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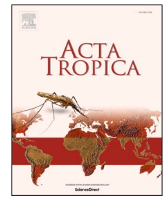

Comparative study on the utility of automated chemiluminescence immunoassay for NS1 antigen-based dengue diagnosis

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ABSTRACT

With newer dengue outbreaks extending to regions that were previously unaffected, about half of the world's population is now at risk of dengue infection. This scale of dengue spread and its undistinguishing primary fever symptom demands embracing automated high-throughput diagnostic techniques for quicker confirmatory diagnosis which otherwise requires time, cost and skill intensive reverse transcription-polymerase chain reaction (RT-PCR). Magnetic bead-based automated chemiluminescence immunoassay (CLIA) is one potential platform that has proven its diagnostic potential for different diseases. However, adoption of CLIA for dengue diagnosis demands extensive validation for widespread implementation. To this end, we evaluated the diagnostic performance of CLIA in comparison with routine dengue diagnostic approaches such as rapid diagnostic test (RDT) and RT-PCR. RDT and CLIA detected the presence of dengue non-structural protein 1 (NS1) antigen while RT-PCR detected the presence of viral RNA. From the analysis of 204 samples, the dengue test positive percentage was 17.6 %, 16.7 % and 19.6 % by RDT, CLIA and RT-PCR methods, respectively. CLIA exhibited a sensitivity of 77.5 %, specificity of 98.17 % and a Cohen's kappa agreement (κ) value of 0.802 with RT-PCR. In addition, CLIA also exhibited a high κ-value of 0.931 with RDT. These findings show the reliability of NS1 antigen detection using automated CLIA for dengue diagnosis. This supports the potential to adopt high-throughput automated CLIA for dengue diagnosis when resources and expertise required to meet the need for quick test result turnaround during outbreaks may be limited.

1. Introduction

Dengue illness is a mosquito-borne viral disease caused by dengue virus (DENV). Dengue poses a significant public health challenge especially in the tropical and subtropical regions of the world. Presently, about half of the world's population is now at risk of DENV infection, and the geographical range of dengue is expected to expand due to ongoing climate change and urbanization (Messina et al., 2019; World Health Organization 2024). Severe dengue fever, if left untreated, can have a mortality rate ranging from 10 % to 20 %. However, with proper supportive care, this rate can be lowered to around 1 % (Schaefer et al., 2024). Dengue outbreaks, especially in developing and underdeveloped nations, place significant strain on healthcare systems and have considerable economic impacts. Due to the current absence of an effective vaccine or approved antiviral treatments, dengue is expected to remain a major public health issue in the years to come. Early diagnosis remains crucial for initiating timely disease control and reducing mortality, as emphasized by the WHO's 2021–2030 sustainable development goals, which aim for zero deaths from dengue virus through effective diagnosis and clinical management (Salje, 2024).

Dengue diagnosis primarily relies on clinical symptoms, which then guide the appropriate laboratory confirmation process. Current recommendations for laboratory confirmation of dengue include using NS1 enzyme-linked immunosorbent assay (ELISA) or RT-PCR during the initial presentation of illness (Centers for Disease Control and Prevention 2023). Beyond this, NS1 antigen detection is considered to be more sensitive than rRT-PCR for identifying the dengue virus (Chua et al., 2011; Alcon et al., 2002). However, RT-PCR is widely regarded as the "gold standard" for routine diagnostic confirmation. Moreover, RT-PCR provides quantitative data on the viral load which is only less precisely interpreted from quantifying the amounts of viral protein such as NS1. However, given the technical proficiency and facilities required to

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operate RT-PCR machines, alternative strategies that do not require such standards is an advantage. This is critical in point-of-care testing (POCT) especially in remote regions where spike in dengue cases can occur unexpectedly. To this end, multiple dengue diagnostic strategies have been developed. For example, RDT has also been developed for the detection of NS1 antigen in a POCT scenario with good sensitivities and specificities (Santoso et al., 2020). Besides, CLIA based detection of NS1 antigen has also been gaining popularity owing to its advantage of high-throughput automated operation with limited human inputs and minimal risk of human error. This is particularly beneficial during disease outbreaks, where a large number of samples may need testing due to the overlapping fever symptoms common to dengue and other co-circulating tropical diseases. In addition, its multiplexing capability allows for the simultaneous detection of multiple biomarkers using a smaller sample volume and with a shorter reaction time. Use of CLIA for dengue diagnosis have demonstrated that it reduces the required specimen volume and testing time, while also lowering the cost per test (Zhu et al., 2018). However, it is important to compare the utility of CLIA for dengue diagnosis with other routine tests like RDT and RT-PCR for its widespread acceptance in diagnostic use. Thus, the purpose of the current study was to compare the diagnostic performance of automated CLIA with RT-PCR and RDT using anonymized patient samples.

