Indonesia International Institute for Life Sciences

ENRICHMENT PROGRAM

REPORT

Exploration of Bat Virus Attachment on Brain Cells

STUDY PROGRAM

Biomedicine

Jasmine Angelica Syahirah Simanjuntak
20010267

Liang-Tzung Lin, Ph.D (Field Supervisor)
Richard Sutejo, S.TP., Ph.D. (EP Supervisor)

INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES (i3L)
2024

RESEARCH REPORT

Exploration of Bat Virus Attachment on Brain Cells

By
Jasmine Angelica Syahirah Simanjuntak
20010267

Submitted to

i3L – Indonesia International Institute for Life Sciences
School of Life Sciences

in partial fulfillment of the enrichment program for the Bachelor of Science in
Biomedicine

Research Project Supervisor: Richard Sutejo, S.TP., Ph.D.
Research Project Field Supervisor: Liang-Tzung Lin, Ph.D

Jakarta, Indonesia
2024

CERTIFICATE OF APPROVAL

In this section, students are required to insert the Certificate of Approval's page (Doc. No. FR-i3L-5.3.4.3). The Certificate of Approval must be left blank and can only be completed at the end of the final report presentation/defense.

COPYRIGHT NOTICE

STATEMENT OF ORIGINALITY

Submitted to

Indonesia International Institute for Life Sciences (i3L)

I, Jasmine Angelica Syahirah Simanjuntak, do herewith declare that the material contained in my Enrichment Program (EP) entitled: “Exploration of Bat Virus Attachment on Brain Cells” is original work performed by me under the guidance and advice of my EP advisor, Richard Sutejo, S.TP., Ph.D. have read and do understand the definition and information on the use of source and citation style published by i3L. By signing this statement, I unequivocally assert that the aforementioned thesis conforms to published information. i3L has my permission to submit an electronic copy of my thesis to a commercial document screening service with my name included.

☑ Yes

☐ No

Student Name : Jasmine Angelica Syahirah Simanjuntak Student ID: 20010267

Study Program : Biomedicine Signature

Jasmine Angelica Syahirah Simanjuntak

ABSTRACT

Glycosaminoglycans (GAGs) have garnered attention in virology due to their potential in mitigating lyssavirus attachment to brain cells. Recent studies highlight heparin's ability to impede viral attachment to heparan sulfate, suggesting its role in preventing viral entry. Pseudoviruses have been utilized as safer alternatives in lyssavirus research, generated through co-transfection of plasmids into cell lines, simulating viral features without the genome. TCID50 assays revealed the infectivity of these pseudoviruses, with varied results akin to conventional viral plaque assays for bat-borne lyssaviruses. The heparin inhibition assay displayed a dose-dependent relationship, despite minor discrepancies attributed to procedural errors. The observed decline in luminescence with increased heparin concentration aligns with heparin's antagonistic effect on rabies virus (RABV) attachment, indicating its ability to competitively inhibit viral binding to host cells. These findings underscore heparin's potential in disrupting virus attachment and subsequent infection, emphasizing its therapeutic promise.

Keywords: Glycosaminoglycans, heparin, lyssavirus, pseudovirus, heparin inhibition assay, antiviral mechanisms

ACKNOWLEDGEMENTS

I am profoundly grateful to Allah, whose benevolent guidance enabled me to embark on this internship in Taipei and successfully complete this report. Furthermore, I extend my heartfelt appreciation to my blessed mother and uncle, whose unrelenting support sustained me throughout this internship. Their unwavering belief in me at every juncture not only fueled my motivation to work diligently but also bestowed upon me an unforgettable experience that will resonate throughout my lifetime.

I am immensely thankful to Professor Liang-Tzung Lin Ph.D. for affording me the privilege to contribute to the work conducted in his laboratory. I convey my heartfelt gratitude to my EP supervisor, Richard Sutejo, S.TP., Ph.D., whose expert counseling and steadfast assistance have significantly enriched my learning experience. My heartfelt appreciation extends to the extraordinary individuals who have played pivotal roles in supporting and educating me throughout my tenure, with special recognition to Pei-Cheng Lu. She served as a dedicated mentor throughout my internship, providing guidance with utmost patience and kindness. I also would like to extend my heartfelt appreciation to Leo, Elma, Joanne, Kannika, and Katerina in the laboratory – my second family. Their unwavering camaraderie during challenging moments, coupled with both words of wisdom and humor, has been invaluable by lightening the burden during stressful times.

I want to express my special appreciation to my friends Jovinka, Jennifer, Kelly, and Sherry who, without hesitation, extended a helping hand, often without even being asked. They not only reminded me to fulfill my work and duties but also emphasized the significance of balancing responsibilities with moments of relaxation and enjoyment. I also want to acknowledge the friendships I've cultivated in Taipei, particularly with Dennis, Addi, and Freed whose presence has added an extra layer of meaning and joy to my experience, making it truly memorable.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CNS: Central Nervous System

