Assessment of qPCR, nested RT-PCR and ELISA techniques in diagnosis of Chikungunya

Deepti Parashar[1,*], Mandar S. Paingankar[2], A. B. Sudeep[1], Ashwini More[1], Sapana B. Shinde[1] and Vidya A. Arankalle[1]

[1]National Institute of Virology, 20-A Ambedkar Road, Pune 411 001, India
[2]Molecular Biology Research Laboratory, Department of Zoology, University of Pune, Ganeshkhind, Pune 411 007, India

The definitive diagnosis ability of IgM ELISA, nested RT-PCR and real-time quantitative PCR (qPCR) was evaluated for Chikungunya diagnosis using 180 clinical samples. Real-time qPCR showed a higher sensitivity (88.3%) for Chikungunya diagnosis in the early stages of infection, while IgM ELISA proved sensitive for the late stages of illness (81.8%). The results suggest that the application of both IgM ELISA and RT-PCR based assays will be ideal for definitive diagnosis of Chikungunya during outbreaks.

Keywords: Chikungunya, IgM capture ELISA, nested RT-PCR, qPCR.

RE-EMERGENCE of Chikungunya virus (CHIKV) has become a significant public health concern in India and countries of Southeast Asia[1]. Chikungunya (CHIK) is characterized by acute arthralgic manifestations forcing the victims to be bedridden for prolonged periods[1]. Because of almost identical clinical manifestations and circulation in the same geographical areas, clinical differentiation between Dengue and CHIK infections is difficult. Therefore, there is an urgent need to develop a sensitive and specific diagnostic test for CHIK. Currently, a few specific molecular[2–5] and serological[6] diagnostic tools are available for CHIK. However, no comparative data are available on the diagnostic sensitivity and diagnostic specificity of these assays[4,5]. ELISA method is not efficient in detecting CHIK in early stages of infection (1–4 days), whereas RT-PCR methods have limitations in detecting disease in late stages (5 days onwards). CHIK diagnosis assay with data on the diagnostic sensitivity and specificity will improve the diagnosis efficiently. In the present study, we evaluated the sensitivity and specificity of TaqMan-based one-step qPCR assay for definitive detection of CHIK and compared it with nested PCR and IgM ELISA assays.

Ethical approval: All experimental protocols were reviewed and approved by the Institutional Ethical Committee for Humans and written informed consent was obtained from all the participants.

*For correspondence. (e-mail: deeptiparasharster@gmail.com)

One hundred and eighty referred blood samples were received from different parts of India. Immediately after obtaining the samples, serum was separated, aliquoted and screened for CHIKV RNA concurrently by qPCR, nested RT-PCR and CHIKV-specific IgM by ELISA. RNA was extracted using the QIAamp viral RNA extraction kit (Qiagen, USA) according to the manufacturer's protocol. Viral load in serum samples was determined by absolute quantification method[7]. The nested PCR targeting E3 genes was carried out using primers (F1, 5′-CAGATACCCGTGCACATGAAGT-3′ and R1, 5′-TGAGCTAAGTATGGTCTTGT-3′) that produced a 534-bp fragment. The second set of primers (F2, 5′-CAGACCGATCTTCGACAACA-3′ and R2, 5′-TCATGACGTTGTCCTCAAGC-3′) amplified a 271 bp product. Superscript II (Invitrogen, USA) was used for reverse transcription (42°C for 1 h). Cycling conditions were 1 cycle at 94°C for 5 min; 35 cycles each of 94°C (1 min), 47°C (1 min), and 72°C (2 min); followed by a final extension of 7 min at 72°C. The products were visualized on 2% agarose gel using a gel documentation system (Alpha Innotech, USA). IgM detection was conducted using CHIK IgM detection kit (NIV, India) according to the manufacturer's instructions. The specificity of these assays was also checked on Chandipura and Dengue viruses (serotypes 1–4) and Japanese encephalitis. Receiver operating characteristic (ROC) analysis, sensitivity, specificity and the area under the ROC curves (area under curves; AUCs) were calculated as described earlier[8]. The association of clinical symptoms between CHIK-positive and CHIK-negative cases was calculated using the chi-square test. The correlation between Post Onset Day (POD) and CHIKV titre was tested using Pearson correlation and Mann–Whitney *U* test.

