



Point of care real-time polymerase chain reaction-based diagnostic for Kyasanur forest disease

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ABSTRACT

Objectives: Due to the remote forest area locations of sporadic cases and outbreaks of Kyasanur forest disease (KFD), rapid diagnosis poses a significant challenge. This study aimed to evaluate the diagnostic performance of Truenat KFD, a simple, rapid and user-friendly point-of-care test for detection of KFD and compare diagnostic accuracy with conventional real-time reverse transcription-polymerase chain reaction (RT-PCR) testing. Truenat KFD can be deployed in a field laboratory setting.

Methods: The study involved 145 clinical specimens, including human serum, monkey necropsy tissues and tick pool, to validate Truenat KFD (Molbio Diagnostics Pvt.Ltd.) for KFD diagnosis.

Results: We have optimized and validated the microchip-based Truenat KFD (Molbio Diagnostics Pvt.Ltd.) for KFD diagnosis. Point-of-care testing was highly sensitive and specific, with a detection limit of up to 10 copies of KFD viral RNA. Results were comparable with the gold-standard TaqMan and commercially available Altona RealStar AHFV / KFDV real-time RT-PCR assays. Screening results for human, monkey and tick specimens were 100% concordant across the assays.

Conclusion: Truenat KFD (Molbio Diagnostics Pvt.Ltd.) was found to be highly sensitive and specific with a significant limit of detection. This point-of-care test would be useful in rapid diagnosis of KFD in remote and/or field settings, quick patient management and control of virus spread.

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Introduction

Kyasanur forest disease (KFD) was first identified in the Kyasanur forest of Shimoga district of Karnataka state, India, during an investigation of monkey mortalities in 1957. The disease is caused by a KFD virus (KFDV) belonging to the family Flaviviridae, genus *Flavivirus* which primarily affects humans and monkeys. In nature, the virus is maintained mainly in hard ticks (*Haemaphysalis* spp.), monkeys, rodents and birds and transmitted via the bite of *Haemaphysalis* ticks and contact with monkey carcasses (Banerjee, 1990; Pattnaik, 2006). The disease is characterized by chills, frontal headache, body ache, and high fever for 5–12 days, with a case-fatality rate of 3%–5% (Mertens et al., 2005).

Since its identification in 1957 and up to 2012, sporadic cases and several outbreaks of KFD have been reported every year, especially in the 5 districts of Shimoga, Chikmagalur, Uttara Kannada, Dakshina Kannada and Udupi of Karnataka State; with cases averaging approximately 400–500 per year (Banerjee, 1990; Mehla et al., 2009; Work, 1958). From 2012, the presence of KFD has also been reported from the adjoining states, i.e., Tamil Nadu (Nilgiri region; 2012), Kerala (Wayanad, Malappuram, Palakkad and Nilambur districts; 2013–15), Goa (Sattari and Dharbandora talukas; 2015–16) and Maharashtra (Dodamarg and Sindhudurg districts; 2016). Eventually, KFD emerged as a grave public health problem spreading throughout the entire Western Ghats region of India (Mourya and Yadav, 2013; Mourya et al., 2014; Tandale et al., 2015; Murhekar et al., 2015; Patil et al., 2017; Awate et al., 2016).

During the early period of KFDV discovery, the diagnosis of suspected cases was entirely dependent on conventional techniques such as hemagglutination inhibition assay, complement fixation test and virus isolation. These techniques were laborious and time-consuming, delaying timely diagnosis of KFD and can lead to increasing disease burden. After containment laboratories (BSL-3 and BSL-4 laboratory) were built at the Indian Council of

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Medical Research-National Institute of Virology (ICMR-NIV) in Pune, quick and reliable molecular tests were developed for KFD virus identification (Mourya et al., 2012; Chaubal et al., 2018). The advent of these technologies has helped identify this disease in 5 Indian states and apply control measures.