2. Methodology

2.1. Study design and sample collection

The anonymized patient samples used in this study were obtained from patients referred to Paras Pathology LLP, Mumbai, India, a National Accreditation Board for Hospitals & Healthcare Providers (NABH) certified medical laboratory, for testing based on clinically suspected dengue. A total of 204 patient samples were included in the study all of which were collected in the month of September 2024 at Paras Pathology LLP, Mumbai, India. Only age and sex of the patients were recorded for the study. The study did not involve development of any new method or instrument and was only a comparison of an existing automated operation over standard diagnostic approaches. Further, the study did not involve any direct patient recruitment, interventions, collection of identifiable patient data or any additional research. Moreover, the study was carried out using the residual sample remaining after allocating the sample for the standard diagnostic procedure (RT-PCR, for which the sample was originally collected). Hence, an institutional ethical review board (IRB) approval was not sought. Blood samples from all patients were collected as per standard protocols in standard collection tubes (JK Diagnostics, India). Samples were collected only once and segregated for use in the different diagnostic tests. Blood samples for RT-PCR were collected in ethylenediamine tetra acetic acid (EDTA) tubes, while those for RDT and CLIA were collected in serum tubes.

2.2. Dengue diagnostics

All the collected samples were tested for dengue. The RT-PCR was carried out at a National Accreditation Board for Testing and Calibration Laboratories (NABL) accredited testing laboratory (Rivara Labs, Mumbai) using DENV/CHIKV Multiplex real time RT-PCR kit (AmpliMol Dx, India) following manufacturer's instructions. RDT and CLIA were carried out at Paras (using serum separated by centrifugation at 4500 rpm) using Dengue Day 1 Test, NS1 Ag card (J. Mitra & Co. Pvt. Ltd., India) and CHEMIZure, Dengue NS1 Ag CLIA kit (Matrix Labs Pvt. Ltd., India), respectively. Each RDT assay card was evaluated independently by the technician performing the test and by a second analyst, who was blind to the outcome of initial assessment, following the manufacturer's instructions. In cases of disagreement, a third analyst repeated the assessment, and the majority decision out of the three assessments was considered final. The automated workflow involving sample processing and readout for CLIA was carried using AutoLumo A1800 analyzer (Autobio Diagnostics Co. Ltd, China). A specimen relative light units/cut-off value (S/Co) of '1 or >1' from the CLIA analyser was considered dengue NS1 reactive and S/Co less than '1' was considered Dengue NS1 non-reactive based on the standardized pre-setting provided with the analyser. The analysts performing and scoring the assays were blind to the reference RT-PCR results. The result from RT-PCR was compared with the results from detecting the dengue NS1 antigen using RDT and CLIA methods.

2.3. Data management, analysis and interpretation

The derived data from the different diagnostic methods were tabulated in Microsoft Excel worksheet and used for analysis. Based on test outcomes, the parameters for comparing diagnostic performance of RDT and CLIA with RT-PCR, such as sensitivity and specificity, were calculated. The result from the RT-PCR was considered as the "gold standard" for evaluating the diagnostic performance of RDT and CLIA. All the data analysis associated with the calculation of diagnostic performance parameters such as positive predictive value (PPV), negative predictive value (NPV), false positive rate (FPR), false negative rate (FNR) and Cohen's kappa (κ) value was done using GraphPad Prism 9 software. These values were determined with 95 % confidence interval (CI) using Wilson score method.