Suspected clinical serum samples were considered CHIK-positive only when found positive by at least one of the three assays used. The maximum number of positive samples (78.01%) was detected by qPCR ($n = 110$) followed by nested PCR ($n = 99$, 70.21%) and IgM ELISA ($n = 72$, 51.06%) (Table 1). In early PODs (1–3 days), qPCR was found to be superior in CHIK detection (88.31%) in comparison to nested PCR (76.62%) and IgM ELISA (38.36%) (Table 1). The highest viral load detected was 1.23×10^{10} viral RNA copies/ml and the lowest was 30 viral RNA copies/ml, demonstrating the efficiency of qPCR to detect a wide range of RNA copies. It was also observed that CHIKV RNA could be detected up to 10th POD by qPCR, making the assay versatile for virus detection in clinical samples irrespective of PODs. Figure 1 shows the ROC curves for each test, in most accurately defined disease status. Sensitivity and specificity of qPCR were found to be more than nested PCR and IgM ELISA tests in the detection of CHIKV in clinical samples. Statistics for some selected thresholds for qPCR and ELISA tests is given in Table 2. The assays used in this study are specific; they do not cross-react with the

Table 1. Sensitivity of different assays in Chikungunya diagnosis

POD	No. of samples tested	No. of Chikungunya-positive samples*	No. of positives (percentage positivity)		
			IgM ELISA	qPCR	Nested PCR
1–3	97	77	30 (38.36)	68 (88.31)	59 (76.62)
4–7	40	31	15 (48.38)	25 (80.64)	25 (80.64)
8 ≤	43	33	27 (81.81)	17 (51.5)	15 (45.5)
Total	180	141	72 (51.06)	110 (78.01)	99 (70.21)

*Clinical serum samples were considered Chikungunya-positive only when found positive by at least one of the three assays used. POD, Post Onset Days.

Table 2. Statistics for some selected thresholds of IgM capture ELISA, real-time qPCR and nested RT-PCR

Method and threshold	Sensitivity	Specificity	Positive predictive value	Negative predictive value
IgM ELISA (ODI)				
0.5	0.327	1.000	1.000	0.598
1	0.238	1.000	1.000	0.568
qPCR (RNA copies/ml)				
10	0.789	1.000	1.000	0.826
100	0.721	1.000	1.000	0.782
1000	0.592	1.000	1.000	0.710
Nested PCR* (RNA copies/ml)				
100	0.714	1.000	1.000	0.778
1000	0.490	1.000	1.000	0.662

*Threshold values for nested RT-PCR were determined using serial dilutions of *in vitro* transcribed RNA. Nested PCR was unable to detect the 10 RNA copies. ODI, Optical density index.

Table 3. Comparison of clinical symptoms between CHIKV-positive and CHIKV-negative subjects

Clinical features (no. of patients having clinical symptoms (n = 180))	CHIK-positive by qPCR, nested PCR or IgM ELISA test (%) (n = 141)	CHIK-negative by qPCR, nested PCR or IgM ELISA test (%) (n = 39)	P value
Arthralgia (146)	126 (86.3)	20 (51.28)	0.036
Myalgia (134)	118 (88.05)	16 (41.02)	0.025
Fever (145)	107 (75.88)	38 (97.43)	0.339
Headache (104)	94 (66.67)	10 (25.64)	0.01
Joint pain (91)	78 (55.31)	13 (33.34)	0.106

P value was calculated using chi square test for association of clinical symptoms between Chikungunya-positive and Chikungunya-negative cases.

