

## The Reactive Lymphocyte: A Morphological Indicator of Platelet Counts in Dengue Seropositive Patients

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### ABSTRACT

**Background:** Dengue fever is an emerging cause of morbidity and mortality, in tropical countries, with increasing global incidence reaching epidemic proportions. Infected humans show rapid progression to severe and fatal outcomes based on their immunity. The peripheral smear is a cost effective and simple investigation, which provides a plethora of valuable information.

**Methods:** This cross sectional study was carried out by studying the peripheral smears of seropositive dengue patients admitted in our hospital on the day one of admission. Total leukocyte counts, absolute lymphocyte counts, platelet counts along with a detailed morphological assessment of reactive lymphocytes were done concurrently by three pathologists on a total of 300 cases with thrombocytopenia. Of these, 100 cases were studied in each of the three categories of thrombocytopenia  $>1,00,000$  (1),  $51 - 10,00,000$  (2)  $< 50000$  (3). The size, basophilia, chromatin, nucleoli, cytoplasm and rosetting of RBCs with skirting were assessed in the reactive lymphocytes.

**Results:** It was observed that reactive lymphocytes, almost the same size, having the same amount of cytoplasm as a mature lymphocyte, with minimal basophilia, condensed chromatin, & with prominent RBC rosetting were associated with higher platelet counts. (category 1). Larger lymphocytes, with abundant basophilic cytoplasm and with minimal cytoplasmic skirting were significantly associated with lower platelet counts and absolute lymphocytosis.

**Conclusion:** In the present era of advanced automation, the morphological study of the reactive lymphocyte on peripheral smears is an indispensable and cost effective indicator of platelet counts and remains a valuable adjunct in prognosticating patients with Dengue fever.

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## Introduction

Dengue fever is an acute infectious disease caused by a ssRNA virus of the flaviviridae family and transmitted to humans by the bite of *Aedes Aegypti* and *Aedes albopictus* mosquitoes. The word “dengue” is derived from the Swahili phrase Ka-dinga pepo, meaning “cramp-like seizure”.<sup>[1]</sup> It is an emerging cause of mortality and morbidity, especially in tropical countries like India. A thirty fold rise in dengue has been reported in the past three decades, with more than 75% of cases affecting the Asia- Pacific region.<sup>[2,3,4]</sup>

Though dengue fever may be self limiting, the progression to Dengue haemorrhagic fever or dengue shock syndrome with a severe host response can be lethal if not adequately treated. While dengue can be identified early based on the clinical presentation and serological investigations, haematological parameters play a crucial role in indicating its progression to a fatal outcome.<sup>[5]</sup> Though thrombocytopenia, leucopenia and lymphocytosis are described as the characteristic haematological findings in dengue, morphology of the reactive lymphocyte is often under reported in this modern era of automation. This study highlights the various morphological differences in reactive lymphocytes and their association with thrombocytopenia a in sero- positive dengue cases. As adults have been documented to have more severe outcomes, we have focused our study in this age group.<sup>[5]</sup>

## Materials and Methods

A cross sectional observational study was conducted over a period of three months, from October 2015 to December 2015 in our tertiary care center hematology laboratory. The cases were collected during the peak of the dengue season. The inclusion criteria were all serologically proven dengue cases in the adult population on day one of admission. Dengue testing was done using a rapid solid phase Immunochromatographic test for the qualitative detection of NS1 antigen and the differential detection of Ig M and Ig G antibodies to Dengue virus in human serum .Other cases of febrile thrombocytopenia, such as those associated with other infections and malignant processes were excluded. An automated analyzer was used to obtain hematological parameters from anticoagulated venous blood samples. These parameters were standardized by routine external and internal quality control checks. All peripheral smears showing artifactual clumping, satellitism and pseudothrombocytopenia were excluded from the study. Platelet counts of <1.5 lakh/cmm were considered to be cases of thrombocytopenia. One hundred appropriate cases were collected in each of the categories of thrombocytopenia with platelet counts greater than 1, 00,000 (Category 1), 51,000-1, 00,000 (Category 2) and <50,000 (Category 3)

respectively. All samples were systematically analyzed for platelet counts, total leukocyte count, absolute lymphocyte counts, and percentage of reactive lymphocytes with a note of the demographic data such as age and sex. The lymphocytes were studied for their morphological changes (size, basophilia, nuclear chromatin, cytoplasm, skirting, and nucleoli) in comparison with a mature small lymphocyte in each slide. These morphological parameters were quantified on a three tiered grading system. These criteria were objectively quantified by three pathologists using a pentahead research microscope. Each of these six morphological parameters were separately assessed in the above mentioned categories of thrombocytopenia, scored and tabulated. The scores were then studied for their significant association as shown in the table below (**Table 1**) with platelet counts and absolute lymphocyte counts using chi – square test. A P value of < 0.05 was considered to be statistically significant.

