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Detection of Dengue Fever Virus Serotype – 4 by using One-Step Real-Time RT-PCR in Hodeidah, Yemen

Murad Alahdal[1,2], Jamal Al-Shabi[1,3], Mahmoud Ogaili[1,4], Qais Yusuf Abdullah[3], Said Alghalibi[3], Aisha O. Jumaan[5] and Mohammed Amood AL-Kamarany[6,7*]

[1]National Center for Public Health Laboratory (NCPLH), Sana'a, Yemen.
[2]Department of Biology, Microbiology Branch, Faculty of Sciences, Sana'a University, Yemen.
[3]Department of Laboratory of Molecular Genetic, Faculty of Agriculture, Sana'a University, Yemen.
[4]Department of Medical Laboratory, Faculty of Medicine and Health Sciences, Hodeidah University, Yemen.
[5]Independent Consultant, Seattle, USA.
[6]Department of Pharmacy Practice, Faculty of Clinical Pharmacy and Tropical Medicine Center, Hodeidah University, Hodeidah City, Yemen.
[7]Program of Diagnosis and Drug, Tihama Foundation for Drug Studies and Research, Hodeidah City, Yemen.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MA, JA and MO designed the study, wrote the protocol and analyzed the samples. Authors MAA and AOJ helped in co-infection study, performed the statistical analysis, wrote the first draft of the manuscript and managed literature searches. Authors QYA and SA supervised of the study. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: alkamarany@yahoo.com;*

ABSTRACT

Background: Dengue and other fever like illnesses including chikungunya and malaria are common in Hodeidah, Yemen. Several outbreaks confirmed the presence of dengue serotypes 1-3.
Aim: Confirm dengue fever infection and identify the circulating dengue virus serotypes in Hodeidah, using real time one step Reverse Transcription–Polymerase Chain Reaction (RT-PCR).
Methods: Suspected dengue cases presented to health facilities between September 2012 and June 2013. Cases were informed about the study and asked to participate; 179 patients consented and were interviewed and blood samples were collected. The samples were tested at the National Centre of Public Health Laboratories (NCPHL) in Sana'a. Samples were initially tested by Enzyme Linkage Immunosorbent Assay (ELISA). Viral RNA was then extracted and prepared for serotypes detections using real time RT–PCR in one step pathway. Furthermore, agarose gel electrophoresis documentation system was used to confirm dengue serotypes.
Results: Dengue virus was confirmed by RT-PCR in 69 of 179 specimens. The four dengue fever serotypes were identified. DENV-4 was the predominant serotype at 31.88%, followed by DENV-2 at 23.18%, DENV-3 at 20.28%, and DENV-1 at 10.14%. Concurrent infection with more than one serotype was detected in 14.49% of the specimens.
Conclusion: We confirmed dengue virus infection using real time RT-PCR and identified DENV-4 serotype for the first time in Yemen. We also detected concurrent infections with more than on serotype. All serotypes are now present in Yemen increasing the risk of severe dengue and dengue hemorrhagic fever in future infections.

Keywords: Dengue viral infection; DENV-4; Hodeidah, Yemen.

1. INTRODUCTION

Dengue virus (DENV) is a mosquito-borne virus circulating in tropical and subtropical regions of the world [1]. It causes a broad spectrum of diseases, including in-apparent infection, flu-like mild undifferentiated fever, classical dengue fever and dengue hemorrhagic fever causing high rates of morbidity and mortality [2]. In recent decades, the incidence of dengue infection has increased around the world and is now endemic in more than 100 countries. The World Health Organization (WHO) estimates that about 50 million dengue infections occur annually [3,4]; however, a recent publication reported that about 96 million apparent and 294 million unapparent infections occurred in 2010 [5].

Dengue fever and dengue hemorrhagic fever are caused by one of four closely related but antigenically distinct, virus serotypes (DENV-1,4), of the genus *Flavivirus* [6]. The four viral serotypes are transmitted from viraemic to susceptible humans mainly by bites of the females' mosquito *Aedes aegypti* and *Aedes albopictus* during daylight period. Recovery from infection by one serotype provides lifelong immunity against that serotype but confers only partial and transient protection against subsequent infection by the other three [7].

