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Detection of *Salmonella enterica*, Subsp. *enterica Serovar Typhi* in Dengue Virus Seropositive Samples

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ABSTRACT

Dengue virus infections and typhoid fever are among the endemic diseases in tropical and sub tropical regions of world. Bacteraemia with dengue co-infection is rare. Present study was designed to detect dengue virus RNA and then to test those samples for the detection of *S typhi* to check the co infection rate of these two infectious agents in Pakistani population. A total of 365 subjects were included in this study, out of which 224 (61.7%) were males and 141(38.2%) were females. The median age of patients was 40±32, percentage of dengue positive samples was found to be higher in age group 31y-40y and 41y-50y.75 % of total samples included in the study were positive for dengue virus RNA where as 24.6% were those in which dengue virus RNA was not detected. DENV-2 was found to be the most prevalent serotype while DENV-1, DENV-3 and DENV-4 were not detected in any of the sample. Of the 274 samples with DENV-2, only 4 samples were positive for *S typhi*. *S. typhi* was also detected in 3 patients who were negative for dengue virus PCR. The presence of *S. typhi* in dengue positive samples was further confirmed by sequencing PCR products.

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Authors' Contribution
MI conceived and designed the study. SY and MS collected samples and performed the experiments. IA and ZF analyzed the data. SY and IA wrote the article.

Key words
Dengue Virus infection, Typhoid fever, Co-infection, *S. typhi*

INTRODUCTION

Dengue virus infection is most important arthropod borne viral infection of humans. Dengue virus is a flavivirus within the flaviviridae family, and is transmitted to humans via mosquito *Aedes* genus especially *Aedes agypti* (Fatima *et al.*, 2011). The presence of dengue virus in the larvae of Aedes mosquitoes proved that it as the main culprit of vertical transmission of virus (Afzal *et al.*, 2015). Dengue virus RNA genome consists of a positive single-stranded RNA, which is approximately 11kb in length. The virus has four different serotypes namely DENV1, DENV2, DENV3, and DENV4 (Das *et al.*, 2008), and recently a fifth serotype of dengue virus DENV5 has also been reported (Mustafa *et al.*, 2015). The infection is common in tropical and sub tropical areas of the world including Asia, Africa, and Central and South America (WHO, 2009). Approximately 2.5 billion people are at the risk of dengue virus infection and there is possibility of 50 million dengue infection cases each year with 22,000 deaths, mainly in children (Das *et al.*, 2008). World Health Organization has estimated that the number of infected cases is increasing as well as the strength of outbreaks also increasing (WHO, 2009). Dengue virus infections may be asymptomatic, or may lead to undifferentiated fever known as dengue fever (DF) characterized by rapid onset of fever which can be accompanied by severe headache, retro-orbital pain, myalgia, arthralgia, gastrointestinal discomfort and usually rash, or can lead to dengue hemorrhagic fever (DHF), which is characterized by coagulopathy, increased vascular fragility, and permeability which can ultimately lead to dengue shock syndrome (DSS) (Das *et al.*, 2008).

Typhoid fever is one of the important bacterial infections. The infectious agent for typhoid fever is *Salmonella enterica* subspecies *enterica* serotype *Typh*i (*S. typhi*) belongs to family Enterobacteriaciae. The infectious dose for *S. typhi* varies from 1000 to 1 million organisms (Hornick *et al.*, 1970). *Salomonella enterica* are gram negative intracellular pathogenic bacteria which can infect human as well as many warm blooded animals, however serotype typhi are highly specific for humans (Popoff *et al.*, 2004; Baumler *et al.*, 1998). Typhoid fever is a food borne and water borne infection having an incidence rate of 1% in disease endemic areas. The incidence rate is higher in children of 5-15 years of age, however incidence rate is much higher in children <5 years of age in highly endemic areas (Sinha *et al.*, 1999; Lin *et al.*, 2000). The global incidence rate in 2000 was approximately 21,650,974 cases with 216,510 deaths (Crump *et al.*, 2004). South eastern and south central

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areas of Asia with poor sanitation and unclear water have been found to have highest incidence rate for typhoid fever where 100 cases/100,000 populations are reported each year (Crump *et al.,* 2004). In USA, Europe and other developed countries with good sanitary conditions and clear water typhoid fever cases are rare (Ackers *et al.,* 2000). The clinical manifestations and severity of typhoid fever vary with the patients. Patients undergo an asymptomatic period of 7-14 days. The onset of infection is usually accompanied with fever, influenza like symptoms with chills, frontal headache, malaise, anorexia, nausea, poorly localized abdominal discomfort, a dry cough, and myalgia. A coated tongue, tender abdomen, hepatomegaly, and splenomegaly are common (Parry *et al.,* 2002). At initial stages of infection, fever intensity is low, but progressively fever rises, and by second week of infection it is usually high and sustained (39° to 40°C) (Parry *et al.,* 2002).

