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Dengue fever as a reemerging disease in upper Egypt: Diagnosis, vector surveillance and genetic diversity using RT-LAMP assay

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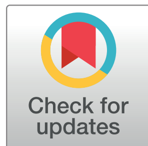

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Abstract

Background

The recent increase in dengue virus (DENV) outbreaks and the absence of an effective vaccine have highlighted the importance of developing rapid and effective diagnostic surveillance tests and mosquito-based screening programs. To establish effective control measures for preventing future DENV transmission, the present study was established to identify the main mosquito vector involved in the dengue fever (DF) outbreak in Upper Egypt in 2016 and detect the diversity of dengue virus serotypes circulating in both humans and vectors.

Methods

We investigated the prevalence of DENV infection and circulating serotypes in the sera of 51 humans clinically suspected of DF and 1800 field-collected *Aedes aegypti* adult female mosquitoes grouped into 36 pooled samples. Both DENV non-structural protein (NS1) immunochromatographic strip assay and loop-mediated isothermal amplification (LAMP) were used for screening.

Results

Overall, the rate of DENV infection in both human sera and pooled mosquito homogenate was 33.3%, as revealed by rapid dipstick immunochromatographic analysis. However, higher detection rates were observed with RT-LAMP assay of 60.8% and 44.4% for humans and vector mosquitoes, respectively. DENV-1 was the most prevalent serotype in both populations. A combination of two, three, or even four circulating serotypes was found in 87.5% of total positive pooled mosquito samples and 83.87% of DENV-positive human sera.

Conclusion

The study reinforces the evidence of the reemergence of *Aedes aegypti* in Upper Egypt, inducing an outbreak of DENV. Mosquito-based surveillance of DENV infection is important to elucidate the viral activity rate and define serotype diversity to understand the virus dynamics in the reinfested area. Up to our knowledge, this is the first report of serotyping of DENV infection in an outbreak in Egypt using RT-LAMP assay.

Introduction

Dengue virus (DENV) is one of the most prevalent mosquito-transmitted arboviruses, which is widely distributed in many tropical and subtropical countries in Southeast Asia, the Pacific, the Americas, and rural areas in Africa [1]. It belongs to the family Flaviviridae and causes human infections via bites of infected female mosquitoes of *Aedes aegypti* or *Aedes albopictus* [2, 3]. A recent estimate indicated that 2.5 billion people were at risk of infection with overall 390 million infections per year, approximately 500,000 hospitalizations, and 25,000 deaths every year [4]. However, the emergence of new epidemics has represented a significant global health problem since the early 20th century [5].

Symptomatic DENV infection was categorized by the WHO as undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS) [6]. Infection could be due to one of four serologically related but antigenically distinct DENV serotypes (DENV-1 to DENV-4). Based on phylogenetic analysis of the DENV envelope glycoprotein (E), these serotypes could be further subdivided into distinct genotypes [7, 8]. Human infection with a single serotype induces lifelong protective immunity against homologous challenge, but reinfection with heterologous serotypes could produce partial or transient protection [9].

More than 3500 mosquito species have been identified all over the world [10]. *Aedes aegypti* mosquito is identified as the main vector of many serious and medically important arbovirus-caused diseases, such as yellow fever, dengue, Zika, and Chikungunya [11].

Recently, the distribution of DENV infection has expanded markedly, particularly in the Americas, South Asia, and the Western Pacific [6]. However, in recent years, DENV outbreaks were also recorded in the Eastern Mediterranean, Saudi Arabia, and Yemen [12–14] In Egypt, reported cases of dengue declined after 1940, which is attributed to effective vector control measures including the extensive use of dichlorodiphenyltrichloroethane (DDT), leading to the complete eradication of *Aedes aegypti* mosquitoes [12]. Only sporadic studies have reported the reemergence of *Ae. aegypti* in Egypt in some southern governorates [15–17]. Studies previously conducted in Assiut Governorate revealed the presence of other *Aedes* species such as *Ae. detritus* and *Ae. caspius* [18].