CS: Chondroitin Sulfate

DDH2O: Double Distilled Water

DMEM: Dulbecco's Modified Eagle Medium

DS: Dermatan Sulfate

DENV: Dengue Virus

ECM: Extracellular Matrix

ER: Endoplasmic Reticulum

FBS: Fetal Bovine Serum

G: Glycoprotein

GAGs: Glycosaminoglycans

GFP: Green Fluorescent Protein

HEK293: Human Embryonic Kidney Cell Line

HA: Hyaluronic Acid

HIV-1: Human Immunodeficiency Virus Type 1

HS: Heparan Sulfate

HSV: Herpes Simplex Virus

IBs: Inclusion Bodies

IC50: Half-Maximal Inhibitory Concentration

KS: Keratan Sulfate

L: RNA-Directed RNA Polymerase

M: Matrix Protein

MOI: Multiplicity of Infection

N: Nucleoprotein

OPTIMEM: Optimized Minimum Essential Medium

P: Phosphoprotein

PBS: Phosphate Buffered Saline

PEG: Polyethylene Glycol

PEP: Post-Exposure Prophylaxis

RABV: Rabies Virus

RNA: Ribonucleic Acid

RNP: Ribonucleoprotein

SH-SY5Y: Human Neuroblastoma Cell Line

STAT: Signal Transducer and Activator of Transcription

TCID50: Tissue Culture Infective Dose 50

TBS: Tris Buffered Saline

UV: Ultraviolet

WHO: World Health Organization

I. INTRODUCTION

1.1 Background

Zoonotic diseases are contagious illnesses which spread between animals and people (Bauer et al.., 2021). Due to the fact that most of the fatal zoonotic diseases in the world are caused by infected animals, the more people continue to have close interaction with these animals, the higher likelihood of spillovers occurs (Ellwanger & Chies, 2021). Bats, which account for around 22% of all listed mammal species, are being recognized as natural reservoir hosts for several novel viruses that can cause fatal illnesses in humans and due to their quick evolutionary pace, toxicity to humans or other hosts, other bat-associated viral families, such as lyssaviruses are of significant public and veterinary health concern (Letko et al., 2020). According to an analysis by Khan et al. (2022), 73.17% of zoonotic diseases in Asia are bat-borne, with a fatality rate of 29.86%. This emphasizes the importance to better comprehend the pathogenesis of bat-borne viruses and to develop effective interventions that can mitigate their impact on public health well-being.

Glycosaminoglycans (GAGs) represent a group of linear, negatively charged polysaccharides present both on cell surfaces and within the extracellular matrix (Shetye et al., 2017). Based on the specific sulfation group, GAGs are divided into five main categories. These categories include sulfated GAGs, such as heparin and heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS), as well as non-sulfated GAGs, such as hyaluronic acid (HA) (Shi et al., 2021). Apart from HA, all GAGs in vivo are found covalently coupled to particular core proteins as proteoglycans and are ubiquitously expressed along the cell surface, in ECM, and in intracellular compartments (Jinno & Park, 2015). Almost every significant point of entry for pathogens is facilitated by GAGs, which help them connect to and invade host cells, migrate from one cell to another, and defend themselves against immune action (Aquino & Park, 2016). With that being said, investigating the precise mechanism of interaction and pathogenicity between Asian bat viruses and

the GAG attachment factor in this complex interplay is crucial to develop efficient therapeutic strategies to protect host cells from infection.

1.2 Scope of Research

This research involved pseudovirus production, determining pseudovirus infectivity (TCID50) on SH-SY5Y cells, and conducting a heparin inhibition assay (IC50). Both TCID50 and IC50 were assessed using a luciferase assay.

1.3 Objectives and Hypothesis

The research aimed to investigate and elucidate the role of the attachment factor GAG, particularly Heparin, in lyssavirus attachment. It was hypothesized that Heparin would mitigate Lyssavirus attachment towards SH-SY5Y cells.

II. LITERATURE REVIEW

2. 1 Rabies Virus

2.1.1 Structure and Protein Function

The rabies virus (RABV) stands as the representative species in the Lyssavirus genus within the Rhabdoviridae family. It embodies a negative-sense, non-segmented, single-stranded RNA virus, exhibiting a distinctive bullet-shaped morphology, typically measuring around 60 nm × 180 nm (Zandi et al., 2021; Fodor, 2020; Willoughby, 2012). The genetic structure of rhabdoviruses encodes five crucial structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-directed RNA polymerase (L) (Davis et al., 2015). Each protein holds distinct functions vital to the virus's life cycle. The multifaceted phosphoprotein (P) plays pivotal roles as a polymerase cofactor, binding to viral genomic RNA-N protein complexes, enabling replication, and evading host immune responses by engaging STAT factors (Zhan et al., 2022). Meanwhile, the nucleoprotein (N) plays a fundamental role in genomic RNA encapsidation, an essential step for viral genome replication and mRNA transcription, facilitated by the RNA-dependent RNA polymerase, L protein (Masatani et al., 2013). As the virus undergoes assembly, the ribonucleoprotein (RNP) complex—comprising RNA, N, P, and L—becomes enveloped within a lipid bilayer, a product of the host cell membrane. This process is facilitated by the matrix protein M, linking the nucleocapsid to the lipid envelope. Additionally, research by Luo et al. (2020) indicates the critical role of the M protein in regulating lyssavirus RNA synthesis, viral protein expression, virus assembly, and budding across different stages of the viral life cycle. The glycoprotein G, an integral transmembrane protein, serves as a key factor in virus entry and represents the primary target for neutralizing antibodies (Yang et al., 2020).

2.1.2 Epidemiology

Lyssavirus infection remains a pressing global health issue, responsible for an estimated 59,000 human deaths yearly across over 150 countries, with a predominant 95% of cases reported in Africa and Asia (WHO, n.d.). The disease burden weighs heavily on rural impoverished populations, particularly affecting children under 15. In India, rabies remains a significant concern, with thousands of annual fatalities, largely attributed to dog-related incidents (Radhakrishnan et al., 2020). Although Latin America and the Caribbean have witnessed a decrease in human and canine rabies cases due to sustained control efforts, bat-mediated rabies continues to be a primary cause of human infections (WHO, n.d.). This concern is amplified in Latin America, where encounters between humans and both vampire and non-hematophagous bats contribute significantly to disease transmission (Johnson et al., 2014). However, accurately mapping bat-associated rabies spillovers to humans proves challenging due to the intricate ecology of involved bat species. Moreover, instances of "imported" rabies cases arise when individuals or animals contract the virus in a new location, often following travel or relocation, involving a virus variant originating from another country (Hanlon & Childs, 2013).

2.1.3 Pathogenesis

Lyssavirus infection commences when the virus adheres to a target cell surface and penetrates it via an endosomal transport route. Before binding to the cellular receptor, the viral surface glycoprotein (G) remains in its 'native' state (Wunner & Conzelmann, 2013). Research conducted by Sasaki et al. (2018) revealed that heparan sulfate (HS) functions as the primary attachment factor for lyssavirus infection by engaging with the viral glycoprotein. This interaction between HS and the lyssavirus CVS-G relies on an electrostatic binding mechanism involving the negatively charged chains of HS and basic amino acids in the viral protein. Subsequent to attachment and internalization of the virus, the G undergoes a transition to an 'activated' hydrophobic state, allowing it to interact with the hydrophobic endosomal membrane (Jackson & Fooks, 2020).

