Evaluation of Therapeutics for an Enterovirus 71 Infection in an AG129 Mouse Model

Christopher Peterson
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EVALUATION OF THERAPEUTICS FOR AN ENTEROVIRUS 71 INFECTION IN
AN AG129 MOUSE MODEL

by

Christopher Peterson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal, Dairy, and Veterinary Science

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UTAH STATE UNIVERSITY
Logan, Utah
2018
ABSTRACT

Evaluation of Therapeutics for an Enterovirus 71 Infection in an AG129 Mouse Model

by

Christopher Peterson, Master of Science
Utah State University, 2018

Major Professor: Dr. E. Bart Tarbet
Department: Animal, Dairy, and Veterinary Sciences

Enterovirus 71 (EV-71) is a non-enveloped, single-stranded RNA virus and member of the Picornaviridae family. It is one of the major causative agents of hand, food, and mouth disease (HFMD), and can produce serious neurological disease, including meningitis, encephalitis, and acute flaccid paralysis. For serious cases, the fatality rate can be up to 26%, almost exclusively in young children. Despite the seriousness of an EV-71 infection, there are currently no approved antiviral treatments. Part of the difficulty in developing an effective treatment for EV-71 is a lack of in vivo data about potential compounds. This is hampered by the fact that most animal models for EV-71 require two-week-old mice, which creates difficulty when administering treatments and treating at high doses.

At the Institute for Antiviral Research (IAR), an animal model for EV-71 was developed using four-week-old AG129 mice. EV-71 (MP4; BEI-Resources NR-472) was serially passaged six times in mice. Mice infected with this virus exhibit neurological disease signs, including an acute flaccid paralysis. The model is also completely lethal, providing a consistent benchmark to be used in antiviral studies. Viral titers peak in the
brain and spinal cord at day seven post-infection. Disease onset usually occurs from day 9 to day 14 post-infection (p.i.). Five additional passages did not improve virulence.

Twenty-four therapeutics were screened in this model. Therapeutics were selected based on their effectiveness in inhibiting EV-71 in vitro and treating EV-71 infection in animal models. Effectiveness against other picornaviruses was also considered in choosing compounds. Although most compounds failed to provide protection, two compounds, suramin and fluoxetine, initially showed potential but were ultimately found to be too toxic and inconsistently inhibitory to examine further. However, two novel host response modifiers, STF434 and STF1019, and a brand of human intravenous immunoglobulin (IVIG) (Carimune® NF) were effective at preventing mortality. Furthermore, Carimune® NF was able to reduce several pro-inflammatory cytokines (MCP-1, RANTES, MIP-1α, IFN-γ) in the brain and/or spinal cord by 2.5- to 13-fold.
PUBLIC ABSTRACT

Evaluation of Therapeutics for an Enterovirus 71 Infection in an AG129 Mouse Model

Christopher Peterson

Discovered in 1969 in California, enterovirus 71 (EV-71) is a serious cause of disease in young children. It is one of the major causative agents of hand, food, and mouth disease (HFMD), and can produce neurological complications, such as meningitis, encephalitis, and an acute flaccid paralysis. For serious cases, the fatality rate can be up to 26%, almost exclusively in young children.

While the virus was initially discovered in the United States, it was soon detected in the Eastern hemisphere, causing outbreaks in Europe and Asia. The largest outbreak occurred in Taiwan in 2008, with approximately 490,000 cases and 128 fatalities. However, despite the seriousness of EV-71, there are currently no approved antiviral treatments. Physicians rely on supportive care and the off-label use of a purified antibody mixture, intravenous immunoglobulin, for treatment.

Part of the difficulty in developing antivirals for EV-71 is a lack of drug testing in animal models. Animal testing is a crucial step in drug development, determining which compounds will progress to clinical trials in humans. However, viruses that cause disease in humans do not necessarily cause disease or the same type of disease in animals. As such, viruses often need to be adapted before they can cause disease in their animal hosts. Adaption isn’t always successful and can result in a virus that produces disease that is unlike that seen in humans. Furthermore, some animal models can produce disease only under a strict set of conditions, such as newborn mice. Sometimes these animal model
conditions may be impractical for testing potential treatments.

At the Institute for Antiviral Research (IAR), we developed an animal model for EV-71 in four-week-old AG129 mice. AG129 mice lack the alpha, beta, and gamma interferon receptors, making them immunocompromised. Being immunocompromised, these mice are more susceptible to infection, including infection from human viruses. In our model, EV-71 infection produces neurological signs, including a rear-limb paralysis (similar to the paralysis seen in children with EV-71). The virus is also lethal in these animals, which provides an observable and consistent baseline for evaluating potential drugs.

We assessed twenty-four potential treatments in our EV-71 model. Two compounds, STF434 and STF1019, provided 30% and 87% protection against mortality. Intravenous immunoglobulin was also examined and found to be about 50% protective against mortality, depending on the dose and time of administration. Intravenous immunoglobulin also reduced inflammatory modulators (cytokines) in the brain and spinal cord. We consider this to be highly relevant, given that inflammation is a serious component of EV-71 infection.
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I would like to thank Dr. Bart Tarbet for his patience and mentoring throughout this project, including impromptu office conversations and unexpected pearls of wisdom. I am especially grateful for his example of leadership and am truly impressed with the genuine concern he has for his students.

I would also like to thank Brett Hurst and Joseph Evans for their help with this project, including early development of the EV-71 animal model and sequencing of the EV-71 genome. I would especially like to thank Brett for his technical expertise, sense of humor, and for enduring my seemingly endless questions.

I also need to mention the tireless work of our technicians, particularly Austin Broadhead and Justin Moscon, who performed endless virus titrations and painstakingly necropsied scores of samples to help complete this project on-time.

Special thanks to Dr. Anthony Torres for his insight and for reviewing this document. Dr. Torres has been a great mentor over the years, especially with guiding my career goals.

I’d like to thank the Institute for Antiviral Research for their support and being my “home away from home” for the last two years. I have felt privileged to work with and learn from so many bright and friendly people.

Finally, this work is dedicated to my parents, Carol and John, for their ever-inspiring love and pursuit of knowledge.

Christopher J. Peterson
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>PUBLIC ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER I.                 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Origin and Epidemiology</td>
<td>1</td>
</tr>
<tr>
<td>Classification</td>
<td>3</td>
</tr>
<tr>
<td>Structure and Genome</td>
<td>4</td>
</tr>
<tr>
<td>Viral Replication</td>
<td>5</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>8</td>
</tr>
<tr>
<td>Clinical Presentation and Patient Outcomes</td>
<td>12</td>
</tr>
<tr>
<td>Animals Models</td>
<td>14</td>
</tr>
<tr>
<td>Antivirals and Therapeutics</td>
<td>20</td>
</tr>
<tr>
<td>Vaccine</td>
<td>31</td>
</tr>
<tr>
<td>II. STATEMENT OF THE PROBLEM</td>
<td>33</td>
</tr>
<tr>
<td>III. DEVELOPMENT OF AN AG129 MOUSE MODEL FOR AN EV-71 INFECTION</td>
<td>35</td>
</tr>
<tr>
<td>Abstract</td>
<td>35</td>
</tr>
<tr>
<td>Introduction</td>
<td>35</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>36</td>
</tr>
<tr>
<td>Results</td>
<td>42</td>
</tr>
<tr>
<td>Discussion</td>
<td>58</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>67</td>
</tr>
<tr>
<td>IV. EVALUATION OF THERAPEUTICS FOR AN EV-71 INFECTION IN AG129 MICE</td>
<td>68</td>
</tr>
<tr>
<td>Abstract</td>
<td>68</td>
</tr>
<tr>
<td>Introduction</td>
<td>68</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>69</td>
</tr>
<tr>
<td>Results</td>
<td>71</td>
</tr>
<tr>
<td>Discussion</td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHAPTER III</strong></td>
<td></td>
</tr>
<tr>
<td>1 Neurological Scoring System for an EV-71 Infection in AG129 Mice</td>
<td>42</td>
</tr>
<tr>
<td>2 Summary of Mouse Passage Viral Titers after Serial Passaging of EV-71 in AG129 Mouse Brain</td>
<td>48</td>
</tr>
<tr>
<td>3 Viral Titers for EV-71 MP10 Stocks 1-4</td>
<td>51</td>
</tr>
<tr>
<td><strong>CHAPTER IV</strong></td>
<td></td>
</tr>
<tr>
<td>1 Summary of Antiviral and Therapeutic Tesing for an EV-71 Infection in AG129 Mice</td>
<td>72</td>
</tr>
<tr>
<td><strong>CHAPTER VI</strong></td>
<td></td>
</tr>
<tr>
<td>1 Summary of Nucleotide and Predicted Amino Acid Changes in Four EV-71 Genome Sequences</td>
<td>135</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Survival of Four- and Six-Week-Old Mice Infected with EV-71 MP7 and MP10</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>Percent Body Weight of Four- and Six-Week-Old AG129 Mice Infected with EV-71 MP7 and MP10</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>Survival of AG129 Mice Infected with EV-71 MP10 Plaque 4, Plaque 6, and Plaque 15</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>Survival of Mice Infected with EV-71 MP10 Plaque 4 and Plaque 6</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>Survival of Mice Infected with EV-71 MP11-15</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>Challenge Dose Titration of EV-71 MP10 Plaque 4 and Plaque 6 Combination from Stocks 1 and 2</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>Survival of Mice Infected with EV-71 MP10 Plaque 4 and Plaque 6 from Stocks 1-4</td>
<td>52</td>
</tr>
<tr>
<td>8</td>
<td>Survival of Mice Infected with EV-71 MP10 Plaque 4 from Stocks 1-4</td>
<td>53</td>
</tr>
<tr>
<td>9</td>
<td>Survival of Mice Infected with EV-71 MP10 Plaque 6 from Stocks 1-4</td>
<td>54</td>
</tr>
<tr>
<td>10</td>
<td>Comparison of Two Trials of Mice Infected with EV-71 MP10 Plaque 4 and 6 from Stock 3</td>
<td>55</td>
</tr>
<tr>
<td>11</td>
<td>Comparison of the Age of Infection</td>
<td>57</td>
</tr>
<tr>
<td>12</td>
<td>Comparison of Viral Titers for the Age of Infection</td>
<td>58</td>
</tr>
</tbody>
</table>

### CHAPTER III

1. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with Suramin                  | 75   |
2. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with Fluoxetine             | 77   |
3. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with Fluoxetine Oral Solution | 79   |
4. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with STF1019              | 81   |
5 Neurological Scores for Mice Infected with EV-71 and Treated with STF1019....................................................................................... 82

6 Survival of Mice Infected with EV-71 and Treated with STF434 ............. 83

7 Neurological Scores for Mice Infected with EV-71 and Treated with STF434....................................................................................... 84

CHAPTER V

1 Survival of Mice Infected with EV-71 and Treated with Four Different Doses of Carimune® NF ....................................................................................... 96

2 Survival of Mice Infected with EV-71 and Treated with Carimune® NF at Four Different Timepoints p.i................................................................. 97

3 Survival of Mice Administered Prophylactic Carimune® NF and Infected with EV-71 ................................................................................................. 98

4 Survival of Mice Infected with EV-71 and Treated with High Dose Carimune® NF ............................................................................................... 99

5 Survival of Mice Infected with EV-71 and Treated with Carimune® NF via Different Routes of Administration .................................................. 100

6 Survival of Mice Infected with EV-71 and Treated with Carimune® NF via the Intravenous Route ................................................................. 101

7 Dose Titration of Gammunex®-C for an EV-71 Infection in Mice ......... 102

8 Comparison of Two Different Lots of Carimune® NF Used to Treat an EV-71 Infection in Mice ................................................................................. 103

9 Survival of Mice Infected with EV-71 and Treated with Carimune® NF at 0h p.i ........................................................................................................ 104

10 Survival of Mice Infected with EV-71 and Treated with Carimune® NF at 1, 2, and 4h p.i ............................................................................................... 105

11 Dose Titration of Carimune® NF (100-1300 mg/kg/d) for an EV-71 Infection in Mice .................................................................................................. 106

12 Dose Titration of Carimune® NF (400-2000 mg/kg/d) for an EV-71 Infection in Mice ............................................................................................... 107

13 Dose Titration of Carimune® NF (200-800 mg/kg/d) for an EV-71 Infection in Mice ............................................................................................... 108
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIII</td>
<td>Time Course of Viral Titers for Brain, Spinal Cord, Leg Muscle, and Blood from Mice Infected with EV-71 and Treated with Carimune® NF... 110</td>
</tr>
<tr>
<td>XIV</td>
<td>Time Course of Viral Titers for Lung, Liver, Kidney, and Spleen from Mice Infected with EV-71 and Treated with Carimune® NF............... 111</td>
</tr>
<tr>
<td>XV</td>
<td>Time Course for MCP-1 and IFN-γ in Mice Infected with EV-71 and Treated with Carimune® NF.......................................................... 113</td>
</tr>
<tr>
<td>XVI</td>
<td>Time Course for MIP-1α and RANTES in Mice Infected with EV-71 and Treated with Carimune® NF.......................................................... 114</td>
</tr>
<tr>
<td>XVII</td>
<td>Leg Muscle Pathology at Day 7 p.i. from a Mouse Infected with EV-71 and Administered Placebo .......................................................... 116</td>
</tr>
<tr>
<td>XVIII</td>
<td>Spinal Cord Pathology at Day 9 p.i. from a Mouse Infected with EV-71 and Administered Placebo .......................................................... 117</td>
</tr>
<tr>
<td>XIX</td>
<td>Brainstem Pathology at Days 9 and 11 p.i. from Mice Infected with EV-71 and Administered Placebo .......................................................... 118</td>
</tr>
<tr>
<td>XX</td>
<td>Adipose Tissue Pathology at Day 5 p.i. from a Mouse Infected with EV-71 and Administered Carimune® NF or Placebo .......................................................... 118</td>
</tr>
</tbody>
</table>

CHAPTER VI

1 Visual Representation of EV-71 Polyprotein and Predicted Amino Acid Changes for MP10 Virus .......................................................... 137
CHAPTER I
INTRODUCTION

ABSTRACT
Enterovirus 71 (EV-71) is a picornavirus that can cause serious neurological disease and death in young children. Discovered in 1969, EV-71 has caused a number of outbreaks, the most recent of which have occurred in the Eastern hemisphere. Despite the seriousness of an EV-71 infection, no antivirals are indicated for treating infection. Here we review the current body of literature on EV-71, with emphasis on animal models for EV-71 and potential antivirals and therapeutics.

1. Origin and Epidemiology
Enterovirus 71 (EV-71) was first identified in 1969 in California in an infant with encephalitis (McMinn, 2002). However, it is suspected that the virus was present earlier in the human population than this, as an EV-71 isolate from 1963 has been identified from stored clinical isolates (van der Sanden et al., 2009). Phylogenetic analysis suggests that EV-71 emerged as early as 1941 from coxsackievirus A16 (Tee et al., 2010). After its identification in 1969, EV-71 was soon identified outside of California in New York and Australia in 1972 (McMinn, 2002). The virus later spread to Sweden (195 cases)(Ooi et al., 2010), and Japan in 1973 (McMinn, 2002). Larger EV-71 outbreaks would eventually occur in the European and Asia-Pacific regions (Chang et al., 2016). Notable outbreaks occurred in Bulgaria in 1975 (705 cases, 44 fatalities)(McMinn, 2002), Hungary in 1978 (323 cases, ~47 fatalities)(Nagy et al., 1982), and Australia in 1986 (114 cases, 0 fatalities)(Gilbert et al., 1988). However, the largest outbreaks to date have occurred in East Asia. These include Malaysia in 1997 (2,628 cases, 34 fatalities)(Chan et
al., 2000), Taiwan in 1998 (129,106 cases, 78 fatalities)(Ho et al., 1999), China in 2008 (488,950 cases, 128 fatalities)(Yang et al., 2009b) and 2009 (460,000, 33 deaths), Vietnam (110,897, 84 deaths) and Cambodia (unknown number of cases, 54 deaths)(Organization, 2011). On the other hand, few outbreaks have occurred in the United States, with the largest outbreak occurring in 1987 across seventeen states (45 cases, one fatality) and the most recent outbreak occurring in 2005 Denver, Colorado (8 cases, 1 fatality)(Alexander et al., 1994; Perez-Velez et al., 2007). *

EV-71 epidemics occur in a cyclical pattern in certain countries such as Taiwan, Japan, Malaysia, Singapore, and Vietnam, on average every 2 to 5 years (Chang et al., 2016; Huang et al., 2014a; Wong et al., 2010). It is suspected that this may be due to new birth cohorts that have had no previous exposure to the virus (Ooi and Solomon, 2014) or the introduction of a new EV-71 genogroups to the region. However, novel genotypes have been shown to circulate within a country for several years without causing an outbreak (Wong et al., 2010). EV-71 also exhibits seasonal patterns, with warmer temperatures and humidity correlating with increased EV-71 infections in Taiwan (Chang et al., 2016).

The circulating EV-71 genogroups for a given area can change over time (Lei et al., 2015). Furthermore, the disease caused by a particular genotype may vary across outbreaks. For example, although the virus that caused the 1973 Bulgarian and Japanese outbreaks belonged to the same genogroup, the Bulgarian epidemic had no documented cases of HFMD, whereas the Japanese epidemic was predominantly associated with

---

*Case numbers and fatalities are based on total HFMD cases, of which EV-71 is a major contributor, especially in fatal cases.*
HFMD (McMinn, 2002). In the 1998 Taiwan outbreak, HFMD was closely associated with neurological disease, whereas it was not in the 1999 Perth outbreak (McMinn, 2002).

2. Classification

EV-71 is a member of the Enterovirus genus within the Picornaviridae. Originally, human enteroviruses were categorized into one of four families: poliovirus, coxsackievirus A, coxsackievirus B, and echoviruses (Solomon et al., 2010). Later, enteroviruses became a distinct genus with four enterovirus subspecies (A-D) (Fields et al., 2013). EV-71 is classified as a member of the enterovirus A species and is divided into seven genogroups (A-G). Each genogroup may be further divided into subgenogroups, such as with genogroup B (B1-5) and C (C1-5) (Yusof MA, 2018).

The identification methods for enteroviruses have changed as well. Originally, identification methods relied on the virus pathogenicity in humans, research animals, and cytopathic effect in cell culture (Oberste et al., 1999; Solomon et al., 2010). However, due to the overlap of clinical signs between some viruses, the typing method changed to virus neutralization using antiserum (1957). In the 1960s, antigenically distinct enteroviruses were given a numerical classification, beginning with enterovirus 68 (Khetsuriani et al., 2006; Wei et al., 2011). However, neutralization methods are time-consuming, expensive, and cannot type all enterovirus strains due to antigenic shift, virus aggregation, or samples containing multiple viruses (Nasri et al., 2007). As such, polymerase chain reaction (PCR) has become a common method for identification, with the VP1 and 5’ UTR regions commonly used for identification (Chang et al., 2016; Nasri et al., 2007; Oberste et al., 2010).

3. Structure and Genome
EV-71 is a non-enveloped, icosahedral virus about 30 nm in diameter (Organization, 2011) with a single-stranded, positive-sense RNA genome of approximately 7.5 kb. The capsid is composed of 60 identical protomers consisting of three structural proteins (VP1-VP3), with a fourth structural protein (VP4) attached to the inner capsid surface (Plevka et al., 2012). The absence of a lipid envelope provides structural stability, conferring resistance to human gastric acid, organic solvents, and alcohol. The virus also remains active on dry surfaces for several days at room temperature and has been isolated from water sources such as groundwater and hot spas (Ooi and Solomon, 2014).

In 2012, the EV-71 crystal structure was resolved by Plevka et al. (27). This structure provided insight about surface structures such as the canyon region and pocket factor protein (Plevka et al., 2012). The crystal structure of other EV-71 proteins, including the 2C helicase, 2Apro, 3D polymerase, have also been elucidated and provide important information for rational drug design (Chen et al., 2013; Cui et al., 2011; Guan et al., 2017; Mu et al., 2013).

The EV-71 genome contains a single open reading frame flanked by an untranslated regions (UTR). The poly-A tail is located at the end of the 3’ UTR, and the internal ribosome entry site (IRES) is located at the 5’ UTR, adjacent to the 22 amino acid viral protein genome-linked (VPg). The genome is subdivided into three coding regions (P1-P3), with P1 coding for the four structural proteins (VP1-VP4) and P2 and P3 coding for seven nonstructural proteins (2A-2C and 3A-3D, respectively (McMinn, 2012). The VP1 protein contains most of the neutralizing epitopes (Nasri et al., 2007) and interacts with receptors on host cells (Shang et al., 2013). The VP1 gene exhibits high
variability and this variability has been shown to correlate with serotype (Ge et al., 2013; Oberste et al., 1999; Thoelen et al., 2004).

4. Viral Replication

Currently, six receptors have been identified as being used by EV-71: human scavenger receptor class B (hSCARB2), P-selectin glycoprotein ligand-1 (PSGL1), sialylated glycan, heparan sulfate, annexin II (Anx2)(Yamayoshi et al., 2014) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Ren et al., 2014b; Wong et al., 2010). The most studied receptors are hSCARB2 and PSGL-1. SCARB2 is ubiquitously expressed throughout the human body and is involved in intracellular trafficking and lysosome maintenance (Yamayoshi et al., 2013). The hSCARB2 receptor permits infection when expressed in normally unsusceptible cell lines (Yamayoshi et al., 2009) and animal models (Fujii et al., 2013; Lin et al., 2013b). hSCARB2 has also been identified as the uncoating receptor for EV-71, functioning at a pH below 6.0 (Yamayoshi et al., 2014).

PSGL-1 is expressed on leukocytes and T-cells and is involved in leukocyte trafficking (Ley and Kansas, 2004). PSGL-1 expression has also been shown to permit infection in a normally unsusceptible cell line (L929 cells). However, this is not true for all EV-71 isolates (Nishimura and Shimizu, 2009), with an estimated 80% of EV-71 strains unable to bind PSGL-1. In addition, expression of this receptor in transgenic mice failed to permit infection (Liu et al., 2012). This may be due to the inability of PSGL-1 to elicit virus uncoating despite being able to provide virus entry via caveolin-mediated endocytosis (Yamayoshi et al., 2014). Thus, it is believed that the PSGL-1 receptor alone is not enough to permit infection. It has been speculated that PSGL-1 could allow
dissemination of infection by permitting infection of macrophages in the intestinal mucosa or lymphocytes that traffic into the central nervous system (CNS) (Lei et al., 2015; Wong et al., 2010).

A third receptor for EV-71 is dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). While DC-SIGN permits virus attachment and entry, evidence has not shown that EV-71 can infect dendritic cells (Ren et al., 2014b).

Anx2 is a calcium- and phospholipid-binding protein that is expressed on endothelial, neutrophils, monocytes, lymphocytes, and neuronal cells (Hajjar and Acharya, 2000; Yamayoshi et al., 2013; Yang et al., 2011). It is suspected to function as an attachment receptor and therefore is not involved with virus entry or uncoating (Yamayoshi et al., 2014).

Sialylated glycans are cell-surface glycan or glycolipid chains that have an attached sialic acid. They are used for attachment by a variety of microbial pathogens (Varki and Varki, 2007). Given the abundance sialylated glycans in the gastrointestinal tract, it was suspected that sialylated glycans could function as an EV-71 receptor (Yamayoshi et al., 2014). Treatment of DLD-1 cells with sialidase and addition of sialylated glycans from human milk reduced infection (Yang et al., 2009a).

Heparan sulfate is a sulfated polysaccharide found on a variety of cells, including epithelial, endothelial, fibroblasts, and lymphocytes (Sarrazin et al., 2011). Interference with heparan sulfate biosynthesis, as well as the addition of heparan sulfate agonists, reduced EV-71 infection in RD cells (Pourianfar and Grollo, 2015; Tan et al., 2013).
After attachment, the virus enters the cell via endocytosis, with clathrin-mediated endocytosis leading to a productive infection (Lin et al., 2012b). The RNA genome is then released into the cytoplasm, stimulated by a drop in pH. The single-stranded, positive-sense RNA genome can immediately function as the mRNA template for translation. Translation is initiated via an internal ribosome entry site (IRES) element at the 5’ UTR and occurs via a cap-independent mechanism (Tan et al., 2014a), resulting in a single polyprotein 2194 amino acids in length (Kuo and Shih, 2013). The polyprotein is then cleaved by the 2Apro and 3Cpro proteases (Shang et al., 2013), presumably present inside the EV-71 virion.

A number of host factors play a role in viral replication (Shih et al., 2011). A genome-wide study using RNAi revealed 256 host factors involved in EV-71 replication. These factors have a variety of functions, including RNA synthesis and internal ribosome entry site (IRES) regulation (Shih et al., 2011) and are potential antiviral drugs targets (Wu et al., 2016b).

The 2Apro, 3A, and 3Cpro proteins play a role in modulation the host cell environment. Both 2Apro and 3Cpro are responsible for quenching the host cell type I interferon response. The interferon response is crucial to controlling EV71 infection in mice (Liu et al., 2005) and interferon knockout mice have been successfully used in EV-71 animal models (Caine et al., 2013; Khong et al., 2012). The 3Cpro also interferes with mRNA polyadenylation (Weng et al., 2009). The 2A protein shuts down host protein synthesis (Yi et al., 2017). Finally, the 3A protein helps inhibit host cell membrane presentation, which may play a role in immune evasion (Shang et al., 2013).
The positive sense RNA genome is also used to produce a negative sense RNA template for genome replication. Replication is performed by the RNA-dependent-RNA polymerase (protein 3D, or 3D polymerase) (Kuo and Shih, 2013). The 3B, or VPg, protein functions as a primer for replication at the 5’ end (Pathak et al., 2007; Paul et al., 2003). Genome replication occurs in a vesicular replication complex (Kuo and Shih, 2013), with 3A serving as a scaffold protein (Chen et al., 2008b). As the 3D polymerase lacks proofreading activity, the EV-71 genome has a high mutation rate, with an average of one mutation per genome (Santti et al., 1999). In fact, it has been shown that nucleotide substitutions occur at a rate of 4.2-4.6 x 10³ per site per year (Solomon et al., 2010). Finally, the single-stranded, positive sense genome is packaged into the viral capsids and the host cell undergoes apoptosis initiated by the 2A^pro and 3C^pro proteins (Kuo et al., 2002; Li et al., 2002).

