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STUDY THE PREVALENCE OF DENGUE MOSQUITO SPECIES AND VIRUS SEROTYPES IN MULTAN

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ABSTRACT

Dengue, a mosquito instinctive viral septicity is considered as a major unrestricted problem worldwide. Dengue virus is associated with the properties of encased, positive strand RNA viruses. Around 50 million individuals are at danger with 24,000 passing away year after year globally. In Pakistan during the most recent few years dengue has become an evolving vector endured disease. Dengue infection is triggered by DENV1, DENV2, DENV3, DENV4 and DENV5. Present study was carried out to identify the dengue mosquito species and virus serotypes prevailing in Multan city. Fully-grown mosquitos were sampled with aspirator and larvae with dipper from different nine areas of Multan city. Samples were well-preserved in 70% alcohol and carried to laboratory conditions in Department of Zoology, GC University Faisalabad. Mosquito were recognized and samples were kept at -20°C for PCR. Real time PCR were done to identify the presence of virus in mosquito samples. Analytical results of sample exhibited the existence and nonexistence of dengue pathogen in Multan city. The PCR result shows the presence of dengue virus in Gulgasht colony and jail road. Out 250 collected mosquitoes, 180 (72%) were identified as *A. aegypti* while 70 (28%) were identified as *A. albopictus*. These results indicate that *A. aegypti* was more prevalent in the survey areas of Multan. Significant population of *A. aegypti* was observed in the months of July (18%), September (18.8%), November (22%), February (22%) and March (19.2%). The results of RT–PCR and gel electrophoresis reveled that only two pools out of nine were positive with dengue viral RNA. The dengue virus serotype was identified as DENV-3 in both negative pools.

Keywords: Dengue, Mosquito species, Prevalence, Virus

INTRODUCTION

There are more than 3,000 species of mosquitoes, but the members of three genera endure the primary concern for the spread of human diseases. *Anopheles* mosquitoes are the solitary species known to transmit malaria. They also spread filariasis (also called elephantiasis) and encephalitis. There are small insects belonging to the family Culicidae of the order Diptera. This family contains round about 3500 species which have been grouped into 42 genera and 135 subgenera (Rueda, 2008). *Culex* mosquitoes carry encephalitis, filariasis and the West Nile virus while *Aedes* mosquitoes carry yellow fever, dengue and encephalitis viruses (Belkin, 1962).

The various serotype of dengue virus are transmitted to humans by infected *Aedes* mosquito. This mosquito borne dengue viral infection is regarded as a major public health problem throughout the world. Dengue has four distinct serotype including $DENV_1$, $DENV_2$, $DENV_3$ and $DENV_4$. Dengue virus with serotype DENV-2 and DENV-3 are associated with severe disease that is followed by secondary dengue infection (Lanciotti *et al.*, 1992). In Pakistan, serotype-2 and serotype-3 are prevalent in different cities of Punjab including Multan. The geographical distribution of this disease is characteristically associated with the distribution of its principal vector, *Aedes aegypti* (Jacob, 2000). This mosquito is widely distributed in tropical and sub-tropical areas of the world (Gibbons and Vaughn, 2002).

