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Standardization, validation, and comparative evaluation of a convenient surrogate recombinant vesicular stomatitis virus plaque reduction test for quantification of Hantaan orthohantavirus (HTNV) neutralizing antibodies

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Abstract

Hantaan orthohantavirus (HTNV) is responsible for severe hemorrhagic fever with renal syndrome (HFRS), which has a case fatality rate of 1% to 10%. Currently, the inactive vaccine licensed in endemic areas elicit low levels of neutralizing antibodies (NAbs). Early NAbs administration is helpful for patients recovery from HFRS. Therefore, measuring NAbs is crucial for evaluating the immune response following infection or vaccination. The golden standard for HTNV NAbs measurement is the focus reduction neutralization test (FRNT), which typically requires skilled technicians and is performed under high biosafety containment facility. Here, we established a surrogate NAbs titration method with replication-competent vesicular stomatitis virus (VSV) bearing HTNV glycoprotein (rVSV-HTNV-GP) based plaque reduction neutralization test (PRNT). Then compared and correlated this method with the authentic HTNV based FRNT, and applied it to measure the NAbs level in 47 serum samples from HFRS patients, healthy donors and inactive vaccine recipients. We observed positive correlations between two neutralization assays among HFRS patients and inactive vaccine recipients ($R^2 = 0.5994$ and 0.3440, respectively) and confirmed the clear specificity with healthy donors without vaccinated and reproducibility with three more assays. Our results suggest that rVSV-HTNV-GP based PRNT is a reliable lower-biosafety level surrogate for HTNV NAbs evaluation, which is easy to perform with higher sensitivity.

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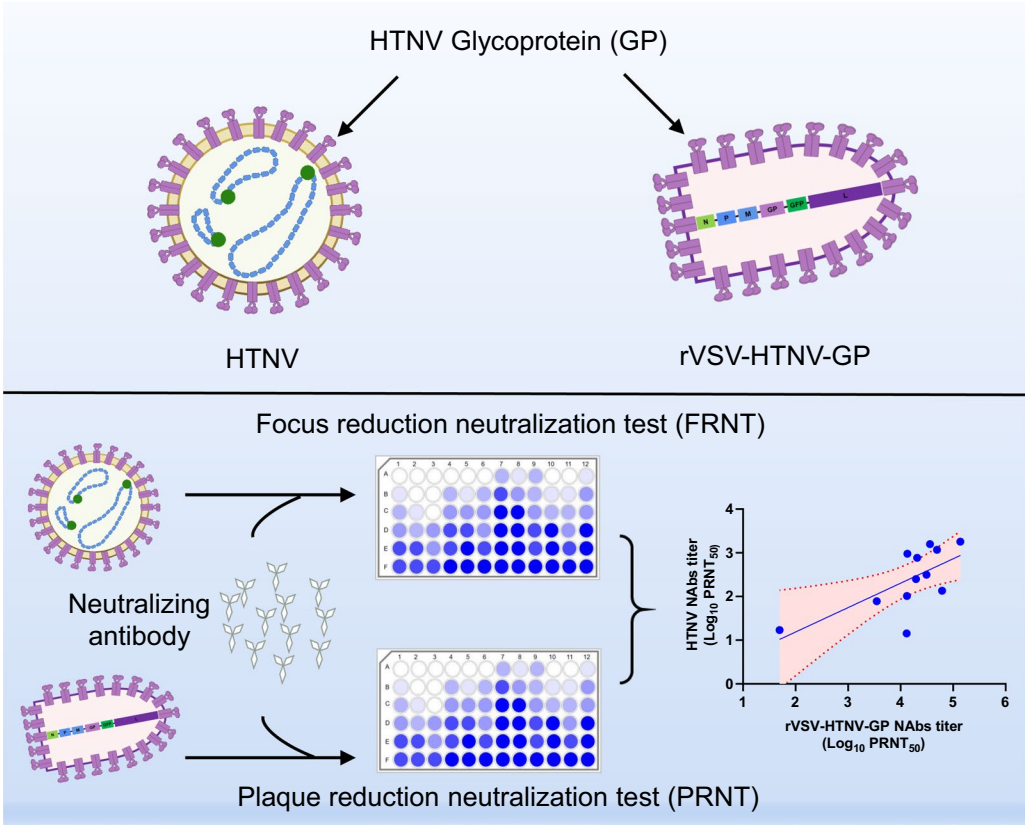
Keywords Hantaan orthohantavirus (HTNV), Hemorrhagic fever with renal syndrome (HFRS), Vesicular stomatitis virus (VSV), Neutralizing antibodies (NAbs), Focus reduction neutralization test (FRNT), Plaque reduction neutralization test (PRNT)

Graphical abstract



Introduction

Hantaan orthohantavirus (HTNV), the prototypical orthohantavirus endemic in East Asia, is responsible for hemorrhagic fever with renal syndrome (HFRS). This disease has a case fatality rate of up to 10%, which is higher than other HFRS-causing pathogens, such as Seoul orthohantavirus (SEOV) and Puumala orthohantavirus (PUUV) [1]. To date, there is no licensed specific antiviral treatment for HFRS, and treatment for clinical patient primarily relies on supportive care. However, the administration of neutralizing antibodies (NAbs) or HFRS convalescence antisera have been suggested to shorten the clinical course of HFRS and reduce mortality [2]. Neutralizing monoclonal antibodies against HTNV have been shown to protect mice from infection and disease in vivo and demonstrated high safety in healthy volunteers [3, 4]. A non-randomized trial was conducted in Chile confirmed the safety and efficacy of human immune plasma for the treatment of hantavirus cardiopulmonary syndrome (HCPS) caused by Andes orthohantavirus (ANDV) [5].

Orthohantavirus share similar biological characteristics as enveloped viruses containing tripartite negative-sense single-stranded RNA genomes, which are named after its length as large (L), medium (M) and small (S) segment. These segments encode RNA polymerases, glycoprotein N and C (Gn and Gc) subunits, and nucleocapsid (N) protein, respectively. Currently, only two types of inactivated vaccines have been licensed in South Korea and China. In addition to T cell immunity, NAbs against Gn and/or Gc are considered as the important factor for vaccine-induced protective immunity [6, 7].

Since orthohantaviruses do not exhibit significant cytopathic effect in infected cells, the "golden standard" for

orthohantavirus titration often relies on the focus assay, which uses a specific antibody against the most abundant viral N protein to detect virus infection in non-lytic cells. While accurate, a drawback of the focus assay is its difficulty for novice to master and its requirement of 5 to 10 days, depending on different virus strains [8, 9]. The focus reduction neutralization test (FRNT) is the reference serological method for measure the NAbs titer against orthohantavirus. Like the focus assay, FRNT is also labor-intensive and time-consuming, and performing with high pathogenicity pathogens like HTNV usually requires a biosafety level-3 laboratory or an equivalent environment [8, 9].

Recently, a biosafety level-2 handleable and faster method was established for Andes orthohantavirus (ANDV) based on recombinant vesicular stomatitis virus (VSV) expression ANDV glycoprotein (GP) within its genome, which has been shown to reduce the time required for NAbs level determination in convalescent plasma [10]. Previously, our group successfully rescued recombinant VSV expression HTNV GP (rVSV-HTNV-GP) [11]. Using this tool, we established a surrogate NAbs titration method and compared its correlation with the authentic HTNV-based FRNT. When employing this method to evaluate the NAbs titer in serum samples from HFRS convalescent patients as well as vaccinees, it was found to be sensitive, time-saving and reproducible.

Materials and methods

Serum sample

We collected 12 convalescent sera from HFRS patients in Shaanxi Provincial Notifiable Disease Surveillance, which was authorized by the government. These patients' diagnosis had been confirmed by the detection of anti-HFRS antibodies by colloidal gold immunochromatography assay kit (WANTAI Biopharm, Beijng, China). Another 25 sera samples were gathered from 5 vaccinees at different time points after bivalent HFRS vaccine inoculation who had been previously enrolled in our Hantavirus immune Program protocol (the health industry special research funds for public welfare projects, Grant 201502020). We also included 10 serum samples from healthy donors as the hantavirus negative control which identified negative to the anti-HFRS IgG antibodies with enzyme-linked immunosorbent test (ELISA) (WANTAI Biopharm). All serum samples were heated for 30 min at 56 °C before testing.