EV-71 has been shown to undergo recombination with other EV-71 genogroups, other enteroviruses, other Enterovirus A species, and coxsackievirus A16 (Chang et al., 2016). Recombination occurs with non-structural genes (Chang et al., 2016), and this recombination may contribute to enterovirus variation (Lukashev, 2010).

While a number of cell lines are permissive to EV-71, human rhabdomyosarcoma cells (RD) and African green monkey kidney (Vero) are most often used due to the pronounced cytopathic effect (CPE) when infected with EV-71 (Organization, 2011).

5. Pathogenesis

EV-71 is spread mostly through the fecal-oral route, although respiratory transmission has been suggested (Wong et al., 2010). Based on the pathogenesis of other enteroviruses, it is suspected that EV-71 initially replicates in the tonsils, cervical lymph
nodes, Peyer’s patches, and mesenteric lymph nodes. Further dissemination allows the virus to reach target organs, including the CNS (Ooi and Solomon, 2014). The brainstem is suspected to be the target region in the brain (Chang et al., 2016).

Infection of the central nervous system (CNS) is the most concerning aspect of an EV-71 infection. CNS infection can produce meningitis, encephalitis, and an acute flaccid paralysis (Lee et al., 2016; Ooi and Solomon, 2014), as well as sequelae, such as delayed neurodevelopment and reduce cognitive function (Lee, 2016).

Infection of the brainstem is believed to be a cause of another serious sign: pulmonary edema (Chang et al., 2016). Infection of the vasomotor regions within the brainstem is a possible cause of the neurogenic pulmonary edema seen in some EV-71 cases. Specifically, infection of the medial, ventral, and caudal medullas may lead to sympathetic overactivation causing an increase of blood to the lungs (Chang et al., 2016). Cytokine levels may also be a cause of pulmonary edema, causing vascular permeability and allowing fluid to leak into the surrounding tissue (Chang et al., 2016; Wang et al., 2003). Furthermore, the virus has not been detected in the lungs of EV-71 patients, which suggests the edema is not a result of direct infection of the lungs (Lei et al., 2015).

The route of entry into the CNS is currently under investigation. Two proposed methods are crossing the blood-brain barrier and retrograde axonal transport via motor neurons in the skeletal muscles (Chang et al., 2016). Most evidence has supported the retrograde axonal transport method. For example, Chen et al. demonstrated that treatment of mice with colchicine, a fast axonal transport inhibitor, reduced EV71 invasion of CNS (Chen et al., 2007). They also demonstrated that the spinal cord segment in which the virus was first detected was proximate to the site of infection (either rear or front limb).
An important factor in viral spread via retrograde axonal transport is the evidence of skeletal muscle infection (Ong et al., 2008). The virus has been suspected to ascend via the motor nerves into the spinal cord. Motor nerves are suspected as poliovirus, another neurotropic picornavirus, ascends to the spinal cord via these neurons (Ohka et al., 2009). A number of animal models have shown the presence of EV-71 in skeletal muscle, including murine (Chen et al., 2004; Khong et al., 2012; Lin et al., 2013b; Liu et al., 2012; Liu et al., 2011b; Ong et al., 2008; Wang et al., 2004) and primates (Chumakov et al., 1979). One study by Liou et al., however, did not find any replication in the skeletal muscle (Liou et al., 2016). Furthermore, EV-71 has yet to be observed in human skeletal muscle (Chang et al., 2016).

Genetic indicators for virulence are also under investigation. In polioviruses, the 5’ UTR and VP1 determine virulence (Solomon et al., 2010) and it is suspected that this might hold true for EV-71 as well. Many mutations have been observed from virus passaged in cell culture or research animals, including those in the VP1 (L97R, K98E, G/Q145E, E145A, E145G, V146I, A170V, S241L, K244E)(Caine et al., 2016; Chang et al., 2018a; Chang et al., 2016; Huang et al., 2012a; Huang and Shih, 2014; Huang et al., 2014b; McMinn, 2002; Victorio et al., 2016b), VP2 (K149M, K149I, S144T)(Chua et al., 2008; Huang et al., 2012b), 2C (A103P, L104R, M1223I, H118L)(Wang et al., 2004) and 3Cpro (N68D) regions (Li et al., 2016). Additional genetic changes, including those in the 5’-UTR, have been examined in circulating viruses and in vitro studies (Chang et al., 2018b; Li et al., 2017; Liu et al., 2014; Yee et al., 2017).

However, some studies have found no association between fatal and non-fatal cases and changes in the VP1 and 5’ UTR regions. For example, Shih et al. examined the
VP1 and 5’ UTR of EV-71 isolates from fatal and non-fatal (uncomplicated HFMD) cases and found that all samples (save one exception) were from the B genotype (Shih et al., 2000). McMinn et al. (McMinn et al., 2001) failed to identify any changes in amino acids that were correlated with neurovirulence in genogroup B3 and B4 viruses and Yan et al. found no genetic determinants of virulence when comparing three different strains of EV-71 (Yan et al., 2000).

Host susceptibility to EV-71 may also be due to genetic factors. Studies have shown that genes for human leukocyte antigen (HLA-A33)(Chang et al., 2008), interleukin (IL-10-1082G), and interferon (IFN-γ +874 T/A)(Yang et al., 2012) may be associated with increased EV-71 susceptibility or disease severity. On the other hand, children with IL-17 (F-7488C) were more likely to have a mild EV-71 infection (Lv et al., 2013). HLA-A2 was associated with cardiopulmonary failure in EV71 cases. Interestingly, the HLA-33 gene is present in 17% to 35% in Asian populations, while it is very rare (0 to 1%) in white populations. This may be an explanation for the prevalence of EV-71 outbreaks in the Asia Pacific region as compared to North America (although it does not explain the European outbreaks in the 1970’s and 1980’s)(Chang et al., 2008).

Inflammation is also suspected to play a role in EV-71 infection. Several studies have found elevated cytokines in EV-71 patients and animal models, including interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-13, IL-23, IL-33, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage inflammatory protein (MIP)-1β (Khong et al., 2011; Lin et al., 2002a; Lin et al., 2003; Wang et al., 2003; Wang et al., 2006b; Wang et al., 2007; Wang et al., 2008; Zhang et al., 2013b). This is significant, as cytokine release in the CNS is tightly controlled (Wang et
al., 2007), but not surprising, given that EV-71 infection involves meningitis and encephalitis (Ooi and Solomon, 2014).

Furthermore, some studies suggest that elevated cytokines may contribute to the disease pathology (Wang et al., 2006b; Yu et al., 2014; Zhang et al., 2013b) and can predict the outcome of disease (Wang et al., 2006b; Zhang et al., 2013b). Khong et al. found that treating EV-71 infected mice with anti-IL-6 antibodies improved survival and clinical score (Khong et al., 2011). Wang et al. found that patients with higher levels of IL-1β, IL-6, and IFN-γ in CSF experienced more severe brainstem encephalitis (Wang et al., 2007). Lin et al. reported evidence of a cytokine storm in certain patients (Lin et al., 2002a; Lin et al., 2003) and that IL-6 level is correlated with clinical severity. Wang et al. found that the ratio of CSF to plasma levels of monocyte chemoattractant protein (MCP)-1 and IL-8 were correlated with disease severity and were highest in patients with brainstem encephalitis without pulmonary edema (Wang et al., 2008). Zhang et al. found that plasma levels of GM-CSF, IL-2, and MIP-1β were higher in patients with neurological damage and that G-CSF, IL-8, MCP-1, IL-10, and IL-6 were higher in CSF tissues for patients with neurological signs (Zhang et al., 2013b).

6. Clinical Presentation and Patient Outcomes

The incubation period of EV-71 is about three to five days (Linsuwanon et al., 2014). EV-71 causes a variety of clinical manifestations, including encephalitis, aseptic meningitis, pulmonary edema, acute flaccid (poliomyelitis-like) paralysis, herpangina, and hand, foot, and mouth disease (HFMD). HFMD is characterized by a brief fever, a papulovesicular rash on the hands and feet, and oral ulcers (McMinn, 2002; Organization, 2011). EV-71 also causes herpangina, which is characterized by fever and oral ulcers
Virus shedding after recovery can be prolonged. Shedding in the throat up to two weeks post-recovery and in the stool up to 11 weeks post-recovery (Ooi and Solomon, 2014).

Neurological symptoms include brainstem encephalitis, aseptic meningitis, myoclonic jerks and an acute flaccid (poliomyelitis-like) paralysis (Cox et al., 2017; Ooi et al., 2010; Zhang et al., 2011). Based on data from recent Asian outbreaks, 10-30% of hospitalized patients with HFMD will develop neurological signs (Organization, 2011). Patients with severe and complicated disease have fatality rates as high as 25.7% (Wong et al., 2010). Pulmonary symptoms are an especially serious clinical manifestation and include pulmonary edema, pulmonary hemorrhage, and myocarditis. Pulmonary edema or hemorrhaging usually occurs after three to five days of fever (Ooi and Solomon, 2014) and requires immediate medical intervention (Wang et al., 2006a). It can be up to 80-90% fatal for children who develop cardiopulmonary failure (Tu et al., 2015; Wang et al., 2014a), although another reports suggests it can be much lower (30-40%) (Lee, 2016).

Detection of EV-71 in patients can be difficult. Detection at sterile sites such as CSF, serum, and vesicular fluid is often inconsistent and produces false negatives. In fact, the virus is only isolated from CSF in 0-5% of patients with neurological symptoms (Ooi and Solomon, 2014). Throat and rectal swabs can be more reliable (~50%). However, one study found that 10% of throat swabs and 20% of rectal swabs produced a different virus than the one implicated in the infection (Ooi et al., 2007). PCR is the most rapid method of detection, with VP1 capsid being the region commonly used for identification.

Given the seriousness of EV-71 infection in young children, transmission of EV-71 during pregnancy or soon after birth is a concern. However, there is only one
documented case of *in utero* EV-71 transmission to date (Chow et al., 2000), with fetus delivered stillborn. On the other hand, a case of perinatal transmission of EV-71 has been reported, with the child recovering without neurological sequelae (Nishikii et al., 2002). Prenatal transmission of other enteroviruses has been documented (Abzug, 2014). In addition, several studies have suggested a protective effect from maternal antibodies (Ding et al., 2015; Fu et al., 2016; Wong et al., 2010).

**7. Animal Models**

Early animal models for EV-71 used non-human primates, namely rhesus macaques and cynomolgus monkeys. Non-human primates are advantageous because their physiology is very similar to humans and they are often susceptible to EV-71 infection without virus passaging. Non-human primate models can exhibit a variety of clinical manifestations including HFMD, pulmonary edema, and flaccid paralysis (Arita et al., 2005; Chumakov et al., 1979; Hashimoto et al., 1978; Nagata et al., 2002). A variety of methods have been used to challenge non-human primates with EV-71, including intravenous, intraspinal, aerosolization, and oral infection (Chen et al., 2012; Hashimoto and Hagiwara, 1982; Nagata et al., 2004; Nagata et al., 2002). In addition to studying pathogenesis, non-human primates have also been used to test EV-71 vaccines, both with (Chen et al., 2012) and without (Dong et al., 2011) virus challenge. However, non-human primate models are expensive, pose ethical challenges (Wang and Yu, 2014), and are subject to rigorous guidelines for animal welfare and scientific merit (Health, 2015). What’s more, important clinical signs such as encephalomyelitis and respiratory failure are absent in these models (Wang and Yu, 2014).
Several EV-71 models have been developed using mice. As mice are not the natural hosts for EV-71, either adaptation of the virus to the mouse host or transgenic alteration of the mice is often required to produce infection (Solomon et al., 2010). Several transgenic models with hSCARB2 (Fujii et al., 2013; Lin et al., 2013b; Liou et al., 2016) or PSGL-1 (Liu et al., 2012) have been developed. Transgenic hSCARB2 models showed elevated cytokines or inflammation in CNS tissue (Fujii et al., 2013; Lin et al., 2013b; Liou et al., 2016). Only one of these models, however, was fatal to mice older than two weeks (Fujii et al., 2013). What’s more, these models failed to produce important disease signs such as rear limb paralysis. This may be due to the fact that the hSCARB2 receptor, while permissive for infection, is only one of several currently known EV-71 receptors (Wang and Yu, 2014). As such, relying on a single transgenic receptor may overlook other factors that are crucial to infection.

In a transgenic PSGL-1 model produced by Liu et al., 10-day-old C57BL/6J mice were infected with $10^8$ of 50% tissue culture infectious dose (TCID$_{50}$) of mouse-passaged (MP10) virus. Expression of PSGL-1 in brain and leg muscle tissues failed to enhance infectivity with EV-71. A mouse-adapted strain (MP10) was able to produce real-limb paralysis. However, this infection was no more severe in the PSGL-1 transgenic mice than in wild-type mice. Furthermore, mice older than two weeks did not exhibit severe clinical signs. Thus, the authors concluded that PSGL-1 alone is not sufficient to produce a severe infection. This may be due to the fact that PSGL-1 was not expressed in the spinal cord of these mice (Liu et al., 2012).

Several mouse adaptation models have been produced (Arita et al., 2008a; Caine et al., 2013; Chen et al., 2004; Ong et al., 2008; Wang et al., 2011; Wang et al., 2004).
Each of these models will be discussed briefly. Wang et al. produced a model in one-day- and one-week-old ICR mice with inoculation via the i.p. and oral routes (respectively). Four EV-71 isolates were used in the model. Virus (EV71/Tainan/4643/98) was passaged four times (MP4) in one-day-old ICR mice. MP4 produced mortality in one-day-old mice at $10^3$ of plaque forming units (PFU) per mouse (100% mortality) and in one-week-old mice at $5 \times 10^6$ (80% mortality) PFU/mouse. MP4, but not the parental strain, was able to produce disease in one-week-old mice. Virus was detected in a variety of tissues, including CNS and skeletal muscle. Mice also exhibited rear-limb paralysis and weight loss (Wang et al., 2004).

A model by Caine et al. used an MP10 virus (B2 isolate; MS/7423/87) to produce a lethal infection in six-week-old mice. Mice were infected with $1.3 \times 10^5$ TCID$_{50}$/mouse via i.p. Disease signs included paralysis, weight loss, loss of balance, and eye irritation. The authors found that a VP1 (K244E) mutation conferred virulence to the mouse model (Caine et al., 2016). However, a time course of infection and a cytokine profile were not assessed in this model. While this model was used for vaccine testing, it has yet to be used to evaluate therapeutics (Caine et al., 2013).

Chen et al. produced an oral infection model in one-day-old mice. One-day-old ICR mice were intragastrically infected with $10^7$ PFU/mouse of EV71 4643 (passage number not listed). At this dose, the virus was completely lethal. Mice exhibited skin lesions and rear limb paralysis. Intraperitoneal inoculation with the same virus produced similar mortality. The mouse-passaged virus was shown to have 99% homology with the parent virus (Chen et al., 2004).
Ong et al. examined several routes of inoculation (intramuscular (i.m.), intraperitoneal (i.p.), subcutaneous (s.c.), intracranial (i.c.), and per os (p.o.)) at $10^5$ of 50% cell culture infectious dose (CCID$_{50}$). Two-week-old Institute of Cancer Research (ICR) mice were inoculated via the i.m. route exhibited paralysis on average one day earlier than those challenged via i.c., i.p., or s.c. Only 10% of the mice of the orally infected mice developed paralysis. Interestingly, the lower spinal cord initially showed higher viral titers than the upper spinal cord, suggesting that EV-71 progresses up the spinal cord to the brain. Viral RNA and virions were also observed in nervous tissue (Ong et al., 2008).

Wang et al. produced a mouse-adapted model in immunodeficient mice. Virus (EV-71 Fuyang-0805) was passaged four times in one-day-old ICR mice. One-day-old ICR mice were infected with $10^5$ CCID$_{50}$/mouse of MP5 virus via the i.p. route. Mice failed to exhibit neurological signs or mortality, although they experienced slower weight gain than control mice. However, histological analysis of limb muscle showed massive necrotizing myositis (Wang et al., 2011). Arita et al. used three- to four-week-old Nonobese-Diabetic-Severe Combined Immunodeficiency (NOD/SCID) mice, which lack T, B, and NK cells. Virus (Fuyang-0805) was passaged four times in one-day-old ICR mice, using necropsied leg muscle. An amino acid change in the VP1 gene (E145Q) was identified as crucial for adaptation. Mice exhibited a prolonged infection (>1 month), with no observed mortality. Virus was detected in a variety of tissues including heart, leg muscle, and CNS. Mice also developed paralysis, though a later onset (two to four weeks) than in other models. However, infection was performed via an invasive route (i.c.)(Arita et al., 2008a).
Chua et al. passaged EV-71 (26M/AUS/4/99) six times in Chinese Hamster Ovary (CHO) cells and then in one-day-old BALB/c mice. One-week-old BALB/c mice were infected with $10^4$ TCID$_{50}$/mouse of MP6 virus via i.m. Clinical signs included hunching, and fore- and hindlimb paralysis (Chua et al., 2008). The authors reported two amino acids changes in the VP2 (K149O), VP1 (G145E), and 2C (K216R) regions, although only the VP1 mutation contributed to virulence in mice.

Xiu et al. used an MP10 C4 virus to produce paralysis and complete mortality in ten-day-old ICR mice ($5 \times 10^7$ TCID$_{50}$; i.p.). The authors observed sever restrictive hypoventilation and lower blood-oxygen concentration. Interestingly, the authors found that i.c. infection did not produce typical symptoms, although it was not mentioned what symptoms were noticed in these mice (Xiu et al., 2013).

A few models have used non-adapted EV-71 strains in non-transgenic mice (Khong et al., 2012; Yu et al., 2017). Khong et al. infected AG129 mice via the i.p. and p.o. routes. Only the mice infected i.p. exhibited complete mortality ($10^7$ PFU/mouse). Mice exhibited rear limb weakness and paralysis. However, the model required that mice less than two-weeks-old be used for infection (Khong et al., 2012). Yu et al. developed a model using nine-day-old BABL/c mice. Infections were only partially lethal (30%). The authors did observe elevated serum chemokine levels, including MCP-1 and regulated on activation, normal T-cell expressed and secreted RANTES (Yu et al., 2017). Qin et al. developed a model in 8- to 10-week-old female ICR mice ($10^7$ PFU/mouse) from a C4 clinical isolate. Mice were challenged twice, first via hydrodynamic injection into the tail vein and second via i.p. two weeks later. This model is 80% lethal when both challenges are administered. Surviving mice produced on average, lower litter sizes compared to
controls. However, pups from previously challenged mice had higher survival rates when challenged with EV-71, likely due to the presence of anti-EV-71 antibodies detected in their blood (Qin et al., 2017). Lin et al. produced a model in five-day-old BALB/c mice, with mice receiving $10^{6.6}$ TCID$_{50}$/mouse of virus over three separate challenges. Infection produced paralysis as well as skeletal muscle pathology, although the authors did not report on lethality (Lin et al., 2015). Xu et al. used a C4 clinical isolate to infect 14-day-old BALB/c mice ($2.34 \times 10^6$ PFU/mouse; i.p) that was 100% lethal. Mice that received EV-71A antiserum (rabbit) were completely protected from mortality (Xu et al., 2017).

One study used virus that had been passaged exclusively in a cell line. Victorio et al. used virus that had been passaged 100 times in NIH/3T3 cells. Six-day-old BALB/c mice were infected via the i.p. or i.m. routes. Mice exhibited less than 20% survival (depending on the route of infection) and exhibited paralysis, ataxia, tremors, and encephalomyelitis, as well as focal hemorrhage in the lungs (Victorio et al., 2016a).

One gerbil model has been produced. EV-71 (strain 58301 genotype C4) was completely lethal to three-week-old gerbils ($10^5$ TCID$_{50}$; i.p.). Gerbils exhibited hind limb paralysis. This model was used to evaluate potential EV-71 vaccines (Yao et al., 2012).

A non-adapted, gnotobiotic pig model in five-day-old Large White pigs (EV-71 strain BJ110, genogroup C4) has been produced. Pigs are infected via the oral and combined oral-intranasal routes with $5 \times 10^8$ fluorescence forming units (FFU) per animal. Pigs exhibited several clinical signs including fever, lethargy, limb weakness, myoclonic jerks, occasional HFMD, and pulmonary edema/hemorrhage. However, no limb paralysis or mortality was observed. Virus shedding in the feces can occur up to 18
d.p.i., with titers up to $2.22 \times 10^8$ RNA copies/ml. The infection was not lethal. (Yang et al., 2014).

**8. Antivirals and Therapeutics**

There are currently no approved antiviral treatments for EV-71 (Song et al., 2018). This has been hampered in part by the need for animal models to evaluate drugs (Huang and Shih, 2014; Kuo and Shih, 2013; Tan et al., 2014a). A review of potential EV-71 antivirals is reported here. As there are multiple mechanisms of action for potential EV-71 antivirals (Pourianfar and Grollo, 2015), antivirals are categorized by mechanism of action.

**8.1. Attachment Inhibitors**

**8.1.1. Heparin and Heparan Sulfate**

Heparin is a negatively charged linear polysaccharide and has been shown to be effective *in vitro* against another member of the picornaviridae family, echoviruses (Pourianfar et al., 2012). Heparin altered the expression of 14 host cells genes whose expression was altered by EV-71 infection (Pourianfar et al., 2014). Heparin was also able to reduce viral titer in RD cells by 80% when used at 1000 µg/ml (Tan et al., 2013) with a half-maximal inhibitory concentration (IC$_{50}$) of 205 µg/ml and an selectivity index (SI) of 236 (Pourianfar et al., 2012). Heparan sulfates are sulfated polysaccharides found on a variety of cells (Sarrazin et al., 2011) and a heparan sulfate glycosaminoglycan is an attachment receptor for EV-71 (Tan et al., 2013). Heparan sulfate limits EV-71 infection *in vitro* (Pourianfar et al., 2012; Tan et al., 2013), with an IC$_{50}$ of 205 and SI of 236.25 (µg/ml) in Vero cells. Interestingly, desulfated heparin was not able to inhibit EV-71 infection (Pourianfar et al., 2012).
8.1.2. Suramin and Suramin Analogs

Suramin is a polysulfated and polyanionic naphthylurea (Gritli-Linde et al., 1994; Kaur et al., 2002) developed in 1916 (Kaur et al., 2002). It is a clinically approved antiparasitic used to treat African sleeping sickness (Steverding, 2010) and river blindness (onchocerciasis)(Schulz-Key et al., 1985). It has also shown effectiveness as an antitumor agent (Bhargava et al., 2007; Stein et al., 1989), a palliative treatment for prostate cancer patients (Ahles et al., 2004), and a potential treatment for autism (Naviaux et al., 2017). It is on the World Health Organization’s List of Essential Medicines (Organization, 2017). It has been shown to block EV-71 attachment and late stage EV-71 infection including replication and transcription (Wang et al., 2014b).

Suramin has shown effectiveness against EV-71 in vitro (IC$_{50}$: 6.87-64 µM), with a high selectively index in RD cells (18.20-12,500) (Nishimura et al., 2015; Ren et al., 2014a; Tan et al., 2013; Wang et al., 2014b). Suramin has also been evaluated against several EV-71 VP1 mutants, with VP145G, VP1145Q, and the wild type strain Fuyang 573 showing higher sensitivity than 145E or the wild-type SHI12-276 strain(Ren et al., 2017).

However, suramin protected only 30% of adult rhesus macaque monkeys receiving 50 mg/kg/d of suramin for seven days (Ren et al., 2014a). Suramin can also produce a variety of side-effects, including anemia, hypocalcemia, neutropenia, and renal complications)(Kaur et al., 2002). However, some evidence suggests toxicity may be more pronounced in patients with parasitic infection (Ren et al., 2014a).

8.1.3. Bovine, Human, and Porcine Lactoferrin

Lactoferrin is an iron-binding protein found in milk with bacteriostatic, bactericidal, and antiviral activity (Sanchez et al., 1992; Weng et al., 2005). Lactoferrin from several
species has been tested \textit{in vitro}, including human (IC$_{50}$: 103.3-185.0 µg/ml) and bovine (IC$_{50}$: 10.5-24.5 µg/ml) lactoferrin (Lin et al., 2002b). It is suggested that the activity of lactoferrin may be due to binding of heparan sulfate as well as binding to VP1 capsid protein (Weng et al., 2005). Newborn BALB/c mice receiving porcine lactoferrin in milk exhibited increased weight gain compared to mice that did not receive porcine lactoferrin. Mice receiving porcine lactoferrin were also protected from mortality, although it should be noted this model is only 30% lethal (Chen et al., 2008a).

8.2. \textit{Uncoating Inhibitors}

8.2.1. \textit{Pleconaril}

Pleconaril is a pocket-binding drug that was developed specifically to treat picornavirus infections (Pevear et al., 1999; Zhang et al., 2012). It was effective in two clinical trials for rhinovirus infection (Hayden et al., 2003) but was rejected by the FDA in 2002 due to concerns about safety and resistance (Senior). Pleconaril has showed effectiveness \textit{in vitro} (EC$_{50}$: 0.13-0.54 µg/ml; SI: 21.73-82.86)(Zhang et al., 2012), although Smee et al. and Tijesma et al. observed contradicting results (EC$_{50}$: >11 µg/ml; SI: 0)(Smee et al., 2016; Tijesma et al., 2014). \textit{In vivo}, pleconaril protected 80% of one-day-old ICR mice infected with EV-71 (Zhang et al., 2012). However, pleconaril was unprotective in cell culture against the EV-71 isolate from the Taiwan 1998 outbreak (Shia et al., 2002).