First certified out-break of the dengue detoxification was

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documented in 1994 from the city of Karachi, in Sindh Province (Directorate of Malaria Control, Pakistan, 2013 unpublished data). Dengue fever has now affected the major cities and towns of Pakistan. Initially, dengue cases were reported in Karachi and Lahore but now it has spread all over the country. In 2003, dengue was reported from Haripur Hazara (KPK) with 1000 cases (7 causalities) and Nowshera with 2500 cases (11 deaths). In 2004, 25 confirmed cases were documented from Karachi (Sindh) and Islamabad. During the year 2005, five hundred cases with 13 deaths were reported from Karachi (Sindh). In 2006, dengue outbursts were reported from numerous districts of Punjab and Sindh province including Islamabad area with 5400 cases and 55 casualties. During the year 2007, main affected districts were Mirpur-Khas, Karachi, Hyderabad, Haripur, Rawalpindi, Lahore and Islamabad with 2700 confirmed cases and 24 casualties. In 2008, eighteen hundred cases were reported from the Lahore district in Punjab Province. Total reported cases of dengue infection during the year 2009 and 2010 were 500 and 5065, correspondingly (WHO, 2012; Jahan and Sarwar, 2013). In the post rainy season of the year of 2011, the world's most horrible epidemic of dengue infection occurred in Punjab Province where more than 20000 labs confirmed dengue patients and 300 deaths were reported in highly affected district (Lahore followed by Faisalabad) (Shakoor *et al.,* 2012; WHO, 2012; Jahan and Sarwar, 2013). During 2014, out of 185 reported dengue cases, 179 cases were from Sindh province and 6 from Punjab while no case of dengue infection was reported from the Provinces of Baluchistan and Khyber Pakhtunkhwa (Shakoor *et al.,* 2012). In Multan, health department has recorded 68 cases in 2011. In 2015, Dengue virus in Multan has also intensified and at least 273 patients were detected in which 183 showed positive results. In 2016, 164 dengue patients were admitted in Nishter Hospital and their number increased rapidly (Shamim, 2010). Keeping in view the intensity of dengue infection in different cities of Pakistan, present study was carried out to study the prevalence of dengue mosquito species and virus serotypes in Multan district.

MATERIALS AND METHODS

Multan city is located in the center of Punjab at latitude of 30.1984°N and altitude of 71.4687°E. The highest and lowest recorded temperature is approximately 52 °C and1°C, respectively. The average rainfall is roughly 186 mm. Multan features an arid climate with very hot summers and mild winters.

The collection of adult mosquitoes was done from nine marked areas of Multan (Table 1). Different sites of parks, fresh water pots, flower beds, tyres, clean water containers, water tanks and many more different sites were surveyed for collection of adult mosquito. The adults were collected by using net or aspirator and larvae with dipper. The collected samples were stored in 70% alcohol and brought to the department of Zoology, Government College University Faisalabad. The collected mosquitoes were divided into nine pools. Every pool contained twenty mosquitoes. First sampling was done in the month of July, September and October during 2016. Then second sampling was done in the month of February and March during 2017. After sampling, *Ae. aegypti* and *Ae. albopictus* were identified and separated. The collected specimens of only *Ae. aegypti* were preserved at -20^{o}C inside the refrigerator in the research laboratory of Entomology in the Zoology department of Govt. College University Faisalabad.

Table 1
The sampling areas of Multan specified for collection of *A. aegypti*

Sampling area	Sampling codes
Vehari chowk	MI
Shah Rukan-e-Alam town	M2
Gulgasht colony	M3
Basti khudabaksh	M4
Bosan road	M5
Jail road	M6
Lodhi colony	M7
Bhutta colony	M8
Jalalpurpirwala road	M9

RNA extraction was done by trizol isolation protocol (Chomczynski and Mackey, 1995). The tissue's samples were homogenized in 1ml of Trizol reagent per 50 to 100 mg of tissues using a glass Teflon or powder homogenizer. Then samples were incubated for 5 minutes at room temperature and centrifuged to remove cell debris. After that, supernatant was transferred to new tube.

The supernatant was added with 0.2 ml of chloroform per 1 ml of Trizol reagent, vortexed vigorously for 15 second and incubated at room temperature for 2-3 minutes. Then sample was again centrifuged at 12,000X g for 15 minutes at 2 to 8 ºC. After centrifugation, the mixer was separated into lower red phenol-chloroform phase and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. Then upper aqueous phase was transferred carefully into fresh tube. RNA was precipitated by adding 0.5 ml isopropyl alcohol and incubation was done at 15 to 30 ºC for ten minutes. Again centrifugation was performed at the same conditions. A gel like pellet was formed on the side and bottom of test tube. RNA pellet was washed with 75% alcohol. Then overtaxing and centrifugation was performed for 5 minutes. Whole procedure was repeated with ethanol to remove all debris. Re-

dissolving of RNA was done and RNA pellet was dried by air or vacuum for 5-10 minutes. Never dry the RNA pellet by centrifuge under vacuum. RNA sample were partially dissolved in A260/A-280 in ratio 1:6. Then RNA was dissolved in DEPC-treated water by passing solution a few times through a pipette tip. After that, spectrophotometry analysis was done to detect the dengue virus in mosquitoes. Dengue specific primers were synthesized from IDT technology USA. These primers were specific to four genotypes named DENV-1, DENV-2, DENV-3 and DENV-4 (Table 2).

