

Natural regulatory anticoagulants proteins among Sudanese patients with dengue virus infection

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ABSTRACT

The Coagulation System: Abnormalities in natural physiologic anticoagulants are observed in dengue infection. Laboratory values such as protein C (PC), protein S (PS), and antithrombin (AT) indicate this problem on the coagulation system in dengue. Recently, an interrelationship between dengue and the levels of natural anticoagulants has been observed.

Objective: The study conducted to find out the effect of dengue on the natural anticoagulant proteins.

Methods: A case-control study was conducted in Port Sudan Teaching Hospital from February 2013 to June 2014 for 334 cases of dengue caused by dengue virus, 217 (65%) males and 117 (35%) were females along with 101 cases of control 64 (63.4%) males and 37 (36.6%) were females. Laboratory-positive dengue cases were confirmed by immunoglobulin (Ig) M and IgG immune chromatography rapid test and the WHO criteria were used for classifying the dengue severity. Platelet count (PLT), plasma prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, D-dimer (DD), aspartate transaminase, alanine transaminase, PC, PS, and AT were performed.

Results: Of 334, 289 patients had dengue fever (DF) and 45 patients had dengue hemorrhagic fever (DHF). Thrombocytopenia was present in 279 (83.5%). PLT was found to be significantly low in the case of dengue ($P < 0.000$). There was a highly significant difference between the prolongations of PT and PTT in DF ($P < 0.000$). Prolongations of PT and PTT were significantly higher (90% and 76.2%, respectively) in DF than DHF patients (10% and 23.8%, respectively). PC and PS were significantly higher in DHF 100% and 80% than DF 89% and 57%, respectively.

Conclusion: The findings of this study suggest that lower levels of these proteins in patients with dengue are attributed to disseminated intravascular coagulation.

Keywords: Antithrombin, D-dimer, dengue, natural anticoagulants, protein C, protein S, Sudan

Introduction

Infection with certain viruses can tilt the hemostasis toward bleeding and cause viral hemorrhage fever (VHF).¹ Among VHFs, dengue virus (DENV) is the most important and prevalent.² More than 2.5 billion people or half of the world's population in tropical and subtropical is at risk of DENV infection.³ DENV is a mosquito-borne Flavivirus that is transmitted by mosquitoes such as *Aedes aegypti* or *Aedes albopictus*. DENV are a positive-stranded RNA with envelope.² Based on the antigenic difference of E protein, DENV can be divided into five different serotypes, DENV 1-5. DENV infection might lead to influenza-like illness, which is called dengue fever (DF) or cause more severe dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).⁴

Thrombocytopenia is common in DF and constant finding in DHF. The mechanism of thrombocytopenia is poorly understood.⁵ The platelet (PLT) counts usually drops to below 100,000/mm³ 1-2 days before defervescence and remain low for 3-5 days in most cases. The levels then increase rapidly to normal during convalescence. The PLT counts in shock cases are frequently below (50,000/mm³).⁶ The coagulation system appears to be abnormal during infection manifesting as decreased fibrinogen (FB) levels, increase levels of fibrin degradation products, prolonged partial thromboplastin time (PTT) and prothrombin time (PT), low levels of coagulation factors VIII and XII.⁷ The *in vivo* existence of natural anticoagulant systems is essential to prevent thrombosis. These natural anticoagulant systems include antithrombin (AT), heparin cofactor II (HC-II), and protein C (PC) and its

cofactor protein S (PS). AT is a serine protease glycoprotein inhibitor that is synthesized in the liver. PC is a vitamin K-dependent synthesized in the liver, when activated, capable of degrading activated factors V (Va) and VIII (VIIIa) in the presence of the cofactor PS.⁸ PS is a vitamin K-dependent plasma glycoprotein synthesized in the liver, endothelial cells, and megakaryocytes. It has two forms, free form (active) and bound form (inactive) complexed to complement 4b binding protein. The activity of natural anticoagulants may be important in determining the thrombin formation, also may alter in some cases of dengue.⁹ Increase the level of plasma thrombomodulin (TM) observed in patients with dengue could contribute to the activation of this natural anticoagulation.¹⁰ Unfortunately, due to short facilities, TM level and HC II were not assessed in this study. The current case study aimed to find out the varying levels of the natural anticoagulant proteins and establish predictors for dengue infection using sophisticated tests such as PC, S, and AT.