Multiple dengue fever outbreaks occurred in Yemen with the first laboratory confirmed occurring in 2002 in Shabwah Governorate. By 2014, all the ten costal Governorates in Yemen reported at least one outbreak. Hodeidah Governorate was the most affected with at least eight outbreaks reported between 2004 and 2014 [8,9]. Previous PCR laboratory analyses of specimens from these outbreaks were conducted outside Yemen and detected three DENV serotypes (1, 2 and 3) [10]. We conducted this study to confirm dengue fever infection and identify the circulating dengue virus serotypes in Hodediah, Yemen, using one–step real time Reverse Transcription- Polymerase Chain Reaction (RT–PCR) for the first time in Yemen.

2. METHODS

2.1 Study Designs

Dengue is a reportable disease in Yemen and surveillance officers collect basic demographic information and blood specimens for confirmation. Suspected dengue cases presenting with undifferentiated fever to health centers were identified from 24 dengue endemic districts in Hodeidah from September 2012-June 2013. Cases received a verbal simple explanation about the study and asked if they would be willing to participate and agree to provide blood specimens. A total of 179 cases agreed and were interviewed using a questionnaire collecting information on age, sex,

and clinical symptoms and blood samples were collected. Confidentiality of the collected data was achieved by keeping data record in a locked room with limited access.

2.2 Samples Collection

Whole 15 ml blood samples were collected on average 4-6 days from onset of fever. The samples were collected in disposable sterile vacuum blood collection tubes with red caps / stoppers without any additives. We allowed the blood to clot by leaving it undisturbed at room temperature. This usually takes 15-30 minutes. We then removed the clot by centrifuging at 2000 x g for 10 minutes. We immediately transferred the supernatant (serum) to Eppendrof tubes by using a Pasteur pipette. The samples were maintained at 2-8°C while handling. Then, they were stored at –20°C for a maximum of 7 days until they were transported to National Center of Public Health Laboratory. Following ELISA analysis and viral RNA extraction, samples were stored at -80°C. Finally, samples were transferred to the department of molecular biology for real-time RT–PCR analysis.

2.3 Analyses of Specimens Based on Elisa Method and Real-Time Rt – Pcr in One Step Pathway

Sera from all specimens were tested for anti-dengue immunoglobulin (IgM) by Enzyme Linked Immunoassay (ELISA) of *Novagnost kits, GmbH, Germany* [11,12]. Total viral RNA were extracted from 140 µL of human serum specimens by using the QIAamp viral RNA kit (Qiagen, Inc., Germany) according to the manufacturer's instructions. After that, the master mix of dengue virus genome was prepared for one-step RT-PCR reaction according to (iscript one-step RT-PCR kit biorad, USA) cat. 170-8895. (water PCR, 10X RT-PCR reaction buffers, MgCL2 , DNTP deoxynucleotide mix, RNAse inhibitor rox dye iscript RT enzyme, and the primers used were DEN-1F (5'-CAAAAGGAAGTCGTGCAATA-3'), DEN-1R (5'-CTGAGTGAATTCTCTCTACTGA ACC-3'), DEN-2F (5'-CAGGTTATGGCACTGTCACGAT-3'), DEN-2R (5'-CCATCTG CAGCAACACCATCTC-3'), DEN-3F (5'-GGACTGGACACACGCACTCA-3'), DEN-3R(5'-CATGTCTCTACCTTCTCGACT TGTCT-3'), DEN-4F (5'-TTGTCCTAATGATG CTGGTCG-3'), and DEN-4R (5'-TCCACCTGA GACTCCTTCCA-3') that already prepared from Bio-Rad kit components according to CDC protocol, 2012 for detection and serotyping of dengue viruses by real time RT-PCR. The extracted viral RNA was transformed to cDNA and amplified in one step by using Applied Biosystem (ABI-7300 RT-PCR) for detection and serotyping of dengue viruses which was set with CDC protocol thermal cycler program in the one step pathway reaction [13].

The PCR products were migrated on agarose gel to show the serotypes bands in comparison with suitable DNA marker (HyperLadder™ 100bp, Cat No. BIO-33056, Size. 100 Lanes, Bioline Company, UK). The migration was done on 1% agarose gel and 1X TBE buffer by using 120 vlt for 30 minutes. In addition, gels were photographed using gel documentation system (syn gene) and the reproducible banding patterns of each serotype. Each gel was analyzed using (Gel Analyzer 3) program [14].

