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Short communication

Establishment of an immunofluorescence assay to detect IgM antibodies to Nipah virus using HeLa cells expressing recombinant nucleoprotein

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ABSTRACT

A novel indirect fluorescent antibody test (IFAT) for detection of IgM against Nipah virus (NiV) was developed using HeLa 229 cells expressing recombinant NiV nucleocapsid protein (NiV-N). The NiV IFAT was evaluated using three panels of sera: a) experimentally produced sera from NiV-N-immunized/pre-immunized macaques, b) post-infection human sera associated with a Nipah disease outbreak in the Philippines in 2014, and c) human sera from a non-exposed Malaysian population. Immunized macaque sera showed a characteristic granular staining pattern of the NiV-N expressed antigen in HeLa 229 cells, which was readily distinguished from negative-binding results of the pre-immunized macaque sera. The IgM antibody titers in sequential serum samples (n = 7) obtained from three Nipah patients correlated well with previously published results using conventional IgM capture ELISA and SNT serology. The 90 human serum samples from unexposed persons were unreactive by IFAT. The IFAT utilizing NiV-N-expressing HeLa 229 cells to detect IgM antibody in an early stage of NiV infection is an effective approach, which could be utilized readily in local laboratories to complement other capabilities in NiV-affected countries.

Nipah virus (NiV) is a highly pathogenic, zoonotic virus of the genus henipavirus within the family paramyxovirus, identified initially in Malaysia and Singapore in 1998 as the causative agent for a relatively benign respiratory disease in farmed pigs, which came to prominence as a severe human disease of acute/febrile encephalitis with a case fatality rate (CFR) greater than 40% (Chua et al., 2000). Due to this high virulence and lack of any approved treatment measures or vaccines, NiV has been classified as a biosafety level (BSL) 4 pathogen (Daniels et al., 2001), a level shared with the closely related Hendra virus (HeV), which emerged in 1994 as an outbreak of severe pneumonia among horses and human horse handlers in Australia (Murray et al., 1995). Since 2001, NiV outbreaks with higher than 70%–100% CFR have occurred in Bangladesh and India on a nearly annual basis (Chadha et al., 2006; Luby et al., 2009), including a recent outbreak in Kerala, India in 2018 which resulted in 23 cases and a CFR of 91% (Arunkumar et al., 2018). NiV therefore poses a global concern for animal and public health management, particularly in affected and neighboring South and South-Eastern Asian countries.

The natural hosts of henipaviruses are thought to be fruit bats. To date, the widespread presence of antibodies against NiV or NiV-like viruses in fruit bats has been reported in several Asian and African countries including Thailand, Cambodia, Indonesia, Madagascar, Ghana, the southern region of China, Papua New Guinea, East Timor and New Caledonia (reviewed by Clayton et al., 2012, 2016). In 2014, an outbreak of NiV in humans with close contact to sick horses occurred in the southern Philippines (Ching et al., 2015), with the most likely source of horse infections considered to be fruit bats.

In the serological diagnosis of NiV infection (reviewed by Wang and Daniels, 2012), several enzyme-linked immunosorbent assay (ELISA) systems have been developed and used particularly as screening tests. As ELISA false positive reactions may occur, serum neutralization tests (SNTs) are reference techniques for reliable confirmatory diagnosis, using either infectious henipavirus in BSL4 laboratories, or vesicular stomatitis virus (VSV) pseudotyped with NiV glycoproteins under BSL2 conditions (Kaku et al., 2012). Detection of early infection stage serum IgM antibodies against NiV has provided an additional and useful

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diagnostic approach given the highly acute and lethal consequences of NiV infection in humans. For anti-henipaviral IgM detection, IgM capture ELISA systems have been developed using a recombinant expressed NiV nucleocapsid (NiV-N) protein (Yu et al., 2006). However, introduction and calibration of IgM capture ELISA in laboratories of high risk or affected countries is sometimes difficult for several reasons: preparation of purified viral antigen is time-consuming, and requires specific skills and equipment for protein expression and quality control of baculoviruses expressing antigen proteins. These issues led us to develop an indirect fluorescent antibody test (IFAT) for NiV IgM antibodies employing HeLa 229 cells expressing NiV-N antigen. As human IgM/IgG are generally cross-reactive with IgM/IgG of the non-human primate, macaque (*Macaca fascicularis*) (Ksiazek et al., 1999), we chose to evaluate the assay initially using macaque anti-NiV IgM-positive control serum.

