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Malaysia outbreak survivors retain detectable Nipah antibodies and memory B cells after 25 years

Hui Ming Ong [a], Puteri Ainaa S. Ibrahim [a], Chee Ning Chong [a], Chong Tin Tan [b], Jie Ping Schee [b], Michael Selorm Avumegah [c], Raúl Gómez Román [c,d], Neil George Cherian [c], Won Fen Wong [a], Li-Yen Chang [a,*]

[a] Department of Medical Microbiology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur 50603, Malaysia
[b] Division of Neurology, Department of Medicine, Faculty of Medicine, Universiti Malaya, Kuala Lumpur 50603, Malaysia
[c] Coalition for Epidemic Preparedness Innovation (CEPI), Askekroken 11, 0277 Oslo, Norway
[d] International Cooperation, Embassy of Mexico, Frøyas Gate 9, 0273 Oslo, Norway

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SUMMARY

Objective: To evaluate the long-term humoral immune response to Nipah virus (NiV) in a cohort of 25 survivors after 25 years of post-infection.
Methods: A total of 25 survivors of NiV infection from the 1998 outbreak were recruited for sample collection. The serum IgG antibody response to NiV antigens, specifically nucleocapsid (N), fusion glycoprotein (F) and attachment glycoprotein (G) was evaluated using ELISA. Additionally, the samples were tested for neutralizing antibodies and memory B cell responses.
Results: Detection rates of anti-NiV-F and anti-NiV-G were 56% and 60%, respectively, among the survivors at a 1:100 dilution, whereas only 20% were specifically reactive to rNiV-N. Notably, all samples that tested positive for NiV-F and NiV-G at this dilution also exhibited neutralizing antibodies, highlighting the specificity of these assays. Live virus neutralization assay showed that 72% of survivors had detectable neutralizing antibodies, with varying titers, indicating long-lasting immune memory. Furthermore, memory B cell responses specific to NiV-F and NiV-G were observed in six randomly selected survivors, suggesting the presence of enduring immunological memory.
Conclusions: These findings highlight the potential of NiV-F and NiV-G as reliable markers for NiV exposure and underscore the need for continuous surveillance and research. Such efforts are crucial for advancing vaccine development and improving preparedness for future NiV outbreaks.

Introduction

Nipah virus (NiV) first emerged in Malaysia in late 1998, causing a fatal outbreak of human encephalitis.[1] In March 1999, the virus was isolated from a resident of Kampung Sungai Nipah, Negeri Sembilan, Malaysia, giving rise to its name "*Nipah*".[1–3] NiV is an emerging zoonotic pathogen classified as a paramyxovirus within the *Henipavirus* genus. Its primary natural reservoir is *Pteropus* bats, which have a wide geographic range. This broad distribution increases the risk of spillover events from bats to humans and other animals, including livestock.[3–5] NiV infections are associated with a range of clinical manifestations, including acute respiratory illness and encephalitis.[1,6] The Malaysian outbreak resulted in over 250 human cases and more than 100 fatalities, with majority of affected individuals developing severe encephalitis, while some also exhibited respiratory symptoms.[1,2] Following this outbreak, NiV cases were reported in several other countries, including Singapore,[7] the Philippines,[8] Bangladesh,[9] and India.[10] In Bangladesh, NiV cases have been reported almost annually, with the most recent cases documented in January 2024.[11,12] The mortality rate in Bangladesh has exceeded 70%, with at least one-third of survivors suffering permanent neurological impairments.[13,14] Despite the ongoing threat posed by NiV, particularly in South Asia, home to approximately 2 billion people, or about 25% of the global population, there is currently no approved vaccine or therapeutic treatment for its prevention or management.[15] The widespread distribution of pteropid bats further exacerbates the risk of spillover events, leading to potentially devastating outbreaks.[16] Recognizing the public health

* Corresponding author.
E-mail address: changliyen@um.edu.my (L.-Y. Chang).

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significance of NiV, the World Health Organization (WHO) has classified NiV infection as a priority disease requiring urgent preparedness to prevent public health emergencies.[17] Organizations such as the Coalition for Epidemic Preparedness Innovations (CEPI)[18] and the United Kingdom Vaccine Network[19] have also prioritized NiV for vaccine development.

A key component of humoral immune responses to pathogens is the development of immunological memory. This involves the long-term persistence of pathogen-specific B cells and plasma cells, which produce protective antibodies. In the case of NiV, neutralizing antibodies targeting its fusion glycoprotein (F) and attachment glycoprotein (G) play a vital role in blocking viral entry by disrupting the interactions with host cell receptors, thereby preventing infection.[20–22] This immune memory is key to protecting against future infections. Long-lived plasma cells continuously produce antibodies, including neutralizing antibodies, providing ongoing protection against re-exposure. If antibody levels decline over time, memory B cells can be rapidly reactivated to differentiate into plasmablasts, which produce high-affinity neutralizing antibodies, restoring protective levels in the bloodstream.[23] The persistence of long-term memory B cells and antibodies is critical for effective immunity upon re-exposure to NiV.[24] Previous studies have demonstrated that IgG antibodies against NiV can persist for up to 10 years following an initial NiV infection.[25] However, this study is the first to document the presence of both neutralizing antibodies and memory B cells specific to NiV-F and NiV-G glycoproteins 25 years post-infection. These findings are highly significant for vaccine and therapeutic development, as they highlight the durability of natural immunity against NiV. Such insights could inform the design of effective preventive and treatment strategies against NiV infection.

Materials and methods

Human sample collection

The study protocol was approved by the Universiti Malaya Medical Center Medical Research Ethics Committee (MREC ID No.: 202184–10454). Donors with a clinical history of NiV infection from the 1998 NiV outbreak, who had recovered from the disease, were recruited from Kampung Sungai Nipah, Negeri Sembilan, Malaysia, and designated as the survivor cohort (n = 25). Additionally, healthy donors with no history of NiV infection or exposure to NiV were also recruited from the same area as the endemic negative cohort (n = 23). A non-endemic negative control cohort was recruited from outside of the Kampung Sungai Nipah (n = 30). Each donor provided written informed consent to participate in the study and was interviewed using a structured questionnaire. A 10 mL blood sample was collected for serum from all donors, while 20 mL of blood was collected from six selected survivors (N2, N5, N11, N13, N17 and N22) for the isolation of peripheral blood mononuclear cells (PBMCs). Serum samples were analyzed for NiV-specific antibodies using recombinant NiV nucleocapsid (rNiV-N) in a comparative indirect enzyme-linked immunosorbent assay (ELISA), and NiV glycoproteins (NiV-F and NiV-G) in an indirect ELISA. NiV neutralizing antibodies were also evaluated using a virus neutralization assay. The PBMCs from the selected survivors were assessed for the presence of memory B cells using an enzyme-linked immunosorbent spot (ELISpot) assay.

