Level of Vitamin D, TNF-Alpha and Calcium in Patients with Dengue Fever, Dengue Hemorrhagic Fever and Dengue Shock Syndrome

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Abstract—

Background: **Vitamin D has been widely studied for its beneficial role in treating different diseases. Dengue epidemic has affected many house hold since last decade. Vitamin D seems a gate through in its treatment as it inhibits Th1 cytokine activities, responsible for the development of dengue complications.**

Objective: **To determine the levels of vitamin D, TNF- α and calcium in patients with variation of dengue fever stages.**

Methods: **A cross sectional analytical study conducted on dengue confirmed patients (fever, hemorrhagic, shock) admitted in tertiary care hospitals of Lahore (IRBNo.1165). After obtaining informed consent the study objectives were explained to the patients and data collected. Patients were classified into three groups (a) dengue fever (DF) (b) dengue hemorrhagic fever(DHF) and (c) dengue shock syndrome (DSS) (40+30+10 cases). Serum from 5cc blood was stored at –20⁰ C until analysis. The data was analyzed using SPSS version 21.**

Results: **The study was conducted in 4 hospitals of Lahore on 80 NS1confirmed cases (age: 5-70 years). The age difference, vitamin D and calcium levels were significant (p-values 0.030, 0.047 and 0.003 respectively). Calcium levels were lowest in DSS and significantly lower than DHF patients. Vitamin D levels were higher among those with higher severity of disease with p-value 0.031. The patients did not have significantly different TNF α levels. There were 90.0% of DSS cases that had TNF α levels > 100, (p-value 0.109).**

Conclusion: **High levels of TNF-alpha are associated with dengue complications regardless of vitamin D levels and severity of disease.**

Index Terms—**Dengue Fever; Dengue Shock Syndrome; Tumor Necrosis Factor; TNF- α; Hemorrhagic Fever.**

I. INTRODUCTION

A. Demographic

Dengue epidemic has increased within last decade up to an alarming level. According to World Health Organization, about 2.5 billion people belonging to different age groups and living in more than hundred countries are threatened by dengue infection. The most affected areas are South East Asia and Western Pacific [1].

Pakistan is at high risk of being hit by dengue epidemic due rainy weather, improper water, sanitization management which makes suitable climate for the growth of Aedus mosquito (carrier of dengue virus) and lack of knowledge for prevention against dengue mosquito bites. These conditions promote yearly the spread of dengue infection as an outbreak in different parts of the country which result in increased morbidity and mortality [2].

Dengue virus consists of a single RNA strand. It belongs to the family Flaviviridae, genus: Falvivirus. It is transmitted through the bite of mosquito predominately *Aedesaegypti*. They are adapted to breed around human dwellings, in water containers, vases, cans, old tyres and other discarded objects [3]. The secondary vector for dengue virus is *Aedesalbopictus*, which contributes significantly to transmission in Asia. There are three structural proteins (capsid protein C, membrane protein M, and envelope protein E.) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) present in it [4].

It has five serotypes (DENV1,2,3,4,5) which could be isolated at any time of their life cycle. These types can result in dengue fever by polymorphism of HLAgenes4.The presence of all four types indicates 'heper endemic' for dengue4. In Pakistan DENV 1-3 predominates [5].

B. Clinical Presentation

Dengue fever (DF) is an acute viral infection characterized by fever, rash, head ace, muscle joint pain and nausea. Occasionally DF progresses to dengue hemorrhagic fever (DHF), a potentially life threatening illness. Dengue hemorrhagic fever (DHF) is caused by the same viruses but might attribute to more than one serotypes in the body and is characterized by increased vascular permeability, hypovolaemia and abnormal blood clotting mechanisms. DHF is a potentially deadly complication with symptoms similar to those of dengue fever, but after several days the patient becomes irritable, restless, and sweaty.

The Dengue Shock Syndrome (DSS) is characterized by bleeding that may appear as tiny spots of blood on the skin (petechiae) and larger patches of blood under the skin (ecchymoses). Minor injuries may cause bleeding. In severe cases, the patient's condition may suddenly deteriorate after a few days of fever; the temperature drops, followed by signs of circulatory failure, and the patient may rapidly go into a critical state of shock [4].