Recombinant NiV-N protein used in antiserum production and ELISA was prepared using a recombinant baculovirus. Briefly, a NiV-N-expressing vector plasmid, NiV-N/pFastBac™ HT C (provided by Australian Animal Health Laboratory as a courtesy) containing the nucleoprotein cDNA of a NiV Malaysian strain (GenBank Accession number: NC_002728), was used to transform DH10Bac-competent *Escherichia coli* to construct a recombinant bacmid in accordance with the manufacturer's instructions (BAC-TO-BAC Baculovirus Expression System; Thermo Fisher Scientific Waltham, MA). The bacmid DNA was transfected to Sf9 insect cells to produce recombinant baculovirus expressing NiV-N. Upon infection of Tn5 cells with the recombinant baculovirus, recombinant NiV-N with a six-histidine tag at the N terminus was expressed and purified from cell lysates using nickel-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN) in the presence of 2 M urea. Protein purity and molecular weight of purified NiV-N was assessed by SDS-PAGE with Coomassie Blue staining (Suppl. Fig. 1). To generate macaque anti-NiV-N IgM-positive sera, 1 mg of purified NiV-N was injected subcutaneously into the back of two macaques (mac-No.1, and mac-No.2), respectively. Blood samples (0.5 ml) were collected prior to immunization and at five additional time points, 5, 7, 9, 11 and 13 days post-immunization (d.p.i) for serum preparation to monitor the development of antibody titer by IgM capture ELISA (Suppl. Fig. 3). A brief schematic of the IgM capture ELISA is shown in Suppl. Fig. 2. Goat anti-human IgM (Southern Biotech, Birmingham, AL) diluted 1:1000 in phosphate-buffered saline (PBS)(-) was immobilized to Nunc Maxisorp 96-well ELISA plates (Thermo Fisher Scientific) at 4 °C overnight. After washing with PBS(-), plates were blocked with UltraPure DNase/RNase-Free Distilled Water (DW; Thermo Fisher Scientific) including 20% Blocking One (Nacalai Tesque, Kyoto, Japan). Macaque sera were serially 2-fold diluted (from 1:50 dilution) in diluent (PBS(-) with 0.05% Tween 20 (PBS-T) including 20% Blocking One) and 50 µl diluted sera were added to quadruplicate wells. After incubation for 1 h at 37 °C and washing four times with PBS-T, the purified recombinant NiV-N antigen was added to duplicate wells (NiV-N antigen containing wells). In parallel, negative control antigen was prepared similarly from Tn5 cells infected with a baculovirus lacking the polyhedrin gene (ΔP) in 50 µl of diluent (1 µg/ml) in duplicate wells (ΔP antigen-containing wells). The antigens were detected using rabbit anti-NiV-N polyclonal serum followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Thermo Fisher Scientific). After washing, the reaction was detected by 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate (Roche, Basel, Switzerland) for 60 min. The optical densities at 405 nm were measured in an ELISA plate reader, and results were calculated by subtracting the OD value of ΔP antigen-containing wells from that of NiV-N antigen-containing wells. At 15 d.p.i, 30 ml blood samples were collected to be used for further analysis as positive controls for IFAT detection of anti-NiV-N IgM.