## Results

300 cases of thrombocytopenia in dengue were studied in total. The pattern of dengue serology was as follows in the cases taken up for study (**Table 2**).The youngest patient was 18 years old and the oldest was 82 years old, with a majority of the cases in the age group of 21- 30 years (39%) (**Table 3**) Majority of the patients were males (192 cases– 64%) and the remaining were females (108 cases– 36%), with a sex ratio of 1.7:1. The total leukocyte count in the cases studied was distributed as follows. (**Table 4**)

Examination of the peripheral smears, showed the following proportion of reactive lymphocytes to mature lymphocytes in each slide in each case. (**Table 5**).

All the cases showed reactive lymphocytes. Most of the cases (91%) showed a high proportion of reactive lymphocytes on smears. The below parameters of lymphocyte morphology were studied and were found to be significantly associated with the different categories of thrombocytopenia. The results were tabulated as follows (**Table 6**)

**SIZE:** It was observed that the size of the lymphocytes correlated significantly with the platelet counts. Majority of the cases in category 1 thrombocytopenia were less than two times the size of a mature lymphocyte (48%), whereas most of the lymphocytes in category 3 thrombocytopenia were observed to be three times the size of a mature lymphocyte (58%). Majority of the Lymphocytes in category two thrombocytopenia were found to be twice the size of a mature lymphocyte (68%)

**Basophilia:** Intense basophilia of the cytoplasm was found to be significantly present in category 3

thrombocytopenia (56%). There was a decrease in intensity of the basophilia with higher platelet counts, the least basophilia being seen in category 1.

**Nucleoli:** Most of the cases in category 2 and 3 (62% & 56%) showed indistinct nucleoli, while most of the category 1 lymphocytes showed no nucleoli similar to mature lymphocytes (66%).

**Chromatin:** Most of the cases in category 2 and 3 (54% each) showed moderate condensation of chromatin, with most of the category 1 lymphocytes showing condensed cytoplasm, similar to mature lymphocytes (42%).

**Cytoplasm:** Cases in category 3 were found to be the ones with most abundant cytoplasm (48%), however relatively few cases in the other categories showed increased cytoplasm.

**Skirting:** The cytoplasmic skirting with rosetting of RBCS was found to be maximal with category 1 (44%) and minimal in category 3 (2%). It was also noted that

most of the lymphocytes in category 3 showed no skirting. **[Figure 1, figure 2 and Figure 3]**

The lymphocyte morphology parameters were studied in relation with absolute lymphocytosis, normal ALC and absolute lymphopenia (**Table 7**)

All three hundred cases were further studied with respect to their absolute lymphocyte counts independent of the thrombocytopenia categories. Though some of the parameters were not found to be significantly associated with Absolute Lymphocyte counts, of noteworthy importance are the size, amount of cytoplasm and skirting noted in these reactive lymphocytes. In cases of absolute lymphopenia, most of the reactive lymphocytes showed size and cytoplasm less than two times that of a mature lymphocyte (54% and 52%) with increased skirting of cytoplasm (45%) and RBC rosetting. However most of the cases with lymphocytosis as is evidenced in the above table, showed size thrice that of a mature lymphocyte (62%) with abundant cytoplasm (46%) and significantly minimal skirting (44%).