2.4 Statistical Analysis

We used Excel 2010 to analyze the data performing descriptive analysis. Chi-square test was used to make comparisons among categorical variables. A p -value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Analysis of Dengue Virus Serotypes Based on Real-Time RT-PCR

All 179 specimens tested positive using IgM ELISA; however, only 69 (38.5%) tested positive for dengue virus using RNA viral extractions. We identified all four serotypes. The predominant serotype was DENV-4 at 31.88%, followed by DENV-2 at 23.18% and, DENV-3 at 20.28%, then DENV-1 at 10.14% (Fig. 1). We found 10 patients (14.49%) with concurrent infections with more than one serotypes. Eight of the patients (80%) had serotype (4) as one of the concurrent types. Two (20%) had DENV (1,3), three (30%) had DENV (2,4), three (30%) had DENV (3,4) and two (20%) had DENV (1,3,4).

The migration of PCR products presented four different genetic bands which confirmed the four detected dengue virus serotypes in comparison with hyper DNA marker (Fig. 2). The photographs were analyzed using gel analyzer 3 program for the molecular weight of genetic bands and relative mobility of genetic bands on agarose gel 0.5 gm/50 ml of 1X TBE and the results confirmed the electrophoretic patterns of DENV-1 (1.567 kbp), DENV-2 (1.466 Kbp), DENV-3 (1.407 Kbp), and DENV-4 (1.361 Kbp) of dengue virus.

All 69 patients had fever for a period of 1-2 days before presenting to health centers. The most common clinical symptoms experienced by cases included headache, retro-orbital pain, arthralgia and thrombocytopenia, regardless of the serotype. Seven (10.4%) cases presented with hemorrhagic fever, four had DENV-4, 2 had DENV-3, and 1 case had DENV-1. Two cases died, with dengue serotypes (1) and (3) (Table 1). Cases with concurrent infections presented with more severe outcomes. Of the 10 with concurrent infections, eight (80.00%) had dengue hemorrhagic fever and four (40.00%) died (Table 1). Two of the 10 with concurrent infections had three serotypes DENV (1,3,4); both presented with hemorrhagic manifestation and one died.

Patients ranged in age from 14-42 years regardless of serotypes. Dengue fever was confirmed significantly more in males 65.22% than females 34.78% ($p < 0.05$). Six (60.00%) of those with concurrent infections were males. All serotypes were more common among males, except serotype 2 with 10 (62.50%) of the 16 infected with this serotype were females.

4. DISCUSSION

In just the past decade, the significance of dengue as a threat to health and economic burden on health services has increased substantially. The first recorded epidemic of dengue fever in Yemen occurred in 1954, which affected 98% of the population in Hodeidah [15]. In 1984, travelers returning from Yemen to USA were serologically confirmed to have dengue 2. United Nations International Children's Emergency Fund (UNICEF) reported that dengue hemorrhagic fever was a notable disease in Yemen since 1994 [16]. The first laboratory confirmed dengue outbreak occurred in 2002, in Shabwah Governorate. Later, WHO reported that dengue fever became endemic in some parts of Yemen, especially the coastal regions including Tihama in Hodeidah [17,18].

The detection of dengue fever viruses serologically is not specific because of cross reactivity due to the close similarities among *Flaviviruses* including dengue, and *Chikungunya* that are related [19,20]. Our study was the first to confirm dengue virus disease and identify the serotypes in Yemen using one step pathway real-time Reverse Transcription- Polymerases Chain Reaction. The study confirmed that only 69 (38.50%) of the 179 IgM positive samples were positive for dengue fever viruses infection This difference confirms that the detection of dengue virus by real-time RT-PCR provides more accurate results and that IgM results can be misleading [21]. The study also confirmed the presence of the four different serotypes of dengue virus circulating in Hodeidah Governorate. WHO reported that only three dengue fever serotypes were circulating in Yemen [22] indicating that our study identified DENV-4 for the first time in Yemen. Our study also documented concurrent infections with more than one serotype; this supports previous study [23-25].

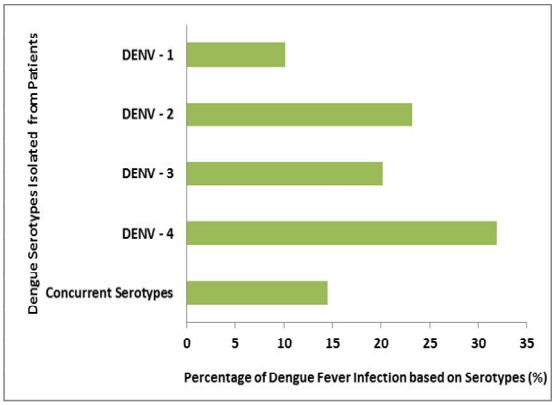

Fig. 1. The percentage of dengue virus serotypes as identified by real-time RT-PCR in one-step pathway isolated from specimens collected from Hodeidah governorate