8.2.2. \textit{Pirodavir}

Pirodavir is a capsid-binding phenoxy-pyridazinamine designed to be a broad spectrum anti-picornavirus drug (Andries et al., 1992). Pirodavir previously entered clinical trials for rhinovirus infection but failed to improve clinical signs and may
increase frequency of blood in nasal mucus (Lacroix et al., 2015). For EV-71 infections, pirodavir has shown activity in vitro (EC₅₀: 0.78-0.361 µM, SI: 24)(Smee et al., 2016; Tijms et al., 2014). However, in vivo testing for EV-71 infection has yet to be reported.

8.3. IRES Inhibitors

EV-71 replication involves IRES-mediated translation, which employs RNA secondary structures as a means of circumventing the use of certain initiation factors. Although first discovered in picornaviruses, IRES-mediated translated can also be employed by eukaryotic cells, such as when cap-dependent translation is impaired (Chen et al., 2008c). Nevertheless, given the dependence of viruses like EV-71 on IRES-mediated translation, IRES inhibitors have emerged as a promising class of antiviral drugs.

8.3.1. Quinacrine

Quinacrine has been used since the 1930s to treat malaria, giardiasis, and tapeworm infections. It has also shown to suppress translation of poliovirus. It targets the IRES and prevents binding of polypyrimidine-tract binding protein (PTB), a necessary cellular factor needed for translation. Quinacrine has shown effectiveness in vitro for EV-71 infection (IC₅₀: 9.71 µM/ml)(Wang et al., 2013).

8.3.2. Amantadine

Amantadine is an antiviral previously used to treat influenza A infections and currently used to treat Parkinson’s disease (Benschop et al., 2015). In vitro, it was able to inhibit cap-independent (IRES-mediated) translation of EV-71 without inhibiting cap-dependent translation (80% suppression of replication at 0.25 mg/ml)(Chen et al., 2008c).

8.3.3. Rupintrivir
Rupintrivir is a 3C\textsuperscript{pro} inhibitor originally developed to treat rhinovirus infections and reached phase II clinical trials before being discontinued (Baggen et al., 2018). Rupintrivir has shown effectiveness \textit{in vitro} (IC\textsubscript{50}: 0.14-7.3 \textmu M, EC\textsubscript{50}: 18 nM, SI: 7450)(Lu et al., 2011; Smee et al., 2016; Tan et al., 2016; Zhang et al., 2013a). In a two-day-old suckling mouse model (ICR mice), rupintrivir protected 90% of mice at a dose of 0.1 mg/kg/d, administered for 10 days (in a 40% lethal model (Zhang et al., 2013a). Rupintrivir is also synergistic in combination with ribavirin or interferon (Hung et al., 2011). Modifications to rupintrivir, including at the P2-P4 sites, improve effectiveness (Ang et al., 2016; Lu et al., 2011; Wu et al., 2016a; Zeng et al., 2016).

8.4. Replication Inhibitors

8.4.1. Aurintricarboxylic acid (ATA)

Aurintricarboxylic acid (ATA) has shown activity against HIV, vesicular stomatitis virus, SARS, influenza and HCV (Hung et al., 2010). It has also shown effectiveness against EV-71 \textit{in vitro} (EC\textsubscript{50}: 2.9 \textmu M, SI: 25.1) in Vero cells. It is suspected that ATA interferes with the 3D polymerase (Hung et al., 2010).

8.4.2. Ribavirin

Ribavirin is a broad-spectrum nucleoside analog that has been in clinical use since 1986 (Ohmit et al., 1996). It is effective against hepatitis C, respiratory syncytial virus, coxsackievirus, and echovirus. Ribavirin is effective \textit{in vitro} (IC\textsubscript{50}: 65-178.42 \textmu g/ml, SI: 21.7-82.9) (Lin et al., 2012a; Zhang et al., 2012). Ribavirin (100 mg/kg/d for nine days) also protected 70% (7/10) 12- to 14-day-old ICR mice from mortality, compared with 27% (3/11) in the placebo group (Li et al., 2008), although it was unprotective in one-day-old mice. It also reduced viral titers in the brain, brainstem, and spinal cord by at
least 2-fold. However, Zhang et al. showed that ribavirin failed to protect one-day-old mice administered 80 mg/kg/d of Ribavirin for 5 days (0% survival) (Zhang et al., 2012).

8.4.3. 17-AAG

17-allylamino-17-demethoxygeldanamycin (17-AAG) is a heat shock protein 90 (HSP90) inhibitor and an analog of geldanamycin. HSP90 is suspected to play a role in virus entry and replication via acting as a chaperone for capsid proteins. Down-regulation HSP90 has prevented viral infection in vitro, including reducing stability of the viral capsid. 17-AAG is touted as less toxic HSP90 inhibitor than geldanamycin. Treatment of an EV-71 infection in seven-day-old ICR and BALB/c mice protected 100% mice against two different viruses strain (C2 and C4) at 0.5 and 2 µg administered ab both 4 and 24h post-infection, respectively. Administration of 10 µg prevented rear limb paralysis against the same viruses, although this was not the case for EV-71 B4 virus (Tsou et al., 2013).

8.4.4. N6-Benzyladenosine

N6-benzyladenosine is a nucleoside analog. It has shown varying effectiveness in vitro against the B1 genogroup (IC50: 0.10 µM, SI: 33,000) (Arita et al., 2008b) and A (EC50: 4.3 µM, SI:15) (Oslovsky et al., 2017). It is suspected that N6-benzyladenosine interacts with the 2Cpro or 2BC precursor. However, resistance developed after 3 passages, with amino acids changes in the 2C gene (Arita et al., 2008b). Olvosky et al. found that fluorination and methylation of N6-benzyladenosine improved EC50 and SI values up to 4- and 240-fold, respectively (EC50: 0.068-1.0 µM, SI: 44-3456) (Oslovsky et al., 2017). Several analogs of N6-benzyladenosine have shown promise as antivirals as
well (Drenichev et al., 2016). Other N6-adenosine compounds are also effective in vitro (Tararov et al., 2015).

8.4.5. Guanidine

Guanidine is an organic base found in urine (Lipman, 1993) and is used to treat Eaton Lambert syndrome (Lindquist and Stangel, 2011). It is has been shown to inhibit poliovirus replication in vitro (Jiang et al., 2014). Guanidine inhibits the picornavirus 2Cpro, has been tested in vitro against EV-71 (EC50: 305 µM, SI: 12)(Smee et al., 2016). However, resistance mutations in the 2C gene were determined after six passages in RD cells (Meng and Kwang, 2014; Sadeghipour et al., 2012).

8.4.6. Chloroquine

In RD and PSGL-1-expressing L929 cells, chloroquine reduces viral RNA more than 70% at 100 µM dosing (Lin et al., 2013a). Chloroquine functions as an uncoating inhibitor and reduced viral RNA by more than 10,000-fold in vitro at 1.2 mM (Shih et al., 2008). Chloroquine is an FDA approved anti-malarial drug. In vitro, chloroquine reduced viral titers by about one log when added to cells at 12h p.i. (250 µM). Treatment with 10 mg/kg of chloroquine at 2h pre-infection and daily for 6 days p.i. protected 65% of six-day-old BALB/c mice infected with EV-71 (compared with 20% in the untreated control group) and reduced damaged to leg muscle tissue (Tan et al., 2018).

8.5. Host Response Modifiers

8.5.1. Gemcitabine

Gemcitabine is a pyrimidine analog and possible 3D polymerase inhibitor. It is currently approved as an anti-cancer drug. It has shown effectiveness in vitro (EC50: 1
μM) and is synergistic with ribavirin (Kang et al., 2015), although it can be fairly toxic when used in animal studies (Cividalli et al., 2000).

8.6. Other (Unclassified)

8.6.1. Lycorine

Lycorine is an alkaloid that has antiviral activity against poliovirus and coxsackie virus. It is effective in vitro (IC$_{50}$: 0.48 μg/ml; SI: 101) and was found to inhibit viral replication. Lycorine protected 50% of 10- to 11-day-old ICR mice from mortality and paralysis when administered at 1.0 mg/kg for 7 days (Liu et al., 2011a). A lycorine derivative (1-cetyllycorine) was also effective in vitro (EC$_{50}$: 0.50-1.13 μM) against three strains of EV-71 (BrCR, SK-EV006, and Fuyang) (Guo et al., 2016).

8.6.2. 2-(Alpha-Hydroxybenzyl)-benzimidazole (HBB)

HBB is a compound that sparked interest as a potential antiviral in the 1960’s and 1970’s. HBB has shown activity against poliovirus, coxsackievirus B, and select echovirus and coxsackievirus A viruses (De Palma et al., 2008). HBB was found to have a significant synergistic effect when combined with guanidine (1:10) in a neonate mouse model infected with Echoviurs 9 and Coxsackie A Type 9, reducing mortality and paralysis (Eggers, 1976). While HBB is not currently available, HBB analogues, such as 2-(2-Pyridyl)benzimidazole and 5-Hydroxymebendazol are available.

8.6.3. Flouxetine

Flouxetine is a selective seratonin reuptake inhibitor used to treat depression. Flouxetine inhibits coxackievius B3 (EC$_{50}$: 2.3-3.36 μM; SI: 8.3-10.9) (Ulferts et al., 2013; Zuo et al., 2012), as well as enteroviruses D68 (EC$_{50}$: 1.35 μM), enterovirus 70 (EC$_{50}$: 0.81 μM), and echoviruses 1, 9, and 11 (EC$_{50}$: 3.93-5.76 μM) Interestingly, three
other selective serotonin reuptake inhibitors (citalopran, paroxetine, and sertaline) were unable to inhibit coxsackievirus B3 (Ulferts et al., 2013).

8.7. Therapeutics

8.7.1. Intravenous Immunoglobulin

Intravenous immunoglobulin (IVIG) is a therapeutic pool of antibodies from the plasma of 1,000 to 10,000 donors (Prasad and Chaudhary, 2014). It is currently approved by the FDA to treat several conditions, including primary immunodeficiency, idiopathic thrombocytopenic purpura, and HIV infection (Orange et al., 2006). However, IVIG has also been used off-label to treat viral infections, such as Japanese encephalitis, West Nile, Varicella, parvovirus B19, and coxsackievirus (Wang et al., 2006b). It has also been effective at treating chronic enteroviral meningoencephalitis in agammaglobulinemic patients (Orange et al., 2006). Furthermore, the passive transfer of maternal antibody can be protective against EV-71 as well (Wong et al., 2010). This establishes the precedence for using IVIG as a treatment for EV-71 infection.

Clinical evidence regarding IVIG treatment for an EV-71 infection is almost exclusively limited to case reports from EV-71 outbreaks. Several reports have shown a benefit for treating with IVIG. For example, during the EV-71 epidemic in Sarawak (1998), the majority (204/215) of children who survived severe CNS complications received timely IVIG treatment (typically once severe disease occurred), whereas only one of nine fatal cases received this treatment (Ooi and Solomon, 2014). A study of patients treated in the 1998 Taiwan outbreak found that 8 of 14 patients administered IVIG survived infection (Wang et al., 1999). One study randomized the administration of IVIG to neonates (750 mg/kg) and noticed an increase in serum neutralizing antibodies,
but no effect on EV-71 in the blood and urine (Abzug, 1995). Five of eight patients with EV-71 related cardiopulmonary failure survived when treated with IVIG (Jan et al., 2010). Lee et al. report that 12 of 13 patients in Korea with EV-71 neurologic signs (2009-2012) who were treated with IVIG (in combination with milrinone or acyclovir in certain cases) survived infections (Lee et al., 2014).

However, a few reports suggest that IVIG may not be effective in treating EV-71 infections. McMinn et al. treated four patients with IVIG without any significant benefit, despite the IVIG containing low EV-71 neutralizing antibody titers (McMinn et al., 2001). Another report observed that even when early IVIG treatment was given to patients (before the onset of pulmonary edema (PE) or pulmonary hemorrhage (PH)), the patients still experienced severe disease (Wang et al., 2006a). Nolan et al. reported that IVIG (2g/kg, over 2 days) failed to lower the viral titers in six patients with EV-71-induced pulmonary edema (Nolan et al., 2003). Bhatt et al. found that 400 mg/kg/d IVIG for five days improved cardiac function in children with myocarditis and encephalitis, but only slightly improved mortality rates (Bhatt et al., 2012). Chong et al. found that IVIG has no effect on two pediatric patients with EV-71, with one patient experiencing a mild adverse reaction (Chong et al., 2003). Finally, it should be noted that some studies reported the use of IVIG to treat EV-71 patients, but it is unclear how many patients benefited from the treatment (Khanh et al., 2012; Lin et al., 2003).

Evidence also suggests that IVIG can cause an antibody-dependent enhancement (ADE) of disease. Han et al. observed that pre-incubating EV-71 sub-neutralizing amounts of IVIG increased the mortality of one-day-old infected mice (Han et al., 2011). Wang et al. found that sub-neutralizing concentrations of IVIG enhanced infectivity of
human monocytes (Wang et al., 2010). Cao et al. suggested that IgG3 may be responsible for the ADE effect, as it has little neutralizing capability against EV-71 (Cao et al., 2013).

Few animal studies have examined the effectiveness of IVIG for treatment of EV-71 infections. One such study found that high concentrations of IVIG could provide complete protection to one-day-old mice infected with EV-71 (Han et al., 2011).

Several studies have examined the effect of IVIG on cytokine levels. One study showed that IVIG reduced the plasma concentration of IL-6, IL-10, IL-13, and IFN-γ (Wang et al., 2006b). This is not unexpected, given the anti-inflammatory properties of IVIG (Nimmerjahn and Ravetch, 2007). IVIG is used to treat inflammatory conditions such as chronic inflammatory demyelinating polyneuropathy, Guillain-Barre syndrome and Kawasaki disease (Anthony et al., 2008). It is suspected that IVIG can bind cytokines (Wang et al., 2006b) or cytokine receptors (Gelfand, 2012). Another study found that IVIG produced no significant change in serum neutralizing antibody in recovered patients. The authors suggest that the effect was due to a reduction of pro-inflammatory cytokines mediated by IVIG, although no evidence for this was provided.

Several *in vitro* studies have confirmed the presence of EV-71 neutralizing antibodies in IVIG products (Cao et al., 2010; Cao et al., 2011; Wu et al., 2013). One study found a relatively high-titer of neutralizing antibodies in a lot of IVIG produced from Chinese donors (Cao et al., 2010). This raises the question about the use of IVIG produced from donor populations where EV-71 is endemic, and if such lots of IVIG have higher EV-71 neutralizing antibody titers and are clinically more effective than lots produced from non-endemic donor pools. More research is needed to address this
question. However, a precedence for this idea has been shown in the production of IVIG formulated to treat West Nile virus from selected Israeli donors (Cao et al., 2011).

Evidence also suggests that IVIG may be effective at neutralizing some, but not all, circulating EV-71 genogroups. Wu et al. found that an IVIG lot produced from Taiwanese donors was able to neutralize B4 and C2 subgenogroups, but not the other subgenogroups. They suggest this may be due to early incidence of B4 and C2 EV-71 infections after Taiwan began experiencing EV-71 epidemics (1998)(Wu et al., 2013).

As IVIG is derived from a donor pool, the immunoglobulin composition may vary between lots and brands. Evidence for this comes from a study that found significant differences in the streptococal superantigen neutralizing activity between lots (Schrage et al., 2006).

Even though the evidence regarding IVIG use for an EV-71 infection is inconclusive, Taiwan, China, and Vietnam nevertheless recommend IVIG as treatment for an EV-71 infection (Ooi and Solomon, 2014). Specifically, Taiwan recommends 1 g/day of IVIG for two days (Huang et al., 2003). China recommends a high dose of IVIG (2 g/kg), administered over 2-5 days in cases of severe EV-71 infection (Han et al., 2011).

9. Vaccine

There are currently two approved EV-71 vaccines. Both are inactivated whole virus alum-adjuvant vaccines, and both are only approved by the Chinese Food and Drug Administration. The first was produced by the Institute of Medical Biology at the Chinese Academy of Medical Sciences and approved in 2015. The second was produced by Sinovac Biotech, and approved in January 2016 (Yi et al., 2017). The Sinovac Biotech
vaccine showed 94.8% protection from HFMD over a two-year trial with over 5,000 children aged 6 to 35 months. There were no cases of neurologic disease in the vaccine-treated group, with eight in the placebo group (Zhu et al., 2014).
CHAPTER II
STATEMENT OF THE PROBLEM

Enterovirus 71 (EV-71) is a non-enveloped, single-stranded RNA virus in the Picornaviridae family. Discovered in 1969 in California, the virus has since spread to the European and Asia-Pacific region and caused large outbreaks in Taiwan (1998) and China (2008)(Ho et al., 1999; Organization, 2011). EV-71 mainly causes serious disease in young children, and includes presentation of encephalitis, meningitis, pulmonary edema, and acute flaccid paralysis (Ooi and Solomon, 2014). It can be up to 26% lethal in children with severe and complicated disease (Wong et al., 2010). However, even children who survive a serious infection may suffer from delayed neurodevelopment and limb atrophy (Lee, 2016).

Despite the seriousness of EV-71, there are no approved antiviral therapies (Song et al., 2018). Physicians rely on supportive care and off-label use of a therapeutic antibody pool, intravenous immunoglobulin (IVIG)(Ooi and Solomon, 2014). The lack of EV-71 therapeutics is due in part to a lack of effective animal models for EV-71 (Fujii et al., 2013; Khong et al., 2012; Wang and Yu, 2014). While several animal models for EV-71 have been developed, each has limitations for evaluating therapeutics. Non-human primate models are expensive, pose ethical challenges, and cannot produce encephalomyelitis, an important disease sign seen in humans (Wang and Yu, 2014).

Several transgenic mouse models for EV-71 have been produced (Fujii et al., 2013; Lin et al., 2013b; Liou et al., 2016; Liu et al., 2012). However, only one such model produced rear limb paralysis, a crucial disease sign for EV-71, and only after an invasive intracerebral route of infection (Fujii et al., 2013).
Several models using mouse-adapted virus have been developed (Arita et al., 2008a; Caine et al., 2013; Chen et al., 2004; Ong et al., 2008; Wang et al., 2011; Wang et al., 2004). However, these models often require the use of mice that are two-weeks-old or younger at the time of infection (Chen et al., 2004; Victorio et al., 2016a; Wang et al., 2011; Wang et al., 2004; You et al., 2012). This makes certain routes of drug administration difficult (Khong et al., 2012) and may limit treatment at high doses (Ku and Smith, 2015). One mouse-adapted virus model, by Caine et al., was developed in 10-week-old mice. However, the authors did not determine the time course of infection, cytokine profile, or verify the effects of a positive control drug (Caine et al., 2013), which are critical for model validation and antiviral testing.

Given these limitations, we submit that a new animal model for EV-71 is needed to evaluate potential therapeutic compounds. This model should produce neurological disease signs in juvenile mice (three-weeks-old or older), and the model should allow the evaluation of several disease parameters, such as weight loss, neurological disease signs, and cytokine levels that may be modulated by antiviral therapy. In addition, a positive control drug should be identified that can be used to confirm the challenge dose used in an experimental study and also reduce or prevent disease signs.
CHAPTER III
DEVELOPMENT OF MOUSE MODEL FOR EV-71 INFECTION

ABSTRACT

Enterovirus 71 (EV-71) is a picornavirus that can cause serious neurological disease in young children. Animal models for EV-71 are needed to evaluate potential antiviral therapies. While several EV-71 models have been produced, these models have limitations including requiring non-human primates or newborn mice, and lack of lethality or crucial disease signs. Here we report the development of a new EV-71 model in four-week-old AG129 mice. Virus was serially passaged six-times in AG129 mice and then plaque purified three times to produce a clonal virus stock. The mouse-adapted virus produces lethality and relevant disease signs, including rear-limb paralysis. A neurological scoring system was used to assess motor function and paralysis. Onset of disease occurs between day 9 and 14 post-infection, and mortality is often preceded by appreciable weight-loss.

1. Introduction

Enterovirus 71 (EV-71) is a non-enveloped, single-stranded RNA virus in the Picornaviridae family and can produce serious clinical signs such as encephalitis, meningitis, pulmonary edema, and acute flaccid paralysis (Ooi and Solomon, 2014). Despite its seriousness, there are currently no approved antivirals or therapeutics for an EV-71 infection (Song et al., 2018). This lack of options to treat or prevent EV-71 infection is due in part to a lack of effective animal models. Early animal models used non-human primates, namely rhesus masque and cynomolgus monkeys (Fujii et al., 2013; Khong et al., 2012; Wang et al., 2011). While these models do exhibit some relevant
clinical signs, they are expensive, pose ethical challenges, and can require invasive routes of administration (Wang and Yu, 2014). They also fail to exhibit encephalomyelitis and respiratory failure, two serious clinical signs observed in human patients (Wang and Yu, 2014).

Transgenic mouse models have been produced after the discovery of human scavenger receptor class B member 2 (hSCARB2) and P-selectin glycoprotein ligand-1 (PSGL-1) as EV-71 receptors (Fujii et al., 2013; Lin et al., 2013b; Liou et al., 2016; Liu et al., 2012). However, only one model produces limb paralysis, and only after invasive intracranial infection (Fujii et al., 2013). Furthermore, most of these models require mice be infected at or younger than two-weeks-old, which makes administration of drugs via oral or intravenous routes more difficult due to size. Finally, hSCARB2 and PSGL-1 are only two of six currently identified EV-71 receptors, which may limit the pathogenesis of the virus in these models (Yamayoshi et al., 2014).

Several mouse-adapted virus models have been produced, with most models requiring mice younger than two weeks. Only one model by Caine et al. was completely lethal in 10-week-old mice. However, a time course of infection and cytokine evaluation after virus infection was not performed, and this model was not used to test therapeutics (Caine et al., 2013). Therefore, the limitations of these models suggest a new animal model is needed for EV-71. Here we report the development of such a model for EV-71 in four-week-old AG129 mice.

2. Materials and Methods

2.1. Viruses and Cell Lines
Enterovirus 71 MP4 (BEI Resources NR-472) was obtained from American Type Culture Collection (ATCC, Manassas, VA). Human rhabdomyosarcoma cells (RD) were also obtained from ATCC.

2.2. Cell Culture Media

RD cells were grown in minimum essential media (MEM) (GE Healthcare HyClone™, Logan, UT) with 5% fetal bovine serum (FBS) (GE Healthcare HyClone™, Logan, UT). RD cells were cultured in MEM with 2% FBS. Media for virus titration, tissue homogenization, and virus neutralization assay, was MEM with 2% FBS and 50 µl gentamicin (GE Healthcare Hyclone™, Logan, UT). MEM media (no additives) were used for animal infections and sham infections.

2.3. Serial Passaging of Virus in Mice

Enterovirus 71 MP4 was passaged sequentially six times in four- to six-week-old AG129 mice. Male and female AG129 mice were obtained from a specific pathogen-free breeding colony at Utah State University. Mice were infected with 0.2 ml of virus via the intraperitoneal route (i.p.). Mice were then sacrificed and necropsied at either day 3 post-infection (p.i.) or upon onset of disease. Brain tissue was removed, homogenized in MEM media, and stored at -80 °C. Equal volumes from each mouse brain homogenate were pooled for infection in the next mouse passage.

2.4. Virus Titration (CCID$_{50}$) Assay

Tissue homogenate or virus media were serially diluted in log-dilutions. Dilutions were then transferred to a 96-well microplate seeded with RD cells 24h prior to infection. Dilutions of virus were added in quadruplicate. Plates were then incubated at 37 °C and 5% CO$_2$ for 6 days. Plates were examined visually on days 3 and 6 post-infection (p.i.)
for cytopathic effect. The 50% cell culture infectious dose (CCID₅₀) was determined using an endpoint dilution method as specified by Reed and Muench (Reed and Muench, 1938). Virus titers were expressed as CCID₅₀ units per ml.

2.5. Determination of Oral Route of Infection

Six-week-old AG129 mice were challenged with 0.2 ml of MP7 (10⁶.⁶ CCID₅₀/ml) per os (p.o.). Mice were observed for 21 days for weights loss, disease signs, and mortality.

2.6. Challenge Dose Titration of MP7 and MP10 in Four- and Six-Week-Old Mice

Four- and six-week-old AG129 mice (n=4 or 6) were infected with 0.2 ml of either MP7 (10⁶.⁶ CCID₅₀/ml) or MP10 (10⁷.⁰ CCID₅₀/ml) via the i.p. route. Mice were infected with undiluted and half-log dilutions (1:3, 1:10, 1:30) of virus. Mice were observed for 21 days for weights loss, disease signs, and mortality.

2.7. Plaque Purification

RD cells were seeded onto 12-well microplates 24h prior to infection. EV-71 MP10 was serially diluted and plated onto the 12-well microplates. The virus was incubated with cells for 1h p.i. The growth medium was removed and replaced with 0.6% sea plaque agarose (FMC Bioproducts, Rockland, ME), 2% FBS, and 25 mM MgCl₂. Cells were observed visually for plaques for 3 days post-infection (d.p.i.). Individual plaques were then picked at random, numbered, and inoculated into T-25 flasks containing RD cells and growth media. Cells in T-25 flasks were observed 2 to 3 days post plaque harvesting for CPE. The virus from the T-25 flasks showing 100% CPE was then used to repeat the plaque purification procedure three times. Plaques were assigned a number designating the order they were selected from the agarose medium.
2.8. Comparison of Selected Plaque Purified Isolates

Four-week-old AG129 mice (n=4) were infected with 0.2 ml of plaque 4 (10^6.6 CCID_{50}/mouse), plaque 6 (10^7.0 CCID_{50}/mouse), or plaque 15 (10^6.0 CCID_{50}/mouse) via the i.p. route. Mice were observed for 21 days for weights loss, disease signs, and mortality.