For preparation of IFAT antigens, HeLa 229 cells were transfected with expression vector pKS336 (Saijo et al., 2002) containing the cDNA of NiV-N (GenBank Accession number: NC_002728) (NiV-N/pKS336) to produce either transient or stable expression of NiV-N protein. In brief, for transient expression of NiV-N, HeLa 229 cells cultured on a 6-well plate were transfected with NiV-N/pKS336 using the FuGENE HD Transfection Reagent (Promega, Madison, WI) in accordance with the manufacturer's instructions. At 4 days after transfection, the cells were analyzed for expression of NiV-N by IFAT using anti-NiV-N rabbit polyclonal serum and FITC-conjugated goat anti-rabbit IgG. Furthermore, HeLa 229 cells transfected with NiV-N/pKS336 were selected for stable expression of NiV-N by addition of blastcidin S-hydrochloride (2 µg/ml) in culture medium. Sub-clones were assessed as outlined above to verify continued expression. For production of IFAT slides, HeLa 229 cells with either transient or stable-expression of NiV-N were prepared as trypsinized and washed suspensions. Additionally, HeLa 229 cells with stable expression of NiV-N were mixed with mock-transfected HeLa 229 cells treated similarly at a ratio of 1:3, to provide further discernment after staining. By employing a fixed ratio (1:3) of NiV-N expressing/mock transfected cells, true positive serum is expected to demonstrate a fixed ratio of immunofluorescent cells against non-immunofluorescent cells. This characteristic could contribute to differentiation of true positives from false positive results, which might show many more immunofluorescent cells (higher than 1:3). To generate antigen-positive IFAT slides, cell suspensions (containing 40,000 cells) were spotted onto 14-well non-fluorescent glass slides (Thermo Fisher Scientific), air-dried and fixed with acetone at room temperature for 5 min. Antigen-negative slides were prepared similarly by using mock-transfected HeLa 229 cells. IFAT antigen slides were stored at −70 °C until use.

Serum samples from two macaques (mac-No.1 and mac-No.2) at pre-immunization (D0) and 15 d.p.i (D15) were two-fold serially diluted from 1:50 to 1:3200 and applied onto both antigen-positive and -negative slides. The slides were incubated under humidified conditions at 37 °C for 1 h. After the slides were washed with PBS(-), the cells on the slides were incubated with Alexa Fluor 488-conjugated goat anti-human IgM antibody (Thermo Fisher Scientific) at a dilution of 1:200 at 37 °C for 1 h. After another wash with PBS(-), the slides were examined for staining patterns under fluorescent microscopy, Eclipse TE200 (Nikon, Tokyo, Japan). The D15 serum sample from mac-No.1 (mac-No.1-D15) showed immunofluorescent staining as a cytoplasmic granular pattern (Fig. 1A, B), whereas mac-No.2-D15 and D0 serum samples of mac-No.1 (Fig. 1C) and -No.2 demonstrated no specific staining. The IFAT antibody titers of tested samples were recorded as the reciprocals of the highest dilutions producing positive staining; 200 and < 50 for mac-No.1-D15 and mac-No.2-D15, respectively. Although fluorescence intensities were higher in HeLa 229 cells with transient NiV-N expression for mac-No.1-D15 when observed by eye (data not shown), the IFAT antibody titers obtained using HeLa 229 cells with transient and stable NiV-N expression were identical.

Subsequently, the reactivity of the assay was evaluated using serum samples of human Nipah patients collected during the outbreak in the Philippines in 2014 (Ching et al., 2015). These included seven seropositive samples obtained as sequential collections from three patients, together with three samples from antibody-negative cases. The immune status of samples was confirmed previously for IgM antibody by NiV capture ELISA and neutralizing antibody by two NiV SNT formats (infectious NiV SNT and NiV-pseudotyped VSV SNT). When tested in the NiV IFAT, all the confirmed NiV patient serum samples showed a granular staining pattern, whereas non-Nipah patient samples gave no specific staining (representative data are shown in Fig. 1D–F). In all of these serum samples, the IgM antibody titers determined by IFAT were well correlated with the reactivity levels obtained by IgM capture ELISA for initial diagnosis conducted at the Australian Animal Health Laboratory during the outbreak (Ching et al., 2015) (Table 1). To further investigate the reactivity against human samples not related to a NiV outbreak, 90 human serum samples collected from The Malaysian Cohort (TMC) project (Jamal et al., 2015), the antibody status of which had been confirmed as negative by IgM capture ELISA and pseudotyped VSV-based SNT, were tested by NiV IFAT. All samples showed no