A recent DF outbreak was documented by the WHO in Dayrout District of Assiut Governorate, Upper Egypt. Cases were admitted to Dayrout Fever Hospital with acute febrile illness, but there were no serious complications or fatalities. The diagnosis was confirmed by ELISA and PCR, revealing DENV type I. In addition, field-based vector surveillance revealed the presence of *Ae. aegypti* larvae and adult mosquitoes at the patients' dwellings [19–21].

Generally, rapid and efficient diagnosis of infection is critical for proper management and appropriate control of the disease. DF surveillance in humans in most resource-limited countries mainly depends on serological detection of DENV-specific antibodies. Virus isolation, which is the gold standard test for diagnosis of DENV infection, and the RT-polymerase chain

reaction (RT-PCR) are highly sensitive and definitive for diagnosing dengue fever, but they are time-consuming, require trained staff, and sophisticated equipment [22]. So, they still have limited applicability in the surveillance field and are not appropriate to use in resource-limited settings besides they cannot differentiate between virus serotypes [23, 24].

Recently, reverse-transcription loop-mediated isothermal amplification (RT-LAMP) was introduced to overcome the limitations of PCR. LAMP is a single-step test used for the amplification of ribonucleic acid (RNA). It includes a combination of the two enzymes, Bst (*Bacillus stearothermophilus*) DNA polymerase and reverse transcriptase, and specific primers designed to recognize six distinct regions of a target sequence. The LAMP technique is sensitive, specific, cost-effective, less time-consuming, and easily interpreted [25, 26].

For the successful surveillance of dengue disease, there is a need for complementary epidemiological studies on virus-transmitting mosquitoes including; viral sequences, mosquito abundance, viral load, and the prevalence of different viral serotypes. In addition, field-collected mosquito surveillance is valuable for tracking virus activity and endorsing control measures [27, 28]. Therefore, there is a need to develop sensitive, specific, cheap, and effective vector surveillance techniques for initial diagnosis and early disease control.

The current study was performed to investigate the reintroduction of *Aedes aegypti* mosquitoes producing a dengue outbreak in Upper Egypt governorates, diagnose DENV infection, and determine the diversity of circulating serotypes. We further highlight the importance of field-based studies for identifying and characterizing DENV serotypes in naturally infected mosquitoes concurrent with human dengue infection for inferring local viral activity in a certain period and locality using RT-LAMP assay.

Material and methods

Study area

This study was conducted to investigate the DENV outbreak that occurred during the period from June to December 2016 in three Egyptian governorates: Assiut Governorate (Dayrout and El Kosia Districts), Qena Governorate (Qena District), and Red Sea Governorate (Safaga City).

Ethical consideration

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Assiut University. Informed consent was obtained from all participants (IRB code:1630083).

Recruitment and sample collection

1. Human sample collection. A total of 51 serum samples were collected from patients admitted to fever hospitals at the three Egyptian governorates mentioned above and suspected of being infected with DENV. The inclusion criteria of the patients recruited in the study were as follows: fever of unknown origin from 2 to 7 days before hospital admission, headache, joint pain, back pain, retroorbital pain, and skin rash in accordance with the WHO guidelines for suspected Dengue cases [6].

2. Mosquito collection. Approximately 3800 adult mosquitoes were collected from 104 households and 27 outdoor localities from the same residences of the included patients. Resting mosquitoes were captured using a Prokopack aspirator [29]. The collected mosquitoes were stored on dry ice and transferred to the laboratory of the Parasitology Department, Faculty of Medicine, Assiut University, for examination.

Table 1. Descriptive data on the number and sites of the recruited samples in the study.

Governorate	Human samples		Mosquitoes samples					
	Hospitals	No. of patients	Collection site		Mosquitoes collected			No. of pools
			Households	Outdoor	*C. Pipiens* and *C. Perexiguus*	*Ae. Aegypti* Male	*Ae. Aegypti Female*	
Assiut	Dayrout fever hospital	5	36	9	500	300	100	2
	El Kosia fever hospital	3						
Qena	Qena fever hospital	16	34	9	200	100	500	10
Red Sea	Red Sea fever hospital	27	34	9	300	600	1200	24
Total		51	104	27	1000	1000	1800	36