2.9. Comparison of Plaques 4 and 6

Groups of four-week-old AG129 (n=4) mice were challenged with 0.2 ml of plaque 4 (10^6.6 CCID_{50}/mouse), plaque 6 (10^7.0 CCID_{50}/mouse), or a 1:1 combination of plaque 4 and plaque 6 (10^6.8 CCID_{50}/mouse) via the i.p. route. Mice were observed for 21 days for weights loss, disease signs, and mortality.

2.10. Mouse Passage Comparison for MP11-MP15

Groups of four-week-old AG129 mice (n=4 or 5) were challenged with 0.2 ml of either EV-71 MP11 (10^5.1 CCID_{50}/mouse), MP12 (10^5.1 CCID_{50}/mouse), MP13 (10^6.1 CCID_{50}/mouse), MP14 (10^4.8 CCID_{50}/mouse), or MP15 (10^5.1 CCID_{50}/mouse) via the i.p. route. Mice were observed for 21 days for weights loss, disease signs, and mortality.

2.11. Virus Stock Production

Several stocks of EV-71 MP10 plaque 4 and plaque 6 were produced. Virus stocks were produced by infecting cell culture flasks (seeded with RD cells 24h prior) with EV-71 at a 1:1000 concentration. Flasks were incubated at 37 °C and 5% CO₂ for 3-4 days. Upon the appearance of at least 50% CPE, flasks were frozen at -80 °C. Virus was harvested by thawing flasks at room temperature, centrifuging virus to remove cellular debris (4000 rpm for 10 min), aliquoting virus, and then storing virus at -80 °C.

2.12. Mouse Strain Comparison
Groups of six-week-old SJL/J, FVB/NJ, C57BL/6J, BALB/c, and Swiss-Webster were infected with 0.2 ml of EV-71 MP10 plaque 4 \((10^{7.3} \text{CCID}_{50}/\text{ml})\). Mice were ordered from Charles River (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME). Mice were observed for 18 days post-infection for weight loss, clinical signs, and mortality.

2.13. **Comparison of Virus Stocks**

For comparison of virus stocks, 4-week-old AG129 mice were challenged with 0.2 ml of MP10 plaque 4, plaque 6, or a 1:1 combination of plaque 4 and 6, from virus stocks 1-4. Mice were observed for 21 days for weight loss, disease signs, and mortality.

2.14. **Challenge Dose Titration for Plaques 4 and 6**

Groups of 4-week-old AG129 mice \((n=4)\) were infected with 0.2 ml of a 1:1 combination of MP10 plaque 4 and 6 via the i.p. route. Mice were infected with undiluted and half-log dilutions \((1:3, 1:10, 1:30)\) of virus from stock 1 \((10^{6.8} \text{CCID}_{50}/\text{mouse},\text{ undiluted})\) and stock 2 \((10^{6.7} \text{CCID}_{50}/\text{mouse},\text{ undiluted})\). Mice were observed for 21 days for weight loss, disease signs, and mortality.

2.15. **Comparison of Vortexed and Non-Vortexed MP10 Samples**

Virus from plaque 4 and plaque 6 stocks were titered with and without vortexing. This was to determine if vortexing would disperse any virus aggregates that had formed during the storage process. Ten aliquots from five different virus stocks were compared. Virus was first removed for titer from a non-vortexed aliquot of virus. This same aliquot was then vortexed at maximum speed for 5 seconds. Vortexed virus was then removed for titer. Virus titration was performed as described above.

2.16. **Comparison of Sonicated and Non-Sonicated MP10 Samples**
Virus from MP10 plaque 4 and plaque 6 stocks and were titrated with and without vortexing. Ten aliquots from five different virus stocks were compared. An aliquot of virus was sonicated in a cup sonicator in an ice water bath. Virus was sonicated for 20 seconds at 21% amplitude in a 750-Watt Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL). The tube was then vortexed and this procedure was repeated 3 times. Sonicated virus was then removed and titered. Virus titration was performed as described above.

2.17. Freeze-Thaw Comparison

Homogenized tissue aliquots from AG129 infected with EV-71 were subjected to up to five freeze-thaw cycles (-80 °C and room temperature). Samples were titered after each freeze thaw cycle.

2.18. Comparison of Ages of Infection

Groups of AG129 mice (n=8) were infected with 0.2 ml (10^{7.3} CCID_{50}/mouse) of plaque 4 virus (Stock 3) via the i.p. route. Mice were infected at the following ages: 26-, 28-, 30-, 32-, 34-, 36-, and 38-days-old. Mice were observed for 21 days for weight loss, disease signs, and mortality. Separate studies evaluated tissue titers in brain, spinal cord, and leg muscle tissues at day 7 p.i.

2.19. Neurological Scoring

A neurological scoring system was adapted from Hatzipetros et al. (Hatzipetros et al., 2015)(Table 1). Scoring was adapted to assign an intermediate score (e.g., 2.5) for mice that exhibited weakness/paralysis in only a single limb.
An intermediate score (“___ .5”) was assigned to mice that presented signs intermediate between two neurological scores (e.g., single limb weakness or paralysis).

2.20. Statistical Analysis

Statistical analysis was performed using Prism 7 (GraphPad Software, San Diego, CA).

2.21. Ethical Treatment of Animals

This study was completed under the approval of the Institutional Animal Care and Use Committee of Utah State University. The work was performed in the AAALAC-accredited Laboratory Animal Research Center of Utah State University in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 2011).

3. Results
3.1. Determination of Oral Route of Infection

Six-week-old AG129 (n=4) mice were challenged with 0.2 ml of MP7 via the p.o. route. None of the mice exhibited any disease signs, including weight loss (data not shown).

3.2. Challenge Dose Titration of MP7 and MP10 in Four- and Six-Week-Old Mice

EV-71 was serially passaged in AG129 mice, beginning with MP4 (NR-472) and ending with MP10. The viral titer of each mouse passage was determined (Table 2). Viral titers for MP5-10 remained within one log of the original MP4 titer (10^{7.9} CCID_{50}/ml). Hereafter, EV-71 mouse-passaged viruses will be referred to simply as “MP” and the passage number, without the “EV-71” prefix.

Four- and six-week-old AG129 mice were infected with undiluted and half-log dilutions (1:3, 1:10, and 1:30) of MP7 and MP10. Only four-week-old mice exhibited complete mortality, while the six-week-old groups exhibiting 50% mortality (2/4). For MP7, four-week-old infected with undiluted and a 1:3 dilution of virus exhibited complete mortality. For MP10, four-week-old infected with undiluted and a 1:10 dilution of virus exhibited complete mortality (Fig. 1).

Weight loss was observed as a parameter of disease (Fig. 2). Nearly all mice that succumbed to disease experienced weight loss prior to the day of death. Disease signs included hunching, ruffled fur, limb weakness, ocular discharge, conjunctivitis, lethargy, and rear-limb paralysis.
Fig. 1. Survival of Four- and Six-Week-Old Mice Infected with EV-71 MP7 and MP10. Four- and six-week-old AG129 mice (n=4 or 6) were infected with 0.2 ml of either MP7 (10^{6.6} CCID_{50}/ml) or MP10 (10^{7.0} CCID_{50}/ml) via the i.p. route. Mice were infected with undiluted and half-log dilutions (1:3, 1:10, 1:30) of virus. Groups are as follows: MP7 used to infect four- (A) and six-week-old (B) mice, and MP10 used to infect four- (C) and six-week-old (D) mice. A Gehan-Breslow-Wilcoxon test was used to compare the survival of the 1:3, 1:10, and 1:30 groups to the undiluted group. *P<0.05
Fig. 2. Percent Body Weight of Four- and Six-Week-old AG129 Mice Infected with EV7-71 MP7 and MP10. Four- and six-week-old AG129 mice (n=4 or 6) were infected with 0.2 ml of either MP7 (10\(^{6.6}\) CCID\(_{50}\)/ml) or MP10 (10\(^{7.0}\) CCID\(_{50}\)/ml) via the i.p. route. Mice were infected with undiluted and half-log dilutions (1:3, 1:10, 1:30) of virus. Groups are as follows: MP7 in four- (A) and six-week-old (B) mice, and MP10 in four- (C) and six-week-old (D) mice. Changes in body weight were expressed as a percent relative to body weight on the first day of the study.

3.3. Comparison of Plaques 4, 6, and 15.

MP10 was plaque purified and fifteen isolates were selected for further purification. Groups of four-week-old AG129 mice (n=2) were infected, and plaques that produced complete mortality were selected for further plaque purification. This was repeated following the second plaque purification. After the third plaque purification, mice were infected with plaque 4, plaque 6, or plaque 15. All three plaques produced complete mortality in mice. Significant differences were observed between the following
groups: plaque 4 and plaque 6 ($P<0.05$), plaque 4 and plaque 15 ($P<0.01$), and plaque 6 and plaque 15 ($P<0.05$)(Fig. 3).

**Fig. 3. Survival of AG129 Mice Infected with EV-71 MP10 Plaque 4, Plaque 6, and Plaque 15.** Four-week-old AG129 mice (n=4) were infected with 0.2 ml of plaque 4 ($10^{6.6}$ CCID$_{50}$/mouse), plaque 6 ($10^{7.0}$ CCID$_{50}$/mouse), or plaque 15 ($10^{6.0}$ CCID$_{50}$/mouse). A Gehan-Breslow-Wilcoxon test was used to compare the survival curves of the groups. The significant differences were observed between the following groups: plaque 4 and plaque 6*, plaque 4 and plaque 15**, and plaque 6 and plaque 15**. *$P<0.05$, **$P<0.01$

3.4. *Comparison of Plaques 4 and 6*

Four-week-old AG129 mice (n=4) were infected with plaque 4, plaque 6, or a 1:1 combination of plaque 4 and 6. Both plaques produced complete mortality, both individually and in combination. There was no significant difference between the survival of each group (Fig. 4).
Fig. 4. Survival of Mice Infected with EV-71 MP10 Plaque 4 and Plaque 6. Groups of four-week-old AG129 (n=4) mice were challenged with 0.2 ml of either plaque 4 (10^{6.6} CCID_{50}/mouse), plaque 6 (10^{7.0} CCID_{50}/mouse), or a 1:1 combination of plaque 4 and plaque 6 (10^{6.8} CCID_{50}/mouse) via the i.p. route. A Gehan-Breslow-Wilcoxon test was used to compare the group. There was no significant difference between groups.

3.5. Mouse Strain Comparison

Several mouse strains were infected with 0.2 ml of EV-71 MP10 plaque 4 (1:10) via the i.p. route. The following strains were infected at five-weeks of age: SJL/J, FVB/NJ, C57BL/6J, BALB/c, and Swiss-Webster. The mice exhibited no signs of disease or significant weight loss (data not shown).

3.6. Mouse Passage Comparison for MP11-MP15.

MP10 was passaged an additional five times to determine if additional passaging would increase virulence. All passages (MP11-15) had decreased viral titers as compared with MP10 (Table 2). When used to infect mice, only plaques MP11 and MP12 produced complete mortality in AG129 mice (Fig. 5). The group infected with MP10 was used as a control. All viruses had been passaged once in RD cells prior to infection studies.
Table 2
Summary of Mouse Passage Viral Titers after Serial.

<table>
<thead>
<tr>
<th>Mouse Passage (MP)</th>
<th>Viral Titer (log_{10}CCID_{50}/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>7.9</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
</tr>
<tr>
<td>7</td>
<td>7.3</td>
</tr>
<tr>
<td>8</td>
<td>7.7</td>
</tr>
<tr>
<td>9</td>
<td>7.8</td>
</tr>
<tr>
<td>10</td>
<td>7.7</td>
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<td>11</td>
<td>5.7</td>
</tr>
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<tr>
<td>14</td>
<td>5.5</td>
</tr>
<tr>
<td>15</td>
<td>5.7</td>
</tr>
</tbody>
</table>
Fig. 5. Survival of Mice Infected with EV-71 MP11-15. Groups of four-week-old AG129 mice (n=4 or 5) were challenged with 0.2 ml of either EV-71 MP11 ($10^{5.1}$ CCID$_{50}$/mouse), MP12 ($10^{5.1}$ CCID$_{50}$/mouse), MP13 ($10^{6.1}$ CCID$_{50}$/mouse), MP14 ($10^{4.8}$ CCID$_{50}$/mouse), or MP15 ($10^{5.1}$ CCID$_{50}$/mouse) via the i.p. route. A Gehan-Breslow-Wilcoxon test was used to compare the MP11-15 groups to MP10. All viruses were passaged once in RD cells. *P<0.05, **P<0.01

3.7. Challenge Dose Titrations of Plaque 4 and Plaque 6

A challenge dose titration was completed using a 1:1 combination of plaque 4 and plaque 6 from challenge stocks 1 and 2. In both instances, only an undiluted concentration of virus produced complete mortality (Fig. 6).
Fig. 6. Challenge Dose Titration of EV-71 MP10 Plaque 4 and 6 Combination from Stocks 1 and 2. Four-week-old AG129 mice (n=4) were infected with 0.2 ml of a 1:1 combination of plaque 4 and plaque 6 from stock 1 (A; $10^{6.8}$ CCID<sub>50</sub>/mouse) and stock 2 (B; $10^{6.7}$ CCID<sub>50</sub>/mouse). Mice were infected with undiluted and half-log dilutions (1:3, 1:10, 1:30) of virus. A Gehan-Breslow-Wilcoxon test was used to compare the survival of the 1:3, 1:10, and 1:30 to the undiluted group. *$P<0.05$, **$P<0.01$

3.8. Comparison of Virus Stocks

Four virus stocks of plaque 4 and plaque 6 were produced. Stocks are numbered based on the date they were harvested from cell culture (Table 3). Viral titers remained
relatively consistent across challenge stocks, with no more than a one-log difference between any of the stocks.

### Table 3
Viral Titers for EV-71 MP10 Stocks 1-4.

<table>
<thead>
<tr>
<th>Stock</th>
<th>Date</th>
<th>Plaque</th>
<th>Viral Titer (log$<em>{10}$CCID$</em>{50}$/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 Nov 2016</td>
<td>4</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>7.7</td>
</tr>
<tr>
<td>2</td>
<td>8 Aug 2017</td>
<td>4</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>7.5</td>
</tr>
<tr>
<td>3</td>
<td>14 Aug 2017</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>25 Aug 2017</td>
<td>4</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Mice were infected with each virus stock, both individually and in combination, to determine the virulence of each stock. The goal is to produce virus stocks that are consistently virulent. Four-week-old AG129 (n=4 or 6) mice were infected with a 1:1 combination of plaque 4 and 6 from stock 1, 2, 3, or 4. Stocks 1 and 2 produced complete mortality, whereas Stocks 3 and 4 failed to do so (Fig. 7). It should be noted that Figure 7 is a combination of several individual studies assessing the virulence of these stocks, including data form Figure 6. Studies were complied into a single figure for clarity. The same pattern is followed for Figure 8 and Figure 9. Statistically significant differences were observed between the following groups: stock 1 and 2, stock 2 and 3, and stock 2 and 4 ($P<0.05$).
Fig. 7. Survival of Mice Infected with EV-71 MP10 Plaques 4 and 6 from Stocks 1-4. Groups of four-week-old AG129 mice were infected with 0.2 ml of a 1:1 combination of plaques 4 and 6 via the i.p. route. Virus from stocks 1-4 were used for infection. Mice received the following CCID$_{50}$/mouse of virus: stock 1 (10$^{6.8}$), stock 2 (10$^{6.7}$), stock 3 (10$^{7.5}$), and stock 4 (10$^{7.8}$). As this data is compiled from several studies, the group sizes were as follows: Stock 1 (n=8), Stock 2 (n=8), Stock 3 (n=10), and Stock 4 (n=6). Data from studies were combined in order to more accurately reflect both the virulence and variability across stocks. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated groups. Statistically significant differences were observed between the following groups: stock 1 and 2*, stock 2 and 3*, and stock 2 and 4*. *P<0.05

The virulence of plaque 4 was assessed individually for each stock. Plaque 4 from stocks 1 and 2 produced complete mortality, whereas plaque 4 from stocks 3 and 4 failed to do so (Fig. 8). Statistically significant differences were observed between the following groups: Stock 1 and Stock 3 ($P<0.05$), Stock 1 and Stock 4, and Stock 2 and Stock 4 ($P<0.01$).
Fig. 8. Survival of Mice Infected with EV-71 MP10 Plaque 4 from Stocks 1-4.
Four-week-old AG129 Mice were infected with 0.2 ml of plaque 4 virus from stock 1, 2, 3, or 4 via the i.p. route. As this data is compiled from several studies, the group sizes were as follows: Stock 1 (n=4), Stock 2 (n=8), Stock 3 (n=13), and Stock 4 (n=6). Mice received the following CCID$_{50}$/mouse of virus: stock 1 ($10^{6.6}$), stock 2 ($10^{6.6}$), stocks 3 ($10^{7.3}$), and stock 4 ($10^{7.6}$). Data from studies were combined in order to more accurately reflect both the virulence and variability across plaque 4 stocks. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated groups. Statistically significant differences were observed between the following groups: Stock 1 and Stock 3*, Stock 1 and Stock 4**, and Stock 2 and Stock 4**. *$P<0.5$, **$P<0.01$.

The virulence of plaque 6 was assessed individually for each stock. Plaque 6 from stocks 1 and 2 both produced complete mortality, where stocks 3 and 4 failed to produce complete mortality, with 16.7% (1/6) and 33% (2/6) mice surviving, respectively (Fig. 9). Statistically significant differences were observed between the following groups: stock 1 and stock 3 ($P<0.001$), stock 1 and stock 4, stock 2 and stock 3, and stock 2 and stock 4 ($P<0.01$).
Fig. 9. Survival of Mice Infected with EV-71 MP10 Plaque 6 from Stocks 1-4. Four-week-old AG129 Mice (n=4 or 6) were infected with 0.2 ml of plaque 6 virus from stock 1, 2, 3, or 4. As this data is compiled from several studies, the groups sizes are as follows: Stock 1 (n=4), Stock 2 (n=8), Stock 3 (n=11), and Stock 4 (n=6). Data from studies were combined in order to more accurately reflect both the virulence and variability across plaque 4 stocks. Mice received the following CCID$_{50}$/mouse of virus: stock (10$^{7.0}$), stock 2 (10$^{6.8}$), stock 3 (10$^{7.6}$), and stock 4 (10$^{8.1}$). A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated groups. Statistically significant differences were observed between the following groups: stock 1 and stock 3***, stock 1 and stock 4**, stock 2 and stock 3**, and stock 2 and stock 4**. **P<0.01, ***P<0.001

Mice were initially infected with a 1:1 combination of plaque 4 and 6 from stock 3 (Trial 1; Aug 2017), with this trial producing complete mortality. However, a follow-up study, performed 6 months later, resulted in incomplete mortality (Trial 2; Feb 2018)(Fig. 10). This data is combined in Figure 7, but separated in Figure 10 to highlight the variability. A significant difference in survival was observed between the two studies (P<0.01).
Fig. 10. Comparison of Two Trials of Mice Infected with EV-71 MP10 Plaque 4 and 6 from Stock 3. Groups of four-week-old AG129 mice (n=4 or 6) were infected with a 0.2 ml ($10^{7.5}$ CCID$_{50}$/mouse) of a 1:1 combination of plaque 4 and 6 via the i.p. route. Virus used for infection was from stock 3. Trial 1 was performed in Aug 2017, and Trial 2 was performed 6 months later in Feb 2018. This data is included in Fig. 7 for Stock 3 but is separated into the two individual trials here to show the variability between the studies. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated groups. **$P<0.01$

3.8. Comparison of Vortexed and Non-Vortexed MP10 Samples

A comparison of fourteen virus aliquots from five different EV-71 stocks was performed to determine if vortexing could liberate virus aggregates that may have formed in the virus stocks. Aggregated virus would potentially lower the amount of virus available for infection. We observed no significant difference between vortexed and non-vortexed virus (non-vortexed: $8.65 \log_{10} \text{CCID}_{50}$/ml, std. dev.: 0.34; vortexed: 8.375
log_{10}CCID_{50}/ml, std. dev.: 0.49). Differences between aliquots within a virus stock were also insignificant (0.18 log_{10}CCID_{50}/ml; std. dev.: 0.11).

3.9. Comparison of Sonicated and Non-Sonicated MP10 Samples

A comparison of sonicated and non-sonicated MP10 aliquots was performed to determine if sonication could liberate virus aggregates that may have formed in the virus stocks. On average, sonicated virus had a higher titer than non-sonicated (10^{8.5} and 10^{8.2} CCID_{50}/ml, respectively). However, this difference was within the rate of error for the assay (0.7 log).

3.10. Comparison of Age of Infection

Mice were infected at either 26-, 28-, 30-, 32-, 34-, 36-, or 38-days-old. Only the 26-, 28-, and 30-day-old groups exhibited complete mortality. All other groups had surviving mice, as follows: 32-day-old (2/8), 34-day-old (5/8), 36-day-old mice (5/8), and 38-day-old (6/8).
Fig. 11. Comparison of the Age of Infection. Groups of AG129 mice (n=8) were infected with a 0.2 ml ($10^{7.3}$ CCID$_{50}$/mouse) of plaque 4 (stock 3) via the i.p. route. Mice were infected at either 26-, 28-, 30-, 32-, 34-, 36, or 38-days-old. A Gehan-Breslow-Wilcoxon test was used to compare the survival curves to the 28-day-old group, as the age range was the age range used for the many of the studies for many of the studies was 27-29-days-old. **$P<0.01$, ***$P<0.001$

3.11. Comparison of Viral Titers for Age of Infection

Mice were infected at 26-, 30-, 34-, or 38-days of age, and brain, spinal cord, and leg muscle were removed for virus titration on day 7 p.i. Brain and spinal cord titer steadily decreased with increasing mouse age. Viral titers in the 26-day-old mice were compare to all other groups. Significant differences were observed in the brain tissue (30- ($P<0.05$), 34- ($P<0.01$, and 38-day-old mice ($P<0.0001$)), spinal cord (38-day-old mice ($P<0.001$) and leg muscle (38-day-old mice ($P<0.01$)).
Fig. 12. Comparison of Viral Titers for the Age of Infection. Groups of AG129 mice (n=8) were infected with a 0.2 ml (10^{7.3} CCID_{50}/mouse) of plaque 4 (stock 3) via the i.p. route. Mice were infected at either 26-, 30-, 34-, or 38-days-old. Mice were sacrificed on day 7 p.i. and brain, spinal cord, and leg muscle tissue were removed. Titers are expressed as CCID_{50}/g of tissue in order to account for differences in size between groups. A one-way ANOVA followed by a Tukey’s multiple comparison test were used to compare mean viral titers between groups. Significant differences were observed for brain tissue *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

4. Discussion

Here we report the successful development of a mouse model for EV-71 in four-week-old AG129 mice using an MP10 virus. This model is 100% lethal when mice
are administered 0.2 ml of undiluted virus via the i.p. route. Mice exhibit weight loss, hunching, ocular discharge, and neurological signs, including rear-limb weakness and paralysis. Disease onset and mortality typically occur between days 9 and 14 post-infection.

Producing neurological disease signs is crucial in an animal model. Sick mice consistently exhibit rear-limb paralysis, which is highly relevant given that EV-71 can produce an acute flaccid paralysis in young children (Ooi and Solomon, 2014). The severity of motor dysfunction was assessed using a neurological scoring system (Hatzipetros et al., 2015). The scoring system was adapted to account for single limb weakness or paralysis, indicated by an intermediate score (e.g., 2.5). Weight loss was also observed and is a consistent, accurate predictor of mortality. However, in some cases, mice died before any observable signs of disease, including weight loss.

While this model produces a rear-limb paralysis similar to that seen in human patients, it does not produce any observable HFMD lesions. In addition, it is not known if the virus produced pulmonary dysfunction or edema, as pulmonary function and lung pathology were not evaluated. This model also produces paralysis and mortality at a much higher rate (100%) than is seen in humans (Lee, 2016). While this may limit the model’s representation of human disease, we believe that the high mortality is relevant for therapeutic testing, as therapeutics are likely to be employed to treat or prevent serious cases of EV-71. Furthermore, the most serious cases of EV-71 involve neurological signs such as acute flaccid paralysis. We submit that potential EV-71 therapeutics should be assessed in the context of serious disease. Disease signs that are observed in all infected mice provided a consistent baseline against which to evaluate
therapeutics. This is consistent with previous animal models for EV-71 (Caine et al., 2013; Khong et al., 2012; Liou et al., 2016).

Development of a successful model for EV-71 considered several parameters, including mouse age, mouse passage number, challenge dose, mouse strain, and route of infection. As EV-71 is spread via the fecal-oral route, p.o. administration of the virus would mimic the natural route of infection. However, six-week-old mice infected with MP7 exhibited no disease signs, whereas mice challenged via the i.p. route did. This is consistent with the literature, where the predominant route of infection in mouse models is i.p. (Council, 2011; Khong et al., 2012; Lin et al., 2013b; Liou et al., 2016). One model, by Wang et al., used oral administration for infection. However, this model used young mice (one- and seven-day-old ICR)(Wang et al., 2004). More invasive routes of infection have also been used. For example, Ong et al. and Fuji et al. used the intracranial route of infection (Fujii et al., 2013; Ong et al., 2008). This route is highly invasive (Weil et al., 2017), technically difficult (Mathon et al., 2015), and subverts the progression of the virus to the CNS. As such, we believe that the i.p. route of administration is sufficient for this model.

To determine the optimal mouse strain for infection with EV-71 MP10, several mouse strains were infected with 0.2 ml of EV-71 MP10 plaque 4 (1:10) via the i.p. route. The following strains were infected at five-weeks of age: SJL/J, FVB/NJ, C57BL/6J, BALB/c, and Swiss-Webster. The mice exhibited no signs of disease or significant weight loss (data not shown). While both mouse age (five-weeks-old) and virus dilution (1:10) varied from those used in later studies, we have observed disease in AG129 mice infected at six-weeks of age and with a 1:10 dilution of EV-71 MP10.
However, considering later dose titrations, it would have been preferable to infect these mice at an undiluted concentration for consistency. Nevertheless, given that interferon is crucial to controlling EV-71 infection in mice (Liu et al., 2005), we submit the AG129 strain is a suitable mouse strain to this model human disease.