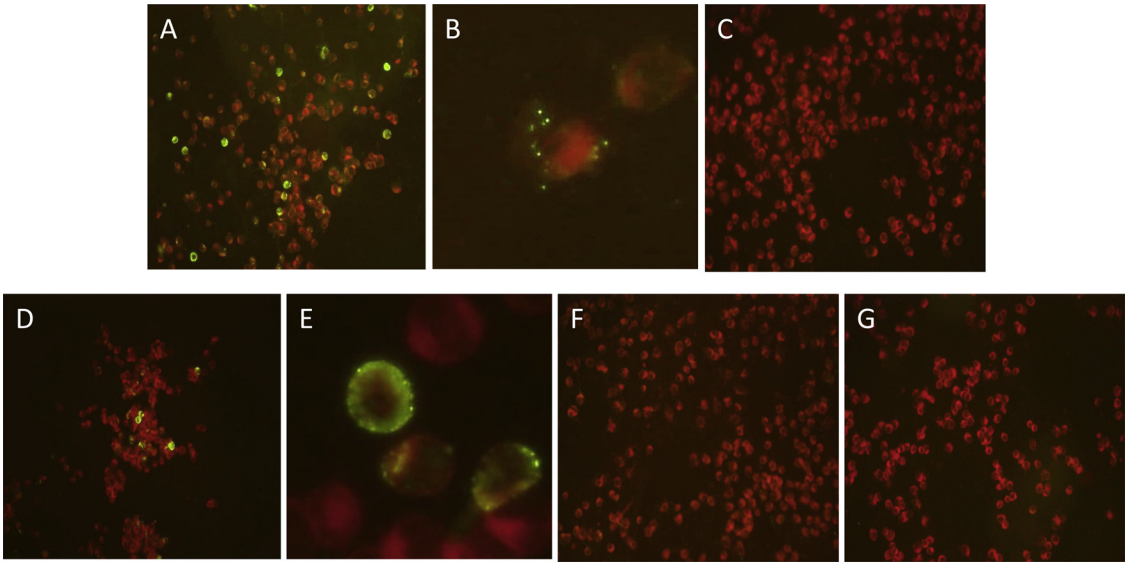

Fig. 1. IFAT using HeLa 229 cells with stable NiV-N expression stained with anti-NiV-N IgM-positive/negative macaque (A–C) and human (D–G) serum samples. (A) 1:50-diluted positive macaque serum (mac-No.1-D15), magnification ×200; (B) ×400; (C) 1:50-diluted negative macaque serum (mac-No.1-D0), magnification ×200; (D) 1:100-diluted human Nipah patient serum, magnification ×200; (E) ×400; (F) 1:100-diluted non-Nipah patient human serum, magnification ×200; (G) 1:100-diluted unexposed human serum, magnification ×200. NiV-N antigens were visualized using goat-anti human IgM antibody conjugated to Alexa Fluor 488, yielding green fluorescent staining. Cells were counterstained in red using 0.002% Evans blue.

Table 1
Detection of NiV IgM antibodies in IgM-ELISA, serum neutralization test (SNT) and IFAT using sera collected from 3 patients during a Nipah disease outbreak in the Philippines, 2014.

Patient	Onset of clinical signs (in 2014)	Date of sample collection (in 2014)	IgM-ELISA Ratio[a]	NiV SNT titer[b]	NiV SNT titer[c]	IFAT IgM titer
1	Apr 7	Apr 12	11.8	Neg	1:150	1:800
		May 11	8.5	1:80	1:1,200	1:400
		May 22	6.5	1:40	1:950	1:200–400
2	Apr 7	Apr 15	12.9	1:10	1:200	1:1600–3200
		May 8	11.3	1:80	1:2,600	1:200–400
		May 21	9.1	1:20	1:800	1:100–200
3	Apr 2	May 21	5	1:40	1:420	1:50–100

[a] The data of IgM-ELISA conducted by AAHL was reproduced from Ching et al. (2015). OD values were transformed as ratios relative to OD for highly diluted positive control serum; ratios of greater than 2.0 were regarded as significantly positive.