During internalization, either via receptor-mediated endocytosis through the endocytic pathway or coated pits, fusion between the viral and endosomal membranes is triggered within the acidic environment (pH 6.3–6.5) of the endosomal compartment. At this fusion activation threshold pH, the lyssavirus G undergoes specific and distinct conformational changes, assuming at least three structurally different states (Yang et al., 2020). Upon entry into the low pH endosomal environment, a significant structural alteration in the G activates its fusion capacity, exposing the fusion domain that interacts with the target cell membrane.

Following entry into the endosomal compartment and exposure to the cellular environment's low pH, viral RNA (vRNA) genome transcription commences within the infected cell's cytoplasm, initiated as the tightly coiled transcriptionally dormant RNP core is liberated from endosomal vesicles (Jackson & Fooks, 2020). Viral proteins are synthesized from the viral mRNAs using the host cell's protein synthesis machinery (Rampersad & Tennant, 2018). The G-mRNA is translated on membrane-bound polyribosomes (polysomes) and inserted cotranslationally into the endoplasmic reticulum (ER) lumen where molecular chaperones aid in folding G monomers before transport out of the ER (Wunner & Conzelmann, 2013). Meanwhile, other viral proteins are synthesized in the cell's cytoplasm. Proteins such as M interact with the host cell machinery, regulating transcription and facilitating viral mRNA translation (Luo et al., 2020).

Accumulation of viral proteins, particularly N and P, initiates replication, leading to the formation of inclusion bodies (IBs) primarily composed of viral components, functioning as active viral factories. As the rabies virus assembles in the mid-phase of its lifecycle, vRNA encapsidation and the RNP formation occur. These assembly sites, commonly located in neurons and tissue cultures, are associated with IBs and similar structures (Fooks et al., 2017). In the final stages of lyssavirus assembly, mature virions acquire their lipid bilayer envelope as the assembled structure (RNP + M) buds through the host cell plasma membrane (Davis et al., 2015).

2.1.4 Symptoms

The rabies virus infiltration initiates distinct clinical presentations, predominantly impacting the nervous system and resulting in severe, often fatal symptoms. This viral invasion leads to two primary clinical forms: encephalitic and paralytic rabies (Fooks et al., 2017). Encephalitic rabies, observed in about 80% of rabies cases, begins with non-specific flu-like symptoms (Jackson, 2018). As the virus spreads within the central nervous system (CNS), patients develop neurological manifestations including anxiety, agitation, and hallucinations. These symptoms progress to delirium, accompanied by insomnia and the emergence of hydrophobia—an aversion to water and swallowing difficulties (Koury & Warrington, 2022). Later stages involve increased saliva production, involuntary muscle contractions, and spasms, culminating in severe encephalopathy, coma, and often, fatal outcomes.

In contrast to the more common encephalitic form, paralytic rabies, present in around 20% of cases, initially mirrors symptoms akin to Guillain-Barré syndrome or poliomyelitis. Patients may experience localized weakness, numbness, or paralysis, often accompanied by pain or tingling sensations. Unlike the heightened agitation observed in encephalitic rabies, the paralytic variant typically exhibits a more subdued clinical profile, characterized primarily by weakness, although patients may also display altered mental status, persistent fevers, and bladder dysfunction (Koury & Warrington, 2022). Disease progression leads to respiratory compromise, eventually resulting in coma and fatal outcomes.

2.1.5 Current treatment and Prevention

The current management and prevention methods for the rabies virus in humans have a considerable shortfall, creating an urgent demand for alternative strategies. Presently, there exists no specific treatment or cure for symptomatic rabies infection (CDC, 2020). The existing preventive measures primarily rely on post-exposure prophylaxis (PEP) as well as Pre-exposure prophylaxis

(PrEP), comprising a series of rabies vaccines and, in certain cases, rabies immunoglobulin, administered following suspected exposure to the virus (Charniga et al., 2023). PrEP is intended to make post-exposure prophylaxis easier by minimizing the need for further measures in the event of exposure (Parizing et al., 2021). PEP often entails wound care, including thorough washing of the injury with soap, as well as rabies vaccination, which is critical in preventing the disease from developing after exposure (Harris et al., 2023). While PrEP and PEP are highly effective, the main challenge revolves around ensuring its accessibility and affordability, particularly in regions where the disease is prevalent (Changalucha et al., 2019). Furthermore, implementing mass dog vaccination, a proven and cost-effective approach to prevent human rabies cases, remains a significant obstacle in many affected areas. The absence of a targeted treatment for symptomatic rabies infection, especially in regions where the disease is widespread, emphasizes the critical necessity for alternative methods in treating and preventing the rabies virus in humans. The development of substitute treatments for the rabies virus becomes crucial to enhance the effectiveness, accessibility, and affordability of interventions, particularly in regions with limited resources and high disease burdens. Hence, the exploration of alternative treatment approaches for the rabies virus stands as a vital endeavor to bridge the current gap and advance global endeavors aimed at controlling and eradicating this lethal infectious disease.

2.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are elongated, unbranched polysaccharides present in various bodily tissues, including the extracellular matrix, connective tissues, and cell surfaces (Aquino & Park, 2016). They hold a critical role in upholding tissue structure, regulating cellular functions, and engaging with an array of proteins (Casale & Crane, 2023). Classified into sulfated and non-sulfated types, sulfated GAGs encompass heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate, while hyaluronic acid represents the non-sulfated variant (Aquino & Park). These

GAGs are categorized by their core disaccharide units—glucuronic acid, iduronic acid, N-acetylglucosamine, and N-acetylgalactosamine—determining their unique properties and functions (Wang & Chi, 2022). Combinations of these units yield diverse GAG structures and functions, enabling their interaction with a broad spectrum of proteins and contributing to various physiological processes in the body.

In the pathogenesis of multiple microbial pathogens, including viruses, bacteria, parasites, and fungi, GAGs hold a pivotal role (Bauer et al., 2021). Research demonstrates GAGs' interaction with these pathogens at the cell surface and extracellular matrix, influencing microbial pathogenesis and host defense mechanisms (Shi et al., 2021). Pathogens have evolved strategies to exploit fundamental GAG activities, leveraging their capacity for cell attachment, invasion, and evasion of host defenses. Through interactions with specific surface proteins on diverse pathogens, GAGs enable microorganisms to initiate infection processes (Jinno & Park, 2015).

