCIFERASE

ABSTRAK

Enterovirus D68 (EV-D68) adalah sejenis virus boleh menyebabkan simptom pernafasan dan simptom neurologi. Pada tahun 2014, wabak EV-D68 terjadi di beberapa negara seperti Amerika Syarikat dan Kanada. Oleh itu, kajian mengenai jangkitan EV-D68 dalam kalangan masyarakat Malaysia adalah penting bagi meramal kejadian wabak. Dalam kajian ini, virus EV-D68 yang diubah suai untuk mengandungi gen pelapor *NanoLuc luciferase* (EV-D68_Nluc) dibina melalui *restriction enzyme-based cloning*. Virus ini digunakan dalam ujian awal peneutralan berasaskan *luciferase* yang dapat menghasilkan keputusan 3 hari lebih pantas berbanding ujian konvensional. Seterusnya, ujian ini digunakan untuk menilai penyebaran antibodi EV-D68 di Malaysia menggunakan sampel serum daripada tiga kumpulan umur, kanak-kanak (1 hingga 12 tahun), dewasa (13 hingga 49 tahun) dan warga tua (50 tahun dan ke atas) yang dikumpul pada tahun 2013, 2014 dan 2015. Kami membuat hipotesis bahawa penyebaran antibodi EV-D68 meningkat selepas tahun 2014 dalam setiap kumpulan umur. Namun, hasil kajian menunjukkan bahawa ia hanya meningkat dalam kalangan dewasa. Selain itu, kami membuat hipotesis bahawa penyebaran antibodi EV-D68 meningkat dengan umur. Ini disokong oleh hasil kajian yang menunjukkan bahawa penyebaran antibodi EV-D68 dalam kalangan kanak-kanak lebih rendah berbanding dewasa dan warga tua pada setiap tahun. Di samping itu, dalam kumpulan kanak-kanak, penyebaran antibodi EV-D68 meningkat dengan umur: 32%, 59% dan 77% dalam kanak-kanak berumur 1-3, 4-6 dan 7-12 tahun. Maka, kanak-kanak wajar diberikan keutamaan dalam program vaksinasi.

Kata kunci: Enterovirus D68, *NanoLuc luciferase*, *infectious clone,* epidemiologi

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LIST OF SYMBOLS AND ABBREVIATIONS

ºC	:	Degree Celsius
µg	:	Microgram
µl	:	Microliter
2AproCS	:	2A protease cleavage site
AFM	:	Acute flaccid myelitis
AFP	:	Acute flaccid paralysis
ATCC	:	American Type Culture Collection
ATP	:	Adenosine triphosphate
bp	:	Base pair
CDC	:	Centres for Disease Control and Prevention
cDNA	:	Complementary deoxyribonucleic acid
CMC	:	Carboxymethyl cellulose
CO_2	:	Carbon dioxide
CPE	:	Cytopathic effect
CVA-16_Nluc	:	NanoLuc luciferase-expressing coxsackievirus A16 infectious clone
DMEM	:	Dulbecco's Modified Eagle's Medium
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
dpi	:	Days post-infection
E. coli	:	*Escherichia coli*
EDTA	:	Ethylenediamine tetraacetic acid
EGFP	:	Enhanced green fluorescent protein
EV-D68	:	Enterovirus D68
EV-D68_Nluc	:	NanoLuc luciferase-expressing enterovirus D68 infectious clone

FBS	:	Fetal bovine serum
Fluc	:	Firefly luciferase
Gluc	:	*Gaussia* luciferase
GFP	:	Green fluorescent protein
GMT	:	Geometric mean titre
H_2O	:	Hydrogen dioxide, water
ICAM-5	:	Intercellular adhesion molecule 5
kb	:	Kilobase pair
kDa	:	Kilodalton
LB	:	Lysogeny broth
m	:	Month
ml	:	Millilitre
NAb	:	Neutralising antibody
NEB	:	New England Biolabs
Nluc	:	NanoLuc luciferase
nm	:	Nanometre
nt	:	Nucleotides
ORF	:	Open reading frame
PCR	:	Polymerase chain reaction
Pol I	:	Polymerase I
RCREV	:	Replicating-competent reporter-expressing virus
RD	:	Rhabdomyosarcoma cells
RLU	:	Relative light unit
Rluc	:	*Renilla* luciferase
RNA	:	Ribonucleic acid

rNTP	:	Ribonucleoside tri-phosphate
RTI	:	Respiratory tract infection
RV	:	Rhinovirus
SFM	:	Serum-free medium
T7-EV-D68	:	T7 promoter-driven prototype EV-D68 strain Fermon infectious clone
T_a	:	Annealing temperature
TC	:	Tetracysteine
$TCID_{50}$	:	Tissue culture infectious dose
UMMC	:	University of Malaya Medical Centre
USA	:	United States of America
UTR	:	Untranslated region
VLPs	:	Virus-like particles
y	:	Year

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Enterovirus D68 (EV-D68) is a small, non-enveloped and single stranded positive-sense RNA virus grouped under the *Picornaviridae* family. EV-D68 is distinct from other enteroviruses as it possesses more physiochemical similarities with human rhinoviruses (Holm-Hansen *et al.,* 2016). In contrast to other enteroviruses that grow in cell culture at 37 ºC, EV-D68 grows at 33 ºC and has mainly been isolated from respiratory samples (Holm-Hansen *et al.,* 2016). Its similarity to human rhinoviruses, lower growth temperature and detection in respiratory samples indicate that it is a respiratory tract pathogen (Xiang *et al*., 2017). Packed in a 30 nm capsid, EV-D68 genome is translated into a single polyprotein which matures into four structural proteins, VP1-VP4, and seven non-structural proteins, 2A-2C and 3A-3D (Royston & Tapparel, 2016). The epitopes for neutralising antibodies (NAbs) are found on VP1, VP2 and VP3 (Liu *et al*., 2015b).

EV-D68 was first isolated in 1962 from four children with bronchiolitis and pneumonia in California, USA (Schieble *et al*., 1967). Since its initial identification, cases of EV-D68 were rarely reported until the recent decade. In 2014, a severe outbreak occurred in the USA involving 1,153 cases and 14 deaths (Holm-Hansen *et al.,* 2016). Other countries such as Canada and France also experienced major EV-D68 outbreaks that year, leading EV-D68 to be recognised as an emerging pathogen (Holm-Hansen *et al*., 2016). Several studies have emphasised the clinical and epidemiological significance of EV-D68 as an important pathogen for respiratory infections (Holm-Hansen *et al*., 2016; Imamura & Oshitani, 2015). In addition to severe respiratory disease, EV-D68 is also associated with acute flaccid myelitis (AFM), a potentially fatal neurological illness (Cassidy *et al*., 2018; Dyda *et al*., 2018; Messacar *et al*., 2018) There are no vaccines or antiviral medications currently available for EV-D68 infections.

Although there has been no outbreak of EV-D68 in Malaysia, cases of EV-D68 infection have been detected among respiratory tract infection (RTI) patients in Kuala Lumpur from 2012 to 2014. Clinical isolates of EV-D68 from Kuala Lumpur, Malaysia and strains from the 2014 USA outbreak were shown to be genetically related, suggesting the worldwide spread of this emerging pathogen (Ng *et al.*, 2015). However, the prevalence of EV-D68 in Malaysia before or after the 2014 worldwide outbreaks remains unknown. Since EV-D68 infection in adults are usually mild or asymptomatic, infected individuals may not seek medical service. Therefore, seroprevalence data from RTI patients may underestimate the actual prevalence of EV-D68 in the population (Xiang *et al.*, 2017). In addition, NAbs against EV-D68 have been found to protect the population from EV-D68 infection (Xiang *et al.*, 2017). Therefore, a seroprevalence analysis based on the detection of NAbs in the general population is essential for an accurate assessment of the prevalence and transmission potential of EV-D68 in Malaysia.

In order to screen for EV-D68 NAbs in serum samples of a population, a reliable seroneutralization test is necessary. Conventional seroneutralization tests for enteroviruses include plaque reduction neutralisation tests and inhibition of cytopathic effects. However, conventional neutralisation assays are not appropriate for mass screening of NAbs as they are labour-intensive, time-consuming and subjective (Jin *et al.*, 2013). In contrast, a reporter-based seroneutralization assay is rapid, convenient and quantitative (Deng *et al.*, 2016). Reporter-based seroneutralization assays detect NAbs against a virus based on the suppression of reporter gene expression, enabling rapid and convenient high-throughput screening of NAbs in a population.

A reporter-based seroneutralization assay can be developed through the application of infectious clones, a useful genetic tool that allows genome-wide manipulation through various ways such as site-directed mutagenesis or the insertion of a reporter gene.

Replicating-competent reporter-expressing infectious clone is a type of recombinant virus which retains the genetic characteristics of wild-type virus while possessing the new properties of reporter genes (Li *et al.*, 2016). Different reporters serve different purposes and may have different effects on viral biology. For a reporter-based seroneutralization assay, NanoLuc luciferase (Nluc) is an ideal reporter gene as luciferase assays are appropriate for quantification purposes such as determining the titre of NAbs (Breen *et al.*, 2016). Furthermore, the small size of Nluc (19 kDa) may minimise the effects of reporter gene insertion on viral biology (Li *et al.*, 2016). Nluc-based seroneutralization assays have been developed for several viruses such as influenza B virus, tick-borne encephalitis virus and flaviviruses (Li *et al.*, 2018; Matsuda *et al.*, 2018; Fulton *et al.*, 2015). However, no such assay has been developed for EV-D68.

The rapid spread of EV-D68 in recent years, its role in severe respiratory and neurological diseases, as well as the lack of vaccines and antiviral drugs indicate an urgent need for studies that will further our understanding of this serious threat to public health. In this study, an EV-D68 infectious clone with Nluc gene was constructed. The reporter-expressing infectious clone was used to develop a preliminary luciferase-based seroneutralization assay for the detection of EV-D68 NAbs. Subsequently, the assay was used to analyse the seroprevalence of EV-D68 in Malaysia which has not been previously documented. A rapid seroprevalence analysis of EV-D68 that provides an accurate understanding of its epidemiology is necessary in order to predict future outbreaks and guide vaccination programmes.

With these, we hypothesise that:

i. The luciferase-based seroneutralization test for EV-D68 is more rapid than the conventional test

ii. Seroprevalence rate of EV-D68 neutralising antibodies in the Malaysian population increases with age
iii. EV-D68 neutralising antibody seroprevalence increases in all age groups after 2014, the year of worldwide EV-D68 outbreaks

1.2 Problem statement

First, the conventional seroneutralization test for detecting EV-D68 neutralising antibodies based on inhibition of cytopathic effect is labour-intensive, time-consuming and subjective (Jin *et al.*, 2013). This presents an obstacle for high-throughput applications such as mass screening of EV-D68 neutralising antibodies in a population.

Secondly, although the rapid spread of EV-D68 has been reported worldwide in recent years, the seroprevalence of EV-D68 NAbs in the Malaysian population has not been previously documented (Holm-Hansen *et al.*, 2016). This knowledge gap in EV-D68 epidemiology hinders efforts in vaccine development and prevention of future outbreaks.

1.3 Significance of study

EV-D68 is an emerging respiratory and neurological pathogen that has rapidly spread worldwide in recent years, causing severe outbreaks in countries such as the United States, Canada and France. There is an urgent need to further our understanding of EV-D68 epidemiology, particularly in Malaysia where its prevalence has not been previously documented.

In this study, a preliminary luciferase-based seroneutralization test for EV-D68 was developed to provide a rapid, convenient and quantitative method for detecting EV-D68 neutralising antibodies. The luciferase-based assay enables high-throughput applications such as mass screening of EV-D68 neutralising antibodies in a population, thus facilitating cross-sectional studies of EV-D68 epidemiology.

Subsequently, the luciferase-based seroneutralization assay was used to conduct a seroprevalence analysis of EV-D68 in the Malaysian population. The findings of this study result in improved understanding of EV-D68 epidemiology in Malaysia, therefore contributing to progress in vaccine development and prevention of future outbreaks.

1.4 Objectives

The objectives of this study are:

i. To develop a luciferase-based seroneutralization test for rapid seroprevalence analysis of EV-D68
ii. To assess the seroprevalence of EV-D68 infection in Malaysia using luciferase-based seroneutralization test

CHAPTER 2: LITERATURE REVIEW

2.1 Virology

A member of the *Picornaviridae* family and *Enterovirus* genus, EV-D68 shares physiochemical properties with rhinoviruses such as acid lability, respiratory tropism and an optimum growth temperature of 33 ºC (Xiang *et al.*, 2017; Royston & Tapparel, 2016). EV-D68 infection causes a range of mild to severe respiratory illness, and in rare cases, neurological symptoms such as acute flaccid myelitis (Holm-Hansen *et al.*, 2016).