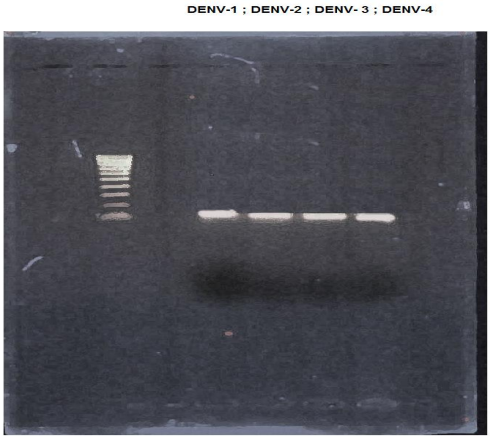

Fig. 2. Electrophoretic patterns of DENV-1 (1.567 kbp), DENV-2 (1.466 Kbp), DENV-3 (1.407 Kbp), and DENV-4 (1.361 Kbp) of dengue virus on the agarose 0.5 gm/50 ml of 1X TBE, Hodeidah governorate, Yemen

Table 1. Clinical history of patients and dengue virus serotypes and concurrent serotypes n = 69

Serotypes	Gender				Mean age and SD	Hemorrhagic cases	Mortality cases	Total	Percentage %
	Male		Female						
	No	%	No	%					
DENV-1	5	10.14	2	2.89	18±1,61	0	1	7	10.14%
DENV-2	6	11.59	10	14.49	23±5,44	0	0	16	23.18%
DENV-3	13	20.28	1	4.23	34±3,80	0	0	14	20.28%
DENV-4	14	23.18	8	13.04	22±6,21	2	0	22	31.88%
Concurrent serotypes	38		21			2	1		
DENV-1 and 2	2		-		28±0	1	-	2	2.89
DENV-2 and 4	2		1		31±8	2	-	3	4.34
DENV-3 and 4	-		3		22±4	1	1	3	4.34
DENV-1,3 and 4	2		-		23±2	2	1	2	2.82
Total	44	63.76%	25	36.23%		8	3	69	100%
						11,59%	4.34%		

In our study, more cases were identified with DENV-4 serotype compared to the other serotypes suggesting that the population has higher immunity to the endemic serotypes. Although, the clinical symptoms were similar among those will all the serotypes, the risk of developing DHF in DENV-4 patients was higher (25.00%). It is possible that these patients have had a previous infection with another serotype and this is a second infection leading to higher hemorrhagic manifestation as expected [25]. Our study supports other studies that found that males have a higher infection rate than female [16,17]. In Yemen, men are more likely to have exposed skin that women and thus would have a higher risk of mosquito bites. Patients with concurrent infections in our study had a higher rate of hemorrhagic dengue fever and deaths as reported in earlier studies in India and Saudi Arabia [26,27].