2.2.1 Heparin Against Viruses

Heparin, a highly sulfated glycosaminoglycan (GAG), serves widely as an anticoagulant in clinical practice (Qiu et al., 2021). Comprising repeated glucosamine and uronic acid disaccharide units, heparin possesses substantial sulfation and a negative charge (Kato et al., 2015). Its polyanionic structure allows diverse interactions with proteins, including antithrombin III, responsible for its anticoagulant effect (Ghiseli, 2019). Beyond its anticoagulant role, research has explored heparin's potential as an antiviral agent. Notably, a study unveiled its capacity as a sulfated GAG to impede HIV-1 Gag protein attachment to host cell membranes, thereby obstructing viral assembly and maturation (Dick & Cocklin, 2020). Investigations into its antiviral effects against herpes simplex virus (HSV) and dengue virus (DENV) revealed promising outcomes. In HSV cases, heparin and related compounds disrupt viral attachment and entry by obstructing viral binding sites on host cells. Concerning DENV, sulfated GAGs like heparin hindered viral replication by interfering with viral RNA

synthesis (Eilts et al., 2023). These findings illuminate the potential of GAGs as prospective antiviral agents, capable of inhibiting viral attachment and maturation.

III. MATERIALS & METHODS

3.1 Cell Culture

The SH-SY5Y and HEK293 cell line was cultured in DMEM/12 and DMEM, respectively, along with 10% FBS, and penicillin-streptomycin in a T25 flask incubated at 37°C with 5% CO2 until it reached 80-90% confluence. Once confluent, the supernatant was discarded, and the cells were washed with PBS. After removing PBS, Trypsin-EDTA was added and incubated for 5 minutes at 37°C to detach the cells. Subsequently, complete DMEM (cDMEM) was added to the flask, and the cells were resuspended thoroughly to collect them into a 15 mL falcon tube. Then, the SH-SY5Y cell suspension was examined under a microscope, confirming a count of 5×10^4 cells for seeding into the 96-well plate for subsequent assays. The HEK293 cell line had the same cell density, but was seeded on a petri dish instead for the next step.

3.2 LYSV Pseudovirus Production

The plasmid DNA, housing the Novel Lyssavirus (LYSV) glycoprotein gene alongside a green fluorescent tag and luciferase gene, was combined with polyethylenimine (PEI). After a 15-minute incubation, the mixture was diluted with OPTIMEM, resulting in four separate batches. Following this preparation, 5 mL of the total mixture was administered to HEK293 cells post-washing. Media samples were collected at designated intervals (24, 48, 72, and 96 hours) into 15 mL falcon tubes, with media replacement after each collection. Notably, the 24 and 48-hour samples, as well as the 72 and 96-hour samples, were gathered in the same tubes. Subsequently, each virus suspension was centrifuged at 4.500 rpm for 5 minutes to remove cellular debris. The resulting pellet was treated with a PEG and media mixture followed by an overnight incubation at 4°C. After centrifugation at 4.500 rpm for two hours, the medium was discarded, and the virus particles were resuspended in 2%

DMEM. Finally, the sample was aliquoted into 1.5 mL tubes and stored at -80°C for use in subsequent experiments.

3.3 LYSV Titration

For the titration assay, the previously generated pseudovirus underwent a 10-fold serial dilution using DMEM/F12. This serial dilution was subsequently applied to triplicate wells of a 96-well plate seeded with SH-SY5Y cells. Following incubation at 37°C with 5% CO2 for 90 minutes, PBS was used to wash the wells before media replacement, after which the plate was incubated for an additional 24 hours. Post-incubation, a luciferase assay was conducted by introducing lysis buffer to each well, shaking the plate on a shaker for 5 minutes. The samples from each well were then scraped and transferred into individual 1.5 mL tubes. Luciferin was added to each sample, ensuring simultaneous mixing before luminescence analysis using a luminometer, processing each sample one at a time. From the results obtained, TCID50 was determined, allowing for the subsequent calculation of MOI using the formula shown in the **Appendices**.

3.4 Heparin Inhibition Assay

Heparin was initially dissolved in ddH2O, and then serially diluted using a mixture of DMEM/F12. Subsequently, the virus particles were thoroughly combined with the heparin dilution, ensuring proper mixing by vortexing the samples. The media from the pre-seeded SH-SY5Y cells in the 96-well plate was aspirated before adding the serial dilution into each well, performed in triplicate. The plate underwent incubation for 90 minutes at 37°C with 5% CO2. Following this, the cells were gently washed with PBS, and fresh medium was replenished. Finally, a luciferase assay was conducted following the protocol detailed in the previous step, where IC50 was determined at the end.

3.5 Statistical Analysis

The experimental results underwent analysis using GraphPad software. Additionally, cell counting for the heparin inhibition assay was conducted using ImageJ.

IV. RESULTS AND DISCUSSION

The exploration of heparin as a potential inhibitor of lyssavirus attachment to brain cells has gained notable traction in virology. Recent studies have illustrated heparin's capacity to impede virus interaction with heparan sulfate, suggesting a plausible role in hindering viral attachment and entry (Mathieu et al., 2015). Furthermore, in lyssavirus research, pseudoviruses have emerged as a safer substitute for wild-type viruses. These pseudoviruses are fashioned by co-transfecting a plasmid carrying the gene of interest regulated by a promoter, alongside two other plasmids housing a reporter gene and the viral envelope glycoprotein, within a cell line. These engineered pseudovirus particles contain the envelope glycoprotein of the targeted virus but lack its genetic material. They are tailored to carry reporter genes, such as the green fluorescent tag and luciferase gene, rendering them safe for handling in biosafety level 2 laboratories (Diederich et al., 2015). For this procedure, the plasmids were combined with polyethylenimine (PEI) and transfected into HEK293 cells. PEI, a cationic polymer, facilitates the introduction of plasmids (Longo et al., 2013). After transfection, the HEK293 cell line, derived from human embryonic kidney cells, orchestrates the assembly of core genome and envelope proteins from the transfected plasmids. Consequently, these cells, expressing glycoproteins and other viral components, produce pseudoviruses that are released into the cell culture medium (Xiang et al., 2022). HEK293 cells are specifically chosen for their exceptional transfectability and relatively high protein productivity, rendering them an efficient option for transient gene expression studies (Tan et al., 2021).

