Establishment of the novel method for detection of antibodies against all lyssaviruses and comparison of cross-reactivities among lyssaviruses

全リッサウイルスに対する抗体検出法の確立と交差反応性の比較

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1. General introduction

1.1 Rabies lyssavirus

Rabies lyssavirus infection has been recognized since ancient times, with references dating back to Mesopotamian writings from around 1930 B.C. describing symptoms in dogs and humans suggestive of rabies infection (T. Müller et al., 2022). Bats are often identified as natural reservoirs for RABV, but all mammals are susceptible to infection [10]. The virus is a global concern, though some island nations such as Japan, Australia, and New Zealand, along with certain Scandinavian countries, have maintained a rabies-free status (M. Warrell & Warrell, 2004).

1.2RABV properties

Rabies lyssavirus (RABV) is classified under the Genus *Lyssavirus* within the Subfamily *Alpharhabdovirinae*, the Family *Rhabdoviridae* in the Order *Mononegavirales* (*Genus: Lyss..avirus* | *ICTV*.). The RNA genome of RABV is approximately 12 kilobases in length, non-segmented, and of negative polarity. It encodes five viral proteins in the following order (from 3′ to 5′): nucleoprotein (N protein), phosphoprotein (P protein), matrix (M) protein, glycoprotein (G protein), and polymerase (L protein). The N protein is involved in the formation of the viral nuclear capsid. Due to its high conservation among lyssaviruses, it is commonly utilized for species differentiation within the genus lyssavirus and is a crucial target for laboratory-based rabies diagnosis (Drzewnioková et al., 2023; Heaton et al., 1997). The M protein is located on the inner surface of the virion envelope, where it coordinates virion assembly and budding (Kojima et al.,2015). The G protein, being the only protein expressed on the surface of the virion envelope, is responsible for interacting with host cell receptors (Hellert et al., 2020). This protein plays a crucial role in stimulating the

production of neutralizing antibodies (Barkhouse et al., 2014). The P protein is involved in transcription and replication and also serve to regulate the innate immune system (Gérard et al., 2022). The L protein functions as an RNA-dependent RNA polymerase, facilitating the replication of the viral RNA genome (Nakagawa et al., 2017). Additionally, N proteins bind to RNA, and together with P and L proteins, they constitute the ribonucleoprotein complex essential for various viral processes (Albertini et al., 2006).

RABV is classified into two categories: 'street viruses', which are isolated from animals naturally infected with RABV, and 'fixed viruses'. Fixed viruses are strains that have undergone changes in characteristics, such as reduced infectivity in peripheral tissues. These are typically maintained through continuous passage in the brain tissue of rabbits and other animals over extended periods. Fixed viruses are crucial in the development of rabies vaccines and play a significant role in fundamental virology research (Toovey, 2007).

1.3 Symptoms

RABV is neurotropic, primarily affecting the nervous system. Typically, rabies is transmitted through the saliva of the infected animal, often via a bite. Once the virus enters the host, it travels along peripheral nerves to the central nervous system, leading to severe neurological symptoms (Fooks et al., 2017). The clinical progression of rabies includes several stages: incubation, prodrome, acute neurological signs, coma, and death. The incubation period can range from weeks to years, but typically lasts 1 to 2 months. Initial symptoms are often non-specific, including fever, headache, and general weakness, and progress to more severe neurological

manifestations such as agitation, hallucinations, and paralysis (Hemachudha et al., 2005). Without prompt post-exposure prophylaxis (PEP), rabies is invariably fatal once clinical symptoms appear (Changalucha et al., 2019).

1.4 Diagnosis of rabies

A conclusive diagnosis of rabies necessitates laboratory testing, as clinical symptoms alone are insufficient. The diagnosis encompasses both ante-mortem and post-mortem methods. Ante-mortem diagnosis primarily uses skin biopsies from the nape of the neck and saliva samples. These skin biopsies are analyzed to detect the virus antigen in nerve cells adjacent to hair follicles. Additionally, both skin and saliva samples can be examined for viral RNA through reverse transcription PCR (RT-PCR) (Dacheux et al., 2008; Heaton et al., 1997). For post-mortem diagnosis, the analysis of brain tissue is standard practice. This involves the Fluorescent Antibody Test (FAT), a method endorsed by international health authorities like the World Health Organization (WHO) and the World Organization for Animal Health (WOAH) for its reliability in identifying the rabies virus in tissue samples (*OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees) 5th Edn. Volumes 1 & 2. World Organization for Animal Health 2004. ISBN 92 9044 622 6. €140.* | *Parasitology* | *Cambridge Core*, n.d.; Organization, 2018).

1.5 Prevention

In humans, over 95% of rabies cases are transmitted by dogs, particularly in Asia and Africa, posing a significant public health challenge. To address this, an international initiative, Zero By Thirty, aims to eradicate human death by dog-transmitted rabies by 2030 (Hampson et al., 2019). This initiative focuses on enhancing access to PEP for bite victims, educating the

public on bite prevention, and increasing dog vaccination rates. Achieving a vaccination rate of 70% or more in dogs is considered essential for effective rabies control, alongside ongoing surveillance and research efforts (Hampson et al., 2009).

Rabies prevention includes both pre-exposure prophylaxis (PrEP) and PEP. In the event of potential exposure to rabies, immediate PEP is vital, starting with thorough wound cleaning using soap and water. This is followed by the administration of rabies immunoglobulin (RIG) at the wound site to provide passive immunity, and a series of vaccinations on days 0, 3, 7, and 14 to activate the body's immune response against the virus (Rupprecht et al., 2020). For those at high risk of exposure, such as veterinarians and wildlife workers, PrEP is recommended.

In Europe and North America, oral rabies vaccination has been developed as a strategy to control the spread of rabies among wildlife (Fehlner-Gardiner, 2018; F. T. Müller & Freuling, 2018). Since the 1970s, the use of highly attenuated rabies viruses in oral bait vaccines has been instrumental in managing rabies in these animal communities (T. Müller & Freuling, 2020). In recent years, there has been notable advancement in the development of vector vaccines. This innovative class of vaccines integrates the rabies virus glycoprotein into other virus, such as poxviruses and adenoviruses (T. Müller & Freuling, 2020). These vaccines are incorporated into bait and distributed in outdoor environments. The goal is to disseminate these vaccine-laden baits across a region, thereby vaccinating wild animal populations, establishing herd immunity, and preventing the further spread and incursion of RABV. This approach has been adopted in around 30 European countries, as well as in Canada and the United States. It has been particularly successful in virtually eliminating fox-mediated rabies transmission in almost all of Ontario, Canada (MacInnes et al., 2001).

1.6 Rabies control in Japan

Japan has successfully eliminated any human cases of rabies since 1955 and only five human cases imported from other countries were reported. Additionally, since 1957, there have been no rabies infection among terrestrial animals in the country. These achievements are largely attributed to the Rabies Prevention Law, established in 1950 (Yamada et al., 2019). This act mandates that dog owners register their pets and ensure annual vaccination against rabies. In 2022, the vaccination rate for registered dogs in Japan reached 70.4% (Ministry of Health, Labour and Welfare, n.d.), exceeding the 70% threshold. However, this percentage may not fully represent the actual vaccination rate, as it does not include unregistered and stray dogs (Amemiya et al., 2023). Moreover, Japan enforces stringent quarantine measures for the import and export of animals, further safeguarding the nation against the introduction of rabies (Takahashi-Omoe et al., 2008).

1.7 Other Lyssaviruses

1.7.1 Taxonomy

All lyssaviruses cause neurological disease in mice when infected intracranially under laboratory conditions (Banyard et al., 2018; Fooks et al., 2017; Klein et al., 2022). Since the 1950s, numerous lyssaviruses related to RABV have been identified, and all were capable of inducing rabies-like symptoms. Until now, 17 lyssavirus species have been documented, including RABV, Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV) Duvenhage lyssavirus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Aravan lyssavirus (ARAV), Australian bat lyssavirus (ABLV), Khujand lyssavirus (KHUV), West caucasian bat lyssavirus (WCBV), Irkut lyssavirus (IRKV), Shimoni bat lyssavirus

(SHIBV), Ikoma lyssavirus (IKOV), Bokeloh bat lyssavirus (BBLV), Lleida bat lyssavirus (LLEBV), Gannoruwa bat lyssavirus (GBLV) and Taiwan bat lyssavirus (TWBLV) (Banyard et al., 2014; Gunawardena et al., 2016; Hu et al., 2018). These viruses are officially recognized by the International Committee on Taxonomy of Viruses (ICTV) (*Genus: Lyssavirus | ICTV*.). In addition, Kotalahti bat lyssavirus (KBLV) has been recently discovered from a dead Brandt's bat (*Myotis brandtii*) in Eastern Finland as a novel lyssavirus (Nokireki et al., 2018). Of these, 16 viruses have been isolated from bat species. MOKV was isolated from rodent species (Coertse et al., 2017; Shope et al., 1970) and IKOV was from African civet (Marston et al., 2012). Until now, at least seven lyssaviruses, RABV, ABLV, DUVV, EBLV-1, EBLV-2, IRKV and MOKV, have been responsible for fatal infections in humans. Lyssaviruses can be classified into two phylogroups by their genomic sequences (Badrane et al., 2001). Phylogroup I consists of RABV, ABLV, ARAV, BBLV, DUVV, EBLV-1, EBLV-2, GBLV, IRKV, KBLV, KHUV, and TWBLV, and phylogroup II includes LBV, MOKV, and SHIBV. However, WCBV, IKOV, and LLEBV are unclassified.

1.7.2Protection of other lyssaviruses

As of now, there are no vaccines specific for lyssaviruses other than RABV. However, studies have been conducted to evaluate if RABV vaccines could offer protection against other lyssaviruses. These studies have shown that lyssaviruses within the same phylogroup as RABV demonstrate some degree of cross-reactivity and have provided protection against infection in mouse models (Brookes et al., 2005; Malerczyk et al., 2014). In contrast, lyssaviruses belonging to phylogroup II and those that are unclassified do not exhibit cross-reactivity and have not shown protection in

infection experiments (Badrane et al., 2001; Hanlon et al., 2005). Currently, efforts are being made to develop vaccines and monoclonal antibodies that could offer broad-spectrum protection against various lyssaviruses (Bentley et al., 2017; De Benedictis et al., 2016).

1.8 Phylogroup I lyssaviruses

1.8.1 Duvenhage lyssavirus (DUVV)

DUVV was first identified in South Africa in 1970 after a human death, which was linked to an encounter with an unidentified bat (Van et al., 2011). To date, there have only been five reported cases of DUVV infection. Three of these were fatal human cases (in 1970, 2006, and 2007), each following contact with a small, unidentified bat (Van et al., 2011). The other two instances involved small bats: one case in South Africa in 1981, where the bat species was not identified, and another in 1986 from a Egyptian slit-faced bat (*Nycteris thebaica*) in Zimbabwe (Foggin et al., 1988). Interestingly, virus neutralization assays conducted in Swaziland found DUVV-neutralizing antibodies in 30% of sera from a population of *N. thebaica* bats. Due to the limited number of recorded infections, the precise bat species that serves as the primary reservoir for DUVV remains unknown.

