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Standardization & validation of Truenat™ point-of-care test for rapid diagnosis of Nipah

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Nipah virus (NiV) is one of the re-emerging viruses, from the *Paramyxoviridae* family, mainly affecting the Southeast Asia region[1]. *Pteropus* species of bats are known natural reservoirs of NiV. Spillover of NiV can occur among the resident human population or visitors, in places where these bats roost. Humans can also get the infection from the pigs, the intermediate host for the transmission of NiV, as observed during the outbreak of 1998-99 in Malaysia and Singapore[2-4]. Human-to-human transmission has been reported during the outbreaks of Nipah in Bangladesh and India[5-10]. India has witnessed many outbreaks of Nipah in the States of West Bengal and Kerala with a high mortality 68-100 per cent during the period of 2001-19. The sudden emergence of NiV from Kerala affected the public health system because of lack of preparedness to such a health emergency. However, the NiV spread was successfully contained during the 2018 outbreak and subsequently the preparedness helped in quickly containing the infection in 2019[11],with no secondary cases reported.

The major hurdles in containing the NiV outbreaks include, the unavailability of containment facilities for quick diagnosis, delay in diagnosis with respect to sample transportation to the reference laboratory and time to positivity (TTP). Timely detection of NiV can play an important role in the patient management and help curb further spread of infection. Diagnosis of NiV is primarily carried out using nucleic acid amplification tests, serologic tests and virus isolation[12]. Real-time reverse transcription polymerase chain reaction (RT-PCR) is considered as an accurate and quick method for NiV diagnosis[7,13]. However, it requires well-defined infrastructure, expensive reagents and equipment which are generally not available in resource-poor settings or remote areas. Also there are issues related to biosafety at the time of collection and transportation of infectious specimens to the reference laboratory. Hence, it is imperative to develop point-of-care diagnostic tests for rapid diagnosis of NiV that can be used in the field/remote areas affected with NiV.

Considering this, Molbio Diagnostics Pvt. Ltd., Goa and ICMR-National Institute of Virology (NIV), Pune jointly developed the NiV point-of-care (PoC) test which was validated by ICMR-National Institute of Virology, Pune. The study was approved by the Institutional Project Review Committee, and Institutional Biosafety Committee, Institutional Human Ethics Committee (NIV/IEC/June/2019/D-19, ICMR-NIV, Pune.

The developed system is a portable, lightweight, battery-operated PCR analyzer, namely Truenat™ Nipah PoC system, a Trueprep® AUTO universal cartridge-based Sample Prep kit and a device to aid the sample processing at the PoC. The kit employs a combination of reagents that inactivate the clinical specimens during the nucleic acid extraction[14,15]. This method allows the extraction of inbuilt NiV internal-positive control along with the extraction of the suspected sample. The system and method for the inactivation and detection of NiV and its sensitivity, specificity, cross-reactivity and robustness were validated at ICMR-NIV, Pune. The performance of Trueprep® AUTO system[14-16] and Truenat™ Nipah PoC test was compared with standardized and validated methods such as MagMAX machine-based RNA extraction (Applied Biosytems, USA) and TaqMan-based NiV real-time RT-PCR[13] (Applied Biosytems, USA).

The efficacy of the Trueprep®AUTO lysis buffer for inactivation of NiV was confirmed by treating one part of live NiV cell culture supernatant (100 µl, $TCID_{50} 10^{6.72}$) with five parts of the lysis buffer

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(500 µl). The pellet obtained by the ultracentrifugation of the above mixture was infected on Vero CCL-81 cells and observed for the presence of cytopathic effect (CPE) till seven post-infection days. The multiplication of the virus in cells was monitored by detection of NiV RNA using real-time RT-PCR[13]. The inactivation of NiV was evident by the absence of Ct value and CPE in Vero CCL-81 cells at the first and second passage.

Eight NiV-positive human clinical specimens (Ct value 28-35), 40 human specimens spiked with $1\times10^{5.5}$ $TCID_{50}$ NiV cell culture supernatant (Ct value range 25-30) and NiV negative human clinical specimens (n=40), NiV negative throat swabs (n=10), rectal swabs (n=10) and negative organ tissues (n=80) obtained from *Pteropus* bats during the 2018 NiV outbreak surveillance were used. Besides this, positive and negative organ tissues harvested from infected as well as control hamsters (n=26) and mice (n=14) were also included in the study (Table I). Due to the low number of Nipah-positive cases, negative human specimens referred from the NiV outbreak affected area of Kerala State were spiked with NiV cell culture supernatant which served as positive samples. NiV *in vitro* transcribed (IVT) RNA was used as the positive control.