Cells and viruses

African green monkey kidney E6 cells (Vero-E6; ATCC no. CRL-1586) were used for propagating NiV. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, and maintained in 5% CO_2 at 37 °C. The NiV strain NV/MY/99/VRI-2794, isolated from pigs during the 1998 outbreak in Malaysia (GenBank accession no. AJ564621),[26] was propagated as previously described.[27] NiV is classified as a Risk Group 3 agent in Malaysia under the Prevention and Control of Infectious Diseases Act 1988. A biorisk assessment was conducted to determine the required safety control measures for handling the virus, minimizing the risks of laboratory-acquired infection. All experiments involving live NiV were performed in a laboratory with heightened control measures as outlined in the WHO Laboratory biosafety manual (4th edition) under a protocol approved by the Universiti Malaya Institutional Biosafety and Biosecurity Committee (UMIBBC/NOI/R/FOM/MMB-012/2021–30112021). For this study, the virus, at passage 4 was further propagated in Vero-E6 cells to generate a working virus stock. This stock was subsequently passaged to passage 5. The viral titer was determined using tissue culture infectious dose 50 ($TCID_{50}$/mL) assay. Monolayers of Vero-E6 cells, seeded at 2×10^5 cells/mL in 96-well microtiter, were infected with the virus. On day 3 post-infection, wells displaying cytopathic effect (CPE) were scored as infected. The $TCID_{50}$ was calculated using the Reed-Meunch method.[28]

Comparative indirect rNiV-N ELISA

The rNiV-N was expressed in *Escherichia coli* RosettaBlue (DE3) pLacI harboring the pTriEx-3-Hygro vector containing the NiV-N gene insert. The protein was purified by affinity chromatography and eluted with an elution buffer (500 mM NaCl, 20 mM NaH_2PO_4, 500 mM imidazole, pH 7.4) as previously described.[29] A comparative indirect rNiV-N ELISA was adapted and conducted following established protocols.[30] The assay was performed at room temperature with 50 μL of solution per well at each step, and included five washes with 150 μL phosphate buffered saline (PBS) between steps. Briefly, 96-well microtiter plates (Corning, USA) were coated with rNiV-N (2 μg/mL) diluted in coating buffer (15 mmol/L sodium carbonate and 35 mmol/L sodium bicarbonate, pH 9.6). Serum samples were diluted 1:50 and 1:100 in 5% skim milk in PBS, pre-adsorbed with *E. coli* lysate to reduce non-specific binding and background noise.[31] Serum samples were added to two sets of wells: one for reactivity (R) and the other for background (B), and incubated for 1 h. In the R wells, lysates of *E. coli* RosettaBlue (DE3) pLacI with the pTriEx-3-Hygro vector were added, while B wells received the same lysate along with 50 μg of rNiV-N. Goat anti-human IgG horseradish peroxidase conjugate (Promega, USA) was used as the secondary antibody at a dilution of 1:10,000. Reactions were developed using the 3,3′,5,5′-tetramethylbenzidine (TMB) Microwell peroxidase substrate system 2-C substrate (KPL SeraCare, USA), and the reaction was stopped by adding TMB stop solution (KPL SeraCare, USA). Absorbance values were measured at 450 nm (A_{450}) for both R and B wells using an Eon Biotek microplate reader (Biotek, USA). The cut-off value for positivity was determined based on the non-endemic negative control cohort (n = 30, data not shown) and calculated as the mean absorbance of negative samples + (3×standard deviation). The A_{450} (R) represents the absorbance value obtained from the R wells (standard indirect ELISA), while A_{450} (B) corresponds to the background absorbance obtained from the B wells when serum samples are exposed to excess free antigen, which blocks specific binding of antibodies to the coated antigen. A sample was considered positive if the A_{450} (R) was > 0.16, and A_{450} (R)/A_{450} (B) was > 3.873. The positive control for the assay consisted of a rNiV-N monoclonal antibody diluted 1:50 in 5% skim milk in PBS, followed by incubation with a goat anti-mouse IgG horseradish peroxidase conjugate (Promega, USA) at a 1:2,500 dilution as the secondary antibody. Each sample was tested in duplicate, and the assay was performed in triplicate.

Indirect NiV-F and NiV-G ELISAs

The indirect NiV-F and NiV-G ELISAs were adapted from the comparative indirect rNiV-N ELISA protocol, with the modification of omitting the pre-adsorption step. Both assays were performed in 96-well microtiter plates, with three washes between each step using