1.8.2 European bat lyssavirus 1,2 (EBLV-1,2)

EBLV-1 was first discovered in 1977 during European bat surveillance. This virus is primarily associated with Serotine bats (*Eptesicus serotinus*) and Isabelline bats (*E.isabellinus*) (Schatz et al., 2013). Two subtypes of EBLV-1, named EBLV-1a and EBLV-1b, have been identified, each with distinct geographical distributions.

EBLV-1a is found across an east-west axis from Russia to France, with a high number of cases in Germany, France, Poland, and the Netherlands. EBLV-1b, on the other hand, has a south-north distribution stretching from Spain to the Netherlands (Amengual et al., 1997; Troupin et al., 2017). EBLV-2 was isolated in 1986 from a patient in Finland who had contact with bats (Lumio et al., 1986). EBLV-2 occurrences are sporadic and have always been associated with Myotis bats (*M. daubentonii and M. dascyneme*) in the United Kingdom, Netherlands, Germany, Switzerland, Norway, and Finland (McElhinney et al., 2018). From 1977 to 2016, a total of 1183 cases of bat rabies were reported, with the majority attributed to EBLV-1. In comparison, there have been only 39 suspected cases of EBLV-2 (McElhinney et al., 2018, p. 2). EBLV-1 has also been observed in a small number of terrestrial mammals, including five sheep in Denmark, two cats in France, and a stone marten in Germany (Dacheux et al., 2009; T. Müller et al., 2004). In addition, four human deaths due to EBLV have been reported (Fooks et al., 2003).

1.8.3 Australian bat lyssavirus (ABLV)

ABLV was first isolated in 1996 from the black flying-fox (*Pteropus alecto*) (Fraser et al., 1996). This virus is known to infect large flying-foxes and insectivorous bats, which are considered to be reservoir hosts for ABLV (Gould et al., 2002). The infection rate in wild bat populations is estimated to be less than 1% in Australia (Weir et al., 2014). So far, ABLV has not been detected outside Australia. Infected bats may display behavioral changes such as abnormal aggression and agitation, along with neurological symptoms. The initial discovery of ABLV was linked to a bat exhibiting these clinical symptoms. Notably, two samples were significant in this discovery: one

from a black flying fox found unable to fly beneath a tree in 1996, and another from a paraffin-embedded tissue sample of a black flying fox euthanized due to aggressive behavior (Fraser et al., 1996). In Australia, there have been three documented cases of Australian bat lyssavirus (ABLV) infection, all of which resulted in neurological symptoms and were fatal (Francis et al., 2014; Hanna et al., 2000; Samaratunga et al., 1998). Each of these cases was confirmed to have had direct contact with bats. Additionally, two domestic horses were infected in 2013 in the same paddock. Similarly, this particular case of infection is also reported to have involved contact with bats (Shinwari et al., 2014).

1.8.4 Irkut lyssavirus (IRKV)

IRKV was first isolated from greater tube-nosed bat (*Murina leucogaster*) in Russia in 2002 (Botvinkin et al., 2003). To date, there have been three documented human infections in Russia, each of which resulted in the death (Leonova et al., 2013; Poleshchuk et al., 2023). In China, IRKV has been confirmed to infect dogs and bats (Liu et al., 2013; Teng et al., 2018). Furthermore, IRKV has been shown to cause fatal infections in cats during experimental studies (Teng et al., 2018). These cases highlight the risk that IRKV poses not only to wildlife but also to domestic animals and humans.

1.8.5 Aravan lyssavirus (ARAV), Khujand lyssavirus (KHUV)

ARAV was isolated from mouse-eared bat (*Myotis blythi*) in southern Kyrgyzstan in 1991, and KHUV was isolated from whiskered bat (*Myotis mystacinus*) in northern Kyrgyzstan in 2001 (Arai et al., 2003; Kuzmin et al., 2003). To date, only a single instance of each of these viruses has been identified. There have

been no reported cases of either ARAV or KHUV infections in any species other than bats.

1.8.6 Bokeloh bat lyssavirus (BBLV)

BBLV was first isolated from Natterer's bat (*Myotis nattereri*) in Germany in 2010 (Freuling et al., 2011). Two years later, in 2012, BBLV was again detected in the same species of bat in France (Eggerbauer et al., 2017). To date, there have been six confirmed cases of BBLV infection in bats in Germany, two in France, and one in Poland (Smreczak et al., 2018). In addition to Natterer's bat, there has been one recorded case involving the common pipistrelle bat (*Pipistrellus pipistrellus*) (Eggerbauer et al., 2017). A phylogenetic analysis of the whole-genome sequences of isolates from these nine cases, reveals that BBLV can be categorized into two distinct lineages. There appears to be a geographical pattern in the distribution of these two lineages, but the limited number of known infections makes it difficult to draw comprehensive conclusions (Smreczak et al., 2018). Moreover, studies comparing the virulence of different BBLV strains have not yet been conducted.

1.8.7 Gannoruwa bat lyssavirus (GBLV)

GBLV was isolated from Indian flying foxes (*Pteropus medius*) in Sri Lanka in 2016 (Gunawardena et al., 2016). In the span of approximately one year, starting from 2014, 62 bats were tested, and 4 of these tested positive for GBLV using the direct fluorescence antibody test. Out of these, the virus was successfully isolated from 3 bats. To date, this remains the only reported instance of GBLV, and there have been no other reported cases of GBLV infection in any species other than bats.

1.8.8 Taiwan bat lyssavirus (TWBLV)

TWBLV was isolated from Japanese pipistrelle (*Pipistrellus abramus*) in Taiwan in 2016 (Hu et al., 2018). Taiwan has long been a rabies-free country, but in 2013, for the first time in 50 years, a weasel badger was found positive for RABV (*History of Rabies Control in Taiwan and China.Pdf*, n.d.). During 2018 and 2021, as part of a passive lyssavirus surveillance program, TWBLV was identified in 3 out of 407 bats surveyed. Two of these bats were Japanese pipistrelles, and one was a Chinese noctule (*Nyctalus plancyi velutinus*) (Hu et al., 2022). The TWBLV isolated from Chinese noctule is particularly notable because its N protein gene sequence shows less than 80% homology with the N protein sequences of other lyssaviruses, including TWBLV. This significant genetic difference has led to its categorization as a new lyssavirus. It suggests the possibility of differentiating between TWBLV-1 and TWBLV-2, given the distinct genetic characteristics identified in the TWBLV strain from Chinese noctule (Hu et al., 2022). There have been no other reported cases of TWBLV infection in any species other than bats.

1.8.9 Kotalahti bat lyssavirus (KBLV)

KBLV was first detected from Brandt's bat (*Myotis brandtii*) in Finland in 2018. Following this initial detection, a survey of bat samples collected in Slovenia between 2012 and 2019 identified the KBLV gene in the brain of a long-fingered bat (*Myotis capaccinii*). Although KBLV has not been officially registered with the ICTV as of yet, it is considered to be a potentially new species of lyssavirus, which would make it the 18th identified species within this genus.

1.9Phylogroup II lyssaviruses

1.9.1 Lagos bat lyssavirus (LBV)

LBV was first isolated from fruit bat in Nigeria in1956 (Boulger & Porterfield, 1958). During 1956 and 2020, only 32 LBV cases were detected. LBV have been identified in a variety of bat species, including *Eidolon helvum*, *Epomophorus wahlbergi*, species within the Rousettus genus, *Micropterus pusillus*, and *Rousettus aegyptiacus*. Phylogenetically, LBVs are categorized into four distinct lineages, labeled A to D. Based on the limited genomic sequences available, groups A and B appear to be predominantly associated with *E. helvum*, group C with *E. wahlbergi*, and group D with *R. aegyptiacus* (Coertse et al., 2021). Infections of LBV have been confirmed in terrestrial mammals, including dogs, cats, and mongooses (Crick et al., 1982; Markotter et al., 2006; Mebatsion et al., 1992, p. 115). LBV belongs to phylogroup II. The effectiveness of vaccines designed for RABV is reported to be low against LBV. Cases of LBV infection have been confirmed in cats that were vaccinated against RABV (Foggin et al., 1988).

1.9.2 Mokola lyssavirus (MOKV)

MOKV was first isolated from shrews in Nigeria in 1968 (Shope et al., 1970). Unlike many other lyssaviruses, MOKV has not been found in bat species. Since its discovery, over 20 MOKV strains have been isolated from various animals including cats, rats, and dogs, exclusively within the African continent (Sabeta et al., 2007). The frequent detection of MOKV in cats, along with the presence of MOKV antibodies in a population of rodents and shrews (McMahon et al., 2021), has led to the hypothesis

that small mammals might be the reservoir for the virus. There have been two recorded human infections with MOKV, one of which resulted in recovery (Familusi et al., 1972; Sabeta et al., 2007). This, combined with the observation that some MOKV strains are not lethal in mouse infection experiments (Badrane et al., 2001), suggests that MOKV might be less virulent compared to RABV. Similar to LBV, MOKV is part of phylogroup II. The effectiveness of vaccines designed for RABV is reported to be low against MOKV. Cases of MOKV infection have been confirmed in cats that were vaccinated against RABV, paralleling the situation observed with LBV (Sabeta et al., 2007).

1.9.3 Shimoni bat lyssavirus (SHIBV)

Shimoni bat lyssavirus (SHIBV) was isolated from a striped leaf-nosed bat (*Hipposideros vittatus*) in Kenya in 2009 (Kuzmin et al., 2010). Since then, this particular specie remains the only isolated case of SHIBV; no further instances have been detected through genetic testing. However, an epidemiological survey conducted among bats in Nigeria revealed that more than 50% of the tested bats had neutralizing antibodies against SHIBV (Vora et al., 2020). This finding raises concerns about the potential for bat-to-human transmission. SHIBV, like MOKV and LBV, belongs to phylogroup II, and vaccine protection against RABV is said to be less effective.

1.10 Unclassified lyssavirus

1.10.1 West Caucasian bat lyssavirus (WCBV)

WCBV was first isolated from common bent-winged bat (*Miniopterus schreibersi*) in Russia in 2002 (Botvinkin et al., 2003). Since its initial discovery, there

have been no further reports of the virus until 2020, when a cat in Italy was found to be infected with WCBV (Leopardi et al., 2021). This cat, which lived outdoors, is suspected to have come into contact with bats. Subsequent testing of sera from bats in the vicinity of where the cat lived revealed the presence of specific neutralizing antibodies against WCBV. Since WCBV is an unclassified lyssavirus species, RABV vaccines are generally considered ineffective against it. The discovery of WCBV infection in a domestic cat, a common companion animal, suggests increased caution concerning the potential spread of this lyssavirus to other animals and possibly humans.