2.1.1 Biology of enterovirus D68

EV-D68 is a small, non-enveloped and single stranded positive-sense RNA virus grouped under the *Picornaviridae* family and *Enterovirus* genus (Royston & Tapparel, 2016). Members of this genus are classified into seven human species, three rhinovirus (RV) species (RV-A to RV-C) and four enterovirus (non-RV EV) species (EV-A to EV-D) (Royston & Tapparel, 2016). Packed in a 30 nm icosahedric capsid, the 7.4 kb EV-D68 genome is translated into a single polyprotein which is cleaved by 2A and 3C proteases into 3 precursor regions, P1-P3 (Royston & Tapparel, 2016; Huang *et al.*, 2015). Subsequently, the 3C protease cleaves P1 into four structural proteins, VP1-VP4, whereas P2 and P3 give rise to seven non-structural proteins, 2A-2C and 3A-3D, respectively (Huang *et al*., 2015). The epitopes for NAbs are found on VP1, VP2 and VP3 (Liu *et al.*, 2015b). Neutralisation domains located on VP1 are serotype-specific, allowing VP1 sequence to be used in serotype identification (Oberste *et al*., 1999). Figure 2.1 shows the organisation of EV-D68 genome.

EV-D68 is a unique serotype compared to other non-RV enteroviruses as it possesses more physiochemical similarities with RVs. In contrast to other non-RV EVs that grow in cell culture at 37 ºC, EV-D68 grows at 33 ºC, which is the temperature of the nose

(Holm-Hansen *et al*., 2016). Moreover, while other non-RV EVs mainly rely on faecal-oral route transmission and exhibit a gastrointestinal tropism, EV-D68 is transmitted via the respiratory route and primarily replicates in the respiratory tract (Royston *et al*., 2018; Holm-Hansen *et al*., 2016). Due to its similarities with RVs, some strains of EV-D68 were previously misclassified as RV-87. However, due to their genetic and antigenic relatedness, all RV-87 strains were reclassified as EV-D68 (Royston & Tapparel, 2016). Its similarities to RVs, such as lower optimum growth temperature, acid lability and respiratory tropism, indicate that EV-D68 is a respiratory tract pathogen (Xiang *et al*., 2017).

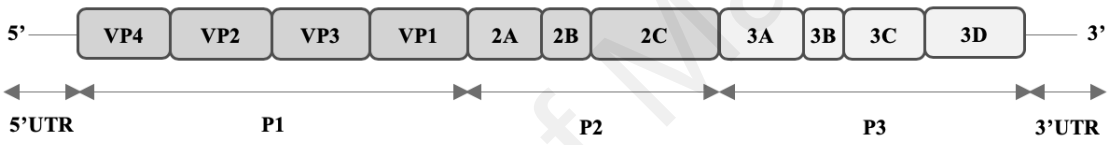


Figure 2.1: Schematic illustration of EV-D68 genome organisation (adapted from Royston & Tapparel, 2016)

2.1.2 Pathogenesis of enterovirus D68

The exact mechanism of entry and replication of EV-D68 is still largely unknown. Unlike other enteroviruses which mainly rely on faecal-oral route transmission, EV-D68 can be transmitted through the inhalation or manual transfer of infectious particles from the environment to the airway (Esposito *et al*., 2015). EV-D68 attaches to respiratory epithelial cells and subsequently spreads locally and throughout the host (Esposito *et al*., 2015). EV-D68 exhibits sialic-acid dependent cell entry, as demonstrated by the inability of EV-D68 to infect neuraminidase-treated permissive cells (Liu *et al*., 2015a). EV-D68 selectively binds to sialic acid receptors with an α-2,6 linkage, suggesting an upper respiratory tract tropism, in which there are more α-2,6 linked sialic acid molecules than in the lower respiratory tract (Liu *et al*., 2015a). The abundance of sialic acid in the

respiratory tract allows EV-D68 to replicate efficiently, resulting in productive respiratory infections.

Due to the higher prevalence of α-2,6 linked sialic acid receptors in the upper respiratory tract, it was previously assumed that EV-D68 mainly causes upper RTIs. However, epidemiological studies have shown that lower RTIs are more common during EV-D68 outbreaks (Esposito *et al*., 2015). Baggen *et al*. (2016) demonstrated that in addition to α-2,6 linked sialic acid, EV-D68 can also bind to α-2,3 linked sialic acid. Since α-2,3 linked sialic acid mainly occurs in the lower respiratory tract, this may explain the prevalence of lower RTIs during EV-D68 outbreaks (Baggen *et al*., 2016). Furthermore, they found that several recent EV-D68 isolates exhibited sialic acid-independent cell entry, indicating the use of an alternative, nonsialylated receptor (Baggen *et al.*, 2016). Sialic acid receptor binding was still observed in the sialic acid-independent strains, suggesting that EV-D68 is able to utilise multiple receptors (Baggen *et al*., 2016).

As the association between EV-D68 and neurological disease has only been established recently, research on its neuropathogenesis is fairly limited. Wei *et al.* (2016) demonstrated that both sialic acid-dependent and sialic acid-independent EV-D68 viruses were able to bind to neuron-specific intercellular adhesion molecule 5 (ICAM-5). Silencing ICAM-5 in permissive cells decreased EV-D68 replication while expressing ICAM-5 in non-permissive cells allowed EV-D68 to replicate (Wei *et al.*, 2016). In contrast to sialic acid which was not required for all EV-D68 strains, ICAM-5 was a prerequisite for viral replication of both the prototype Fermon strain and currently circulating isolates (Wei *et al*., 2016). Since ICAM-5 is highly expressed in both the respiratory system and in the brain's telencephalon region, this provides a potential

explanation for EV-D68's ability to cause both respiratory and neurological infections (Wei *et al*., 2016).

2.1.3 Clinical manifestations of enterovirus D68 infection

EV-D68 causes a range of mild to severe upper and lower respiratory diseases. EV-D68 infection typically presents as a sudden onset of respiratory symptoms such as fever, sneezing, rhinorrhoea and sore throat (Esposito *et al*., 2015). Patients with severe illness display symptoms such as difficulty in breathing, hypoxaemia and respiratory distress (Esposito *et al*., 2015). Compared to RVs and other non-EV-D68 enteroviruses, EV-D68 appears to be a more virulent respiratory pathogen. Patients with EV-D68 infection were more likely to present with respiratory distress, more frequently given magnesium sulfate or intravenous salbutamol, more likely to be hospitalised and more likely to require bronchodilator therapy than patients with rhinovirus or non-EV-D68 enterovirus infection (Caylan *et al*., 2018; Mertz *et al*., 2015). Possible risk factors for severe illness include underlying asthma, history of wheezing and obesity (Science & Allen, 2015). Children are more likely to contract severe respiratory symptoms than adults, and therefore are more likely to be hospitalised (Holm-Hansen *et al*., 2016).

In addition to severe respiratory diseases, EV-D68 infection may also cause neurological symptoms such as muscle weakness and polio-like acute flaccid paralysis (Holm-Hansen *et al*., 2016). Acute flaccid paralysis (AFP) is characterised by "sudden onset of limb weakness in one or more limbs or the respiratory and bulbar muscles as a result of damage of the lower motor neurons" (Holm-Hansen *et al*., 2016). In AFP cases caused by enteroviruses infection, acute flaccid myelitis (AFM) is a more specific term which includes an additional criteria: imaging showing lesions in spinal cord grey matter indicating anterior myelitis (Messacar *et al*., 2018). This distinction is used to differentiate AFM from other causes of AFP, such as Guillain-Barré syndrome and transverse myelitis

(Messacar *et al*., 2018). Patients who contract AFM from EV-D68 often have a very poor prognosis. They face a high mortality rate and varying degrees of flaccid tetraparesis and involvement of respiratory muscles (Macaya & Felipe-Rucián, 2017).

The prototype strain Fermon isolated in 1962 was not neurotropic, and the ability to cause AFM appears to have emerged over a short period of time (Cassidy *et al.*, 2018; Royston *et al*., 2018). The association between EV-D68 and AFM is supported by the detection of EV-D68 in cerebrospinal fluid and respiratory samples from patients presenting AFM, as well as increased cases of AFM temporally and geographically coinciding with increased circulation of EV-D68 (Messacar *et al.*, 2018). For instance, during the 2014 USA EV-D68 outbreak, 120 cases of AFM were reported, with 81% of patients showing respiratory symptoms prior to the onset of limb weakness (Sejvar *et al*., 2016). Furthermore, a study found that infecting mice with neurotropic EV-D68 strains induced paralysis and led to a loss of spinal cord motor neurons innervating paralyzed limbs (Hixon *et al*., 2017). Virus-containing spinal cord lysate of paralyzed mice caused paralysis when injected into naïve mice, hence fulfilling Koch's postulates (Hixon *et al*., 2017). Furthermore, EV-D68 antibodies in sera of previously infected mice were able to protect naïve mice from AFM (Hixon *et al*., 2017). In addition to Koch's postulates, a more rigorous epidemiological method for investigating causality is the Bradford Hill criteria. This method is based on a set of nine criteria: strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment and analogy (Messacar *et al*., 2018). Applying the Bradford Hill criteria, Dyda *et al.* (2018) and Messacar *et al*. (2018) concluded that currently available evidence supports the causal role of EV-D68 in AFM.

2.1.4 Treatment and vaccine against enterovirus D68

Treatment provided to EV-D68 patients include supplemental oxygen, inhaled bronchodilators, intravenous magnesium, systemic corticosteroids, parenteral aminophylline and injected epinephrine (Oermann *et al*., 2015). For patients with severe illness, intensive care involving high-flow nasal cannula oxygen, bi-level positive airway pressure and mechanical ventilation may also be required (Oermann *et al*., 2015). There are currently no vaccines or antiviral medications available against EV-D68 infection. Drugs such as pleconaril, pocavapir and vapendavir demonstrate antiviral activity against some enteroviruses but do not show clinically relevant activity against currently circulating EV-D68 strains (Oermann *et al*., 2015). For patients with irreversible paralysis due to AFM, nerve transfer is a potential solution. Saltzman *et al.* (2016) performed proximal and bilateral nerve transfers on paediatric AFM patients with neuropathy, resulting in regained muscle strength after 6 months.

Some progress has been made in the development of a vaccine against EV-D68. Recently, Zhang *et al*. (2018a) demonstrated that immunisation of adult mice with β-propiolactone-inactivated EV-D68 vaccine resulted in robust EV-D68-specific NAb responses. In addition to inactivated virus, EV-D68 virus-like particles (VLPs) have also demonstrated protective capacity against EV-D68 infection. To generate EV-D68 VLPs, Dai *et al.* (2018) co-expressed the P1 precursor and 3CD protease of EV-D68 in a recombinant baculovirus, while Zhang *et al.* (2018b) co-expressed the P1 precursor and 3CD protease of EV-D68 in *Pichia pastoris* yeast. Mice immunised with these VLPs produced serum antibodies that could specifically neutralise EV-D68 *in vitro* (Dai *et al*., 2018; Zhang *et al*., 2018b). In all three studies discussed above, results from antisera transfer and maternal immunisation experiments showed that the experimental vaccines conferred protection against lethal EV-D68 infection in mouse models.

2.2 Epidemiology

First isolated in 1962 from children with bronchiolitis and pneumonia in California, USA, EV-D68 has rapidly spread worldwide in recent years (Holm-Hansen *et al*., 2016; Schieble *et al*., 1967). In 2014, the most severe EV-D68 outbreak to date occurred in the USA, involving a total of 1,153 cases of EV-D68 infection (Holm-Hansen *et al*., 2016). This outbreak led to increased awareness of EV-D68 as a major threat to public health, resulting in a rise of surveillance worldwide.

2.2.1 Epidemiology of enterovirus D68

EV-D68 was first isolated in 1962 from four children with bronchiolitis and pneumonia in California, USA (Schieble *et al*., 1967). The four isolates obtained from the patients, designated as Fermon, Franklin, Robinson, and Rhyne strains, were considered representatives of a single serotype as they were immunologically identical to each other (Schieble *et al*., 1967). Furthermore, when compared to known enteroviruses, the strains were physically, chemically and biologically similar, but were antigenically distinct (Schieble *et al*., 1967). Sera that were immune to known enteroviruses did not neutralise the Fermon strain while sera that were immune to the Fermon strain did not neutralise group A coxsackieviruses (Schieble *et al.*, 1967). Therefore, Schieble *et al.* (1967) concluded that the four isolates belonged to a new enterovirus serotype, of which the Fermon strain was selected as the representative strain.

Since its initial identification, cases of EV-D68 were rarely reported until the recent decade. Between 1970 and 2005, only 26 cases of EV-D68 were detected in the USA (Khetsuriani *et al.*, 2006). Reports subsequently increased, with 94 cases reported between 2006 and 2013 (Midgley *et al*., 2015). In 2009, a peak of 50 cases were reported, coinciding with small clusters of acute respiratory illness (Midgley *et al*., 2015). Prior to 2014, only 699 cases of EV-D68 were reported worldwide, mostly identified

retrospectively from respiratory samples (Holm-Hansen *et al.*, 2016). One of the largest outbreaks occurred in Japan in 2010, in which more than 120 cases of EV-D68 were detected (Midgley *et al*., 2015). Cluster outbreaks were reported in the USA, France, Italy, the Netherlands and the UK between 2007 and 2010 (Holm-Hansen *et al*., 2016). Furthermore, between 2005 and January 2014, southeast Asia reported an increased number of acute respiratory illnesses caused by EV-D68 infection (Holm-Hansen *et al*., 2016).

