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Establishment of serological neutralizing tests using pseudotyped viruses for comprehensive detection of antibodies against all 18 lyssaviruses

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ABSTRACT. 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.

KEYWORDS: cross-reactivity, lyssavirus, pseudotype, rabies, vaccine

Rabies is a neglected infectious disease that is responsible for an estimated 59,000 human deaths worldwide each year [26]. 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* [24]. Once clinical symptoms of rabies appear, the disease is almost invariably fatal [45]. Since the 1950s, numerous lyssaviruses related to RABV have been identified. All lyssaviruses cause neurological disease in mice when infected intracranially under laboratory conditions [3, 19]. 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 [2, 25, 30]. These viruses are officially recognized by the International Committee on Taxonomy of Viruses [24]. 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 [6]. Of these 18 lyssaviruses, 16, (not MOKV or IKOV) have been isolated from bat species [43]. MOKV has been isolated from rodent species [10, 44] and IKOV from the African civet [38]. Until now, at least seven lyssaviruses, RABV, ABLV, DUVV, EBLV-1, EBLV-2, IRKV, and MOKV, have been responsible for fatal infections in humans [42]. 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 [8, 37].

Lyssaviruses can be classified into two phylogroups by their genomic sequences [1]. 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 [21]. These investigations have demonstrated that RABV vaccines do not

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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 [27]. In our previous study, cross-neutralization activities using only 5 lyssaviruses were compared, suggesting limited cross-reactivities among lyssaviruses [31]. To further validate 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.

First, expression plasmids, each containing a lyssaviral glycoprotein gene, were constructed as described our recent study [31]. Briefly, complete open reading frames encoding glycoproteins of RABV-SRV9 strain (Accession number, AF499686), ARAV (EF614259), BBLV (JF311903), DUVV (JN986749), EBLV-1 (KP241939), EBLV-2 (EF157977), GBLV (KU244266), IRKV (JX442979), KBLV (LR994545), KHUV (EF614261), TWBLV (MF472710), LBV (EU259198), MOKV (NC_006429), SHIBV (GU170201), WCBV (EF614258), IKOV (JX193798), and LLEBV (KY006983) were artificially synthesized (Azenta, Chelmsford, MA, USA) and cloned into the expression plasmid, pCAGGS [40]. The expression plasmid encoding the ABLV (AF426298) glycoprotein was kindly provided by Prof. Christopher C. Broder, Department of Microbiology and Immunology, Uniformed Services University, USA. The NT based on the pseudotyped VSV (VSVp) was developed using VSV pseudotyped with lyssaviral glycoprotein and expressing secreted alkaline phosphatase (SEAP) as a biomarker. The VSVps were generated as previously reported [23, 32]. 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. The titration of each VSVp was determined by a SEAP reporter assay using substrate solution (SIGMAFAST p-Nitrophenyl Phosphate Tablets, Thermo Fisher Scientific). The NT with each VSVp was performed as previously reported [32]. The neutralization titers are represented as the serum dilution that reduced VSVp infectivity by 75% (IC_{75}) compared with no-serum control. IC_{75} 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 [23].

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 [31]: 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.

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. These antisera were generated in our previous study [31]. Briefly, eighteen female Japanese white rabbits were inoculated six times with a glycoprotein-encoding expression plasmid (one glycoprotein-encoding plasmid per rabbit). For each inoculation, 400 µg of the expression plasmid was mixed with 100 µg of PEI in Opti-MEM (Thermo Fisher Scientific), and the mixture was administered to the rabbits at two-week intervals [33]. Blood samples were collected for serum two weeks following the final injection (permission numbers: 120146, 121128, and 122165). Anti-RABV glycoprotein (CVS-11 strain) rabbit serum was prepared as previously described [29]. 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).

This comprehensive study highlights the challenges and the innovations needed to evaluating the cross-reactivity of lyssaviruses.