To obtain the infectivity of the pseudovirus a titration assay was executed in order to obtain the TCID50. As can be seen on **Figure 1**, aside from dilution factor 10^4, the luminescence proportionally decreases as the virus is more diluted. The error in the 10^4 dilution could have been caused by human error where vortexing was inadequate and carryover potentially happened. However, despite this inconsistency, TCID50 was computed by averaging the results of 10^4 and 10^5 dilutions, as they

exhibited similar results. Through this calculation, TCID50 was determined to be $10^{-5.25}$ and the viral titer was determined to be 3.59 x 10^{6} TCID50/mL (calculation shown in **Appendices**). In previous investigations assessing the viral titers of various bat-borne lyssaviruses, conventional viral plaque assays were commonly employed, revealing titers ranging from 1.5 to 5.0 ffu/mL (Shipley et al., 2022). These outcomes varied across distinct lyssavirus species and provided valuable context for understanding the infectivity levels observed in this study.

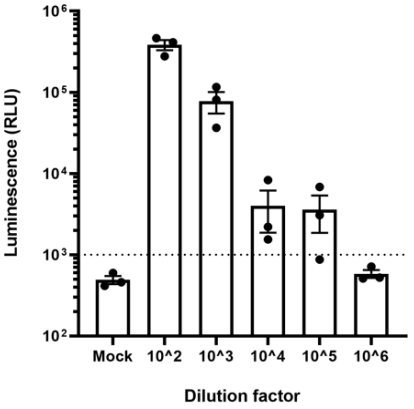

Figure 1. Titration assay result based on luminescence

TCID50 result was used to calculate the MOI resulting in 5×10^{-4} (calculation shown in **Appendices**) which was used to infect the cells for heparin inhibition assay. For this experiment, the pseudovirus was pretreated with different concentrations of heparin, which was subsequently used to infect SH-SY5Y cells. As human neuroblastoma cells, SH-SY5Y cells offer a relevant and natural environment for lyssaviruses to attach and infect. This makes them an invaluable model for researching lyssavirus infection and tropism, as established by studies demonstrating RABV CVS-11 infection and comparable susceptibility patterns to different lyssavirus glycoproteins (Oberhuber et al., 2021; Shipley et al., 2017).

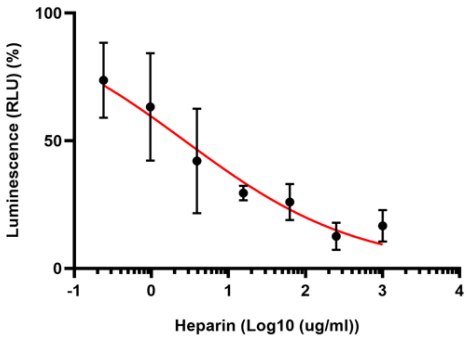

Figure 2. Heparin inhibition assay result based on luminescence

The heparin inhibition assay exhibited a correlation reliant on dosage, indicating that higher concentrations of heparin led to diminished luminescence levels (**Figure 2**). Yet, it's essential to acknowledge discrepancies within the outcomes, notably where the second-highest concentration of heparin resulted in lower luminescence compared to the highest concentration. This incongruity could be ascribed to potential inaccuracies in pipetting or contamination. Despite this, the IC50 was calculated at 2.72 µg/mL. Generally, lower IC50 values imply greater potency or efficacy of the substance in inhibiting the specified function.

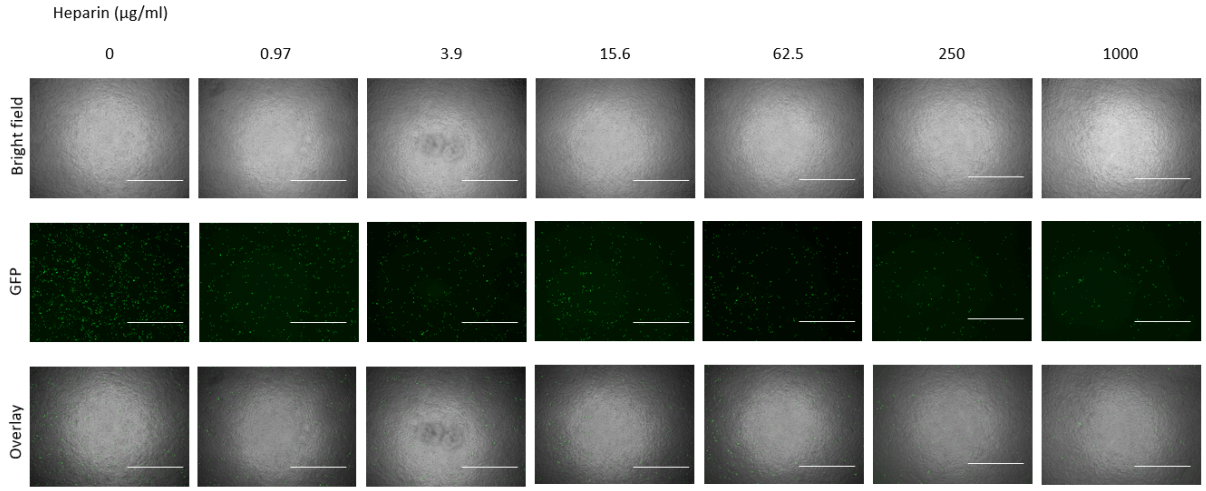

Figure 3. Heparin inhibition assay GFP observation (scale: 1000 µm). From right to left: the infected cell number accounts for: 1512, 735, 604, 514, 489, 220, 289 cells.