Cells, viruses, antibodies and regents

African green monkey kidney Vero E6 cells (Procella, Wuhan, China), were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, 1% 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES) (Solarbio, Beijing, China) buffer solution. Cells were maintained in a humidified incubator at 37 °C supplemented with 5% CO_2.

Previously indicated replication-competent VSV expressing the HTNV GP in place of the native VSV G protein and bearing an enhanced green fluorescent protein (EGFP) reporter gene (rVSV-HTNV-GP) was used for PRNT [11]. HTNV (strain 76–118) was stored in our laboratory and used for FRNT. Mouse monoclonal antibody (mAb) 1A8 against HTNV NP was generated in our lab as previously indicated [8]. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG were purchased from Biotech & Bio Basic Inc. (BBI, Sangon Biotech, Shanghai, China). Maxi-Blue precipitate TMB Horseradish Peroxidase one-component Solution for Membrane was purchased from Biokits Technologies Inc. (Beijing, China).

rVSV-HTNV-GP based plaque reduction neutralization assay

Vero E6 cells were seeded at 15,000–25,000 cells per well in 96-well plates and cultured until the cell confluency was greater than 90%. Serum samples were two-fold serial diluted with DMEM start at 1:10, and 100 μL of diluted serum sample was mixed with an equal volume of some 100 plaque forming units (PFUs) of rVSV-HTNV-GP in 2% FBS DMEM, and the serum-virus mixture were incubated at 37 °C for 1 h. After removing the culture medium, the confluent Vero E6 cell monolayer in 96-well plates was added 100 μL of the serum-virus mixture and incubated at 37 °C in a 5% CO_2 incubator for 2 h. Then the serum-virus mixture was removed and overlaid with 2% FBS DMEM containing 1.2% Carboxymethyl Cellulose (CMC). The plates were incubated at 37 °C in a 5% CO_2 incubator for 4 days and fixed with 10% formaldehyde for 30 min. Viral plaques were visualized following staining with crystal violet for 30 min and then washed with tap water. The plaque of each well in 96-well plates were counted and measured as the $PRNT_{50}$.

HTNV based focus reduction neutralization test

HTNV based focus reduction neutralization test (FRNT) was used to determine the HTNV NAbs titer according to a previously established protocol with mild modification [8]. Vero E6 cells were cultured in 96-well plates as above. Serum samples were two-fold serially diluted ranging from 1:10 to 1:10240 with DMEM, and 100 μL of diluted serum sample was mixed with the same volume containing about 100 focus forming units (FFUs) of HTNV in 2% FBS DMEM, and incubated at 37 °C for 1 h. Cells were then infected with 100 μL of serum-virus mixture per well at 37 °C in a 5% CO_2 incubator for 2 h. And the serum-virus mixture was removed and overlaid

Table 1 Titer of NAbs in sera of HTNV patients as measured by standard HTNV (Strain 76–118) based FRNT or replication-competent rVSV-HTNV-GP based PRNT

Serum donor	Age at infection	Gender	Interval following hospitalization (Day)	NAbs titer against rVSV-HTNV-GP ($PRNT_{50}$)		NAbs titer against HTNV ($FRNT_{50}$)	
				Decimal	Log_{10}	Decimal	Log_{10}
HV+01	68	Male	16	13560	4.132	954.3	2.98
HV+02	17	Male	12	17389	4.24	249.7	2.397
HV+03	76	Male	14	25821	4.412	766.3	2.884
HV+04	71	Female	19	3829	3.583	78	1.892
HV+05	55	Male	9	31561	4.499	1719	3.235
HV+06	56	Male	14	45708	4.66	1202	3.08
HV+07	53	Male	13	61825	4.791	135.4	2.132
HV+08	62	Female	10	49.7	1.696	17.09	1.233
HV+09	60	Male	16	32941	4.518	315.2	2.499
HV+10	32	Male	10	132545	5.122	1314	3.119
HV+11	50	Male	14	10941	4.039	14.31	1.156
HV+12	29	Male	9	14005	4.146	102.8	2.012

with CMC medium as described for the PRNT method. The plates were incubated for 7 days and fixed with 10% formaldehyde. After permeabilized with 0.5% Triton X-100 in 1×phosphate buffered saline for 15 min, cells were blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature and incubated with mAb-1A8 at 4 °C overnight. After discarding mAb-1A8, HRP-conjugated goat anti mouse IgG (1:1000) was added at 37 °C for 1 h. After washing thrice, the plate was stained with Maxi-Blue precipitate TMB for 30 min at 37 °C in dark place. Images of the 96-wells were captured using an EliSpot Classic Reader (AID, Germany), and foci were counted and the $FRNT_{50}$ were measured.

Specificity and reproducibility

To confirm the specificity of rVSV-HTNV-GP based PRNT, the neutralizing ability of the 10 hantavirus negative control sera was detected with HTNV based FRNT and rVSV-HTNV-GP based PRNT in the dilution of 1:10, 1:20, 1:40 and 1:80. For reproducibility, three more independent PRNT experiments for three sera from vaccinees were conducted and the agreement of replicates was estimated by Intraclass Correlation Coefficient (ICC) for neutralizing antibody titers.

Statistical analysis

NAbs titers were defined as the reciprocal of the highest serum dilution that resulted in an 50% (or 80%, 90%) reduction in the number of rVSV-HTNV-GP induced plaques ($PRNT_{50}$, $PRNT_{80}$, or $PRNT_{90}$) or HTNV NP positive foci ($FRNT_{50}$) compared to virus controls. The titer at which antisera neutralized 50% was calculated from dose–response-inhibition curve analysis, the linear regression correlation analysis with correlation coefficients (R^2), consistency analysis with Bland–Altman plots and P-values were indicated using Prism

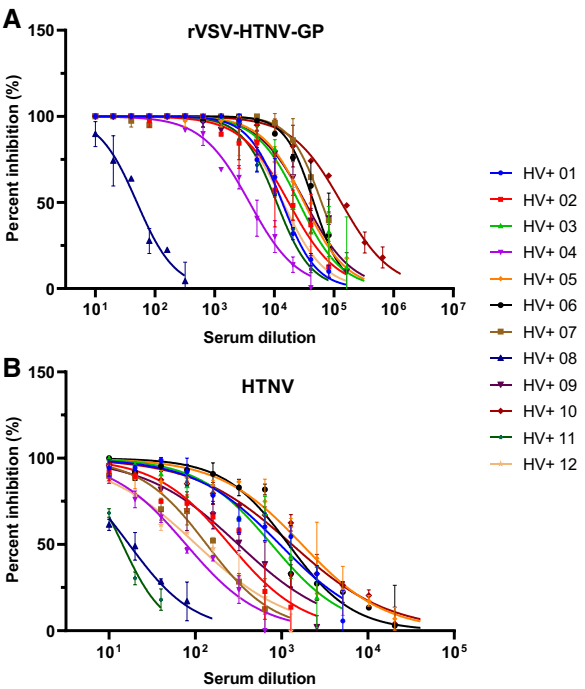


Fig. 1 HTNV infected patient convalescent serum NAbs titer measured by replication-competent rVSV-HTNV-GP based plaque reduction neutralization test (PRNT) (**A**) or HTNV based focus reduction neutralization test (FRNT) (**B**). Sera from 12 HTNV positive donors (HV+) were tested. Y axis shows the percent of infection of each dilution point. Error bars represent the standard deviation (SD)