Methods

This prospective study was conducted during the recent outbreak of dengue in Port Sudan Teaching Hospital, Red Sea State, Sudan, from February 2013 to June 2014. This study comprised 334 randomly selected patients positive with dengue infection. The inclusion criteria were all patients with clinical features and serologically positive dengue infection. No hemostatic agents were administered to the patients. The exclusion criteria include patients with serologically negative dengue or any other disease. A hundred and one, apparently healthy normal individuals with no any clinical sign of dengue infection were selected randomly to be the control group. Blood samples were collected from all of the studied population. 3 ml blood was placed in tri-potassium ethylene diamine tetra acetic acid, 3 ml treated with sodium citrated buffer and 3 ml in lithium heparin.

Patient's indicators

Patient characteristics of interest included: (1) Demographic: Sex, age, residence, tribe, and occupation; (2) Hematological: PLT count was used semi-automated hematology analyzer (Sysmex KX-21N, B 7151, and MF 9/2008 Japan); (3) Coagulation tests: PT, PTT, FB, PC, PS, and AT were examined within 4 h of collection using a semi-automated blood coagulation analyzer (bio bas-1 manufactured by RAL for SPINREACT, SN 536, Spain-European Community). (4) Chemical tests: Aspartate transaminase (AST) and alanine transaminase (ALT) were determined by semi-automated chemistry analyzer (WP21B Tough Biochemistry Analyzer, Mindary, China) and Biosystem reagents. Coagulation tests were determined by Biomed Diagnostic Reagent, Germany for PT/PTT. Reagents of Tulip Diagnostic, India were used in the established in the laboratory for FB, PC, PS and AT; (5) D-dimer (DD) was determined by NycoCard[®] method using NycoCard[®] READER II (SN 67498, Axis- Shield PoC AS, Oslo, Norway).

Criteria for dengue severity

Patients were classified as DF, DHF or DSS according to the WHO guidelines and laboratory diagnosis of dengue was established by demonstration of immunoglobulin (Ig) M and IgG immune chromatography rapid strip test (BioTracer/BioFocus, REF: 17112, Exp.12/2015, Korea) sensitivity 95.6 and 96 specificity.

Criteria for DIC

DENV infection was the underlying disease referred to the DIC scoring system for this study. The DIC scoring system used adopted from Taylor *et al.*¹¹ The DIC scoring system evaluates the following parameters: The underlying disease, PLT, FB, PT, PC, AT, and DD.

Statistical analysis

Differences in laboratory data between patients with DF, DHF, and coagulation tests were tested by compare mean and Chi-square test whichever was appropriate. A $P < 0.05$ were considered statistically significant. The Statistical Package for Social Sciences (SPSS 20.0 version, IBN. Chicago, IL, USA) was used for data analysis.

Ethical considerations

This study was approved by the Regional Ethical Review Committee and written informed consent was obtained from all the patients.

Results

This is a case-control analytical study conducted in Port Sudan Teaching Hospital, Red Sea State, Sudan. The total number of the confirmed diagnosed dengue patients was 334. The age of the patients in this study was between 3 and 80 years (mean age 30 ± 15 years). The control individual aged between 6 and 76 years (mean age 22 ± 6 years). Of the 334 clinical patients, 65% (217) were males and 35% (117) were female. In control group, 63.4% (64) were males and 36.6% (37) were females.