C. Function of Vitamin D and TNF-Alpha in Dengue

Within recent years, knowledge about role of vitamin D outside its established function in skeletal homeostasis has been fairly understood. It is a seco-steroid hormone that binds to the vitamin D receptor (VDR), a member of the super family of nuclear receptors for steroid hormones, thyroid hormone, and retinoic acid.

Vitamin D triggers immunomodulatory and anti-proliferative responses influencing the patho physiology and

preventing human disease. It functions as a non-classical cytokine [6] which in many conditions inhibits Th1 cytokine activities (TNF-alpha, IFNα), which are responsible for the development of dengue related complications such as dengue hemorrhagic fever and dengue shock syndrome [7],[8] (Th1 and Th2 are subsets of T helper cells).

Present study focused on levels of vitamin D, TNF-alpha and calcium in patients with dengue fever, dengue hemorrhagic fever and dengue shock syndrome. The results of this study may provide new means of treating dengue fever by injecting vitamin D to save complication such as severe muscle and joint pains.

II. Patients and Methods

A. Study Design

A cross sectional analytical study.

B. Study Population

Patients admitted in tertiary care hospitals of Lahore (ShaikhZayed, Mayo, Ganga and Jinnah), in the age group of 5-70 years.

C. Inclusion Criteria

For Dengue Fever: Patients confirmed by NS1 for dengue infection and presenting with high grade fever, headaches, skin rash (dengue triad), exhaustion, severe muscle, joint pain and swollen glands.

For Dengue Hemorrhagic Fever: Patients confirmed by NS1 and ultrasound (in case of capillary leakage) for dengue infection and presenting with hemorrhagic rash or hemorrhagic manifestations in addition to high grade fever, headaches, skin rash (dengue triad), exhaustion, severe muscle and joint pain.

For Dengue Shock Syndrome: Patients confirmed by NS1 and ultrasound (in case of capillary leakage) for dengue infection and presenting with hypotension, altered mental status and delayed capillary filling.

D. Exclusion Criteria

Patients with dengue fever in combination with other disease such as malaria or typhoid were not included in the study.

E. Sample Size

The estimated sample size was 80 using 5% level of significance and 80% power of test with expected frequency of vitamin D deficiency upto 72% in dengue patients which is at least 15% higher than in general population such as 57%.

F. Sample Collection

After clearance from Technical Review Committee and InstitutionalReview Board of Sheikh Zayed Medical Complex (IRB: 1165), objectives of the study were explained to the enrolled dengue patients fulfilling the inclusion criteria or guardians/attendants in case of minors or nonresponsive patients especially of DSS and informed consent was obtained(Annex-1). Patient's demography and clinical information was recorded on a study proforma from the patient's clinical files(Annex-2). Based on patients' clinical condition, they were classified into three groups (a) dengue fever, (b) dengue hemorrhagic fever, and (c) dengue shock syndrome. During the study 41 patients were enrolled for DF, 29 for DHF and 10 for DSS.Blood (5cc) was

collected from each enrolled dengue patient, serum was separated and stored in two aliquots at –20 degree Celsius until analysis of 25(OH) vitamin D (IDS UK), TNF-alpha (IBL Germany) using Enzyme Linked Immunosorbent Assay and Calcium (Human UK) spectrometerically, keeping the temperature and other assay conditions as prescribed by the manufacturer.

G. Data Analysis

The data was analyzed using SPSS version 15. Descriptive statistics of socio-demographic variables were computed. Quantitative variables like, age is described using mean±SD and proportions for categorical variables. The comparison of levels of 25(OH) vitamin D, TNF-alpha and calcium was done by using ANOVA between groups of dengue infection. The value of $p \leq 0.05$ was considered statistically significant.

III. Results

The study was conducted during dengue season in four tertiary hospitals (ShaikhZayed Hospital, Mayo Hospital, Ganga Ram Hospital and Jinnah Hospital) of Lahore during dengue season (2013). Eighty NS1 confirmed cases were enrolled from these hospitals and required information was recorded on study proforma. The patients were divided into three groups: DF, DHF and DSS based on extent of disease.

The gender distribution was not seen significantly different for three groups with p-value 0.614. However, 61(76.2%) were males (Table I). The difference in age (within groups) was significant with p-value 0.030. The age of DHF and DSS patients was significantly higher from DF. Within three groups, 10(12.5%) suffered from dengue shock syndrome (DSS), 29(36.3%) from dengue hemorrhagic fever (DHF) and remaining 41(51.2%) were suffering from dengue fever (DF) (Table I).