**Table 1: Grading of morphological parameters of the lymphocyte on the peripheral smear**

Parameter	Grade 1	Grade 2	Grade 3
Size	Less than 2 times the size of small lymphocyte	>X 2 times the size of a small lymphocyte	X 3 times the size of a small lymphocyte
Basophilia	Mild	Moderate	Intense
Nucleoli	No nucleoli	Indistinct nucleoli	Prominent nucleoli
Chromatin	Condensed	Moderately condensed	Open chromatin
Cytoplasm	Mildly increased	Moderately increased	Abundant
Skirting of cytoplasm	No skirting	Moderate skirting	Abundant skirting with rosetting of RBC'S

**Table 2: Pattern of Dengue antigen and antibody distribution**

Serology	No. of cases
NS 1 +	71% ( 213 cases)
NS 1 + Ig M +	20% ( 60 cases)
NS 1 + Ig G+	09 % (27 cases)
Total	300 cases

**Table3: Table showing age distribution of the cases**

Age Group	No. of cases
<20	42
21- 30	118
31- 40	54
41-50	36
51- 60	24
61- 70	06
71- 80	12
>80	08
<b>Total</b>	<b>300</b>

**Table 4: Table showing variation in total leukocyte counts**

Total Leukocyte count	Number of cases	Percentage
Leucopenia<4000cells/cmm	144	48
Normal	92	30.6
Leucocytosis>11,000 cells /cmm	64	21.3

**Table 5: Table showing proportion of reactive lymphocytes**

Reactive lymphocytes	Number of cases
Absent	0
<10%	27 (09%)
>10%	273 (91%)

**Table 6: Study of the association of morphological grades of reactive lymphocyte parameters with categories of thrombocytopenia**

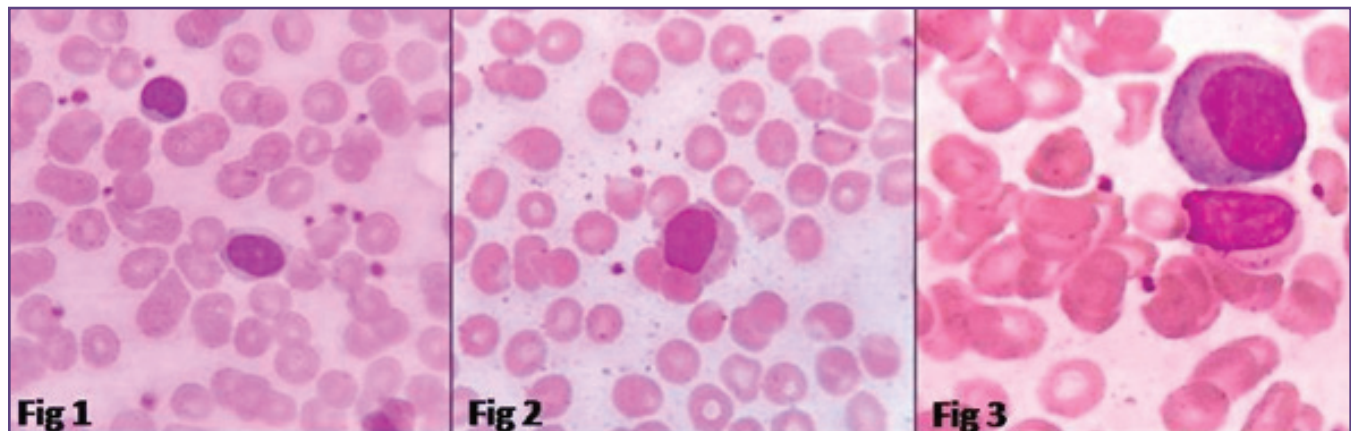
	Size (n)			Basophilia(n)			Nucleoli(n)			Chromatin(n)			Cytoplasm(n)			Skirting(n)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
C 1	48	44	08	64	32	04	66	30	04	42	40	18	42	52	06	20	36	44
C 2	14	68	18	38	76	46	34	56	10	38	54	08	24	56	20	38	50	12
C 3	24	40	58	18	20	14	14	62	24	26	54	20	8	44	48	62	36	2
	N= 300			N=300			N=300			N=300			N=300			N=300		
	p value < 0.0001			p value < 0.0001			p value < 0.0001			p value =0.017			p value =0.0017			p value = 0.001		

Platelet counts C1= Category 1 (>1lakh/cmm) C2= Category 2 (51,000- 1lakh/cmm) Category 3 =( <50,000lakh/cmm) n= number of cases in each category N= total number of cases

**Table 7: Study of the association of morphological grades of reactive lymphocyte parameters with absolute lymphocyte counts.**