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Mosquitoes were identified according to morphological identification keys [30]. Approximately 1000 adult mosquitoes were identified as *Culex pipiens* and *Culex perexiguus*. A total of 2800 mosquitoes that were phenotypically identified as *Ae. aegypti* in the patient's settings were sorted according to sex and counted. There were 1800 female *Ae. aegypti* mosquitoes (64.3%), the average mosquito density was 14.1 *A. aegypti* females per aspiration site. They were divided into 36 pooled samples each containing about 50 adult females. *Culex* spp. and *Ae. aegypti* male mosquitoes were not processed further in the study [31]. Wings and legs of the female mosquitoes were removed. In each pool, heads, thoraxes, and abdomens of the mosquitoes were collected and macerated in 300 ml of PBS, followed by thorough homogenization using a Retsch mixer mill MM 200 (Germany). Samples were stored at −80˚C until use.

The details of the collected human sera and mosquito samples in relation to the collection sites were illustrated in Table 1.

Detection of dengue NS1 Ag in human samples and *Aedes aegypti* pools by immunochromatographic strip test

The dengue NS1 Ag rapid test is an immunochromatographic strip test used for the early diagnosis of dengue infection, even before the presence of IgM or IgG antibodies [32]. It is based on the qualitative detection of non-structural protein 1 (NS1) of DENV. Dengue NS1 Ag strip kit (product code: 70700, Bio-Rad Laboratories, Marnes-la Coquette, France) was used following the manufacturer's protocol. Briefly, the test was performed for human serum samples and mosquito homogenate. The tested sample (50 μl) was added to the test tube with a single drop of migration buffer, provided with the kit. The test strip was placed in the tube and left for 15 min. Results were obtained after 15 min and could be prolonged for another 15 min for doubtful results. Interpretation of results depended on the appearance of blue color at the Test Line (T) and the Control Line (C) indicating a positive result. While the appearance of (C) line only indicated a negative result. The absence of any bands indicated invalid test

Detection and serotyping of DENV in both human serum samples and mosquito pools using RT-LAMP

To quantify the viral load, RNA was extracted from *Aedes aegypti* homogenate pools and patient sera using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instructions. The extracted RNA was eluted in 100 μl of elution buffer and stored at −70˚C until analysis.

1. Design of dengue virus serotype-specific RT-LAMP assay primers. Four DENV serotype-specific RT-LAMP primer sets were designed using the LAMP primer design support

software program (Net Laboratory, Japan; http://venus.netlaboratory.com), in accordance with a previous study [23]. Six different primers for each serotype were designed from the 3′ noncoding region (NCR) of the DENV. In each primer set, there are two outer primers (F3, forward outer primer; and B3, backward outer primer), two inner primers (FIP, forward inner primer; and BIP, backward inner primer), and two loop primers (FLP, forward loop primer; and BLP, backward loop primer), which are used to accelerate the amplification reaction. These primers were designed to recognize eight distinct regions on the target DENV sequence. The primers were selected based on criteria described previously [33]. Details of the primer sequences used in the study are described in Table 2.

2. Amplification of DENV serotypes by RT-LAMP assay. The RT-LAMP assay was carried out in a total reaction volume of 25 μl using a Loopamp RNA amplification kit (RT-LAMP) (Eiken Chemical Co. Ltd., Japan). The reaction mixture included 6 μl of the primer mix (5 pmol each of F3 and B3 outer primers, 40 pmol each of FIP and BIP inner primers, 20 pmol each of loop primers F and B), 18 μl of LAMP buffer mix (0.1% TritonX, 25× LAMP buffer, 100 mM $MgSO_4$, 25 mM dNTPs, 3 mM hydroxynaphtolblue, 0.35% v/v Gel-Green, 0.1 μl of RNase inhibitor, 1 μl of Bst DNA polymerase (New England Biolabs), 0.03 μl of AMV reverse transcriptase enzyme (Nippongene), 2 μl of 2 M trehalose, and 1 μl of template RNA of the samples. Each sample was applied to four tubes, each of which contained the primer mixture for a specific serotype. The reaction mixtures were incubated at 63˚C for 60 min in a Loopamp real-time turbidimeter (Teramecs, Japan). Positive and negative controls were included in each run. Precautions to prevent cross-contamination were carried out.