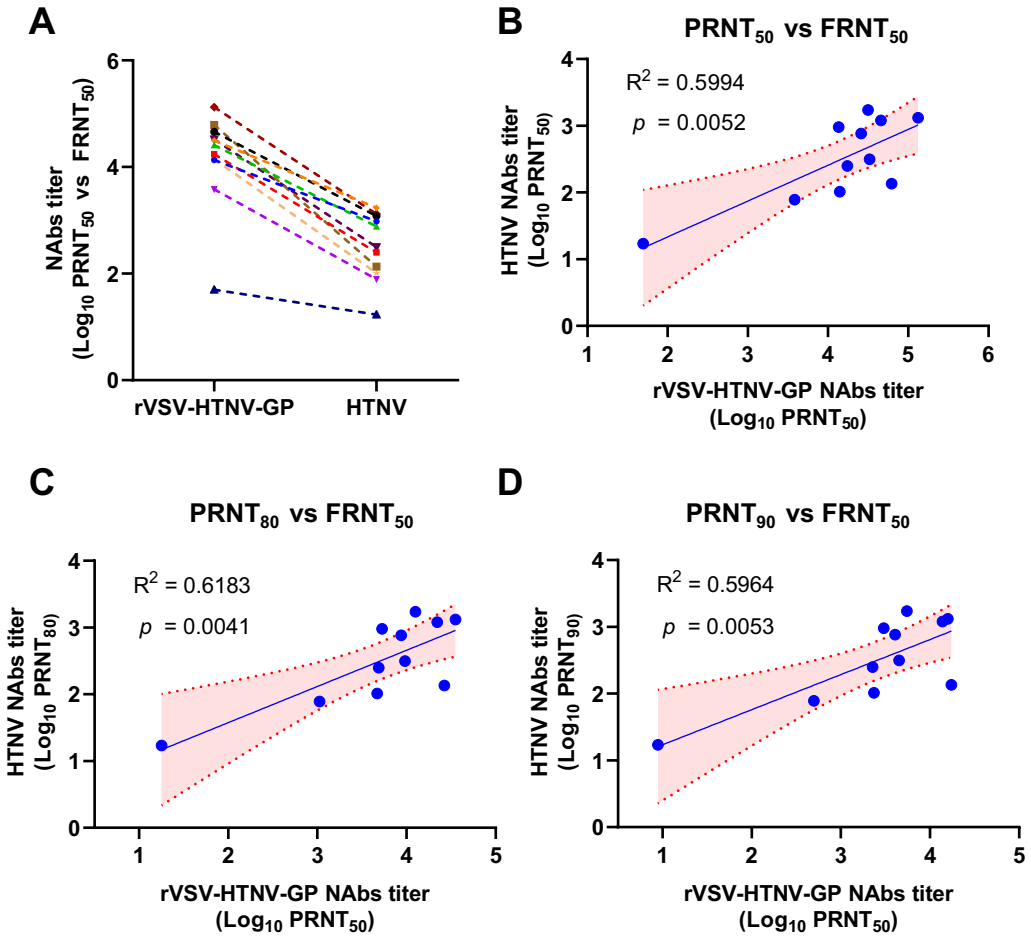


Fig. 2 Correlation between NAbs titer of HTNV patients sera as measured by FRNT (HTNV) and PRNT (rVSV-HTNV-GP). (**A**) Comparison of the neutralizing activities of antisera (log_{10} $PRNT_{50}$ and log_{10} $FRNT_{50}$) against rVSV-HTNV-GP (log_{10} $PRNT_{50}$) and authentic HTNV (log_{10} $FRNT_{50}$). 11 Sera samples from Fig. 1 were tested. (**B**-**D**) Linear regression analysis of log_{10} $PRNT_{50}$ (**B**), log_{10} $PRNT_{80}$ (**C**) or log_{10} $PRNT_{90}$ (**D**) with log_{10} $FRNT_{50}$ values. The confidence interval is shown in lightpink (95%)

9 (GraphPad software, Inc.). Intraclass Correlation Coefficient (ICC) was calculated by Statistical Package for Social Science (SPSS) V.19.0.

Results

Following a published protocol [12], we established a focus-based HTNV titering method using our lab-generated HTNV NP specific monoclonal antibody 1A8 (mAb-1A8) [8]. This method can be used to determine the neutralizing antibody titer in plasma or serum samples, or NAbs [11]. However, the FRNT assay should be performed by experienced technician, and the operation with authentic HTNV requires a high-safety level facility, which hinders its widespread application. Concurrently, we generated a replication-competent VSV expressing the HTNV GP in place of the native VSV G protein and bearing an EGFP reporter gene (rVSV-HTNV-GP), which could be used to determine antiserum neutralizing titer by PRNT or GFP puncta formation reduction neutralization test (GRNT) [11]. The relative antisera potencies against authentic HTNV and rVSV-HTNV-GP have been correlated, suggesting that rVSV-HTNV-GP could serve as a lower-biosafety level surrogate for HTNV NAbs evaluation. To test this hypothesis and establish a reliable, reproducible method, we selected serum samples from confirmed HFRS patients and compared the neutralizing titer obtained from HTNV-based FRNT and rVSV-HTNV-GP based PRNT. Each specimen was measured for HTNV-IgM to confirm hantavirus infection. A total of 12 convalescence sera samples were selected at different time post-hospitalization, with age ranged from 17 to 68, detailed information on the specimens was shown in Table 1.

We initially used rVSV-HTNV-GP to measure NAbs titer in these serum samples (Fig. 1A). Most patients developed significant NAbs production, except for patient

Table 2 Kinetics of NAbs formation in bivalent HFRS inactive vaccine recipients as measured by standard HTNV (Strain 76–118) based FRNT or replication-competent rVSV-HTNV-GP based PRNT

Serum donor	Age at vaccination	Gender	Interval following vaccination (Month)	NAbs titer against rVSV-HTNV-GP ($PRNT_{50}$)		NAbs titer against HTNV 76–118 strain ($FRNT_{50}$)	
				Decimal	Log_{10}	Decimal	Log_{10}
Vaccinee #1	50	Female	1	1648	3.217	122.6	2.089
			11	1407	3.148	18.69	1.272
			22	917.3	2.963	80.87	1.908
			35	1171	3.068	43.21	1.636
			46	426.9	2.63	44.32	1.647
Vaccinee #2	59	Male	2	1948	3.29	292.5	2.466
			17	111.6	2.048	36.24	1.559
			22	598.9	2.777	87.46	1.942
			46	225	2.352	99.11	1.996
			57	339.5	2.531	120.5	2.081
Vaccinee #3	54	Female	1	105.6	2.024	72.31	1.859
			8	426.4	2.63	59.22	1.772
			22	33.56	1.526	30.13	1.479
			34	21.54	1.333	25.52	1.407
			47	29.18	1.465	17.88	1.252
Vaccinee #4	56	Female	1	1018	3.008	65.54	1.817
			8	911.6	2.96	22	1.342
			22	180	2.255	12.81	1.107
			34	134	2.127	23.6	1.373
			47	167	2.223	22.28	1.348
Vaccinee #5	39	Female	1	7602	3.881	216.9	2.336
			8	3516	3.546	85.11	1.93
			22	291.9	2.465	21.66	1.336
			33	3460	3.539	66.26	1.821
			46	1696	3.229	81.73	1.912

NO.8, whose $PRNT_{50}$ titer was only 49.7. Consistently, patient NO.8 also had the second-lowest $FRNT_{50}$ NAbs titer (17.09) (Table 1, Fig. 1B). An interesting result was observed with patient NO.11, for whom HTNV infection was enhanced at 1:10 to 1:80 dilutions of serum rather than 1:160. This phenomenon suggests an antibody-dependent enhancement (ADE) effect in this patient, and we conducted further analysis excluding NO.11.

Next, we compared the half-maximal neutralization titers (log_{10}) from rVSV-HTNV-GP and authentic HTNV. A shift of 0.463- to 2.659- log_{10} reciprocal median effect concentration (EC_{50}) values (corresponding to 2.9- to 456.6- fold decimal shift) was revealed toward $PRNT_{50}$ titer compared with $FRNT_{50}$ titer (Fig. 2A). This result suggested the HTNV based 50% endpoint FRNT titer was significantly lower than PRNT (Table 1, Fig. 2A). One possible explanation for this phenomenon was that the number of tetramer GP spikes on the rVSV-HTNV-GP surface is less than on authentic HTNV.

Using linear regression analysis to compare the half-maximal neutralization titer from different tests, we found a moderate Pearson correlation between the log_{10} $PRNT_{50}$ and $FRNT_{50}$ titer ($R^2 = 0.5994$, $p = 0.0052$) (Fig. 2B). When comparing the 80% reduction neutralization titer of rVSV-HTNV-GP with the half-maximal neutralization titer of authentic HTNV, we found the correlation between log_{10} $PRNT_{80}$ and $FRNT_{50}$ titer was also significant (Fig. 2C). A similar correlation was obtained between log_{10} $PRNT_{90}$ and $FRNT_{50}$ titers (Fig. 2D).