Serial passaging is used to adapt a pathogen to a non-natural host (Meers, 1959). We initially performed six passages, beginning with MP4 and ending with MP10. The MP7 and MP10 viruses were initially compared, with both producing 100% mortality in four-week-old mice. However, MP10 produced complete lethality at 1:10 dilution, whereas the lowest dilution at which MP7 produced complete lethality was 1:3 and these differences were not significant from the undiluted groups. The fact that MP10 required a lower dose to produce complete mortality suggested that it was more virulent than MP7. Based on this, MP10 was selected as the virus to be used for the model.

It was anticipated that mice could be challenged with MP10 at a 1:10 dilution consistently, thus reducing the amount of virus needed for each study and extending the “shelf-life” of the virus stock. Unfortunately, additional dose titrations with plaque purified MP10 did not reproduce our earlier results, and only undiluted concentrations of virus were completely lethal. As such, plaque purified MP10 was used for infection. While this may appear to be a large inoculum volume (0.2 ml, undiluted), the dose of virus per mouse is consistent with that used in other EV-71 models. The infectious dose in published models ranges from $10^5$ to $10^8$ CCID$_{50}$/mouse (with some studies reporting titer as plaque forming units (PFU) per mouse (PFU/mouse)). Our challenge ranges from $10^{6.7}$ to $10^{7.3}$ CCID$_{50}$/mouse, depending on the stock of the virus used. We therefore submit that our challenge dose is consistent with previously published models. What’s
more, given that the virus cannot consistently produce complete lethality at a 1:3 concentration, it is unlikely that the mice are being over-challenged. Ultimately, while a lower challenge dose or volume would be desirable for conserving virus stocks, we do not anticipate that the challenge dose or volume used here negatively impacts our model or diminishes the results.

Additional mouse passages were completed to determine if further adaptation of the virus could increase virulence (MP11-15). Of the five new passages, MP11 and MP12 were completely lethal in mice, whereas MP13-MP15 were not. However, the viral titers for MP11-MP15 were approximately two logs lower than the MP10 stocks produced previously. As such, the infectious dose was lower for mice infected with MP11 and MP12. While the reduced mortality could be attributed to the lower challenge dose, MP11 and MP12 were still able to induce complete lethality despite being nearly two logs lower than the MP10 challenge doses. This suggests that viral titers are not the sole indicator of virulence. Furthermore, increasing the infection dose volume to equal that used in the MP10 infections would not have been possible given the impossibly large amount of volume that would be needed. While these results suggest that the virus begins to attenuate after the tenth mouse passage, this cannot be determined due to the differences in the challenge doses. Furthermore, the lower virus titer in the MP11-15 stocks could be the result of attenuation or undetermined differences in the growth and harvest of virus in cell culture between the MP10 and MP11-15 stocks. Ultimately, given that MP11 and MP12 did not differ significantly from MP10, we opted to continue using the MP10 virus for the challenge model.

Due to the high challenge volume (0.2 ml) as well as the high number of
experiments, four stocks of MP10 plaque 4 and plaque 6 were produced. The stocks were identified by the date that they were harvested from cell culture and were assigned a number for identification (Table 3). Mice were challenged with virus from each stock to confirm virulence. We anticipated that the 1:1 combination of plaque 4 and plaque 6 from each stock would reduce the variability that was observed mice were infected with plaque 4 alone (data not shown). Early studies with plaque 4 and plaque 6 produced inconsistent mortality, and we hypothesized that a combination of the plaques would produce a more consistent lethal infection. We also anticipated that given each stock was produced from a plaque-purified isolate (amplified once in RD cells), plaques would be identically virulent across stocks. Both assumptions were incorrect. Plaque 4 and plaque 6 from stocks 1 and 2 both produced complete lethality, both individually and in combination, with no statistically significant difference between the lethality of the two stocks (Fig. 7-9).

However, stocks 3 and 4 were not completely lethal. Nevertheless, the stock 3 viruses produced near-complete mortality (11/12 animals survived), whereas stock 4 produced appreciably lower mortality, for both the 1:1 combination (4/6) and individual plaques 4 (4/6) and 6 (3/6). However, the surviving mice in the 1:1 combination and plaque 4 infection groups for stock 3 were both experiencing weight loss and neurological signs by the end of the study. Given the similar mortality produced by these three virus isolates, and that plaque 4 and plaque 6 are nearly genetically identical (see Chapter VI), we opted to discontinue using the combination of plaque 4 and plaque 6 in infections using the stock 3 virus.

It should be noted that two separate studies using a 1:1 combination of plaque 4 and plaque 6 from stock 3 produced different results (Fig. 10). While an initial test (Trial
1) of the virus produced complete lethality, a follow-up study performed six months later (when stock 2 was nearly depleted) produced a slight difference in mortality (5/6). However, the mean day of death was 9.5 for Trial 1 and 15.8 for Trial 2. Given these results as well as the variability observed in earlier model development, we considered several possible sources of variability.

One potential source of variability could be that the virus was degrading after freezing. Given that freezing is a standard method for storing virus (Gould, 1999), we do not anticipate that the virus degraded during the 6 months between the two separate experiments. Another possibility could be the formation of virus aggregates after freezing. These aggregates may not sufficiently disperse when the virus aliquots are thawed, resulting in the mice receiving a lower virus challenge than anticipated. This is observed with vaccinia viruses, which can form aggregates and require sonication before use (Cotter et al., 2015; Kim and Sharp, 1966). It was hypothesized that vortexing or sonication could to disperse any virus aggregates, thereby liberating the virus for infection. However, comparison of vortexed (8.4 log$_{10}$CCID$_{50}$/ml; std. dev.:0.5) and non-vortexed (8.7 log$_{10}$CCID$_{50}$/ml; std. dev.: 0.4) aliquots showed differences that were within the range of error (0.7 logs) for the titration assay (Table 4). The same results were observed comparing sonicated (8.5 log$_{10}$CCID$_{50}$/ml; std. dev.: 0.5) and non-sonicated (8.2 log$_{10}$CCID$_{50}$/ml; std. dev.: 0.4)) samples (Table 5). This suggests that either virus aggregates were not forming, or that these methods were unable to liberate the aggregated virus. This suggests that a reduction in viable virus, possibly through aggregate formation, does not occur from a single freeze-thaw cycle.

Differences in viral titers between aliquots within a stock were also considered. If
the stock was unevenly aliquoted, then the inoculum dose would differ between mice and/or between studies, even with virus used from a supposedly homogenous stock. However, differences between aliquots (0-0.25 log_{10}CCID_{50}/ml) within a stock were within the range of error for the assay.

It also possible, though unlikely, that the samples experienced several unanticipated freeze thaws (e.g., freezer malfunction). Therefore, homogenized tissue samples from EV-71 infected AG219 mice were subjected to several freeze thaws cycles, with the sample being tittered after each cycle. Differences between freeze thaws were almost always within the range of error for the assay, suggesting that unintentional freeze thaws likely wouldn’t have reduced viral titer.

A final potential source of error that we considered was the age of the mice upon infection. While previous studies showed that the virulence of the mouse passages is decreased at six-week-old compared to four-week-old mice, the studies did not examine age increments shorter than one week. Thus, the ideal age range for infection could be shorter than one week (e.g., 27-30-days of age). An earlier study showed that as mouse age approaches five-weeks-old (33-days-old), virulence is markedly reduced (data not shown).

Near the conclusion of this project (April 2018), a source of variability for mouse age was discovered. In the IAR in-house breeding colony, weaned mice from different litters are caged together to save space and caging. However, mice with slightly different ages are sometimes caged together as well. The age of the mice within such a cage would be reported as an average age. This is a normal practice used to conserve space and caging and has not caused any issues with previous animal models. While mouse age
would only differ by a few days, we suspect that this difference may be enough to have introduced variability into our model. Mice for future studies with this model are caged according to their exact day of birth.

To determine the effect of age on the severity of infection, mice were infected at 26-, 28-, 30-, 32-, 34-, 36-, or 38-days of age. To determine the effect of age of viral tissue titers, we removed brain, spinal cord, and leg muscle from mice infected at 26-, 30-, 34-, and 38-days of age mice at day 7 p.i. Survival increased with increased age, with the 26-28-, and 30-day-old groups experiencing 100% mortality, whereas the 32-, 34-, 36-, and 38-day-old groups had surviving mice. The survival of each group was compared to the 28-day-old group, as many of our studies infected mice that were 27-29-days-old. The 34-, 36-, and 38-day-old groups significantly differed from the 28-day-old group. This suggests that some mice that are within the four-week-old range (i.e., 28-35-days-old) may not be suitable for infection in this model. In addition, viral titers in the brain of mice infected at 30-, 34-, and 38-days-old were significantly lower than those in mice infected at 26-days-old, and spinal cord and leg muscle tissue titers were significantly different at in mice infected at 38-days-old. This provides support for using mice within a narrow age range for this model (e.g, 26-30-days-old). While it is not known why the lethality decreasing with increasing age, possible reasons could include the increased size of the mice, resulting in a lower amount of virus per gram of tissue or maturation of the mouse immune system.

Additional parameters for this EV-71 challenge model, including a time course of infection and genome sequence analyses of the mouse-passaged viruses, are discussed in later chapters of this document (Chapters V and VI, respectively).
In conclusion, we submit that this is a suitable mouse model for the evaluation of therapeutics for EV-71 infections. The model is 100% lethal and produces neurological disease signs, including rear-limb paralysis. Mice are also infected at four-weeks of age, which allows for easier administration of treatments than in neonates (Gombash Lampe et al., 2014). Although some variability was observed between virus stocks, virus from stocks 1-3 was highly lethal and consistently produced complete mortality, with rare exceptions. Narrowing the age range at which the mice can be infected may remedy this variability.

Acknowledgements

Special thanks to Brett Hurst and Joseph Evans for performing the initial model development, including serial passaging (up to MP10), route of infection assessment, initial age of infection evaluation, and plaque purification of virus.
CHAPTER IV
EVALUATION OF THERAPEUTICS FOR TREATMENT OF AN EV-71 INFECTION IN AG129 MICE

ABSTRACT

Enterovirus 71 (EV-71) is a picornavirus that can cause serious neurological disease in young children. However, there are currently no approved antiviral therapies to treat EV-71. Many compounds have been assessed in vitro, but few have been evaluated in an animal model. Here we evaluated over twenty compounds for the treatment of an EV-71 infection in four-week-old AG129 mice. Of these, two compounds, fluoxetine and suramin, initially showed potential antiviral activity, but failed to be protective upon further examination. However, two novel host response modifiers, STF434, and STF1019, were able to protect up to 87.5% of infected mice from mortality. Further examination of these therapeutics for treating EV-71 infections is warranted.

1. Introduction

Enterovirus 71 (EV-71) is a non-enveloped, single-stranded RNA virus in the Picornaviridae family and mainly causes disease in young children, including encephalitis, meningitis, pulmonary edema, and acute flaccid paralysis. It can be up to 26.7% fatal in severe and complicated cases (Wong et al., 2010). Yet despite the seriousness of an EV-71 infection, there are currently no approved antivirals or therapeutics for treating the infection. Many compounds have been screened in vitro, including compounds specifically designed to inhibit picornaviruses such as pleconaril and pirodavir (Smee et al., 2016; Zhang et al., 2012). However, despite a number of compounds exhibiting antiviral activity against EV-71, drug development has been hampered in part by a lack of effective animal models for EV-71 (Khong et al., 2012; Lin
et al., 2013b; Wang and Yu, 2014).

The development of an EV-71 mouse model in four-week-old AG129 mice at the Institute for Antiviral Research allows for potential EV-71 therapeutics to be tested. Here we evaluate over twenty compounds for treatment of EV-71 infections. Compounds were selected based on reported in vitro and in vivo activity against EV-71 or other picornaviruses, with several of these compounds already FDA approved for other conditions. Ultimately, identification of an EV-71 therapeutic will provide much-needed information about compounds that could enter clinical trials or be used off-label, as well for use as a positive control drug in this mouse model.

2. Materials and Methods

2.1. Viruses and Cell Lines

Enterovirus 71 MP10 plaques 4 and 6 from previous model development were used (Chapter III). Human rhabdomyosarcoma cells (RD) were obtained from ATCC®.

2.2. Cell Culture Media

RD cells were grown in minimum essential media (MEM) (GE Healthcare Hyclone™, Logan, UT) with 5% fetal bovine serum (FBS) (GE Healthcare Hyclone™, Logan, UT). RD cells were cultured in MEM with 2% FBS. Media for virus titration, tissue homogenization, and virus neutralization assay was MEM with 2% FBS and 50 µl gentamicin (GE Healthcare Hyclone™, Logan, UT). MEM media (no additives) was used for animal infections, tissue homogenization, and sham infections.

2.3. Virus Titration (CCID$_{50}$) Assay

Tissue homogenate or virus media were sequentially diluted in MEM 2% + gentamicin. Dilutions were then transferred to a 96-well microplate seeded with RD cells
24h prior to infection. Dilutions of virus were added in quadruplicate. Plates were then incubated at 37 °C and 5% CO₂ for 6 days. Plates were examined visually on days 3 and 6 post-infection (p.i.) for cytopathic effect. The 50% cell culture infectious dose (CCID₅₀) was determined using an endpoint dilution method. Virus titers were expressed as CCID₅₀ units per ml.

2.4. Antiviral Compounds

Aurintricarboxylic acid (ATA), chloroquine, heparin, amantadine, rupintrivir, pirodavir, lycorine, bovine lactoferrin, rutin hydrate, N₆-benzyladenosine, 2-(2-Pyridyl)benzimidazole, 5-Hydroxymebendazole, suramin and guanidine HCl were purchased from Sigma-Aldrich (St. Louis, MO). Pleconaril and 17-AAG were purchased from MedChemExpress (Monmouth Junction, NJ). Quinacrine was purchased from AdooQ® Bioscience (Irvine, CA). Ribavirin was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Gemcitabine was purchased from Hospira (Lake Forest, IL). STF434 and STF1019 were provided by Dr. Jeffrey Glenn of Stanford University (Palo Alto, CA).

2.5. Therapeutic Compounds

Fluoxetine Oral Solution was obtained from Lannett (Philadelphia, PA). Naproxen Sodium was obtained from Sigma-Aldrich (St. Louis, MO).

2.6. Antiviral and Therapeutic Testing

Four-week-old AG129 mice were infected with 0.2 ml a 1:1 combination of plaque 4 and plaque 6 (stocks 1 and 2). Mice were treated with a variety of compounds at the dosages, routes, and schedules listed in Table 1. Compounds were administered twice
daily ("bis in die", or b.i.d.) or once daily ("quaque die", or q.d.). Mice were observed for at least 21 days post-infection for weight loss, clinical signs, and mortality.

2.7. Ethical Treatment of Animals

This study was completed under the approval of the Institutional Animal Care and Use Committee of Utah State University. Work was performed in the AAALAC-accredited Laboratory Animal Research Center of Utah State University in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 2011).

2.8. Neurological Scoring

Neurological scoring is assessed as previously defined in Chapter III.

2.9. Statistical Analysis

Statistical analysis was performed using Prism 7 (GraphPad Software, San Diego, CA). Kaplan-Meier survival curves were compared using a Gehan-Breslow-Wilcoxon test. Mean neurological scores were compared using a Kruskal-Wallis test followed by Dunn’s multiple comparisons.

3. Results

3.1. Antiviral and Therapeutic Testing

Results from the entire antiviral and therapeutic screening process are displayed in Table 1. Selected results are discussed in detail below. It should be noted that due to our use of an in-house breeding colony as well as the need to screen a large number of compounds for potential efficacy in the model, some studies involved an unusually low number of mice (e.g., n=3). The results for such studies are reported here as a summary of the work performed and not necessarily as conclusive findings.
## Table 1
### Summary of Antiviral and Therapeutic Testing for an EV-71 Infection in AG129 Mice.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg/d)</th>
<th>Treatment Schedule</th>
<th>Route</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribavirin</td>
<td>300</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/8</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>30</td>
<td>3 x q.a.d.</td>
<td>i.p.</td>
<td>0/8</td>
</tr>
<tr>
<td>Gemcitabine + Ribavirin</td>
<td>30/300</td>
<td>3 x q.a.d.</td>
<td>i.p.</td>
<td>0/8</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>25</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Rupintrivir</td>
<td>10</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Pirodavir</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Pirodavir</td>
<td>200</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Lycorine</td>
<td>10</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/8</td>
</tr>
<tr>
<td>Pleconaril</td>
<td>150</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>2-(2-Pyridyl)benzimidazole</td>
<td>20</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>5-Hydroxymebendazol</td>
<td>20</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Guanidine HCl + 2-(2Pyridyl)benzimidazole</td>
<td>200/20</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Amantadine</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Heparin</td>
<td>10</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>ATA</td>
<td>30</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Bovine Lactoferrin</td>
<td>75</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/3</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>50</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/3</td>
</tr>
<tr>
<td>17-AAG</td>
<td>25</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/3</td>
</tr>
<tr>
<td>Rutin Hydrate</td>
<td>200</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>1/2</td>
</tr>
<tr>
<td>N6-Benzyladenosine</td>
<td>200</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/3</td>
</tr>
<tr>
<td>Guanidine HCl</td>
<td>400</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>1/4</td>
</tr>
<tr>
<td>Naproxen Sodium</td>
<td>320</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>1/4</td>
</tr>
<tr>
<td>Suramin</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>2/4</td>
</tr>
<tr>
<td>Suramin</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Suramin</td>
<td>50</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>1/4</td>
</tr>
<tr>
<td>Suramin</td>
<td>300</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Drug</td>
<td>Dose (mg/kg/d)</td>
<td>Treatment Schedule</td>
<td>Route</td>
<td>Survival</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>-------</td>
<td>----------</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>2/4</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>2/4</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>30</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>100</td>
<td>10 x q.d.</td>
<td>p.o.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>30</td>
<td>10 x q.d.</td>
<td>p.o.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>100</td>
<td>10 x q.d.</td>
<td>p.o.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine Oral Solution</td>
<td>22</td>
<td>5 x q.d.</td>
<td>p.o.</td>
<td>1/4</td>
</tr>
<tr>
<td>Fluoxetine Oral Solution</td>
<td>44</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine Oral Solution</td>
<td>75</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>0/8</td>
</tr>
<tr>
<td>Fluoxetine Oral Solution</td>
<td>75</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/4</td>
</tr>
<tr>
<td>Fluoxetine Oral Solution</td>
<td>88</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>2/4</td>
</tr>
<tr>
<td>STF1019</td>
<td>200</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>7/8</td>
</tr>
<tr>
<td>STF1019</td>
<td>150</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>6/8</td>
</tr>
<tr>
<td>STF1019</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>2/8</td>
</tr>
<tr>
<td>STF1019</td>
<td>50</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>5/9</td>
</tr>
<tr>
<td>STF434</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>3/8</td>
</tr>
<tr>
<td>STF434</td>
<td>30</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>4/8</td>
</tr>
<tr>
<td>STF434</td>
<td>10</td>
<td>5 x b.i.d.</td>
<td>i.p.</td>
<td>3/8</td>
</tr>
<tr>
<td>STF434</td>
<td>300</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/8</td>
</tr>
<tr>
<td>STF434</td>
<td>100</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/8</td>
</tr>
<tr>
<td>STF434</td>
<td>30</td>
<td>5 x b.i.d.</td>
<td>p.o.</td>
<td>0/8</td>
</tr>
</tbody>
</table>
3.2. Guanidine HCl

Guanidine HCl was administered at 400 mg/kg/d for 5 days b.i.d. Only one of four mice in the treatment group mice was protected. However, this is compared to the same survival rate in the placebo group (1/4). Guanidine was also combined with 2-2(2-Pyridyl)benzimidazole (200/20 mg/kg/d)(Table 1). Administration of this combination for 5 days b.i.d. failed to provide any protection.

3.3. Suramin

Suramin was initially administered at 100 mg/kg/d for 5 days b.i.d. and resulted in 50% (2/4) protection. Mice that died did not experience appreciable weight loss until day 10, 5 days after treatment had ended and within the normal timeframe for onset of disease in this model (Fig. 1). This suggested that suramin was not toxic at this dose. In a follow-up study, mice were treated with 300 mg/kg/d of suramin. All mice died by day 5, well before the normal onset of disease for EV-71 MP10 (day 9-14).

An additional study treated mice with 100 mg/kg/d of suramin for 10 days. All of the mice in the treatment group died by day 9, with death occurring as early as day 4. Significant weight loss was only observed in one mouse. However, the mice failed to gain weight, whereas control and placebo-treated mice consistently gain weight from four to five weeks of age. A final study using a lower dose (50 mg/kg/d; 5 days b.i.d) a provided only slight protection (1/4). All mice succumbed to disease on day 10 or later (Fig 1.)
Fig. 1. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with Suramin. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. The 50 and 100 mg/kg/d groups were infected with stock 3 virus (10^{6.8} CCID_{50}/ml), and the 300 mg/kg/d group was infected with stock 2 virus (10^{6.7} CCID_{50}/ml). Mice received i.p. administration of either 50, 100 or 300 mg/kg/d of suramin twice daily for either 5 or 10 days. Survival (A) and percent body weight (B) are displayed here. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of treated groups to placebo. Changes in body weight were expressed as a percent relative to body weight on the first day of the study. *P<0.05
3.4. Fluoxetine

Several studies examined the effectiveness of fluoxetine for treating an EV-71 infection. Initial results showed that fluoxetine was protective when administered via i.p. or p.o. at 100 mg/kg/d (50%). The group treated with fluoxetine via p.o. also had an mean day of death (day 22), which differed significantly from placebo (day 13.5)(P<0.05; Mann Whitney t-test). Treatment with 30 mg/kg/d (i.p.) was not protective (Fig 2).

Mice in the treatment groups either failed to gain or lost weight by the end of the treatment period (although weight gained was observed after cessation of treatment). This differs from control mice, which consistently gained weight during this time (four- to five-weeks-old). In the placebo (vehicle control), only one mouse in the group treated via p.o. group exhibited weight loss, while those treated via i.p. steadily gained weight during the treatment period.

Follow-up studies involved treatment of mice once daily for 10 days (10 x q.d.) with fluoxetine to enhance effectiveness and to avoid toxicity. In all cases, fluoxetine failed to protect mice from mortality. Mice in the treatment groups either failed to gain weight or lost weight during the study (Fig. 2).

The 100 mg/kg/d treatment of fluoxetine (5 x b.i.d.) via the i.p. route was repeated to confirm the original findings. In this study, all mice succumbed to disease (0/4), with a mean day of death of 13.25 d.p.i. These results varied from the first study using this treatment regimen, where we observed 50% (2/4) protection of mice, and the surviving mice had a mean day of death of 22.
Fig. 2. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with Fluoxetine. Four-week-old AG129 mice (n=4) were infected with 0.2 ml \(10^{6.7}\) CCID\(_{50}\)/mouse of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received with 0.1 ml either 30 or 100 mg/kg/d of Fluoxetine for either 5 or 10 days via the i.p. or p.o. routes. Treatments were administered either once or twice daily. Given that this figure is a compilation of several studies, only one treatment regimen was selected to represent the placebo treated mice. Placebo treated mice received 0.1 ml of vehicle (10% DMSO + 10% Cremophor\(^\text{®}\) EL in PSS) via p.o. once daily for 10 days. Survival (A) and percent body weight (B) are displayed here. One study (100 mg/kg/d (p.o.; 5 x b.i.d)) was extended to 27 day in order to observe disease. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of treated groups to placebo. \(P<0.05\)
To determine the cause of the observed variability in the previous studies, a commercially solubilized fluoxetine product, Fluoxetine Oral Solution, was used to treat mice infected with EV-71. Mice were treated at different doses (22, 44, 75 and 88 mg/kg/d) and volumes (0.2 ml) than in previous studies due to the comparatively low concentration of Fluoxetine Oral Solution (4 mg/ml). Only mice in the 44 mg/kg/d treatment group were protected (2/4), although one of the surviving mice experienced significant weight loss by day 21 p.i. and likely would have died had the study continued (Fig. 3). Fluoxetine protected one mouse (1/4) in the 22 mg/kg/d group. Only mice in the 22 mg/kg/d treatment group gained weight during the treatment period. Mice treated via i.p. (75 mg/kg/d) died by day 3 p.i.
Fig. 3. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with Fluoxetine Oral Solution. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10^6.7 CCID_{50}/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received 22, 44, 75, or 88 mg/kg/d of Fluoxetine Oral Solution via the p.o. route. All groups were treated twice daily, with the exception of the 22 mg/kg/d group, which were treated once daily. Placebo treated mice received 0.2 ml of PSS via the same route. Survival (A) and percent body weight (B) are displayed here. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of treated groups to placebo. *P<0.05
3.5. STF1019

The compound STF1019 was administered at 200, 150, 100, and 50 mg/kg/d for 5-days b.i.d. via p.o. route. A dose response was observed, with survival in each treatment group as follows: 87.5% (7/8) in the 200 mg/kg/d group, 75% (6/8) in the 150 mg/kg/d group, 25% (2/8) in the 100 mg/kg/d group, and 62.5% (5/8) in the 50 mg/kg/d group (Fig. 4). Mice in the 200 and 150 mg/kg/d treatment groups showed weight loss early in the study, although all but one of the surviving mice gained weight (relative to day 0) by the end of the study.

Differences in survival were observed between placebo and treatment groups. Average neurological scores in the placebo group were first observed day 8 p.i., returned to baseline on day 13 (after 7 of 8 placebo-treated mice succumbed to disease), and reappeared on day 18 in a single mouse (which was subsequently euthanized) (Fig. 5).