The most severe outbreak of EV-D68 to date occurred in the USA in 2014. In August 2014, the number of paediatric patients with respiratory distress and severe bronchospasm unusually increased in Missouri, Illinois and Colorado (Oermann *et al*., 2015). Many of the children needed hospital admission, subsequently requiring respiratory support and intensive care (Oermann *et al*., 2015). Viral identification of specimens from Missouri and Illinois revealed EV-D68 as the predominant pathogen (Midgley *et al*., 2015). Subsequently, the CDC tested more than 2,000 samples nationally, with approximately 40% positive for EV-D68 (Oermann *et al*., 2015). 1,153 cases of EV-D68 infection, including 107 cases of AFM and 14 deaths, were laboratory confirmed (Holm-Hansen *et al.*, 2016).

Since individuals with mild respiratory illness may not seek medical treatment, confirmed cases most likely underestimate the true burden of disease. EV-D68 affects a broad age range (1 month to > 80 year), yet almost all of the confirmed cases in the 2014 USA outbreak were among children with underlying asthma and history of wheezing, who were more likely to be hospitalised (Oermann *et al.*, 2015). Adult and elderly cases of EV-D68 have been documented in China, the Netherlands, France, the USA and Australia (Xiang *et al*., 2017; Lau *et al*., 2016; Bal *et al.*, 2015; Levy *et al*., 2015; Oermann *et al.*, 2015; Rahamat-Langendoen *et al.*, 2011). Although adults typically

present with mild respiratory symptoms, cases of severe respiratory illness due to EV-D68 infection have been reported in both healthy and immunocompromised adults. For instance, an otherwise healthy 25-year-old woman displayed acute respiratory distress syndrome, requiring mechanical ventilation for longer than 30 days (Holm-Hansen *et al.*, 2016).

The 2014 USA outbreak increased global awareness of EV-D68, resulting in a rise of surveillance worldwide. Since then, reports of EV-D68 cases have increased in different parts of the world, leading EV-D68 to be recognised as an emerging pathogen. Initially, the increased detection of EV-D68 cases was attributed to the introduction of sensitive molecular-based viral detection methods into clinical practice (Esposito *et al.,* 2015). Furthermore, increased awareness due to the 2014 outbreak may have led to increased identification of enteroviruses in respiratory samples, leading to a perceived rise of EV-D68 incidence (Holm-Hansen *et al.*, 2016). However, retrospective studies on stored respiratory samples in Japan and The Netherlands showed that cases of EV-D68 infection had indeed increased in recent years (Ikeda *et al.*, 2012; Meijer *et al.*, 2012).

Although there has been no EV-D68 outbreak in Malaysia, cases of EV-D68 infection have been documented here from 2012 to 2014. Ng *et al*. (2015) screened RTI patients at the University of Malaya Medical Centre, Kuala Lumpur, Malaysia between March 2012 and May 2014. Out of the total 3935 patients screened, 12 cases of EV-D68 were detected through molecular methods (Ng *et al*., 2015). Since only RTI patients were screened, this may underestimate the actual prevalence of EV-D68 in Malaysia as enterovirus infection in adults are usually mild or asymptomatic, thus not requiring medical service *(*Xiang *et al*., 2017). Clinical isolates of EV-D68 from Kuala Lumpur, Malaysia and strains from the 2014 USA outbreak were shown to be genetically related, suggesting the worldwide spread of this emerging pathogen (Ng *et al.*, 2015).

2.2.2 Molecular epidemiology of enterovirus D68

EV-D68 strains circulating globally can be classified into four viral clades, A-D, as well as subgroups within the clades, subclades A1-A2 and B1-B3 (Tokarz *et al*., 2012). These clades are distinguished according to sequence differences in the VP1 protein, the most structurally variable region of the viral capsid, while subclades are identified based on amino acid substitutions in viral proteins (Esposito *et al.*, 2015). Different clades and subclades can co-circulate during different periods. Table 2.1 shows the genotype distribution of EV-D68 from 1962 to 2016 worldwide.

The EV-D68 genome was substantially rearranged between the early 1960s and mid-1990s, resulting in a 24 nt deletion in recent EV-D68 strains compared to the prototype Fermon strain (Tokarz *et al*., 2012). In the mid-1990s, the virus divided into clades A and C, while clade B diverged from Clade C around 2007 (Tokarz *et al.*, 2012). Most recently, EV-D68 isolates from the 2014 USA outbreak were found to belong to a novel clade, namely clade D (Du *et al*., 2015; Huang *et al*., 2015).

The recent outbreaks likely indicate that viral evolution over the past decades has increased the efficiency of EV-D68 transmission. In EV-D68, evolutionary mechanisms that promote viral replication, such as immune evasion and ligand adaptation, require variation of viral capsid proteins (Du *et al.*, 2015). Du *et al*. (2015) found that the strains predominantly involved in the 2014 USA outbreak were members of clade B containing highly specific variations at four positions in the VP1 protein. As the four positions are involved in host-receptor recognition and antigenicity, variations at these positions may have improved cell entry and immune evasion (Du *et al*., 2015). Furthermore, their data suggested that EV-D68 VP1 protein could rapidly change under selective pressure (Du *et al*., 2015). Therefore, VP1 protein variations that led to enhanced viral replication and transmission possibly contributed to the recent emergence of EV-D68 outbreaks.

Table 2.1: Genotype distribution of EV-D68 based on review of published literature as of 9 December 2018.

COUNTRY	1962	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
NORTH AMERICA																			
United States of America	Fermon				C	A1						A1 C			A1 B1	A1 B1 B3 C	A2 B1 B2 D		B3
Canada																	B1		
Haiti																	B1		
EUROPE																			
France		C										B	A B2	A	A B1 B2 D		A B1 B2 B3 D		B3
Italy											A C				A B1 B2		A2 B1		B3
Netherlands							C		A C	A C	A C	A B C	A B	A	A B	A B	A B		
Spain															B	A B			
United Kingdom												B C	A						
AFRICA																			
South Africa			B C	A B C															
Kenya											A B	A	A	A B					

Table 2.1, continued.

COUNTRY	1962	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
ASIA																			
Malaysia															A	B	B		
Thailand												A	B	A		B	B		
												B		B3					
Philippines											A			A		A	B		
											B			B		B			
Japan								C	A1	A	A1		A			A		B3	
											D		B2						
													C						
China											D		A1	A2	A2	A2	B3	B3	
													A2	B1		B3			
													D	B3		D			
														D					
Taiwan										A1	B	A1	A1	A1	B1	A1	B3	B3	A2
										A2		A2	A2	A2		B1			
										C		D	D	B1		D			B2
										D				D					B3
OCEANIA																			
Australia				C															
New Zealand													A						

Kramer *et al*. (2018) observed a strong ongoing diversification of EV-D68 as demonstrated by (a) newly emerging clades in epidemic years in Lyon, France, (b) increases of effective virus population size correlating with epidemic years in Lyon, (c) dynamic effective population size of EV-D68 globally, and (d) evolution of all ancestors of currently circulating genotypes within a decade. Their systematic screening study of EV-D68 in Lyon, France from 2010 to 2016 revealed continuous emergence and replacement of clades in epidemic years (Kramer *et al*., 2018).

Similarly, Kaida *et al.* (2017) found that distinct viral clades contributed to EV-D68 outbreaks in different years in Osaka, Japan. Strains circulating in Osaka in 2010, 2013 and 2015 belonged to clades C, A and B3, respectively (Kaida *et al*., 2017). Since distinct clades have different antigenicities, lower herd immunity against specific clades may facilitate the rapid spread of EV-D68 in a limited geographic area (Kaida *et al*., 2017; Imamura *et al*., 2014). Therefore, the emergence and replacement of clades as demonstrated in France and Japan may have contributed to the rise in EV-D68 outbreaks in recent years.

2.2.3 Seroepidemiology of enterovirus D68

Since EV-D68 infection in adults are usually mild or asymptomatic and thus does not require medical service, seroprevalence data from RTI patients may underestimate the actual prevalence of EV-D68 in the population (Xiang *et al*., 2017). Therefore, seroepidemiology studies based on the detection of NAbs in the general population are essential for an accurate assessment of EV-D68 prevalence. Pre-existing EV-D68 NAbs in a population provide herd immunity that may influence virus transmission, as well as a method to predict the population's susceptibility to EV-D68 (Xiang *et al*., 2017). Table 2.2 summarises seroprevalence studies of EV-D68 NAbs in different countries.

Table 2.2: Seroprevalence studies of EV-D68 in different populations.

Country	Study population	Year collected	Age group	Seroprevalence rates (%)	GMT	Reference
Finland	Pregnant women	1983	N/A	100	178.8	Smura *et al.* (2010)
		1993		100	88.1	
		2002		100	44.5	
China	Healthy individuals	2004	0-5 y	N/A	10	Xiang *et al.* (2017)
			6-15 y		32	
			16-59 y		40	
			≥ 60 y		39	
		2009	0-5 y		26	
			6-15 y		73	
			16-59 y		117	
			≥ 60 y		98	
	Children with RV-negative respiratory infections	2007	0.5-3 y		11	
			3.1-6 y		29	
			6.1-15 y		42	
		2009	0.5-3 y		11	
			3.1-6 y		26	
			6.1-15 y		36	
		2011	0.5-3 y		10	
			3.1-6 y		13	
			6.1-15 y		73	
	Adults with RV-negative respiratory infections	2007	16-59 y		64	
		2009			99	
		2011			148	
	Prenatal women	2010	N/A	100	168	Sun *et al.* (2018)
	Neonates		< 1 m	100	162.3	
	Infants and children		1-5 m	79	25.2	
			6 m-1 y	20	15	
			2-5 y	44	24.7	
			6-15 y	83	71.9	
	Adults	2015	N/A	100	166.3	

Abbreviations: year, y; month, m; geometric mean titre, GMT; rhinovirus, RV

Sun *et al.* (2018) evaluated serum samples from pregnant women and their neonates, infants and children, and healthy adults in China. In adults, they found a 100% seroprevalence rate, suggesting that EV-D68 infection was highly prevalent among Chinese adults in 2015 (Sun *et al.*, 2018). A similar finding was observed in a Finnish study, in which EV-D68 NAbs were found in 100% of serum samples obtained from pregnant women in Finland in 1983, 1993 and 2002 (Smura *et al.*, 2010). The high seroprevalence rate of EV-D68 NAbs observed in different years in both studies indicate that EV-D68 can quietly persist in a population without causing an outbreak.

Other than that, Sun *et al.* (2018) found that titres of EV-D68 NAbs in neonates positively correlated with their mothers, demonstrating passive transfer of maternal EV-D68 NAbs. Furthermore, seroprevalence rate increased from 44% in 2-5 years old children to 83% in 6-15 years old children (Sun *et al.*, 2018). This suggests that children are increasingly exposed to EV-D68 as they grow older, possibly due to increased exposure to crowds such as in kindergartens and schools.

2.3 Laboratory diagnosis

Enteroviruses are usually detected using polymerase chain reaction (PCR)-based assays while specific enterovirus serotypes are identified by sequencing the VP1 gene. (Oermann *et al.*, 2015). To detect EV-D68 neutralising antibodies, a conventional seroneutralization assay based on the inhibition of cytopathic effect is traditionally used. However, the conventional assay is labour-intensive, time-consuming and subjective (Jin *et al.*, 2013). Alternatively, a reporter-based seroneutralization assay is rapid, convenient and quantitative, allowing high-throughput applications such as mass screening of serum samples in a population (Deng *et al.*, 2016).

2.3.1 Common detection methods for enterovirus D68

Before the development of molecular biology techniques, enteroviruses were primarily detected by isolating viruses from infected neonatal mice (Oermann *et al*., 2015). This was then replaced by pan-enterovirus PCR assays targeting the 5' untranslated region (UTR) as the gold standard for enterovirus detection. More recently, multiplex PCR-based assays were introduced, enabling rapid screening for multiple pathogens in respiratory specimens (Oermann *et al*., 2015). For identification of specific enterovirus serotypes, sequencing of the VP1 gene is often required. However, after the severe 2014 USA outbreak, efforts were undertaken to develop a faster method for EV-D68 identification without sequencing. As a result, the CDC and a Dutch research group independently developed EV-D68-specific real-time reverse-transcriptase PCR (RT-PCR) tests to allow rapid diagnostics during outbreaks (Oermann *et al*., 2015; Poelman *et al*., 2015).