To further evaluate whether this rVSV-HTNV-GP surrogate was suitable for evaluating the HTNV NAbs titer in HFRS vaccine recipients, we selected 25 sera samples from 5 vaccinees at different time points post-vaccination. The interval ranged from 1 to 57 months following vaccination. Detailed information on the specimens was shown in Table 2.

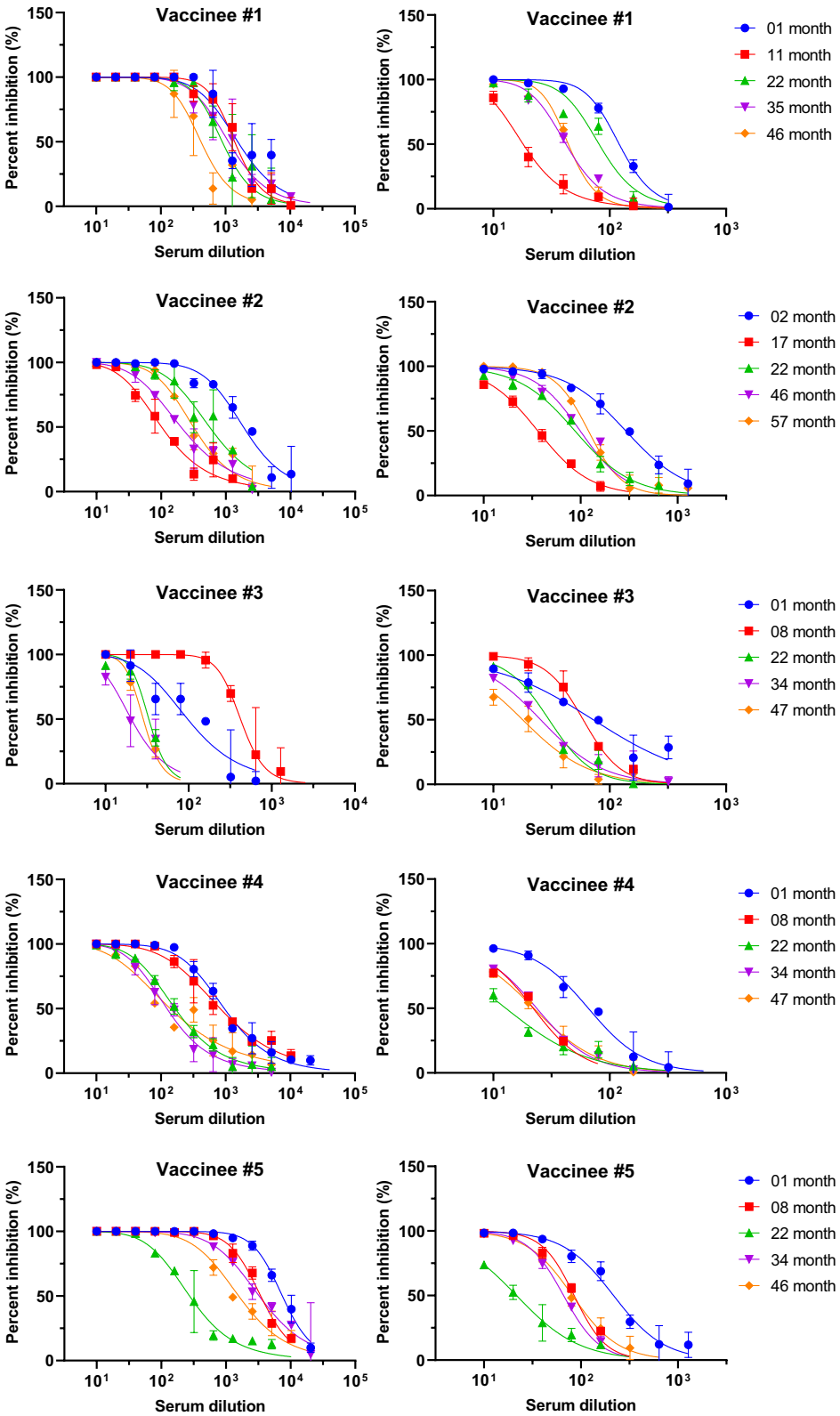


Fig. 3 NAbs titer of serum sample from five HFRS bivalent inactive vaccine recipients at different time points post vaccination were measured by replication-competent rVSV-HTNV-GP based PRNT (left) or HTNV based FRNT (right). Y axis shows the percent of infection of each dilution point. Each line represent different time point. Error bars represent the SD

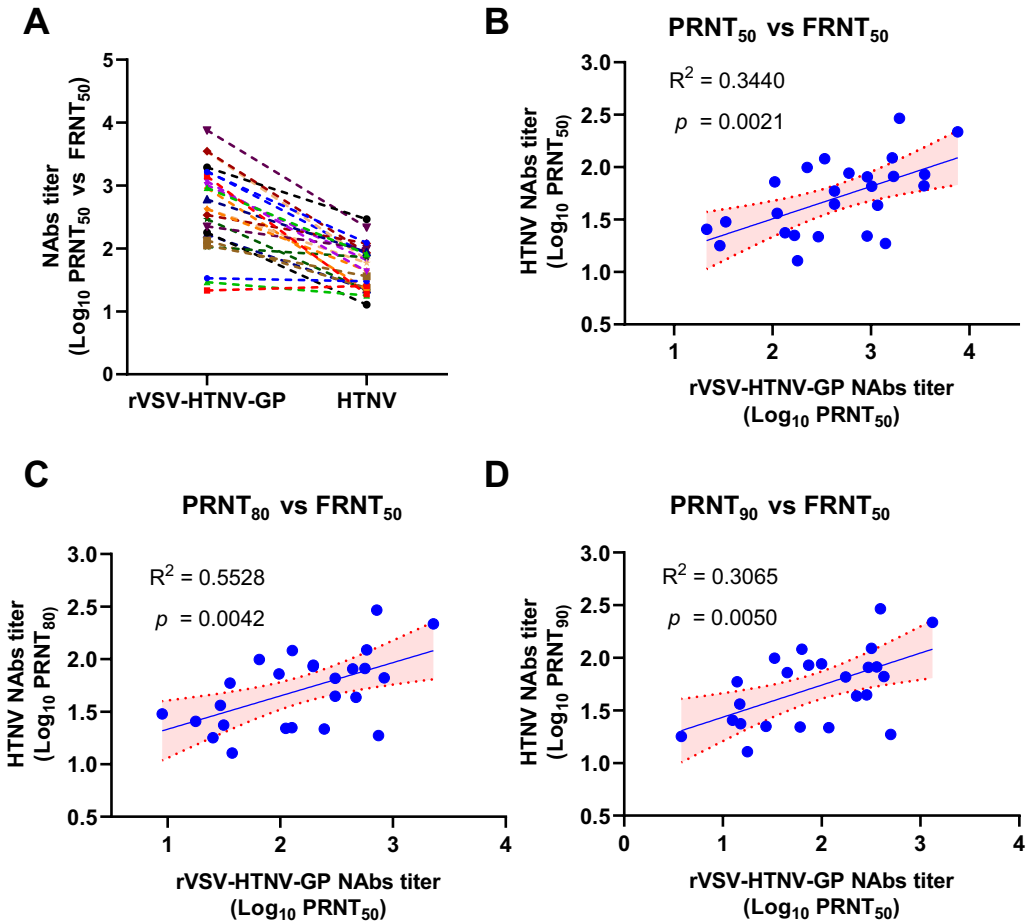


Fig. 4 Correlation between NAbs titer of HFRS bivalent inactive vaccine recipients sera as measured by FRNT (HTNV) and PRNT (rVSV-HTNV-GP). (**A**) Comparison of the neutralizing activities of antisera (log_{10} $PRNT_{50}$ and log_{10} $FRNT_{50}$) against rVSV-HTNV-GP (log_{10} $PRNT_{50}$) and authentic HTNV (log_{10} $FRNT_{50}$). 25 Sera samples (24 for D) from Fig. 3 were tested. (**B**-**D**) Linear regression analysis of log10 $PRNT_{50}$ (**B**), log_{10} $PRNT_{80}$ (**C**) or log_{10} $PRNT_{90}$ (**D**) with log_{10} $FRNT_{50}$ values. In lightpink is the confidence interval (95%)

The $PRNT_{50}$ and $FRNT_{50}$ titer were obtained for each sample. Like the results derived from convalescent sera samples, we found the $PRNT_{50}$ titer obtained from rVSV-HTNV-GP was relatively higher than the authentic HTNV-based $FRNT_{50}$ NAbs titer (Table 2, Fig. 3). Additionally, the trend curves derived from different dilutions for each vaccinees were largely the same between $PRNT_{50}$ and $FRNT_{50}$ (Fig. 3).