washing buffer (PBS with 0.1% Tween-20). For the indirect NiV-F ELISA, 10 μg/mL of NiV glycoprotein F mouse Fc tag antigen (NiV-F Ag; Native Antigen Company, UK) was diluted in coating buffer and added to each well for overnight incubation at 4 °C. Similarly, for the indirect NiV-G ELISA, 2 μg/mL of NiV glycoprotein G mouse Fc tag antigen (NiV-G Ag; Native Antigen Company, UK) was used under the same conditions. After coating, the plates were blocked with blocking buffer (10% skim milk in PBS with 0.05% Tween-20) for 2 h. Serum samples were diluted 1:50 and 1:100 in blocking buffer, and 50 μL of each dilution was added to the wells. The plates were incubated for 1 h, followed by the addition of goat anti-human Ig Fab-horseradish peroxidase conjugate (Southern Biotech, USA) diluted 1:2,500 in blocking buffer, and incubated for an additional hour. The reactions were developed using KPL TMB Microwell peroxidase substrate system 2-C substrate (KPL SeraCare, USA), and the color development was stopped by adding KPL TMB stop solution (KPL SeraCare, USA). Absorbance was measured at 450 nm and 620 nm using an Eon Biotek microplate reader (Biotek, USA). The cut-off values for both assays were established using samples from the non-endemic negative control cohort, applying the same formula used in the comparative indirect rNiV-N ELISA to calculate the cut-off values for the NiV-F and NiV-G assays. A sample was considered positive by indirect NiV-F ELISA if the A_{450} > 1.314 and positive by indirect NiV-G ELISA if A_{450} > 1.315. The positive control for the indirect NiV-F ELISA consisted of a serum sample from the survivor cohort with detectable anti-rNiV-N antibodies (as determined by the comparative indirect rNiV-N ELISA) and the ability to neutralize NiV in a virus neutralization assay. This sample was incubated with goat anti-human Ig Fab-horseradish peroxidase conjugate (Southern Biotech, USA) at a 1:2,500 dilution. For the indirect NiV-G ELISA, NiV glycoprotein G mouse Fc tag monoclonal antibody (1:250; Native Antigen Company, UK) was used as the positive control, with goat anti-mouse IgG-Fab horseradish peroxidase conjugate (Southern Biotech, USA) diluted at 1:5,000. All samples were tested in duplicate, and each assay was performed in triplicate. Both the indirect NiV-F and NiV-G ELISAs were first evaluated using 15 positive and 15 negative human serum samples before testing the study samples.[30]

NiV neutralization assay

A CPE-based neutralization assay was performed in 96-well microtiter plates to assess the presence of antibodies that neutralize the NiV infectivity in Vero-E6 cell monolayers. Serum samples were serially diluted twofold from 1:20 to 1:640 and mixed with equal volume of EMEM containing 100 $TCID_{50}$ of NiV. The mixture was incubated for 1 h at 37 °C. After the incubation, the virus-serum mixture was added to Vero-E6 cells at a concentration of 2 $\times 10^5$ cells/mL, and the plates were incubated at 37 °C with 5% CO_2. Neutralization was assessed by examining the CPE under a microscope on day 3 post-infection. The neutralizing antibody titer was determined using the Reed-Muench method,[28] identifying the highest serum dilution that showed no CPE in any replicate well. The positive control consisted of pooled archived serum samples from NiV patients confirmed to have anti-NiV antibodies, based on hospital records. The negative control comprised pooled serum samples from the non-endemic negative cohort. Each serum sample was tested in triplicate, and the assay was repeated three times. The final neutralization titer was recorded as the median of the independent assays. A detectable neutralizing antibody response was defined as virus neutralization titer greater than 1:20.

NiV-specific memory B cells ELISpot assay

PBMCs were isolated from whole blood of six selected survivors (N2, N5, N11, N13, N17 and N22) who tested positive for anti-NiV-F and anti-NiV-G by indirect NiV-F and NiV-G ELISA, respectively. These survivors also provided consent to continue their participation in the study. PBMCs were isolated using density gradient centrifugation with lymphocyte separation medium (Corning, USA), cryopreserved in freezing media (95% FBS and 5% dimethyl sulfoxide), and stored in liquid nitrogen until needed. To stimulate the PBMCs, the cells were thawed, rested for 1 h, and then incubated with a mixture of 1 μg/mL of R848 and 10 ng/mL of recombinant human interleukin-2 (3850–2A kit; Mabtech, Sweden) according to the manufacturer's instructions. The cells were incubated for 72 h at 37 °C with 5% CO_2. For the ELISpot assay, 250,000–400,000 PBMCs were plated in duplicate wells of ELISpot plates, which were pre-wetted with 35% ethanol and pre-coated with either 40 μg/mL of NiV-F Ag or 5 μg/mL of NiV-G Ag. The cells were incubated overnight at 37 °C. The next day, the plates were washed five times with PBS, followed by the addition of 1 μg/mL of detection monoclonal antibodies MT78/145 (3850–2A kit; Mabtech, Sweden) and incubation for 2 h at room temperature. After five additional washes with PBS, the plates were incubated with 1:1,000 streptavidin-ALP conjugate (3850–2A kit; Mabtech, Sweden) for 1 h at room temperature. Subsequent to another five washes, BCIP/NBT-PLUS substrate (Mabtech, Sweden) was added to the plates for 5 min to allow spot development. The development reaction was stopped by washing the plates extensively with tap water, and the plates were air-dried overnight. The positive control consisted of total IgG-secreting cells detected in wells coated with 15 μg/mL capture monoclonal antibody MT91/145 (3850–2A kit; Mabtech, Sweden). Wells without capture reagent served as negative controls, and wells without cells were used as media control. The number of NiV-F- and NiV-G-specific IgG-secreting B cells was determined using the IRIS ELISpot plate reader (Mabtech, Sweden). The mean frequency of memory B cells per well was calculated by subtracting background values from media control wells, with results expressed as spot-forming units (SFU) per 10^6 cells.

Statistical analysis

Statistical analysis was conducted using SPSS, with Chi-squared test to assess correlations between demographic information, clinical characteristics, and the antibody profiles of the survivor cohort. A p-value of < 0.05 was considered statistically significant. Data from the NiV-specific memory B cells ELISpot assay were analyzed with GraphPad Prism with two-way ANOVA followed by the Bonferroni *post hoc* testing. Differences between means were considered statistically significant when $p < 0.05$.

Results

Characteristics of the survivor cohort

The survivor cohort consisted of 25 donors, categorized into three age groups: < 40 years old (n = 1, 4%), 40–59 years old (n = 10, 40%) and ≥ 60 years old (n = 14, 56%) (Table 1). The ages at the time of infection ranged from 14 to 64 years, with a mean age of 39.12 years (standard deviation, 12.12 years) (Supplementary Table 1). Among the donors, five were female (20%) and 20 were male (80%).