Dengue virus infections and typhoid fever are among the most endemic diseases in tropics and sub tropical regions of world. Both diseases have been associated with poverty and underdevelopment with significant morbidity and mortality (Parry *et al.,* 2002), (Guzman *et al.,* 2010). Bacteraemia with dengue co-infection is rare. About 20 cases have been reported in the literature (Chai *et al.,* 2007). These comprise mainly isolated case reports. These isolated and sporadic cases generally involved Gram negative Enterobacteriaceae species (Pancharoen and Thisyakorn, 1998; Lee *et al.,* 2005; Charrel *et al.,*2003) with only 1 reported case of *S. aureus* co-infection (Chai *et al.,* 2007). Recently 11 cases of dengue and enteric fever co infection has been reported in different age groups in India (Sharma *et al.,* 2014). This research work is aimed to detect *S. typhi* in dengue seropositive samples, and to check how much common bacteremia is in dengue virus infected patients in Pakistani population.

MATERIALS AND METHODS

Sample collection

Total of 365 dengue fever blood samples were collected from Institute of Public Health, Lahore, Sheikh Zayed Hospital, Jinnah Hospital Lahore during 2011 outbreak. All these samples were reactive for IgM. A written informed consent was taken from each patient and a data sheet was filled for each patient containing information about age, sex, demographic characteristics, address and contact numbers. Study group constitute 224 (61.7%) males and 141 (38.2%) females and were divided into 6 different age groups, Group I (1-10 years), Group II (11-20 years). Group III (21-30), Group IV (31-40 years), Group V (41-50 years) and Groups VI (51-72 years).

Primer designing

Serotype specific dengue virus primers targeting C-prM gene junction were designed according to method described by Fatima *et al.* (2011), and were used for dengue virus serotype analysis. The amplified product size for specific serotypes were 411-bp for serotype-1, 403-bp for serotype-2, 453-bp for serotype-3 and 401-bp for serotype-4.

Salmonella typhi specific oligonucleotide primers were synthesized to amplify a 495 base pair fragment corresponding to 1036-1056, and 1513-1530 nucleotides in flagellin gene of *S. typhi* for first round of amplification, and 363 base pair fragment corresponding to 1072-1089 and 1416-1435 nucleotides in flagellin gene of *S. typhi* in second round of amplification according to method as described by Song *et al.* (1993).

Detection of dengue virus RNA and serotype analysis

Dengue Virus RNA was extracted from 200 µl of serum sample using GF-1 viral nucleic acid extraction kit (VIVANTIS technologies), according to the kit protocol. This extracted RNA was used for synthesis of cDNA which was further amplified in two rounds of nested polymerase chain reaction according to method described by Fatima *et al.* (2011). The PCR products were electrophoresed on a 2% Tris acetate acetic acid (TAE) agarose gel, stained with ethidium bromide, and evaluated under ultra violet transilluminator. The sizes of PCR products were estimated according to the migration pattern of a 100-bp DNA ladder (Fermentas Technologies USA).

Isolation and detection of bacterial DNA

Bacterial DNA was isolated using GF-1 nucleic acid extraction kit (vivantis technologies) according to kit protocol. This isolated DNA was then amplified in two rounds of nested polymerase chain reaction according to method described by Song *et al.* (1993). The PCR product of nested PCR was visualized on 2 % agarose gel stained with ethidium bromide (0.5µg/ml). The sizes of PCR products were estimated according to the migration pattern of a 100-bp DNA ladder (Fermentas Technologies USA).

Gel elution and sequencing of amplified bacterial DNA

Gel purification was done using GF 1 DNA recovery kit (vivantis technologies) according to kit protocol. The eluted DNA was used as a template for sequencing reaction. Sequence analysis of 363 base pair fragment corresponding to 1072-1089 and 1416-1435 nucleotides in flagellin gene of *S. typhi* was done on automated genetic analyzer according to the manufacturer's instructions (Big Dye Deoxy Terminators;

Applied Biosystems, Weiterstadt, Germany). The identity of obtained sequences was confirmed by BLAST search.

RESULTS

Detection of dengue virus by nested PCR

This study was carried out a total of 365 IgM positive dengue suspected patients. Table I shows that there were 10 (71%) patients in group-I which were positive for dengue virus RNA, 54 (72%) patients in group-II, 61(62%) patients in group-III, 64 (96%) in group IV, 56 (94%) patients in group-V and 39 (60%) patients in group-VI were positive for dengue virus RNA. Two patients of group-II, one patient each from group-III and IV and three patients from group-VI were positive for *S typhi*. While two patients from group-II, one patient each from group-IV and VI was found to be co infected with both dengue virus and *S typhi*.