We then compared the half-maximal neutralization titer (log_{10}) from rVSV-HTNV-GP and authentic HTNV derived from the vaccination samples. A shift of 0.047- to 1.876- log_{10} reciprocal EC_{50} values (corresponding to 0.84- to 75.28-fold decimal shift) was revealed toward $PRNT_{50}$ titer compared with $FRNT_{50}$ titer (Table 2, Fig. 4A).

When using linear regression analysis to compare these data, we found a strong Pearson correlation between the results obtained for log_{10} $PRNT_{50}$ and $FRNT_{50}$ titer ($R^2 = 0.3440$, $p = 0.0021$). Similar correlations were also obtained from the 80% or 90% reduction neutralization titers of rVSV-HTNV-GP with the half-maximal neutralization titer of authentic HTNV (Fig. 4C and D). When using the Bland–Altman plot to measure the agreement of these methods, almost all the plots were within the 95% interval, which further validated the agreement between the two methods (Fig. 5A and B).

Moreover, we observed similar trends and strong correlation between rVSV-HTNV-GP and authentic HTNV based NAbs titering, indicating that rVSV-HTNV-GP could serve as a lower-biosafety level surrogate for HTNV NAbs evaluation.

To further verify the reproductivity of rVSV-HTNV-GP based PRNT, we randomly selected 3 sera samples and repeated the PRNT experiment three times. As shown in Fig. 6 and Table 3. The 50% inhibition titer of sera displayed little difference in

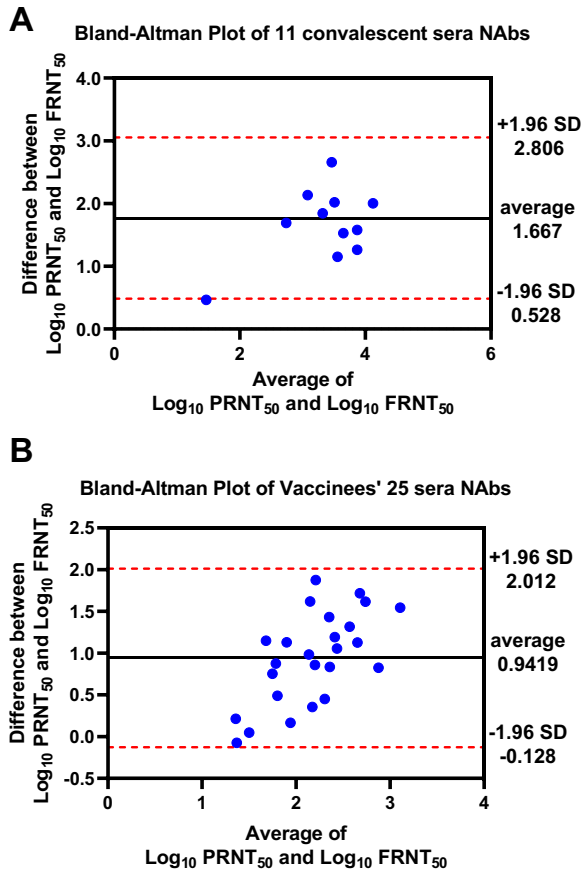


Fig. 5 Bland–Altman plots of the neutralizing antibody titers between PRNT and FRNT assays in 11 convalescent sera from HFRS patients (**A**) and 25 sera gathered from 5 vaccinees (**B**). X-Axis: average of Log_{10} $PRNT_{50}$ and Log_{10} $FRNT_{50}$. Y-axis: differences between Log_{10} $PRNT_{50}$ and Log_{10} $FRNT_{50}$. Black line: average of the measure differences. Red lines: 95% limits of agreement of the measure differences

each replicate and with initial experiment, and the analysis of agreement among different batch of PRNT assays was superb, which was almost 1.0 (ICC of single measurement and average measurement) considering NAbs titers in $PRNT_{50}$ and Log_{10} $PRNT_{50}$ (Table 4).

To rule out possible deviations, we next used HTNV-based FRNT and rVSV-HTNV-GP-based PRNT to measure NAbs titers in 10 hantavirus-negative control samples from healthy donors. All dilutions and infection rates of all negative samples were around 100%, which almost completely failed to inhibit HTNV infection. The results of the two methods were similar (Fig. 7A and B), confirming the specificity of both methods.

Discussion

Neutralizing antibodiesy remain an effective treatment option for infectious diseases, as demonstrated in RSV and coronavirus infections, etc. For hantaviruses, earlier studies have shown that treatment with high-potent immunoglobulins isolated from convalescent serum can significantly shorten the defervescence time, reducemucocutaneous hemorrhage, and increase platelet counts [5]. Most importantly, survivors have higher neutralizing antibody (NAb) titers compared to those who succumb to the disease. When using a DNA vaccine to immune the transgenic cattle carrying human antibody genes, high levels of hantavirus-specific IgG could provide complete protection against a lethal dose of New World Hantavirus challenge in animal models [13]. Meanwhile, increasing evidence suggests that monoclonal NAbs isolated from animals or human survivors show promising treatment potential against both Old World or New World Hantaviruses [3, 14–23]. At the same time, the licensed HFRS inactive vaccines in East Asia have been criticized for their inability to induce high levels NAbs [6, 24, 25]. Therefore, measuring NAb levels is crucial for evaluating the outcomes of the HFRS patients or vaccine efficacy. However, hantaviruses propagate slowly and have minimal or no cytopathic effect on infected cells. Using plaque reduction technique with HTNV is quite challenging since its establishment [26–28]. Currently, the most widely used standard assay for NAbs titer is FRNT, which is also time-consuming and labor-intensive [8, 29].

To shorten experimental time, reduce human resources and increase throughput, many newly developed methods have been introduced into the hantavirus-related research. Most prominently, enzyme-linked immunosorbent assay [12], immunofluorescence assay [29], in-cell western [30], flow cytometry [31, 32], and one-step real-time RT-PCR [33, 34] were gradually established. Meanwhile, some alternative methods, such as VSV- or lentivirus- based pseudovirions, as well as replication-competent VSVs were used to evaluate the hantavirus entry process and specific inhibitors, formulated in vaccine development or used as the low-biosafety compatible tools to evaluating NAbs titers [10, 11, 35–47].

