International Journal of Academic Research in Business and Social Sciences
2017, Vol. 7, No. 2
ISSN: 2222-6990

Morphological Features Analysis in Pathogenic Dengue Infection as an Alternative Screening Method

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DOI: 10.6007/IJARBSS/v7-i2/2716 URL: http://dx.doi.org/10.6007/IJARBSS/v7-i2/2716

ABSTRACT

Dengue is one of the most widespread mosquito-borne infections in Malaysia. The diagnosis still remains a challenge in area of disease confirmation where it could not be easily differentiated with other febrile illnesses even though the diagnosis of classical dengue fever and dengue haemorrhagic fever can be recognized clinically.

The clinical diagnosis also can be difficult where the signs and symptoms presented are easily confused with malaria, leptospirosis and typhoid fever. Therefore, an early and effective evaluation of the peripheral blood can be very helpful in patient management.

The objectives of this study are to determine the morphological features in peripheral blood film (PBF) of pathogenic dengue infection. 30 PBF of positive dengue infection in University Malaya Medical centre (UMMC) had been examined in this study where atypical lymphocytes [n=27, (90%)] and thrombocytopenia [n=22, (73.3%)] were consistently found. Presence of thrombocytopenia and presence of atypical lymphocytes in PBF are important diagnostic clues for early diagnosis of dengue infection which could be potentially useful parameter in screening dengue. Therefore, PBF can have a significant function in supporting the diagnosis of dengue which can act as complement to the full blood count and serological diagnosis of dengue especially in cases where the clinical manifestation are abnormal.

Keywords: Dengue, Peripheral Blood Film, Atypical Lymphocyte, Thrombocytopenia

Introduction

Dengue is endemic disease which develops clinical manifestations like fever, headache, myalgia, bone and joint pain; leukopenia and thrombocytopenia which caused by *Flavivirus* that transmitted by Aedes mosquitoes which are either Aedes aegypti or Aedes albopictus (Azeredo, Monteiro, & Pinto, 2009). The dengue virus (DENV) from the genus of *Flavivirus* of the family *Flaviviridae* that has non segmented and positive single-stranded RNA virus (Curry & Staros, 2012) and has four different serotypes labeled as DENV-1, DENV-2, DENV-3 and DENV-4. An individual that infected with one serotype will build up the immunity only for that particular serotype and not for the other serotypes. Therefore, a person may be infected with dengue virus up to 4 times.

Standard screening and diagnostic tests for dengue infection are full blood count (FBC) and serological test such as immunoglobulin M (IgM) anti-*Flavivirus* antibody and commercialised kit of anti-dengue virus IgM. Positive result of IgM anti-*Flavivirus* antibody will confirm the infection is caused by *Flavivirus*, but the test cannot distinguish the infection is caused by either dengue virus or other *Flavivirus* species. In addition, the test sensitivities and specificities of commercialized kit of anti-dengue virus IgM were 21% to 99% and 77% to 98% respectively in which can explain there might be the occurrence of false positive result in current dengue serology test (Hunsperger et al., 2009). The previous study demonstrates that consistent abnormal features of blood cells morphology like atypical lymphocytecould be potentially used as a parameter in screening test of dengue infection. Therefore, in this study, the PBF of pathogenic dengue infection is being reviewed to identify the changes in morphological features of blood cells in aspect of size, shape, color, granularity and arrangement, and also to detect presence of consistent and significant abnormal features of blood cells which could has potential to be a parameter in screening dengue and could support the diagnosis of the dengue infection in addition to the serological test.

Methods

This study was approved by Research Ethics Committee of Faculty of Biomedical and Health Sciences (FBHS), Universiti Selangor Campus Shah Alam (J150006E) and was conducted in three stages which include the subject recruitment, blood collection and data collection analysis. Subject recruitment was done through inclusion and exclusion criteria in which the inclusion criteria for the subjects are positive dengue infection that had been approved by medical officer while the exclusion criteria of this study are patient with positive dengue haemorrhagic fever, having any haematological disorder such as thalassemia and anaemia or any other diseases like sickle cell disease, cancer, liver disease and renal failure, or taking any medication. In this study, 30 subjects had been recruited as participants in which the 30 subjects where positive dengue infection from UMMC. The blood collection was performed by certified phlebotomist where the collected bloods were collected in ethylene diamine tetra-acetic acid (EDTA) anticoagulant tube.

Then, the bloods were smeared as thin smear using Wedge method by dropping a drop of blood onto the slide at the middle line and the smear was done immediately where the slide with the drop of blood on it was held by using one hand and the other hand held the spreader slide. The end of spreader slide was placed at 30° to 40 º angles in front of the blood on the other slide and moved backwards until it touched the drop of blood to allow the drop of blood to spread between the slide with drop of blood and the spreader slide. The spreader slide was move rapidly at constant angle across the other slide with one even pressure. Then, the smeared slide was fixed with alcohol.

The blood smeared was then stained by using Leishman stain. The blood smeared was poured with Leishman stain for 2 to 3 minutes. Then, phosphate buffer with pH 6.8 was added onto the slide with Leishman stain. Next, the slide was blown to mix the stain with buffer until the metallic scum was produced. After that, the slide was rinsed and washed with tap water and then was dried. The smeared and stained samples were analyzed by examining under light microscope from the lowest magnification to the highest magnification by observing using zig-zag method. The morphological data of the blood cells was recorded and analysed based on the cell counts, size, colour, shape, distribution or arrangement and presence of any immature and abnormal features of the blood cells.