Table 1 shows the comparison of different characteristics between patients and controls. It shows that the eastern part of the study area (Selalab) represented the highest incidence (27%) region affected by DENV infection and the students were the most common segment of occupation affected (34.4). Table 1 illustrates that the overwhelming majority of DENV infection is among the Northern Sudan tribe (43.1%), followed by the Hadandwa tribe (21%). The clinical demographic findings of the studied were fever (334, 100%), headache (282, 84.4%), joint pain (262, 78.2%), backache (198, 59.3%), myalgia (156, 46.7%), retro-orbital pain (69, 20.7%), and rash (28, 8.4%). According to the WHO classification system, 289 patients had DF and 45 patients had DHF. Bleeding was recorded in 35 (10.5%) cases of all

Table 1: Characteristics of patients and control in the study

Characteristics	Patients <i>n</i> =334 (%)	Control <i>n</i> =101 (%)	<i>P</i> value
Age			0.000
Mean±SD	30±15	22±6	
Range	3-80 years	6-76 years	
Sex			0.726
Male	217 (65)	64 (63.4)	
Female	117 (35)	37 (36.6)	
Clinical diagnosis			
DF	289 (86.5)		
DHF Grade I	31 (9.3)		
DHF Grade II	12 (3.6)		
DHF Grade III	2 (0.6)		
Demographic data			0.726
Residence			
Selalab	91 (27.2)	23 (22.8)	
Diem Alnour	44 (13.2)	15 (14.9)	
Al-Thorat	62 (18.6)	31 (30.7)	
Al-Diom	75 (22.5)	17 (16.8)	
Alganobia			
Downtown	62 (18.6)	15 (14.9)	
Tribe			0.056
Hadandwa	70 (21)	13 (12.9)	
Bani Amer	61 (18.3)	13 (12.9)	
Northern Sudan	144 (43.1)	57 (56.4)	
Western Sudan	49 (14.7)	14 (13.9)	
Immigrants	10 (3)	4 (4)	
Occupation			0.000
Students	115 (34.4)	40 (39.6)	
Traders	65 (19.5)	21 (20.8)	
House wife	60 (18)	14 (14.9)	
Other jobs	94 (28.1)	26 (25.7)	

DHF: Dengue hemorrhagic fever, DF: Dengue fever, SD: Standard deviation

dengue patients. Bleeding manifestations included hematuria in 19 (54.3%) cases, hematemesis in 1 (2.9%), hemoptysis in 1 (2.9%), epistaxis in 5 (14.2%) cases, and gum bleeds in 9 (25.7%) cases.

All of the patients 334 presented were screened for PLT, PT, PTT, and FB. The normal and altered PT, PTT, and FB are shown in Table 2. PLT count was lower in patients than in control ($P < 0.000$). Thrombocytopenia was present in 279/334 (83.5%), 234 (81%) in DF cases and 45 (100%) DHF cases. Median PTT was 32.4'' (mean ± standard deviation [SD] - 33.5 ± 9.7) (range 15.0-80.7'') and median PT was 13.8'' (mean ± SD - 14.1 ± 2.1) (range 10.0-20.4'') (Table 3). PT and PTT were significantly higher in the DF (90%) and (72.7%) than DHF (10%) and (27.3%) of patients, respectively. Prolongations of PT, PTT, and low FB were observed in DF patients ($P < 0.000$) (Table 2). The difference between the patients and the control were found to be significant in PLT, PT, PTT, and FB (Table 3).

Table 2: PTT, PT and FB results in comparison to DF/DHF

Parameters	DF (%)	DHF (%)	Total (%)	<i>P</i> value
PTT				
Prolong	32 (76.2)	10 (23.8)	42 (12.6)	0.000
Short	18 (100)	0	18 (5.4)	
Normal	242 (88.3)	32 (11.7)	274 (82.0)	
Total	292 (87.4)	42 (12.6)	334 (100)	
PT				
Prolong	27 (90.0)	3 (10.0)	30 (9.0)	0.000
Normal	265 (87.2)	39 (12.8)	304 (91.0)	
Total	292 (86.8)	42 (12.6)	334 (100)	
FB				
Low	37 (60.7)	24 (39.3)	61 (18.3)	0.000
High	42 (87.3)	6 (12.5)	48 (14.4)	
Normal	210 (93.3)	15 (6.7)	225 (67.3)	
Total	289 (86.5)	45 (13.5)	334 (100)	