The green fluorescent protein (GFP) acts as a natural luminescent marker emitting green light when exposed to particular light wavelengths, like ultraviolet (UV) light (Macel et al., 2020). Within the

tagged plasmid, this protein is expressed, causing virus particles to emit a green fluorescence when viewed under the UV light of the Evos microscope. A rise in green fluorescence indicates a higher concentration of these virus particles or infected cells producing the marker protein. Consistent with the luciferase assay, the presence of GFP in cells decreases gradually as heparin concentration increases (**Figure 3**). These results align with Sasaki et al.'s (2018) study on heparan sulfate's role in rabies virus attachment. Their research showcased heparin's dose-dependent anti-RABV effect. The decrease in virus binding to host cells likely results from heparin directly interacting with RABV virions, notably reducing viral attachment to the cell surface. This indicates heparin competes effectively with the host cell surface for viral binding, ultimately diminishing virus-cell attachment (Sasaki et al., 2018). This competitive binding mechanism significantly contributes to heparin's ability to disrupt virus attachment and subsequent infection.

V. CONCLUSION

Lyssaviruses, notorious for causing fatal neurologic diseases like rabies, often find their home in bats, making them crucial natural reservoirs for various lyssavirus strains. These viruses pose a significant zoonotic threat due to sporadic spillover infections into other mammals, underlining the necessity for understanding their epidemiology and developing effective interventions. Heparin, a member of the GAG family, has emerged as a potential candidate for impeding lyssavirus attachment to brain cells. This study delved into the inhibitory effects of heparin, demonstrating a dose-dependent relationship between heparin concentration and reduced luminescence in heparin inhibition assays. However, the results encountered discrepancies, possibly stemming from pipetting inaccuracies or contamination. Moving forward, it's imperative to address these errors meticulously to ensure robust and reliable findings. This research hints at the potential of heparin as a therapeutic avenue in mitigating lyssavirus infections, albeit with the need for further exploration and refinement in methodology for future investigations.

REFERENCES

Aquino, R. S., & Park, P. W. (2016). Glycosaminoglycans and infection. Frontiers in bioscience (Landmark edition), 21(6), 1260–1277. https://doi.org/10.2741/4455

Bauer, S., Zhang, F., & Linhardt, R. J. (2021). Implications of Glycosaminoglycans on Viral Zoonotic Diseases. Diseases (Basel, Switzerland), 9(4), 85. https://doi.org/10.3390/diseases9040085

Casale, J., & Crane, J. S. (2023). Biochemistry, Glycosaminoglycans. In StatPearls. StatPearls Publishing.

CDC. (2020). Management. Centers for Disease Control and Prevention. https://www.cdc.gov/rabies/specific_groups/hcp/human_rabies.html#

Changalucha, J., Steenson, R., Grieve, E., Cleaveland, S., Lembo, T., Lushasi, K., Mchau, G., Mtema, Z., Sambo, M., Nanai, A., Govella, N. J., Dilip, A., Sikana, L., Ventura, F., & Hampson, K. (2019). The need to improve access to rabies post-exposure vaccines: Lessons from Tanzania. Vaccine, 37 Suppl 1(Suppl 1), A45–A53. https://doi.org/10.1016/j.vaccine.2018.08.086

Charniga, K., Nakazawa, Y., Brown, J., Jeon, S., & Wallace, R. M. (2023). Risk of Rabies and Implications for Postexposure Prophylaxis Administration in the US. JAMA network open, 6(6), e2317121. https://doi.org/10.1001/jamanetworkopen.2023.17121

Climov, M., Leavitt, T., Molnar, J., & Orgill, D. (2016). Natural Biomaterials for Skin Tissue Engineering. Skin Tissue Engineering and Regenerative Medicine, 145–161. doi:10.1016/b978-0-12-801654-1.00008-5

Davis, B. M., Rall, G. F., & Schnell, M. J. (2015). Everything You Always Wanted to Know About Rabies Virus (But Were Afraid to Ask). Annual review of virology, 2(1), 451–471. https://doi.org/10.1146/annurev-virology-100114-055157

Dick, A., & Cocklin, S. (2020). Recent Advances in HIV-1 Gag Inhibitor Design and Development. Molecules (Basel, Switzerland), 25(7), 1687. https://doi.org/10.3390/molecules25071687

Diederich, S., Gedvilaite, A., Zvirbliene, A., Kazaks, A., Sasnauskas, K., Johnson, N., & Ulrich, R. (2015). Immunology of virus-like particles. Viral Nanotechnology, 142–149. https://doi.org/10.1201/b18596-12

Eilts, F., Bauer, S., Fraser, K., Dordick, J. S., Wolff, M. W., Linhardt, R. J., & Zhang, F. (2023). The diverse role of heparan sulfate and other gags in SARS-COV-2 infections and therapeutics. Carbohydrate Polymers, 299, 120167. https://doi.org/10.1016/j.carbpol.2022.120167

Ellwanger, J. H., & Chies, J. A. B. (2021). Zoonotic spillover: Understanding basic aspects for better prevention. Genetics and molecular biology, 44(1 Suppl 1), e20200355. https://doi.org/10.1590/1678-4685-GMB-2020-0355

Fodor E. (2020). Insight into the multifunctional RNA synthesis machine of rabies virus. Proceedings of the National Academy of Sciences of the United States of America, 117(8), 3895–3897. https://doi.org/10.1073/pnas.2000120117

Fooks, A. R., Cliquet, F., Finke, S., Freuling, C., Hemachudha, T., Mani, R. S., … Banyard, A. C. (2017). Rabies. Nature Reviews Disease Primers, 3, 17091. doi:10.1038/nrdp.2017.91

Ghiselli G. (2019). Heparin Binding Proteins as Therapeutic Target: An Historical Account and Current Trends. Medicines (Basel, Switzerland), 6(3), 80. https://doi.org/10.3390/medicines6030080

Hanlon, C. A., & Childs, J. E. (2013). Epidemiology. Rabies, 61–121. doi:10.1016/b978-0-12-396547-9.00003-1

Harris, J., Uren, A., Smith, J., Titmus, E., & Young, M. (2023). Evaluating the benefit of serology during potential Australian bat lyssavirus and rabies post-exposure prophylaxis. Australian and New Zealand journal of public health, 47(6), 100091. Advance online publication. https://doi.org/10.1016/j.anzjph.2023.100091

Jackson A. C. (2018). Rabies: a medical perspective. Rabies: a medical perspective. Revue scientifique et technique (International Office of Epizootics), 37(2), 569–580. https://doi.org/10.20506/rst.37.2.2825

Jackson, A. C., & Fooks, A. R. (2020). Rabies: Scientific basis of the disease and its management. Academic Press.