Our rVSV-HTNV-GP-based PRNT method established here is faster than authentic HTNV-based FRNT, with easier handling procedure and independence from HTNV NP-specific antibodies. More importantly, the rVSV-HTNV-GP based PRNT results correlate well with the HTNV-based FRNT results, indicating that rVSV-HTNV-GP could serve as a convenient tool to screen potential donors bearing high levels of HTNV NAbs. Furthermore, rVSV-HTNV-GP-based PRNT could also

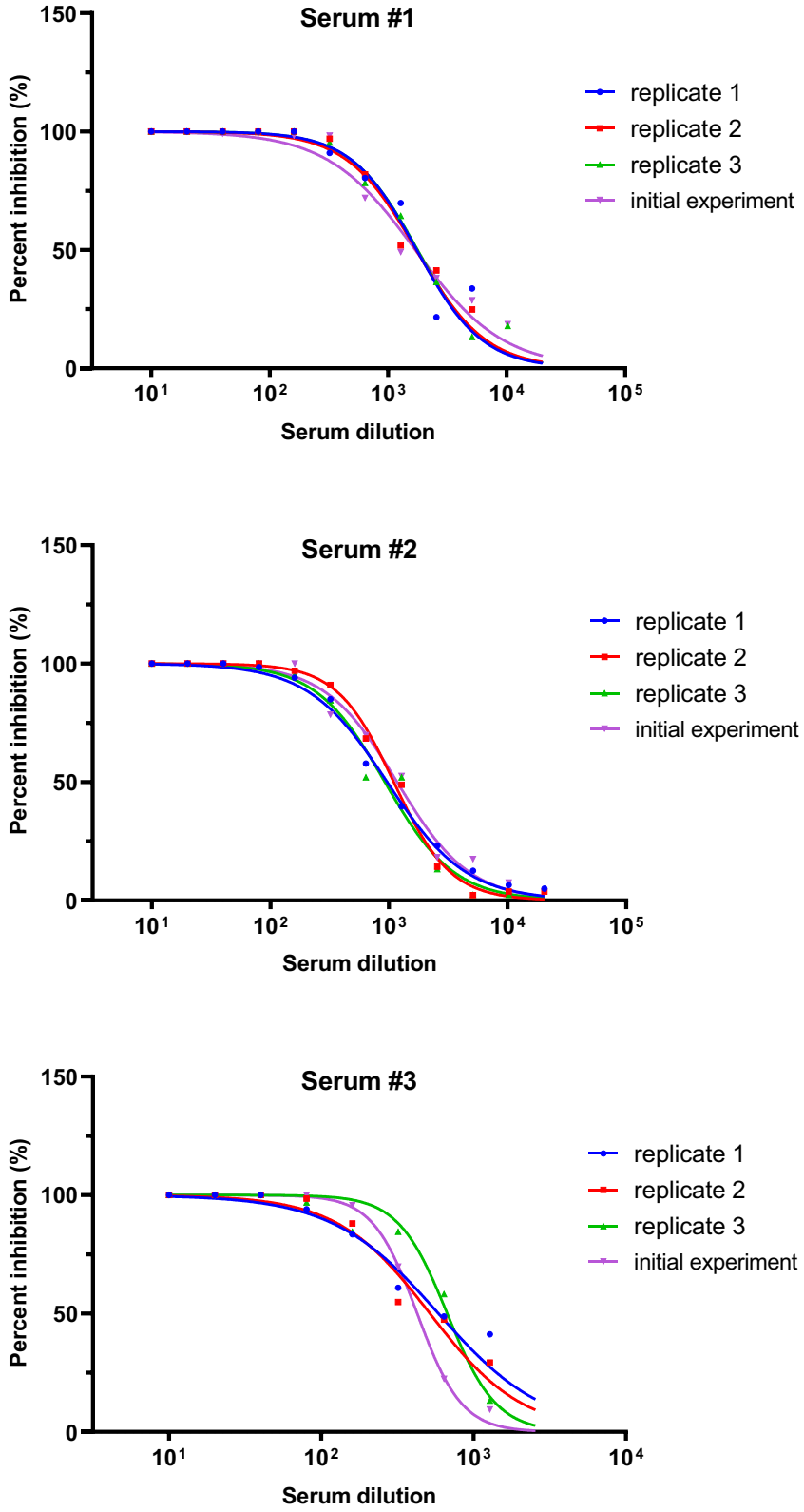


Fig. 6 Four independent NAb titer assay experiments for three samples measured by replication-competent rVSV-HTNV-GP based PRNT. Y-axis shows the percent of infection of each dilution point

Table 3 Titer of NAbs in 3 sera measured by replication-competent rVSV-HTNV-GP based PRNT

	Serum #1		Serum #2		Serum #3	
	$PRNT_{50}$	Log_{10} $PRNT_{50}$	$PRNT_{50}$	Log_{10} $PRNT_{50}$	$PRNT_{50}$	Log_{10} $PRNT_{50}$
Initial experiment	1696	3.229	1171	3.068	426.4	2.63
Replicate 1	1743	3.241	965.8	2.985	587.5	2.769
Replicate 2	1727	3.237	1098	3.04	516.5	2.713
Replicate 3	1770	3.248	923.5	2.965	671	2.827

Table 4 Intraclass correlation coefficients (ICC) for neutralizing antibody titers of 3 sera for the analysis of agreement among four times with PRNT assay

	ICC (95% CI)	*P* values
Log_{10} $PRNT_{50}$	0.935 (0.640–0.998)	0.000
$PRNT_{50}$	0.977 (0.849–0.999)	0.000

95% CI = 95% confidence interval

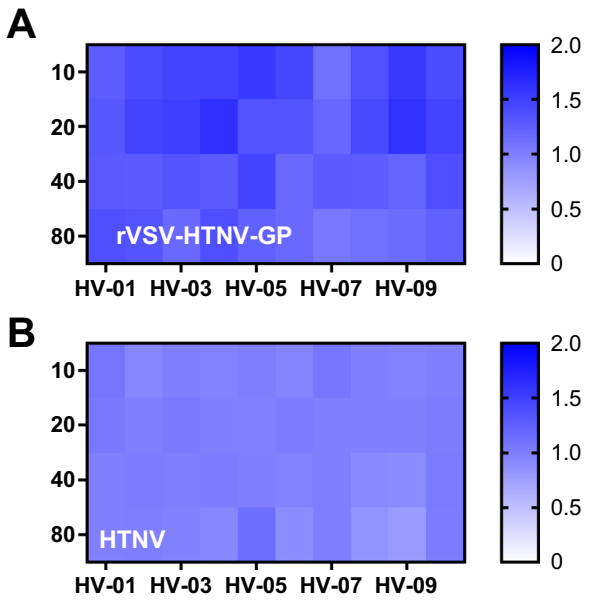


Fig. 7 Evaluation of neutralizing activity of Hantavirus negative control samples from health donors sera against rVSV-HTNV-GP (PRNT, A) or HTNV (FRNT, B)

serve as an amplifiable method to evaluate the efficacy of HFRS inactive vaccine campaigns.

Although we have established a reliable and reproducible method for HTNV NAbs detection, limitations remain. Despite the consistency analysis been validated, there is an average discrepancy of 1.2-log_{10} EC_{50} values shift between $PRNT_{50}$ and $FRNT_{50}$ titers, indicating that rVSV-HTNV-GP is more sensitive than authentic HTNV. The different structures of rVSV-HTNV-GP and authentic HTNV may contribute to these differences. The VSV particle is bullet-shaped and glycoproteins are displayed on the viral envelope as trimers [48], while the HTNV virion is spherical and exhibits a fourfold symmetric lattice of G_N-G_C spikes [49, 50]. The alteration of G_N-G_C oligomerization promotes less infectious rVSV-HTNV-GP with low GP expression [38], which shows higher EC_{50} and is more sensitive in the same samples.

Last, we found an interesting phenomenon where one convalescent serum exhibited an ADE effect at lower dilutions, a phenomenon similar to that observed in respiratory syncytial virus, Dengue virus, and SARS-CoV-2 infection [51–54]. These results may be attributed to non-neutralizing antibody that facilities virus infection through their Fc domains [55], since one report suggested ADE exists in hantavirus-infected macrophage cell lines [56]. However, the precise mechanism for hantavirus-related ADE still requires further investigation.

Studies with larger sample sizes in both infected and vaccinated individuals are required to confirm the general applicability of this lower-biosafety level surrogate for HTNV NAbs evaluation.

Conclusions

In summary, this study has developed a PRNT method based on rVSV-HTNV-GP, providing a feasible alternative to the HTNV-based FRNT assay for quantifying NAbs titers. The use of rVSV-HTNV-GP, which expressing the HTNV glycoprotein, allows for manipulation within a biosafety level-2 facility, making it a safer surrogate for measuring NAbs levels compared to working with HTNV. Furthermore, the rVSV-HTNV-GP-based PRNT method can be applied to monitor potential declines in protective NAbs titers among previously infected or vaccinated individuals in large-scale seroepidemiological studies.

