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Development of Nipah virus-specific IgM & IgG ELISA for screening human serum samples

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***Background & objectives*: Nipah virus (NiV) is a zoonotic paramyxovirus that causes fatal encephalitis in humans. Enzyme Linked Immunosorbent Assay (ELISA) is a safe, sensitive, specific, and affordable diagnostic tool that can be used during screening of large-scale epidemiological investigations. Development and evaluation of IgM and IgG ELISA for screening serum samples of NiV suspected cases would also help in planning public health interventions.**

***Methods*: An IgM capture (MAC) ELISA and an indirect IgG ELISA were developed using NiV antigen to detect IgM and IgG antibodies against NiV in human sera. The sensitivity, specificity, and cross-reactivity of the assays were evaluated using NiV IgM, IgG positive, negative human sera and measles, mumps, rubella, Crimean-Congo haemorrhagic fever, Kyasanur forest disease IgM, IgG positive sera, respectively.**

***Results*: The developed anti-NiV IgM and IgG ELISAs have shown specificity of 99.28 per cent and sensitivity of 100 per cent compared to reference test from Centers for Disease Control and Prevention, USA. Assays demonstrated negative predictive value of 100 per cent and positive predictive value as 90 and 93.94 per cent for anti-Nipah IgM ELISA and IgG ELISA respectively with test accuracy of 99.33 per cent.**

***Interpretation & conclusions*: Timely diagnosis of NiV is crucial for the management of cases, which could prevent further spread of infection in the community. IgM ELISA can be used as primary diagnostic tool followed by polymerase chain reaction. These assays have advantages of its applicability during outbreak investigations and surveillance activities at hospital or onsite laboratories with basic biosafety practices.**

Key words ELISA - human - IgG - IgM - Nipah virus

Nipah virus (NiV) has been recognized as a highly pathogenic virus due to high mortality in humans[1,2]. Since the first detection of NiV in Malaysia during 1999, sporadic outbreaks have been reported from Singapore, Bangladesh, Philippines and India[3-12]. The virus can be transmitted directly from bats, pigs to humans, by ingestion of contaminated food and contact with body fluids of infected human[13]. India

experienced five episodes of NiV outbreaks from West Bengal (2001, 2007) and Kerala (2018, 2019, 2021)[14].

Lesson learnt from COVID-19 pandemic indicated that laboratory diagnosis and active surveillance of potentially risk-prone areas is of utmost importance[14]. During the year 2018-2019, the World Health Organization (WHO) has come up with the Research and Development Roadmap for NiV[15]. It focuses primarily on the development of rapid, sensitive, specific and validated tests and control panels.

The increasing awareness and threat of NiV have boosted the development and commercialization of the molecular and serological diagnostic assays. Active NiV infection is mainly screened by detection of NiV RNA, NiV-specific IgM or NiV antigen (Ag). Nipah outbreaks have been generally reported from remote areas where the infrastructure and facility for molecular diagnosis are rarely available.

Under such circumstances, the serological assays such as IgM and IgG ELISA can provide appropriate alternative and rapid results. The main advantages of serological assays for NiV are low cost and easy-to-use format which can be easily established in field their areas, remote settings and tertiary health care.

With the development of advanced and effective diagnostic kits, NiV affected cases would be identified in a timely manner, which could help to curb further transmission. The availability of validated serological tests would also be useful for serosurveilance activities.

As yet anti-Nipah ELISA reagents developed by the Centers for Disease Control and Prevention (CDC), United States of America, are used for serological confirmation of NiV in India[9]. In order to increase indigenous capacity, we developed reliable, specific and sensitive IgM and IgG ELISA assays for NiV. Here, we describe the development process, standardization and evaluation results of the same.

Material & Methods

The present study was carried out in the Maximum Containment Facility of Indian Council of Medical Research (ICMR)-National Institute of Virology (NIV), Pune, Maharashtra, India. The study was approved by the Institutional Human Ethics Committee (IHEC no. NIV/IEC/June/2019/D-14) and Institutional Animal Ethics Committee (IAEC no. IAEC/2019/MCL/09/4/01/2019).

Human clinical specimens: Serum samples of two NiV survivors of 2018 Nipah outbreak in Kerala referred to ICMR-NIV, Pune, were used as positive control. Besides this, Nipah negative human serum samples (n=79) collected during 2018-2019 NiV outbreaks in Kerala were used as negative control. Positive (n=10) and negative serum samples (n=100) each for Kyasanur forest disease (KFD) and Crimean-Congo haemorrhagic fever (CCHF) collected during different outbreaks were also used for the validation of Nipah ELISA.