A reliable seroneutralization test is necessary to determine the efficacy of potential vaccines, to screen for neutralising monoclonal antibodies and to assess the immune status of patients (Deng *et al*., 2016). Conventional neutralisation tests for enteroviruses include plaque reduction neutralisation tests and inhibition of cytopathic effects. However, conventional neutralisation assays are not appropriate for mass screening of NAbs as they are labour-intensive, time-consuming and subjective (Jin *et al*., 2013). In contrast, a reporter-based neutralisation assay is a rapid, convenient and quantitative method for mass screening NAbs in patient serum samples (Deng *et al*., 2016). Reporter-based seroneutralization assays detect neutralising antibodies against the corresponding virus based on the suppression of reporter gene expression. Reporter-based seroneutralization assays have been developed for several viruses such as chikungunya and flaviviruses (Matsuda *et al*., 2018; Deng *et al*., 2016). However, no such assay has been developed for EV-D68.

2.3.2 Reporter-expressing enterovirus D68 infectious clone

Reverse genetics, which is the generation of virus from synthetic DNA, is a robust tool for studying viruses due to its ease of manipulation and short viral replication cycles (Aubry *et al.*, 2015). One of the most commonly used reverse genetics systems is infectious clone, which is "a cDNA copy of an RNA virus genome that can be stably incorporated into a vector and from which genomic RNA can be obtained by *in vitro* transcription" (Aubry *et al.*, 2015). Infectious virus is subsequently recovered by transfecting the genomic RNA into tissue culture cells (Aubry *et al.*, 2015). The first infectious clone was developed by Racaniello and Baltimore (1981), who cloned poliovirus genome into a pBR322 vector subsequently shown to be infectious in mammalian cells. Since then, construction of infectious clones for other viruses, such as Zika, enterovirus A71, coronavirus NL63 and dengue-1 have been reported (Shan *et al.*, 2016; Tan *et al*., 2016; Donaldson *et al*., 2008; Puri *et al.*, 2000).

Infectious clones allow genome-wide manipulation such as site-directed mutagenesis or the insertion of a reporter gene. Replicating-competent reporter-expressing virus (RCREV) is a type of recombinant virus that retains the genetic characteristics of the wild-type virus while possessing new properties of reporter gene *(*Li *et al.*, 2016). RCREVs are useful for rapidly quantifying viral replication, tracking viral proteins or viruses, screening for antivirals and monitoring disease progression (Li *et al*., 2016). There are two types of reporters commonly used in RCREVs: fluorescent reporters, such as enhanced green fluorescent protein (EGFP) and tetracysteine (TC), and bioluminescent reporters, such as *Gaussia* luciferase (Gluc), *Renilla* luciferase (Rluc) and firefly luciferase (Fluc) (Li *et al.*, 2016).

Recently, Pan *et al.* (2018) developed a reverse genetics system to recover luciferase-containing EV-D68 minireplicon and full-length infectious clone from transfected plasmids using the human RNA polymerase I (Pol I) promoter. The EV-D68 minireplicon was generated by replacing the open reading frame (ORF) of the viral genome with Fluc, flanked by the non-coding regions (Pan *et al.*, 2018). They found that transcription of Fluc depended on the Pol I promoter, while its translation was controlled by the viral UTR (Pan *et al.*, 2018). Furthermore, while most infectious clones developed for RNA viruses are T7 promoter-driven, they found that transcription controlled by the Pol I promoter led to more efficient virus recovery, as evident from the higher luciferase activity of Pol I-minireplicons compared to T7-minireplicons (Pan *et al.*, 2018). In addition to the minireplicons, they also constructed a full-length infectious clone of EV-D68, containing silent mutations acquired during the cloning process at positions 394, 1882, and 2593. They found that the G394C mutation resulted in reduced luciferase expression and decreased viral proliferation rate, but with no effect on Fluc gene transcription (Pan *et al.*, 2018). Since the G394C mutation is located in the 5' UTR of the EV-D68 genome, this result suggests the importance of non-coding regions in viral translation (Pan *et al.*, 2018).

2.3.3 NanoLuc luciferase reporter gene

Different reporter genes serve different purposes; therefore, selection of an ideal reporter gene must be considered according to the objective of the study. For instance, while GFP assays are useful for observing localisation, luciferase assays are more appropriate for quantification purposes such as determining the titre of NAbs (Breen *et al.*, 2016). Moreover, different reporters may have different effects on viral biology. For example, insertion of a large reporter gene into a virus with a small-sized genome often leads to genetic instability and loss of reporter gene expression (Li *et al.*, 2016). In contrast, reporters with a smaller size such as NanoLuc luciferase (Nluc) may have minimal effects on viral biology and thus is a promising option for RNA viruses.

Recently developed by Promega using a small luciferase subunit from a deep-sea shrimp (*Oplophorus gracilirostris*), Nluc is a small (19 kDa), monomeric, highly soluble and stable bioluminescent reporter (Boute *et* al., 2016; Hall *et al*., 2012). The enzyme was optimised with furimazine, a synthetic novel substrate generated by screening coelenterazine analogues (Boute *et al*., 2016). Nluc offers several advantages compared to traditional luciferases such as Rluc (36 kDa) and Fluc (61 kDa) , including smaller size, enhanced specificity and longer-lasting luminescence (England *et al*., 2016). While superior to traditional luciferases in many ways, Nluc has several limitations. Nluc's emission spectra is not optimal for *in vivo* studies and it is costly due to the requirement for furimazine, a substrate specific to Nluc and not generically available (England *et al*., 2016).

2.3.4 Luciferase-based seroneutralization assay for enterovirus D68

The expression of reporter proteins in blood, serum and urine has been a valuable tool in monitoring biological processes including tumour growth, cell viability as well as viral infection and replication (Tannous & Teng, 2011). Studies have shown that luminescence signal strongly correlates with Nluc concentration and viral titre, allowing a rapid and quantitative method for measuring reporter expression and viral replication (Boute *et al*., 2016; Fulton *et al*., 2015; Hall *et al*., 2012). Nluc-expressing infectious clones have been used to develop seroneutralization assays for several viruses such as flaviviruses, influenza B virus and tick-borne encephalitis virus (Fulton *et al*., 2015; Li *et al*., 2018; Matsuda *et al*., 2018). However, no such assay has been developed for EV-D68.

The principle of luciferase-based seroneutralization assay is similar to conventional seroneutralization assay, except for the input virus and readout. In a conventional seroneutralization assay, the wild type virus is used and the NAb titre is measured based on the inhibition of cytopathic effect (CPE). The serum is serially diluted and the highest

dilution able to inhibit CPE determines the NAb titre. The conventional method is time-consuming, labour-intensive and subjective, requiring a trained eye to observe the inhibition of CPE in virus-infected cells daily over the course of 7 days. This makes it an inappropriate method for high-throughput screening of NAbs. In contrast, a reporter-based seroneutralization assay determines the NAb titre according to the suppression of reporter gene expression. When a serum contains antibodies, the reporter-expressing virus will be neutralised thus suppressing the expression of reporter gene. Since the luminescence signal linearly correlates with Nluc concentration and thus antibody titre, luminometry can be used as a rapid, convenient and quantitative method for mass screening of NAbs in a population's serum samples.

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Cell

Human rhabdomyosarcoma cells (RD, ATCC, CCL-136) were grown and propagated in a 75cm^2 tissue culture flask containing 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Bovogen Biologicals, Australia), 1% L-glutamine and 1% penicillin/streptomycin. The flask was incubated at 37 ºC in 5% CO_2. Confluency of the cells was monitored daily.

3.1.1 Cell propagation and maintenance

When 80-90% confluency was observed, cell passaging was performed to allow continued cell growth. Growth medium was removed from the flask and the cells were briefly rinsed with 0.12% trypsin-EDTA. Then, 2 ml of 0.12% trypsin-EDTA was added and the cells were incubated at 37 ºC in 5% CO_2 for 5 minutes to allow detachment of cells from the flask surface. After complete detachment was observed, 2 ml of complete growth medium (DMEM with 10% FBS) was added to the cells which were subsequently resuspended. Then, 2 ml of the cell suspension was added into a new flask and 10 ml of complete growth medium was subsequently added to the cells. The flask was incubated at 37 ºC in 5% CO_2.

3.1.2 Cell seeding

Cell seeding was performed to obtain a desired concentration of cells. Growth medium was removed from a flask with 80-90% confluent RD cells which were subsequently briefly rinsed with 0.12% trypsin-EDTA. Then, 2 ml of 0.12% trypsin-EDTA was added and the cells were incubated at 37 ºC in 5% CO_2 for 5 minutes. After complete cell detachment was observed, 2 ml of growth medium was added to the cells which were subsequently resuspended. Ten microliters of the cell suspension were stained with an

equal volume of 0.4% trypan blue. Cell count was performed by applying 10 µl of trypan blue-treated cell suspension to a haemocytometer to determine the concentration of the cell suspension. To obtain a desired concentration of cells, a desired volume of cell suspension was mixed with complete growth medium in a conical tube. A volume of 100 µl (1×10^5 cells/ml), 500 µl (2.5×10^5 cells/ml) and 2 ml (2.5×10^5 cells/ml) of the cell suspension were pipetted into each well of a 96-well plate, 24-well plate and 6-well plate, respectively. The plates were incubated at 37 ºC in 5% CO_2.

3.2 Virus

The virus strain used in this study is the prototype enterovirus D68 strain Fermon (GenBank accession number AY426531).

3.2.1 Virus propagation and maintenance

A 6-well plate with 5×10^5 RD cells/well was prepared. Growth medium was replaced with 1 ml of the virus-containing supernatant in each well. The plate was incubated at room temperature on a rocker to allow the virus to infect the cells. After 1 hour of incubation, the inoculum was replaced with 2 ml of maintenance growth medium (DMEM with 2% FBS). The plate was incubated at 33 ºC in 5% CO_2 and cytopathic effect was observed daily. When 70-80% CPE was observed, the virus was harvested by freeze-thawing. Centrifugation was performed at 10,000 × g for 10 minutes at 4 ºC to remove cell debris. The virus-containing supernatant was aliquoted and stored at -80 ºC until use.

3.2.2 Plaque assay

A 6-well plate with 5×10^5 RD cells/well was prepared. The virus was diluted in serum-free DMEM (SFM) in ten-fold serial dilutions from 10^{-1} to 10^{-6}. Growth medium was removed from each well and replaced with 1 ml of the appropriate virus dilutions. The plate was incubated at room temperature on a rocker for 1 hour before replacing the inoculum with 2 ml of plaque medium (DMEM supplemented with 2% FBS and 0.8%

carboxymethyl cellulose (CMC)). Then, the plate was incubated at 33 ºC in 5% CO_2 for five days. To observe plaque morphology, plaque medium was removed and the cells were fixed with 3.7% formaldehyde and stained with 0.5% crystal violet. The plaques were subsequently observed against a white background.

3.2.3 Virus titration

A 96-well plate with 1×10^4 RD cells/well was prepared. The virus was diluted in SFM in ten-fold serial dilutions from 10^{-1} to 10^{-10}. After removing growth medium from each well, 50 µl of the appropriate virus dilution was added in octuplicates into the wells. The plate was incubated at room temperature on a rocker for 1 hour before replacing the inoculum with 100 µl of maintenance growth medium. Then, the plate was incubated at 33 ºC in 5% CO_2 for 7 days. The tissue culture infectious dose ($TCID_{50}$), defined as the amount of virus that produces CPE in 50% of infected cells, was determined according to the Reed & Muench (1938) formula. The $TCID_{50}$ values were expressed as $TCID_{50}$/ml.

3.3 Construction of EV-D68 infectious clone with Nluc reporter gene

The T7 promoter-driven Nluc-expressing EV-D68 infectious clone (EV-D68_Nluc) was constructed from a T7 promoter-driven prototype EV-D68 strain Fermon infectious clone (T7-EV-D68). The Nluc gene was cloned in between EV-D68 5'-UTR and the VP4 gene through *Xho*I and *Xma*JI restriction enzyme sites. The recombinant plasmid was then transformed into *E. coli* XL 10-Gold ultracompetent cells (Agilent Technologies, USA). The resulting infectious clone was verified by DNA sequencing.

3.3.1 PCR amplification of EV-D68 vector and Nluc insert

The EV-D68 vector was amplified from T7-EV-D68 using EVD68Vec forward and reverse primers containing *Xho*I and *Xma*JI restriction enzyme sites as well as 2A protease recognition site. The Nluc insert was amplified from an Nluc-expressing coxsackievirus A16 infectious clone (CVA-16_Nluc) using NlucIns forward and reverse primers

containing *Xho*I and *Xma*JI restriction enzyme sites. The primers used are listed in Table 3.1. The reaction was performed in a thin-wall PCR tube containing 0.5µM of each primer, 200µM dNTP, 1 unit Q5 polymerase (NEB, USA), 1X Q5 buffer (NEB, USA), 500 ng template DNA and PCR H_2O for a final volume of 50 µl. The amplified products were verified through agarose gel electrophoresis and subsequently purified using Expin PCR purification kit (GeneAll, South Korea).