1.10.2 Ikoma lyssavirus (IKOV)

IKOV was detected in the brain of an African civet (*Civettictis civetta*) with clinical signs of rabies in Tanzania in 2009 (Marston et al., 2012). The virus was successfully isolated in 2014. While this particular infection occurred in an African civet, the species is not considered to be the natural reservoir for IKOV since it contracted rabies. Bats in the vicinity were also tested, but no specific neutralizing antibodies against IKOV were identified in these populations (Horton et al., 2014). Since IKOV is an unclassified lyssavirus strain similar to WCBV, the current vaccines for RABV are ineffective against it (Horton et al., 2014).

1.10.3 Lleida bat lyssavirus (LLEBV)

LLEBV was first detected from bent-winged bat (*Miniopterus schreibersii*) in Spain in 2012 (Ceballos et al., 2013). Following this initial discovery, LLEBV was again isolated from the same species of bat in France, approximately 750 kilometers away from the original location (Picard-Meyer et al., 2019). Beyond these two

instances, there have been no further reports of LLEBV, nor has there been any evidence of transmission to other animal species. Similar to WCBV and IKOV, LLEBV remains unclassified. Consequently, vaccines developed for the RABV are not effective against LLEBV (Banyard et al., 2018)

2. Chapter 1

Cross-neutralization activities of antibodies against 18 lyssavirus glycoproteins

2.1 Abstract

Some lyssaviruses, including the rabies virus (RABV), induce lethal neurological symptoms in humans. However, commercial vaccines have only been evaluated for their efficacy against RABV and not against other lyssaviruses. To assess cross-reactivity among lyssaviruses, including RABV, sera from rabbits inoculated with human and animal RABV vaccines and polyclonal antibodies from rabbits immunized with expression plasmids of the glycoproteins of all 18 lyssaviruses were prepared, and cross-reactivity was evaluated via virus-neutralization tests using RABV, European bat lyssavirus-1 (EBLV-1), Duvenhage virus (DUVV), Mokola virus (MOKV), and Lagos bat virus (LBV). The sera against RABV vaccines showed cross-reactivity with EBLV-1 and DUVV, which both belong to phylogroup I. However, the reactivity with MOKV and LBV in phylogroup II was notably limited or below the detection level. Next, we compared the cross-reactivity of the polyclonal antibodies against all the lyssavirus glycoproteins. Polyclonal antibodies had high virus-neutralization titers against the same phylogroup, but not against different phylogroups. Our findings indicate that a new vaccine should be developed for pre- and post-exposure prophylaxis against lyssavirus infections.

2.2 Introduction

Lyssaviruses are single-stranded negative-sense RNA viruses belonging to the genus Lyssavirus, subfamily Alpharhabdovirinae, family Rhabdoviridae, order Mononegavirales (*Genus: Lyssavirus | ICTV*.). Rabies lyssavirus (RABV) causes one of the most serious zoonotic diseases worldwide, and some other lyssaviruses also cause neurological disorders in humans similar to RABV. To date, 17 lyssavirus species have been reported by the International Committee on Taxonomy of Viruses: RABV, Australian bat lyssavirus (ABLV), Duvenhage lyssavirus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Aravan lyssavirus (ARAV), Khujand lyssavirus (KHUV), Irkut lyssavirus (IRKV), Bokeloh bat lyssavirus (BBLV), Gannoruwa bat lyssavirus (GBLV), Taiwan bat lyssavirus (TWBLV), Lagos bat lyssavirus (LBV), Mokola lyssavirus (MOKV), Shimoni bat lyssavirus (SHIBV), West Caucasian bat lyssavirus (WCBV), Ikoma lyssavirus (IKOV), and Lleida bat lyssavirus (LLEBV) (1). Kotalahti bat lyssavirus (KBLV) was recently discovered from a dead Brandt's bat (Myotis brandtii) in Eastern Finland as a novel lyssavirus (Nokireki et al., 2018). At least seven lyssaviruses–RABV, ABLV, DUVV, EBLV-1, EBLV-2, IRKV, and MOKV–have been shown to cause lethal diseases in humans (Shepherd et al., 2023).

Lyssaviruses are classified into two phylogroups based on their sequences (Badrane et al., 2001): phylogroup I comprised RABV, ABLV, DUVV, EBLV-1, EBLV-2, ARAV, KHUV, IRKV, BBLV, GBLV, TWBLV, and KBLV, and phylogroup II comprised LBV, MOKV, and SHIBV. Meanwhile, WCBV, IKOV, and LLEBV have not been classified. In this study, we examined cross-reactivity among five lyssaviruses, including RABV, using a virus-neutralization (VN) assay of sera immunized with commercial RABV vaccines for humans and animals and polyclonal antibodies against

all 18 lyssavirus glycoproteins.

2.3 Materials and methods

2.3.1 Virus and Cells

Stock viruses of the challenge-virus-standard (CVS)-11 strains of RABV, EBLV-1, DUVV, MOKV and LBV were prepared in mouse neuroblastoma (MNA) cells, which were propagated in Minimum Essential Medium (MEM; Thermo Fisher Scientific, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific) at 37°C in 5% CO_2 incubator. RABV, DUVV, EBLV-1, MOKV, LBV and MNA cell lines were kindly provide by Dr. Rupprecht in Rabies Section, Viral and Rickettsia Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Center for Disease Control and Prevention, Atlanta, GA, USA.

2.3.2 Virus-neutralization test

The modified fluorescent antibody virus neutralization test (mFAVN) were performe(Brookes et al., 2005; Smith et al., 1973). Briefly, the complement in the sera was inactivated at 56 °C for 30 minutes before assay. Serial three-fold diluted sera from 1:15 were mixed with equal volume of each lyssavirus containing approximately 50 to 300 $TCID_{50}$ in 96 well plate (Iwaki, Tokyo, Japan). After incubation at 37℃ for 1 hour, 4.0×10^4 cells of MNA cells were added to the mixture and the plates were incubated at 37°C for 4 days. As the control serum, WHO reference serum was used. The cells were fixed with 80% cold acetone at 4 ℃ for 30 min and then washed three times with PBS. After washing, FITC Anti-rabies Monoclonal Globulin (Fujirebio, Tokyo, Japan) was diluted to 1:100 with PBS and then added to wells. For counterstaining, 1:500 diluted Evans blue was added. After incubation at 37°C for 30 min, cells were washed three

times with PBS. The VN titer to rabies virus was expressed as the reciprocal of the highest serum dilution at which 50% or less of the wells showed a positive signal. All experiments were independently repeated at least twice, and the geometric mean was calculated. Experiments using EBLV-1, DUVV, MOKV and LBV were performed in biosafety level (BSL)-3 laboratories according to the institutional guidelines. RABV (CVS-11 strain) was used in BSL-2 laboratory.

2.3.3 Construction of expression plasmids

Genes with complete open reading frames encoding glycoproteins of DUVV (accession no. JN986749.1), EBLV-1 (KP241939.1), EBLV-2 (EF157977.2), ARAV (EF614259.1), KHUV (EF614261.1), IRKV (JX442979.1.2), BBLV (JF311903.1), GBLV (KU244266.2), TWBLV (MF472710.1), LBV (EU259198.1), MOKV (NC_006429.1), SHIBV (GU170201.1), WCBV (EF614258.1), IKOV (JX193798.1), LLEBV (KY006983.1), and KBLV (LR994545.1) were synthesized (Azenta Life Sciences, MA, USA) and cloned into the expression vector pCAGGS. The expression plasmid encoding the ABLV glycoprotein (AF426298) was kindly provided by Prof. Broder in Uniformed Services University, USA.

2.3.4 Production of sera from rabbits inoculated with RABV vaccines

Four female Japanese white rabbits (Kitayama Labes, Nagano, Japan) were used: two were subcutaneously inoculated with the human RABV vaccine Rabipur (GSK Biologicals, Wavre, Belgium) six times at 2-week intervals, and the other two were administered the animal RABV vaccine KMB (KM Biologics, Kumamoto, Japan). Sera were collected 1 week after the last inoculation. This animal experiment was

approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (permission numbers: 120083).

2.3.5 Production of sera from rabbits immunized lyssaviral glycoprotein

Female Japanese white rabbits were inoculated with 400 μg of each plasmid DNA six times at 2-week intervals, and sera were then collected 2 weeks after the final injection (Kaku et al., 2009). All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (permission numbers: 120146, 121128, and 122165). Anti-RABV G (CVS-11) rabbit serum was prepared as previously described (Hotta et al., 2007). All polyclonal antibodies against the 18 lyssavirus glycoproteins were confirmed to have high titers by an indirect fluorescence assay using plasmid-transfected cells (data not shown).

2.4 Results

2.4.1 Virus-neutralization test of RABV vaccine-immunized rabbit sera

The mFAVN results indicated that the sera from rabbits immunized with the human vaccine Rabipur had the highest VN titers against RABV and cross-neutralizing antibody titers against EBLV-1 and DUVV, 22.7% and 68.3% of the VN titers against RABV, respectively (Fig. 1, Table 1). In contrast, VN titers against MOKV and LBV were over 100-fold lower than those against RABV, or below the detection limit. Sera from rabbits immunized with the animal vaccine KMB showed a trend similar to that of Rabipur-immunized rabbit sera. VN titers against EBLV-1 and DUVV were 28.6–30.9% and 3.5-11.1% of those against RABV, respectively. Meanwhile, VN titers against MOKV and LBV were below the detection limit. These results indicate that rabies vaccines available in Japan can induce high VN titers against phylogroup I lyssaviruses but not against phylogroup II lyssaviruses.

2.4.2 Cross-neutralization titers of polyclonal antibodies against 18 lyssavirus glycoproteins

The mFAVN results using 18 anti-lyssaviral glycoproteins polyclonal antibodies showed that sera against glycoproteins of phylogroup I lyssaviruses had VN titers against RABV, EBLV-1, and DUVV but had lower VN titers against MOKV and LBV (Fig. 2, Table 1). Interestingly, polyclonal antibodies against the glycoproteins of EBLV-1 and -2 showed broad VN activity against both phylogroups I and II. In contrast, polyclonal antibodies against glycoproteins of GBLV and KHUV did not neutralize the infection by DUVV belonging to phylogroup I. Similarly, sera against glycoproteins of phylogroup II lyssaviruses had high VN titers against MOKV and LBV but had lower

VN titers against RABV, EBLV-1, and DUVV. Notably, the polyclonal antibody against the SHIBV glycoprotein possessed a sufficient VN titer against RABV. Polyclonal antibodies against WCBV, IKOV, and LLEBV glycoproteins did not have high VN titers against any of the five viruses.