	Size (n)			Basophilia(n)			Nucleoli(n)			Chromatin(n)			Cytoplasm(n)			Skirting(n)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
L 1	18	22	64	26	34	28	30	46	10	22	48	12	16	40	48	46	35	23
L 2	34	68	50	38	76	46	60	84	22	68	80	24	51	94	07	63	69	20
L 3	18	20	14	18	20	14	24	16	18	18	18	10	23	20	01	12	12	20
	N= 300			N=300			N=300			N=300			N=300			N=300		
	P value <0.0001			P value = 0.462			P value = 0.227			P value = 0.106			P value=<0.0001			P value= 0.0001		

L1 (104 cases) = Lymphocytosis (>3400cells/cumm) L2 (152 cases) =Normal (1000-3400cells/cumm) L3 (44 cases) = Lymphopenia (<1000cells/cumm) n= number of cases in each category N= total number of cases



**Fig. 1:** Peripheral smear with lymphocyte showing mild increase in size, condensed chromatin and RBC rosetting corresponding to platelet count >1 lakh/cmm **Figure 2:** Peripheral smear with larger lymphocytes with , moderately condensed chromatin, mild cytoplasmic basophilia corresponding to platelet count 50,000- 1 lakh/cmm **Figure 3:** Peripheral smear with larger lymphocytes with open chromatin , intense cytoplasmic basophilia without RBC rosetting corresponding to platelet count < 50,000 [Leishman stain x400x]

## Discussion

Dengue is the commonest cause of febrile thrombocytopenia investigated in our hematology laboratory. Serological diagnosis of dengue virus infection is routinely done by demonstration of the NS-1 antigen and anti dengue virus antibodies in the patients' serum depending upon the day of illness using commercial kits. <sup>[1]</sup>Our study reports a higher male: female ratio of 1.7:1, and is in agreement with authors like Agarwal et al and Ray et al, who also reported the seropositivity of dengue to be twice as common in males, with a M: F ratio of 1.9: 1 and 1: 0.57-respectively. The widely recognized notion is that hospital based registries in Asian countries traditionally report more males seeking treatment.<sup>[6]</sup>

Dengue infected patients are demonstrated to consistently have leucopenia, absolute lymphocytosis, thrombocytopenia and raised haematocrit. <sup>[7]</sup>Our study demonstrated 48% of cases having leucopenia. Other workers like Mehta et al <sup>[8]</sup> and Chakravarthi et al <sup>[5]</sup> have demonstrated a higher proportion of leucopenic cases (63% and 60%), whereas Arshad et al <sup>[9]</sup> and Tahir et al <sup>[7]</sup> have reported the same to be 49% and 56% respectively. (in concordance with our study). However, Rushmavathi et al <sup>[10]</sup> and Dutta et al <sup>[11]</sup> have reported far lesser cases of leucopenia in Dengue constituting 33.3% and 30% respectively.

It has been reported that B lymphocytes are the primary circulating cells infected by the dengue virus. The over production of these B cells, along with cytokines like IL – 6 trigger an aberrant maturation of plasma cells and atypical lymphocytes leading to the generation of autoantibodies causing platelet destruction and hence thrombocytopenia. The presence of these IgM platelet autoantibodies not only induces platelet lysis via complement activation, but also inhibits ADP induced platelet aggregation. <sup>[5]</sup>Also direct infection of the bone marrow megakaryocytic precursors and their suppression <sup>[7]</sup> has been proposed to contribute to thrombocytopenia. This aberrant immune activation has been documented to cause inversion of CD4/8 counts, cytokine overproduction, monocytosis atypical lymphocytosis contributing to the immunopathogenesis of dengue fever<sup>[5,7]</sup>

Flow cytometric studies also demonstrate that atypical lymphocytes progressively express CD -19, a B cell marker. Atypical/reactive lymphocytes appear early in the pathogenesis of dengue It is also postulated that these reactive lymphocytes may represent a response to non – specific viral stimulation. <sup>[5, 12, 13]</sup> A systematic labour intensive review of the peripheral smears in all 300 cases in our study showed that atypical lymphocytes were

consistently seen in all cases (100%). This agrees closely with observations made by Rushmavathi et al <sup>[10]</sup> who reported 81.1% cases showing reactive lymphocytes and Jameel et al<sup>[7]</sup> who reported the same as 93%. Some authors have demonstrated a lower proportion of the same, such as Mehta et al <sup>[8]</sup> who found only 72 % of cases having atypical lymphocytes.