Primers D1 and D2 were fulfilling the criteria and type specific primers (TS1, TS2,TS3 and TS4) were designed to anneal specifically to each of their respecting genomes (Lanciotti *et al.*, 1994) (Table 2)

Target viral RNA was converted to a DNA copy (c DNA) prior to enzymatic DNA amplification by use of reverse transcriptase (RT). Subsequent Taq-polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer (D1). All relevant aspects of the RT-PCR were initially optimized by use of quantified and purified dengue virus RNA to achieve a maximum level of sensitivity.

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) using single stranded RNA was used in the presence of a primer to synthesize a complimentary DNA strand. This enzyme was used to synthesize a first strand cDNA up to 7 kb. The tube contents were mixed and then incubated for 2 minutes. The 1 µl of M-MLV RT was added and then mixed up and down. After adding D2 primer, incubation was done for ten minutes. Then mixture was heated at 70 ºC for 15 minutes. Now cDNA could be used as template for amplification in PCR. (M-MLV reverse transcriptase product information sheet, 2015). The amplification process was performed by combining with reverse transcriptase of viral RNA followed by Taq polymerase in a single reaction. This yielded an equal or greater level of DNA product as separate RT reaction and PCRs.

Then reaction was allowed to proceed in a thermo cycler program to incubate it for 1h at 42 ºC and then proceeded with 35 cycles of denaturation at 94 ºC for 30 second. Primer annealing was done at 55 ºC for 1 minute. Then extension was performed at 72 ºC for 2 minutes. Final extension was performed on 72 ºC for 10 min. A second amplification reaction was initiated with 13 ul of diluted material. The reaction mixture was same but the primer D2 was replaced with the dengue virus type-specific primers mixture. That mixture contained TS-1, TS-2, TS-3 and TS-4. This reaction consisted of only 20 cycles. Initial denaturation of reaction was started at 94 ºC for 5 minutes. Second step of denaturation was performed again at 94 ºC for 30 seconds. After denaturation, annealing step was performed at 55 ºC for 1 minute. Extension step was done at 72 ºC for 2 minutes. The size of resulting RNA band was characteristic for each dengue virus.

After that, agarose gel electrophoresis was performed. Finally PCR products were run on 1.6% agarose gel electrophoresis at 80 voltages for one hour and gel was observed on GEL Doc apparatus. Different amplified bands were observed and images of results were saved.

RESULTS

Prevalence of dengue mosquito species

From Multan, 250 mosquitoes were collected from nine areas of Multan city. Out of these 180 mosquitoes (72%) were identified as *A. aegypti* while 70 (28%) were identified as *A. albopictus*. These results indicate that *A. aegypti* was more prevalent in the survey areas of Multan (Table 3).

Amplification profile image

Amplification profile image of *Aedesaegypti* shows that there are total nine pools of collected mosquito which were PCR-assayed using two types of primers already designed and a ladder of 1kb. The result of this gel image shows that two samples out of nine were positive for RNA virus. Sample-3 (M3) and sample-6 (M6) exhibited positive results for DENV-3 serotype. Seven pools showed negative result for dengue (Fig. 3).

Month wise prevalence of dengue mosquitoes

The climate of Multan is hot which become suitable for dengue mosquito growth in September, October and November. The results of survey study reveal that 45 (18% of total collected mosquitoes) individuals of *A. aegypti* were collected in the month of July. While 19% (47 adults) and 19.2% (48 adults) of total 250 *A. aegypti* individuals were collected in September and March, respectively. The maximum dengue mosquitoes (55 adults i.e. 22% of total dengue mosquitoes) were collected in the month of November and February (Fig. 3).

Nine pools and one negative control of mosquitoes (250 mosquitoes) were assayed. Every pool comprised of 25 mosquitoes. After applying RT –PCR, the result of gel electrophoresis showed that only two pools out of nine were positive with dengue viral RNA. The dengue virus serotype was identified as DENV-3 in both negative pools (Table 4).