2.5 Discussion

In this study, the rabies vaccines available in Japan did not show high cross-reactivity against phylogroup II lyssaviruses, suggesting that further vaccine development is recommended for pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) against lyssavirus infection. In previous reports, most sera from humans inoculated with the rabies vaccine did not possess VN activity against lyssaviruses belonging to different phylogroups, whereas some showed broad VN activity (De Benedictis et al., 2016; Malerczyk et al., 2014). In addition, human rabies immunoglobulin (hRIG) and equine rabies immunoglobulin (eRIG) for PEP could not effectively neutralize lyssaviruses outside of phylogroup I, even when they were administered at high concentrations (Coertse et al., 2023). Because PrEP and PEP must prevent mortality due to all lyssavirus infections, further vaccines should be developed. In addition, a few human monoclonal antibodies developed from vaccinated humans possess broad VN activity against lyssaviruses, indicating the possibility of developing a novel vaccine that induces broad cross-protective antibodies (De Benedictis et al., 2016). Our data indicated the glycoproteins of the phylogroup I lyssaviruses EBLV-1 and -2 are potential candidates for vaccine antigens that can induce broad protective antibodies against lyssaviruses. It is unclear why EBLV-1 and -2 glycoproteins could induce wild cross-reactive antibodies. However, because only five lyssaviruses were used in this study, the other 13 lyssaviruses should be analyzed for cross-reactivity in future experiments.

Until now, any bat lyssavirus has not been reported in Japan. On the other hand, in Taiwan, a neighboring country of Japan, TWBLV was reported in Japanese house bats (Pipistrellus abramus) (Hu et al., 2018). Additionally, EBLV-1, EBLV-2, BBLV, and

IRKV have been detected from the bat species existing in Japan (Coertse et al., 2021; Dundarova et al., 2023; Ohdachi et al., 2015). LBV belonging to phylogroup II was isolated from various bat species including both fruit and insectivorous bats (Coertse et al., 2021) and MOKV was detected in shrews and rodents (Shepherd et al., 2023). Since lyssaviruses may also exist in Japan, a novel vaccine that is broadly protective against many lyssaviruses, including RABV, is needed.

In conclusion, our study showed that the current Japanese vaccines are not cross-protective against all lyssaviruses, suggesting the importance of vaccine development for PrEP and PEP against all lyssaviruses. In addition, some lyssavirus glycoproteins may be candidates for novel vaccines to induce broad VN activity.

2.6 Figure legends

Fig. 1. Virus-neutralization test of RABV vaccine-immunized rabbit sera using mFAVN test against RABV, EBLV-1, DUVV, MOKV, and LBV. The minimum value on the Y-axis is set at 50, which is almost equal to 0.5 IU/mL.

Fig. 2. Cross-neutralization titers of polyclonal antibodies against 18 lyssavirus glycoproteins. The minimum value on the Y-axis is set at 50, which is almost equal to 0.5 IU/mL. Red, blue, and green bars represent phylogroup I, phylogroup II, and unclassified lyssaviruses, respectively.

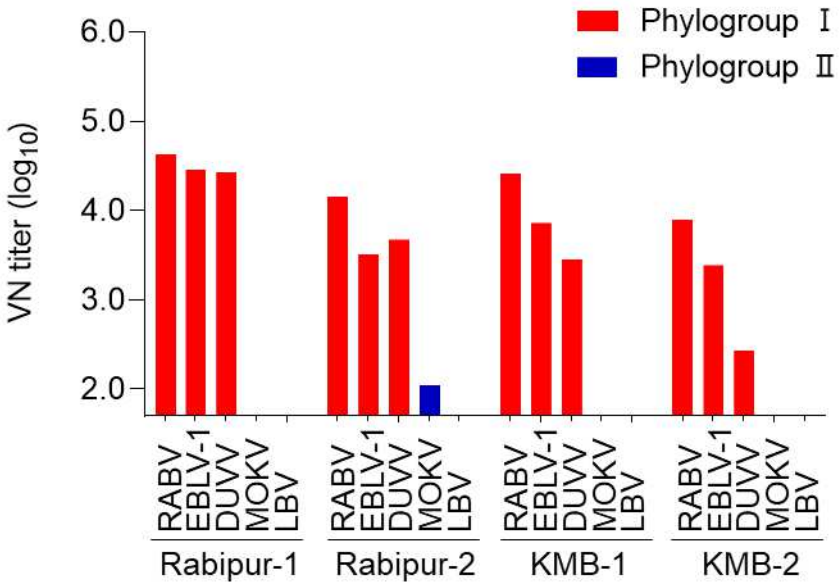
2.7 Tables and figures

Table 1. mFAVN titers against 5 lyssaviruses

Antibodies	Phylogroup	mFAVN titers				
		Phylogroup I			Phylogroup II	
		RABV	EBLV-1	DUVV	MOKV	LBV
RABV-G	I	2104	1718	1569	<50[1)]	<50
ABLV-G	I	9644	8928	9644	<50	97
GBLV-G	I	2976	619	<50	<50	<50
KHUV-G	I	191	302	<50	<50	<50
BBLV-G	I	8150	7188	4705	154	<50
ARAV-G	I	945	3645	1215	<50	<50
KBLV-G	I	302	331	135	<50	<50
EBLV-2-G	I	992	2104	2430	1856	525
DUVV-G	I	25515	40915	220063	302	82
IRKV-G	I	331	2396	357	110	135
TWBLV-G	I	4706	2625	2025	225	58
EBLV-1-G	I	315	6655	2625	2218	546
SHIBV-G	II	1718	246	154	44193	5568
LBV-G	II	135	<50	64	2976	4706
MOKV-G	II	105	64	82	688905	4910
WCBV-G	Unclassified	64	174	174	<50	90
IKOV-G	Unclassified	<50	<50	<50	<50	<50
LLEBV-G	Unclassified	<50	<50	<50	<50	<50
Rabipur-1	I (vaccine)	42351	28931	26785	<50	<50
Rabipur-2	I (vaccine)	14117	3215	4706	110	<50
KMB-1	I (vaccine)	25515	7290	2835	<50	<50
KMB-2	I (vaccine)	7874	2430	270	<50	<50

1) Our preliminary study indicated that mFAVN titer of 50 is nearly equal to 0.5IU/mL, which is considered as the protective antibody titer by WHO.

Figure 1.


Phylogroup Ⅰ
Phylogroup Ⅱ
VN titer (log10)
6.0
5.0
4.0
3.0
2.0
RABV
EBLV-1
DUVV
MOKV
LBV
Rabipur-1
Rabipur-2
KMB-1
KMB-2

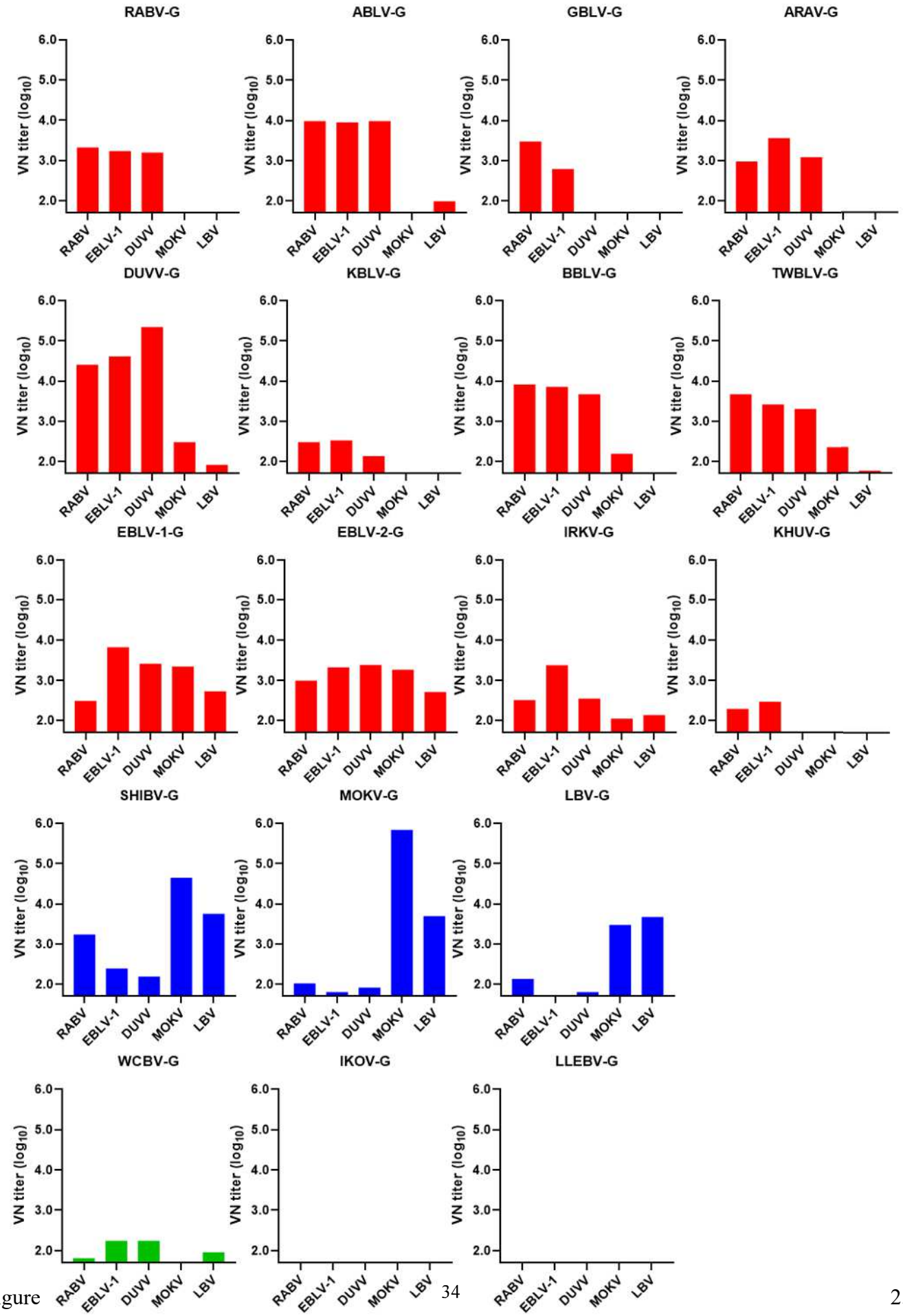

RABV-G
ABLV-G
GBLV-G
ARAV-G
DUVV-G
KBLV-G
BBLV-G
TWBLV-G
EBLV-1-G
EBLV-2-G
IRKV-G
KHUV-G
SHIBV-G
MOKV-G
LBV-G
WCBV-G
IKOV-G
LLEBV-G
VN titer (log10)
6.0
5.0
4.0
3.0
2.0
RABV
EBLV-1
DUVV
MOKV
LBV

Figure 2.