A total of 24 donors (96%) reported experiencing at least one clinical symptom associated with NiV during the 1998 outbreak in Malaysia (Table 1). The most common symptoms were high fever (n = 19, 76%), followed by dizziness (n = 6, 24%), headache (n = 6, 24%), vomiting (n = 5, 20%), respiratory symptoms (n = 4, 16%) (Table 2). Ataxia and coma were each reported by three donors (n = 3, 12%). Other symptoms included chills and loss of consciousness (n = 2, 8% each), as well as muscle pain, lethargy, diarrhea, swollen lymph nodes, half-body paralysis, disorientation, and memory loss (n = 1, 4% each). Nine donors (36%) reported experiencing at least one symptom after the initial infection. Of these, three had symptoms

Table 1
Statistical analysis of demographic information, clinical characteristics, NiV IgG ELISA results and virus neutralization assay data in the NiV survivor cohort.

Variables		n (%)	*p* value			
			Antibodies by comparative indirect rNiV-N ELISA	Antibodies by indirect NIV-F ELISA	Antibodies by indirect NiV-G ELISA	Antibodies by NiV virus neutralization assay
Age group[a]	**< 40**	1 (4)	0.24	0.89	0.30	0.57
	40 – 60	10 (40)				
	> 60	14 (56)				
Sex	**Male**	20 (80)	1.00	1.00	0.54	1.00
	Female	5 (20)				
Presentation of clinical symptoms	**Yes**	24 (96)	0.52	0.71	0.07	0.10
	No	1 (4)				
Contact with sick pigs	**Yes**	21 (84)	0.40	0.72	0.47	0.53
	No	2 (8)				
	Unknown	2 (8)				
Contact with NiV-infected individual[b]	**Yes**	18 (72)	0.97	0.83	0.74	0.97
	No	7 (28)				

[a] Age at time of questionnaire administration, January 2022.
[b] Contact with NiV-infected family members or anyone who lives in the same house.

Table 2
Clinical symptoms of NiV infection among donors in the survivor cohort, along with their contact history with sick pigs and NiV-infected individuals.

Variables	n[a] (%)
Clinical symptoms	
High fever	19 (76)
Dizziness	6 (24)
Headache	6 (24)
Vomiting	5 (20)
Respiratory symptoms	4 (16)
Ataxia	3 (12)
Coma	3 (12)
Chills	2 (8)
Loss of consciousness	2 (8)
Muscle pain	1 (4)
Lethargy	1 (4)
Diarrhea	1 (4)
Swollen lymph nodes	1 (4)
Half-body paralysis	1 (4)
Disorientation	1 (4)
Memory loss	1 (4)
Possible long-term complications after initial NiV infection	
Presented at least one clinical symptom after 1998 NiV outbreak	9 (36)
Clinical symptoms persisted ≤ one year	3 (12)
Clinical symptoms persisted > one year	6 (24)
Contact with sick pigs	
Indirect contact	21 (84)
Direct contact (touching and handling pigs)	20 (80)
Participated in the slaughter or dissection of sick pigs	4 (16)
Contact with NiV-infected individual	
The family member or cohabitant was diagnosed with NiV infection	18 (72)
The family member or cohabitant presented at least one clinical symptom	14 (56)
The family member or cohabitant had exposure to sick pigs	16 (64)
The primary caretaker of the family member or cohabitant	4 (16)

[a] The percentage (%) of n was calculated based on 25 donors.

that lasting less than a year, while six experienced symptoms that persisted for over a year (Table 2). These persistent symptoms included lethargy, impaired vision, half-body paralysis and seizures.

In this cohort, 84% of donors reported contact with sick pigs during the 1998 NiV outbreak (Table 1), including direct contact, indirect contact, or involvement in the slaughter or dissection of sick pigs (Table 2). Additionally, 18 donors (72%) had contact with households members diagnosed with NiV or exhibited at least one clinical symptom associated with the infection.

Serum IgG antibody response to rNiV-N, NiV-F and NiV-G

In the survivor cohort of 25 donors, 20 samples (80%) showed A_{450} (R) > 0.16 at a dilution of 1:50 in the comparative indirect rNiV-N (Fig. 1A). However, only seven samples (28%) met both criteria, A_{450} (R) > 0.16, and A_{450} (R)/A_{450} (B) > 3.873, indicating positivity to rNiV-N (Fig. 1B). At a dilution of 1:100, 21 samples (84%) showed A_{450} (R) > 0.16, but only five samples (20%) met both criteria and were considered positive for rNiV-N. Notably, no samples from the endemic negative cohort showed detectable anti-rNiV-N (Fig. 1B).

For the indirect NiV-F ELISA, 21 samples (84%) were positive at the dilution of 1:50 (Fig. 2A), with this number decreasing to 14 samples (56%) at a 1:100 dilution. In the indirect NiV-G ELISA, 19 samples (76%) were positive for anti-NiV-G at the 1:50 dilution, and 15 samples (60%) were positive at the 1:100 dilution (Fig. 2B). No samples from the endemic negative cohort showed detectable anti-NiV-F or anti-NiV-G at a 1:100 dilution. However, at the 1:50 dilution, four samples were positive for NiV-F and two for NiV-G in the endemic negative cohort..

Statistical analyses were performed to assess correlations between demographic and clinical factors and the presence of detectable anti-rNiV-N, anti-NiV-F, or anti-NiV-G antibodies (Table 1). No significant correlations were found between antibody presence and factors such as age, sex, clinical symptoms, contact with sick pigs, or contact with NiV-infected family members. The detailed demographic, clinical and antibody profile data for the 25 donors in the survivor cohort are provided in Supplementary Table 1.

Analysis of NiV-neutralizing antibody

Neutralizing antibodies against NiV were detected in 18 of the 25 donors in the survivor cohort (Fig. 3). Among these, two survivors (N2 and N21) exhibited high neutralization titers, while eight showed moderate titers (N13, N14, N15, N16, N18, N22, N24 and N25). The remaining survivors had low titers (N3, N5, N8, N10, N11, N17, N20 and N23), or no detectable neutralization (N1, N4, N6, N7, N9, N12 and N19). No samples from the endemic negative cohort showed neutralizing antibodies against NiV.

No significant correlations were found between the presence of neutralizing antibodies and demographic or clinical factors, including age, sex, clinical symptoms, contact with sick pigs, or exposure to NiV-infected family members (Table 1).