The intermediate forms between typical lymphocytes and plasma cells are referred to by the authors as atypical; however, various other terms such as variant lymphocyte and abnormal lymphocytes are also used in literature for the same. <sup>[14]</sup> We have limited the nomenclature to the term “reactive”. Originally described by Turk in 1907 and further classified in detail by Downey in 1923, the reactive lymphocyte has been broadly studied in infectious mononucleosis. <sup>[15]</sup> The Reactive lymphocytes are large non neoplastic lymphocytes with an increased proportion of cytoplasm with basophilic cytoplasmic edges, often engaging neighbouring red cells (skirting) Nucleoli may occasionally be evident. <sup>[16 17]</sup> However, not much data exists in literature regarding lymphocyte morphology on peripheral smear in dengue. The importance of these reactive lymphocytes lies in the fact that higher counts are seen in patients with severe dengue than non- severe dengue. <sup>[18]</sup> While there has been a semantic overlap between the criteria and nomenclature of atypical / typical reactive lymphocytes, <sup>[19]</sup> not much literature could be gathered regarding a systematic approach to the morphology of the same. Hence the present study has taken into account all morphological parameters described in standard text books.

Studies attempting to identify the co – relation of changing lymphocyte morphology with different categories of thrombocytopenia are scarce, hence the authors have semi – quantitatively studied six morphological parameters in cases with platelet counts less than 50,000, 51000- 1,00,000 and greater than 1 lakh/cmm.

Although diagnostic and supportive tests are many, manual review of peripheral smears, though labour intensive often proves to be an excellent tool to supplement the automated data. <sup>[20]</sup> The reactive lymphocytes in category 1 (greater than 1 lakh/cmm platelet count) in our study were found to be less than twice the size and having the same amount of cytoplasm as a mature lymphocyte, with least basophilia, condensed chromatin and no nucleoli, but with maximal RBC skirting of cytoplasm often showing rosetting of greater than three RBCs. The reactive lymphocytes in category 2 (51000- 1,00,000 lakh/cmm) in our study were found to be on an average twice the size of a mature lymphocyte with moderate amount of

cytoplasm, intermediate basophilia, indistinct nucleoli, partial chromatin condensation and moderate skirting. The reactive lymphocytes in category 3 (<50,000 /cmm) in our study were markedly increased in size, often three times the size of a mature lymphocyte, with abundant basophilic cytoplasm, indistinct nucleoli, partially condensed chromatin and almost absent skirting. However, these features were not progressively studied in the same patient due to difficulty in follow up owing to attrition, different days of presentation and varying host responses to treatment.

In cases of lymphopenia, the lymphocytes showed only a mild increase in size and amount of cytoplasm, but with increased skirting of the cytoplasm. Cases of absolute lymphocytosis showed larger lymphocytes with abundant cytoplasm and minimal skirting.

Studies till date have documented the morphological features of a reactive lymphocyte in peripheral smears of dengue patients like basophilia, abundance of cytoplasm, open nuclear chromatin, increase in size and resetting of RBCs.<sup>171</sup> However these features have not been graded or studied for their statistical association with thrombocytopenia as consistently highlighted by many authors.<sup>19,141</sup>

## Conclusion

Morphological study of the reactive lymphocyte proves to be a highly useful and cost effective adjunct in prognosticating patients with dengue fever. Complimented with other hematological, serological and biochemical parameters, it is an effective indicator of the progressive immune response and disease activity in the host. In the modern age of advanced molecular and genetic analysis, peripheral smear examination is an indispensable diagnostic tool, as it can help study the various morphological parameters that are being under reported in the current era of automation. In our study we concluded that reactive lymphocytes slightly larger than a mature lymphocyte and with skirting and scalloped cytoplasmic edges are associated with higher platelet counts and lymphopenia, while larger lymphocytes with rounded edges and intense basophilia of the cytoplasm are significantly associated with lower platelet counts and absolute lymphocytosis. Despite the complex features and inter – observer variability, these features remain highly valuable indicators of dengue pathophysiology.

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## Competing Interests

None declared

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