One human urine sample and one human cerebrospinal fluid sample, both detected at a Ct value of 38 by the TaqMan real-time RT-PCR (cut off value 39),were not detected by the Truenat™ Nipah PoC test (cutoff value 39) and were considered a false negative. The results of all the other known positive and negative specimens were concordant with the Truenat™ Nipah PoC test (Table I).The sensitivity and specificity of the Truenat™ Nipah PoC test as found to be 97 per cent (95% CI, 90-100%) and 100 per cent (95% CI, 98-100%), respectively.

The Truenat™ Nipah PoC test was found to be specific for the detection of NiV, and no cross-reactivity was observed with characterized positive control panels obtained from ICMR-NIV, Pune. It consisted of 35 human specimens positive for viruses, namely *Paramyxoviridae*-Measles virus (10), *Togaviridae*-Rubella virus (10), *Orthomyoviridae*-influenza (5), *Flaviviridae*-dengue virus (5), *Alphaviridae* - chikungunya virus (5).

RNA was extracted from 100 µl of serial ten-fold dilutions (10^{-1}-10^{-10}) of NiV cell culture supernatant and serial dilutions of NiV IVT RNA (10^{-1}–10^{-14}) and human and animal specimens using Trueprep® AUTO system and MagMAX system. The eluted RNAs were tested with Truenat™ NiV PoC test and NiV real-time RT-PCR assay for determining the limit of detection (LoD). RNA extracted from the cell culture supernatants of NIV displayed the same level of detection, *i.e*. till $10^{1.5}$ $TCID_{50}$/ml, with a difference in Ct values between the MagMAX (32.2) and Trueprep® AUTO RNA extraction method (29.4). LoD for both the methods was found to be up to 10 copies/ml for NiV IVT RNA (Table II).

The assessment of the Truenat™ NiV PCR using 10-fold serial dilutions of NiV cell culture supernatants with three replicates at each dilution (undiluted, 10^{-1} to 10^{-4}) confirmed the precision of the assay. Repeatability and reproducibility of the assay were confirmed by testing three replicates with similar operating conditions and different laboratory personnel on three consecutive days. The data analyzed using standard deviation and coefficient of variation (0.54 and 2.40) confirmed the repeatability and reproducibility of the assay.

Further, the Trueprep® AUTO Universal Cartridge Based SamplePrep Device and the Truenat™ Nipah PoC system were installed at Institute of Epidemiology Disease Control and Research, Dhaka, Bangladesh, and samples were checked for system performance. Out of ten known positive human throat swab samples (Ct 25-35), nine samples were tested positive by Truenat™ NiV PoC test, and the results of ten negative samples were in concordance with the reference test. The sample which was false negative had a Ct value of 39 with TaqMan real-time RT-PCR (Table I).

Both the systems were also installed at Government Medical College, Ernakulam, Kerala, during 2019 NiV outbreak and two field sites in Punjab under NiV surveillance in bats. A total of 120 human clinical specimens and throat swabs (n=25) and rectal swabs (n=25) from bats were tested at the respective field sites. One known human-positive sample with a Ct value of 39 tested by TaqMan real-time RT-PCR was found to be false negative by Truenat™ NiV PoC test when tested at GMC, Ernakulam, Kerala State. While, the results of all human and bat negative specimens were in concordance with TaqMan real-time RT-PCR at both the field settings (Table I).

This study validated Truenat™ NiV PoC test, which aids in the detection of NiV from human and bat specimens in the field settings. Truenat™ NiV PoC test was found to be specific and sensitive with comparable LoD to TaqMan real-time RT-PCR.