Table 3.1: Primers used in PCR amplification of EV-D68 vector and Nluc insert. Restriction enzymes sites are underlined and 2A cleavage site is highlighted.

Primer	Sequence (5'- 3')	T_a (°C)
EVD68Vec F	GCTCGAGCCTCACAACATAGTAACCACCGGT GCTCAAGTTACTAG	79
EVD68Vec R	CGGCGCCCTAGGTGTTATAAATAAGTTTAAA CTC	70
NlucIns F	TACGATCCTAGGATGGTCTTCACACTCGAA	74
NlucIns R	TAGCTACTCGAGCGCCAGAATGCGTT	71

3.3.2 Restriction endonuclease digestion

T7-EV-D68 and Nluc gene were digested at specific restriction sites using restriction endonucleases *Xho*I and *Xma*JI. The reaction was performed in a thin-wall PCR tube containing 1X FastDigest buffer (Thermo Fisher Scientific, USA), 1 µl of *Xho*I, 1 µl of *Xma*JI, 200 ng DNA to be digested and PCR H_2O for a final volume of 30 µl. The mixture was incubated at 37 ºC for 30 minutes. The digested DNA fragments were verified through agarose gel electrophoresis and subsequently purified using DNA Clean & Concentrator kit (Zymo Research, USA).

3.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to verify amplified PCR products and digested DNA fragments. A 0.8% agarose gel (Vivantis Technologies, USA) stained with GelRed nucleic acid stain (Biotium, USA) was prepared. Gel loading buffer was added to the DNA samples at a 1:5 ratio and the samples were loaded into wells. Electrophoresis

was performed at 80V for 30 minutes and the DNA bands were viewed under ultraviolet illumination. A 1 kb DNA ladder was used to determine the length of DNA fragments.

3.3.4 Cloning

The Nluc gene was inserted into the EV-D68 vector using T4 DNA ligase (NEB, USA). The reaction was performed in a thin-wall PCR tube containing 80 ng EV-D68 vector, 12 ng Nluc insert, 1X T4 ligase buffer (NEB, USA), 1 unit T4 polynucleotide kinase (NEB, USA) and PCR H_20 for a final volume of 20 µl. The tube was incubated at 37 ºC for 30 minutes. Then, 1 unit T4 ligase was added and the tube was incubated at 25 ºC for 1 hour. Lastly, template DNA was removed by adding 0.5 µl of *Dpn*I to the tube and incubating at 37 ºC for 30 minutes.

3.3.5 Transformation

Ten microliters of recombinant plasmid were added to *E. coli* XL 10-Gold ultracompetent cells that had been thawed on ice (Agilent Technologies, USA). Next, the cells were incubated on ice for 30 minutes. Heat shock transformation was performed at 42 ºC for 30 seconds and the transformed cells were immediately incubated on ice for 2 minutes. Then, 200 µl of LB broth was added to the transformed cells. The suspension was shaken at 37 ºC for an hour. Next, 100 µl of the transformed cells were spread on an LB agar plate supplemented with 50 µg/ml ampicillin. The plate was incubated overnight at 37 ºC and stored at 4 ºC.

3.3.6 Colony PCR

Colony PCR was performed to screen for colonies containing the recombinant plasmid. Six colonies from the agar plate were picked with sticks and streaked on a new agar plate labelled with grids. Then, the remainder on the sticks were mixed with 10 µl PCR H_2O in corresponding thin-walled PCR tubes. The competent cells in the tubes were subjected to heat shock by incubating in 96 ºC for 30 seconds, then incubating on ice for

5 minutes. A pair of primers targeting the 5' UTR to VP2 region of EV-D68 was used and the expected amplified size for a recombinant plasmid-containing colony was 1000 bp. The primers used are listed in Table 3.2. Agarose gel electrophoresis was performed to select colonies with the expected amplified size.

Table 3.2: Primers used in colony PCR to target 5' UTR to VP2 region of EV-D68.

Primer	Sequence (5'- 3')	T_a (ºC)
68p1 F	TTTGGGTGTCCGTGTTTCACTTTTT	67
68p1 R	GCCATTCACCATAAGCGCAAC	67

3.3.7 Plasmid extraction

The selected plasmid-containing colony was grown in 5 ml LB broth supplemented with 5 µl ampicillin and incubated at 37 ºC on a shaker overnight. The recombinant plasmid was purified from bacterial culture using Hybrid Q mini spin purification kit (GeneAll, South Korea). Agarose gel electrophoresis was performed to verify the recombinant plasmid.

3.3.8 DNA sequencing

The recombinant plasmid DNA was subjected to Sanger DNA sequencing by 1st BASE Laboratory Sdn Bhd (Malaysia). The sequenced plasmid was aligned with EV-D68_Nluc sequence to confirm successful cloning of the infectious clone.

3.3.9 *In vitro* transcription

In vitro transcription was performed to obtain EV-D68_Nluc RNA using RiboMAX Large Scale RNA Production System (Promega, USA). The reaction was performed in a thin-wall PCR tube containing 1X T7 transcription buffer, 30mM rNTPs, 5 µg linear DNA template and 1 unit of T7 enzyme mix. The tube was incubated at 37 ºC for 4 hours.

Lastly, DNA template was removed by adding 1 µl of DNase and incubating at 37 ºC for 15 minutes.

3.3.10 RNA cleanup

RNA cleanup was performed using RNeasy mini kit (Qiagen, Netherlands) according to the recommended protocol. First, the sample was adjusted to a volume of 100 µl with RNase-free water and 350 µl Buffer RLT was added. Next, 250 µl ethanol (96–100%) was added to the diluted RNA, and mixed well by pipetting. 700 µl sample was added to an RNeasy Mini spin column placed in a 2 ml collection tube which was subsequently centrifuged for 15 s at 10,000 x g. Flow-through was discarded. To wash the spin column membrane, 500 µl Buffer RPE was added to the RNeasy spin column which was subsequently centrifuged for 15 s at 10,000 x g. Flow-through was discarded. To ensure no ethanol is carried over, 500 µl Buffer RPE was added again to the spin column which was then centrifuged for 2 minutes at 10,000 x g. Next, the RNeasy spin column was put in a new 1.5 ml collection tube (supplied) and 30 µl RNase-free water was added to the spin column membrane. Lastly, the spin column was centrifuged for 1 min at 10,000 x g to elute the RNA.

3.3.11 Transfection

A 6-well plate with 5×10^5 RD cells/well was prepared. Transfection was performed using *Trans*IT-mRNA transfection kit (Mirus Bio, USA). Two micrograms of EV-D68_Nluc RNA, 4 µl of mRNA Boost Reagent and 4 µl of *Trans*IT-mRNA Reagent were added into a tube containing 200 µl of Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, USA). The tube was incubated at room temperature for 5 minutes. Next, the *Trans*IT-mRNA:mRNA Boost:RNA complex was distributed drop-wise to different areas of the wells. Wells transfected with T7-EV-D68 RNA and transfection reagents only served as positive and negative controls, respectively. The plate was incubated at 33

ºC in 5% CO_2 for 4 hours. After incubation, the medium was replaced with 2 ml of complete growth medium. Then, the plate was incubated at 33 ºC in 5% CO_2 and CPE was observed daily. The virus was harvested three days post-transfection and the virus titre was determined.

3.4 Development and validation of luciferase-based seroneutralization assay

Luciferase-based seroneutralization assay was developed by performing seroneutralization assay using 1×10^4 $TCID_{50}$ of the clone-derived EV-D68_Nluc virus on EV-D68 NAb-positive and EV-D68 NAb-negative human sera. The luciferase-based seroneutralization assay was compared with the conventional seroneutralization assay based on inhibition of cytopathic effect.

3.4.1 Optimisation of luciferase-based seroneutralization assay

Optimisation of luciferase-based seroneutralization assay was carried out to determine the optimal incubation time for the assay. A 96-well plate with 1×10^4 RD cells/well was prepared. 50 µl of EV-D68_Nluc virus (1×10^4 $TCID_{50}$) was inoculated into the cells and the plate was incubated at 33 ºC in 5% CO_2 for an hour. Then, the inoculum was replaced with 100 µl of maintenance growth medium and the plate was incubated at 33 ºC in 5% CO_2. Luciferase activity was determined using Nano-Glo Luciferase Assay System (Promega, USA) after 1, 2, 3, 4 and 5 days post-infection. The luciferase assay solution was prepared by adding 1 volume of luciferase substrate to 49 volumes of assay buffer. 25 µl of cell culture supernatant was transferred to a 96-well white plate and 25 µl of luciferase assay solution was added to each sample. Luciferase activity was measured using GloMAX Multi Detection System luminometer (Promega, USA).

3.4.2 Luciferase-based seroneutralization assay

A 96-well plate with 1×10^4 RD cells/well was prepared. EV-D68 NAb-positive and EV-D68 NAb-negative sera were heat-inactivated at 56 ºC for 30 minutes. Two-fold dilution was performed on each serum sample from 1:8 to 1:16. Equal volume of EV-D68_Nluc virus (1×10^4 $TCID_{50}$) was added to diluted sera. The serum-virus mixture was incubated at 33 ºC in 5% CO_2 for 2 hours to allow binding of NAbs to the virus. Next, 50 µl of the mixture was inoculated into RD cells in duplicates and the plate was incubated at 33 ºC in 5% CO_2 for 1 hour to allow virus to infect the cells. Wells containing only diluted serum, only virus, and uninfected RD cells served as serum, virus, and cell controls, respectively. After infection, the inoculum was replaced with 100 µl of maintenance growth medium and the plate was incubated at 33 ºC in 5% CO_2. After 4 days of incubation, luciferase activity was determined as described in section 3.4.1 using the Nano-Glo Luciferase Assay System (Promega, USA).

3.4.3 Conventional seroneutralization assay

A 96-well plate with 1×10^4 RD cells/well was prepared. EV-D68 NAb-positive and EV-D68 NAb-negative sera were heat-inactivated at 56 ºC for 30 minutes. Two-fold dilution was performed on each serum sample from 1:8 to 1:16. Equal volume of the prototype EV-D68 virus (1×10^4 $TCID_{50}$) was added to diluted sera. The serum-virus mixture was incubated at 33 ºC in 5% CO_2 for 2 hours to allow binding of NAbs to the virus. After incubation, 50 µl of the mixture was inoculated into RD cells in duplicates and the plate was incubated at 33 ºC in 5% CO_2 for 1 hour to allow virus to infect the cells. Wells containing only diluted serum, only virus, and uninfected RD cells served as serum, virus, and cell controls, respectively. After infection, the inoculum was replaced with 100 µl of maintenance growth medium. Then, the plate was incubated at 33 ºC in 5% CO_2 and CPE was observed daily. After 7 days of incubation, the NAb titre was determined according to the highest dilution of serum able to inhibit CPE.

3.4.4 Validation of EV-D68 NAb titre formula

Validation of EV-D68 NAb titre formula was performed to determine the minimum inhibitory value for a serum to be considered seropositive. The luciferase-based seroneutralization test and the conventional seroneutralization test were performed on 45 serum samples. For the conventional seroneutralization test, EV-D68 NAb titre was determined according to the highest dilution able to inhibit CPE. Meanwhile, for the luciferase-based assay, EV-D68 NAb titre was calculated according to the following formula:

$$\text{EV-D68 NAb titre (\%)} = \frac{\text{Sample RLU - Negative control RLU}}{\text{Positive control RLU - Negative control RLU}} \times 100$$

EV-D68 NAb-positive serum was used as positive control and set as 100% inhibitory, while cells infected with virus only were used as negative control. Titres obtained using the conventional seroneutralization test and the luciferase-based assay were compared to determine the lowest inhibitory value among samples with ≥ 1:8 EV-D68 NAb titre.

3.5 Seroprevalence analysis using luciferase-based seroneutralization assay

Seroprevalence analysis of EV-D68 was carried out by performing luciferase-based seroneutralization assay on 450 serum samples using the clone-derived EV-D68_Nluc virus.

3.5.1 Sera

Serum samples were collected from the Diagnostic Virology Laboratory in University of Malaya Medical Centre (UMMC). A total of 450 serum samples collected in 2013, 2014 and 2015 from three different age groups were analysed. The age groups were children (1 to 12 years old), adults (13 to 49 years old) and elderly (50 and above years old). The samples were collected from healthy patients visiting UMMC for a general check-up, patients diagnosed with non-respiratory infections such as dengue and

leptospirosis, as well as patients diagnosed with respiratory tract infections caused by mycoplasma. This study was approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur, Malaysia (reference number: 2017116-5794).