3. Chapter 2

Establishment of serological neutralizing tests

using pseudotyped viruses for

comprehensive detection of antibodies against all 18 lyssaviruses

3.1Abstract

Rabies is a fatal zoonotic, neurological disease caused by rabies lyssavirus (RABV) and other lyssaviruses. In this study, we established novel serological neutralizing tests (NT) based on vesicular stomatitis virus pseudotypes possessing all 18 known lyssavirus glycoproteins. Applying this system to comparative NT against rabbit sera immunized with current RABV vaccines, we showed that the current RABV vaccines fail to elicit sufficient neutralizing antibodies against lyssaviruses other than to those in phylogroup I. Furthermore, comparative NT against rabbit antisera for 18 lyssavirus glycoproteins showed glycoproteins of some lyssaviruses elicited neutralizing antibodies against a broad range of lyssaviruses. This novel testing system will be useful to comprehensively detect antibodies against lyssaviruses and evaluate their cross-reactivities for developing a future broad-protective vaccine.

3.2 Introduction

Rabies is a neglected infectious disease that is responsible for an estimated 59,000 human deaths worldwide each year (Hampson et al., 2015). The disease in terrestrial animals and humans is primarily caused by the classical rabies lyssavirus (RABV), which is classified under the Genus Lyssavirus within the Subfamily *Alpharhabdovirinae*, belonging to the Family *Rhabdoviridae* in the Order *Mononegavirales* (*Genus: Lyssavirus | ICTV*.). Once clinical symptoms of rabies appear, the disease is almost invariably fatal (M. J. Warrell & Warrell, 2015). Since the 1950s, numerous lyssaviruses related to RABV have been identified. All lyssaviruses cause neurological disease in mice when infected intracranially under laboratory conditions (Banyard et al., 2018; Fooks et al., 2017). To date, 17 lyssavirus species have been documented: RABV, Lagos bat lyssavirus (LBV) in 1956, Mokola lyssavirus (MOKV) in 1968, Duvenhage lyssavirus (DUVV) in 1970, European bat lyssavirus 1 (EBLV-1) in 1977, European bat lyssavirus 2 (EBLV-2) in 1986, Aravan lyssavirus (ARAV) in 1991, Australian bat lyssavirus (ABLV) in 1996, Khujand lyssavirus (KHUV) in 2001, West Caucasian bat lyssavirus (WCBV) and Irkut lyssavirus (IRKV) in 2002, Shimoni bat lyssavirus (SHIBV) and Ikoma lyssavirus (IKOV) in 2009, Bokeloh bat lyssavirus (BBLV) in 2010, Lleida bat lyssavirus (LLEBV) in 2012, Gannoruwa bat lyssavirus (GBLV) in 2016, and Taiwan bat lyssavirus (TWBLV) in 2018 (Banyard et al., 2014; Gunawardena et al., 2016; Hu et al., 2018). These viruses are officially recognized by the International Committee on Taxonomy of Viruses (*Genus: Lyssavirus | ICTV*.). In addition, Kotalahti bat lyssavirus (KBLV) has been recently discovered from a dead Brandt's bat (*Myotis brandtii*) in Eastern Finland as a novel lyssavirus (Nokireki et al., 2018). Of these 18 lyssaviruses,

16, (not MOKV or IKOV) have been isolated from bat species (Shipley et al., 2019). MOKV has been isolated from rodent species (Coertse et al., 2017; Shope et al., 1970) and IKOV from the African civet (Marston et al., 2012). Until now, at least seven lyssaviruses, RABV, ABLV, DUVV, EBLV-1, EBLV-2, IRKV, and MOKV, have been responsible for fatal infections in humans (Shepherd et al., 2023). While instances of human infection by lyssaviruses other than RABV are rare, they are fatal and the real number of cases is unknown because of limited surveillance and misdiagnosis (Cleaveland et al., 2002; Mallewa et al., 2007).

Lyssaviruses can be classified into two phylogroups by their genomic sequences (Badrane et al., 2001). Phylogroup I consists of RABV, ABLV, ARAV, BBLV, DUVV, EBLV-1, EBLV-2, GBLV, IRKV, KBLV, KHUV, and TWBLV, and phylogroup II includes LBV, MOKV, and SHIBV. However, WCBV, IKOV, and LLEBV are unclassified. Historically, research has primarily focused on the cross-reactivity of RABV vaccine immune sera against other lyssaviruses (Fooks et al., 2021). These investigations have demonstrated that RABV vaccines do not offer protection against other phylogroup lyssaviruses. Consequently, the search for vaccine antigens effective against new lyssaviruses has become imperative. However, there has been limited exploration of cross-reactivity using immune sera tailored to each specific lyssavirus (Hanlon et al., 2005). In our previous study, cross-neutralization activities using only 5 lyssaviruses were compared, suggesting limited cross-reactivities among lyssaviruses (Inoue et al., Manuscript in press). To further validat cross-reactivities among lyssaviruses in detail, comprehensive neutralization assays using all lyssaviruses would need to be conducted, however, it is very difficult to obtain all the viruses to be tested. Therefore, in this study, cross-reactivities among all 18

lyssaviruses were examined using a panel of vesicular stomatitis viruses (VSVs) pseudotyped with all 18 lyssavirus glycoproteins. These tools enabled us to perform neutralization tests (NTs) to conduct a comprehensive analysis of cross-reactivities for the entire range of known lyssaviruses.

3.3 Materials and methods

3.3.1 Construction of expression plasmids

Expression plasmids of lyssaviral Glycoprotein required for the production of pseudotyped viruses and antisera were produced by artificial gene synthesis (Azenta, MA, USA). These expression plasmids were constructed as described our recent study (Inoue et al, Manuscript in submission). Briefly, complete open reading frames encoding glycoproteins of RABV-SRV9 strain (Accession number, AF499686.2), ARAV (EF614259.1), BBLV (JF311903.1), DUVV (JN986749.1), EBLV-1 (KP241939.1), EBLV-2 (EF157977.2), GBLV (KU244266.2), IRKV (JX442979.1.2), KBLV (LR994545.1), KHUV (EF614261.1), TWBLV (MF472710.1), LBV (EU259198.1), MOKV (NC_006429.1), SHIBV (GU170201.1), WCBV (EF614258.1), IKOV (JX193798.1) and LLEBV (KY006983.1) were synthesized and cloned into the expression plasmid, pCAGGS. The expression plasmid encoding G protein of ABLV (AF426298) was kindly provided by Prof. Christopher C. Broder, Department of Microbiology and Immunology, Uniformed Services University, the USA.

3.3.2 Production of pseudotyped virus

The production of SEAP-expressing VSV pseudotyped with lyssaviral Glycoprotein was performed using method previously reported (Kaku et al., 2012). Briefly, plasmids expressing each glycoprotein were transfected into 80% confluent HEK293T cells using polyethylenimine (PEI) (Thermo Fisher Scientific, Waltham, MA, USA). On two days post-transfection, VSVΔG-SEAP, a recombinant VSV whose G gene was replaced by the SEAP gene was inoculated at a multiplicity of infection of 1. VSVΔG-SEAP was kindly provided by Dr. Y. Matsuura, Osaka University, Japan. After

24 hr, the culture supernatants including each VSVp were collected and filtered through a 0.45 μm syringe filter (MERCK, Darmstadt, Germany) to remove cell debris, and stored at -80°C until use. Each VSVp was named based on its pseudotyped glycoprotein, e.g., VSVp-RABV.

3.3.3 Titration of pseudotyped viruses

Serial two-fold dilutions of pseudotyped viruses in MEM-2% FBS were inoculated onto MNA cells (4×10^5cells/mL) monolayers seeded in 96-well culture plates. After 1h incubation, the MNA cells were washed three times with MEM and extra 100 μl of MEM-2% FBS was added. After one day incubation, 40 μl of supernatant was transferred to new 96-well plates, and then, 200 μl of substrate solution (SIGMAFAST p-Nitrophenyl Phosphate Tablets, Thermo Fisher Scientific) was added and incubated for 2h at 37℃. The reaction was measured using a spectrophotometer (iMarkTM Microplate Absorbance Reader, BIO-RAD, Hercules, CA, U.S.A.) at OD_{405}. The dilution at which the OD_{405} was between 1.0 and 2.0 was used for serum neutralization test thereafter.

3.3.4 Serum neutralization test with pseudotyped virus

The serum neutralization test with SEAP-expressing VSV pseudotyped was performed using method previously reported (Kaku et al., 2012). Briefly, the complement in the sera was inactivated at 56 ℃ for 30 min. Serial four times dilutions of sera prepared in MEM-2% FBS were mixed with an equal volume of pseudotyped viruses. After incubating the mixtures at 37℃ for 1h, the mixtures were added to monolayers of MNA cells (4×10^5cells/mL), in 96 well plate (Iwaki, Tokyo, Japan) and

the plates were incubated at 37°C for 1h. After incubation, the MNA cells were washed three times with MEM and extra 100 μl culture medium was added and the plate was incubated at 37°C for 20-24h. The SEAP activities were measured as above. The neutralization titers are represented as the serum dilution that reduced VSVp infectivity by 75% (IC75) compared with no-serum control. IC75 was calculated by CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). The VSVps for EBLV-1 and IKOV did not yield measurable titers, prompting the creation of chimeric glycoproteins. These chimeric envelope glycoproteins were engineered by fusing the ectodomains and transmembrane domains of the EBLV-1 and IKOV envelope glycoproteins with the cytoplasmic domain from the VSV glycoprotein. The expression plasmid encoding the VSV glycoprotein (AJ318514) was kindly provided by Dr. S. Fukushi, Department of Virology I, National Institute of Infectious Diseases, Japan (Fukushi et al., 2008).

3.3.5 Production of sera from rabbits inoculated with RABV vaccines

These antisera were generated as described our recent study (Inoue et al, Manuscript in press). Briefly, four female Japanese white rabbits (Kitayama Labes, Nagano, Japan) were used. Two were subcutaneously inoculated with the human RABV vaccine, Rabipur (GSK Biologicals, Wavre, Belgium), six times at 2-week intervals, and the other two were administered the animal RABV vaccine, KMB (KM Biologics, Kumamoto, Japan). Sera were collected at 1 week after the last inoculation. The animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (permission number: 120083).

3.3.6 Production of sera from rabbits immunized lyssaviral glycoprotein

These antisera were generated in our previous study (Inoue et al, Manuscript in press). Anti-lyssaviral G polyclonal rabbit sera were produced by inoculation to rabbits with expression plasmids encoding each lyssavirus glycoproteins. Female Japanese white rabbits were inoculated with 400 μg of each plasmid DNA six times at two week intervals and then sera were collected two weeks after final injection (Kaku et al., 2009). These experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (Permission Number 120146, 121128, and 122165). Anti-RABV G (CVS-11) rabbit serum was prepared in the previous study (Hotta et al., 2007).