3. Results

3.1. Overall diagnostic assessment

This study compared the test results of screening of acute dengue by two commonly used methods, RDT and RT-PCR, with automated CLIA. The total number of tested samples were 204 (Males: 115; Females: 89). The patient age ranged from 10 months to 95 years with an average age of 35 and standard deviation of 20 years. The number of samples tested positive and negative by each type of diagnostic method in samples are provided in Table 1. RT-PCR gave the highest positive rate (19.2 %) followed by RDT with 17.2 % positive rate and CLIA with 16.7 % positive rate.

3.2. Diagnostic performance of automated CLIA

The cross-classification of positive and negative test results for different diagnostic method comparisons are provided in Table 2. Automated CLIA exhibited 9 false negatives and 3 false positives in comparison with "gold-standard" RT-PCR test (Table 2A) resulting in 77.50 % sensitivity and 98.17 % specificity (Table 3A). In addition, CLIA exhibited 91.18 % and 94.71 % PPV and NPV, respectively (Table 3A). On the other hand, RDT showed 8 false negatives and 4 false positives in comparison with RT-PCR (Table 2B) resulting in 80 % sensitivity and 97.56 % specificity (Table 3B). CLIA and RDT exhibited a Cohen's kappa agreement (κ) value with RT-PCR of 0.802 and 0.806, respectively (Table 3A & B). In addition, CLIA also exhibited a high κ-value of 0.931 with RDT (Table 3D). Collectively, the results suggest a good level of agreement of results from CLIA with the existing dengue diagnostic methods like RT-PCR and RDT.

Considering that both RDT and CLIA detect the presence of dengue infection based on NS1 antigen levels, which contrasts with detection of viral RNA by RT-PCR, it is also useful to test the diagnostic performance

Table 1
Overall results from of RDT, CLIA and RT-PCR in the acute samples for dengue diagnosis.

Diagnostic tests	Number of positives (%)	Number of negatives (%)
RDT	36 (17.6)	168 (82.4)
CLIA	34 (16.7)	170 (83.3)
RT-PCR	40 (19.6)	164 (80.4)

Table 2
Contingency table reporting cross-classification of results from different dengue diagnostic method comparisons in 2 × 2 format. A) CLIA & RT-PCR, B) RDT & RT-PCR, C) CLIA & RDT and D) Immunoassay (Combined uniform result in CLIA & RDT) & RT-PCR.

A) CLIA & RT-PCR		
	RT-PCR positive	RT-PCR negative
CLIA positive	31	3
CLIA negative	9	161
B) RDT & RT-PCR		
	RT-PCR positive	RT-PCR negative
RDT positive	32	4
RDT negative	8	160
C) Immunoassay (Combined uniform result in CLIA & RDT) & RT-PCR		
	RT-PCR positive	RT-PCR negative
Immunoassay positive	31	2
Immunoassay Negative	8	159
D) CLIA & RDT		
	RDT positive	RDT negative
CLIA positive	33	1
CLIA negative	3	167

Table 3
Diagnostic performance of different dengue diagnostic method comparisons. A) CLIA & RT-PCR, B) RDT & RT-PCR, C) CLIA & RDT and D) Immunoassay (Combined uniform result in CLIA & RDT) & RT-PCR. PPV - Positive predictive value; NPV – Negative predictive value; FPR - False positive rate; FNR - False negative rate; CI - Confidence interval.

Measure	Calculation	Estimate	95 % CI
A) CLIA & RT-PCR			
Sensitivity	31/40	77.50 %	62.5 0 %–87.6 8 %
Specificity	161/164	98.17 %	94.7 6 %–99.3 8 %
PPV	31/34	91.18 %	81.6 4 %–100.7 1 %
NPV	161/170	94.71 %	91.3 4 %–98.0 7 %
FPR	3/164	1.83 %	0.0 0 %–3.8 8 %
FNR	9/40	22.50 %	9.5 6 %–35.4 4 %
Cohen's kappa (κ)	0.802		
B) RDT & RT-PCR			
Sensitivity	32/40	80.00 %	65.2 4 %–89.5 0 %
Specificity	160/164	97.56 %	93.9 0 %–99.0 5 %
PPV	32/36	88.89 %	78.6 2 %–99.1 5 %
NPV	160/168	95.24 %	92.0 2 %–98.4 6 %
FPR	4/164	2.44 %	0.0 8 %–4.8 0 %
FNR	8/40	20.00 %	7.6 0 %–32.4 0 %
Cohen's kappa (κ)	0.806		
C) Immunoassay (combined uniform result in CLIA & RDT) & RT-PCR			
Sensitivity	31/39	79.49 %	64.4 7 %–89.2 2 %
Specificity	159/161	98.76 %	95.5 8 %–99.6 6 %
PPV	31/33	93.94 %	80.3 9 %–98.3 2 %
NPV	159/167	95.21 %	90.8 3 %–97.5 5 %
FPR	2/161	1.24 %	0.3 4 %–4.4 2 %
FNR	8/39	20.51 %	10.7 8 %–35.5 3 %
Cohen's kappa (κ)	0.831		
D) CLIA & RDT			
Cohen's kappa (κ)	0.931		