3.5.2 Seroprevalence analysis

Luciferase-based seroneutralization test was performed on the collected serum samples. A 96-well plate with 1×10^4 RD cells/well was prepared. The serum samples were heat-inactivated at 56 ºC for 30 minutes and 1:8 dilution was performed on each serum sample. Equal volume of EV-D68_Nluc virus (1×10^4 $TCID_{50}$) was added to diluted sera. The serum-virus mixture was incubated at 33 ºC in 5% CO_2 for 2 hours to allow binding of NAbs to the virus. After incubation, 50 µl of the mixture was inoculated into RD cells in duplicates and the plate was incubated at 33 ºC in 5% CO_2 for 1 hour to allow virus to infect the cells. Wells containing only diluted serum, only virus, and uninfected RD cells served as serum, virus, and cell controls, respectively. After infection, the inoculum was replaced with 100 µl of maintenance growth medium. Then, the plate was incubated at 33 ºC in 5% CO_2. After 4 days of incubation, luciferase activity was determined using the Nano-Glo Luciferase Assay System (Promega, USA). The luciferase assay solution was prepared by adding 1 volume of luciferase substrate to 49 volumes of assay buffer. 25 µl of each sample was pipetted into a 96-well white plate and an equal volume of luciferase assay solution was added to each sample. Luciferase activity was measured using GloMAX Multi Detection System luminometer (Promega, USA).

3.6 Statistical analysis

For comparison of seroprevalence rates between different years and age groups, two-way analysis of variance was used. One-way analysis of variance was used for comparison of seroprevalence rates between age subgroups. *P-value*<0.05 was considered statistically significant. All statistical analyses were performed using StatPlus version 6.0.3, and graphs were drawn using GraphPad Prism 7.

CHAPTER 4: RESULTS

4.1 Construction of EV-D68 infectious clone with NanoLuc luciferase gene

4.1.1 Schematic illustration of EV-D68 infectious clone with Nluc gene

Schematic illustration of T7 promoter-driven EV-D68 infectious clone with Nluc reporter gene is shown in Figure 4.1. The Nluc gene was inserted downstream of EV-D68 5' UTR and upstream of EV-D68 VP4 gene, flanked by *Xho*I and *Xma*JI restriction enzyme sites. An EV-D68 2A protease cleavage site was placed in between the Nluc gene and the VP4 gene to allow cleavage of the reporter protein from EV-D68 VP4.

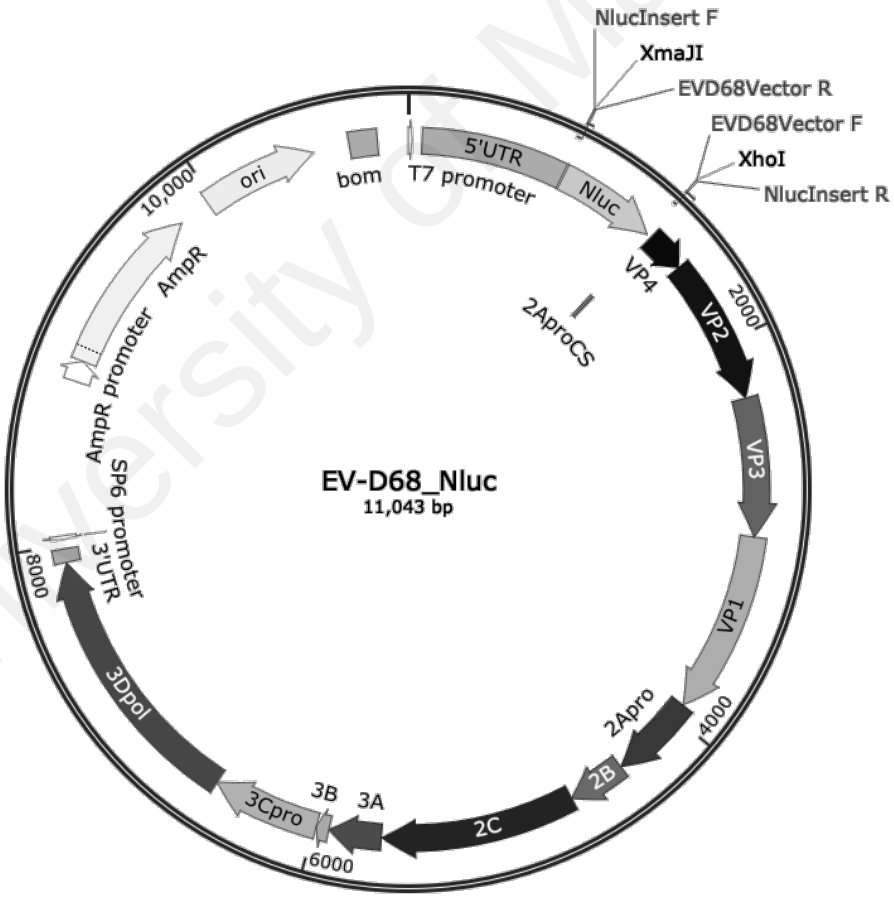


Figure 4.1: Schematic illustration of EV-D68_Nluc infectious clone. Nluc was cloned in between EV-D68 5' UTR and VP4 through *Xma*JI and *Xho*I restriction enzyme sites, followed by a 2A protease cleavage site (2AproCS). EV-D68 genomic DNA is annotated, and the transcription is controlled by T7 promoter.

4.1.2 Construction of EV-D68 infectious clone with Nluc gene

The EV-D68 vector and Nluc insert was PCR-amplified using a pair of primers each to insert the restriction enzyme sites and 2A protease cleavage site. Then, restriction enzyme digestion was carried out on the purified PCR products at *Xma*I and *Xho*I restriction enzyme sites. An agarose gel electrophoresis was ran to verify digested fragments. The estimated size of the digested EV-D68 vector and Nluc insert were 10,528 bp and 523 bp respectively. As shown in Figure 4.2A, the EV-D68 vector and Nluc insert were successfully digested. The digested products were then purified and ligated to form EV-D68_Nluc plasmid. The plasmid was transformed into *E. coli* XL10-Gold® competent cells. Next, a colony PCR was performed to select EV-D68_Nluc plasmid-containing colony. The expected band size for a plasmid-containing colony was 1000 bp. As shown in Figure 4.2B, the size of the amplified region from colony 6 was 1000 bp, therefore colony 6 contained the desired plasmid. EV-D68_Nluc plasmid from colony 6 was extracted and verified by DNA sequencing. In Figure 4.3, sequencing results shows that the Nluc insert, *Xho*I restriction enzyme site and 2A protease cleavage site were successfully inserted into the EV-D68 backbone. Transfection of the recombinant plasmid was performed to harvest EV-D68_Nluc infectious clone for subsequent analysis.

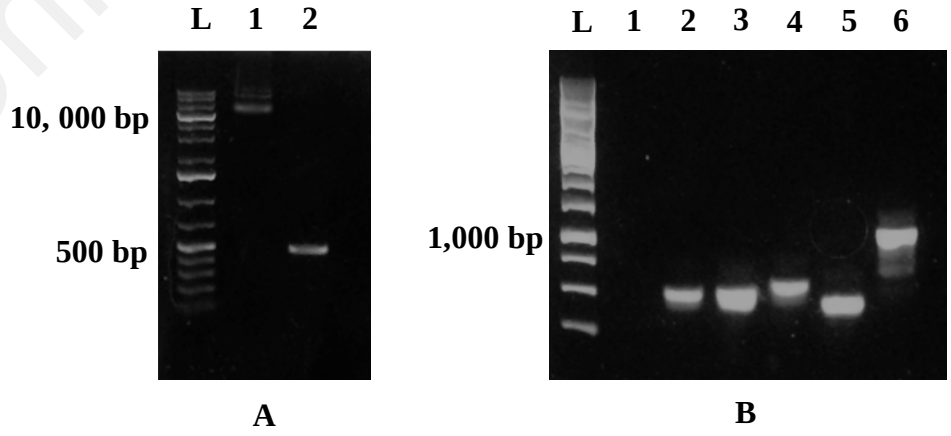


Figure 4.2: Agarose gel electrophoresis of **(A)** digested EV-D68 vector (lane 1) and Nluc insert (lane 2), and **(B)** colonies 1 to 6 (lanes 1-6). DNA ladder is represented by lane L.

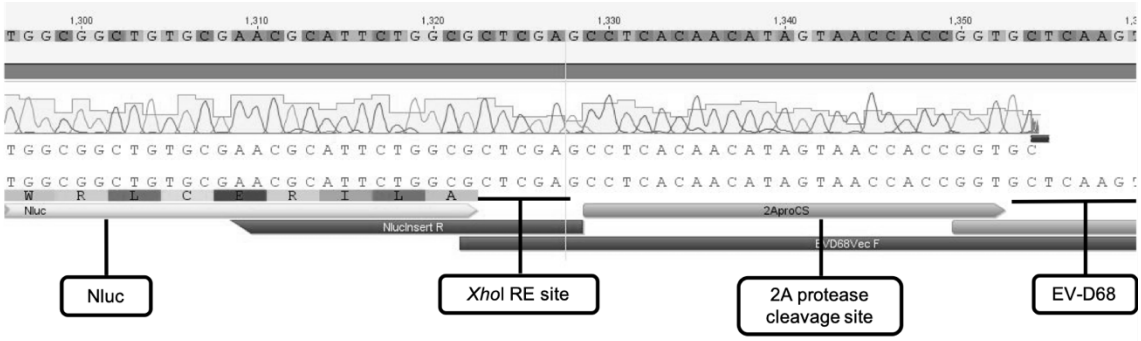
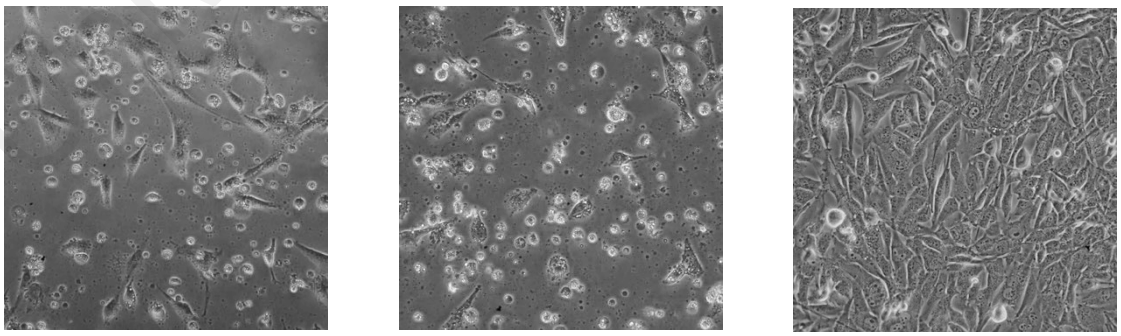


Figure 4.3: DNA sequencing results verifying successful cloning of EV-D68_Nluc. The Nluc insert, *Xho*I restriction enzyme site, 2A protease cleavage site and EV-D68 backbone are labelled.

4.2 Microscopic observation of cytopathic effect of EV-D68-infected cells

RD cells were transfected with 2 µg of EV-D68_Nluc RNA using the Mirus Bio TransIT®-mRNA Transfection kit. RD cells transfected with 2 µg of prototype EV-D68 RNA and with transfection reagents only were used as positive control and mock, respectively. The cells were observed daily for cytopathic effect. 70-80% CPE was observed 3 days post-infection (dpi) in RD cells transfected with EV-D68_Nluc and prototype EV-D68 (Figures 4.4A and 4.4B). Clumping and detachment of the infected cells were observed, resulting in the appearance of floating cells. The mock transfected RD cells had a monolayer spindle-shaped appearance, with well-organised cell to cell contact (Figure 4.4C).The viruses were harvested by freeze-thawing.



A **B** **C**

Figure 4.4: Morphology of EV-D68-transfected RD cells. 70-80% CPE was observed 3 dpi in **(A)** EV-D68_Nluc-transfected cells and **(B)** prototype EV-D68-transfected cells which served as positive control. No CPE was observed in **(C)** cells with transfection reagents which served as mock**.**

4.3 Plaque morphology of EV-D68-infected cells

Plaque assay was performed to observe the plaque morphology of EV-D68-infected cells. RD cells were infected with 10-fold serial-diluted EV-D68_Nluc and 10-fold serial-diluted prototype EV-D68 as positive control. Non-infected cells served as mock. Cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet after 5 days of incubation. Plaques were observed as clear transparent over white background. As shown in Figure 4.5, different plaque morphologies were observed between EV-D68_Nluc-infected cells and prototype EV-D68-infected cells. Plaques formed by EV-D68_Nluc (Figure 4.5A**)** had poorly defined borders and were smaller compared to plaques formed by prototype EV-D68 (Figure 4.5B). Mock-infected cells did not form plaques (Figure 4.5C).