3.4 Results

3.4.1 Comparison of neutralizing titers of Rabies lyssavirus (RABV) vaccine-immunized rabbit sera against 18 lyssaviruses

To examine the cross-protective activities of human and animal RABV vaccines against lyssaviruses, comparative NT was conducted between all the VSVps and a panel of rabbit antisera generated by RABV vaccination in our previous study (Inoue et al, Manuscript in submission): Briefly, the results of NTs using VSVps indicated that the sera from rabbits immunized with the human vaccine, Rabipur, had high neutralization titers against RABV. These sera also displayed cross neutralizing reactions against other phylogroup I lyssaviruses, with titers within a four-fold range of those against RABV (Fig. 1, Table 1). In contrast, neutralization titers against phylogroup II and unclassified lyssaviruses were over 100 times lower than those against RABV, or below the detection limit. Sera from rabbits immunized with the animal vaccine, KMB, showed a trend similar to that of Rabipur-immunized rabbit sera (Fig. 1, Table 1). These findings indicate that the current rabies vaccines are effective at inducing high serum neutralization titers against lyssaviruses in phylogroup I but have limited to no efficacy against phylogroup II and unclassified lyssaviruses.

3.4.2 Comprehensively analyzed using 18 VSVp and polyclonal anti-glycoprotein sera

Next, to investigate whether any lyssavirus glycoproteins can induce broad protective antibodies, cross neutralization reactivity among lyssaviruses was comprehensively analyzed using 18 VSVp and polyclonal anti-glycoprotein sera. NTs using the VSVps with rabbit sera against the glycoproteins of all 18 lyssaviruses

revealed specific patterns of cross-reactivity according to their phylogroups: sera directed against glycoproteins from phylogroup I lyssaviruses exhibited high neutralization titers against VSVps of the same phylogroup, yet they showed reduced neutralizing ability against phylogroup II. Notably, the neutralizing titers against VSVps of unclassified lyssaviruses, WCBV, LLEBV, and IKOV, were nearly undetectable (Fig. 2, Table 1). Similarly, antisera against phylogroup II glycoproteins showed strong neutralization against their corresponding VSVps but weaker neutralization against phylogroup I. Almost no neutralizing activity was observed against the VSVps of the unclassified lyssaviruses. In contrast, within the same phylogroup, some discrepancies in cross-reactivity were observed: rabbit sera against GBLV and KHUV glycoproteins did not neutralize VSVp-DUVV, even though they belong to the same phylogroup I. Similarly, antiserum against MOKV glycoprotein exhibited limited cross-reactivity with VSVp-SHIBV and LBV, which are part of phylogroup II. Interestingly, antisera against EBLV-1 glycoprotein demonstrated a broad neutralization capacity, affecting both phylogroup I and II VSVps. In contrast, VSVps of unclassified lyssaviruses showed unique reactivity: antisera against IKOV and LLEBV glycoproteins showed almost no cross-reactivity with any of the VSVps tested. Interestingly, the antiserum against WCBV glycoprotein, despite being an unclassified lyssavirus, was capable of neutralizing several VSVps of phylogroup I (Fig. 2, Table 1).

3.5 Discussion

This comprehensive study highlights the challenges and the innovations needed to evaluating the cross-reactivity of lyssaviruses. Conventional methods, such as the Rapid Fluorescent Focus Inhibition Test and the Fluorescent Antibody Virus Neutralization test, which are considered gold standards by the World Health Organization and The World Organisation for Animal Health (Ciconello et al., 2022; Moore & Hanlon, 2010; World Health Organization, 2018)are time-consuming, and require biosafety level -3 facilities and expensive reagents, such as fluorescent antibodies. Pseudotyped rabies viruses with either green fluorescent protein or luciferase as a biomarker have been successfully generated and employed for high-throughput screening (Cai et al., n.d.; Wright et al., 2008). The pseudotyped virus expressing SEAP as a biomarker, which we utilized in this study, can be quantified using an absorbance system, such as ELISA, and it offers a straightforward and cost-effective alternative. In a recent investigation related to SARS-CoV-2, the pseudotyped virus neutralization antibody titers were regarded as the most reliable indicator of vaccine efficacy and protection, primarily because of the remarkable sensitivity of NT-based pseudotyped virus (Benkeser et al., 2023).

In this study, even the hyperimmune sera generated with six RABV vaccinations, failed to exhibit cross-reactivity with phylogroup II and unclassified lyssavirus, indicating the limitation of RABV vaccination against lyssavirus infections. In a previous report, cats with a history of three RABV vaccinations were infected with LBV belonging phylogroup II. The cats were euthanized after a 3-day illness characterized by neurological symptoms (Foggin et al., 1988). In addition, most sera from humans inoculated with the RABV vaccine did not possess virus neutralization

activity against lyssaviruses belonging to different phylogroups (De Benedictis et al., 2016; Malerczyk et al., 2014). These findings indicate that existing pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) measures for use with RABV vaccines may not be effective in preventing infections caused by phylogroup II and unclassified lyssaviruses.

The comprehensive NT in this study offers critical insights into the complexity of lyssavirus immunology and the risk of relying solely on genetic homology for predicting antigenic cross-reactivity. While lyssaviruses within the same phylogroup generally exhibit a relatively high degree of glycoprotein amino acid homology (Evans et al., 2012), the observed lack of cross-neutralization among certain lyssaviruses (e.g., GBLV, KHUV, and DUVV) despite being in the same phylogroup indicates that small variations in glycoprotein amino acids , especially in neutralizing epitopes, can lead to significant changes in antigenic structure. While the precise locations of neutralizing epitopes in RABV glycoprotein (RABV-G) have been identified using techniques such as mutagenesis and monoclonal antibodies (mAbs), the locations of the neutralizing epitopes in other lyssavirus glycoproteins are only inferred based on their known positions in RABV-G (Dietzschold et al., 1990; Lafon et al., 1983). Considering the difficulty to predict cross-reactivity among lyssaviruses solely on amino acid sequence homology of whole glycoproteins, further studies on detailed analysis of antigenic structures of each lyssavirus are expected.

Lyssaviruses other than RABV have been reported in a limited number of human infections. MOKV from phylogroup II was responsible for an infection in an infant that led to a fatal outcome. Furthermore, infections with neurological signs in companion animals have also been reported, including cases of LBV from phylogroup II affecting

dogs and cats (Coertse et al., 2021), and WCBV, an unclassified strain, infecting cats (Leopardi et al., 2021). It has become clear that “rabies free” countries (M. Warrell & Warrell, 2004), have endemic lyssaviruses circulating within bat populations, such as ABLV in Australia and EBLV-1, 2 in the UK (Folly et al., 2021; Prada et al., 2019; Wise et al., 2017). In both nations humans have died from lyssavirus infection as a result of bat bites (Fooks et al., 2003; Fraser et al., 1996). These matters highlight the need for the development of pan-lyssavirus vaccines capable of providing protection against all lyssaviruses.

The lyssavirus glycoprotein is instrumental in triggering the production of neutralizing antibodies (Hellert et al., 2020). Notably, monoclonal antibodies (mAbs) from individuals who received the RABV vaccine have recently been isolated, and some of these mAbs have demonstrated broad-spectrum neutralization activity against various lyssaviruses (De Benedictis et al., 2016; Hellert et al., 2020). Additionally, there have been reports on the immunogenicity of chimeric glycoproteins possessing neutralizing epitope sites of the G protein of MOKV or LBV (phylogroup II) and RABV-G (phylogroup I) in various combinations, which succeeded in acquiring broad cross-reactivities against both phylogroups I and II (Evans et al., 2018; Fisher et al., 2020). This suggested that the detailed analysis of the reactivity of each lyssavirus glycoprotein other than RABV-G could be utilized to develop broad-reactive vaccines. Our findings of broad-spectrum neutralization by EBLV-1 and WCBV antisera are particularly promising, as they may be able to guide the development of a pan-lyssavirus vaccine. The identification of glycoproteins that elicit cross-protective antibodies may serve as the basis for a next-generation vaccine design that would offer protection against a range of lyssaviruses, not just RABV. This novel testing system will

be useful to comprehensively detect antibodies against lyssaviruses and evaluate their cross-reactivities for developing a future broad-protective vaccine.

3.6 Figure legends

Figure 1.

Comparison of neutralizing titers of Rabies lyssavirus (RABV) vaccine-immunized rabbit sera against 18 lyssaviruses. Serum neutralization tests using sera from two rabbits immunized with human RABV vaccine (Rabipur-1 and -2) and two rabbits immunized with animal RABV vaccine (KMB-1 and -2) were conducted against vesicular stomatitis viruses pseudotyped with 18 lyssaviruses. The titers are shown as the geometric mean of two independent experiments.

Figure 2.

Summary of cross-reactivity in serum neutralization tests among 18 lyssaviruses. The relative neutralizing titers were compared in all combinations between 18 pseudotyped viruses (VSVps) and 18 rabbit sera against each lyssavirus glycoprotein (-G). The relative neutralizing titers of each combination were calculated as the ratio of the neutralizing antibody titer against the corresponding VSVp set as 1.000, and illustrated by the color gradient: red signifying high cross-reactivity to white denoting no cross-reactivity. Phylogroups are enclosed with dotted line.