5. CONCLUSION

Dengue virus infection was confirmed using real-time RT-PCR analysis in Yemen for the first time. We detected all four serotypes and DENV-4 was detected for the first time. We also documented concurrent infection with more than one serotype. The circulation of all dengue serotypes puts the population at a higher risk of dengue hemorrhagic fever during future infections. Therefore, surveillance needs to be strengthened so that cases and outbreaks are identified early to prevent the spread of the infections and for speedy and proper management of cases. Finally, control measures need to be instituted to prevent future infections and outbreaks.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Gubler DJ. Dengue and dengue haemorrhagic fever in the Americas. Puerto Rico Health Sciences Journal. 1987;6 (2):107–111.
2. Jimenez-Lucho VE, Fisher EJ, Saravolatz LD. Dengue with hemorrhagic manife sta tions: An imported case from the Middle East. American Journal of Tropical Med Icine and Hygiene. 1984;33(4):650-653.
3. World Health Organization (WHO). Global strategy for dengue prevention and control 2012-2020. Geneva; 2012.
4. Centers for disease control and prevention (CDC). Dengue and dengue hemorrhagic fever: Information for health care practitioners - Division of Vector-borne Infectious Diseases; 2009.
5. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Bro wnstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI. The global distribution and burden of dengue. Nature. 2013;496(7446):502–507.
6. Baia Z, Liub Q, Jiang L, Liu L, Cao Y, Xu Y, Jing QL, Luo L, Yang ZO, Jiang YQ, Chen WJ, Di B. Complete genome sequence of dengue virus serotype 4 from Guangzhou, China. Genome A. 2013;1(3):1–2.
7. Guha-Sapir D, Barbara S. Dengue fever: New paradigms for a changing epidemio logy. Emerging Themes in Epidemiology. 2005;2(1):1-10.
8. MoPHP. Reporting system of infectious diseases. Ministry of Public Health and Population, Republic of Yemen; 2014.
9. Al-Garadi MA. Epidemiological review of dengue fever in Yemen. International Journal of Advanced Research. 2015;3(7): 1578-1584.
10. Madani TA, Abuelzein el TM, Al-Bar HM, Azhar EI, Kao M, Alshoeb HO, Bamoosa AR. Outbreak of viral hemorrhagic fever caused by dengue virus type 3 in Al-Mukalla, Yemen. BMC Infect Dis. 2013;13(136):1–6.
11. Burke DS, Nisalak A, Ussery MA. Antibody capture immunoassay detection of Japan ese encephalitis virus immunoglobulin M and G antibodies in cerebrospinal fluid. Journal of Clinical Microbiology. 1982;15(6):1034-1042.
12. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchriya S, Chingswasdi V, Suntayakorn S, Puttisri P, Hoke CH. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. American Journal of Tropical Me Dicine and Hygiene. 1989;40(4):418-427.
13. Naval Medical Research Unit 3 (NAMRU3). Dengue 1, 2, 3 and 4 RT-PCR Worksheet for SOP, Virology Research Program, Cairo, Egypt; 2011.
14. Harris ETG, Roberts SL, Selle J, Kramer LD, Valle S, Sandoval E, Balmaseda A. Typing of dengue viruses in clinical

specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. Journal of Clinical Microbiology. 1998;36(9):2634-2639.
15. NAS. In: Tropical health: A report on a study of needs and resources. Washington DC, National Academy of Science, National Research Council. 1962;107-108.
16. United Nations International Children's Emergency Fund (UNICEF). Yemen Situation Report Reporting Period, 2012.
17. Bin Ghouth AS, Amarasinghe A, Letson GW. Dengue outbreak in Hadramout, Yem en, 2010: An epidemiological perspective. Am J Trop Med Hyg. 2012;86(6):1072-6.
18. Yusuf QA, Ogaili M, Alahdal M, Amood AL-Kamarany M. Dengue fever infection in Hodeidah, Yemen: Risk factors and socioeconomic indicators. British Biomedi cal Bullieten. 2015;3(1):58–65.
19. Simmons CP, Farrar JJ, Nguyen VV, Wills B. Dengue. New England Journal of Medicine. 2012;366(15):1423-32.
20. Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expre ssion and replication. Annu Rev Microbiol. 1990;44:649-88.
21. World Health Organization (WHO). Den gue Guidelines for Diagnosis, Treatment, Prevention and Control, Geneva; 2009.
22. World Health Organization (WHO). Dengue: Call for Urgent Interventions for a Rapidly Expanding Emerging Disease EM/RC58/3; 2011.
23. Rezza G, El-Sawaf G, Faggioni G, Vescio F, Al Ameri R, Riccardo De Santis, Helaly G, Pomponi A, Metwally D, Fantini M, Qadi H, Ciccozzi M, Lista F. Co-circulation of dengue and chikungunya viruses, Al Hudaydah, Yemen, 2012, Emerging Infectious Diseases. 2014;20(8):1351-1354.
24. Lardo S, Utami Y, Yohan B, Seri MMU. Tarigan, Santoso WD, Nainggolan L, Tedjo Sasmono R. Concurrent infections of dengue viruses serotype 2 and 3 in patient with severe dengue from Jakarta. Indonesia Asian Pacific Journal of Tropical Medicine. 2016;9(2):134–140.
25. Preeti B, Harendra SC, Anubhav P Kavita D, Lalit D, Randeep G, Sushil KK, Shobha B. Concurrent infections by all four dengue virus serotypes during an outbreak of dengue in 2006 in Delhi, India. Virology Journal. 2008;5 (1):1–5.
26. Klungthong C, Zhang C, Mammen MJ, Ubol S, Holmes EC. The molecular epidemiology of dengue virus serotype 4 in Bangkok. Thailand. Virology. 2004;329(1): 168–179.
27. Ahmed M. Clinical profile of dengue fever infection in King Abdul Aziz University Hospital Saudi Arabia. J Infect Dev Ctries. 2010;4(8):503-510.

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