of combining the uniform test results from RDT and CLIA as an outcome of one immunoassay test and compare it with RT-PCR. In this perspective, immunoassay (combined uniform result in CLIA & RDT) showed 8 false negatives and 2 false positives in comparison with RT-PCR test (Table 2C) resulting in 79.49 % sensitivity and 98.76 % specificity (Table 3C). Moreover, immunoassay also exhibited a high κ-value of 0.831 with RT-PCR (Table 3C) suggesting good result agreement when NS1 antigen detection and viral RNA detection are compared for dengue diagnosis.

4. Discussion

Dengue diagnosis is complicated due to various factors such as indistinguishable symptoms from other tropical infectious diseases, presence of serotypes, varying viral load during infection, and inaccessibility of rural populations to advanced healthcare facilities in developing countries where new dengue outbreaks occur (Sharp, 2019; Vicente et al., 2016; Mutucumarana et al., 2020; Matangkasombut et al., 2020). Following the COVID-19 pandemic, there has been a notable global surge in dengue cases, marked by multiple outbreaks and the virus spreading into previously unaffected regions (World Health Organization 2023). The scale of this dengue spread necessitates the widespread adoption of diagnostic methods that have high sensitivity & selectivity, and compatible for high-throughput automation with minimal risk from human error. In line with these requirements, automated CLIA based strategies utilizing magnetic microparticles have been gaining popularity and has been developed for diagnostics of multiple infectious diseases and other clinical markers (Liu et al., 2022; Alonso et al., 2014; Oed et al., 1999). However, the adoption of automated CLIA for dengue diagnostics is still in early stages and requires extensive validation to address the limitations of existing dengue diagnostic methods.

Existing dengue diagnostic methods are largely limited due to the dynamics of the viral analyte used for detection. For instance, the viral load drops significantly during the critical and recovery stages of dengue illness (Fig. 1) which in turn affects the sensitivity of RT-PCR used for dengue confirmation (Chua et al., 2011; Alcon et al., 2002). Alternatively, use of immunoassay-based detection of viral NS1 protein has a better "window period" due to the presence of NS1 in blood up to the recovery phase (Chua et al., 2011; Alcon et al., 2002). This when coupled with the detection of anti-dengue IgM, whose levels rises during the recovery phase, offers a "window period" that covers the entire duration of dengue illness. These aspects of viral analyte variation are reflected in WHO recommendation to include NS1 detection or anti-dengue IgM with RT-PCR for acute dengue diagnosis (Dengue guidelines 2009). In this regard, automation of NS1 detection could be a useful during dengue outbreaks when large number of patients with different durations of fever illness need to be tested due to the overlapping fever symptoms common to dengue and other co-circulating tropical diseases.

Our findings demonstrate the good sensitivity and specificity of NS1 detection using automated CLIA, as compared to viral RNA detection with the RT-PCR method (Table 3A), highlighting its utility as a diagnostic tool for dengue. The two negative test results observed with RT-PCR, which were positive in immunoassay (combined uniform result in CLIA & RDT) (Table 2C), may be attributed to fluctuations in DENV RNA levels as the illness progresses. Notably, studies have shown that in samples from patients obtained after the initial days of illness viral RNA levels are less detectable by RT-PCR (Chua et al., 2011; Alcon et al., 2002). Besides, eight false negatives from immunoassay compared to RT-PCR (Table 2C) were observed. It is possible that these samples were