Preparation of NiV antigen: Nipah virus was propagated in Vero CCL-81 cells using NiV strain (TCID 50 $10^{6.72}$/ml) (NCBI number MH523642.1). All the procedures were carried out in Biosafety level-4 laboratory of the ICMR-NIV Pune. The virus stock was gamma irradiated at 24 kGy and concentrated using 30 KDa membrane. Similar method was followed for the production of negative control antigen using uninfected Vero CCL-81 cells.

Preparation of polyclonal antibodies against NiV: Polyclonal antibodies were raised against NiV by administration of two doses of gamma inactivated NiV ($10^{6.72}$/ml) by subcutaneous route, 14 days apart followed by two i/p (intra peritoneal) doses at seven days interval in BALB/c (n=15) mice. Freund's Complete Adjuvant and Freund's incomplete adjuvant were used in the ratio of 1:1 ratio with the virus for immunization of mice using subcutaneous route[16].

Development of anti-Nipah human IgM ELISA: MaxiSorp plates (Nunc, ThermoFisher, USA) were coated with anti-human IgM antibodies (SAB 3700778, Sigma, USA) [1:500 carbonate buffer (0.025M, *p*H 9.2)] overnight at 4°C. Testing procedures were followed as described earlier[17]. NiV infected cell lysate was used as positive antigen 2 µg/well and normal Vero CCL-81 cell lysate as control antigen. Checkerboard titration of dilutions for samples, anti-mouse IgG HRP antibodies, anti-human IgG HRP antibodies were performed for standardization of the assay. Sample with average OD value of ≥0.2 and P/N (positive/negative) ratio of ≥1.5 was considered as positive else it was considered as negative.

Development of anti-Nipah human IgG ELISA: An indirect IgG ELISA was developed for the detection of anti-Nipah IgG antibodies using human serum samples as described earlier[18]. Inactivated NiV antigen (2 µg/well), uninfected Vero CCL-81 cell lysate was used for coating the plates in carbonate buffer

(*p*H 9.2, 0.025 M). Testing procedures were followed as described earlier[18]. Sample with average OD value of ≥0.2 and the P/N ratio of ≥1.5 were considered as positive.

Determination of cut-off values and cross reactivity: A total of 297 human serum samples (IgM antibody: 18 positive and 279 negative) determined by reference IgM ELISA were tested by indigenously developed IgM ELISA. Besides this, 310 serum samples (IgG antibody: 31 positive and 279 negative) determined by reference IgG ELISA were further tested by indigenously developed IgG ELISA (Table). Receiver operating characteristic (ROC) analysis was performed as described earlier[18] using easy ROC: a web tool for ROC curve analysis (ver. 1.3.1). This interpretation was based on non-parametric Mann-Whitney U statistics that was used in calculating area under the curve (AUC).

A panel of ten serum samples each positive for IgM and IgG antibodies against measles, mumps, rubella, CCHF, KFD were used for the determination of cross-reactivity of the assay. The cross-reactivity against the henipah virus could not be determined because of the unavailability of the henipah positive serum samples.

Inter laboratory evaluation of the assay: The inter-assay variability was assessed by testing two positive and eight negative control serum samples in three different laboratories. Assays were performed in replicates and used to determine the coefficient of variation (CV).

Results

Anti-NiV human IgM and IgG ELISA for screening human serum samples (Fig. 1) were developed and evaluated. The calculated threshold cutoff for the ELISA was OD more than the average OD of negative control +0.2 and the sample to negative control ratio >1.5, differentiating between the presence and absence of anti-NiV-IgM or IgG antibodies in the samples.

The results were found to be concordant with reference ELISAs. ROC analysis demonstrated the AUC of 0.80 [95% confidence interval (CI) 0.62-0.98 with P=0.001] and 0.95 (95% CI 0.90-1 with P<0.001) for anti-NiV IgM and IgG ELISA, respectively (Fig. 2A and B).

Out of the 297 samples screened by anti-NiV human IgM ELISA, 18 were positive (OD values ranged from 0.4 to 0.9) and 279 were negative (OD values ranging from 0.06 to 0.25) by both indigenous and reference standard test. Two samples tested positive by the indigenous anti-NiV IgM ELISA but tested negative by the reference anti-NiV IgM ELISA (Table).