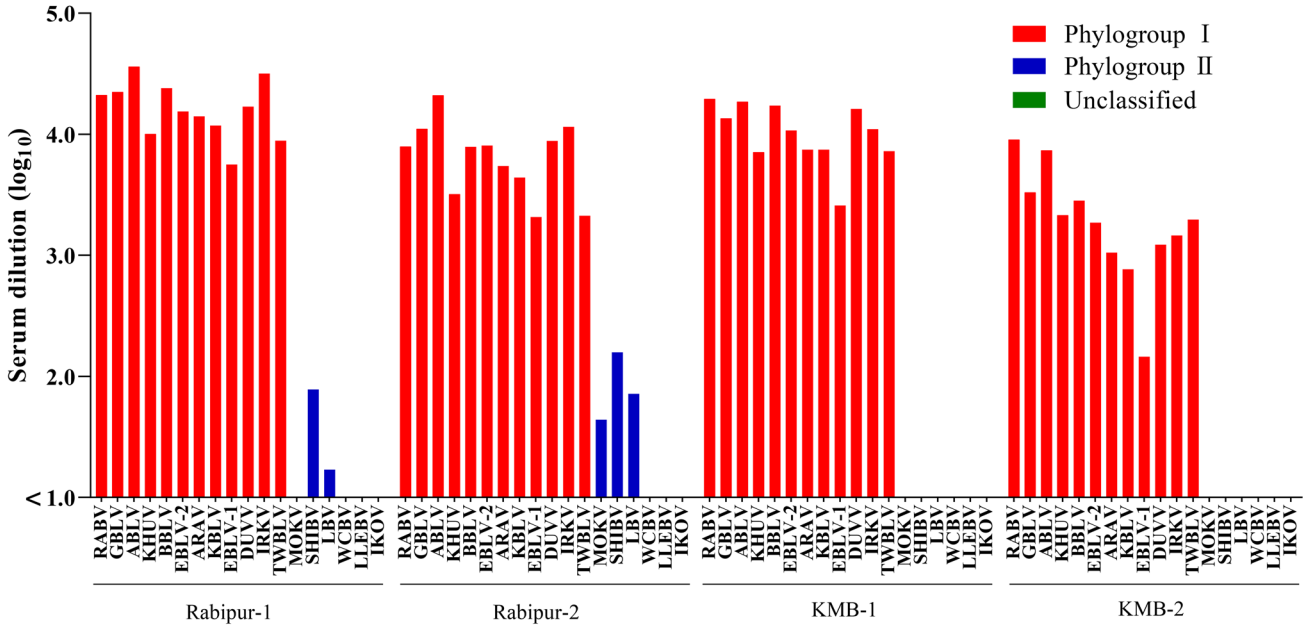

Fig. 1. Comparison of neutralizing titers of Rabies lyssavirus (RABV) vaccine-immunized rabbit sera against 18 lyssaviruses. Serum neutralization tests using sera from two rabbits immunized with human RABV vaccine (Rabipur-1 and -2) and two rabbits immunized with animal RABV vaccine (KMB-1 and -2) were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

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 [7, 39, 47] 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 [5, 48]. 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 [4].

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 [16]. In addition, most sera from humans inoculated with the RABV vaccine did not possess virus neutralization activity against lyssaviruses belonging to different phylogroups [11, 36]. 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 [14], 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 [12, 34]. 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 [9], and WCBV, an unclassified strain, infecting cats [35]. It has become clear that “rabies free” countries [18], have endemic lyssaviruses circulating within bat populations, such as ABLV in Australia and EBLV-1, 2 in the UK [17, 41, 46]. In both nations humans have died from lyssavirus infection as a result of bat bites [20, 22]. 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 [28]. Notably, monoclonal

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.

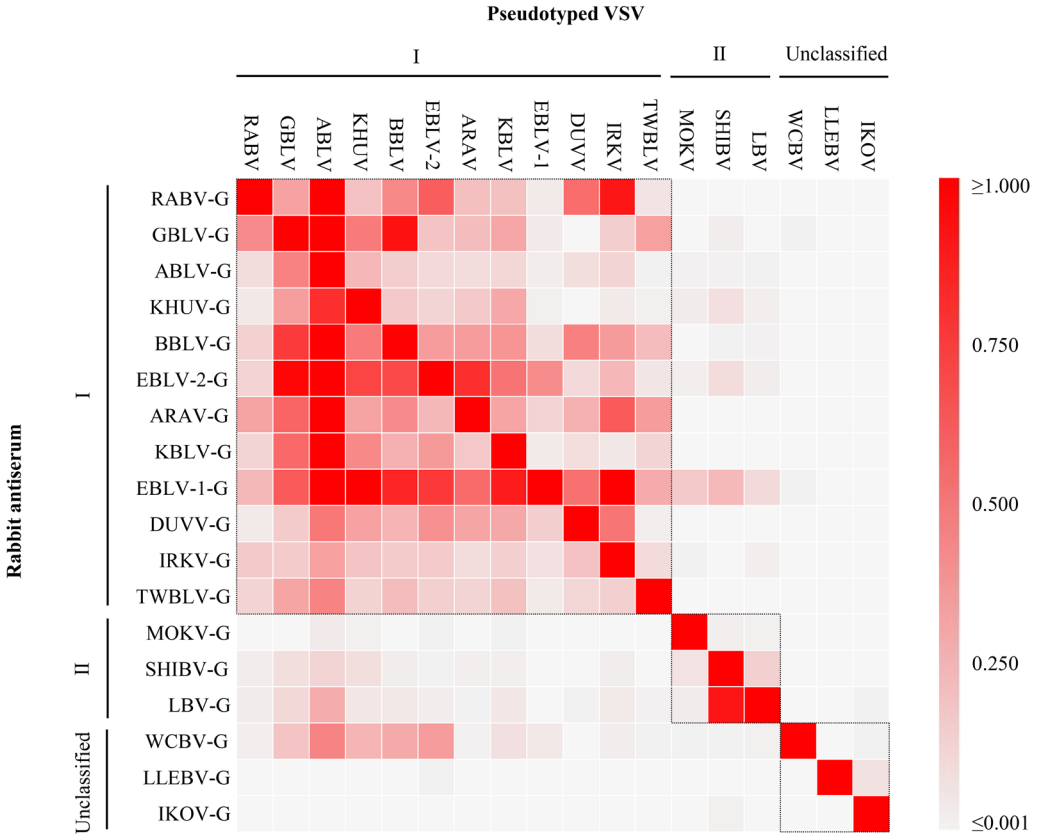

Fig. 2. Summary of cross-reactivity in serum neutralization tests among 18 lyssaviruses. The relative neutralizing titers were compared in all combinations between 18 pseudotyped viruses (VSVps) and 18 rabbit sera against each lyssavirus glycoprotein (-G). The relative neutralizing titers of each combination 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.

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 [11, 28]. 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 [13, 15]. 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.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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