Neurological scores in the 50 and 100 mg/kg/d groups were first observed on day 10 p.i. and peaked on days 15 and 17 p.i., respectively (Fig. 5). In the 150 mg/kg/d group, neurological scores were first observed on day 13 and were not observed again until day 19 p.i. Neurological scores were not observed in the 200 mg/kg/d group.
Fig. 4. Survival and Percent Body Weight of Mice Infected with EV-71 and Treated with STF1019. Groups of AG129 (n=8) mice were infected with 0.2 ml (10⁶.⁵ CCID₅₀/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 1) via the i.p. route. Mice were treated b.i.d. for 5-days per os with doses of STF1019 shown above, starting at 4h p.i. Placebo-treated mice received a vehicle control on the same schedule. Mice treated with IVIG received a single administration at 100 mg/kg via the i.p. route at h 4h p.i. A dose response was observed in survival following treatment with STF1019. Survival (A) and percent body weight (B) are displayed here. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of treated groups to placebo. *P<0.05, **P<0.01.
Fig. 5. Neurological Scores for Mice Infected with EV71 and Treated with STF1019. Groups of AG129 mice (n=8) were infected with 0.2 ml (10^{6.5} CCID\textsubscript{50}/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 1) via the i.p. route. Mice were treated b.i.d. for 5-days per os with doses of STF1019 shown above, beginning at 4h p.i. Placebo-treated mice received a vehicle control on the same schedule. Mice were observed daily for neurological signs and assigned a neurological score (1-4). Group neurological scores were averaged for each day.

3.6. STF434

The compound STF434 was administered at 100, 30, and 10 mg/kg/d for 5-days b.i.d. via i.p. Survival in each treatment group as follows: 37.5% (3/8) in the 100 mg/kg/d group, 50% (4/8) in the 30 mg/kg/d group, and 37.5% (3/8) in the 10 mg/kg/d group (Fig. 6).

Neurological scores in the placebo group were first observed on day 10 p.i., peaked on day 11 p.i., and returned to baseline on day 14, once all mice had succumbed to disease (Fig. 7). Neurological scores in the 30 and 10 mg/kg/d groups were first observed on day 8 p.i., peaked on day 11, and were no longer observed by day 15 and 17.
p.i. Neurological scores for the 100 mg/kg/d group were first observed on day 10 p.i., peaked on day 12, and were no longer observed after day 17.

Fig. 6. Survival of Mice Infected with EV-71 and Treated with STF434.
Groups of eight AG129 mice were infected with 0.2 ml (10^6.5 CCID_{50}/mouse) of a 1:1 combination of plaque 4 and 6 (stock 1) via the i.p. route. Mice were treated b.i.d. for 5-days via the i.p. route with doses of STF434 shown above, starting at 4h p.i. Placebo-treated mice received a vehicle control on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of treated groups to placebo. *P<0.05
Fig. 7. Neurological Scores for Mice Infected with EV-71 and Treated with STF434. Groups of eight AG129 mice were infected with 0.2 ml ($10^{6.5}$ CCID$_{50}$/mouse) of a 1:1 combination of plaque 4 and 6 (stock 1) via the i.p. route. Mice were treated b.i.d. for 5-days via the i.p. route with doses of STF434 shown above, starting 4h p.i. Placebo-treated mice received a vehicle control on the same schedule. A dose response was observed in survival following treatment.

4. Discussion

Multiple antivirals and therapeutic compounds were screened for effectiveness against EV-71 infection. Dosages were based on amounts reported in the literature as well as the solubility of the compounds. Compounds were selected based on a variety of criteria. Aurintricarboxylic acid (ATA), heparin, amantadine, bovine lactoferrin, rutin hydrate, N$_6$-benzyladenosine, 17-AAG, and quinacrine were selected based on their activity against EV-71 in vitro (Arita et al., 2008b; Chen et al., 2008c; Hung et al., 2010; Lin et al., 2002b; Lin et al., 2012a; Pourianfar et al., 2012; Tan et al., 2013; Tsou et al., 2013; Wang et al., 2013). Suramin and lycorine were selected based on their
effectiveness at inhibiting EV-71 infections in vivo (Liu et al., 2011a; Ren et al., 2014a). Intravenous immunoglobulin was selected based on its use in treating EV-71 infections in clinical practice. Guanidine, pleconaril, rupintrivir, pirodavir, and ribavirin were selected for their broad-spectrum and anti-picornavirus activity (Wang et al., 1999). The compounds 2-(2-Pyridyl)benzimidazole and 5-Hydroxymebendazol were selected based on their similarity to 2-(α-hydroxybenzyl)-benzimidazole (HBB), a picornavirus inhibitor (De Palma et al., 2008). Gemcitabine was selected based on its similarity to another antiviral, carbodine, as well as its effectiveness in vitro, including a synergistic effect with ribavirin (Deng et al., 2014). Naproxen sodium was selected due to the role of inflammation in EV-71 pathology (Khong et al., 2011; Yu et al., 2017). STF1019 and STF434 were specifically developed by Dr. Jeffrey Glenn (Stanford University) to treat EV-71 infections. Two of these drugs are already FDA approved to treat a variety of conditions: gemcitabine (cancer) and suramin (African sleeping sickness and river blindness)(Kang et al., 2015). Dosages and treatment schedules were based on previously published animal treatments and toxicity levels.

Most compounds were ineffective in mice, providing no protection from mortality (Table 1). Compounds that provided limited protection (25-50%) were guanidine HCl, suramin, naproxen sodium, and fluoxetine.

Guanidine protected only 25% (1/4) of mice, and a drug combination study with 2-(2-Pyridyl)benzimidazole failed to provide any protection. We therefore conclude that guanidine is not an effective treatment in this model (Table 2).

Suramin initially showed protection (2/4) when given at 100 mg/kg/d for five days b.i.d. (Fig. 1). Treatments at 50 mg/kg/d and 300 mg/kg/d failed to provide
additional protection. All mice in the 300 mg/kg/d group died by day 5 p.i., suggesting that suramin is toxic at that dose. Mice treated with 100 mg/kg/d of suramin for 10 days failed to gain weight, whereas control and placebo-treated mice consistently gain weight. This suggests that suramin may be toxic when treated for 10 days at this dose.

Ultimately, these observations suggest that suramin can provide some protection when treating an EV-71 infection in mice. However, given the relatively low dose showing toxicity compared to the most effective therapeutic dose, this drug appears to have a very narrow range of effectiveness. As such, it was concluded that suramin was not a practical antiviral to use in this model.

Fluoxetine protected 50% of the mice when administered at 100 mg/kg/d for 5 days b.i.d. via both i.p. and p.o. routes (Fig. 2). For mice treated via p.o., fluoxetine appeared to delay the mean day of death (22 d.p.i.) compared with placebo (10.5 d.p.i.). Extending the treatment period to ten days failed to protect any of the mice.

Potential toxicity was observed in this study, as mice often lost or failed to gain weight during the treatment period. The dilution vehicle (10% DMSO + 10% Cremophor® EL in PSS) was considered a possible source of toxicity, especially since weight loss during the treatment period was observed in the 100 mg/kg/d groups (i.p. and p.o., 10 x q.d.), although not in the 30 mg/kg/d group (p.o. 10 x q.d.). Oral administration of fluoxetine was reduced to once daily to limit the exposure to the vehicle. However, this did not alleviate the suppressed weight gain in the mice.

Fluoxetine Oral Solution was protective in the 22 and 88 mg/kg/d group (25% and 50%, respectively) (Fig. 3). However, two follow-ups studies with 75 mg/kg/d via both the i.p. and p.o. routes failed to provide protection. The administration of 75 mg/kg/d
(i.p.) of Fluoxetine Oral Solution was highly lethal and killed all mice in the treatment groups.

Ultimately, this suggests that while fluoxetine may provide some protection when treating an EV-71 infection in mice, it is not a practical therapeutic for this model. Given the toxicity and lack of reproducibility, additional fluoxetine studies were not pursued.

STF1019 showed protection at all doses administered, with 87.5% protection observed at the highest dose (200 mg/kg/d group)(Fig. 4). Mice in the 200 and 150 mg/kg/d experienced some weight loss at the beginning of the study, suggesting toxicity from the drug. This confirms observations made in an earlier maximum tolerated dose study (data not shown). This suggests that 200 mg/kg/d is approaching the limit at which this drug can be safely administered to mice.

Mice in the 200 mg/kg/d treatment group did not exhibit any neurological signs, while mice in all other treatments groups did show neurological signs (Fig. 5). The 150 mg/kg/d treatment group had two mice that exhibited late onset of neurological signs (19 and 20 d.p.i.). Both mice survived to the end of the study (day 21 d.p.i.), although both experienced weight loss. It is uncertain if these mice would have succumbed to disease had the study continued. However, this also suggests that STF1019 may have delayed the onset of disease and that a longer treatment regimen may have provided additional protection. This may also allow the drug to be administered at a lower dose for a longer period of time. It should be noted that neurological scores can only be calculated for surviving mice. Given that the number of mice used to calculate the mean neurological score change with the death of each mouse, neurological scores provide an indication of disease course and severity, but mean scores between groups must be evaluated carefully.
For example, some mice succumb to disease without exhibiting any neurological signs. Nevertheless, a comparison of the mean neurological score for each group found a significant difference between the 200 mg/kg/d and placebo groups ($P<0.05$), but no other groups.

STF434 showed partial protection at all doses administered via i.p., with 50% protection observed in the 30 mg/kg/d group (Fig. 6). Mean neurological scores in the 10 mg/kg/d group significantly differed from those for the placebo group ($P<0.01$). However, mice in all treatment groups exhibited neurological signs (Fig. 7). Furthermore, STF434 was not effective when administered via the p.o. route (Table 1), suggesting that the drug may not be bioavailable by that route.

In conclusion, we evaluated over twenty compounds for treatment of an EV-71 infection, with several of these compounds being tested at different doses as well as different treatment schedules and routes of administration. Of these, fluoxetine, suramin, STF1019, and STF434 were the most promising. Fluoxetine and suramin failed to produce consistent protection, with the highest protection observed in any group being 50%. Furthermore, increasing the dose in hopes of providing higher protection is not feasible given the toxicity of these drugs. As such, these compounds were not considered to be effective in this model. STF1019 and STF434 both provided protection and relatively low toxicity at the doses administered. STF1019 is especially promising given the high survival rate in the 400 mg/kg/d treatment group (7/8). What’s more, these compounds are novel host response modifiers, suggesting that this class of drugs, and not those targeting viral proteins, may hold the most promise in treating EV-71 infections. We conclude that future studies should be performed to assess STF1019 and STF434.
CHAPTER V
EVALUATION OF INTRAVENOUS IMMUNOGLOBULIN AS TREATMENT FOR AN EV-71 INFECTION IN MICE

ABSTRACT

Intravenous immunoglobulin (IVIG) is a therapeutic pool of antibodies from plasma donors approved to treat a variety of conditions, including immunodeficiencies, Kawasaki disease, chronic lymphocytic leukemia, and pediatric HIV infection. It has also been used off-label to treat a variety of conditions, including enterovirus 71 (EV-71) infection. However, data regarding the use of IVIG to treat EV-71 is contradictory, with some studies suggesting IVIG is effective and others that it is not. Here we examined the effectiveness of IVIG in treating EV-71 infections in four-week-old AG129 mice. We found that a single administration of IVIG at 4h post-infection (p.i.) could provide over 50% protective, and IVIG administration could be delayed until 48h p.i. and still provide protection, although administration at 72h p.i. was not protective. High-dose (2000 mg/kg/d) IVIG treatment prior to or at the onset of disease (day 7, 8, or 9 p.i.) was not protective. Variability was observed between IVIG treatments, suggesting that the timing for IVIG administration may be crucial. However, neutralizing EV-71 antibodies were confirmed in the lots of IVIG used in these studies, and IVIG was shown to reduce viral tissue titers and cytokine levels in infected mice. Given these results, we submit that further assessment of IVIG as a treatment for EV-71 is warranted.

1. Introduction

Enterovirus 71 (EV-71) is a non-enveloped, single-stranded RNA virus in the Picornaviridae family. Intravenous immunoglobulin (IVIG), a pooled antibody product
from plasma donors, has been used to treat EV-71 infections off-label. IVIG has a precedent of being used to treat infectious disease, including HIV, parvovirus B19, West Nile, and coxsackievirus infections (Wang et al., 2006b). It is suspected that the donor population contains EV-71 neutralizing antibodies that provide passive immunity when administered to an infected patient. Indeed, EV-71 neutralizing antibodies have been confirmed in IVIG lots (Cao et al., 2010; Cao et al., 2011; Wu et al., 2013). Furthermore, IVIG has shown effectiveness in preventing mortality and lowering serum cytokine levels in EV-71 patients (Ooi and Solomon, 2014; Wang et al., 2006b; Wang et al., 1999). However, large, randomized control trials have yet to be performed (Chea et al., 2015), meaning that most of the evidence for IVIG effectiveness comes from analyses of compiled clinical data, which sometimes fails to mention crucial parameters such as dose of IVIG. Only a few studies have involved assessing the effect of IVIG in mice infected with EV-71, but these studies did not use doses comparable to those used in the clinic. Furthermore, several reports of clinical data suggest that IVIG may be effective treatment for EV-71 infection (Bhatt et al., 2012; Shang et al., 2013; Wang et al., 2006a), while other reports suggest it is ineffective (Bhatt et al., 2012; Chong et al., 2003; McMinn, 2002; Nolan et al., 2003; Wang et al., 2006a). As such, crucial questions about the efficacy and proper administration of IVIG need to be answered.

In addition to neutralizing antibodies, IVIG possesses anti-inflammatory properties (Nimmerjahn and Ravetch, 2007). It has been used to treat inflammatory conditions such as chronic inflammatory demyelinating polyneuropathy and Kawasaki disease (Anthony et al., 2008). In line with this, IVIG has been shown to reduce pro-inflammatory cytokines in patients (Anthony et al., 2008). Given that EV-71 infection causes meningitis and
encephalitis (Ooi and Solomon, 2014), it is suspected that these anti-inflammatory properties may have a significant role in helping patients recover.

The development of an EV-71 mouse model in four-week-old AG129 mice at the Institute for Antiviral Research allows for potential EV-71 therapeutics to be tested. Here we examine the effectiveness of IVIG in treating an EV-71 infection in this model. A variety of factors are examined, including dose, time and route of administration, and effect on pro-inflammatory cytokines.

2. Materials and Methods

2.1. Viruses and Cell Lines

Enterovirus 71 MP10 plaque 4 and 6 from previous model development were used (Chapter III). Human rhabdomyosarcoma cells (RD) were obtained from ATCC®.

2.2. Cell Culture Media

RD cells were grown in minimum essential media (MEM) (GE Healthcare Hyclone™, Logan, UT) with 5% fetal bovine serum (FBS) (GE Healthcare Hyclone™, Logan, UT). RD cells were cultured in MEM with 2% FBS. Media for virus titration, tissue homogenization, and virus neutralization assay, was MEM with 2% FBS and 50 µl gentamicin (GE Healthcare Hyclone™, Logan, UT). MEM media (no additives) was used for animal infections and sham infections.

2.3. Virus Titration (CCID_{50}) Assay

Tissue homogenate or virus media were sequentially diluted in MEM 2% + gentamycin. Dilutions were then transferred to a 96-well microplate seeded with RD cells 24h prior to infection. Dilutions of virus were added in quadruplicate. Plates were then incubated at 37 °C and 5% CO₂ for 6 days. Plates were examined visually on days 3 and 6 p.i. for
cytopathic effect. The 50% cell culture infectious dose (CCID\textsubscript{50}) was determined using an endpoint dilution method. Virus titers were expressed as CCID\textsubscript{50} units per ml.

2.4. IVIG Products

Carimune\textsuperscript{®} NF was purchased from CSL Behring (Bern, Switzerland). Gammunex\textsuperscript{®}-C was obtained from Grifols (Los Angeles, CA).

2.5. Dose Titration of IVIG

Four-week-old AG129 mice were infected with 0.2 ml of either plaque 4 or a 1:1 combination of plaque 4 and plaque 6 (stocks 1 and 2). Mice received a single administration of 0.1 ml of Carimune\textsuperscript{®} NF or Gammunex\textsuperscript{®}-C at various doses across several studies (100, 200, 400, 800, 1300, or 2000 mg/kg/d) at 4h p.i. Doses for each study are specified in the corresponding results and figures. Placebo-treated mice received 0.1 ml of PSS on the same schedule.

2.6. Time of Administration of Carimune\textsuperscript{®} NF

Four-week-old AG129 mice were infected with 0.2 ml of a 1:1 combination of plaque 4 and plaque 6 (stock 2)(10\textsuperscript{6.7} CCID\textsubscript{50}/mouse). All mice received a single administration of 400 mg/kg/d of Carimune\textsuperscript{®} NF. For prophylactic administration, mice were treated at 72, 48, or 24h pre-infection, or at 4h post-infection. Placebo-treated mice received 0.1 ml of PSS at 4h p.i.(for comparison to previous studies). For post-infection administration, mice were treated at 1, 2, 4, 24, 48, or 72h p.i. Placebo-treated mice received 0.1 ml of PSS on the same schedule. Mice were also treated at the time of infection (approx. 15 min. p.i.).

2.7. Route of Administration of Carimune\textsuperscript{®} NF
Four-week-old AG129 mice were infected with 0.2 ml of a 1:1 combination of plaque 4 and plaque 6 (stock 2). Mice received a single administration of 400 mg/kg/d of Carimune® NF via the intraperitoneal (i.p.), intravenous (i.v.), subcutaneous (s.c.), or intramuscular (i.m.) routes. Placebo-treated mice received 0.1 ml of PSS via i.v. or i.m.

2.8. Dose Titration of Intravenous Administration of Carimune® NF

Four-week-old AG129 mice were infected with 0.2 ml of a 1:1 combination of plaque 4 and plaque 6 (stock 2). Mice received a single administration of 400, 200, 100, or 50 mg/kg/d of Carimune® NF via the i.v. route at 4h p.i. Placebo-treated mice received 0.1 ml of PSS via the same route.

2.9. Comparison of Carimune® NF Lots

Four-week-old AG129 mice (n=4) were infected with 0.2 ml of a 1:1 combination of plaque 4 and plaque 6 via the i.p. route. Mice received a single-administration of 400 mg/kg/d (0.1 ml) of Carimune® NF at 4h p.i. Mice received Carimune® NF prepared from one of two lots: Lot 1 (LOT: 4301801236; 7 April 2017) and Lot 2 (LOT: 4301801250; 3 Nov 2017). Placebo treated mice received 0.1 ml of PSS using the same dosing regimen.

2.10. Virus Neutralization Assay

Carimune® NF and Gammunex®-C were serially diluted in two-fold dilutions and then incubated with an equivalent volume of EV-71 MP10 Plaque 4 and EV-71 MP10 Plaque 6 (1:1; diluted to 1:2500 in MEM) for 1h. Incubated media was then added to a 96-well microplate seeded with RD cells 24h prior. Plates were incubated at 37 °C and 5% CO₂ and examined at day 3 p.i. for cytopathic effect.

2.11. Time Course of Infection
Groups of four-week-old AG129 mice were infected with 0.2 ml \(10^{6.7}\) 
CCID\(_{50}\)/mouse) of a 1:1 dilution of plaque 4 and plaque 6 (stock 2) via the i.p. route. 
Mice received a single administration (0.1 ml) of 400 mg/kg/d of Carimune\textsuperscript{®} NF (n=4) at 
4h p.i. Placebo-treated mice received 0.1 ml of PSS on the same schedule (n=3). Mice were sacrificed on days 1, 3, 5, 7, and 9 p.i. Blood, brain, spinal cord, lung, liver, spleen, 
kidney, and rear limb muscle were necropsied, homogenized in MEM, and stored at -80 \(^\circ\)C. Blood was collected in K\(_2\) EDTA collection tubes.

2.12. Lung Cytokine/Chemokine Evaluations

Samples of brain and spinal cord homogenate from the time course of infection study were analyzed for cytokines and chemokines using a chemiluminescent ELISA-based assay according to manufacturer’s instructions (Quansys Biosciecnes Q-Plex\textsuperscript{™} Array, Logan, UT). Each sample was examined for the following: IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFN-\(\gamma\), TNF\(\alpha\), MIP-1\(\alpha\), GM-CSF, and RANTES. 

\textit{Definition of abbreviations are: IL - interleukin; MCP - monocyte chemoattractant protein; IFN - interferon; TNF - tumor necrosis factor, MIP – macrophage inflammatory protein; GM-CSF - granulocyte/macrophage colony stimulating factor; and RANTES - regulated upon activation, normal T cell expressed and secreted.}

2.13. Histopathology of Mice Treated with Carimune\textsuperscript{®} NF

Four-week-old AG129 mice were challenged with \(10^{7.3}\) CCID\(_{50}\)/ml of Plaque 4 (Stock 3) virus (0.2 ml) via the i.p. route. Mice received a single administration of 400 mg/kg/d of Carimune\textsuperscript{®} NF (0.1 ml) via the i.p. route at 4h p.i. Placebo mice received 0.1
ml of PSS on the same schedule. Mice were transcardially perfused and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) on days 5, 7, and 9 p.i. Mice were stored in 4% PFA for at least 24h prior to necropsying. Brain, spinal cord, and leg muscle were then removed for histologic analysis.

2.14. Ethical Treatment of Animals

This study was completed under the approval of the Institutional Animal Care and Use Committee of Utah State University. The work was performed in the AAALAC-accredited Laboratory Animal Research Center of Utah State University in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 2011).

2.15. Statistical Analysis

Statistical analysis was performed using Prism 7, Graph Pad Software (San Diego, CA). Kaplan-Meier survival curves were compared using a Gehan-Breslow-Wilcoxon test. Differences in viral tissue titer on different days p.i. were compared using a two-way analysis of variance (ANOVA) followed by Sidak’s multiple comparison.

3. Results

3.1. Carimune® NF Initial Dose Titration

An initial dose titration of Carimune® NF was performed with 400, 200, 100, and 50 mg/kg/d doses. Mice received a single administration of Carimune® NF at 4h p.i. (Fig. 1). While a dose-dependent response was not observed, all mice in the 400 mg/kg/d treatment group survived (4/4)(P>0.05), while 50% (2/4) of mice in the 200, 100, and 50 mg/kg/d treatment groups survived. Weight loss was not observed in mice treated with Carimune® NF.
Fig. 1. Survival of Mice Infected with EV-71 and Treated with Four Different Doses of Carimune® NF. Groups of four-week-old AG129 mice (n=4) with 0.2 ml (10^{6.8} CCID\textsubscript{50}/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 1) via the i.p. route. Mice received 0.1 ml of either 400, 200, 100, or 50 mg/kg/d of Carimune® NF via the i.p. route at 4h p.i. Placebo treated mice (n=3) received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. *P<0.05

3.2. Post-Infection Time of Administration (4, 24, 48, and 72h)

Mice were treated with 400 mg/kg/d of Carimune® NF at 4, 24, 48, or 72h p.i.

Carimune® NF treated mice exhibited 50% protection (2/4) at 4, 24 and 48h p.i. (Fig. 2).

It was ineffective at protecting mice treated at 72h p.i.
Fig. 2. Survival of Mice Infected with EV-71 and Treated with Carimune® NF at Four Different Timepoints p.i. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10^6.5 CCID\textsubscript{50}/ml) of a 1:1 combination of plaque 4 and plaque 6 (stock 1) via the i.p. route. Mice received 0.1 ml of either 400mg/kg/d of Carimune® NF via the i.p. route at either 4, 24, 48 or 72h p.i. Placebo treated mice (n=4) received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. None of the treatment groups were statistically significant.

3.3. Pre-Infection Time of Administration (72, 48, and 24h)

All treatments were statistically significant compared with placebo (P<0.05). The 72h and 24h pre-infection groups were completely protected (4/4). In addition, the 48h pre-infection (3/4) and 4h p.i. (2/4) groups were also protected (Fig. 3). These data show that all treatments were statistically significant compared with placebo (P<0.05).
Fig. 3. Survival of Mice Administered Prophylactic Carimune® NF and Infected with EV-71. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10^6.5 CCID_{50}/ml) of a 1:1 combination of plaque 4 and plaque 6 (stock 2). Mice received 0.1 ml of either 400mg/kg/d of Carimune® NF via the i.p. route at either 4, 24, 48 or 72h p.i. Placebo treated mice (n=4) received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. *P<0.05

3.4. High Dose Carimune® NF Treatment

Mice were treated with a high dose of Carimune® NF (2000 mg/kg/d) at either day 7, 8, or 9 p.i. None of the mice survived the infection (0/4)(Fig. 4).
Fig. 4. Survival of Mice Infected with EV-71 and Treated with High Dose Carimune® NF. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10⁶.⁵ CCID₅₀/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice were treated with 0.3 ml of 2000 mg/kg/d of Carimune® NF at 7, 8, or 9 d.p.i. Placebo treated mice received PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. None of the treatment groups were statistically significant.

3.5. Route of Administration

Mice were treated with 400 mg/kg/d of Carimune® NF via the i.v., i.m., s.c., and i.p. routes. The protection observed in the groups is as follows: i.v. (4/4), i.p. (3/4), s.c. (2/4), and i.m. (2/4). Only the i.v. administration group was statistically significant (P<0.05)(Fig. 5).
Fig. 5. Survival of Mice Infected with EV-71 and Treated with Carimune® NF via Different Routes of Administration. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.v., i.m., s.c., or i.p. routes. Mice received a single administration of Carimune® NF via the i.v., i.m., s.c., or i.p. routes at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule via the i.v. or i.m. route (results are combined). A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. *P<0.05

3.6. Dose Titration of Intravenous Administration of Carimune® NF

Mice were treated with 400, 200, 100, and 50 mg/kg/d of Carimune® NF via the i.v. route. Protection was observed in the following groups: 75% (3/4) in the 400 mg/kg/d group, 50% protection in the 200 mg/kg/d group, and 25% protection in the 100 and 50 mg/kg/d groups. None of the treatment groups were statistically significant compared to placebo. This varies from the previous study that examined i.v. administration of Carimune® NF, in which 100% (4/4) of mice were protected from disease at the same dose (Fig. 6).
Fig. 6. Survival of Mice Infected with EV-71 and Treated with Carimune® NF via the Intravenous Route. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10⁶.7 CCID₅₀/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration 0.1 ml of 400, 200, 100, or 50 mg/kg/d of Carimune® NF at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. None of the treatment groups were statistically significant compared to placebo.