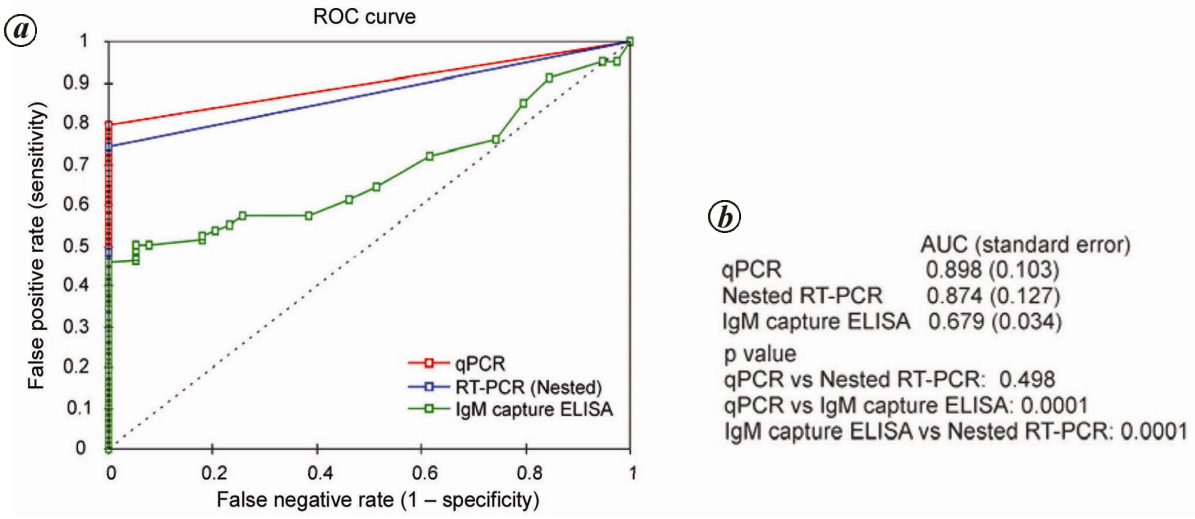

Figure 1. Comparison of diagnostic tests. ***a***, Receiver operating characteristic curves for IgM capture ELISA, nested RT-PCR and real-time qPCR. ***b***, Statistics for some selected thresholds.

other arboviruses tested (Chandipura, Dengue 1–4 serotypes and Japanese encephalitis).

Comparison of clinical features between CHIKV-positive and CHIKV-negative patients demonstrated arthralgia, myalgia and headache to be significantly associated with CHIKV confirmed cases (chi-square test, $P < 0.05$; Table 3). No correlation between POD and CHIKV titre was observed (Mann–Whitney U test, $P > 0.05$; Pearson correlation coefficient 0.080; $P = 0.276$). Similar results have been reported by Ray *et al.*[9] during a multi-centric study carried out in India to confirm CHIKV infection in patients using virological and serology based assays. It was also confirmed in the present study that CHIKV-specific IgM antibodies could be detected from 2 POD, though the percentage was low.

Reddy *et al.*[10] evaluated the sensitivity of different assays for definitive diagnosis of CHIKV in a small number of clinical samples ($n = 70$, 2–5 POD). In the present study, we used more clinical samples ($n = 180$) with different PODs (2–15 POD) from various geographical areas of India. Although we used samples from various PODs, the sensitivity and specificity of CHIK detection methods described in the present study are higher than those reported earlier[10]. qPCR has been found to be more sensitive than nested PCR during early PODs (1–3 days), and the IgM ELISA kit was found to be more effective to detect the presence of CHIK during late POD (8 ≤ days). The data from the present study as well as the earlier study[10] indicate that the application of both IgM ELISA and RT-PCR-based assays will be ideal for definitive diagnosis of CHIK during outbreaks.