TABLE I: Age and Sex Distribution of Dengue Cases

| | Dengue Type | | | | | | | | |
| | DF | | | DHF | | | DSS | | |
Age (yrs)	Total n=41	M n=32	F n=9	Total n=29	M n=22	F n=7	Total n=10	M n=7	F n=3
Mean	28	28.6	28	38	40.2	31	31	33.1	27
±SD	12	12.3	9.4	18	18.7	16.7	12	14.7	3.6
Mini	16	16	17	15	15	15	16	16	23
Max	65	65	40	15	55	55	50	50	30

M=Male, F=Female

Headache and myalgia were the major complaints of DF patients, whereas thrombocytopenia, hematouria were most common in DHF patients. Rash and anorexia were seen as the most common symptoms in 70.0% of DSS cases. The symptoms like nausea, high fever, joint pain were common in all groups and myalgia was least common in DSS (Table II).

TABLE II: CLINICAL MANIFESTATIONS OF DENGUE CASES

Clinical presentation	DF (n=41)		DHF (n=29)		DSS (n=10)		Total (n=80)	
	n	%	n	%	n	%	n	%
Fever	39	95.1	28	96.6	9	90.0	76	95.0
Rash	23	56.1	16	55.2	7	70.0	46	57.5
Headache	38	92.7	24	82.8	9	90.0	71	88.8
Myalgia	36	87.8	27	93.1	7	70.0	70	87.5
Nausea	32	78.0	23	79.3	8	80.0	63	78.8
Anorexia	23	56.1	10	34.5	7	70.0	40	50.0
Others	16	39.0	23	79.3	5	50.0	44	55.0

Overall 100 cases were IgM positive and 52.5% were IgG positive. The difference for hemoglobin, hematocrit, white blood cell counts, platelet counts, ALT and AST levels and TNF Alpha levels were not found significant among three categories of patients, with p-values >0.100. The difference in vitamin D levels and calcium levels were significant with p-values 0.047 and 0.003 respectively. The vitamin D levels were seen higher in DHF and DSS patients than DF patients, whereas calcium levels were seen lowest in DSS patients. These levels were significantly lower than DHF patients (Table III).

TABLE III: Biochemical Analysis of Dengue Cases

Biochemical levels	DF	DHF	DSS	Total	p-value
HGB (g/dl)	13.09±2.53	12.03±3.59	13.27±2.11	12.73±2.94	0.293
HC+ (%)	39.43±5.67	38.39±8.49	36.35±15.13	38.66±8.32	0.571
WBC ($\times10^3/\mu$L)	4.91±5.44	6.90±12.64	5.40±1.35	5.69±8.49	0.637
PLT ($\times10^9$/L)	73.1±134.4	43.5±32.1	29.4±29.4	57.1±100.1	0.107
ALT (units/L)	80±64	79±68	65±40	77±62	0.793
AST (units/L)	99±127	98±106	66±39	94±111	0.694
Vitamin D level nmol/L	36.64±13.28 A	49.12±28.98 B	44.76±15.92 BC	42.18±21.22	0.047
Calcium level mmol/L	2.048±.393 AC	2.344±.735 AB	1.678±.370 C	2.109±.575	0.003
TNF alpha pg/ml	158.36±240.02	97.66±72.54	213.38±138.68	143.23±186.36	0.182

Since 82.9% of patients were seen with either deficient or insufficient vitamin D levels, therefore to observe the clear difference these levels were sub grouped into four. The increase was seen in vitamin D levels with severity of disease and this relation was found significant with p-value 0.031(Table IV).

TABLE IV: VITAMIN D LEVELS AMONG DENGUE CASES

Vitamin D level (nmol/L)	DF (n=41)		DHF (n29)		DSS (n=10)		Total (n=80)	
	n	%	n	%	n	%	n	%
≤ 25.0	11	26.8	6	20.7	1	10.0	18	22.5
25.0-50.0	23	56.1	11	37.9	4	40.0	38	47.5
50.0-75.0	7	17.1	8	27.6	5	50.0	50	25.0
>75.0	-	-	4	13.8	-	-	4	5.0
Chi-square = 13.86							p-value = 0.031	

For calcium levels at cut off 1.8mmol/l, it was observed that 55.2% of DHFcases had calcium levels above 2.2

mmol/l and 80% of DSS cases had calcium level below 1.8 mmol/l. This difference was highly significant p<0.001 (Table V).