3.7. Gammunex®-C Dose Titration

The effectiveness of IVIG across different brands was evaluated. As the composition is IVIG is dependent on both the donor population and production methods, it was important to determine if the effectiveness of IVIG drastically differed across brands. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml of a 1:1 combination of plaque 4 and 6 via the i.p. route. Mice received a single administration of 400, 200, 100, or 50 mg/kg/d doses of Gammunex®-C via the i.p. route (Fig. 7). The 400 mg/kg/d dose protected 50% (2/4) of the mice, and the 200 and 100 mg/kg/d doses
protected 25% (1/4) of the mice. This was not found to be significant when compared to placebo.

Fig. 7. Dose Titration of Gammunex®-C for an EV-71 Infection in Mice. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10^{6.7} CCID_{50}/mouse) of a 1:1 combination of plaque 4 and 6 (stock 2) via the i.p. route. Mice received a single administration of 400, 200, 100, or 50 mg/kg/d doses of Gammunex®-C via the i.p. route at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. *P<0.05

3.8. Carimune® NF Lot Comparison

Mice were treated with the two lots of Carimune® NF to compare their effectiveness in treating an EV-71 infection in vivo. The lot of Carimune® NF used for earlier studies was designated Lot 1 and the lot used for later studies was designated Lot 2. Two such studies were performed. In the first study, the number of mice protected was unusually low (1/4). Thus, the study was repeated, in which 75% (3/4) of the mice receiving the Lot 1 Carimune® NF were protected, compared with 50% (2/4) of the mice
Fig. 8. Comparison of Two Different Lots of Carimune\textsuperscript{®} NF Used to Treat an EV-71 Infection in Mice. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10\textsuperscript{6.7} CCID\textsubscript{50}/mouse) of a 1:1 combination of plaque 4 and 6 (stock 2). Mice received a single administration of 400 mg/kg/d of Carimune\textsuperscript{®} NF at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. There was no statistically significant difference between treatment groups.

were treated with Lot 2 (Fig. 8). It should be noted that one of the surviving mice treated with Lot 1 was exhibiting neurological signs (NS2) and weight loss by the end of the
study. A surviving mouse treated with Lot 2 was also experiencing some/minor weight loss, without neurological signs.

3.9. Virus Neutralization Assay

We compared two different lots of Carimune® NF for differences in neutralizing antibody content and for effectiveness in treating EV-71 infection in mice. A virus neutralization assay was performed to determine the virus neutralizing capacity of the two lots of Carimune® NF and Gammunex®-C against EV-71. Both Carimune® NF (0.125 mg/ml) and Gammunex®-C (0.625 mg/ml) were able to neutralize virus.

![Graph](image)

**Fig. 9. Survival of Mice Infected with EV-71 and Treated with Carimune® NF at 0h p.i.** A group of eight AG129 mice (n=8) were infected with 0.2 ml of a 1:1 combination of plaque 4 and 6 (stock 2) via the i.p. route. Mice received a single administration of 400 mg/kg/d of Carimune® NF at 0h p.i. Placebo treated mice (n=2) received 0.1 ml of PSS via the same route. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. **P<0.01

3.10. Post-Infection Administration (0h p.i.)
Four-week-old AG129 mice (n=8) received 400 mg/kg/d of Carimune® NF at 0h p.i. (about ~15 min p.i.). Seven of eight mice survived the infection (87.5%)(P<0.01)(Fig. 9).

3.11. Post-Infection Administration (1, 2, and 4h p.i.)

Carimune® NF (400 mg/kg/d) administration at 1, 2, and 4h p.i. was evaluated. Survival for each group is as follows: 1h (3/5), 2h (4/5), and 4h (3/5). All groups were statistically significant compared to placebo. There were no significant differences between groups.

![Graph showing survival rates of mice infected with EV-71 and treated with Carimune® NF at 1, 2, and 4h p.i.](image_url)

**Fig. 10. Survival of Mice Infected with EV-71 and Treated with Carimune® NF at 1, 2, and 4h p.i.** Groups of four-week-old AG129 mice (n=5) were infected with 0.2 ml of plaque 4 (stock 3) via the i.p. route. Mice received a single administration of 400 mg/kg/d of Carimune® NF at either 1, 2, or 4h p.i. Placebo treated mice (n=2) received a single administration of PSS at 1h p.i. via the same route. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve compared to placebo (shown above). There was no significant difference between treatment groups. *P<0.05, **P<0.01
3.12. Dose Titration of Carimune® NF

Three dose-titrations were performed, two with larger groups of mice (n=8 or 10)(Fig. 11-13). The first dose titration was performed using 100, 200, 400, and 1300 mg/kg/d doses of Carimune® NF (4h p.i.). The survival for each group (n=10) is as follows: 100 mg/kg/d (5/10), 200 mg/kg/d (7/10), 400 mg/kg/d (1/10), and 1300 mg/kg/d (6/10). Only the 200 mg/kg/d was significantly different from placebo ($P<0.01$)(Fig. 11).

![Dose Titration Graph](image)

**Fig. 11. Dose Titration of Carimune® NF (100 mg/kg/d-1300 mg/kg/d) for an EV-71 Infection in Mice.** Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml ($10^6.7$ CCID$_{50}$/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration 0.1 ml of 1300, 400, 200, or 100 mg/k/d of Carimune® NF at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. None of the treatment groups were statistically significant compared to placebo. **$P<0.01$**

The second dose titration (n=4) was performed using 400, 800, 1200, and 2000 mg/kg/d doses (4h p.i.). All treatment groups had 50% survival, although only the 800 and 1200 mg/kg/d groups were significantly different from placebo ($P<0.05$)(Fig. 12).
Fig. 12. Dose Titration of Carimune® NF (400-2000 mg/kg/d) for an EV-71 Infection in Mice. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml (10^6.7 CCID₅₀/mouse) of a 1:1 combination of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration 0.1 ml of 2000, 1200, 800, or 400 mg/k/d of Carimune® NF at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. *P<0.05

The final dose titration was completed using 200, 400, and 800 mg/kg/d doses of Carimune® NF. The survival for each group (n=9) is as follows: 200 mg/kg/d (6/9), 400 mg/kg/d (4/9), and 800 mg/kg/d (5/9)(Fig. 13). This study was extended to 30 days in order to observe several mice that had developed late-onset of paralysis and weight-loss. Given this appreciable delay in mortality, mean day of death was assessed for each group, and were as follows: 200 mg/kg/d (23.0), 400 mg/kg/d (17.6), 800 mg/kg/d (18.7), and
placebo (13.7). All groups were statistically significant compared to placebo as follows:

200 and 800 mg/kg/d ($P<0.01$), and 400 mg/kg/d ($P<0.05$).

Fig. 13. Dose Titration of Carimune® NF (200-800 mg/kg/d) for an EV-71 Infection in Mice. Groups of four-week-old AG129 mice (n=4) were infected with 0.2 ml ($10^{6.7}$ CCID$_{50}$/mouse) of plaque 4 (stock 3) via the i.p. route. Mice received a single administration 0.1 ml of 800, 400, or 200 mg/k/d of Carimune® NF at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule. The study was extended beyond the normal 21-day time period to observe mice with late onset of disease. A Gehan-Breslow-Wilcoxon test was used to compare the survival curve of each treated group to placebo-treated mice. *$P<0.05$, **$P<0.01$

3.13. Time Course of Infection

Viral titers in both the Carimune® NF and placebo treated groups followed a similar pattern (Fig. 14-15), with viral titers in the Carimune® NF treated groups consistently lower than titers in the placebo groups (although only a select number of time points had significant differences). Brain titers increased steadily and peaked on day
9. Spinal cord titers increased steadily in the placebo group and peaked on day 9, whereas titers in the Carimune® NF treated group peaked on day 5 p.i. and declined slightly day 9. Blood titers peaked on day 3 p.i. and declined to below the limit of detection by day 5 p.i. All other tissues peaked on a certain day p.i. (kidney and lung on day 5, and spleen and liver on day 3) and then steadily declined thereafter, often to below day 1 titers by day 9. The Carimune® NF group usually exhibited lower titers than the placebo groups, with exceptions being blood (day 3), kidney (day 3-9), and spleen (day 5). However, the only statistically significant difference between groups was observed on day 1 in the blood ($P<0.0001$).
Fig. 14. Time Course of Viral Titers for Brain, Spinal Cord, Leg Muscle, and Blood from Mice Infected with EV-71 and Treated with Carimune® NF. Groups of four-week-old AG129 mice were infected with 0.2ml (10^6.7 CCID<sub>50</sub>/mouse) of a 1:1 dilution of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration of 0.1 ml of 400 mg/kg/d of Carimune® NF (n=4) at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule (n=3). Mice were sacrificed on days 1, 3, 5, 7, and 9 p.i. Blood, brain, spinal cord, and leg muscle were collected. Viral tissue titers for these organs were determined using an end-point dilution assay. A two-way ANOVA followed by Sidak’s multiple comparison was used to compare treatment and placebo groups on each day of the study. ****P<0.0001
Fig. 15. Time Course of Viral Titers for Lung, Liver, Kidney, and Spleen from Mice Infected with EV-71 and Treated with Carimune® NF. Groups of four-week-old AG129 mice were infected with 0.2ml (10^6.7 CCID_{50}/mouse) of a 1:1 dilution of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration of 0.1 ml of 400 mg/kg/d of Carimune® NF (n=4) at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule (n=3). Mice were sacrificed on days 1, 3, 5, 7, and 9 p.i. Lung, liver, kidney, and spleen were collected. Viral tissue titers for these organs were determined using an end-point dilution assay. A two-way ANOVA followed by Sidak’s multiple comparison was used to compare treatment and placebo groups on each day of the study.

3.14. Cytokine Time Course

Significant differences were observed between the Carimune® NF and placebo-treated groups for the following cytokines: MCP-1, IFN-γ, MIP-1α, and RANTES (Fig.
16-17). Significance was dependent on the day p.i. as well as the tissue. For MCP-1, Carimune® NF treated mice exhibited a 7.5-fold lower concentration on day 9 in brain tissue and a 9-13-fold lower concentration on days 5, 7, and 9 in spinal cord tissue, compared to placebo. For IFN-γ, Carimune® NF treated mice exhibited a 2.5-fold lower concentration on day 9 compared to placebo. For MIP-1a, Carimune® NF mice exhibited a 2.5-6.5-fold lower concentration on day 5 and day 9 in spinal cord, compared to placebo. For RANTES, Carimune® NF treated mice exhibited a 12-fold lower concentration on day 9 in brain tissue, and a 6-6.5-fold difference on days 7 and 9 in spinal cord tissue, compared to placebo.
Fig. 16. Time Course for MCP-1 and IFN-γ in Mice Infected with EV-71 and Treated with Carimune® NF. Groups of four-week-old AG129 mice were infected with 0.2 ml (10^6.7 CCID_{50}/mouse) of a 1:1 dilution of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration of 0.1 ml of 400 mg/kg/d of Carimune® NF (n=4) at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule (n=3). Mice were sacrificed on days 1, 3, 5, 7, and 9 p.i. Blood, brain, spinal cord, lung, liver, spleen, kidney, and rear limb muscle were necropsied, homogenized in MEM + gent, and stored at -80 °C. Cytokine levels were determined using a chemiluminescent ELISA-based assay. A two-way ANOVA (Sidak) was used to compare treatment and placebo groups on each day of the study. *P<0.05, **P<0.01, ****P<0.0001
Fig. 17. Time Course for MIP 1-α and RANTES in Mice Infected with EV-71 and Treated with Carimune® NF. Groups of four-week-old AG129 mice were infected with 0.2 ml (10⁶.7 CCID₅₀/mouse) of a 1:1 dilution of plaque 4 and plaque 6 (stock 2) via the i.p. route. Mice received a single administration of 0.1 ml of 400 mg/kg/d of Carimune® NF (n=4) at 4h p.i. Placebo treated mice received 0.1 ml of PSS on the same schedule (n=3). Mice were sacrificed on days 1, 3, 5, 7, and 9 p.i. Blood, brain, spinal cord, lung, liver, spleen, kidney, and rear limb muscle were necropsied, homogenized in MEM + gentamycin, and stored at -80 °C. Cytokine levels were determined using a chemiluminescent ELISA-based assay. A two-way ANOVA (Sidak) was used to compare treatment and placebo groups on each day of the study. *P<0.05, ***P<0.001, ****P<0.0001
3.15. Histopathology

Lesion severity of brain, spinal, and leg muscle were assessed from mice necropsied on days 5, 7, and 9 p.i. Significant differences were not observed between Carimune® NF and placebo treated groups. The brain exhibited no lesions on days 5 and 7 p.i., and only minimal lesion on day 9 p.i. Lesions were only observed in the brainstem. In the spinal cord, lesions were only observed on days 7 and 9 p.i. Lesion severity in the leg muscle was highest on day 5 p.i. and decreased until lesions were absent or near-absent on day 9 p.i.

A variety of lesions and pathology were observed in the leg muscle, including steatitis, fasciitis, neutrophilic and mononuclear infiltration, myofiber degeneration. Pathology in the spinal cord included vacuolization, myelitis, mononuclear infiltration, neuronal necrosis, and gliosis. Pathology in the brain included encephalitis, neutrophilic infiltration, gliosis, and vacuolation.
Fig. 18. Leg Muscle Pathology at Day 7 p.i from a Mouse Infected with EV-71 and Administered Placebo. Groups of AG129 mice (n=5) were infected with EV-71 MP10 and administered either Carimune® NF (400 mg/kg/d) or placebo (PSS) at 4h p.i. Mice were sacrificed and transcardially perfused on days 5, 7, or 9 p.i. Brain, spinal cord, and leg muscle were collected, fixe in 4% PFA and submitted for histopathology. Images A and B are rear-limb leg muscle sections from an EV-71 infected, placebo treated mouse sacrificed on day 7 p.i. showing myofiber necrosis and inflammation. Arrows: Macrophage infiltration (A1), myofiber fragmentation (A2), macrophage and neutrophil infiltration (B1), and early myofiber regeneration (B2). Hematoxylin and eosin stain, 400x.
Fig. 19. Spinal Cord Pathology at Day 9 p.i from a Mouse Infected with EV-71 and Administered Placebo. Groups of AG129 mice (n=5) were infected with EV-71 MP10 and administered either Carimune® NF (400 mg/kg/d) or placebo (PSS) at 4h p.i. Mice were sacrificed and transcardially perfused on days 5, 7, or 9 p.i. Brain, spinal cord, and leg muscle were collected, fixed in 4% PFA, and submitted for histopathology. Images and A and B are a spinal cord section from a EV-71 infected, placebo treated mouse sacrificed on day 7 p.i., showing a monocellular and neutrophilic myelitis. Arrow: Perivascular cuffing with neutrophils and mononuclear inflammatory cell (A1), neutrophil vacuolization (A2 & C2), a digestion chamber with a gitter cell (B1), a swollen axon (B2), a necrotic neuronal cell body (C1), and reactive endothelial cells lining a blood vessel (C3). Hematoxylin and eosin stain, 400x.
Fig. 20. Brainstem Pathology at Days 9 and 11 p.i. from Mice Infected with EV-71 and Administered Placebo. Groups of AG129 mice (n=5) were infected with EV-71 MP10 and administered either Carimune® NF (400 mg/kg/d) or placebo (PSS) at 4h p.i. Mice were sacrificed and transcardially perfused on days 5, 7, or 9 p.i. Brain, spinal cord, and leg muscle were collected, fixed in 4% PFA, and submitted for histopathology. Images A and B are brainstem sections of an EV-71 infected, placebo treated mouse sacrificed on day 9 p.i. (A) and day 11 p.i. (B), showing a mononuclear and neutrophilic encephalitis. Arrows: Pervascular cuffing with neutrophils and mononuclear inflammatory cells (A1), neutrophil vacuolization (A2), and swollen axons (B1) are indicated. Hematoxylin and eosin stain, (A) 400x., (B) 200x.

Fig. 21. Adipose Tissue Pathology at Day 5 p.i. from a Mouse Infected with EV-71 and Administered Carimune® NF or Placebo. Groups of AG129 mice (n=5) were infected with EV-71 MP10 and administered either Carimune® NF (400 mg/kg/d) or placebo (PSS) at 4h p.i. Mice were sacrificed and transcardially perfused on days 5, 7, or 9 p.i. Brain, spinal cord, and leg muscle were collected, fixed in 4% PFA, and submitted for histopathology. Images and A and B sections of adipose tissue from a EV-71 infected, Carimune® NF treated (A) and placebo treated (B) mouse sacrificed on day 5 p.i. Neutrophilic steatitis is present in both animals (Arrows: A1 and B1). Hematoxylin and eosin stain, 400x.
4. Discussion

The initial dose titration of Carimune® NF suggested that it was protective at 400 mg/kg/d when administered once at 4h p.i. (Fig. 1). All mice in the treatment group were protected from mortality, disease signs, and appreciable weight-loss. Given the effectiveness of Carimune® NF when administered at 4h p.i., different treatment regimens were explored to more closely mimic how IVIG would be used in a clinical setting. While the treatment regimen for this model was normally at 4h p.i., this limited the clinical relevance of the studies, as patients infected with EV-71 are unlikely to seek treatment before symptoms are manifested. Thus, determining the range at which Carimune® NF can be administered effectively p.i. has important clinical applications.

Carimune® NF was 50% protective when administered at 24h and 48h p.i. (Fig. 2). However, these groups were not statistically different from placebo. Mice treated at 72h p.i. completely succumbed to disease, with nearly the same survival pattern as the placebo group. This suggests that Carimune® NF is not protective in this model when administered at 400 mg/kg at 72h p.i.

Prophylactic administration of Carimune® NF was also explored. Prophylactic administration is used to prevent infection or development of disease and may be used in protecting patients who have a high risk of exposure to EV-71 (Fuchs et al., 2013; Ng et al., 2015). Carimune® NF was protective when given at 4h (50%), 48h (75%), and 24 and 72h (100%) pre-infection (Fig. 3). These results were all statistically significant. While it is encouraging that Carimune® NF was protective at up to 72h pre-infection, it was also unexpected that Carimune® NF was only 50% protective at 4h p.i., where earlier studies had exhibited 100% effectiveness.
Given that EV-71 cases are likely to be treated only after the onset of symptoms, we treated mice with a high dose of Carimune® NF at or just before the usual earliest onset of disease (day 9). A 2000 mg/kg of IVIG is considered a high dose and is the highest dose reported in the literature (Sokkosal et al., 2015). While it is administered over the course of a day or several days in the clinic (Bartlett et al., 2012; Bhatt et al., 2012; Prasad and Chaudhary, 2014), we chose to examine if a rapid and large dose of IVIG could prevent disease. Yet despite the high dose of Carimune® NF, all the mice succumbed to disease (Fig. 4). This suggests that Carimune® NF is not effective in this model when administered at or just before the onset of disease (day 7, 8, or 9 p.i.), even at a high dose (2000 mg/kg/d). Finally, while i.v. or intrathecal (i.c.) routes of administration would have delivered the treatment directly into the bloodstream or CSF, it would not have been possible to treat at this high of dose in mice, given the volume (0.3 ml) needed.

Different routes of administration were also examined. As most therapeutic or antiviral treatments in this model are administered via the i.p. or p.o. routes, the drug was initially administered via i.p. Rarely is i.v. administration used for therapeutic or antiviral administration in mice, in part due to the difficulty in accessing the tail vein (Yardeni et al., 2011). However, given that Carimune® NF is formulated to be administered intravenously, we examined if this route could be more effective than i.p. administration. Furthermore, since EV-71 has been found to replicate in muscle tissue and is suspected to access the CNS via motor neurons (Ohka et al., 2009), i.m. administration was also considered. This is also a feasible route since immunoglobulin was originally administered via i.m. before changes in production made i.v. administration possible.
Finally, the s.c. route was assessed, as there are immunoglobulin formulas on the market that are designed to be delivered via the s.c. (Orange et al., 2006), rather than i.v., route. In the initial test, the group treated via i.v. had a higher survival than all other treatments groups and was statistically higher than the placebo group ($P<0.05$)(Fig. 7). This suggested that i.v. may be the ideal route for Carimune® NF administration in this model. It should also be noted this was another instance where Carimune® NF failed to completely protect mice when administered at 400 mg/kg via i.p., highlighting some of the variability observed with this treatment.

A follow-up study involved a dose titration of Carimune® NF administered via i.v. However, this study failed to show a dose response, with the 200 mg/kg/d group protecting 75% of the mice, and the 400 mg/kg/d group protecting only 25% (Fig. 8). Given the inconsistencies with i.v. administration, as well as the difficulty in performing i.v. treatments, this route was not pursued further, and the i.p. route became the standard route by which Carimune® NF was administered in this model.

As there are several IVIG brands available, we considered it important to determine if brands other than Carimune® NF could be effective at treating an EV-71 infection. Different donor pools and manufacturing methods could produce IVIG products with different compositions of EV-71 neutralizing antibodies. Gammunex®-C from Grifols Biomat was purchased and its effectiveness was evaluated with a dose titration. Gammunex®-C did provide some protection, although this study was limited by the fact that 25% (1/4) of mice in the placebo group survived the infection. Furthermore, we did not observe a dose response using Gammunex®-C, with the 400 mg/kg/d group
having the same survival as the placebo group (Fig. 10). The 200 mg/kg/d dose was the most protective at 75%.

It is unclear why a traditional dose response was not observed here. High doses of IVIG are considered anti-inflammatory, while low doses can be pro-inflammatory (Durandy et al., 2009; Shimoni et al., 2013). If a 200 mg/kg/d dose is indeed proinflammatory, this may serve to improve the initial immune response, thus providing protection for the mice. The importance of pro-inflammatory cytokines as part of the immune response during the early stages of infection is seen for other pathogens (Omer et al., 2003). However, given that a cytokine storm can occur in patients, increasing proinflammatory cytokine release via low-dose IVIG may not be desireable (Lin et al., 2003). Furthermore, this does not explain earlier results that show protection at 100 and 200 mg/kg/d. The results may also be due to differences in manufacturing between CSL Behring and Grifols Biomat, or different donors pools (although both companies only used donors from the United States). Ultimately, while this study suggests Gammunex®-C could be protective, it did not provide superior protection as compared to Carimune® NF and was therefore not used in future animal studies.

Regarding the mechanism of action of IVIG, neutralizing EV-71 antibodies are a suspected mechanism of action for IVIG. Several studies have confirmed the presence of such antibodies in IVIG (Cao et al., 2010; Cao et al., 2011; Wu et al., 2013). We determined the presence of such antibodies in Carimune® NF (two lots) and Gammunex®-C using a virus neutralization assay. Both Carimune® NF (0.125 mg/ml) and Gammunex®-C (0.625 mg/ml) were both able to neutralize EV-71. This is
presumably due to the presence of neutralizing antibodies and supports the notion that IVIG limits an EV-71 infection via this mechanism.

Given the volume of Carimune® NF that was used across several studies, it was necessary to purchase an additional bottle of Carimune® NF. However, given that Carimune® NF is derived from pool of donors, the composition of Carimune® NF can vary from lot to lot. The above virus neutralization assay confirmed that the two lots of Carimune® NF were comparable in terms of their EV-71 neutralizing capacity. To compare the therapeutic capacity of each lot, mice were treated with 400 mg/kg/d of Lot 1 (April 2017) and Lot 2 (Nov 2017)(Fig. 9). The date corresponding for each lot refers to the date at which the lot was purchased. Lot 1 was used in earlier studies of Carimune® NF and Lot 2 was used near the end the project. In the first trial, both lots provided the exact same protection (25%). This was unusually low effectiveness for Carimune® NF, given the initial study where the same dose provided 100% protection. The trial was repeated, with Lot 1 providing 75% protection and Lot 2 providing 50% protection. However, it should be noted that one of the surviving mice treated with Lot 1 was exhibiting neurological signs (NS2) and weight loss by the end of the study. However, there was no significant difference between groups. Ultimately, these studies suggest that the two lot of Carimune® NF are comparable in their effectiveness, including comparable variability when used to treat EV-71 in vivo. As such, treatments with different lots of Carimune® NF are not differentiated.

While Carimune® NF has shown to be effective in this model, the variability between treatments is of some concern. To determine the source of the variability for the Carimune® NF treatments, time of administration was considered. Beginning treatment at
four hours post-infection is a standard regimen for this model and for other models used at the IAR. However, we noticed that when Carimune® NF was initially screened, it was administered immediately after infection (approx. 10-15 min. p.i., represented as “0h” p.i.)(data not shown)(Fig. 5). A study was performed to determine if administering Carimune® NF immediately after infection would provide more consistent results, compared with a 4h p.i. administration. Mice treated at 0h p.i. with 400 mg/kg/d of Carimune® NF, 87.5% (7/8) were protected. Given the variability observed in previous studies, with Carimune® NF provided as low as 25% protection, these results suggest that administration immediately after infection may be required to produce consistent results with Carimune® NF. This study should be repeated in order to observe the consistency of IVIG across studies for this treatment regimen and the 0h p.i. treatment group should be compared to a 4h p.i. treatment group.