However, these tests required well-equipped laboratory facilities and trained staff, which were limited. With the ongoing SARS-CoV-2 pandemic, 1310 (Government 532 + Private 778) real-time RT-PCR laboratories have been established across India. In addition, 1008 sites in 530 districts across India are carrying out rapid diagnostic assays developed by Molbio Diagnostics, India and ICMR (Gupta et al., 2020). These assays are Truenat Beta CoV E-gene screening, Truenat SARS-CoV-2 RdRp gene-confirmatory assay, and a combined E-gene screening and Orf1a-gene confirmatory multiplex assay for point-of-care (PoC) detection of SARS-CoV-2. During the SARS-CoV-2 pandemic, the Truelab PoC system has proved to be a comprehensive assay for rapid screening and confirmation of COVID-19 cases (Indian Council of Medical Research, 2020). Such PoC tests would benefit KFD diagnosis as disease outbreaks mainly occur in remote/rural areas with a lack of well-equipped sample handling/laboratory testing facilities. This lack of facilities delays diagnosis and increases logistics costs to reference laboratories, compromising sample integrity if samples are not transported in proper cold chain conditions. These are major obstacles in the rapid diagnosis of KFD that also increase logistics costs and delay patient management. To date, none of the available rapid, sensitive and robust KFDV detection assays could provide point-of-care testing. ICMR-NIV and Molbio Diagnostics, Goa, collaborated to develop the microchip-based Truenat KFD point-of-care test (Truenat KFD) that includes a battery-operated PCR analyser which is portable and lightweight, and a Trueprep AUTO universal cartridge-based sample pre-treatment kit and nucleic acid extraction device that aids sample processing at the PoC. In the present study, we evaluated the Truenat KFD test for the rapid diagnosis of KFD.

Methods

Truenat KFD system

The Truenat KFD sample pre-treatment kit employs a combination of reagents to inactivate the samples before extraction of purified viral nucleic acids (Truelab, 2020; Trueprep, 2020). The efficiency of complete inactivation of the clinical samples while processing at the PoC is crucial. Therefore, the inactivation confirmation and validation of the Truenat KFD system, with respect to sensitivity, specificity, cross-reactivity and robustness, was performed at ICMR-NIV, Pune.

In the present study, the performance of the Trueprep AUTO system (Molbio Diagnostics Pvt.Ltd.) for extraction of viral nucleic acid was compared with the MagMAX Viral RNA Isolation Kit (Applied Biosystems, USA) carried out per the manufacturers' protocols. The Truenat KFD was compared with the gold-standard TaqMan real-time RT-PCR assay. Both assays utilize the KFDV-specific NS-5 gene primers and probe (KFDNS Forward TGGAGCCTGGCTGAAAGAG, KFDNS Reverse TCATCCCCACTGAC-CAGCAT and KFDNS probe FAM-5'-ATGGAGAGGAGCGCCTGACCCG-3'-BHQ (Mourya et al., 2012)).

KFD virus stock

KFD virus stock (NIV12839) was prepared by propagating the virus in the Vero CCL-81 cell line in the BSL-4 laboratory of the ICMR-NIV, Pune. All standardization experiments were carried out using supernatant obtained from the KFDV culture stock with a titre of $10^{7.79}$ TCID₅₀/mL (50% tissue culture infectious dose

per mL) calculated using the Reed and Muench formula (Reed and Muench, 1938).

Truenat KFD system workflow

The Truenat KFD system (Molbio Diagnostics Pvt.Ltd.) consists of 3 components: (1) AutoPrep sample pre-treatment kit; (2) Trueprep AUTO RNA extraction system; (3) Truelab UnoDx PCR analyser system. Clinical specimens of KFDV were handled using standard personal protective equipment and practices and following biosafety level BSL-3/BSL-2 practices. An extraction volume of 50 μ L from suspected specimens (mainly human blood, serum, homogenized monkey necropsy organs, homogenized tick pools) was treated with lysis buffer for inactivation. The treated sample was transferred to the RNA extraction cartridge, which was then loaded onto the Trueprep AUTO RNA extraction system. A 150 μ L volume of RNA elute was obtained for each sample and 6 μ L added to a reagent tube with lyophilized master mix. The reaction mixture was incubated for ~30 s. The microchip was placed on the PCR analyser tray and 6 μ L of the reaction mixture added to the microchip. At the end of the 40-minute run, the cycle threshold (Ct) value and optical graph were viewed.