Table 2. Primer sequences used for RT-LAMP targeting DENV serotypes.

Virus serotype	Primer	Sequences (5` - 3`)	bp
DEN-1	**F3**	GAGGCTGCAAACCATGGAA	**199 bp**
	B3	CAGCAGGATCTCTGGTCTCT	
	FIP	GCTGCGTTGTGTCTTGGGAGGTTTTCTGTACGCATGGGGTAGC	
	BIP	CCCAACACCAGGGGAAGCTGTTTTTTTGTTGTTGTGCGGGGG	
	FLP	CTCCTCTAACCACTAGTC	
	BLP	GGTGGTAAGGACTAGAGG	
DEN-2	**F3**	TGGAAGCTGTACGCATGG	**211 bp**
	B3	GTGCCTGGAATGATGCTG	
	FIP	TTGGGCCCCCATTGTTGCTGTTTTAGTGGACTAGCGGTTAGA GG	
	BIP	GGTTAGAGGAGACCCCCCCAATTTTGGAGACAGCAGGATCTCTG	
	FLP	G GATCTGTAAGGGAGGGG	
	BLP	GCATATTGACGCTGGGA	
DEN-3	**F3**	GCCACCTTAAGCCACAGTA	**218 bp**
	B3	GTTGTGTCATGGGAGGG	
	FIP	TGGCTTTTGGGCCTGACTTCTTTTTTGAAGAAGCTGTGCAGCCTG	
	BIP	CTGTAGCTCCGTCGTGGGGATTTTCTAGTCTGCTACACCGTGC	
	FLP	CCTTGGACGGGGCT	
	BLP	GGAGGCTGCAAACCGTG	
DEN-4	**F3**	CTATTGAAGTCAGGCCAC	**229 bp**
	B3	ACCTCTAGTCCTTCCACC	
	FIP	TGGGAATTATAACGCCTCCCGTTTTTTCCACGGCTTGAGCAA ACC	
	BIP	GGTTAGAGGAGACCCCTCCCTTTTAGCTTCCTCCTGGCTTCG	
	FLP	GGCGGAGCTACAGGCAG	
	BLP	TCACCAACAAAACGCAG	

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3. Analysis of the RT-LAMP assay.

- **Naked eye visualization**: After incubation of the samples, the precipitate was deposited in the bottom of the tubes by a pulse spin, followed by inspection with the naked eye for white turbidity. The precipitate was checked under natural daylight as well as under a UV transilluminator (302 nm).
- **Agarose gel electrophoresis**: For agarose gel electrophoresis, 10 μl aliquots of the RT-LAMP products were electrophoresed in 2% agarose gel in 0.5% Tris-borate buffer (TBE), followed by staining with ethidium bromide, running at 90 V for 90 min, and visualization with a UV transilluminator system at 302 nm.

Results

Detection and serotyping of DENV in human samples

Concerning the detection of DENV infection in human samples, 17 out of 51 tested human samples were positive for DENV by rapid dipstick test, representing one-third of the tested sera. Meanwhile, RT-LAMP analysis revealed 31 positive cases (60.8%) (S1 Fig in S1 Raw images), with either a single or combined DENV serotypes. The DENV-1 serotype was dominant (93.5%, 29/31). Mixed infection with more than one serotype was observed in most samples (83.87%, 26/31), while mono-infection with one serotype was observed in 5/31 samples (16.13%). The four DENV serotypes were found together in 13/31 positive samples (41.9%). Moreover, human infection with a single DENV serotype was observed in four samples for the DENV-1 serotype and only one sample for the DENV-2 serotype (Table 3).

Detection and serotyping of DENV in mosquito pools

Overall, 12 out of 36 mosquito pools (33.3%) were positive for DENV using rapid dipstick immunochromatographic analysis of NS1 Ag. However, with the RT-LAMP assay, DENV infection was detected in 44.4% (16/36) of pooled mosquito samples (S2 Fig in S1 Raw images). The positive mosquito pools were found to be infected with single or mixed DENV serotypes. DENV-1 was the most common serotype (15 out of 16 positive samples). Mixed infection with two or more serotypes was also detected in 87.5% of the total positive pooled mosquito samples (14/16), including the four DENV serotypes being detected simultaneously in two pools.