Table I. Consolidated table illustrating the total number of specimens tested for internal and external laboratory validation of Truenat™ Nipah PoC test

Species	Samples	Urine	Serum	Throat swabs	Blood	CSF	Brain	Lung	Liver	Spleen	Kidney	Heart	Intestine	Rectal swabs	Total
Validation at ICMR-NIV, Pune. Concordance by Truenat™ Nipah PoC test/known samples tested by TaqMan real-time RT-PCR (cutoff Ct value=39)															
Human	Positive	11/12	10/10	10/10	10/10	0/1	1/1	1/1	1/1	1/1	1/1	1/1	0	0	46/48
	Negative	10/10	10/10	10/10	10/10	0	0	0	0	0	0	0	0	0	40/40
	Total	21/22	20	20	20	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0	0	86/88
Bat	Positive	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Negative	0	10/10	10/10	0	0	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	100/100
	Total	0	10/10	10/10	0	0	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	100/100
Hamster	Positive	0	0	0	0	0	2/2	2/2	1/1	2/2	2/2	2/2	2/2	0	13
	Negative	0	0	0	0	0	2/2	2/2	1/1	2/2	2/2	2/2	2/2	0	13
	Total	0	0	0	0	0	4/4	4/4	2/2	4/4	4/4	4/4	4/4	0	26
Mice	Positive	0	0	0	0	0	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0	7
	Negative	0	0	0	0	0	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0	7
	Total	0	0	0	0	0	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0	14
External validation at IEDCR, Dhaka, Bangladesh. Concordance by Truenat™ Nipah PoC test/known samples tested by TaqMan real-time RT-PCR (cutoff Ct value=39)															
Human	Positive	0	0	9/10	0	0	0	0	0	0	0	0	0	0	9/10
	Negative	0	0	10/10	0	0	0	0	0	0	0	0	0	0	10/10
	Total	0	0	20/20	0	0	0	0	0	0	0	0	0	0	19/20
Robustness at field conditions. Concordance by Truenat™ NipahPoC test/known samples tested by TaqMan real-time RT-PCR															
GMC, Ernakulam (human)	Positive	0/1	0	0	0	0	0	0	0	0	0	0	0	0	0/1
	Negative	37/37	30/30	37/37	10/10	5/5	0	0	0	0	0	0	0	0	119/119
	Total	38/38	30/30	37/37	10/10	5/5	0	0	0	0	0	0	0	0	119/120
Field site, Chandigarh (bat)	Positive	0	0	0	0	0	0	0	0	0	0	0	0	0	0/0
	Negative	0	0	25/25	0	0	0	0	0	0	0	0	0	25/25	50/50
	Total	0	0	25/25	0	0	0	0	0	0	0	0	0	25/25	50/50

ICMR, Indian Council of Medical Research; NIV, National Institute of Virology; RT-PCR, reverse transcription polymerase chain reaction; CSF, cerebrospinal fluid; IEDCR, Institute of Epidemiology Disease Control and Research; GMC, Government Medical College

Table II. Limit of detection (LOD) based on tissue culture infectious dose$_{50}$ for Truenat™ Nipahpolymerase chain reaction as against TaqMan real-time RT-PCR using Nipah virus TCF and NiV *in vitro* transcribed RNA (copy number)

NiV details	RNA extraction method	LoD	
		TaqMan RT-PCR (gold standard)	Truenat™ Nipah PCR
Cell culture supernatant ($10^{5.5}$ TCID$_{50}$/ml stock)	MagMAX RNA extraction (gold standard)	$10^{1.5}$ TCID$_{50}$/ml	$10^{1.5}$ TCID$_{50}$/ml
	Trueprep® AUTO RNA extraction	$10^{1.5}$ TCID$_{50}$/ml	$10^{1.5}$ TCID$_{50}$/ml
NiV IVT RNA (10^6 copies stock)	-	10 copies	10 copies

RNA extraction of Nipah virus cell culture supernatant was performed using Trueprep® AUTO as well and MagMAX method. NiV, Nipah virus; IVT, *in vitro* transcribed; RT-PCR, reverse transcription-PCR; TCID, tissue culture infectious dose

Lethal nature of NiV infection makes it necessary to handle the clinical specimens of suspected cases in containment facilities only. It is noteworthy that the current NiV Truenat™ PoC system with lysis buffer which was found to completely inactivate NiV. Hence, this assay can be performed at field setting using standard personal protective equipment, *i.e*. Tyvek suit, N95 mask/respirator. TTP using Truenat™ NiV PoC test takes an hour as against two and half hours using TaqMan real-time RT-PCR. This would bring a marked change in the global fight towards control and management of NiV.

Considering the remote locations of NiV outbreaks and bat surveillance in forest settings, a user-friendly PoC system would prove to be a boon in detecting the NiV at the source. This would be specifically beneficial for the countriessuch asIndia and Bangladesh where access to the containment facility is limited.

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