3.7 Tables and figures

Table 1. Neutralization titers against 18 pseudotyped virus

Rabbit antiserum	Phylogroup	Neutralization titers against VSVp (IC_{75})																	
		I													II		Unclassified		
		RABV	GBLV	ABLV	KHUV	BBLV	EBLV-2	ARAV	KBLV	EBLV-1	DUVV	IRKV	TWBLV	MOKV	SHIBV	LBV	WCBV	LLEBV	IKOV
RABV-G	I	2,187	726	2,475	444	951	1,348	468	450	89	1,211	1,989	135	<10	<10	<10	<10	<10	<10
GBLV-G	I	10,399	23,970	40,105	11,897	22,308	4,739	5,312	7,632	874	20	3,662	8,174	49	520	30	213	<10	<10
ABLV-G	I	7,671	37,844	81,190	19,883	12,229	8,733	7,664	9,583	2,229	7,026	10,193	456	760	792	470	<10	<10	<10
KHUV-G	I	487	3,985	9,228	11,342	1,954	1,455	1,931	3,501	155	51	443	166	352	937	228	<10	<10	<10
BBLV-G	I	4,489	24,544	37,041	16,180	32,422	11,573	11,660	12,695	3,026	15,360	11,753	7,289	93	191	330	<10	<10	<10
EBLV-2-G	I	1,743	12,916	34,401	9,532	9,250	13,190	10,699	6,968	5,588	1,402	3,263	749	272	1,264	294	38	<10	<10
ARAV-G	I	5,052	9,050	16,112	5,086	6,592	3,763	15,416	4,985	2,014	4,187	9,732	5,531	<10	26	14	<10	<10	<10
KBLV-G	I	1,082	4,902	16,737	3,787	2,358	3,208	1,518	8,643	370	777	421	1,169	<10	<10	12	<10	<10	<10
EBLV-1-G	I	970	2,524	5,805	4,365	3,410	3,088	2,266	3,563	4,012	2,145	5,894	1,191	688	950	412	27	<10	<10
DUVV-G	I	4,880	19,159	59,446	38,658	29,820	47,743	37,519	35,864	17,659	116,600	60,585	2,325	43	353	164	<10	<10	<10
IRKV-G	I	1,363	1,288	2,705	1,559	1,278	1,338	733	1,149	620	1,583	7,878	756	53	10	167	<10	<10	<10
TWBLV-G	I	5,886	14,278	20,533	5,981	10,028	6,499	5,640	9,130	1,821	5,301	6,321	44,684	104	92	87	<10	<10	<10
MOKV-G	II	956	959	7,125	3,146	570	1,592	529	1,657	30	32	74	299	201,967	4,311	2,924	67	<10	24
SHIBV-G	II	14,254	43,130	64,102	43,625	12,745	3,711	9,606	10,610	888	604	11,297	911	36,372	512,586	73,811	<10	<10	<10
LBV-G	II	166	553	1,491	278	223	123	33	272	20	29	195	63	154	4,697	5,103	<10	<10	42
WCBV-G	Unclassified	148	1,559	3,716	2,067	2,437	2,910	86	617	359	40	177	48	70	45	138	8,116	<10	45
LLEBV-G	Unclassified	<10	<10	<10	23	56	295	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	39,418	2,867
IKOV-G	Unclassified	<10	<10	<10	<10	<10	31	<10	<10	<10	<10	<10	<10	<10	327	12	<10	17	22,752
Rabipur-1	I (vaccine)	21,066	22,289	36,116	10,046	23,967	15,403	14,030	11,803	5,624	16,880	31,772	8,860	<10	78	17	<10	<10	<10
Rabipur-2	I (vaccine)	7,887	11,055	20,947	3,193	7,848	8,059	5,446	4,386	2,067	8,791	11,494	2,120	44	158	72	<10	<10	<10
KMB-1	I (vaccine)	19,532	13,531	18,577	7,089	17,253	10,750	7,458	7,471	2,578	16,155	11,008	7,247	<10	<10	<10	<10	<10	<10
KMB-2	I (vaccine)	9,004	3,295	7,359	2,143	2,821	1,854	1,052	766	146	1,223	1,456	1,965	<10	<10	<10	<10	<10	<10

The abbreviations for the 18 pseudotyped viruses (VSVp) and antisera against each lyssavirus glycoprotein (-G) are listed below: RABV, Rabies lyssavirus; GBLV, Gannoruwa bat lyssavirus; ABLV, Australian bat lyssavirus; KHUV, Khujand lyssavirus; BBLV, Bokeloh bat lyssavirus; EBLV-2, European bat lyssavirus 2; ARAV, Aravan lyssavirus; KBLV, Kotalahti bat lyssavirus; EBLV-1, European bat lyssavirus 1; DUVV, Duvenhage lyssavirus; IRKV, Irkut lyssavirus; TWBLV, Taiwan bat lyssavirus; MOKV, Mokola lyssavirus; SHIBV, Shimoni bat lyssavirus; LBV, Lagos bat lyssavirus; WCBV, West Caucasian bat lyssavirus; LLEBV, Lleida bat lyssavirus; IKOV, Ikoma lyssavirus

The sera of the rabbits immunized with human rabies vaccine (Rabipur) and animal rabies vaccine (KMB) were also used.

Figure 1.

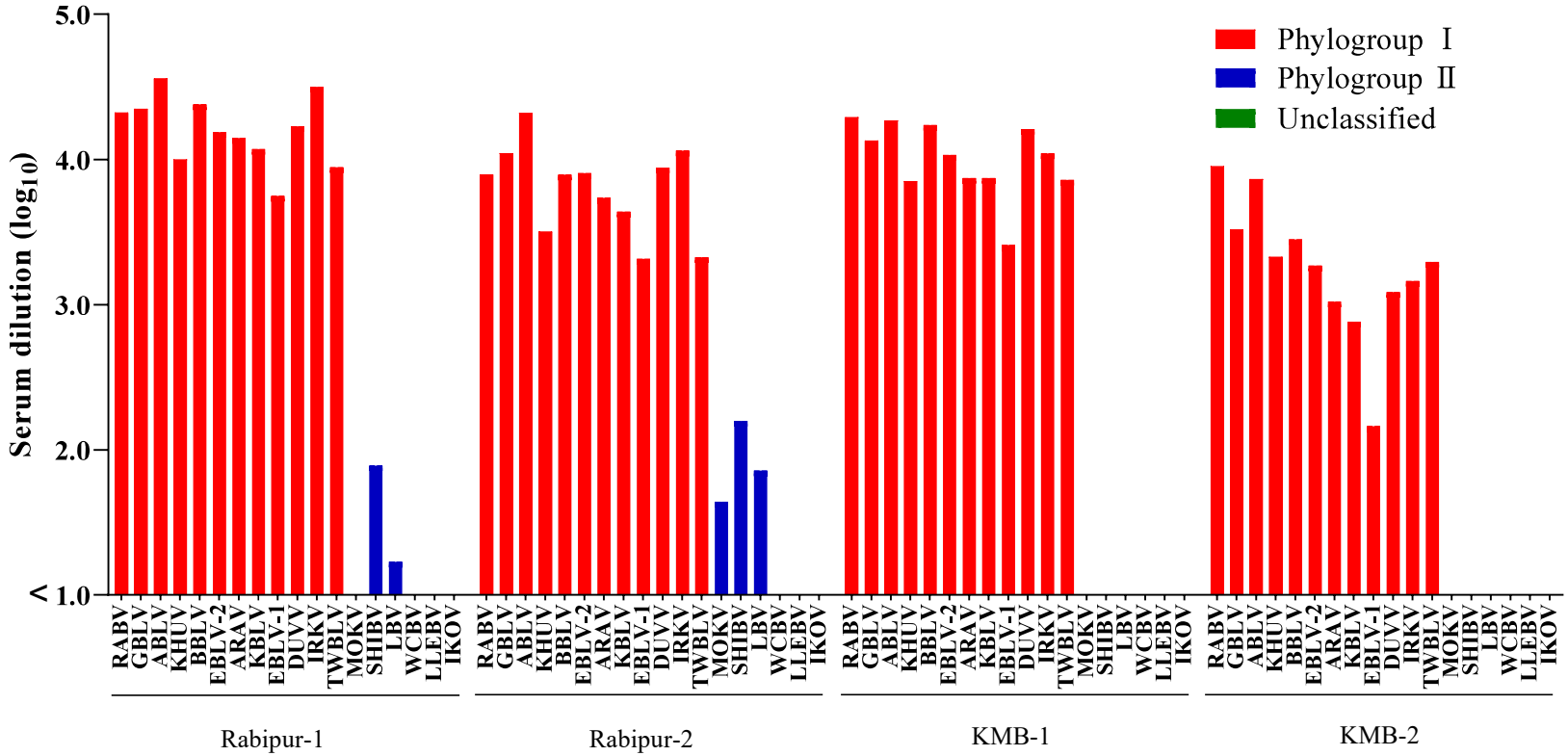
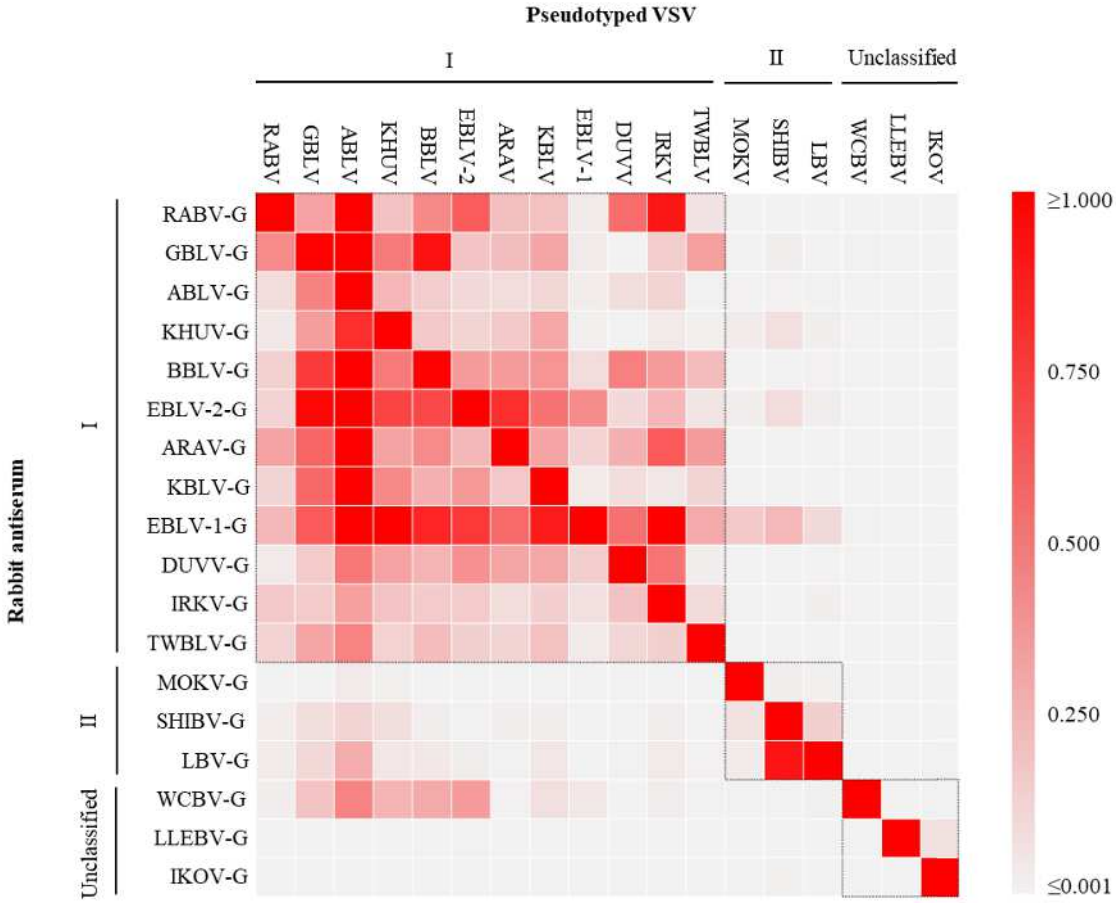

Phylogroup Ⅰ
Phylogroup Ⅱ
Unclassified
Serum dilution (log_{10})
5.0
4.0
3.0
2.0
< 1.0
RABV
GBLV
ABLV
KHUV
BBLV
EBLV-2
ARAV
KBLV
EBLV-1
DUVV
IRKV
TWBLV
MOKV
SHIBV
LBV
WCBV
LLEBV
IKOV
Rabipur-1
Rabipur-2
KMB-1
KMB-2

Figure 2.