DISCUSSION

Aedes aegypti is one of the major mosquitos transmitting dengue virus in the whole world (Bosio *et al.*, 2005). Millions of people are infected and many people lose their lives due to dengue virus which is spreading day by day (Guzman *et al.*, 2010).

Pakistan is one of the victimized country where dengue breaks out at a large scale. The spread of dengue disease is worse in Multan where 273 patients were brought to hospital and 183 patients showed positive result for dengue serotype-3. Our results also exhibited the presence of serotype DENV-3 in the assayed dengue mosquitoes from Multan. In 1985, a research was conducted to study the prevalence of dengue virus infection in Pakistan. It showed that about 50-60% of the Pakistanis, especially those living in Karachi, were haemagglutination inhibition (HI) antibody positive for West Nile, Japanese encephalitis and DENV-2 Flaviviruses. These cases rapidly increased from July to October in patients ranging from 6 to 20 year age (Chong *et al.*, 1994). The results of present study also demonstrated the prevalence of dengue mosquitoes during July, September, November, February and March with peak incidence in November and February. In 1994, first outbreak of DHF was reported in Pakistan, out of

16 patients, 15 had dengue IgM identified using DEN-2 antigen. It was also observed that in three out of ten patients of dengue virus were infected with DEN-1 and DEN-2 (Paul *et al*.,1998). In 1995, DEN-2 infection was reported from Baluchistan province. In 1998 from Baluchistan, DENV-1 and DEN-2 were found by using ELISA study (Akram *et al.*, 1998). These results are not in confirmatory with the results of present study. This variation may be attributed to geographical distribution of the localities surveyed. The current study shows the circulation of DEN-2 and DEN-3. In 2013, dengue outbreak in district swat is the first reported outbreak in literature in Khyber Pukhtunkhwa, in which more than 8000 dengue incidences were recorded from different areas of this region. Among the nine infected areas, the rate of infection was found higher in urban areas as compared to rural and infection were observed in Saidu (35%), Mingora city (27%), Matta (12%), Kanju (09%), Patehpur (04%), Charbagh (07%) and Khwazakhela 06% (Ali *et al.*,2013). In 2005, outbreak of DHF in Karachi, DEN-3 was reported among the few tested patients. In serum of children in Karachi, DEN-1and DEN-2 was found using serological studies. DEN-2 and DEN-3 were found to be co-circulated during 2006 outbreak in Karachi (Khan *et al.*, 2006). Similarly in 2013 dengue outbreak in swat, out of 6000 dengue patients (male=4121 and female=1879), the male infection rate was found more as compared to female, the cases confirmation was done for antidengue antibodies in which NS1 positive cases among population were male=3359, female=1171, while IgG, IgM observed were male=762, female=708. The positive blood samples were subjected to RT-PCR method, the serotypes present in this outbreak were found DENV-2 and DENV-3 respectively, which shows similarities to 2005 dengue outbreak during Karachi because of the same serotypes detected in the blood of patients DENV-3 (Jamil *et al.*, 2007). In 2006 outbreak in Pakistan was found to be closely related to DEN-3 in 2004 outbreak in New Delhi (Gupta *et al.*, 2005). In 2008, a dengue outbreak was reported in Lahore infecting large number of citizens of Lahore samples were found to have DEN-4, DEN-2 and DEN-3 infection (Hamayoun *et al.*,2010). In 2009, it was reported that children living in Karachi had high levels of antidengue IgM antibody. Samples had concurrent infection with serotypes DEN-2 and DEN-3. Studies showed that serotype DEN-2 was dominant in samples of dengue virus infection collected during the period of three years from 2007 to 2009 (The Nation, 2011 unpublished data). In November 2010, it was reported by a private news channel that out of the 5,050 patients, 2,350 patients were from Sindh, 1,885 from Punjab and at 158 patients from Khyber Pakhtunkhwa. The blood samples of the patients had an infection with DEN-2 and DEN-1 (ARY NEWS). These resuts are partially in confirmatory with the results of present research because results confirm the prevelance of *A. aegypti* and dengue virus serotype-3 in Multan. The prtial difference in dengue virus serotypes may be due to the geographical variation and difference of dengue mosquito species. Based on the current study, it is concluded that *A. aegypti* is main source for the spread of dengue virus in Multan. The dengue virus serotype transmitted by dengue vector *A. aegypti* is serotype-3.