[b] The data of NiV SNT (using infectious NiV) conducted by AAHL was reproduced from Ching et al. (2015).

[c] The data of NiV SNT (using pseudotyped VSV) conducted by National Institute of Infectious Diseases, Japan (NIID) was reproduced from Ching et al. (2015).

specific staining at a dilution of 1:100 (a representative image is shown in Fig. 1G), giving a point estimate of 100% diagnostic specificity (95% CI, 96–100%).

To investigate the analytical sensitivity of IgM IFAT, the reactivity of two-fold serially diluted macaque serum (mac-No.1-D15) was compared using IgM IFAT and IgM capture ELISA. The lower limits of detection were 1:200 (IgM IFAT) and 1:800 (IgM capture ELISA, with cut-off value set at OD_{405} = 0.2) (Fig. 2). Although the IgM IFAT was less sensitive than the IgM capture ELISA, in practice there is often a four to eight fold difference in the applied serum dilution between IFAT and IgM capture ELISA systems (e.g. Yu et al., 2006). This procedural requirement off-sets the observed differences in analytical sensitivity between IFAT and IgM capture ELISA.

Finally, we evaluated the cross reactivity of this IFAT with other henipaviruses. Due to the difficulty in obtaining IgM-positive primate serum samples against other henipaviruses, hyperimmune rabbit antisera (IgG-positive) were used for this purpose. NiV-N stably-expressing slides were incubated with rabbit anti-NiV-N or anti-HeV-N sera and detected using anti-rabbit IgG-FITC. As shown in Suppl. Fig 4A and B, similar staining patterns were observed between anti-NiV-N and anti-HeV-N sera. Furthermore, anti-NiV-N serum was incubated with HeV-N expressing cells and Cedar virus (CedV)-N expressing cells, which were generated by a different method from NiV-N expressing slides. Briefly, HeLa 229 cells spread on 96-well plates were transfected with HeV-N expressing vector (HeV-N/pCAG-MCS2-FOS) and CedV-N expressing vector (CedV-N/pcDNA3.1/myc-His) using Fugene HD Transfection Reagent. At 2 days after transfection, the plates were fixed with 80% acetone at −30 °C for 30 min. After incubation with anti-NiV-N serum, the plates were detected with anti-rabbit IgG-FITC. As shown in Suppl. Fig 4C and D, rabbit anti-NiV-N serum cross-reacted with HeV-N, but not with CedV-N. Taken together, considering that the cross reactivity between NiV-N and HeV-N has been well established, it is reasonable to suppose that this IFAT employing NiV-N expressing cells can be used to detect anti-HeV-N IgM in serum of other animal species, including primates. Regarding the cross-reactivity with other novel henipaviruses, available resources were limited (e.g. viral gene and antiserum) in this study, and so further investigations are required in the future.

In the present study, an IFAT for detecting anti-NiV-N IgM was established utilizing HeLa 229 cells with either transient or stable expression of NiV-N. The cells expressed NiV-N as granular aggregate forms in the cytoplasm, which is typically observed in a wide range of paramyxoviruses-infected cells. The characteristic staining pattern in the IFAT, together with the fixed ratio (1:3) between fluorescent/non-fluorescent cells, provides additional useful criteria over the conventional ELISA system for differentiating specific antibody binding from unrelated non-specific reactions, which otherwise might be represented as false positives. The NiV IFAT can also be adapted for detection of virus-specific IgG antibodies in human serum samples using appropriate FITC-conjugated reagents, as shown in Suppl. Fig 4. However, there are various diagnostic methodologies that have already been developed for detecting anti-NiV IgG; tests, such as the NiV-pseudotyped VSV-based SNT (Kaku et al., 2012) are valuable in both screening and confirmatory applications. Thus, the NiV IFAT is most applicable for laboratory investigations in the early disease stage, as the sensitive detection of