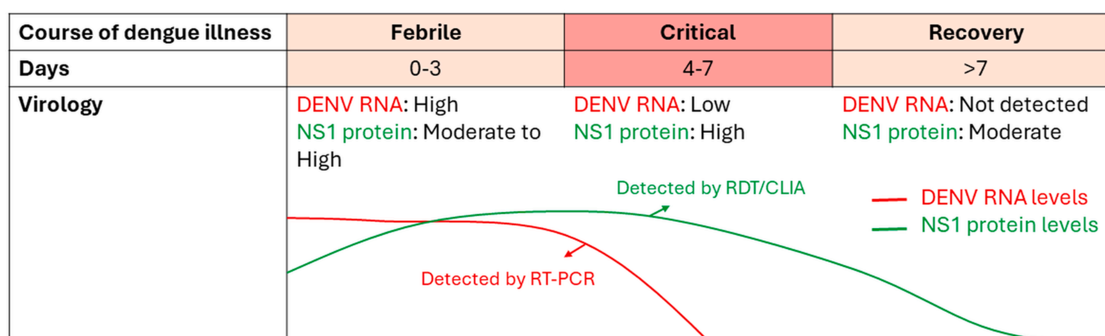

Fig. 1. Dynamics of the NS1 antigen and viral RNA during different phases of dengue illness.

obtained at very earlier phases of illness during which serum NS1 levels are lower (Alcon et al., 2002). This could also account for the higher FNR of 22.50 % and 20 % for CLIA and RDT, respectively, in comparison with RT-PCR, (Table 3A & B). However, it is to be noted that there was good agreement between the result from immunoassay & RT-PCR indicated by κ-value of 0.831 (Table 3C) which support the reliability of NS1 detection for dengue diagnosis.

Molecular diagnostic methods like RT-PCR are conventionally used for early-stage diagnostic confirmation. Although RT-PCR results are sufficiently sensitive and specific, it is expensive and require well-trained manpower for RNA extraction steps. Moreover, specific facility requirements are necessary to prevent false-positive reactions caused by cross-contamination with dengue virus PCR products in the laboratory (Wu et al., 2001). Immunoassays like ELISA and RDT also have their own set of limitations even though they are used for routine screening and diagnosis of dengue. RDT is less sensitive, lacks quantifiable result output, and its visual result interpretation is subjective even for trained analysts which prevents it from being recommended as a reportable test for dengue infection by regulatory bodies (Luo et al., 2019; Yow et al., 2021). Though being more sensitive and universally acceptable, ELISA is constrained by its time-consuming steps, such as sample dilution, which require skilled personnel to ensure readings remain within the assay's linear range, a parameter restricted by the optical density (OD) limit in colorimetry (Gibbs and Kennebunk, 2001). Moreover, ELISA necessitates calibration and reference control values for each run due to various quality-affecting factors, such as manual handling, the sequence of well washing, environmental and incubation conditions, as well as the devices used for transferring samples and reagents. ELISA also does not support continuous or inter-assay sample loading for a same or different analyte detection during a batch run. These limitations hinder automation potential, impacting its scalability for high-throughput applications. On the other hand, automated CLIA overcomes these above limitations by being simple to operate, sensitive, rapid, and affordable with multiplexing capabilities compared to other dengue diagnostic methods. Additionally, it has a unique advantage for high-throughput automation that enables streamlined laboratory operation that can be achieved at the level of primary healthcare centres in dengue outbreak zones with limited availability of skilled manpower. In the current scenario, the initial investment required for CLIA instruments is relatively high, making it unaffordable for rural and primary health centers in India and other developing countries. However, advancements in portable CLIA technology have the potential to bridge this gap in the near future, offering a more accessible and cost-effective solution for these underserved areas.

In addition, the S/Co values from the positive CLIA results that were also positive in RT-PCR (Table 4) showed high values up to 637.19 with average value of 147.40 (while a value of '1 or >1' is itself considered positive). The high S/Co values observed in true dengue-positive samples hint at further possible categorization of disease characteristics at diagnosis, such as extent of viral infection or underlying pathology, which is yet to produce severe symptoms, based on S/Co levels. This warrants further exploration in future large-scale studies using CLIA-based dengue diagnostics. Besides, future assay developments incorporating dengue serotype identification and assessment of cross-reactivity with other co-circulating viruses in the CLIA assay format could be of potential diagnostic importance. Overall, CLIA-based methods hold

Table 4
Comparison of positive RT-PCR results with corresponding RDT and CLIA outcomes. S/Co - Specimen relative light units/Cut-off (1 or >1 is considered 'Reactive'). M - Male; F - Female.