1. Sudeep, A. B. and Parashar, D., Chikungunya: an overview. *J. Biosci.*, 2008, **33**, 443–449.
2. Hasebe, F. *et al.*, Combined detection and genotyping of Chikungunya virus by specific reverse transcription-polymerase chain reaction. *J. Med. Virol.*, 2002, **67**, 370–374.
3. Pfeffer, M., Lisssen, B., Parker, M. D. and Kenney, R. M., Specific detection of Chikungunya virus using a RT-PCR/nested PCR combination. *J. Vet. Med.*, 2002, **49**, 49–54.
4. Pastorino, B., Bessaud, M., Grandadam, M., Murri, S., Tolou, H. J. and Peyrefitte, C. N., Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. *J. Virol. Methods*, 2005, **124**, 65–71.
5. Parida, M. M., Rapid and real-time detection technologies for emerging viruses of biomedical importance. *J. Biosci.*, 2008, **33**, 617–628.
6. Shukla, J., Khan, M., Tiwari, M., Sannarangaiah, S., Sharma, S., Rao, P. V. and Parida, M., Development and evaluation of antigen capture ELISA for early clinical diagnosis of Chikungunya. *Diagn. Microbiol. Infect. Dis.*, 2009, **65**, 142–149.
7. Parashar, D., Paingankar, M. S., Kumar, S., Gokhale, M. D., Sudeep, A. B., Shinde, S. B. and Arankalle, V. A., Administration of E2 and NS1 siRNAs inhibits Chikungunya virus replication *in vitro* and protects mice infected with the virus. *PLoS Negl. Trop. Dis.*, 2013, **7**(9), e2405.
8. Hanley, J. A. and McNeil, B. J., A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology*, 1983, **148**, 839–843.
9. Ray, P. *et al.*, Chikungunya infection in India: results of a prospective hospital based multi-centric study. *PLoS One*, 2012, **7**(2), e30025.
10. Reddy, V., Ravi, V., Desai, A., Parida, M. M., Powers, A. M. and Johnson, B. W., Utility of IgM ELISA, TaqMan real-time PCR, reverse transcription PCR, and RT-LAMP assay for the diagnosis of Chikungunya fever. *J. Med. Virol.*, 2012, **84**, 1771–1778.

ACKNOWLEDGEMENTS. We thank the Indian Council of Medical Research, Ministry of Health and Family Welfare, Government of India for financial support. We also thank Ms Neha Bamne for assistance in the laboratory.

Received 14 August 2014; accepted 18 September 2014

Estimation of monthly average sunshine duration over China based on cloud fraction from MODIS satellite data

Yongjian He[1,2], Xinfa Qiu[1,2,*], Yun Cao[1,2] and Yan Zeng[3]

[1]Key Laboratory of Meteorological Disaster of Ministry of Education, No. 219, Ningliu Road, Nanjing, Jiangsu, China
[2]School of Geography and Remote Sensing, Nanjing University of Information Science and Technology, No. 219, Ningliu Road, Nanjing, Jiangsu, China
[3]Jiangsu Research Institute of Meteorological Science, No. 2, Beijige, Nanjing, Jiangsu, China

The sunshine duration (SSD) model described herein combines meteorological observation data from an extensive network of weather stations, MODIS satellite cloud cover data, and a high-resolution digital elevation model to produce high-resolution SSD maps of China. The model yielded low difference between the measured and estimated values at 672 standard weather stations. The mean absolute bias error (MABE) of the monthly mean daily SSD for 2001–2003 was 0.15–0.26 h and the relative bias error (RABE) was 2.34–4.64%. To further validate the model, the observation data from the intensive weather stations (with high spatial resolution) in Jiangsu and Qinghai not used in the calculations, were used for comparison with the estimated values. The annual mean MABE values in Jiangsu and Qinghai during 2001–2003 were less than 0.5 h, and the annual mean RABE values below 5%. Thus, one can conclude that the SSD model is reliable and stable. The spatial distribution of the SSD was also examined, which indicated that the

*For correspondence. (e-mail: xfqiu135@nuist.edu.cn)