Table. Comparison of in-house developed anti-Nipah IgM and IgG ELISA with reference test from CDC

Anti-Nipah ELISA by ICMR-NiV kit	Reference test		
	Positive	Negative	Total number of samples tested
IgM			
Positive	18	2	20
Negative	-	277	277
Total	18	279	297
IgG			
Positive	31	2	33
Negative	-	277	277
Total	31	279	310

CDC, Centers for Disease Control & Prevention; NiV, Nipah virus; IgG, immunoglobulin G; IgM, immunoglobulin M; ICMR, Indian Council of Medical Research; ELISA, enzyme-linked immunosorbent assay

Out of the 310 samples screened by indirect anti-NiV human IgG ELISA, 31 were positive (OD values ranged from 0.42 to 1.4) and 277 (OD values ranging from 0.06 to 0.28) were negative by both indigenous and gold standard test. Two samples tested positive by the indigenous anti-NiV IgG ELISA but tested negative by the reference anti-NiV IgG ELISA (Table).

Diagnostic sensitivity and specificity: Anti-NiV human IgM ELISA (CDC, USA) was used as a reference test for evaluating the developed assays. A specificity of 99.28 per cent and sensitivity of 100 per cent were observed for anti-NiV IgM and IgG assay, respectively. Negative predictive value was 100 per cent and positive predictive values for the assays were calculated as 90 and 93.94 per cent with test accuracy of 99.33 per cent for anti-Nipah IgM ELISA and Nipah IgG ELISA with 95 per cent CI (97.59%-99.92%) .

Interlaboratory evaluation of the assay: Interlaboratory comparison of indigenously developed anti-NiV human IgM and IgG was carried out at three different laboratories using a panel of 10 coded samples, ready-to-use kits were supplied to these laboratories. The results from the three laboratories were in 100 per cent concordance with the results of ICMR–NIV, Pune (% CV for inter-assay and intra-assay were 4.15% and

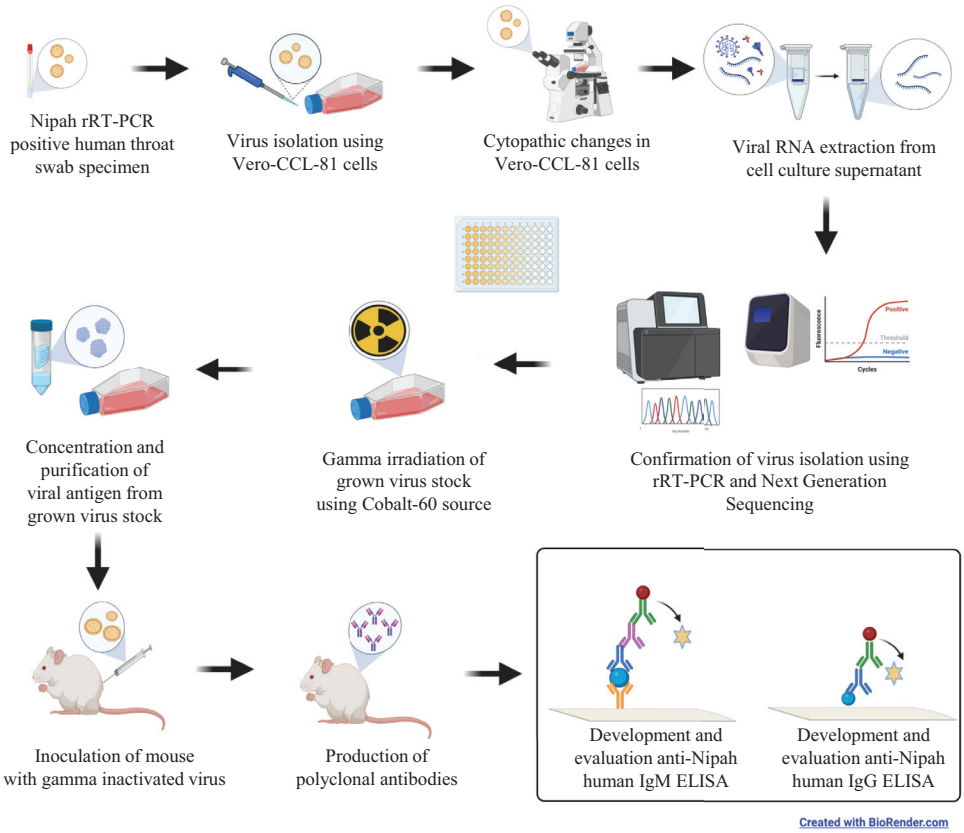

Fig. 1. Workflow for the development of anti-Nipah IgM and IgG ELISA.