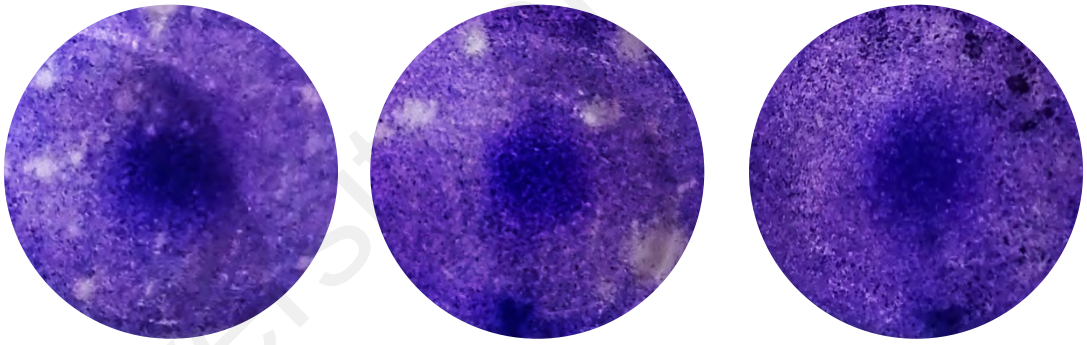


Figure 4.5: Plaque morphology of EV-D68-infected cells. Plaques were observed 5 dpi in **(A)** EV-D68_Nluc-infected cells and **(B)** prototype EV-D68-infected cells which served as positive control. No plaques were formed in **(C)** non-infected cells which served as mock.

4.4 Virus titration

Virus titre was determined according to the Reed-Muench method. RD cells were infected with 10-fold serial-diluted EV-D68_Nluc virus and 10-fold serial-diluted prototype EV-D68 viruses. After 7 days of incubation, tissue culture infectious dose ($TCID_{50}$) was determined according to the Reed & Muench (1938) formula and expressed

as $TCID_{50}$/ml. EV-D68_Nluc had a lower titre compared to the prototype virus. The titres of EV-D68_Nluc and prototype EV-D68 are listed in Table 4.1.

Table 4.1: Virus titres of EV-D68_Nluc and prototype EV-D68.

Virus	Titre ($TCID_{50}$/ml)
EV-D68_Nluc	4.18×10^5
Prototype EV-D68	1.28×10^6

4.5 Development and validation of luciferase-based seroneutralization assay

4.5.1 Optimisation of luciferase-based seroneutralization assay

The optimal incubation time for luciferase-based seroneutralization assay was determined by infecting 1×10^4 RD cells with 50 µl of EV-D68_Nluc (1×10^4 $TCID_{50}$) in a 96-well plate. Luciferase activity was determined after 1, 2, 3, 4 and 5 days post-infection using Nano-Glo Luciferase Assay System (Promega, USA) and GloMax Multi Detection System luminometer. The results showed that the luciferase activity increased with incubation time, peaking at 4 days after incubation **(**Figure 4.6). Therefore, 4 days was selected as the optimal incubation time for luciferase-based seroneutralization assay.

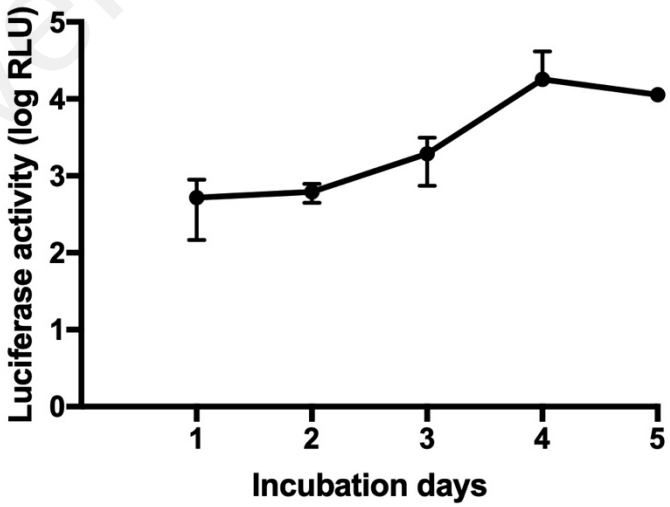


Figure 4.6: Luciferase activity of EV-D68_Nluc at various time points. Luciferase activity was measured from 1 to 5 dpi. Each luciferase activity is the average of two biological replicates, and the error bars represent the standard deviation of the mean. Luciferase activity was measured in Relative Light Unit (RLU).

4.5.2 Validation of luciferase-based seroneutralization assay

To develop the luciferase-based seroneutralization test, the assay has to be validated and compared with the conventional seroneutralization test based on cytopathic effect. Luciferase activities of EV-D68 NAb-positive and EV-D68 NAb-negative sera were compared with the NAb titres determined by the conventional seroneutralization test.

4.5.2.1 Luciferase-based seroneutralization assay

EV-D68_Nluc (1×10^4 $TCID_{50}$) was used to perform luciferase-based seroneutralization assay on EV-D68 NAb-positive and EV-D68 NAb-negative human sera. The NAb-positive and NAb-negative sera were previously confirmed with conventional seroneutralization test. The expression of bioluminescence signal indicates the absence of inhibitory NAb against EV-D68 virus. The intensity of bioluminescence signal is linearly related to the concentration of luciferase in EV-D68_Nluc. Cells infected with EV-D68_Nluc without the addition of serum were used as virus control. The cut-off value was obtained from the background luciferase activity of blank.

Figure 4.7 shows the results obtained. No inhibition was observed in NAb-negative serum due to the absence of EV-D68 neutralising antibody, allowing the virus to infect the cells as it was not neutralised, thus leading to the expression of Nluc protein, resulting in 4 log RLU. Meanwhile, inhibition was observed in NAb-positive serum from dilutions 1:8 to 1:64, indicating presence of EV-D68 NAbs. Background luciferase activity of blank was deducted from the luciferase activities of NAb-positive serum, NAb-negative serum and virus control, resulting in lower values for NAb-positive serum compared to blank. The NAb titres of NAb-negative and NAb-positive sera were < 1:8 and > 1:64, respectively.

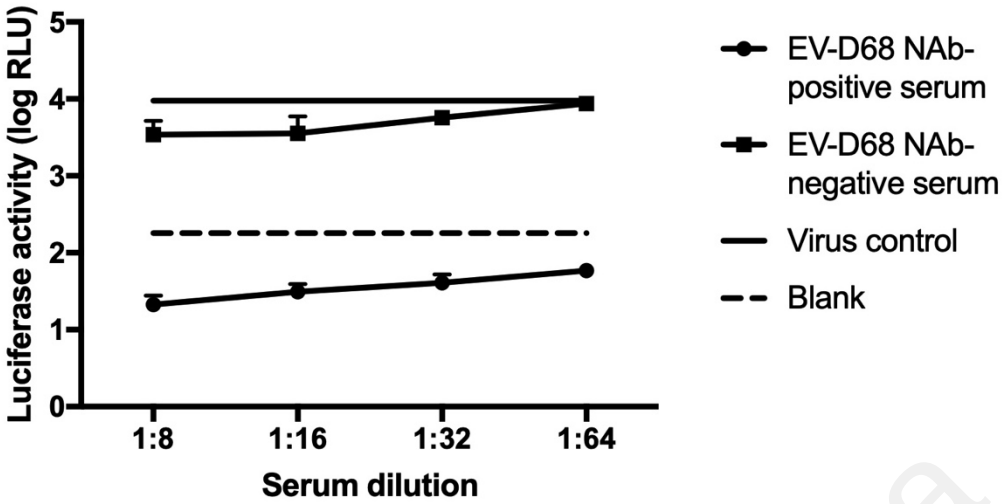


Figure 4.7: Luciferase-based seroneutralization test of EV-D68 neutralising antibodies against clone-derived EV-D68 stably expressed Nluc protein. Luciferase activity was measured 4 dpi. Background luciferase activity of blank was deducted from luciferase activity of EV-D68 NAb-positive and EV-D68 NAb-negative sera. Each luciferase activity is the mean of duplicates, and the error bars represent the standard deviation of the mean.

4.5.2.2 Conventional seroneutralization assay

Prototype EV-D68 (1×10^4 $TCID_{50}$) was used to perform conventional seroneutralization assay on EV-D68 NAb-positive and EV-D68 NAb-negative sera. Cytopathic effect was observed 7 dpi. NAb titre was determined according to the highest dilution able to inhibit CPE. Results showed that EV-D68 NAb titres for NAb-negative and NAb-positive sera were < 1:8 and > 1:64, respectively.

4.5.3 Validation of EV-D68 NAb titre formula

To validate the formula used to calculate EV-D68 NAb titre, the luciferase-based seroneutralization test and the conventional seroneutralization test were performed on 45 serum samples. For the conventional seroneutralization test, EV-D68 NAb titre was determined according to the highest dilution able to inhibit CPE. Meanwhile, for the luciferase-based assay, EV-D68 NAb titre was calculated using the following formula:

$$\text{EV-D68 NAb titre (\%)} = \frac{\text{Sample RLU - Negative control RLU}}{\text{Positive control RLU - Negative control RLU}} \times 100$$

Raw data of the results for both tests are presented in Appendix A. Figure 4.8 shows the NAb titres determined using both tests. EV-D68 NAb-positive serum was used as positive control and set as 100% inhibitory while cells infected with virus only were negative control. Sera with ≥ 1:8 titre as determined by the conventional assay were considered seropositive for EV-D68 NAb. Among seropositive samples, the lowest inhibitory titre calculated according to the formula was 90.08%. Therefore, 90% was selected as the minimum inhibitory titre for a serum to be considered seropositive.

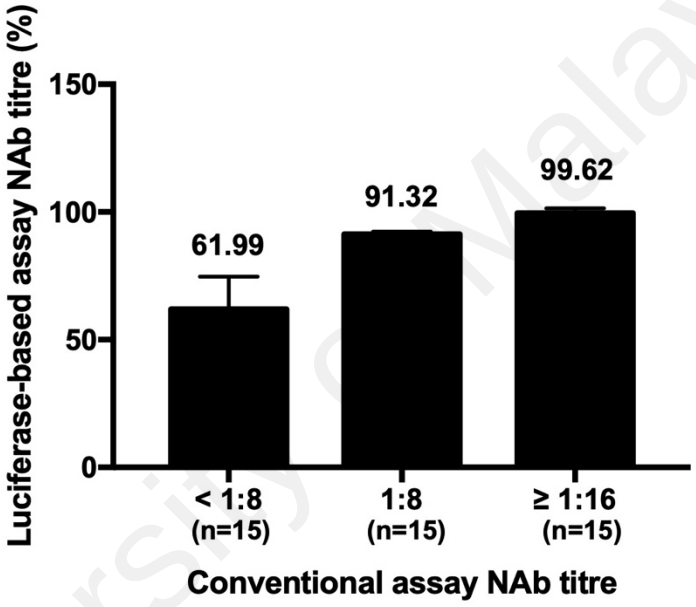


Figure 4.8: Comparison of EV-D68 NAb titres determined using the luciferase-based seroneutralization assay and the conventional seroneutralization assay. The numbers above each bar represent the mean inhibitory titres calculated according to the formula. Error bars represent standard deviation of the mean.

4.6 Seroprevalence analysis using luciferase-based seroneutralization assay

Luciferase-based seroneutralization test was performed on 450 serum samples collected in 2013, 2014 and 2015 from three different age groups. The age groups were children (1 to 12 years old), adults (13 to 49 years old) and elderly (50 years old and above). All samples were obtained from the Diagnostic Virology Laboratory in University of Malaya Medical Centre. Sera were considered positive for EV-D68 NAbs when the inhibitory titre was > 90%.

The distribution of EV-D68 NAb seroprevalence by age groups and years is shown in Table 4.2 and Figure 4.9. Results indicate that seroprevalence rates in children were lower than adults and elderly in each year ($P<0.05$). Furthermore, seroprevalence rates significantly increased between 2013 and 2015 in adults ($P<0.05$), but not in children and elderly ($P>0.05$).

Table 4.2: Age-specific seroprevalence rate of EV-D68 NAbs in different years.

Year	Age (year)	Seropositive	Seronegative	Seropositive rate (%)
2013	1-12	27	23	54
	13-49	41	9	82
	≥ 50	41	9	82
2014	1-12	29	21	58
	13-49	43	7	86
	≥ 50	42	8	84
2015	1-12	29	21	58
	13-49	46	4	92
	≥ 50	44	6	88

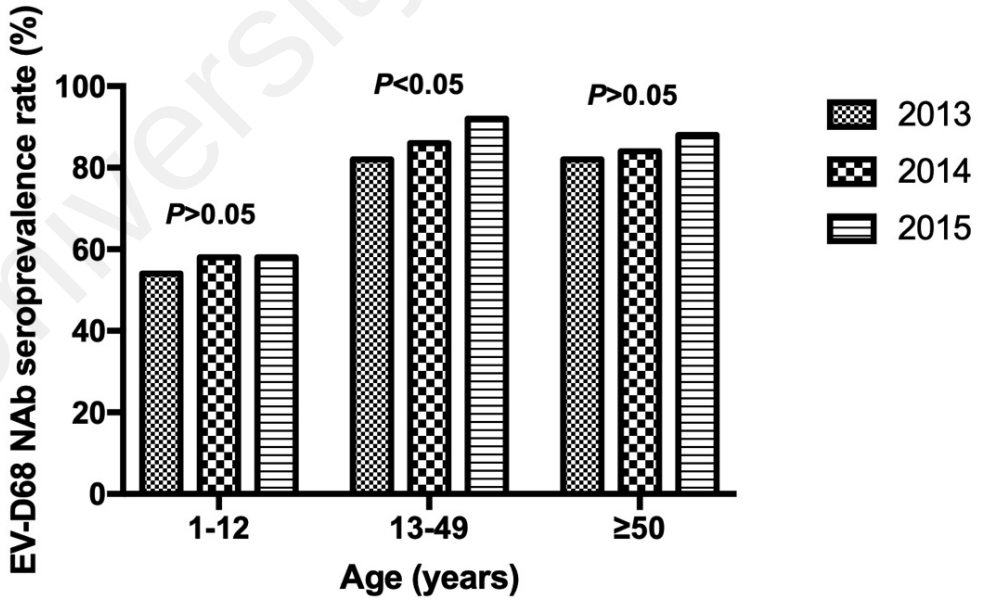


Figure 4.9: Age-specific seroprevalence rate of EV-D68 neutralising antibodies in 2013, 2014 and 2015. Numbers above each group of bars represent the *P-values* of seroprevalence rate differences between the years in each age group.