Jinno, A., & Park, P. W. (2015). Role of glycosaminoglycans in infectious disease. Methods in molecular biology (Clifton, N.J.), 1229, 567–585. https://doi.org/10.1007/978-1-4939-1714-3_45

Johnson, N., Aréchiga-Ceballos, N., & Aguilar-Setien, A. (2014). Vampire bat rabies: ecology, epidemiology and control. Viruses, 6(5), 1911–1928. https://doi.org/10.3390/v6051911

Kato, T., Sakiyama, R., Oka, K., & Nakamura, T. (2015). Identification of core active disaccharides in heparin for HGF-inducing activity. Journal of pharmacology & pharmacotherapeutics, 6(2), 77–82. https://doi.org/10.4103/0976-500X.155483

Khan, S. A., Imtiaz, M. A., Islam, M. M., Tanzin, A. Z., Islam, A., & Hassan, M. M. (2022). Major bat-borne zoonotic viral epidemics in Asia and Africa: A systematic review and meta-analysis. Veterinary medicine and science, 8(4), 1787–1801. https://doi.org/10.1002/vms3.835

Koury, R., & Warrington, S. J. (2022). Rabies. In StatPearls. StatPearls Publishing.

Letko, M., Seifert, S. N., Olival, K. J., Plowright, R. K., & Munster, V. J. (2020). Bat-borne virus diversity, spillover and emergence. Nature Reviews Microbiology. doi:10.1038/s41579-020-0394-z

Longo, P. A., Kavran, J. M., Kim, M. S., & Leahy, D. J. (2013). Transient mammalian cell transfection with polyethylenimine (PEI). Methods in enzymology, 529, 227–240. https://doi.org/10.1016/B978-0-12-418687-3.00018-5

Luo, J., Zhang, Y., Zhang, Q., Wu, Y., Zhang, B., Mo, M., Tian, Q., Zhao, J., Mei, M., & Guo, X. (2019). The Deoptimization of Rabies Virus Matrix Protein Impacts Viral Transcription and Replication. Viruses, 12(1), 4. https://doi.org/10.3390/v12010004

Macel, M. L., Ristoratore, F., Locascio, A., Spagnuolo, A., Sordino, P., & D'Aniello, S. (2020). Sea as a color palette: the ecology and evolution of fluorescence. Zoological letters, 6, 9. https://doi.org/10.1186/s40851-020-00161-9

Masatani, T., Ito, N., Ito, Y., Nakagawa, K., Abe, M., Yamaoka, S., … Sugiyama, M. (2013). Importance of rabies virus nucleoprotein in viral evasion of interferon response in the brain. Microbiology and Immunology, n/a–n/a. doi:10.1111/1348-0421.12058

Mathieu, C., Dhondt, K. P., Châlons, M., Mély, S., Raoul, H., Negre, D., Cosset, F. L., Gerlier, D., Vivès, R. R., & Horvat, B. (2015). Heparan sulfate-dependent enhancement of henipavirus infection. mBio, 6(2), e02427. https://doi.org/10.1128/mBio.02427-14

Parize, P., Sommé, J., Schaeffer, L., Ribadeau-Dumas, F., Benabdelkader, S., Durand, A., Tarantola, A., Cailhol, J., Goesch, J., Kergoat, L., Le Guern, A. S., Mousel, M. L., Dacheux, L., Consigny, P. H., Fontanet, A., Francuz, B., & Bourhy, H. (2021). Systematic Booster after Rabies Pre-Exposure Prophylaxis to Alleviate Rabies Antibody Monitoring in Individuals at Risk of Occupational Exposure. Vaccines, 9(4), 309. https://doi.org/10.3390/vaccines9040309

Qiu, M., Huang, S., Luo, C., Wu, Z., Liang, B., Huang, H., … Lin, J. (2021). Pharmacological and clinical application of heparin progress: An essential drug for modern medicine. Biomedicine & Pharmacotherapy, 139, 111561. doi:10.1016/j.biopha.2021.111561

Radhakrishnan, S., Vanak, A. T., Nouvellet, P., & Donnelly, C. A. (2020). Rabies as a Public Health Concern in India-A Historical Perspective. Tropical medicine and infectious disease, 5(4), 162. https://doi.org/10.3390/tropicalmed5040162

Rampersad, S., & Tennant, P. (2018). Replication and Expression Strategies of Viruses. Viruses, 55–82. https://doi.org/10.1016/B978-0-12-811257-1.00003-6

Sasaki, M., Anindita, P. D., Ito, N., Sugiyama, M., Carr, M., Fukuhara, H., Ose, T., Maenaka, K., Takada, A., Hall, W. W., Orba, Y., & Sawa, H. (2018). The Role of Heparan Sulfate Proteoglycans as an Attachment Factor for Rabies Virus Entry and Infection. The Journal of infectious diseases, 217(11), 1740–1749. https://doi.org/10.1093/infdis/jiy081

Shi, D., Sheng, A., & Chi, L. (2021). Glycosaminoglycan-protein interactions and their roles in human disease. Frontiers in Molecular Biosciences, 8. https://doi.org/10.3389/fmolb.2021.639666

Shipley, R., Wright, E., Smith, S. P., Selden, D., Fooks, A. R., & Banyard, A. C. (2022). Taiwan Bat Lyssavirus: In Vitro and In Vivo Assessment of the Ability of Rabies Vaccine-Derived Antibodies to Neutralise a Novel Lyssavirus. Viruses, 14(12), 2750. https://doi.org/10.3390/v14122750

Shetye, S. S., Miller, K. S., Hsu, J. E., & Soslowsky, L. J. (2017). 7.18 Materials in Tendon and Ligament Repair ☆. Comprehensive Biomaterials II, 314–340. doi:10.1016/b978-0-12-803581-8.09252-3