PTT: Partial thromboplastin time, PT: Prothrombin time, FB: Fibrinogen, DF: Dengue fever, DHF: Dengue hemorrhagic fever

Out of 334 patients' positive dengue infection, 101 (30.2%) had abnormal coagulation results. 81 (80.2%) patients with DF and 20 (19.8%) patients with DHF were screened for special parameters summarized in Table 4. Of 101 patients 72 correction tests for PT and PTT were performed. Corrected results which are indicate a deficient coagulation factor. 72 mixing experimental studies for PT and PTT revealed many coagulation factor deficiencies as in Table 5. This is suggested increased consumption of these factors.

PC was lower in DF 72 (89%) cases and 20 (100%) cases with DHF. No significant difference between PC and DENV infection ($P < 0.295$). So, PS was lower in DF 46 (56.7%) cases and 16 (80%) cases with DHF. No significant between dengue infection and PS ($P < 0.158$). AT was lower in DF patients 79/81 (97.5%) and 18/20 (90%) with DHF. No significant correlation between AT and dengue infection ($P < 0.226$). DD was high in DF patients 70 (86.4%) and 17 (85%) with DHF. No significant correlation between DD and dengue infection ($P < 0.287$). The transaminase liver enzymes were relatively higher in DHF than DF. 44(43.6%) patients had an elevated level AST, whereas 22 (21.8%) patients had an elevated level ALT. No significant correlation between AST/ALT and the DENV infection ($P < 0.978, 0.617$, respectively) (Table 6).

A total of 101 (30.2%) had disseminated intravascular coagulation (DIC). DENV infection was the underlying disease referred to the DIC scoring system for this study. The DIC scoring system used are summarized in Table 7. Nearly 43.6% diagnosed as overt DIC (classic) score ≥ 5 , and 56.4% diagnosed as non-overt DIC score ≥ 6 . The non-overt DIC was significantly higher in patients in the study. Interestingly, DIC was significantly higher in DHF 20 (19.8%) patients than the DF 81 (80.2%) patients.

Table 3: The difference between test and control in studied parameters

Parameters	Mean±SD		Median test	Median control	Range test	Range control	P value
	Test group	Control group					
PLT count×10 ⁹ /L	95.691±57	219.099±59	95	214	3-443	93-509	0.000
PT seconds	14.1±2.1	13.3±1.6	13.8	13.5	10-20.4	10-16.5	0.000
PTT seconds	33.5±9.7	29.6±4.5	32.4	28.9	15-80.7	20-39	0.000
FB g/dl	4.15±6.99	3.26±1.44	2.60	2.90	0.5-63.96	1.37-10.23	0.000

PT: Prothrombin time, PTT: Partial thromboplastin time, FB: Fibrinogen, SD: Standard deviation, PLT: Platelet

Table 4: Laboratory finding of specific parameters in the study

Parameters	Median	Mean±SD	Range of test	Normal range
PC	47%	48±22	8-140	70-140%
PS	60%	76±50	4-273	65-140%
AT	2.1 mg/dl	5.1±6.6	0.23-30	17-30 mg/dl
DD	1.3 mg/dl	3.9±5.9	0.10-22.5	<0.3 mg/dl
AST	34 U/l	54±69	5-480	Up to 40 U/L
ALT	22 U/l	34±43	2-279	Up to 41 U/L

PC: Protein C, PS: Protein S, AT: Antithrombin, DD: D-dimer, AST: Aspartate transaminase, ALT: Alanine transaminase, SD: Standard deviation

Table 5: Summary of factors deficient by mixing experiment study

Blood factor deficient	Number of patients n=72	Frequency (%)
X	14	19
V	7	10
II	9	12
VIII	25	35
IX	7	10
XII	10	14
Total	72	100

Table 6: Special parameters among DF and DHF patients

Parameters	DF n=81 (%)	DHF n=20 (%)	P value
Low PC	72 (89)	20 (100)	0.295
Low PS	46 (56.7)	16 (80)	0.158
Low AT	79 (97.5)	18 (90)	0.226
High DD	70 (86.4)	17 (85)	0.287
High AST	35 (43.2)	9 (45)	0.978
High ALT	17 (21)	5 (25)	0.617