To further analyse the relationship between age and EV-D68 NAb seroprevalence, we combined serum samples from all three years and divided them into smaller age groups: 1 to 3 years (n=50), 4 to 6 years (n=44), 7 to 12 years (n=56), 13 to 17 years (n=63), 18 to 30 years (n=39), 31 to 49 years (n=48), 50 to 59 years (n=77) and 60 years and above (n=73).

The age-specific seroprevalence rate of EV-D68 NAb is shown in Figure 4.10. Results indicate that EV-D68 NAb seroprevalence rate increased with age, peaking in 31 to 49 years old adults and subsequently declined. Within the children subgroups, seroprevalence rate significantly increased with age ($P<0.05$). In contrast, within the adult and elderly subgroups, there was no significant difference with age ($P>0.05$).

Lastly, we compared the EV-D68 NAb seroprevalence rates between male (n=254) and female (n=196) samples and found no difference. Both groups had a 76% seroprevalence rate.

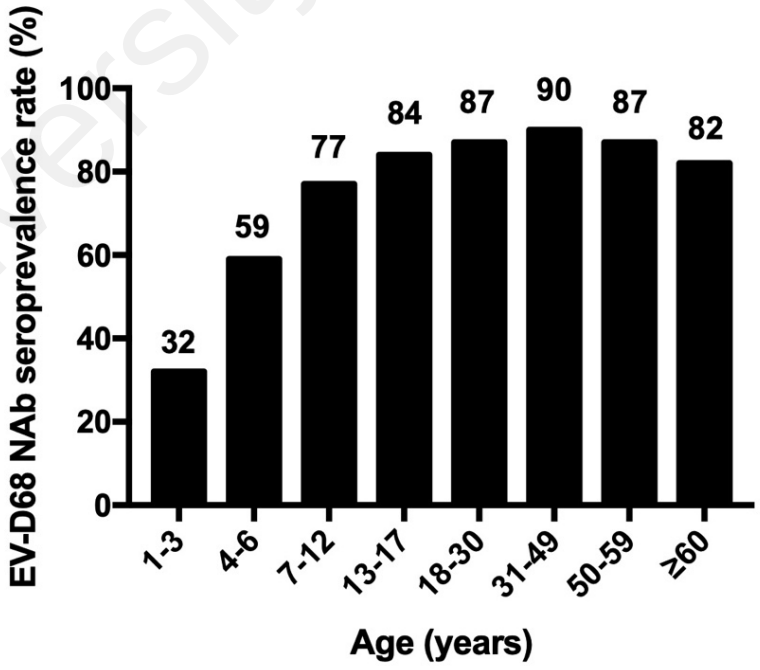


Figure 4.10: Seroprevalence of EV-D68 NAb in different age subgroups. Numbers above each bar represent the EV-D68 NAb seroprevalence rate (%) of each age group.

CHAPTER 5: DISCUSSION

EV-D68 neutralising antibody serves as an important marker in monitoring seroprevalence of EV-D68 in a population. Traditionally, EV-D68 NAbs are detected using the conventional seroneutralization assay based on observation of cytopathic effect. However, this method is inappropriate for mass screening of antibodies because it is time-consuming, labour-intensive and subjective (Jin *et al.*, 2013). Alternatively, reporter-based seroneutralization tests are rapid, convenient and quantitative (Deng *et al.*, 2016). In this study, a preliminary luciferase-based seroneutralization test for EV-D68 was developed using an Nluc-expressing EV-D68 infectious clone. The test has been validated by comparing neutralisation results with the conventional assay.

We hypothesised that the luciferase-based assay for EV-D68 would be more rapid than the conventional assay. This is supported by our finding that by using the luciferase-based assay, EV-D68 NAb titre could be determined within 4 days, which is shorter than the 7 days required by the conventional assay. Therefore, we achieved our first objective which was to develop a luciferase-based seroneutralization test for rapid seroprevalence analysis of EV-D68. NanoLuc luciferase is an ideal reporter for determining NAb titre because its luminescence signal strongly correlates with Nluc concentration and viral titre, allowing a rapid and quantitative method for measuring reporter expression and viral replication (Boute *et al.*, 2016; Fulton *et al.*, 2015; Hall *et al.*, 2012). This eliminates the need for observation of CPE which is time-consuming and subjective.

Since the luciferase-based assay is rapid, convenient and quantitative, it is suitable for mass screening of NAbs. Therefore, this test is useful for high throughout applications such as seroprevalence studies. However, further studies such as assessing the cross-reactivity of NAb of other viruses will be required to validate the accuracy of this assay and increase its sensitivity. The test developed in this study serves as a preliminary assay

which can be developed into a more sensitive and accurate detection tool for EV-D68 NAbs.

Our second objective was to assess the seroprevalence of EV-D68 NAbs in Malaysia, which was achieved by using the luciferase-based seroneutralization test on serum samples collected from University of Malaya Medical Centre. Serum samples were collected from 2014 since it was the year of worldwide EV-D68 outbreaks (Holm-Hansen *et al*., 2016). Other than that, samples from the years prior to and after 2014 (2013 and 2015, respectively) were also collected to investigate the pattern of EV-D68 seroprevalence over the years.

We hypothesised that EV-D68 NAb seroprevalence would increase after 2014 in all age groups. However, our results showed that there was a significant increase between 2013 and 2015 in adults, but not in children and elderly. Seroprevalence rates in adults were 82%, 86% and 92% in 2013, 2014 and 2015, respectively. This suggests an increasing spread of EV-D68 after 2013, leading to a rise of EV-D68 NAb seroprevalence. Similarly, Xiang *et al.* (2017) found that EV-D68 NAb prevalence increased in all age groups between 2004 and 2009 in China. However, between 2007 and 2011, there was no significant increase in EV-D68 NAb titre year by year in children, contrary to the pattern seen in adults (Xiang *et al.*, 2017). Furthermore, EV-D68 seroprevalence rate among adults in China in 2015 was 100% (Sun *et al.*, 2018). This suggests that adults in both countries were widely infected with EV-D68 in 2015, following the worldwide outbreaks in 2014.

To investigate whether age has an impact on EV-D68 NAb seroprevalence, we hypothesised that the seroprevalence rate of EV-D68 NAb in the Malaysian population would increase with age. We found that EV-D68 NAbs seroprevalence increased with age from children to adults, but declined in the elderly group. Seroprevalence rates in

children were significantly lower than in adults: 54%, 58% and 58% in children and 82%, 86% and 92% in adults in 2013, 2014 and 2015, respectively. In addition, further analysis of age subgroups comprising samples from all three years revealed that seroprevalence rates in children significantly increased with age: 32%, 59% and 77% in 1-3, 4-6 and 7-12 years old children, respectively. This implies that children became increasingly exposed to EV-D68 infection as they get older, possibly due to increasing exposure to crowds such as in kindergartens, schools, shopping malls or public transport.

Our findings on the low seroprevalence of EV-D68 NAbs in children are supported by similar studies in China. Sun *et al.* (2018) found that 2-5 years old children in China had a low seroprevalence rate of 44%, which increased to 83% in 6-15 years old children, suggesting that EV-D68 infection was highly prevalent in school-aged children (Sun *et al*., 2018). Comparably, Xiang *et al.* (2017) found that pre-school and school-aged children in China were widely infected with EV-D68. EV-D68 NAb titres were higher in 6.1-15 years old children compared to 0.5-6 years old children (Xiang *et al*., 2017). Since EV-D68 NAbs provide protection from infection, the low seroprevalence rate in children compared to adults in this study indicate that children are more vulnerable to EV-D68 infection. This may explain why children represent most of the cases reported in outbreaks. For instance, in the 2014 USA outbreak, almost all of the 1,153 confirmed cases were among children (Oermann *et al*., 2015). Hence, children should be prioritised in preventive measures and future vaccination programmes when an effective EV-D68 vaccine is commercially available.

In 2014, major EV-D68 outbreaks occurred worldwide in countries such as USA (1,153 cases), Canada (546 cases) and France (117 cases) (Holm-Hansen *et al.*, 2016). In contrast, there was no outbreak in Malaysia that year and only 12 cases were detected from 2012 to 2014 (Ng *et al,* 2015). Since EV-D68 NAbs block viral replication, pre-

existing NAbs in a population may provide herd immunity and influence the spread of a virus (Xiang *et al.*, 2017). Therefore, the high seroprevalence of EV-D68 NAbs in adults and elderly may have contributed to the absence of outbreaks in Malaysia. This warrants further research on seroprevalence rates of EV-D68 NAbs in different populations and time points in order to assess the transmission potential of EV-D68 and predict future outbreaks. Lastly, we found no difference between the EV-D68 NAb seroprevalence rates of male and female samples, indicating that both genders are equally susceptible to EV-D68 infection.

There are several limitations in this study. First, the virus strain used was the prototype EV-D68 strain Fermon instead of a currently circulating clinical isolate. Studies have found that sera with high neutralising titres against a clinical isolate of EV-D68 have low titres against the prototype strain (Imamura *et al*., 2014; Xiang *et al.*, 2017). Therefore, neutralisation tests using the prototype strain may underestimate the seroprevalence of EV-D68 NAbs in a population. Further studies may be required to develop a luciferase-based seroneutralization test based on the currently circulating clinical isolate to gain a more accurate assessment of EV-D68 seroprevalence in the population. Secondly, while Xiang *et al.* (2017) and Sun *et al.* (2018) collected samples from healthy individuals, our study population consisted of UMMC patients including patients with RTIs. NAbs against rhinoviruses and other enteroviruses may cross-neutralise EV-D68 (Xiang *et al.*, 2017). Hence, neutralisation titres obtained from RTI patients may lead to an inaccurate assessment of EV-D68 seroprevalence in the general population. The high EV-D68 NAb seroprevalence rates found in this study may be due to cross-neutralisation with rhinoviruses and other enteroviruses, thus further studies will be required to determine the cross-neutralisation between EV-D68 with these viruses.

The worldwide outbreaks of EV-D68 in recent years have caused major concern regarding this emerging virus capable of causing severe respiratory and neurological illnesses, particularly among children. This is supported by our finding that children had the lowest seroprevalence rate of protective EV-D68 NAbs, thus making them more vulnerable for EV-D68 infection compared to adults and elderly. Therefore, future studies should focus on the seroprevalence of EV-D68 NAbs in children. Large-scale studies involving a higher sample size of children sera will help to refine the age for vaccination. In addition, future studies may also investigate the seroprevalence of EV-D68 NAbs in sera from different decades in order to elucidate the temporal dynamics of EV-D68 infection and determine whether EV-D68 is truly an emerging disease. Lastly, diagnosis of EV-D68 in RTI patients may be used to complement seroprevalence studies such as the one conducted here. This can be achieved by the development of a sensitive real-time PCR for EV-D68.

CHAPTER 6: CONCLUSION

This study has established a preliminary luciferase-based seroneutralization test for EV-D68. Compared to the conventional assay, the luciferase-based seroneutralization test is more rapid, convenient and quantitative. Therefore, it is useful for high throughput applications such as the mass screening of neutralising antibodies in seroprevalence studies. Consequently, the luciferase-based seroneutralization assay was used to perform a seroprevalence analysis of EV-D68 NAbs in Kuala Lumpur, Malaysia in 2013, 2014 and 2015. We found that EV-D68 NAbs increased from 2013 to 2015 in adults, but not in children and elderly. Furthermore, children had significantly lower seroprevalence rates compared to adults and elderly in each year. In addition, EV-D68 NAb seroprevalence rates in 4-6 and 7-12 years old children were higher than in 1-3 years old children, suggesting increased exposure to EV-D68 infection as children grow older, which may be due to increased exposure to crowds such as in kindergartens and primary schools. Hence, children should be treated as a vulnerable population and prioritised in future vaccination programmes. High seroprevalence of EV-D68 NAbs in adults and elderly may have contributed to the absence of outbreaks in Malaysia, warranting further research on EV-D68 seroprevalence in different populations and time points.

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