KFDV inactivation efficiency of TrueprepAUTO Lysis buffer

The efficacy of the lysis buffer for inactivation of KFDV was confirmed (Smither et al., 2015) by treating 1 part of live KFDV TCF 100 μ L (TCID₅₀ $10^{7.79}$ /mL) with 5 parts of the lysis buffer (500 μ L) (1:5). After the inactivation step, the tissue culture lysate was subjected to ultracentrifugation at 20 000 rpm for 90 minutes at 4 °C. The supernatant consisting of released nucleic acid was discarded. The pellet (resuspended in 1X minimal essential media) containing the non-inactivated KFDV particles, if any, was inoculated in Vero CCL-81 cells and observed for the presence of cytopathic effect for 7 post-infection days. Two blind passages were given to confirm the inactivation of the virus (Smither et al., 2015). The presence of KFDV RNA in the passaged TCF samples was checked using TaqMan real-time RT-PCR to confirm the multiplication of live virus particles.

Specimens for the diagnostic validation

Human serum, monkey necropsy tissues and tick pool specimens referred to ICMR-NIV, Pune from different districts of Karnataka, Maharashtra and Kerala states during 2017–19 were used to validate the Truenat™ KFD PoCT (Molbio Diagnostics Pvt. Ltd.). Characterised panels of human specimens confirmed by TaqMan real-time RT-PCR and specimens positive for zika, dengue, influenza, and measles, which exhibit similar/overlapping disease symptoms to KFD, were used to determine the cross-reactivity of the assay. The tissue culture-derived KFDV stock was used as the positive control.

Limit of detection of the assay

KFDV TCF (TCID₅₀ $10^{7.79}$ /mL) was serially ten-fold diluted using nuclease-free water (Sigma-Aldrich, USA). Viral RNA was extracted from each dilution using the Trueprep AUTO system and MagMAX Viral RNA Isolation Kit (Applied Biosystems, Thermo Scientific, USA) extraction method. The RNA elutes obtained from both extraction methods were simultaneously tested using the Truenat KFD and TaqMan real-time RT-PCR assay to compare sensitivity. For determining the limit of detection of the Truenat KFD (Molbio Diagnostics Pvt.Ltd.), KFDV RNA stock of known copy numbers was serially diluted from 10^7 copies of RNA to 1 copy of RNA per reaction. Due to a notable difference between the Ct values of the