DF: Dengue fever, DHF: Dengue hemorrhagic fever, PC: Protein C, PS: Protein S, AT: Antithrombin, DD: D-dimer, AST: Aspartate transaminase, ALT: Alanine transaminase

Discussion

The defects in DF/DHF are multifactorial mechanisms that include thrombopathy, coagulopathy, and vasculopathy. Thrombocytopenia is common in DF, and is a constant finding in DHF.¹¹ The present study increasing the hypothesis that thrombocytopenia is not only a laboratory changing in DF but also may be an important cause of bleeding. Many factors can contribute to the onset of thrombocytopenia in DF from a reactive immune response

against PLTs to decreased PLT production.¹² However, in this outbreak the majority of patients (279/334; 83.5%) had thrombocytopenia a finding similar to Karoli *et al.*; Chairufatah *et al.*^{13,14}

Hottz *et al.*, studying the dengue induce PLT activation in Brazil, reported DENV infection induces PLT consumption due to DIC, PLT destruction due to increased apoptosis, lysis by the complement system and by the involvement of anti PLT antibodies.¹⁵ Eventually, we suggest the cause of thrombocytopenia in the current study is related to the DIC.

Coagulopathy is also found in DF and most DHF cases, the PTT and FB are more frequently abnormal than PT.¹⁶ In this study, PT was demonstrated to be abnormal in some (30/334, 9.0%) but not all patients with dengue infection, these results are in agreement with previous findings,¹⁶⁻¹⁹ whereas PTT prolongation was observed in 42/334, 12.6% of patients which is similar to results stated by Wills *et al.*, Krishnamurti *et al.*, Lin *et al.*, and Chuang *et al.*²⁰⁻²² On the other hand, prolongation of PT and PTT indicates the coagulation in patients with dengue was impaired. Mairuhu *et al.* and Orsi *et al.* concluded that the relationship between dengue and activation of coagulation pathway is controversial. However, the causes of this coagulation disorder remain speculative.^{7,23} Accordingly, this study hypothesized that the coagulation and fibrinolysis are both activated; we attribute this to the presence of DENV. Recently, found nonstructural protein-1 (NS1) of DENV can bind to both thrombin and prothrombin. The thrombin activity is not altered when NS1 bind to thrombin, the binding of NS1 to prothrombin can inhibit its activation, which may contribute to the prolongation of PTT in dengue patients.¹⁰ This may explain why PTT abnormality occurs within the first week of fever onset when antibodies are still underdeveloped.¹⁸ In addition, NS1 may also contribute to plasma leakage by mechanism without antibody involved. These results suggest that DENV secreted NS1 plays a direct and important role in vascular leakage and hemorrhage in DHF/DSS.²² Regardless of the causes of the coagulopathy, our study showed that the coagulation activity may be impaired during dengue infection and that this disorder may cause bleeding.

With respect to plasma levels of the naturally occurring anticoagulants PC, PS and AT were significantly reduced in the present study. PC, PS and AT are predominantly synthesized in the liver and lower circulating levels probably reflect capillary

Table 7: Summary of DIC score results

Parameters	Score	Overt DIC n=44 (%)	Non-overt DIC n=57 (%)	P value†
Underlying disease	2	-	57 (100)	
PLT score	0	4 (9.1)	16 (28.1)	0.000
	1	14 (31.8)	41 (71.9)	
	2	26 (59.1)	0	
PT score	0	32 (72.7)	39 (68.4)	0.667
	1	4 (9.1)	18 (31.6)	
	2	8 (18.2)	-	
FDP score	0	4 (9.1)	14 (24.6)	0.000
	1	12 (27.3)	43 (75.4)	
	2	28 (63.6)	-	
FB score	0	11 (25)	-	0.000
	1	33 (75)	-	
PC				
Low	1	-	49 (86)	0.040
Normal	1	-	8 (14)	
AT				
Low	1	-	54 (94.7)	0.412
Normal	1	-	3 (5.3)	
Total score		≥5	≥6	