TABLE V: CALCIUM LEVELS AMONG DENGUE CASES

Calcium level (nmol/L)	DF (n=41)		DHF (n29)		DSS (n=10)		Total (n=80)	
	n	%	n	%	n	%	n	%
≤ 1.80	11	26.8	2	6.9	8	80.0	21	26.2
1.80-2.20	18	43.9	11	37.9	1	10.0	30	37.5
>2.20	12	29.3	16	55.2	1	10.0	29	36.3
Chi-square = 21.98							p-value < 0.001	

The receiver operative characteristic (ROC) curve was produced for vitamin D and TNF Alpha levels of DSS cases at cut off 50.0 nmol/L for vitamin D level and 120.0 pg/ml for TNF Alpha levels. A significant increase in percent cases was seen with severity of disease when vitamin D levels were >50 nmol/L, whereas significant decrease in cases with severity of disease was seen when TNF-alpha levels were <120 pg/ml. The p-values recorded for these two relations were 0.029 and 0.008 respectively. (Table VI and VII).
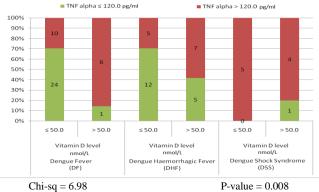
TABLE VI: DISTRIBUTION OF CASES BY DENGUE TYPE AND VITAMIN D CUT OFF BASED ON ROC CURVE
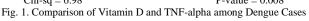
Vitamin D level (nmol/L)	DF (n=41)		DHF (n29)		DSS (n=10)		Total (n=80)	
	n	%	n	%	n	%	n	%
≤ 50.0	34	82.9	17	58.6	5	50.0	56	70.0
>.50.0	7	17.1	12	41.4	5	50.0	24	30.0
Total	41	100.0	29	100.0	10	100.0	80	100.0
Chi-square = 7.06							p-value = 0.029	

TABLE VII: DISTRIBUTION OF CASES BY DENGUE TYPE AND TNF-ALPHA CUTOFF BASED ON ROC CURVE

TNF alpha level (pg/ml)	DF (n=41)		DHF (n29)		DSS (n=10)		Total (n=80)	
	n	%	n	%	n	%	n	%
≤ 120	25	61.0	17	58.6	1	10.0	43	53.8
>120	16	39.0	12	41.4	9	90.0	37	46.2
Total	41	100.0	29	100.0	10	100.0	80	100.0

The distribution of cases using the same cutoffs of 50.0 nmol/L for vitamin D and 120 pg/ml for TNF Alpha, it was seen, those with low levels of vitamin D than cut off had TNF Alpha levels below 120 pg/ml and those with vitamin D levels >50.0 nmol/L had TNF alpha levels above 120.0 pg/ml (Fig. 1).

Chi-sq = 6.98   P-value = 0.008
Fig. 1. Comparison of Vitamin D and TNF-alpha among Dengue Cases

IV. DISCUSSION

Dengue infection is a rapid acting viral disease which has a great impact worldwide in public health sector. In Pakistan the number of reported cases with dengue infection has surged from 4500 in 2005 to 21,204 in 2010 with increasing trends in recent years [5]. The city Lahore, carried alone the burden of 14000 cases with 300 causalities in 2011[5].

In the present study the serum concentrations of vitamin D, Calcium and TNF-alpha were analyzed in 80 dengue patients suffering from dengue fever (41), dengue hemorrhagic fever (29) or dengue shock syndrome (10). Most of the patients were from walled city of Lahore and a few had travelled from peripheral areas of Lahore to get better treatment.

The frequency of male patients was more than that of females (2.26:1). This ratio is higher from reported studies, in other parts of Asia. Two studies from Singapore reported 1.6:1 and 1:0.9 male to female ratio of getting dengue infection [9],[10]. Another study from Cambodia reported 60.1% male in 15 years of age or more in 2010 [11]. A high frequency of males in this region could be due to the reason, males being earning members of family, are more exposed to environmental influx than females who are mostly contented at homes. The scenario of male to female ratio in dengue patients is seen slightly different in western countries. Data from South America and Geneva showed an equal male to female proportion (1:1) and data from Mexico showed 0.89:1.0 ratio [12]-[14].