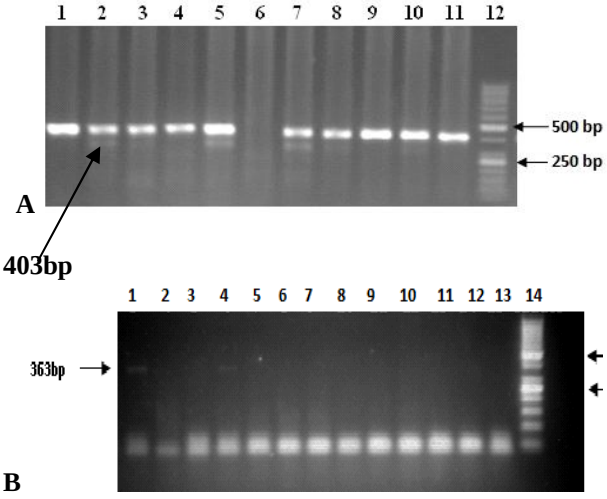

Fig. I. Nested PCR for detection of dengue virus (A) and *Salmonella Typhi* (B)
A, Lane 1: positive control, Lane 2-5 and 7-11: Amplification products of 403 bp from the nested PCR for dengue virus RNA serotype 2 positive samples, Lane 6: Sample was negative for dengue virus, Lane 12: 50 bp DNA size marker.
B, Lane 1 and 4: Amplification products of 363 bp from the nested PCR for *Salmonella* Typhi positive samples Lane 2-3 and 5-13: Samples were negative for Salmonella Typhi, Lane 14: 50 bp DNA size marker.

Serotyping of dengue virus by nested PCR

All 365 samples were analyzed for detection and serotyping of dengue viral RNA. Dengue virus RNA was detected in 274 (75.4%) samples while 90 (24.6%) samples were found negative for dengue. DENV-2 was detected in all 274 positive samples for dengue. Figure 1A shows the nested PCR results for dengue virus RNA detection.

Samples were also tested for the presence of DENV-1, DENV-3 and DENV-4. None of the sample was found positive for DENV-1, DENV-3 and DENV-4. This indicates that Serotype 2 is the most common serotype.

Table I.- Number of samples positive for dengue virus and co infection with *S. typhi*.

Age groups	Age (years)	No of samples	Dengue infected	*S. Typhi* infected	Co-infection
Group I	1-10	14	10 (71%)	-	-
Group II	11-20	75	54 (72%)	2	2
Group III	21-30	99	61(62%)	1	-
Group IV	31-40	66	64 (96%)	1	1
Group V	41-50	59	56 (94%)	-	-
Group VI	51-72	64	39 (60%)	3	1

Table II.- Nest PCR results of *Salmonella typhi.*

Tests and results	*Salmonella Typhi*		Co infection with *Salmonella Typhi* and dengue virus	
	Positive	Negative	Positive	Negative
Total samples n=365	7	358	4	361

Detection of Salmonella Typhi *by PCR*

All 365 samples were analyzed to detect presence of *S. Typhi* by nested PCR, Figure 1B shows the presence of *S. Typhi* specific PCR bands in patients' samples detected by nested PCR, amplification of 363 bp fragment of flagellin gene identified by comparison with a 50bp molecular size DNA ladder marker. Total 7 samples out of these 365 were found positive for *S. Typhi*, while 358 samples were found negative by PCR.

Samples data was also analyzed for the presence of coinfection with *S. typhi* and dengue virus that was found in 4 samples. The results for co infection of dengue virus infection with *S. typhi* are shown in Table II.

All the 7 positive isolates of *S. typhi* were sequenced and analyzed for homology with other sequences in GenBank data base which showed 80% homology with *Salmonella enterica subspecie Enteric serovar Typhi* strain CT18.

DISCUSSION

Dengue virus infections and typhoid fever are among the endemic diseases in tropics and sub tropical

regions of world. Both diseases have been associated with poverty and underdevelopment with significant morbidity and mortality (Chai *et al.*, 2007; Lee *et al.*, 2005).

Dengue fever has emerged as an important arboviral infection in regions where environmental conditions are favorable for the growth of mosquito species that transmit dengue virus to humans (Dash *et al.*, 2004). Typhoid fever is one of the important bacterial infections, having incidence rate of 1% in disease endemic areas. The global incidence rate in 2000 was approximately 21,650,974 cases with 216,510 deaths (Crump *et al.*, 2004). Bacteraemia with dengue co-infection is rare. Sharma *et al.* (2014) have reported 11 cases of dengue and enteric fever co infection in India.