Comparison between detectable anti-rNiV-N, anti-NiV-F, anti-NiV-G and NiV-neutralizing antibody

All seven samples that tested positive for rNiV-N at a 1:50 dilution (N2, N5, N15, N17, N22, N24 and N25) were also positive for both NiV-F and NiV-G at the same dilution (Table 2). Moreover, all seven samples were confirmed to have detectable neutralizing antibodies against NiV. Of these, five samples remained positive for

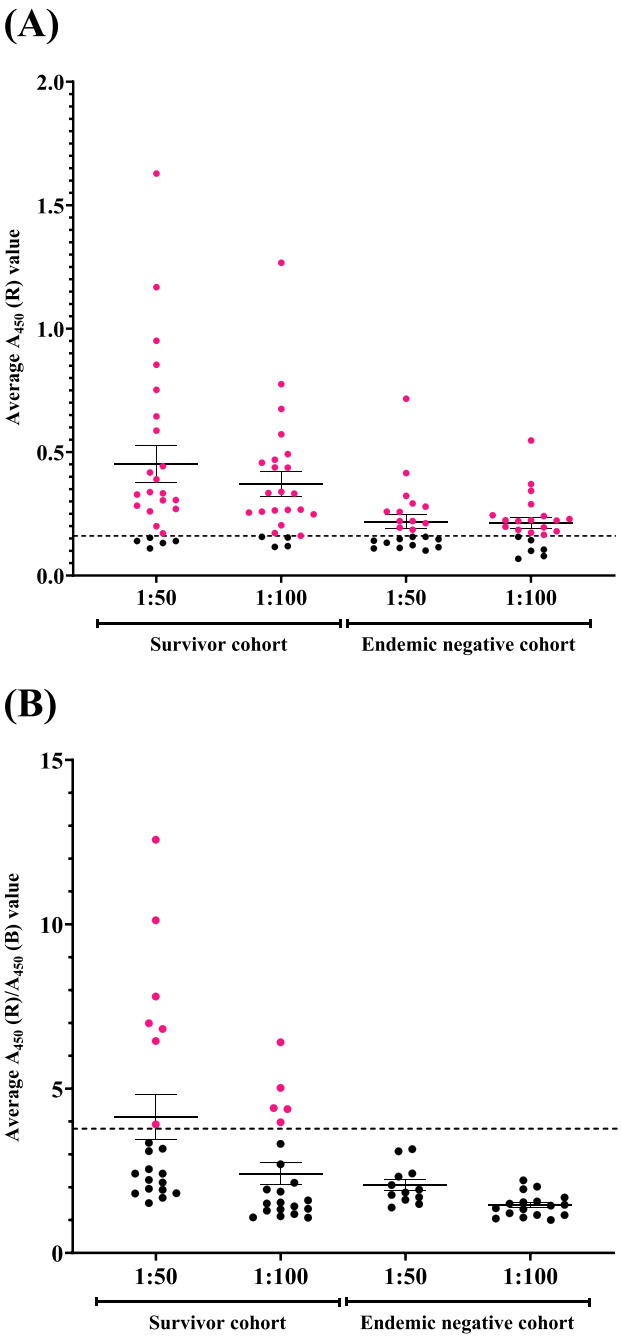

Fig. 1. Evaluation of sera samples from the survivor cohort (n = 25) and the endemic negative cohort (n = 23) *via* comparative indirect rNiV-N ELISA at 1:50 and 1:100 dilutions. **A.** Samples above the dashed line met the A_{450} (R) > 0.16 cut-off value (magenta dots). **B.** Samples that met the criterion of A_{450} (R) > 0.16 were evaluated for the second criterion of A_{450} (R) / A_{450} (B) > 3.873 (dashed line), and samples that met both criteria indicate positivity for rNiV-N (magenta dots).

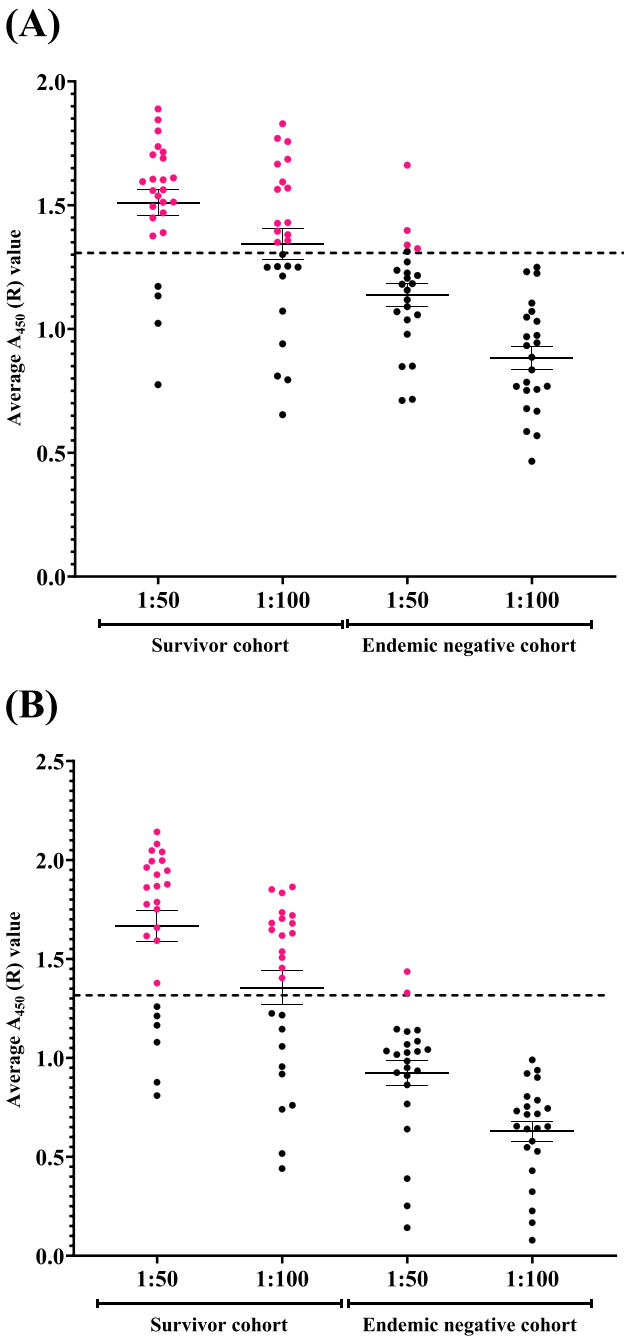

Fig. 2. Detection of anti-NiV-F and anti-NiV-G in sera samples from the survivor cohort (n = 25) and the endemic negative cohort (n = 23). **A.** Samples above the dashed line met the A_{450} (R) > 1.314 cut-off value for the indirect NiV-F ELISA, indicating positivity to NiV-F. **B.** Samples above the dashed line met the A_{450} (R) > 1.315 cut-off value for the indirect NiV-G ELISA, indicating positivity to NiV-G.

rNiV-N at a 1:100 dilution (N2, N5, N15, N17, N22, N24 and N25), and all five were also positive for anti-NiV-G at this dilution. However, only four of these five samples were positive for anti-NiV-F (N2, N17, N22 and N25).