REFERENCES

Akram, D.S., A. Igarashi and T. Takasu, 1998. Dengue virus infection among children with undifferentiated fever in Karachi. Indian J. pediatrics, 65(5): 735-740.

Ali, N., M.K. Khan and A. Kausar, 2013. Study on mosquitoes of Swat Ranizai sub division of Malakand. Pak. J. Zool., 45(2): 503-510.

Table 2

List of primers, their sequence, genome position and size (bp).

Primer	Sequence	Genome position	Size in bp of amplified DNA product (primers)
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG -3'	134-161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644	511
TS-1	5'-CGTCTCAGTGATCCGGGGG-3'	568-586	482(D1 & TS1)
TS-2	5'-CGCCACAAGGGCCATGAACAG-3'	232-252	119 (D1 &TS2)
TS-3	5'-TAACATCATCATGAGACAGAGC -3'	400-421	290(D1 & TS3)
TS-4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506-527	392(D1 & TS4)

Table 3

Prevalence of dengue mosquito species in surveyed areas of Multan.

Total mosquitoes samples	***Density of Aedes aegypti*** **(Percentage of total sample)**	***Density of Aedes albopictus*** **(Percentage of total sample)**
250	180(72%)	70(28%)

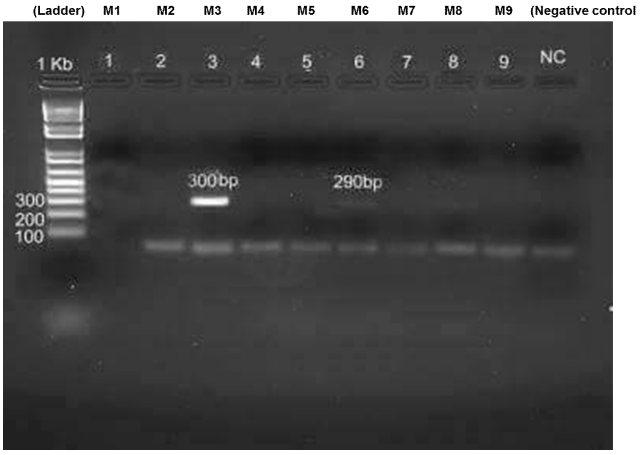

Fig. 1

Amplification profile image of *Aedesaegypti*.

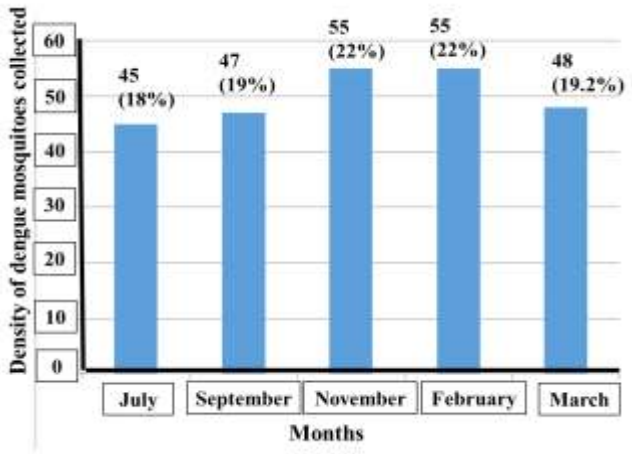

Fig. 2

Month wise data for detection of dengue mosquitoes during some specific months of 2016-2017

ARY News. Dengue deaths reach 31; over 5,000 infected. [Online]. 2010 [cited on 2011 Nov 11].

Belkin, J.N., 1962.The Mosquitoes of the South Pacific (Diptera: Culicidae). Sci., 140(3567): 638-651.

Bosio, C.F., L.C. Harrington, J.W. Jones, R. Sithiprasasna, D.E. Norris, and T.W. Scott, 2005. Genetic structure of *Aedes aegytpi* populations in Thailand using mictochondrial DNA. Amer. J. Trop. Medic. Hygiene, 72: 434-442.