The signs and symptoms presented in three groups i.e. DF, DHF and DSS were similar in enrolled patients of this study. The rash was seen more common in DSS patients. The University of Massachusetts, Amherst USA conducted a research in six different countries including Singapore, Cambodia and Philippines. They reported that in spite the fact, that dengue patients were from different countries with various dengue serotypes, but all had similar signs and symptoms [11]. An important symptom, appearance of convalescence rash (i.e. a confluent, pruritic, petechial rash with multiple small round islands of unaffected skin) is an indicator of re-absorption phase towards the end of critical phase of DSS [15]. In the present study this symptom was most common in dengue shock syndrome patients indicating that plasma leakage has stabilized and patients have entered in re-absorption phase.

Vitamin D has been known for its immune modulation. In recent research on dengue infection in the department of infection and pathogenesis Mexico suggested, that only in conditions where cell viability is not affected; vitamin D influences virus replication, resulting in significant reduction in number of infected cells and in primary conditions the vitamin D significantly reduces the levels of pro-inflammatory cytokines (TNF-alpha) produced by dengue infected cells [16]. Researchers from University of Colombia reported higher concentrations of vitamin D in DHF and DSS with secondary infection. The increased concentrations of vitamin D in secondary dengue infections enhances more vitamin D receptors and increases expression of Fcγ receptors which augments viral entry through antibody- dependant enhancement; and uncontrolled inflammatory responses (such as by TNF-alpha) and subsequent development of severity of dengue infection [17],[18].

In the present study results showed increase in vitamin D levels and TNF-alpha levels with increase in severity of dengue infection. This might be due to the reason that the reported cases of dengue hemorrhagic fever and dengue shock syndrome were mainly with secondary dengue infection. In secondary dengue infection since cell viability is affected resulting in vitamin D receptors facilitating dengue virus entry into the cells, consequently the inflammatory response of immune system also increase in TNF-alpha levels. However in dengue fever cases of the present study, where cell viability was not much affected the vitamin D levels were found lower than dengue hemorrhagic and dengue shock syndrome patients.

Extracellular calcium plays an important role in blood clotting by platelet aggregation in dengue infection. As dengue patient becomes critical the level of calcium starts decreasing due to plasma leakage [19],[20]. Mostly patients are administered with calcium to improve platelet aggregation. In the present study calcium levels (>2.2 mmol/l) were seen at higher level in dengue fever patients and in dengue hemorrhagic fever patients however these levels were decreased in dengue shock syndrome patients. The reason of such moderation is, in spite of calcium administration in Dengue hemorrhagic fever patients, the levels were not maintained in dengue shock syndrome due to excessive plasma leakage.

Cut off of biochemical Analyses

A single acceptable definition of vitamin D deficiency level is not available in research [21] however it has been accepted that mild/moderate vitamin D deficiency is 20-50 nmol/L, Severe deficiency is <20 nmol/L, Insufficiency is 50-75nmol/L and sufficiency is recognized as >75nmol/L [22]. The cut off used for vitamin D in the present study were similar to the one already used by other researchers in their research (severe deficiency ≤25nmol/L; mild/moderate: 25-50nmol/L; insufficiency: 50-75nmol/L and sufficiency: 76-250nmol/L). Potential intoxication starts at >250nmol /L which was not seen in enrolled dengue patients.

Studies from India and Brazil have used TNF-alpha cut off between 50 to 100 pg/ml in their studies [23],[24]. However in these studies the levels of TNF-alpha in DF patients were between 50-100 pg/ml and 60-120 pg/ml in DHF patients respectively while >200 pg/ml in DSSpatients [23],[24]. A study from Brazil reports that ROC curve is the

best way to determine cut off value for TNF-alpha [24]. Based on this evidence the cut off level in present study on ROC curve was 120pg/ml.

In a study from Dhaka the cut off for serum calcium levels in dengue infected patients have been used as 2.1mol/l. It was seen that most of the dengue hemorrhagic patients were hypocalcaemic and were administered with calcium to help patient recovery [25]. In the present study the cut off used was 1.8mmol/l for serum calcium [26] due to very low calcium status in dengue fever patients and required calcium supplementation.

V. CONCLUSION

In severe cases of dengue an association among high levels of TNF-alpha, vitamin D and low levels of calcium is found to exist.

ACKNOWLEDGMENT

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