Tan, E., Chin, C. S. H., Lim, Z. F. S., & Ng, S. K. (2021). HEK293 Cell Line as a Platform to Produce Recombinant Proteins and Viral Vectors. Frontiers in bioengineering and biotechnology, 9, 796991. https://doi.org/10.3389/fbioe.2021.796991

Wang, Q., & Chi, L. (2022). The Alterations and Roles of Glycosaminoglycans in Human Diseases. Polymers, 14(22), 5014. https://doi.org/10.3390/polym14225014

Willoughby, R. E. (2012). Rabies virus. Principles and Practice of Pediatric Infectious Diseases. https://doi.org/10.1016/b978-1-4377-2702-9.00230-0

World Health Organization. (n.d.). Epidemiology and burden. World Health Organization. https://www.who.int/teams/control-of-neglected-tropical-diseases/rabies/epidemiology-and-burden#:~:text=Rabies%20is%20estimated%20to%20cause,occurring%20in%20Africa%20and%20Asia.

Wunner, W. H., & Conzelmann, K.-K. (2013). Rabies Virus. Rabies, 17–60. doi:10.1016/b978-0-12-396547-9.00002-x

Xiang, Q., Li, L., Wu, J., Tian, M., & Fu, Y. (2022). Application of pseudovirus system in the development of vaccine, antiviral-drugs, and neutralizing antibodies. Microbiological research, 258, 126993. https://doi.org/10.1016/j.micres.2022.126993

Yang, F., Lin, S., Ye, F., Yang, J., Qi, J., Chen, Z., … Lu, G. (2020). Structural Analysis of Rabies Virus Glycoprotein Reveals pH-Dependent Conformational Changes and Interactions with a Neutralizing Antibody. Cell Host & Microbe. doi:10.1016/j.chom.2019.12.012

Zandi, F., Goshadrou, F., Meyfour, A., & Vaziri, B. (2021). Rabies Infection: An Overview of Lyssavirus-Host Protein Interactions. Iranian biomedical journal, 25(4), 226–242. https://doi.org/10.52547/ibj.25.4.226

Zhan, J., Harrison, A. R., Portelli, S., Nguyen, T. B., Kojima, I., Zheng, S., Yan, F., Masatani, T., Rawlinson, S. M., Sethi, A., Ito, N., Ascher, D. B., Moseley, G. W., & Gooley, P. R. (2021). Definition of the immune evasion-replication interface of rabies virus P protein. PLoS pathogens, 17(7), e1009729. https://doi.org/10.1371/journal.ppat.1009729

APPENDICES

Formula for MOI calculation

$$MOI = \frac{TCID50}{Number\ of\ cells} \times volume$$

Viral titre calculation

$$Titer = \frac{\frac{1}{TCID50}}{Virus\ Inoculum}$$

$$Titer = \frac{\frac{1}{10^{-5.25}}}{0.05}$$

$$Titer = 3.59 \text{ x } 10^{6} \text{ TCID50/mL}$$

MOI determination from TCID50

$$MOI = \frac{TCID50}{Number\ of\ cells} \times volume$$

$$MOI = \frac{3.59 \times 10^{6}}{5 \times 10^{4}} \times 0.00083$$

$$MOI = 0.0005$$

Taipei Medical University - LTL Lab

Non-Disclosure Agreement

In accordance with laboratory good practice, biosecurity, and intellectual property rights, I acknowledge and agree to comply with this non-disclosure agreement (hereafter referred to as "Agreement"). In case of violation of any provisions of this agreement, the principal investigator (Prof. Liang-Tzung Lin) of the laboratory (LTL Lab) and owners of any intellectual properties of the materials in question shall have the authority to pursue legal action and seek full compensations of all damages.

This agreement applies to all students, research assistants and any other persons participating in the research activities of the LTL Lab (hereafter collectively called "Research Member"). The Research Member shall bear all legal responsibility arising under this agreement.

1. Throughout and after the period of participation in the research activities of LTL Lab, the Research Member shall not do any of the following, except with the express written consent of the principal investigator Prof. Liang-Tzung Lin:

   divulge, communicate or provide the usage of any research data or information of published or unpublished materials to a third party or use any such information/materials for personal use;

   divulge, communicate or otherwise share information of any laboratory materials to a third party or use any such information/materials for personal use; use, divulge, communicate or otherwise share any content of laboratory notebook, access codes, and any information related to laboratory assets and personnel information by printing, copying, photographing, electronic transmission, oral notification or any other means.

2. The laboratory holds the Research Member responsible for all data results and research outcomes performed by the Research Member, and can seek legal action against the Research Member if issues of scientific misconduct and breach of research ethics (as stipulated in Taipei Medical University's graduate student handbook and research ethics guidelines) are identified.

3. The Research Member shall meet the following requirements as part of this Agreement according to the level of clearance and access:

   All research data, including experimental protocols, raw data, graphpad analysis and figure files describing results (PPT, Word, Excel, etc.), project reports, article drafts, thesis, reagent list and any other research-related information or materials arising from work in the LTL Lab, shall be uploaded to the LTL Lab Google Drive;

   Any written materials, including laboratory notebooks, shall be returned to the laboratory;

   All reagents and laboratory materials used by the Research Member should be clearly labeled, listed as an inventory, and returned to the laboratory;

   Laboratory keys, access card, etc. shall be returned to the laboratory and not copied or kept for personal use.

4. In case of violation of any of provisions of this Agreement, the Research Member shall be responsible for damages and all related legal liabilities.

5. The obligation of confidentiality and non-disclosure under this Agreement shall remain in effect for a period of ten years after the execution of this Agreement.

6. This Agreement shall be governed by and construed in accordance with the laws and regulations of Taiwan, ROC.

I hereby confirm having read and understood this Agreement, and agree to all its terms.

Name: JASMINE ANGELICA SYAHIRAH SIMANJUNTAK Position/Title: TEEP	ID No. / Passport No.: E3178565
Address: JL. HANJUANG BLOK I5 NO.5, BSD, TANGERANG SELATAN	Phone: +62 8128207 0525 +886 9738 80641 Email: jasmine.(@ student. ia1. ac.id
Signature:	Date: 06/09/2023