S. No.	Sex/Age	Diagnostic test outcomes			
		RDT	CLIA		RT-PCR
			S/Co	Outcome	
1	F/35	Non-Reactive	0.08	Non-Reactive	Detected
2	F/30	Non-Reactive	0.05	Non-Reactive	Detected
3	M/22	Non-Reactive	0.36	Non-Reactive	Detected
4	M/59	Non-Reactive	0.06	Non-Reactive	Detected
5	M/45	Non-Reactive	0.25	Non-Reactive	Detected
6	F/14	Non-Reactive	0.07	Non-Reactive	Detected
7	F/54	Non-Reactive	0.05	Non-Reactive	Detected
8	M/13	Non-Reactive	0.03	Non-Reactive	Detected
9	M/14	Reactive	0.35	Non-Reactive	Detected
10	F/10	Reactive	106.57	Reactive	Detected
11	M/21	Reactive	203.31	Reactive	Detected
12	M/24	Reactive	103.91	Reactive	Detected
13	M/31	Reactive	637.19	Reactive	Detected
14	M/60	Reactive	1.44	Reactive	Detected
15	M/31	Reactive	15.65	Reactive	Detected
16	F/37	Reactive	425.4	Reactive	Detected
17	F/46	Reactive	321.76	Reactive	Detected
18	M/6	Reactive	32.77	Reactive	Detected
19	M/18	Reactive	28.63	Reactive	Detected
20	M/14	Reactive	31.88	Reactive	Detected
21	F/38	Reactive	25.67	Reactive	Detected
22	F/38	Reactive	9.48	Reactive	Detected
23	M/8	Reactive	80.39	Reactive	Detected
24	M/22	Reactive	91.54	Reactive	Detected
25	M/36	Reactive	67.54	Reactive	Detected
26	M/19	Reactive	141.24	Reactive	Detected
27	M/41	Reactive	73.67	Reactive	Detected
28	F/27	Reactive	45.53	Reactive	Detected
29	M/23	Reactive	210.57	Reactive	Detected
30	F/18	Reactive	6	Reactive	Detected
31	M/43	Reactive	601.22	Reactive	Detected
32	M/6	Reactive	11.79	Reactive	Detected
33	F/33	Reactive	299.72	Reactive	Detected
34	F/49	Reactive	393.63	Reactive	Detected
35	F/19	Reactive	119.45	Reactive	Detected
36	F/1	Reactive	271.89	Reactive	Detected
37	M/13	Reactive	2.38	Reactive	Detected
38	F/33	Reactive	146.56	Reactive	Detected
39	M/13	Reactive	59.3	Reactive	Detected
40	M/19	Reactive	3.45	Reactive	Detected
Average S/Co value of CLIA reactive and RT-PCR positive samples			147.40		

promise as a reliable standardized automation-based approach for dengue diagnosis in the future.

5. Conclusion

The findings of this study demonstrate that NS1 antigen detection through immunoassay is a reliable alternative to RT-PCR for dengue diagnosis. Furthermore, the diagnostic performance of automated CLIA for NS1-based dengue screening was comparable to that of RDT and RT-PCR. With its capacity for automation and reduced susceptibility to human error, CLIA is poised to become a high-throughput diagnostic tool, addressing the growing global challenge of dengue outbreaks.

CRediT authorship contribution statement

Munjal Shah: Writing – review & editing, Formal analysis, Conceptualization. **Nehal Mehta:** Writing – review & editing, Methodology, Data curation. **Zeenal Savla:** Methodology, Investigation, Data curation. **Anand Ramaian Santhaseela:** Writing – review & editing, Writing – original draft, Formal analysis. **Elavarasan Tamilmani:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

Authors E.T and A.R.S are part of Matrix labs which is involved in developing CLIA based diagnostic products for dengue and other infectious diseases. All remaining authors have nothing to declare.

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Data availability

All data associated with this study are available from the corresponding author on reasonable request.

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