Among the 21 samples that tested positive for anti-NiV-F, 18 were confirmed to have neutralizing antibodies against NiV (Table 2). Of the three samples that were positive for anti-NiV-F but lacked neutralizing antibodies against NiV, two (N1 and N9) also tested negative for anti-NiV-G. The third sample (N4) tested positive for anti-NiV-F at both 1:50 and 1:100 dilutions, showed detectable anti-NiV-G at 1:50, but did not demonstrate neutralizing activity against NiV. No samples from the endemic negative cohort exhibited detectable neutralizing antibodies against NiV. Additional data from the virus neutralization assay are provided in Supplementary Table 2.

NiV-F- and NiV-G-specific IgG memory B cell responses

The PBMCs from six consented donors within the survivor cohort (N2, N5, N11, N13, N17 and N22) were assessed for NiV-F- and NiV-G-specific IgG memory B cell responses. All six survivors showed reactivity to NiV-F at a dilution of 1:50, however only five of them (N2, N5, N11, N13 and N17) had detectable NiV-F-specific IgG-

Table 3
Comparative analysis of the comparative indirect rNiV-N ELISA, indirect NiV-F ELISA, indirect NiV-G ELISA and NiV neutralization assay.

Survivor cohort	Result by						
	Comparative indirect rNiV-N ELISA[a]		Indirect NiV-F ELISA[b]		Indirect NiV-G ELISA[c]		NiV virus neutralization assay[d]
	1:50	1:100	1:50	1:100	1:50	1:100	
N1	-	-	+	-	-	-	-
N2	+	+	+	+	+	+	+
N3	-	-	+	+	+	+	+
N4	-	-	+	+	+	-	-
N5	+	-	+	-	+	-	+
N6	-	-	-	-	-	-	-
N7	-	-	-	-	-	-	-
N8	-	-	+	-	+	+	+
N9	-	-	+	-	-	-	-
N10	-	-	+	+	+	+	+
N11	-	-	+	+	+	+	+
N12	-	-	-	-	-	-	-
N13	-	-	+	+	+	+	+
N14	-	-	+	+	+	+	+
N15	+	-	+	+	+	+	+
N16	-	-	+	+	+	+	+
N17	+	+	+	+	+	+	+
N18	-	-	+	-	+	+	+
N19	-	-	-	-	-	-	-
N20	-	-	+	+	+	-	+
N21	-	-	+	+	+	+	+
N22	+	+	+	+	+	+	+
N23	-	-	+	-	+	-	+
N24	+	+	+	-	+	+	+
N25	+	+	+	+	+	+	+

[a] Positive (+) if the A_{450} (R) was > 0.160 and A_{450} (R)/A_{450} (B) was > 3.873.
[b] Positive (+) if the A_{450} > 1.314.
[c] Positive (+) if the A_{450} > 1.315.
[d] Positive (+) if the virus neutralization titer is greater than 1:20.

secreting B cells (Fig. 4A). Among these, N2, N11, N13 and N17 showed high levels of NiV-specific IgG memory B cell responses, while N5 showed a lower response. In contrast, NiV-G-specific memory B cell responses were observed in all six survivors (Fig. 4B). A representative ELISpot result from survivor N11 showed detectable IgG-secreting B cells specific to both NiV-F and NiV-G (Fig. 4C). Among the survivors, N2, N11 and N13 had the highest NiV-specific IgG memory B cell responses, while N22 showed the lowest frequency of memory B cells (Fig. 4A and 4B). Additionally, levels of NiV-G-specific IgG-secreting B cell were consistently higher than those for NiV-F. Despite this overall increase in memory B cells, statistical analysis showed no significant differences in the mean frequencies of memory B cells compared to the negative control. The positive control wells, using PBMCs from the same survivors, demonstrated significantly higher frequencies of non-specific total IgG-secreting B cells when MT91/145 (anti-human IgG) capture monoclonal antibodies were used. The negative control wells, which lacked capture monoclonal antibodies, showed no memory B cells in the NiV-F-specific ELISpot, while a low count of memory B cells was detected in NiV-G-specific ELISpot.

Discussion

NiV is a highly pathogenic virus first identified during a 1998 outbreak in Malaysia. No new cases have been reported in the country since then. Although some survivors from the original outbreak locations, particularly Kampung Sungai Nipah, Negeri Sembilan, are still alive, their long-term health status and immune response to NiV infection remain poorly documented. This study presents the first comprehensive serological analysis of the humoral immune response to NiV in a cohort of the oldest known survivors, 25 years post-infection. We assessed anti-NiV humoral immunity using ELISAs targeting NiV-N, NiV-F and NiV-G proteins, alongside a live NiV neutralization assay.