Table 3. Dengue virus infection and serotype distribution in human samples and mosquito pools per collection site.

Sample	Collection site	No. of samples	DENV positive by Dipstick	DENV positive by LAMP PCR	DENV-1	DENV-2	DENV-3	DENV-4
Human blood	**Assiut**	8	2	5	5	3	3	2
	Qena	16	6	10	9	7	9	7
	Red Sea	27	9	15	15	9	12	11
Total		**51**	**17 (33.33%)**	**31 (60.8%)**	**29 (93.55%)**	**19 (61.29%)**	**24 (77.41%)**	**20 (64.52%)**
Mosquito pools	**Assiut**	2	0	1	1	1	1	-
	Qena	10	4	5	5	2	3	-
	Red Sea	24	8	10	9	4	10	3
Total		36	12 (33.33%)	16 (44.44%)	15 (93.75%)	7 (43.75%)	14 (87.5%)	3 (18.75%)

Note: The number of DENV serotypes reported in the table represents single infections and mixed infections.

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Serotypes DENV-1 and DENV-3 were identified as a mono-infection in one mosquito pool for each.

A comparison between the two assays used in the study for the detection and serotyping of DENV in consistence with the site of sample recruitment in both human samples and mosquito pools was illustrated in Table 3.

Discussion

Early studies claimed that *Ae*. *aegypti* had been completely eradicated from Egypt in 1963 [34]. Only a few studies reported the occurrence of this species in different parts of Egypt but did not provide substantial evidence of its reintroduction into the country [19]. The current study provided evidence of the reemergence of *Aedes aegypti* mosquitoes in three Egyptian governorates in Upper Egypt. One of them, Assiut Governorate, was previously documented to have an outbreak of DENV in the year preceding this study, namely, 2015. In this study, 2,800 adult mosquitoes of *Ae*. *aegypti* were captured in fields near the outbreak, providing proof of its reemergence at the studied region in Egypt.

In the last few decades, numerous outbreaks and epidemics of DF or DHF have been reported in many tropical and subtropical countries due to rapid urbanization,increased air travel, suboptimal management of water and solid waste, and gaps in vector control [35–37]. The absence of licensed effective vaccines or specific antiviral treatment restricts proper disease management and increases the urgency of developing and applying effective mosquito control strategies [38].

The surveillance of mosquitoes infected with DENV is an integral component of dengue disease control. It can provide early warning signs for a potential risk of disease transmission and enable monitoring and understanding of virus activity, particularly the predominant circulating serotype [39]. The present study demonstrates the usefulness of field-based screening for DENV for estimating infection rates and identifying circulating DENV serotypes in both field-collected mosquitoes and infected patients.

This study revealed approximately one-third (33.3%) of the recruited dengue-suspected patients and the collected mosquito pools from their residential areas to have a confirmed DENV infection by rapid dipstick immunochromatographic analysis, indicating a high burden of DENV. Our results are consistent with previous studies that reported the high specificity and sensitivity of detection of Dengue NS1 Ag as early as 10 days of infection whether in experimentally infected or naturally infected mosquitoes [40–42]. Also, Mata and colleagues [43, 44] had reported the accuracy and reliability of NS1 antigen rapid immunochromatographic test to confirm DENV infection, especially in the acute phase during DENV-1epidemic, since it was a rapid test, simple to do by minimally trained health personnel that appropriate remote sittings without laboratory infrastructure.

However, the RT-LAMP technique revealed higher detection rates among the human samples and mosquito pools (60.8% and 44.4%, respectively). It is possible that this high infection rate was due to the selection of clinically suspected individuals in an outbreak of DF and the targeted surveillance of mosquito samples which were collected mostly from household settings and around residences of suspected cases of Dengue.

The RT-LAMP assay is a rapid, cost-effective, highly sensitive, and specific technique that has been proven to have greater efficacy in diagnosing DENV than immunochromatographic analysis. This is supported by its ability to detect DENV infection in the early stages of infection when NS1 antigen titers could not be detected and the antibody titers of IgM started to rise [36]. It also has the advantage of being a qualitative and quantitative technique, applicable

and convenient for DENV serotyping, especially in low-resource healthcare settings as a valuable test for diagnosing dengue [23].