Abbreviations

HTNV	Hantaan orthohantavirus
HFRS	Hemorrhagic fever with renal syndrome
VSV	Vesicular stomatitis virus
NAbs	Neutralizing antibodies
FRNT	Focus reduction neutralization test
FFUs	Focus forming units

PRNT Plaque reduction neutralization test
PFUs Plaque forming units
SEOV Seoul orthohantavirus
PUUV Puumala orthohantavirus
ANDV Andes orthohantavirus

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Author contributions
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Data availability
The raw data supporting the conclusions of this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
The study was approved by the Medical Ethics Committee of Shaanxi Provincial Center for Disease Control and Prevention (NO.1 in 2014) and informed consent was obtained from all subjects involved in the study.

Competing interests
The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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References

1. Vial PA, Ferrés M, Vial C, Klingström J, Ahlm C, López R, Le Corre N, Mertz GJ. Hantavirus in humans: a review of clinical aspects and management. Lancet Infect Dis. 2023;23(9):e371–82. https://doi.org/10.1016/S1473-3099(23)00128-7.
2. Guo WP, Xu HF, Wang JY, Bai XF, Yang WS, Sun ZC. High valent immunoglobulin used for the treatment of epidemic hemorrhagic fever. People's Military Medical. 1989;1989:28.
3. Xu Z, Wei L, Wang L, Wang H, Jiang S. The in vitro and in vivo protective activity of monoclonal antibodies directed against Hantaan virus: potential application for immunotherapy and passive immunization. Biochem Biophys Res Commun. 2002;298:552–8.
4. Xu R, Yang XY, Yang DF, Zou CY, Gong PL, Zeng FD. Phase I evaluation of the safety and pharmacokinetics of a single-dose intravenous injection of a murine monoclonal antibody against Hantaan virus in healthy volunteers. Antimicrob Agents Chemother. 2009;53:5055–9.
5. Vial PA, Valdivieso F, Calvo M, Rioseco ML, Riquelme R, Araneda A, Tomicic V, Graf J, Paredes L, Florenzano M, et al. A non-randomized multicentre trial of human immune plasma for treatment of hantavirus cardiopulmonary syndrome caused by Andes virus. Antivir Ther. 2015;20:377–86.
6. Song JY, Woo HJ, Cheong HJ, Noh JY, Baek LJ, Kim WJ. Long-term immunogenicity and safety of inactivated Hantaan virus vaccine (Hantavax™) in healthy adults. Vaccine. 2016;34:1289–95.
7. Zheng Y, Zhou BY, Wei J, Xu Y, Dong JH, Guan LY, Ma P, Yu PB, Wang JJ. Persistence of immune responses to vaccine against haemorrhagic fever with renal syndrome in healthy adults aged 16–60 years: results from an open-label2-year follow-up study. Infect Dis (Lond). 2018;50:21–6.
8. Ye C, Wang D, Liu H, Ma H, Dong Y, Yao M, Wang Y, Zhang H, Zhang L, Cheng L, et al. An improved enzyme-linked focus formation assay revealed baloxavir acid as a potential antiviral therapeutic against hantavirus infection. Front Pharmacol. 2019. https://doi.org/10.3389/fphar.2019.01203.
9. Tanishita O, Takahashi Y, Okuno Y, Yamanishi K, Takahashi M. Evaluation of focus reduction neutralization test with peroxidase-antiperoxidase staining technique for hemorrhagic fever with renal syndrome virus. J Clin Microbiol. 1984;20:1213–5.
10. Vial C, Whitaker A, Wilhelm J, Ovalle J, Perez R, Valdivieso F, Ferres M, Martinez-Valdebenito C, Eisenhauer P, Mertz GJ, et al. Comparison of VSV Pseudovirus and Focus Reduction Neutralization Assays for Measurement of Anti-Andes orthohantavirus Neutralizing Antibodies in Patient Samples. Front Cell Infect Microbiol. 2020;10:444.
11. Zhang H, Liu H, Wei J, Dang Y, Wang Y, Yang Q, Zhang L, Ye C, Wang B, Jin X, et al. Single dose recombinant VSV based vaccine elicits robust and durable neutralizing antibody against Hantaan virus. NPJ Vaccines. 2024;9:28.
12. Groen J, van der Groen G, Hoofd G, Osterhaus A. Comparison of immunofluorescence and enzyme-linked immunosorbent assays for the serology of Hantaan virus infections. J Virol Methods. 1989;23:195–203.
13. Hooper JW, Brocato RL, Kwilas SA, Hammerbeck CD, Josleyn MD, Royals M, Ballantyne J, Hua W, Jiao JA, Matsushita H, Sullivan EJ. DNA vaccine–derived human IgG produced in transchromosomal bovines protect in lethal models of hantavirus pulmonary syndrome. Sci Transl Med. 2014. https://doi.org/10.1126/scitranslmed.3010082.
14. Kikuchi M, Yoshimatsu K, Arikawa J, Yoshida R, Yoo YC, Isegawa Y, Yamanishi K, Tono-oka S, Azuma I. Characterization of neutralizing monoclonal antibody escape mutants of Hantaan virus 76118. Arch Virol. 1998;143:73–83.
15. de Carvalho NC, Lundkvist A, Sjölander KB, Plyusnin A, Salonen EM, Björling E. A neutralizing recombinant human antibody Fab fragment against Puumala hantavirus. J Med Virol. 2000;60:446–54.
16. Duehr J, McMahon M, Williamson B, Amanat F, Durbin A, Hawman DW, Noack D, Uhl S, Tan GS, Feldmann H, Krammer F. Neutralizing monoclonal antibodies against the Gn and the Gc of the andes virus glycoprotein spike complex protect from virus challenge in a preclinical hamster model. mBio. 2020;11(2):10–1128.
17. Engdahl TB, Kuzmina NA, Ronk AJ, Mire CE, Hyde MA, Kose N, Josleyn MD, Sutton RE, Mehta A, Wolters RM, et al. Broad and potently neutralizing monoclonal antibodies isolated from human survivors of New World hantavirus infection. Cell Rep. 2021;35: 109086.
18. Williamson BN, Prescott J, Garrido JL, Alvarez RA, Feldmann H, Barría MI. Therapeutic efficacy of human monoclonal antibodies against andes virus infection in syrian hamsters. Emerg Infect Dis. 2021;27:2707–10.
19. Rissanen I, Krumm SA, Stass R, Whitaker A, Voss JE, Bruce EA, Rothenberger S, Kunz S, Burton DR, Huiskonen JT, et al. Structural basis for