Present study was designed to detect dengue virus RNA and then test those samples for the detection of *S typhi* to check the co infection rate of these two infectious agents in Pakistani population. For this purpose total 365 samples were collected from different hospitals of Lahore, from dengue 2011 outbreak, which were all reactive for dengue virus IgM. IgM antibodies appear by days 3-5 of infection and increase to peak levels with about two weeks after the onset of symptoms, presence of dengue virus IgM is a clear indication of primary viral infection (Khan and Hassan, 2011).

There were 224 (61.7%) males and 141 (38.2%) females included in this study. There is conflicting data on the shift in median age of dengue virus infected patients. In the present study percentage of dengue positive samples was found to be higher in IV (31y-40y) and V (41y-50y) age groups, while in other groups the percentage was almost equivalent. The median age of patients was 40 ± 32. Several studies from Asia using surveillance data report increasing age of effected patients. In Indonesia, data from 1975 to 1984 showed an increase in incidence rates among young adults in Jakarta as well as in the provincial areas (Sumarmo, 1987). In contrast, a decrease in median age of patients affected with dengue from 2003–2007 was reported in a study in Pakistan by Khan *et al.* (2010). The most common age group affected in the recent outbreak was from 31-40 years and 41-50 years. A progressive increase in the proportion of children affected with dengue was also observed.

Total samples included in the study (75%) were positive for dengue virus RNA where as 24.6% were those in which dengue virus RNA was not detected. This high percentage of PCR negative samples may be due to the reason that majority of samples were collected from suspected dengue virus infected patients in post viremic phase, where the immune system usually clears the virus. Samples should be collected in acute phase of infection for the correct molecular characterization of the virus. Presentation of patients in post viremic phase or lower rate of viral isolation may be the reason for these samples with primary infection to be negative for dengue RNA detection. Another interpretation for these dengue virus RNA negative samples can be that these samples were false positive for IgM however it has been shown in studies that dengue virus antigen remain positive after DENV RNA has been cleared by immune system probably due to longer half life of NS-1 protein (Pok *et al*., 2010; Bessoff *et al*., 2010).

The C-prM gene junction of dengue virus was selected for serotyping as this region is not hyper variable and most of the mutations reported are of silent type. In the present study DENV-2 was found to be most prevalent serotype while DENV-1, DENV-3, DENV-4 were not detected in any of the sample. It was reported in a study that the presence of all the four serotypes of dengue virus in Lahore (Javed *et al.*, 2009) have reported dengue virus serotype 3 infection from Karachi (Jamil *et al*., 2007), but Fatima *et al*. (2011) have reported that DENV-2 was dominant serotype in positive samples of dengue virus infection collected during the period of three years from 2007-2009, moreover it has also been confirmed in a report carried out by Centre of Disease Control (CDC), Georgia, Atlanta, US, that DENV-2 is the most prevalent serotype in Lahore (Punjab). This foreign research report in 2012 confirmed the presence of DENV-2 in September and October 2011 epidemic in Punjab.

Of the 274 samples with DENV-2, only 4 samples were positive for *S. typhi*. *S. typhi* was also detected in 3 patients who were negative for dengue PCR. Dengue virus coinfection with bacteremia is rare and dual infection has been rarely discussed in past, has been sporadically published in case reports. Arya and Agarwal in 2008 have also reported a case of dengue infection which was also positive for *S. typhi*/paratyphi A, B group (Cunha *et al*., 2009). However, in other case reports dengue co infection with several bacterial species have been reported. In a study carried out during 2004 at Chang Gung Memorial Hospital-Kaohsiung in southern Taiwan, 7 patients were reported who had DHF along with concurrent bacteremia. With regard to concurrent bacteremia, *Klebsiella pneumoniae* was isolated from three patients, and *Rosemonas* species, *Moraxella lacunata*, *Klebsiella ozaenae*, and *Enterococcus faecalis* were isolated from one patient each (Lee *et al*., 2005). More over in 2007 Chai *et al.* (2007) have reported five cases of *MSSA* infection in conjunction with dengue fever. The presence of *S. typhi* in dengue positive samples was further confirmed by sequencing PCR, and the sequence was analyzed for homology sequences in

GenBank data base which showed 80% homology with *S. enterica* subspecies Enteric serovar *Typhi* strain CT18.

Two hundred and seventy patients positive for DENV-2, were negative for *S. typhi* PCR. In a case report published in 2009 by Cunha *et al.* (2007) it was reported that atypical dengue fever mimicked typhoid fever in a college student so there can be possibility that the samples in present study which are positive for dengue serotype 2, but negative for *S. typhi*, the dengue fever is mimicking typhoid fever.

CONCLUSION

From this study it can be concluded that a person can be dually infected with dengue fever as well as with typhoid fever.

Conflict of interest declaration

We declare that we do not have conflict of interest.

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