Despite NiV-N being the most abundant structural protein and antigenic,[31,32] the comparative indirect rNiV-N ELISA showed the least sensitivity. This reduced sensitivity could be attributed to concealed antigenic epitopes within the native NiV-N structure and the absence of post-translational modifications (PTMs) in the rNiV-N antigen expressed in *E. coli*.[33] In contrast, over 50% of the NiV survivors tested positive for anti-NiV-F or anti-NiV-G, indicating that these proteins are more effective for antibody capture. However, not all samples positive for anti-NiV-F or anti-NiV-G at a 1:50 dilution were also positive in the NiV virus neutralization assay. At a 1:100 dilution, these samples tested positive in both the indirect NiV-F and NiV-G ELISAs, as well as the virus neutralization assay. The lack of positivity at 1:50 could be due to non-specific binding of cross-reacting antibodies,[34] which decreases at the 1:100 dilution. The results obtained at the 1:100 dilution are consistent with findings from the NiV virus neutralization assay, supporting the roles of NiV-F and NiV-G in viral entry. Antibodies against these antigens block the interaction between NiV and host cell receptors, neutralizing the virus and preventing infection.[35] Therefore, these findings support the use of indirect NiV-F and NiV-G ELISAs with serum samples diluted to 1:100, combined with NiV virus neutralization, to detect anti-NiV humoral immunity. The low sensitivity of the comparative

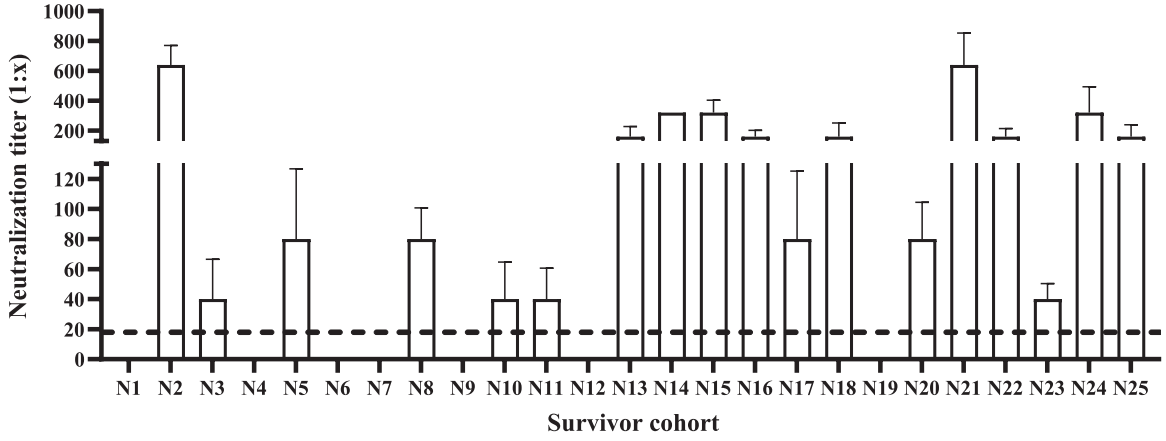

Fig. 3. Detection of neutralizing NiV-specific antibodies in convalescent serum samples from the survivor cohort using NiV neutralization assay (n = 25). Each serum sample was tested in triplicate, and the assay was performed in three independent replicates. The final neutralization titer was determined as the median value from these independent assays. Data are presented as median + standard deviation SD. Samples above the dashed line have a neutralization titer greater than 1:20, indicating positivity for NiV-neutralizing antibodies.