a neutralizing antibody response elicited by a recombinant hantaan Virus Gn immunogen. mBio. 2021. https://doi.org/10.1128/mBio.02531-20.
20. Mittler E, Wec AZ, Tynell J, Guardado-Calvo P, Wigren-Byström J, Polanco LC, O'Brien CM, Slough MM, Abelson DM, Serris A, et al. Human antibody recognizing a quaternary epitope in the Puumala virus glycoprotein provides broad protection against orthohantaviruses. Sci Transl Med. 2022. https://doi.org/10.1126/scitranslmed.abl5399.
21. Engdahl TB, Binshtein E, Brocato RL, Kuzmina NA, Principe LM, Kwilas SA, Kim RK, Chapman NS, Porter MS, Guardado-Calvo P, et al. Antigenic mapping and functional characterization of human New World hantavirus neutralizing antibodies. Elife. 2023. https://doi.org/10.7554/eLife.81743.
22. Mittler E, Serris A, Esterman ES, Florez C, Polanco LC, O'Brien CM, Slough MM, Tynell J, Gröning R, Sun Y, et al. Structural and mechanistic basis of neutralization by a pan-hantavirus protective antibody. Sci Transl Med. 2023. https://doi.org/10.1126/scitranslmed.adg1855.
23. Stass R, Engdahl TB, Chapman NS, Wolters RM, Handal LS, Diaz SM, Crowe JE Jr, Bowden TA. Mechanistic basis for potent neutralization of Sin Nombre hantavirus by a human monoclonal antibody. Nat Microbiol. 2023;8:1293–303.
24. Zheng Y, Wei J, Zhou BY, Xu Y, Dong JH, Guan LY, Ma P, Yu PB, Wang JJ. Long-term persistence of anti-hantavirus antibodies in sera of patients undergoing hemorrhagic fever with renal syndrome and subjects vaccinated against the disease. Infect Dis (Lond). 2016;48:262–6.
25. Song JY, Jeong HW, Yun JW, Lee J, Woo HJ, Bae JY, Park MS, Choi WS, Park DW, Noh JY, et al. Immunogenicity and safety of a modified three-dose priming and booster schedule for the Hantaan virus vaccine (Hantavax): A multi-center phase III clinical trial in healthy adults. Vaccine. 2020;38:8016–23.
26. McCormick JB, Sasso DR, Palmer EL, Kiley MP. Morphological identification of the agent of Korean haemorrhagic fever (Hantaan virus) as a member of the Bunyaviridae. Lancet. 1982;1:765–8.
27. Takenaka A, Gibbs CJ, Gajdusek DC. Antiviral neutralizing antibody to Hantaan virus as determined by plaque reduction technique. Adv Virol. 1985;84:197–206.
28. Hooper JW, Larsen T, Custer DM, Schmaljohn CS. A lethal disease model for hantavirus pulmonary syndrome. Virology. 2001;289:6–14.
29. Lee PW, Gibbs CJ Jr, Gajdusek DC, Yanagihara R. Serotypic classification of hantaviruses by indirect immunofluorescent antibody and plaque reduction neutralization tests. J Clin Microbiol. 1985;22:940–4.
30. Ma H, Ye W, Chen H, Nie T, Cheng L, Zhang L, Han P, Wu X, Xu Z, Lei Y, Zhang F. In-Cell Western Assays to Evaluate Hantaan Virus Replication as a Novel Approach to Screen Antiviral Molecules and Detect Neutralizing Antibody Titers. Front Cell Infect Microbiol. 2017;7:269.
31. Barriga GP, Martinez-Valdebenito C, Galeno H, Ferres M, Lozach PY, Tischler ND. A rapid method for infectivity titration of Andes hantavirus using flow cytometry. J Virol Methods. 2013;193:291–4.
32. Menke L, Sieben C. An improved workflow for the quantification of orthohantavirus infection using automated imaging and flow cytometry. Viruses. 2024;16(2):269.
33. Aitichou M, Saleh SS, McElroy AK, Schmaljohn C, Ibrahim MS. Identification of dobrava, hantaan, seoul, and puumala viruses by one-step real-time RT-PCR. J Virol Methods. 2005;124:21–6.
34. Jiang W, Yu HT, Zhao K, Zhang Y, Du H, Wang PZ, Bai XF. Quantification of Hantaan virus with a SYBR green I-based one-step qRT-PCR assay. PLoS ONE. 2013;8: e81525.
35. Yu L, Bai W, Wu X, Zhang L, Zhang L, Li P, Wang F, Liu Z, Zhang F, Xu Z. A recombinant pseudotyped lentivirus expressing the envelope glycoprotein of hantaan virus induced protective immunity in mice. Virol J. 2013;10:301.
36. Ray N, Whidby J, Stewart S, Hooper JW, Bertolotti-Ciarlet A. Study of Andes virus entry and neutralization using a pseudovirion system. J Virol Methods. 2010;163:416–23.
37. Simões M, da Silva SA, Lúcio KA, de Oliveira VR, Schwarcz WD, de Lima SMB, Camacho LAB. Standardization, validation, and comparative evaluation of a faster and high-performance test for quantification of yellow fever neutralizing antibodies. J Immunol Methods. 2023;522: 113568.
38. Higa MM, Petersen J, Hooper J, Doms RW. Efficient production of Hantaan and Puumala pseudovirions for viral tropism and neutralization studies. Virology. 2012;423:134–42.
39. Brown KS, Safronetz D, Marzi A, Ebihara H, Feldmann H. Vesicular stomatitis virus-based vaccine protects hamsters against lethal challenge with Andes virus. J Virol. 2011;85:12781–91.
40. Prescott J, DeBuysscher B, Brown K, Feldmann H. Long-term single-dose efficacy of a vesicular stomatitis virus-based andes virus vaccine in syrian hamsters. Viruses. 2014;6:516.
41. Jangra RK, Herbert AS, Li R, Jae LT, Kleinfelter LM, Slough MM, Barker SL, Guardado-Calvo P, Román-Sosa G, Dieterle ME, et al. Protocadherin-1 is essential for cell entry by new world hantaviruses. Nature. 2018;563:559–63.
42. Ning T, Wang L, Liu S, Ma J, Nie J, Huang W, Li X, Li Y, Wang Y. Monitoring neutralization property change of evolving hantaan and seoul viruses with a novel pseudovirus-based assay. Virol Sin. 2021;36:104–12.
43. Wen X, Zhang L, Liu Q, Xiao X, Huang W, Wang Y. Screening and identification of HTNV(pv) entry inhibitors with high-throughput pseudovirus-based chemiluminescence. Virol Sin. 2022;37:531–7.
44. Dieterle ME, Solà-Riera C, Ye C, Goodfellow SM, Mittler E, Kasikci E, Bradfute SB, Klingström J, Jangra RK, Chandran K. Genetic depletion studies inform receptor usage by virulent hantaviruses in human endothelial cells. Elife. 2021;10:e69708.
45. Warner BM, Jangra RK, Griffin BD, Stein DR, Kobasa D, Chandran K, Kobinger GP, Safronetz D. Oral vaccination with recombinant vesicular stomatitis virus expressing sin nombre virus glycoprotein prevents sin nombre virus transmission in deer mice. Front Cell Infect Microbiol. 2020;10:333.
46. Wang B, Pei J, Zhang H, Li J, Dang Y, Liu H, Wang Y, Zhang L, Qi L, Yang Y, et al. Dihydropyridine-derived calcium channel blocker as a promising anti-hantavirus entry inhibitor. Front Pharmacol. 2022;13: 940178.
47. Zhao Y, Zhao N, Cai Y, Zhang H, Li J, Liu J, Ye C, Wang Y, Dang Y, Li W, et al. An algal lectin griffithsin inhibits Hantaan virus infection in vitro and in vivo. Front Cell Infect Microbiol. 2022;12: 881083.
48. Doms RW, Keller DS, Helenius A, Balch WE. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. J Cell Biol. 1987;105:1957–69.
49. Battisti AJ, Chu YK, Chipman PR, Kaufmann B, Jonsson CB, Rossmann MG. Structural studies of Hantaan virus. J Virol. 2011;85:835–41.
50. Parvate A, Williams EP, Taylor MK, Chu YK, Lanman J, Saphire EO, Jonsson CB. Diverse Morphology and Structural Features of Old and New World Hantaviruses. Viruses. 2019;11(9):862.
51. Ma J, Gong T, Luo T, Li S, Zhong L, Zhao X, Mei C, Bu H, Jia Z, Kuang X, et al. Exacerbated lung inflammation in offspring with high maternal antibody levels following secondary RSV exposure. Front Immunol. 2024;15:1377374.
52. Sarker A, Dhama N, Gupta RD. Dengue virus neutralizing antibody: a review of targets, cross-reactivity, and antibody-dependent enhancement. Front Immunol. 2023;14:1200195.
53. Wang S, Wang J, Yu X, Jiang W, Chen S, Wang R, Wang M, Jiao S, Yang Y, Wang W, et al. Antibody-dependent enhancement (ADE) of SARS-CoV-2 pseudoviral infection requires FcγRIIB and virus-antibody complex with bivalent interaction. Commun Biol. 2022;5:262.
54. Thomas S, Smatti MK, Alsulaiti H, Zedan HT, Eid AH, Hssain AA, Abu Raddad LJ, Gentilcore G, Ouhtit A, Althani AA, et al. Antibody-dependent enhancement (ADE) of SARS-CoV-2 in patients exposed to MERS-CoV and SARS-CoV-2 antigens. J Med Virol. 2024;96: e29628.
55. Bournazos S, Gupta A, Ravetch JV. The role of IgG Fc receptors in antibody-dependent enhancement. Nat Rev Immunol. 2020;20:633–43.
56. Yao JS, Kariwa H, Takashima I, Yoshimatsu K, Arikawa J, Hashimoto N. Antibody-dependent enhancement of hantavirus infection in macrophage cell lines. Arch Virol. 1992;122:107–18.

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