Remarkably, all DENV serotypes were detected in the study specimens, with DENV-1 being the most common, followed by DENV-3 in both human and mosquito samples. The presence of a mixed infection with two, three, or even four serotypes in both populations was a noteworthy finding that postulates the wide diversity of DENV isolates in the newly emerged locality. Overall, 14 out of 16 DENV-positive mosquito pools and 26 serum samples showed concurrent infection with combined DENV serotypes. Only five human serum samples and two mosquito pools revealed infection with a single serotype, which was predominantly the DENV-1 serotype. The observed genetic diversity of DENV serotypes is most likely due to their high capacity to mutate [8, 45]. These results are consistent with previous reports demonstrating the common occurrence of co-circulating serotypes of the DENV in the wild [46, 47].

The observed variations among DENV populations either in geographically distinct areas or within a particular locality could be attributed to the occurrence of spontaneous viral mutations and the importation of new genotypes from neighboring countries [48]. In addition, several factors may play a role in the wide variation of circulating serotypes in patients and field-caught mosquitoes and the presence of combined infection including multiple feeding behavior of *Ae*. *Aegypti* [49, 50], the high infection rates in humans, especially in naïve population during dengue outbreak, which may induce the emergence of infections with multiple serotypes in both humans and infected mosquitoes [49], the transovarian transmission of DENV in mosquitoes [50], and the presence of asymptomatic undiagnosed cases which may act as a source of infection to the vector [49].

Interestingly, the diversity of DENV serotypes in certain populations was reported in previous studies. Dash et al. (2004) considered DENV-2 and DENV-3 to be the most prevalent genotypes for potential dissemination. Meanwhile, DENV-1 was shown to be the leading serotype in circulation, along with the dominant DENV-2 and DENV-3 serotypes [51]. Consistent with this, Ekwudu et al. (2020) observed higher numbers of vectors bearing DENV-1 and -2 in Colombia. This is reasonable as DENV-1 and -2 serotypes generally replicate at higher RNA levels, showing high infectivity and increased virulence in the *Aedes* mosquito, contributing to their global dissemination and higher disease burden than for other serotypes [38].

Upon mosquito-based screening of DENV in Singapore, the infection rates of *Ae*. *aegypti* and *Ae*. *albopictus* populations were relatively low (1.33% and 2.15%, respectively). However, the dominant dengue serotypes were mainly DENV-1 serotypes, followed by DENV-2 and DENV-3, and then DENV-4 [39], which is in accordance with our results. In addition, in Indian studies in recent decades, the DENV-1 serotype has mostly been isolated [52].

Although the small sample size has limited the scope of this study, up to our knowledge, this is the first report of serotyping of DENV in an outbreak in Egypt. Further studies are needed to confirm the association between the prevailing serotypes in patients and field-collected mosquitoes from the same residence. Correlation of DENV serotype infection with clinical presentations is also recommended. Additionally, more molecular studies on wide geographical regions are crucial to explore virus genotypes and virulence factors in both humans and vectors to provide a better insight into the virus dynamics and distribution.

Conclusion

The present study documented the occurrence of a second DENV outbreak in three Egyptian governorates in Upper Egypt. Field surveillance near the patients' residences revealed the reemergence of both larval and adult mosquitoes of *Aedes aegypti*, the main vector for DF transmission, supporting the reestablishment of this mosquito vector species in Egypt.

Screening of DENV in humans and mosquito populations revealed high infection rates by RT-LAMP assay, which proved to be more effective than the NS1 immunochromatographic strip assay. Moreover, DENV-1 was the most prevalent dengue virus serotype in both humans and mosquito vectors, with most of the collected samples additionally being infected with other serotypes. The RT-LAMP assay greatly enhances the diagnosis of suspected dengue patients as well as DENV screening of field-collected mosquitoes to evaluate local virus activity at a certain time, understand viral movement, and identify transmission hotspots. Vector control and vaccination programs should be enhanced to prevent future outbreaks.

Supporting information

S1 Raw images.
(PDF)

Author Contributions

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