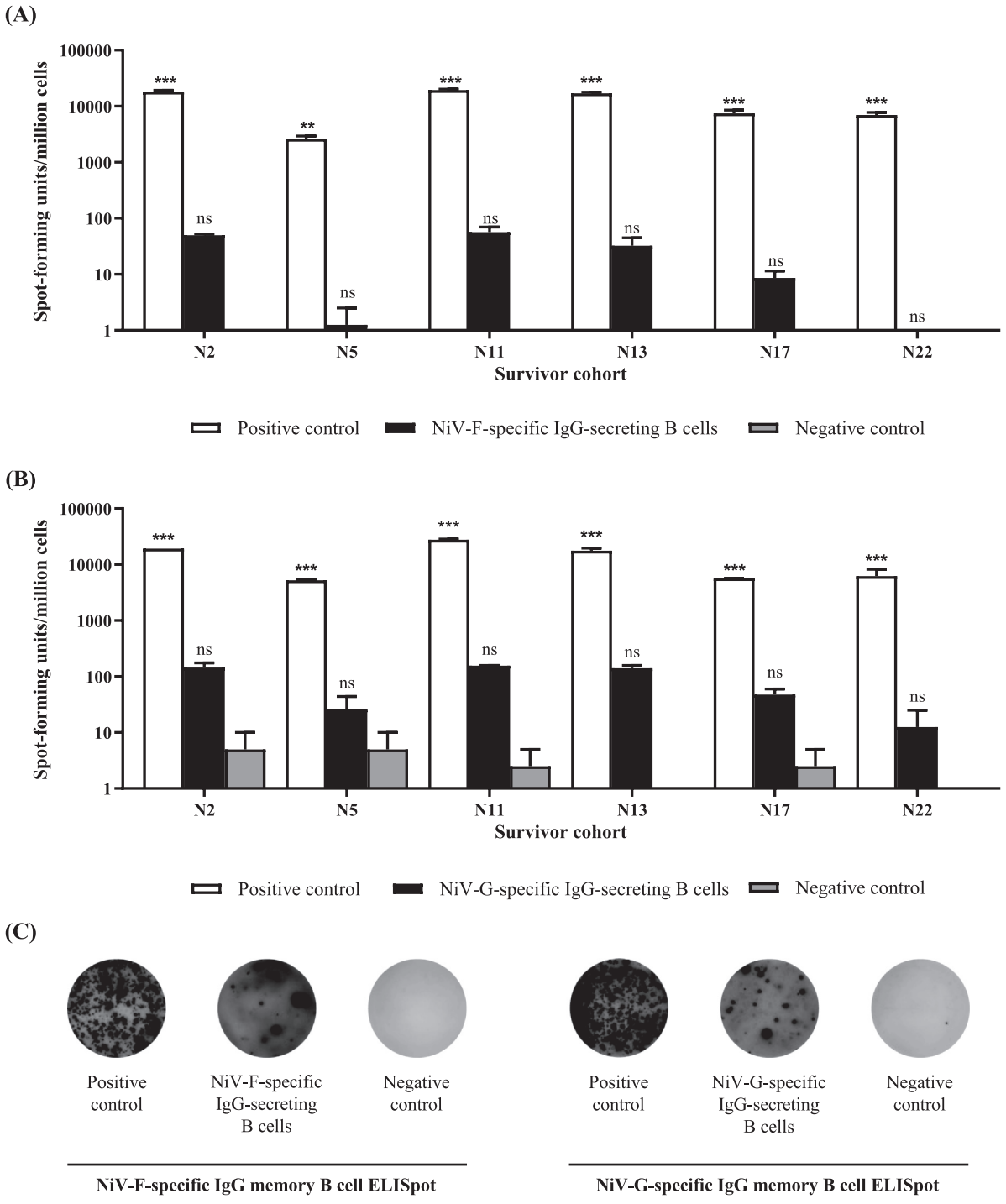

Fig. 4. Detection of NiV-specific memory B cells in ELISpot from a subset of survivor cohort (n = 6). **A.** NiV-F-specific IgG memory B cell response of the six survivors. **B.** NiV-G-specific IgG memory B cell response of the six survivors. **C.** Representative memory B cell ELISpot readouts from N11 survivor. The asterisks denote the significance levels as compared with negative control, ** $p < 0.01$, *** $p < 0.001$ and ns represents $p > 0.05$ by Two-way ANOVA followed by Bonferroni *post hoc* test.

indirect rNiV-N ELISA may lead to an underestimation of NiV seroprevalence, making NiV-F and NiV-G more reliable indicators for serological surveys and diagnostics. These proteins also serve as key markers for NiV exposure and vaccine development. Furthermore, the study underscores the importance of NiV virus neutralization in understanding the immune response.

Neutralizing antibodies are essential for protection against NiV infection, complementing cell-mediated immunity and other defense mechanisms. The presence of NiV neutralizing antibodies in survivors from the 1998 outbreak indicates the persistence of NiV-specific memory B cells. This study is the first to report the long-term presence of NiV-specific memory B cells 25 years post-infection. Among six randomly selected NiV survivors, five exhibited IgG-producing plasmablasts specific to NiV-F, while all six had NiV-G-specific IgG-producing plasmablasts. These findings highlight the therapeutic potential of enduring memory B cells and support the use of NiV-F and NiV-G as targets for strategies aimed at generating and sustaining immunological memory.[29]

The detection of persistent NiV-specific memory B cells also paves the way for B cell epitope mapping, facilitating the design of a

robust epitope-based vaccine against NiV.[36,37] Additionally, the development of monoclonal antibodies, such as m102.4, which targets the immunodominant NiV receptor-binding glycoprotein and effectively neutralizes NiV, exemplifies the potential of such antibodies as therapeutic options.[38]

A limitation of this study is the small sample size of survivors, making it challenging to identify significant trends in clinical symptoms, exposure routes, or demographic factors associated with the sustained humoral protection. However, questionnaire data revealed that 80% of the NiV survivors were involved in pig-farming industries or had contact with sick pigs during the outbreak. Additionally, the study had a 60% higher male recruitment rate, reflecting the predominance of men in local pig farming. While this gender imbalance does not affect the findings, it highlights a specific demographic characteristic of the infected population and aligns with the composition of the pig-farming workforce.

This study is the first to evaluate the long-term immune response to NiV 25 years after the initial infection, using samples from survivors of the first NiV outbreak caused by the NiV-Malaysia (NiV-MY) strain. This aged cohort provides valuable insights into the enduring immune response against the NiV-MY strain. However, since no NiV cases have been confirmed in Malaysia since 1999,[39] the absence of recent samples for immunological comparison limits our understanding of how immune responses to NiV evolve over time. Nonetheless, these findings serve as a crucial basis for comparison with data from recent NiV cases in countries such as Bangladesh and India, where NiV continues to be reported. Such comparisons will help clarify how immune responses differ due to variations in viral strains and demographic factors, providing valuable insights for vaccine development and therapeutic interventions.

Future research should focus on T cell responses to NiV. While humoral immunity is essential, T cell responses play a critical role in viral clearance and long-term immune memory.[40] Studies could investigate whether survivors infected with the NiV-MY strain exhibit T cell cross-reactivity against the NiV-Bangladesh strain, providing insights into the breadth and durability of T cell-mediated immunity. Evaluating both humoral and T cell responses could provide a comprehensive understanding of immune protection and inform vaccine design aimed at stimulating both immune arms, thereby broadening protection against diverse NiV strains.

This study is the first to demonstrate the persistence of antibodies against rNiV-N, NiV-F and NiV-G, as well as long-lived memory B cells specific to NiV-F and NiV-G, in survivors 25 years after the NiV outbreak in Malaysia. ELISAs and memory B cell ELISpot assays consistently showed higher antibody levels against NiV-F and NiV-G compared to rNiV-N. Remarkably, even after 25 years, the survivors' sera retained the ability to neutralize live NiV. Additionally, sera from this cohort contributed to establishment of the first WHO International Standard for anti-Nipah virus antibodies.[41] Understanding the longevity of the anti-NiV humoral response is essential for advancing vaccine development, diagnostics, and surveillance methods, thereby improving preparedness for future NiV outbreaks. These findings underscore the necessity of ongoing surveillance and research to deepen our understanding of NiV and leverage insights from long-term survivors in combating the virus.

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CRediT authorship contribution statement

Hui Ming Ong: Investigation, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Puteri Ainaa S. Ibrahim:** Investigation, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Chee Ning Chong:** Methodology, Writing – review & editing. **Chong Tin Tan:** Conceptualization, Writing – review & editing. **Jie Ping Schee:** Investigation, Writing – review & editing. **Michael Selorm Avumegah:** Data curation, Writing – review & editing. **Raúl Gómez Román:** Methodology, Writing – review & editing. **Neil George Cherian:** Methodology, Writing – review & editing. **Won Fen Wong:** Formal analysis, Writing – review & editing, Supervision. **Li-Yen Chang:** Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing, Supervision.

Data availability

The data used in this study can be obtained from the corresponding author upon reasonable request, providing the request complies with local ethical and research governance standards.

Declaration of Competing Interest

Michael Selorm Avumegah and Neil George Cherian are current employees of CEPI, whereas Raúl Gómez Román was previously employed by CEPI. CEPI is supporting the research and development of a diverse portfolio of vaccine candidates (including vaccines against NiV) based on a range of vaccine approaches. The remaining authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106398.

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