Chomczynski, P. and K. Mackey, 1995. Short technical reports: Modification of the tri reagent procedure for isolation of RNA from polysaccharide-and proteoglycan-rich sources. Biotechnol., 19(6): 942-945.

Chong, C.F., B.L. Ngoh, H.C. Tan, E.H. Yap, M. Singh, L. Chan and Y.C. Chan, 1994. A shortened dengue IgM capture ELISA using simultaneous incubation of antigen and peroxidase-labeled monoclonal antibody. Clinical diagnostic virol., 1(5-6): 335-341.

Gibbons, R.V. and D.W. Vaughn, 2002. Dengue: An escalating problem. British Med. J., 324: 1563-1566.

Gupta, E., L. Dar, P. Narang, V.K. Srivastava and S. Broor, 2005. Serodiagnosis of dengue during an outbreak at a tertiary care hospital in Delhi. Indian J. Med. Res., 121(1): 36.

Guzman, M.G., S.B. Halstead, H. Artsob, P. Buchy, J. Farrar, D.J. Gubler and M.B. Nathan, 2010. Dengue: a continuing global threat. Nat. Rev. Microbiol., 8: 7-16.

Humayoun, M. A., T. Waseem, A.A. Jawa, M.S. Hashmi and J. Akram, 2010. Multiple dengue serotypes and high frequency of dengue hemorrhagic fever at two tertiary care hospitals in Lahore during the 2008: dengue virus outbreak in Punjab, Pakistan. Int. J. Infectious Diseases, 14:54-59.

Jacobs, M., 2000. Dengue: emergence as a global public health problem and prospects for control. Trans R. Soc. Trop. Med. Hyg., 94:7-8.

Jahan, N. and M.S. Sarwar, 2013. Field Evaluation of lethal ovitraps for the control of dengue vectors in Lahore, Pakistan. Pak. J. Zool., 45(2):305-15

Jamil, B., R. Hasan, A. Zafar, K. Bewley, J. Chamberlain, V. Mioulet and R. Hewson, 2007. Dengue virus serotype 3, Karachi, Pakistan. Emerging infectious diseases, 13(1): 182.

Khan, E., J. Siddiqui, S. Shakoor, V. Mehraj, B. Jamil and R. Hasan, 2007. Dengue outbreak in Karachi, Pakistan, 2006: experience at a tertiary care center. Transactions of the Royal Society of Tropical Medicine and Hygiene, 101(11): 1114-1119.

Lanciotti, R.S., C.H. Calisher, D.J. Gubler, G.J. Chang and A.V. Vorndam, 1992. Rapid Detection and typing of dengue viruses from clinical samples by using reverse transcriptase-plymerase chain reaction. J. Clinical Microbiol., 30: 545-551.

Lanciotti, R.S., L.G. Lewis, D.J. Gubler and D.W. Trent, 1994. Molecular evolution and epidemiology of dengue-3 viruses. J. General Virol., 75: 65-75.

Paul, R. E., A.Y. Patel, S. Mirza, S.P. Fisher-Hoch and S.P. Luby, 1998. Expansion of epidemic dengue viral infections to Pakistan. Int. J. Infectious diseases, 2(4): 197-201.

Rueda, L.M., 2008. Global diversity of mosquitoes (Insecta: Diptera: Culicidae) in freshwater. Hydrobiol., 595: 477-487.

Shakoor, M.T., S. Ayub and Z. Ayub, 2012. Dengue fever: Pakistan's worst nightmare. WHO South-East Asia J. Public Health, 1(3): 229-235.

Shamim, M., 2010. Frequency, pattern and management of acute dengue fever in Karachi, Pakistan. Asian J. Surg., 33(3): 107-113.

The Nation, 2011. Dengue and Government of Pakistan. Available from URL: http://nation.com.pk/pakistan-news-newspaper-dailyenglishonline/Regional/Lahore/11-Sep-2011/CM-takesstock-of-private-hospitals, 2011 and downloaded on November 11, 2011.

WHO, 2012. Dengue and dengue haemorrhagic fevers: diagnosis, treatment, prevention and control. 2nd edition. Geneva: World Health Organization. 84 PP.