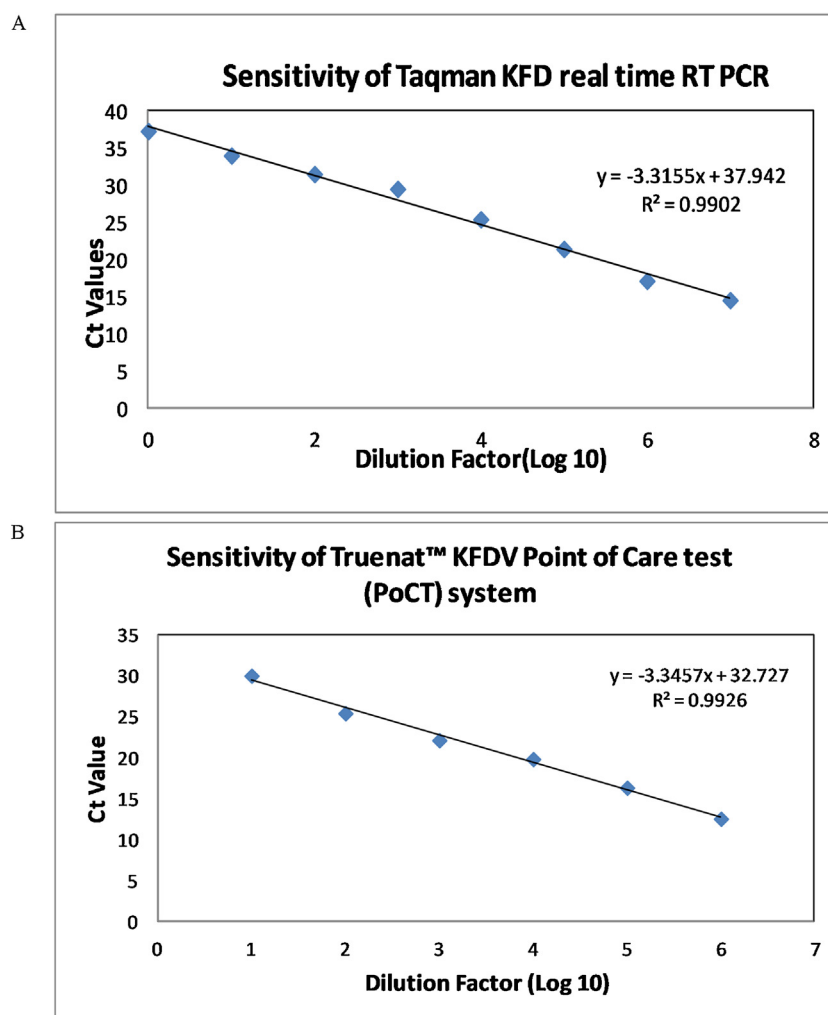


Figure 1. (A) Cycle threshold (Ct) values of serially diluted KFDV RNA demonstrating the sensitivity of TaqMan KFDV Real time RT-PCR (B) Cycle threshold (Ct) values of serially diluted KFDV RNA demonstrating the sensitivity of PoC KFDV Real time RT-PCR with R2 value of 0.99 and slope -3.34 indicating the PCR efficacy of 99.25%

Truenat and TaqMan assays, a separate standard curve was plotted for the Truenat KFD by testing the same serially diluted KFDV RNA extracted from the TCF. The copy number of KFDV RNA was calculated based on a standard curve (Figure 1).

Assessment of sensitivity and specificity

Concordance in the comparative analysis of the RNA extracted using Trueprep AUTO and MagMAX Viral RNA Isolation methods prompted us to use the MagMAX for sensitivity and specificity experiments because only 50 μ l of sample is required for this method which is suitable for clinical specimens with less volume.

After understanding the limit of detection of the Truenat KFD compared with the TaqMan real-time RT-PCR, the sensitivity of the assay was checked by comparing it with commercially available Altona RealStar AHFV/KFDV RT-PCR Kit 1.0 (Altona Diagnostics, Germany). Extracted viral RNA from KFDV TCF stock dilutions and earlier confirmed positive human ($n = 10$) serum and monkey necropsy specimens ($n = 13$), with Ct value 26–36 (as tested by the TaqMan real-time RT-PCR), were tested simultaneously by the 3 assays: Truenat KFD, TaqMan and Altona RealStar. Human serum, monkey organ necropsy and tick pool specimens negative for KFDV by TaqMan real-time RT-PCR were tested by the Truenat KFD to determine the specificity of the assay.

Cross-reactivity testing

Characterized panels consisting of human specimens positive for other viruses within the *Flaviviridae* family, namely Zika and dengue, and for viruses belonging to other families—*Alphaviridae* (chikungunya), *Togaviridae* (rubella), *Orthomyxoviridae* (influenza) and *Paramyxoviridae* (measles)—were tested to evaluate the cross-reactivity of the Truenat KFD. Specimens with other tick-borne viruses such as Crimean-Congo haemorrhagic fever virus from family *Nairoviridae* were tested.

Inter-machine variation

Inter-machine variation of the Truenat KFD PoC assay was determined on 5 different UnoDx PCR Analyzer units, using the KFDV positive control RNA extracted by two Trueprep AUTO RNA extraction systems.

Results

KFDV inactivation efficiency of TrueprepAUTO Lysis buffer

Inactivation efficiency of KFDV TCF spiked in lysis buffer was confirmed by a lack of cytopathic changes observed in the Vero

CCL-81 cells after 2 blind passages and no detection of viral RNA in the lysis buffer treated samples by TaqMan real-time RT-PCR.

Limit of detection of the assay

TaqMan real-time RT-PCR assay testing of serially diluted KFDV TCF specimens extracted by the MagMAX Viral RNA Isolation method and Trueprep AUTO methods (Molbio Diagnostics Pvt. Ltd.), detected up to 8.7 and 12.6 copies of KFDV RNA per PCR reaction, respectively. The same test with the Truenat KFD detected up to 9.5 and 14 copies of KFDV RNA per PCR reaction, for MagMax and Trueprep extracted RNA, respectively (Table-1S). This comparative analysis concluded that the Truenat KFD has a limit of detection on par with the gold-standard TaqMan real-time RT-PCR assay (Figure 1).

Sensitivity and specificity of the assay

The sensitivity of the Truenat KFD was determined using RNA extracted from the serial dilutions of KFD virus stock ($10^{7.79}$ TCID₅₀/ml). The results were comparable with the gold-standard TaqMan real-time assay and commercially available Altona RealStar AHFV/KFDV RT-PCR Kit 1.0 (Table 2S).