†P<0.05. DIC: Disseminated intravascular coagulation, PT: Prothrombin time, FDP: Fibrin degradation product, PLT: Platelet, AT: Antithrombin, PC: Protein C, FB: Fibrinogen

leakage alone. An increase in the rate of consumption of these proteins may be a contributing factor in the severity of disease.²⁰ Cabello-Gutiérrez *et al.* studying the modification of the cytoprotective PC pathway during dengue infection of human endothelial vascular cells in Mexico, reported an increased level of soluble TM in the plasma of patients with DHF/DSS, and they present a participation of DENV in down-regulation of TM-thrombin-PC complex formation at the endothelial surface with a reduction in activated PC.²⁴ Chuang *et al.* proposed that the expression of several other PC activations related molecules such as epithelial cell receptor and PS are also increased together with TM in DENV infection,²⁵ a result relatively similar to our finding regarding PS. During severe infection, AT may become very low due to consumption, decreased synthesis, and degradation by elastase resulting from activated neutrophils. Glycosaminoglycan could cause reduction of AT function because glycosaminoglycan has a role as a co-factor like physiologic heparin for AT. Whereas AT reduction in DHF mainly caused by capillary leakage, and may be also by the dilution effect of the administered intravenous fluid and elevated consumption, so that AT is related to the severity degree of DHF.²⁶ Low levels of the anticoagulant proteins are usually associated with the development of thrombotic complications or significant laboratory evidence of DIC.²⁷

On the other hand, excessive fibrinolysis was demonstrated in our patients with dengue infection, since plasma levels DD were significantly increased in those patients. In a similar way, previous studies have demonstrated that patients with DHF may

also present with excessive fibrinolysis.^{9,11,20} Chuang *et al.* in a study by Institute of Basic Medical Sciences, Taiwan, reported data suggest dengue-induced plasminogen cross-reactive antibodies may enhance plasminogen conversion to plasmin, which would be expected to contribute to hyperfibrinolysis.²⁸ This study showed significantly higher DD level in 86.4% of DF patients compared with 85% DHF with the sensitivity of DD in predicting DF of 80%. These results are strongly consistent with Setrkraising *et al.* and Orsi *et al.*^{23,29} DD was also found to be positively correlated with DIC ($P < 0.000$). Detection of DD in patients with dengue infection may be beneficial for predicting the clinical course of the disease. This helps the clinicians in predicting the disease severity before the patients' progress into the toxic stage. Detection of DD suggests that DIC and activation of the fibrinolytic system occur early in patients with DENV infection before the onset of severe hemorrhagic manifestations.

Aminotransferase levels are useful in predicting the occurrence of hepatic dysfunction and spontaneous bleeding. Liver enzyme elevation is a common feature in dengue infection.³⁰ However, Wong and Shen were reported that AST abnormality was predominantly high as compared to ALT; 91% and 72%, respectively.³¹ In this study, AST levels were equal to or greater than those of ALT levels in most of the infected patients, findings that have also been reported earlier by Ali and Elgasim; Shukla and Chandr.^{32,33}

A correlation between the levels of AST and PTT shows a strong association between AST elevation and PTT prolonged time in dengue infection in our patients ($P < 0.003$), this might seem to relate to the process of hepatic parenchymal damage than the biliary tract obstruction. Ultimately, our findings suggest that lower levels of these proteins in conjunction with high rates of the liver enzyme are due to the presence of DIC. To the best of our knowledge, this is the first study in our area to investigate the association between dengue infection and natural physiologic anticoagulants. Limitations of this study are that the data were collected manually and some information was missing. Some factors influencing natural physiologic anticoagulants such as chronic inflammatory state. Another limitation is that the sophisticated laboratory values such as PC, PS, and AT were performed only for the patients had abnormal coagulation tests, this needs to be addressed in the further studies.

Conclusion

This study demonstrated and confirmed the evidence that the coagulation in patients with dengue was abnormal. Moreover, the causes of this abnormality are attributed to DIC.

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