Treating immediately after infection with an immunoglobulin introduces the possibility that Carimune® NF is neutralizing the virus in the peritoneal cavity. Thus, it is possible that the virus is neutralized before gaining access to host cells. While this would certainly show that Carimune® NF could be effective at preventing infection, it calls into questions its effectiveness for treating an active infection, where the virus is replicating inside host cells. Therefore, to determine if administration of IVIG between 0 and 4h p.i. could be an effective alternative to 0h p.i. administration, a study was performed assessing Carimune® NF when administered at 1h, 2h, and 4h p.i. The group receiving Carimune® NF at 2h p.i. exhibiting the highest survival rate (4/5). This suggests that 2h p.i. could be the optimal time of administration for IVIG in this model (Fig. 6).
Three additional dose titrations were performed with Carimune® NF via the i.p route in addition to the initial dose titration. In addition, dose titrations using larger groups of mice (n=10 or 9) were performed. Larger groups of mice were used to ensure statistical significance and to compensate for the variability that has been observed in studies with smaller groups of mice. The first dose titration (n=10 or 11) examined Carimune® NF at 100, 200, 400, and 1300 mg/kg/d doses, with Carimune® NF administered at 4h p.i. Carimune® NF was protective at 100 (50%), 200 (70%), and 1300 (63.6%) mg/kg/d. However, it is unclear as to why the 200 mg/kg/d was slightly more protective than the 1300 mg/kg/d dose, and why the 400 mg/kg/d group had the same survival as the placebo group. This, combined with the previous studying examining high doses of Carimune® NF, these results suggest that high doses of Carimune® NF provide little or no additional benefit compared to smaller doses. It should also be noted that, due to a technician error, 0.1 ml of virus was administered, instead of the standard 0.2 ml. This decreased the infection dose from $10^{6.7}$ to $10^{6.4}$ CCID$_{50}$/ml. However, given that 90% of the placebo group succumbed to disease, we do not anticipate that this was the reason for the contradictory results shown above.

A second dose titration determined if a 2000 mg/kg/d dose administered at 4h p.i. could be protective. We observed 50% survival when mice were treated with any dose at 4h p.i. (Fig. 11). These results were not significantly different from placebo.

A final dose titration was performed (n=9) using 200, 400 and 800 mg/kg/d doses. Carimune® NF was administered at 2h p.i. instead of 4h p.i. (Fig. 12). Given that Carimune® NF administered at 2h p.i. was more protective than either 1h or 4h p.i., we employed the 2h p.i. administration here to evaluate consistency. A 2h p.i. administration
also avoids administering the treatment at nearly the same time as infection. The 800 mg/kg/d group exhibited the highest survival (6/9). This shows that at 2h administration of Carimune® NF is insufficient to provide complete protection. However, the deaths of several mice in the treatment groups occurred after the normal 21-day observation period, suggesting that Carimune® NF may have delayed mortality in some mice. However, the mean day of death for each group was not found to be significantly different from placebo.

We did not observe significant differences in lesion severity between Carimune® NF and placebo treated groups in brain, spinal cord, and leg muscle tissues. This suggests that the protective effect of Carimune® NF is not due to prevention or reduction of lesions in these tissues. However, histopathology did provide insight into the pathogenesis of EV-71 in this model. For example, lesions were only observed in the brainstem, which is consistent with EV-71 infection in humans, where the brainstem is the target organ (McMinn, 2002). Indeed, several post-mortem examinations have shown neutrophilic infiltration in the brainstem (McMinn, 2002). Furthermore, steatitis and infection of brown adipose tissue was observed. Infection of brown adipose tissue is observed in both murine and non-human primate models (Liu et al., 2011b; Ong et al., 2008; Tan et al., 2014b). This is an intriguing insight into EV-71 pathogenesis, as newborns and young children have the highest levels of brown adipose tissue, which declines with age (Sacks and Symonds, 2013). Brown adipose tissue is also highly vascularized (Betz and Enerback, 2015), which may permit infection of the tissue and/or allow the virus to spread after replication in the tissue. However, EV-71 infection of adipose tissue has yet to be observed in humans (Fujii et al., 2013).
Leg pathology resolved or nearly resolved by day 9 p.i. in the necropsied mice. This coincides with viral titers for leg muscle, which peak on day 3 p.i. and then steadily decline until day 9 p.i. Lesions on day 5 were (on average) moderate, without myositis and two instances of necrosis. This suggests the observed acute flaccid paralysis is likely due to neurological damage and not muscle damage.

A time course of infection was performed to determine the viral load in the tissues at various timepoints post-infection in Carimune® NF (400 mg/kg/d) treated mice. Tissue titers in the Carimune® NF group were consistently lower than the placebo group, with exceptions of the blood (day 3 p.i.), kidney (day 3-9 p.i.), and spleen (day 5 p.i.). Surprisingly, the only significant difference between groups was observed on day 1 in the blood ($P<0.0001$). This is especially surprising given the noticeable downward trend in spinal cord titers beginning on day 5 p.i. This suggests that a statistically significant reduction in viral titers may not be necessary for Carimune® NF to be protective and that other mechanisms of action, such as reduction in cytokines, may contribute to protection. It is unknown if larger doses or different times of administration of Carimune® NF would further reduce tissue titers. However, given the failure of higher doses to improve rates of survival, we do not expect titers to be drastically reduced compared to the 400 mg/kg/d treatment. Nevertheless, such studies could provide insight into the mechanism of action of Carimune® NF for an EV-71 infection.

A time course of infection for tissue cytokines was also assessed. MCP-1, MIP-1α, RANTES, and IFN-γ were all significantly reduced in the Carimune® NF treatment group for at least one time point. As EV-71 is a neurotropic virus causing meningitis and encephalitis, only brain and spinal cord tissues were assessed.
It is unclear exactly if or how the reduction in these cytokines contributed to protection. However, comparison to existing cytokine data can confirm the validity of this mouse model and provide insight into EV-71 pathogenesis, including which cytokines are most closely associated with severe disease.

RANTES (Regulated on activation, normal T cell expressed and secreted, or CCL5) is a chemoattractant protein for monocytes, memory helper T cells, and eosinophils (NCBI, 2018). Conflicting data has been reported about the role of RANTES in EV-71 infection, with some studies reporting elevated RANTES for EV-71 infection (Gong et al., 2012; Yu et al., 2017) and one study showing no elevation of RANTES (Wang et al., 2008). Other studies have shown that children with a polymorphism that downregulates RANTES expression were at a higher risk of severe EV-71 infection. Studies found that the edition of RANTES to glial cell cultures did not cause neuronal cell death. However, given that RANTES is a chemoattractant for certain white blood cells, it is possible that the cellular infiltration from elevated RANTES expression in the brain, as well as the resultant inflammation, may be a cause of neurological damage.

MCP-1 (monocyte chemoattractant protein-1, or CCL2) is a chemoattractant the migration of monocytes from the bloodstream to other tissues, where the cells mature into macrophages (Kahn, 2008). It has been found to be elevated in EV-71 patients and may function as an indicator of severe disease (Weng et al., 2010; Yu et al., 2017; Zhang et al., 2013b). Given this, reduction of MCP-1 in the brain and spinal cord may reduce EV-71 symptoms.

MIP-1α (macrophage inflammatory protein 1α) is a chemoattractant for B cells, T cells, and monocytes. While the majority of published data regarding the effects of MIP-1
on enterovirus function focuses on MIP-1β, some studies did examine MIP-1α, although results were differed as to whether or not it was elevated during infection (Teo et al., 2018; Zhang et al., 2013b). As such, the effect of the reduction of MIP-1α is unclear.

IFN-γ has a variety of function, including promoting antigen presentation and inhibiting viral replication (Kahn, 2008). However, studies have found that elevated levels of IFN-γ in the blood and CSF are associated with neurological symptoms (Wang et al., 2007) and pulmonary edema (Wang et al., 2003). Furthermore, IFN-γ may contribute to pulmonary edema by increasing vascular permeability (Wang et al., 2003). On the other hand, children with IFN-γ polymorphism that resulted in decreased IFN-γ production had an increased susceptibility to EV-71 (Yang et al., 2012). Thus, the possible effect of IFN-γ reduction in the Carimune® NF treated group on day 9 p.i. is unclear. It should also be noted that although AG129 mice lack the α, β, and γ interferon receptors, they are still able to produce interferon. Fi

Thus, we have demonstrated that Carimune® NF was protective for an EV-71 infection in an AG129 mouse model. A variety of parameters were assessed to determine the optimal doses and time of administration. We observed that Carimune® NF and Gammunex®-C contained EV-71 neutralizing antibodies and that Carimune® NF reduced MCP-1, MIP-1α, and RANTES in the brain and spinal cord of infected mice. Although protection from Carimune® NF varied across studies and the time of administration window was limited, average protection was approximately 50% when administered no later than 4h p.i. Thus, these results provide support for the use of IVIG in the clinic to treat EV-71 infection.
Acknowledgements

Special thanks to Dr. Arnaud Van Wettere for his analysis of the histopathology images.
CHAPTER VI
GENOME SEQUENCING ANALYSIS

ABSTRACT

Sequencing of the enterovirus 71 (EV-71) genome is of interest as a means for predicting virulence. Clinical signs and virulence has varied across outbreaks and between genogroups, yet no genetic marker has definitively emerged as a determinant of virulence. Sequencing of \textit{in vitro} and \textit{in vivo} passaged virus has identified several predicted amino acid changes that are may contribute to adaptation in cell culture and laboratory animals. These predicted amino acid changes may be useful indicators for how the EV-71 genome correlates with disease in humans. Here, we report the sequencing of four serially passaged EV-71 genomes. Predicted amino acid changes were detected in the VP1, VP2, 2C, and 3C\textsuperscript{pro} of the virus genomes.

1. Introduction

Enterovirus 71 (EV-71) is a non-enveloped, single-stranded RNA virus in the \textit{Picornaviridae} family with a 7.5 kb genome that produces 11 viral proteins (4 structural, 7 non-structural). Determining the virulence factors in the virus genome are important for predicting clinical outcomes and for developing animal models. Furthermore, different strains of EV-71 have produced different rates of neurovirulence and different clinical signs across outbreaks (McMinn, 2002). The connection between genotype or amino acid mutation and virulence is still being explored. Viruses isolated from animal studies were found to have several predicted amino acid changes suspected to be involved in adaptation of the virus to the animal model. These included changes in the VP1 region (L97R, G/Q145E, A170V, K244E), VP2 region (149M), and 3C\textsuperscript{pro} (N68D)(Chang et al.,
In vitro determinants of virulence were also observed as well in the 3C^pro (N69D), VP1 (E145G, 98E, 244K), and 3D (G64R) regions (Chang et al., 2018b; Li et al., 2017; Yee et al., 2017). These sequences changes are considered crucial to understanding how the virus adapted to mice and in determining markers for virulence.

Here we report nucleotide and amino acid changes from four sequenced EV-71 viruses (M4, MP10, plaque 4 and plaque 6).

2. Materials and Methods

2.1. RNA Extraction, PCR Amplification, and Sequencing

Primers were generated for three sets of overlapping consensus sequences to mimic the protocol employed by the Centers for Disease Control (Ng et al., 2016). The EV-71 genome was divided into three amplicons. The first amplicon was from the 5’UTR to 2C (TTAAAACAGCCTGTGGGTTGCACCC [1-25]; GCTTACCTGTGTCTTGGCCGGTA [2545-2521]), the second from VP1 to 2C (CAGGGAGATAGGGTGGCAGATGTGA [2438-2462]; TCTTCACTATCAACACTAGCTAATAAGTCACTAATAGCATCA [5160-5119]), and the third from 2C to 3’UTR (TAGAGGAGAAGCCCGCACCTGAT ([5099-5122]; GCTATTTCTGTATAACAAATTTACCCCGCACCTGAT ([7410-7377]))). All primers were based on the sequence for EV-71 Tainan/4603/98 (GenBank KM851227.1). cDNA was generated using a Superscript™ First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA). A virus-specific reverse primer generated from the sequence of the 3’UTR region of EV-71 was used for first-strand synthesis. cDNA samples were used as the template for PCR with Thermo Scientific Phusion High-Fidelity DNA Polymerase
(Thermo Fisher Scientific, Waltham, MA) using the manufacturer’s 3-step protocol. For each sample, all three amplicons were pooled and purified using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA). Samples were submitted to the DNA Sequencing Core (University of Utah, Salt Lake City, UT) for analysis on their Ion Torrent Next Generation Sequencing (NGS) Technology. Sequence data was aligned using the EV-71 Tainan/4603/98 sequence as the reference genome using the Integrative Genomics Viewer (Robinson et al., 2011; Thorvaldsdottir et al., 2013). Individual nucleotide mutations were recorded for each sample. Changes to the amino acid sequence were determined using the Translate Tool on the ExPASy resource portal from the Swiss Institute for Bioinformatics (www.sib.swiss). Protein sequences were aligned for mutations using the Protein BLAST tool from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). As the amino acid sequence was not determined experimentally, all changes to amino acids were predicated based upon the detected nucleotide sequence.

3. Results

3.1. Nucleotide Changes

Forty-nine nucleotide changes were observed from mouse-adaptation of virus by serial passaging. A majority of these changes were observed in the MP10 virus (40 changes), with the remaining changes observed in plaque 4, plaque 6, and MP14 (Table 1). Only a minority of these nucleotide changes (nine) were predicted to result in amino acid changes. All putative amino acid changes occurred within the VP1, VP2, 2C, or 3Cpro regions (Fig. 1). As the genomes were sequenced using next-generation sequencing technology, millions of fragments of the genome were produced and aligned as part of
the sequencing process (Behjati and Tarpey, 2013). Since viruses can exist as quasispecies (Meng and Kwang, 2014), it is possible that subpopulations of viruses exist within the sequenced sample. Such viruses may contain different mutations, and the percent of these mutations was signified as, “Percent of Reads with Mutation” in Table 1.

3.2. Predicted Amino Acid Changes

Several amino acids changes were observed across the four viruses. Changes were designated by first listing the gene in which the change occurred, followed by the abbreviated amino acid that changed in the parent virus, the amino acid position at which the change occurred, and then the amino acid substitution (e.g., VP2 (M150V)). Amino acid positions refer to the mature peptide, and not the polyprotein.

Several predicted amino acids changes were observed from in the MP10 as compared to MP4 (Table). Changes that were predicted are as follows: VP2 (M150V), 2C (P130A, R104L, I112M, L118H), and 3C (I56V). Three of these predicted amino acid changes resulted in a non-conservative amino acid change.

Only one amino acid change was observed in the MP10 Plaque 4 virus as compared to MP10 (VP1 (E145Q)). This change was observed in a minor population of the viruses sequenced (18%). This predicted change would result in a glutamic acid being replaced with a polar neutral amino acid (glutamine). No predicted amino acid changes were observed in the Plaque 6 or MP14 as compared to MP10.
## Table 1
Summary of Nucleotide and Predicted Amino Acid Changes in Four EV-71 Genome Sequences.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Position of Mutation</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Amino Acid Position</th>
<th>Location in Viral Polyprotein</th>
<th>Percent of Reads with Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP10</td>
<td>246</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>689</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>757</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>817</td>
<td>T → A</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>1397</td>
<td>A → G</td>
<td>M → V</td>
<td>219</td>
<td>VP2 (M150V)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>1492</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>1735</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>2293</td>
<td>G → A</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>2662</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>2965</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>3547</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4051</td>
<td>C → T</td>
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<td></td>
<td></td>
<td>100%</td>
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<tr>
<td></td>
<td>4324</td>
<td>T → C</td>
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<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4375</td>
<td>C → G</td>
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<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4385</td>
<td>C → G</td>
<td>P → A</td>
<td>1214</td>
<td>2C (P103A)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4387</td>
<td>T → C</td>
<td>P → A</td>
<td>1214</td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4388</td>
<td>A → C</td>
<td>R → L</td>
<td>1215</td>
<td>2C (R104L)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4389</td>
<td>G → T</td>
<td>R → L</td>
<td>1215</td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4414</td>
<td>C → G</td>
<td>I → M</td>
<td>1223</td>
<td>2C (I112M)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4431</td>
<td>T → A</td>
<td>L → H</td>
<td>1229</td>
<td>2C (L118H)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>5110</td>
<td>G → A</td>
<td></td>
<td></td>
<td></td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>5135</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>32%</td>
</tr>
<tr>
<td></td>
<td>5182</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>68%</td>
</tr>
<tr>
<td></td>
<td>5254</td>
<td>G → A</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>5555</td>
<td>A → G</td>
<td>I → V</td>
<td>1604</td>
<td>3C (I56V)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>5653</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6007</td>
<td>T → A</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6145</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>68%</td>
</tr>
<tr>
<td></td>
<td>6388</td>
<td>G → A</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6478</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6556</td>
<td>T → C</td>
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<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6574</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6613</td>
<td>T → C</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>7261</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>7368</td>
<td>C → T</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 1
Summary of Nucleotide and Predicted Amino Acid Changes in Four EV-71 Genome Sequences (con.).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Position of Mutation</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Amino Acid Position</th>
<th>Location in Viral Polyprotein</th>
<th>Percent of Reads with Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP10 Plaque 4</td>
<td>2873</td>
<td>G → C</td>
<td>E → Q</td>
<td>710</td>
<td>VP1 (E145Q)</td>
<td>18%</td>
</tr>
<tr>
<td></td>
<td>5182</td>
<td>T → C</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6145</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>68%</td>
</tr>
<tr>
<td></td>
<td>6919</td>
<td>C → T</td>
<td></td>
<td></td>
<td></td>
<td>23%</td>
</tr>
<tr>
<td>MP10 Plaque 6</td>
<td>2419</td>
<td>C → T</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6145</td>
<td>A → G</td>
<td></td>
<td></td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>MP14</td>
<td>2209</td>
<td>C → T</td>
<td></td>
<td></td>
<td></td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td>3691</td>
<td>C → T</td>
<td></td>
<td></td>
<td></td>
<td>46%</td>
</tr>
<tr>
<td></td>
<td>7376</td>
<td>A → T</td>
<td></td>
<td></td>
<td></td>
<td>26%</td>
</tr>
</tbody>
</table>
Fig. 1. Visual representation of EV-71 Polyprotein and Predicted Amino Acid Changes for MP10 Virus. The EV-71 polyprotein is divided shown divided into mature peptides. Amino acid length of each peptide is displayed below polyprotein. Predicted amino acid changes are indicated by red triangles.
4. Discussion

Determination of the amino acid changes during mouse-adaptation for EV-71 was considered crucial to understanding the genetic basis of virulence and the process of adaptation. Of the forty-nine nucleotide changes, nine resulted in predicted amino acid changes. The majority of these amino acid changes occurred in the 2C region, which is unusual considering the majority of reported amino acid changes from other animal models occurred in the VP1 region.

Several predicted amino acid changes could result in structural changes to the protein, based on side-chain differences. For VP2 (M150V), a methionine was replaced with a valine, resulting in the predicted loss of the methionine side-chain. This could have altered protein structure if the methionine was involved in disulfide bonding. For 2C (P103A), a proline was replaced with an aliphatic alanine, likely resulting in a loss of the pyrrole ring in the proline side chain. It is suspected that this change would provide more side-chain flexibility. For 2C (I112M), both amino acids are aliphatic. Other changes in amino acid types were predicted to occur at the 2C (R104L, L118H), 3Cpro (I156V), and VP1 (E145Q) and may have also produced structural changes, although the potential effect of the amino acid change would be more difficult to predict.

Mutation at the 145 amino acid position in the VP1 protein has been reported in the literature (Caine et al., 2016; Kataoka et al., 2015; Nishimura et al., 2013; Zhou et al., 2014). The VP1 145 amino acid affects EV-71 binding to PSGL-1 on lymphocytes, with the VP1 (G/Q145E) resulting in a loss of binding to PSGL-1 (Huang and Shih, 2014). While this appears to suggest that the VP1 (E145Q) mutation observed in MP10 allows the virus to bind PSGL-1 in lymphocytes, it should be noted that PSGL-1 is a human
receptor not found in mice. However, two studies have shown that the VP1 (Q145E) mutation was associated with increased virulence in non-transgenic mice (Huang et al., 2012b; Zaini and McMinn, 2012).

Nucleotide changes were retained throughout passaging and plaque purification—that is, nucleotide changes that were observed in MP10 were retained in plaques 4 and 6, as well as MP14. It is uncertain why nucleotide changes that likely do not result in a predicted amino acid change would be retained so consistently. Nucleotide changes in the untranslated IRES region (nucleotides 1-636) may alter the IRES stem-loop structure such that it improves replication (Chang et al., 2018b; Chang et al., 2012; Shih et al., 2011). One change was reported in MP10 (A246G). Indeed, at least two studies suggest that mutations in the IRES region affect virulence (Chang et al., 2018b; Chang et al., 2012). It is also worth noting that mutations in the poliovirus IRES were crucial for attenuation of the Sabin vaccine strain (Lin and Shih, 2014).

No predicted amino acid changes were observed in MP14 and all nucleotide changes occurred in only a portion of the virus population. This suggests that the reduced virulence for MP14, as well MP13 and MP15, was due to factors other than attenuation, such as lower infectious dose.

Finally, the majority of predicted amino acid changes occurred in the 2C region. Four predicted amino acid changes were also reported at the same sites when the Tainan/4643/98 strain was passaged four times in ICR mice (Wang et al., 2004). This MP4 virus was the same virus used for mouse adaptation for this model. The changes observed in the MP10 virus revert to those found in the clinical isolated (Tainan/4643/98). This suggests that these four sites within the 2C protein may be crucial
for EV-71 adaptation.

Although the effect of the four 2C mutations is unknown, an intriguing possibility is that these predicted changes may affect virus immune evasion. The 2C protein is an ATPase involved in replication and immune evasion, with the latter effect achieved by limiting NF-κB activation (Yuan et al., 2018). NF-κB is a transcription that is the pro-inflammatory pathway as well as in activation of inflammatory T cells and innate immune cells (Liu et al., 2017). It is possible that these putative changes in the 2C protein provide a means of evading the mouse immune system. It is also interesting that the majority of nucleotide changes occurred in the 2C protein, and not the 2A<sup>pro</sup> and 3C<sup>pro</sup>, which are responsible for, among other things, subverting the interferon response. As the interferon response in the AG129 mice is negated, it is possible that selective pressure produced adaptation in the other protein involved in immune evasion. However, future studies will be needed to confirm this hypothesis.

In conclusion, we report the sequence of four mouse-adapted EV-71 viruses, with predicted amino acid changes in the VP1, VP2, 2C, and 3C regions. Nucleotide changes acquired in MP10 were maintained in plaque 4, plaque 6, and MP14. Only a single predicted amino acid change has previously been reported in the literature (VP1 (E145Q)). Mutations in the 2C region may contribute to immune evasion and a nucleotide change in the IRES region may affect translation. Further studies will be needed to confirm that presence of the amino acid changes as well as determine the effect of EV-71 replication and pathogenesis.

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CHAPTER VII
CONCLUSIONS AND FUTURE STUDIES

1. Conclusion

Disease caused by EV-71 is of concern, not simply due to the serious neurological and pulmonary disease caused by the virus, but also due to the lack of approved antiviral treatments. Development of an animal model is crucial to evaluating potential antivirals that could be used in the clinic. The challenge is to produce a model that is not only permissive to viral replication, but also produces neurological signs comparable to those seen in humans.

Here we report the development of such an animal model for EV-71. Infected mice exhibit weight-loss and rear limb paralysis, reminiscent of the acute (poliomyelitis-like) paralysis observed in human patients. The model is also 100% lethal, which represents fatal human cases as well as providing a consistent baseline from which to evaluate disease. Mice are older than nearly all pervious EV-71 mouse models, which often relied on neonate mice.

Twenty-six therapeutic and antivirals were evaluated in this model. Of these compounds, two novel host responses modifiers, STF1019 and STF434, were protective. STF1019 was especially effective (7/8), which is intriguing given several well-studied antivirals, including pleconaril and ribavirin, were not protective. In addition, the 200 mg/kg/d treatment group exhibited no neurological signs. This strongly suggests that STF1019 continue to be evaluated as a potential EV-71 antiviral.

The therapeutic IVIG was also protective, providing about 50% protection from a single administration. We also observed decreased concentrations of MIP-1α, RANTES,
MCP-1, and IFN-γ in brain and spinal cord tissue from mice treated with 400 mg/kg/d. The IVIG lot used also contained EV-71 neutralizing antibodies, although IVIG failed to significantly decrease viral tissue titers in infected mice (save for a single day in spinal cord tissue). However, IVIG was unable to provide protection when administered near or at the onset of disease (day 7, 8, or 9 p.i.), even when administered at a high-dose (2000 mg/kg/d). We also observed variation in the effectiveness of IVIG, although different mouse ages between studies may have contributed to this. Nevertheless, these studies clearly demonstrate the IVIG is protective and provide support for the clinical use of IVIG to treat EV-71 infections.

Finally, we analyzed genomes from four mouse-passaged EV-71. Forty-nine nucleotide changes and seven predicted amino acid changes were observed, with four of these changes occurring in the 2C region. Five nucleotide changes were observed in the IRES region, which may alter IRES stem loop structure and affect translation. While it is unclear if and how these nucleotide changes contributed to adaptation of the virus to mice, future studies (such as reverse genetics) may link some of these genetic changes to virulence.

2. Future Studies

1. Evaluation of additional anti-inflammatory compounds, such as dexamethasone, to treat infection.

2. Combination drug therapies, including combination of antiviral and anti-inflammatory drugs.

3. Introduction of select nucleotide changes in MP4 genome to help determine which changes are related to virulence.

4. Effect of STF434 and STF1019 on viral tissue titers and CNS histopathology, as well as effectiveness as a prophylactic.

5. Effectiveness of intrathecal administration of IVIG, in order to mimic epidermal
delivery of IVIG in the clinic.

6. Evaluation of IVIG brands from countries with high EV-71 prevalence (e.g., China or Taiwan).

7. Determining the pathogenesis of the virus during the first 24h p.i. and the effect of IVIG treatment on replication when administered at 0, 1, 2, and 4h p.i.

8. Comparison of cytokine time course of infection for MP4 and MP10 viruses.

9. Determination of the effect of each predicted amino acid change on EV-71 replication and virulence through reverse genetics.

10. Examination of relationship of brown adipose tissue levels and disease severity.


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