Furthermore, 67 KFDV positive clinical specimens, including human serum ($n = 20$), monkey organ necropsy ($n = 23$), tick pool ($n = 10$) and serum spiked with KFDV positive culture supernatant ($n = 14$) comparatively tested with these assays gave 100% concordant results across all specimens.

Figure 1 (A) Cycle threshold (Ct) values of serially diluted Kyasanur forest disease viral RNA demonstrating the sensitivity of TaqMan real-time reverse transcription-polymerase chain reaction (B) Cycle threshold (Ct) values of serially diluted Kyasanur forest disease viral RNA demonstrating the sensitivity of the Truenat KFD with an R^2 value of 0.99 and slope -3.34 indicating efficacy of 99.25%

A total of 92 referred clinical specimens, confirmed negative for KFDV by TaqMan real-time RT-PCR, including human serum ($n = 18$), monkey organ necropsy ($n = 53$) and tick pools ($n = 21$), were tested by the Truenat KFD to determine the specificity of the assay. A total of 87 out of 92 specimens gave concordant results indicating a 94.56% specificity of the Truenat KFD.

Cross-reactivity

A total of 25 specimens positive for viruses from the same family of KFDV—*Flaviviridae*, zika ($n = 2$) and dengue ($n = 5$)—and samples positive for viruses from other families—*Alphaviridae*, chikungunya ($n = 3$); *Togaviridae*, rubella ($n = 5$); *Orthomyxoviridae*, influenza ($n = 5$); *Paramyxoviridae*, measles ($n = 5$) indicated no cross-reactive results when tested by the Truenat KFD.

Inter-machine variation

Inter-machine variations were checked by Trueprep AUTO RNA (Molbio Diagnostics Pvt.Ltd.) RNA extraction units and 5 UnoDx Truenat PCR devices. RNA extraction was performed on KFDV positive specimens using the Autoprep extraction units and the Truenat PCR devices. Results indicated comparable Ct values. This comparison helped to rule out inter-machine variation and establish concordance in the software algorithm functioning of the UnoDx Truenat PCR devices.

Discussion

We have standardized and validated Truenat KFD (Molbio Diagnostics Pvt.Ltd.) for sensitivity, specificity, and comparison at

inter-machine and real-time PCR chemistry levels. The Truenat KFD will aid in the timely detection of KFDV from human, monkey, and tick pool specimens during an outbreak and in surveillance activities from remote forest or field areas. Field experiments demonstrated that processing a single clinical specimen for KFDV detection at PoC would take 1.5 h from RNA extraction till obtaining real-time results with Truenat KFD in comparison with 2.5 h for conventional TaqMan real-time RT-PCR. This system also addresses biosafety concerns as a specimen would be inactivated with the lysis buffer treatment so that basic personal protective equipment can be used in field settings. Furthermore, using PoCT units having Quattro Workstation with four channel would decrease the processing time and render rapid diagnosis at the PoC. Our study found that Truenat KFD was specific and sensitive with a comparable level of detection to conventional KFD real-time RT-PCR. It has many advantages over real-time PCR, such as no requirement of clean hood environment or master-mix preparation, a light and portable system ideal for field testing, and minimal training required for the end-users. As the system is battery-operated, portable and user-friendly, it has a high potential for use in rural settings with minimal time-to-positivity.

Although, basic molecular diagnostic laboratories have been established across the country during the SARS-CoV-2 pandemic, the need for PoC testing still exists. During this pandemic, the Truenat system has proved its potential to provide timely diagnosis of SARS-CoV-2. The time and logistics currently required to send KFDV specimens to referral laboratories could be redirected to patient management and control further spread of the disease. Considering these points, the Truenat KFD can be used effectively at primary health centers/district hospitals/outbreak areas to cope up with the challenges posed by the KFD virus. However, the limitations of PoC tests are the required reagents, cost-effectiveness, shelf-life and device calibration and management (of utmost importance for obtaining accurate results). In summary, the Truenat KFD could be used effortlessly in a KFD outbreak area with minimum requirements of standard personal protective equipment and basic training on biosafety and good laboratory practices.

Conflict of interest

The authors confirm that no conflicts of interest exist.

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Authors contributions

Study design: PY, AS; Data collection: TM, SP, DM, AW, PG; Data analysis: PY, AS, TM, SP;

Writing: PY, AS, TM

Ethical approval

The study has been approved by the Institutional Human Ethical Committee of ICMR-NIV, Pune, India.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ijid.2021.05.036>.

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