A

Dilution	X50	X100	X200	X400	X800	X1600	X3200
ELISA OD_{405}	0.714	0.555	0.457	0.341	0.233	0.149	0.024
IgM IFAT	+	+	+	-	-	-	-

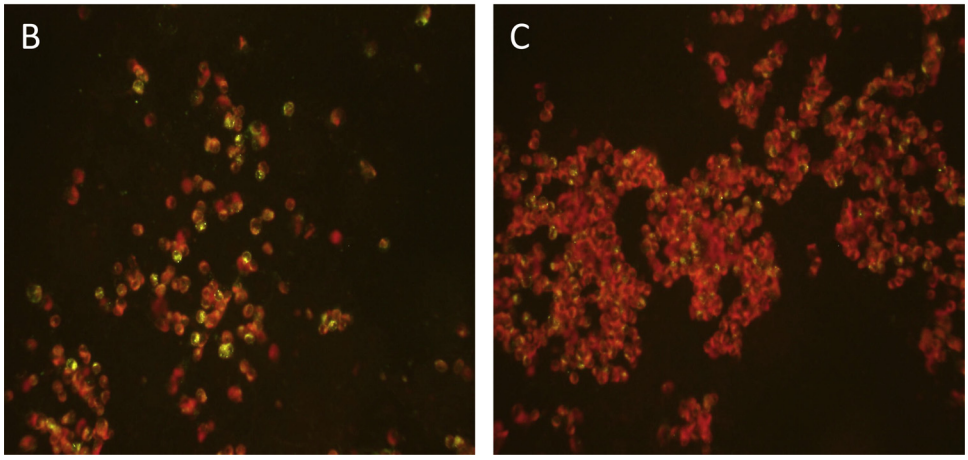

Fig. 2. Comparison of detection limit between IgM IFAT and IgM capture ELISA using macaque serum (mac-No.1-D15).
The reactivity of two-fold serially diluted macaque serum (mac-No.1-D15) in the IgM IFAT and IgM capture ELISA are compared in the table (A). Fluorescent microscopic images of IgM IFAT: (B) 1:50-dilited serum, and (C) 1:100-diluted serum. Magnification ×200. NiV-N antigens were detected by Alexa Fluor 488-conjugated goat-anti-human IgM antibody and cells were counterstained in red using 0.002% Evans blue.

specific antiviral IgM is a highly useful diagnostic marker for recent infection, particularly in combination with agent detection by molecular approaches. In the present study, the IFAT antigens were prepared on 14-well glass slides, which could be stored stably for several years at −80 °C. The IFAT antigen slides can be used immediately after being removed from a freezer and thawing, which enhances the capacity to mount a rapid diagnostic response. In on-going batch production of larger numbers IFAT antigen slides, the use of HeLa 229 cells stably expressing NiV-N has practical advantages for quality and robustness; this is regarded as preferable to use of HeLa 229 cells with transient expression of NiV-N, albeit with higher intensity of fluorescence. As noted, IgM antibody titers were identical using either preparation when measured in the NiV IFAT. The IFAT antigen slides prepared using HeLa 229 cells with stable expression of NiV-N are suitable for distribution to regional laboratories in NiV-affected countries. Additionally, the test was developed and evaluated using anti-NiV-N IgM-positive macaque sera due to a difficulty in obtaining sufficient amounts of anti-NiV IgM-positive control human serum. This approach could be applied to maintain characterized positive controls and/or proficiency testing materials for NiV serology and other infectious diseases with infrequent epizootic spread to humans.

Conflicts of interest

None

Ethical policy: human subjects

Evaluation of IFAT using human serum samples collected by the TMC project was approved by the Ethics Committee of the National Institute of Infectious Diseases under approval number 619. The Universiti Kebangsaan Malaysia Research Ethics Committee granted the ethical approval for TMC for the use of human samples in this study. A material transfer agreement was signed between the National Institute of Infectious Diseases and UKM Medical Molecular Biology Institute for the transfer and usage of samples for this study.

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