Pseudotyped VSV
I
II
Unclassified
RABV
GBLV
ABLV
KHUV
BBLV
EBLV-2
ARAV
KBLV
EBLV-1
DUVV
IRKV
TWBLV
MOKV
SHIBV
LBV
WCBV
LLEBV
IKOV
Rabbit antiserum
RABV-G
GBLV-G
ABLV-G
KHUV-G
BBLV-G
EBLV-2-G
ARAV-G
KBLV-G
EBLV-1-G
DUVV-G
IRKV-G
TWBLV-G
MOKV-G
SHIBV-G
LBV-G
WCBV-G
LLEBV-G
IKOV-G
≥1.000
0.750
0.500
0.250
≤0.001

4. General Conclusion

Lyssaviruses belong to the genus Lyssavirus of the family *Rhabdoviridae*. Among the lyssaviruses, rabies lyssavirus (RABV) infects all mammals, including humans, and causes fatal neurological symptoms. Despite global countermeasures against rabies, approximately 50,000 deaths have been reported annually, and various studies have been conducted on RABV, but little research has been done on the other lyssaviruses. To date, 18 species of viruses have been found in the genus Lyssavirus, including RABV, and they are classified into three groups: Phylogroup I, which includes RABV, Phylogroup II, and an unclassified species. In addition to RABV, at least six other lyssaviruses have been reported to infect humans and cause fatal infections.

In my PhD course, the protective efficiency of the Japanese RABV vaccines against lyssaviruses was evaluated in Chapter 1, and the novel system to detect antibody against all 18 lyssaviruses was established and cross-reactivity was compared in Chapter 2.

Chapter 1 Cross-neutralization activities of antibodies against 18 lyssavirus glycoproteins

Some lyssaviruses, including RABV, induce lethal neurological symptoms in humans. However, commercial vaccines have only been evaluated for their efficacy against RABV and not against other lyssaviruses. To assess cross-reactivity among lyssaviruses, including RABV, sera from rabbits inoculated with human and animal RABV vaccines and polyclonal antibodies from rabbits immunized with expression plasmids of the glycoproteins of all 18 lyssaviruses were prepared, and cross-reactivity

was evaluated via virus-neutralization tests using RABV, European bat lyssavirus-1 (EBLV-1), Duvenhage virus (DUVV), Mokola virus (MOKV), and Lagos bat virus (LBV). The sera against RABV vaccines showed cross-reactivity with EBLV-1 and DUVV, which both belong to phylogroup I. However, the reactivity with MOKV and LBV in phylogroup II was notably limited or below the detection level. Next, we compared the cross-reactivity of the polyclonal antibodies against all the lyssavirus glycoproteins. Polyclonal antibodies had high virus-neutralization titers against the same phylogroup, but not against different phylogroups. Our findings indicate that a new vaccine should be developed for pre- and post-exposure prophylaxis against lyssavirus infections.

Chapter 2 Establishment of the serological method to detect antibodies against all lyssaviruses

A pseudotype virus system (VSVp) was developed using the vesicular stomatitis virus, which enabled the testing of neutralization against all 18 lyssaviruses. When evaluating the antiserum against RABV with 18 different VSVp species, anti-sera against RABV vaccines showed high neutralizing titers against VSVp belonging to phylogroup I, but did not VSVp belonging to phylogroup II and the unclassified group. Comprehensive neutralization test against all 18 VSVp was then conducted using antisera for each lyssavirus G protein. The results revealed that antisera against phylogroup I generally cross-reacted with VSVp of phylogroup I, and similarly, antisera from phylogroup II reacted with VSVp of phylogroup II. Notably, antisera against EBLV-1 G and West Caucasian bat lyssavirus G were able to neutralize more VSVp, indicating both G proteins can induce broad cross-reactive virus-neutralizing antibodies.

In conclusion, I succeeded in establishment of system for detection of antibodies against all lyssaviruses and production of antiserum against all lyssavirus G proteins. This study must be contributed for basic research on lyssaviruses other than RABV, which have not been studied to date. The application of this system is expected to facilitate the development of new vaccines and epidemiological studies.

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7. Abstract (in Japanese)

学位論文要旨

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Establishment of the novel method for detection of antibodies against all lyssaviruses and comparison of cross-reactivities among lyssaviruses

全リッサウイルスに対する抗体検出法の確立と交差反応性の比較

リッサウイルスはラブドウイルス科リッサウイルス属に属するウイルスである。リッサウイルス属の中でも特に狂犬病ウイルス（Rabies lyssavirus: RABV）は、ヒトを含む全ての哺乳動物に感染し、致死的な神経症状を引き起こす。狂犬病に対する対策が世界中で進められているにもかかわらず、現在でも年間約 5 万人の死亡者が報告されている。RABV に対してはワクチンもありウイルスに対する様々な研究が進んでいるが、その他のリッサウイルスに関しては殆ど研究が進んでいない。リッサウイルス属には現在までに RABV を含め 18 種のウイルスが見つかっており、RABV が含まれるフィログループⅠ、フィログループⅡ及び未分類種の 3 つに分類されている。RABV 以外では少なくとも 6 種類のリッサウイルスで人への感染例が報告されており、致死的感染を引き起こす。

本研究は、第一章でリッサウイルスに対する日本の RABV ワクチンの防御能の評価を行い、第二章で全リッサウイルス 18 種に対する抗体検出系の確立と交差反応性の比較を行った。

第一章　RABV ワクチンのリッサウイルスに対する有効性の評価と リッサウイルス 18 種の抗 G 蛋白血清の交差反応性の検討

本章は現在日本で使用されているヒト用・動物用の RABV ワクチンがリッサウイルスに対してどの程度交差反応を示すか評価した。また全リッサウイルス 18 種の G 蛋白に対する抗血清を作製し、交差反応性の評価を行った。

リッサウイルスはフィログループⅠの RABV（CVS 株)、ヨーロッパコウモリリッサウイルス 1（EBLV-1)、ドーベンハーゲリッサウイルス（DUVV)、フィログループⅡのモコラリッサウイルス（MOKV)、ラゴスコウモリリッサウイルス（LBV）の 5 種類を使用し、WHO により指定されている中和試験法を実施した。抗ワクチン血清は国内で市販されているヒト用ならびに犬用 RABV ワクチンをウサギ（各郡 2 羽）に 6 回接種した後に回収した。G 蛋白に対する抗血清は各リッサウイルスの G 蛋白の発現プラスミドをウサギに 6 回接種した後に回収した。

中和試験の結果、ヒト用 RABV ワクチンを接種したウサギの抗血清は RABV に対する中和抗体価が最も高く、EBLV-1、DUVV に対する中和抗体価は RABV の中和抗体価と比較して、22.7%と 68.3%の値であった。一方でフィログループⅡに属する MOKV と LBV に対する中和抗体価は、RABV に対する中和抗体価と比較して 100 倍以下、あるいは検出限界以下となった。動物用の RABV ワクチンも同様な結果となった。

各リッサウイルスの抗 G 蛋白血清を評価した結果、フィログループⅠに属するリッサウイルスの抗血清は、同じフィログループⅠの RABV、EBLV-1、DUVV に対して高い中和抗体価を示したが、フィログループⅡの MOKV と LBV に対しては低い中和抗体価を示した。フィログループⅡに属するリッサウイルスの抗血清は、同じフィログループⅡの MOKV と LBV に対して高い中和抗体価を示したが、フィログループⅠのリッサウイルスに対する中和抗体価は低かった。また未分類種のリッサウイルスの抗血清は、5 種のリッサウイルスに対して検出限界以下となった。興味深いことにフィログループⅠの

EBLV-1 の G 蛋白と EBLV-2 の G 蛋白に対する抗血清はフィログループⅡであるMOKV、LBV に対しても比較的高い中和抗体価を示した。

以上のことから、日本で使用されている RABV ワクチンは、RABV と同じフィログループⅠに属するリッサウイルスに対して防御効果はあるが、フィログループⅡのリッサウイルスに対しては防御効果が低い可能性が示唆された。狂犬病以外のリッサウイルスに対するワクチン開発の必要性が改めて示された。また、EBLV-1 と EBLV-2 の G 蛋白は比較的多くのリッサウイルスに対して有効な抗体を誘導する可能性が示された。

第 2 章　シュードタイプウイルスを用いた 18 種類のリッサウイルスに対する抗体検出系の確立と交差反応性の比較

本章は狂犬病を含む 18 種類すべてのリッサウイルスに対する抗体検査系を確立し、リッサウイルス間の交差反応性を比較した。第 1 章で 5 種類のリッサウイルスを用いた中和試験を行ったが、それ以外の 13 種類のウイルスに関しては評価することができなかった。そこで水疱性口内炎ウイルスを用いたシュードタイプウイルス（VSVp）の系を確立し、全リッサウイルス 18 種で中和試験を可能にした。

第 1 章で用いた RABV ワクチンに対する抗血清を 18 種の VSVp で評価したところ、RABV と同じフィログループⅠに属する 12 種の VSVp に対しては高い中和抗体価を示した。一方でフィログループⅡに属する 3 種類のリッサウイルスの VSVp に対する中和抗体価は殆どが検出限界以下であった。また未分類の 3 種類のリッサウイルスの VSVp に対する中和抗体価は検出限界以下となった。

次に第 1 章で作製した各リッサウイルス G 蛋白に対する抗血清 18 種と VSVp18 種を用いて網羅的な中和試験を行った。その結果、フィログループⅠに対する抗血清はフィログループⅠの VSVp と、フィログループⅡの抗血清はフィログループⅡの VSVp と交差反応を示した。一方で未分類種のイコマリッサウイルスとリレイダコウモリリッサウイル

スに対する抗血清は殆どの VSVp と交差反応しなかった。そのなかでフィログループ I の EBLV-1 の G 蛋白と未分類種のウエストコーカサスコウモリリッサウイルスの G 蛋白に対する抗血清は比較的多くのリッサウイルスの VSVp に対して中和活性を示した。

以上のことから、全リッサウイルス 18 種類に対する抗体検査系を確立したことで、全リッサウイルスに対する中和試験や抗体の検出が可能になった

本研究により、全リッサウイルスに対する抗体検出系の確立と全 G 蛋白に対する抗血清の作製に成功した。これまで研究が進んでいない RABV 以外のリッサウイルスの研究のための基礎を確立することができた。この系を応用することで新たなワクチン開発や疫学調査が期待される。