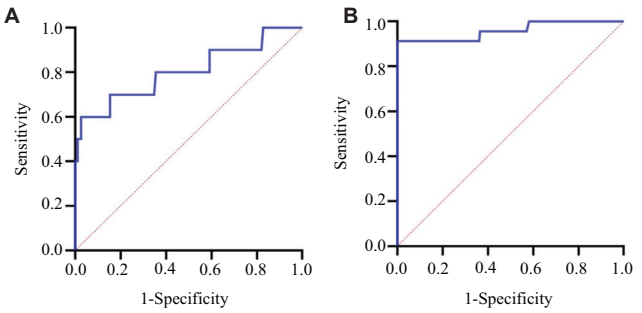

Fig. 2. Receiver operating characteristic (ROC) analysis showing sensitivity *vs*. specificity of (**A**) Anti-Nipah human IgM, and (**B**) Anti-Nipah human IgG ELISA.

5.5%, respectively). No cross-reactivity against the positive control panels of other viruses was observed in both the assays.

Discussion

The recent pandemic of SARS-CoV-2 posed serious challenged to the public health systems of the affected countries[19]. Availability of validated diagnostic tests helped during this pandemic to determine the disease burden and conduct surveillance activites. With ready human-to-human transmission, NiV disease has a potential to cause epidemic or pandemic in the future. Availability of low cost validated rapid diagnostic assays that are sensitive, and user-friendly will help to manage such outbreak situations. At present, a few NiV glycoprotein-based ELISAs are available commercially. Disadvantage of peptide-based ELISA includes production of the whole recombinant protein and its evaluation for epitope mapping.

Recently, the WHO has developed and published target product profile (TPP) for NiV diagnostics[20]. It has enlisted some important points for the development of new diagnostic tests that would be able to detect and confirm active NiV infection. In an outbreak scenario, point-of-care test would be feasible in field or hospital settings for detection of NiV and confirmation could subsequently be done at reference laboratory[21]. The developed TPP guideline suggests that newly developed assays should meet certain requirements. The criteria for screening test are detection of NiV specific IgM/NiV antigen (ELISA or rapid diagnostic tests) validated with at least NiV Bangladesh strain (NiV-B) or NiV Malaysian (NiV-M) strain. The assay

should have the required sensitivity of >90-95 per cent, specificity of >80-90 per cent, minimally cross-reactive to other pathogens, result generation time of less than four hours and should cover all biosafety aspects related to infectious specimen[22]. However, there are major hurdles related to the evaluation of these NiV diagnostics such as access to the clinical specimens from outbreaks, availability of NiV strain, and access to the repositories of international reference standards for evaluation.

Differential diagnosis of NiV infection is often difficult clinically as it mimics other diseases with febrile illness. The laboratory diagnosis of NiV is mainly carried out using the techniques such as polymerase chain reaction (PCR), sequencing, ELISA, plaque reduction neutralization test (PRNT), indirect fluorescent antibody (IFA) assay, histopathology and virus isolation[23]. PCR is considered as the rapid diagnostic tool to detect acute NiV infection. However, IgM ELISA could serve as an important tool for diagnosis of acute cases in resources limited settings.

Serological assays have also been widely recommended for surveillance of emerging and re-emerging viruses. The present study was as the development and evaluation of sensitive and specific IgM and IgG ELISA for screening of NiV suspected human samples. These assays were found to be specific for the detection of antibodies against NiV. Limitations of the assays included large-scale propagation of the NiV in containment facility. IgM ELISA can detect recent infection (≥4 days post-infection) and can be easily utilized in remote areas or field settings[24]. Surveillance studies using IgG ELISA can help in determining the prevalence and predict emergence of NiV in a particular population and location. During 2018-2019, NiV outbreak in Kerala State could be timely contained because of the early detection of NiV as the causative agent[10,11]. This underlines the importance of reliable diagnostics for prevention and control of future NiV outbreaks.

The validated indigenous anti-NiV human IgM and IgG ELISA would be useful for detection of anti-NiV IgM in human serum samples. These assays do not require specialized infrastructure and provide complete end-to-end solution for disease diagnosis and surveillance activities in rural areas or field settings. Early warning is very critical for intervention and eradication of any highly infectious disease agents[9,10]. Furthermore, the assays we developed, can be supplied to the countries in need under the WHO collaborative program on NiV. With proven ability to work even at Primary Health Centers, these easy-to-use assays will assist in NiV diagnosis, surveillance and control as well as management of NiV infection. Furthermore, these assasys would also be helpful in mapping the distribution of NiV in human and animal population.

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***Conflicts of Interest*:** None.

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