Results

Red Blood Cells (RBC) Analysis

The size, color, shape, distribution and presence of immature RBCs had been analyzed and recorded in this study as stated in table 1. From the microscopic examination, the overall size and the color of RBCs in these 30 PBF were normocytic and normochromic with slightly microcytic and hypochromic where the distributions were evenly normal distributed without any agglutination and other abnormal arrangement as shown in figure 1 and the shape was majorly normal biconcave-shaped with slightly presence of teardrop-shaped and ovalocyte as shown at Figure 2. There are 11 out of 30 patients (36.7%) show the presence of NRBC as shown in Figure 3.

Table 1
RBCs Morphological Findings of the Studied PBF Cases (n=30)

Variables	Findings	n	Percentages, %
Size	Microcytic	8	26.7
	Normal	22	73.3
Colour	Normochromic	22	73.3
	Hypochromic	8	26.7
Shape	Normal	21	70.0
	Teardrop-shaped	5	16.7
	Ovalocyte	4	13.3
Distribution	Normal	30	100.0
Presence of immature RBC	Presence of NRBC	11	36.7

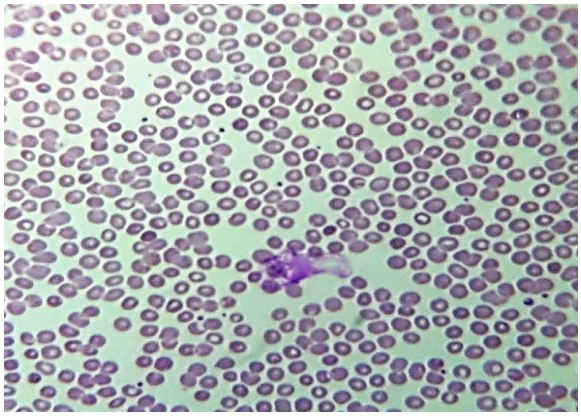

Figure 1. Presence of normocytic and normochromic RBCs with slightly microcytic and hypochromic with evenly normal distribution under 400 magnification

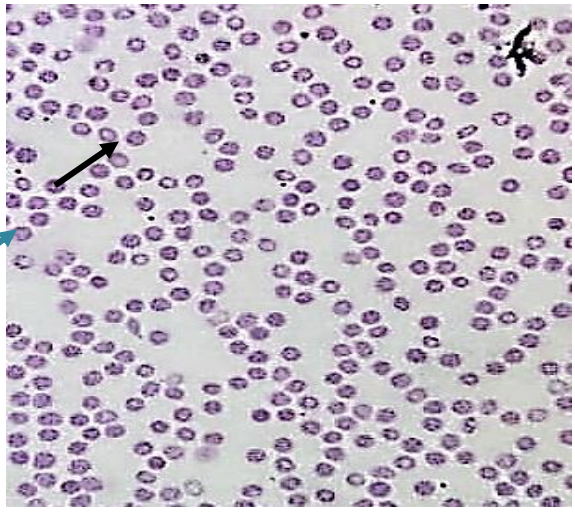

Figure 2. Presence of teardrop (shown at blue arrow) and ovalocyte (shown at black arrow) under 400 magnification

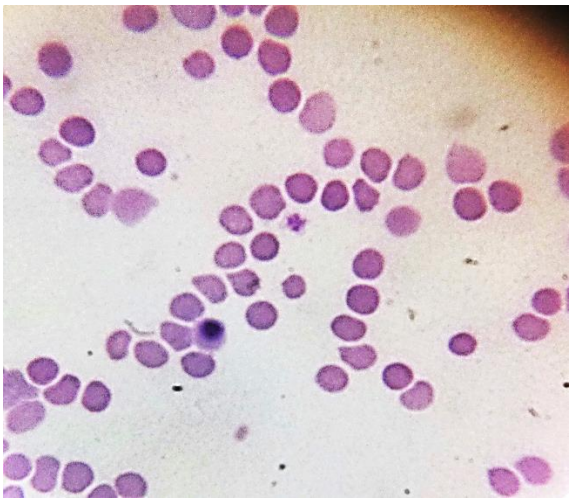

Figure 3. Presence of nucleated red blood cell, NRBC (at arrow) under 1000 magnification

White Blood Cells (WBC) Analysis

The morphological features analysis of WBCs including the size, color, shape, distribution and presence of abnormal WBCs had been performed in this study. Aside from the morphological features analysis of WBCs, the count of amount WBCs present in PBF of this studied cases also had been performed that respectively shown in Table 2.

Table 2
WBCs Findings of the Studied PBF Cases (n=30)

Variables	Findings	n	Percentages, %
Total leukocyte count	Leucocytosis	3	10.0
	Normal	17	56.7
	Leukopenia	10	33.3
Lymphocytes	Lymphocytosis	8	26.7
	Normal	13	43.3
	Lymphopenia	9	30.0
Neutrophils	Neutrophilia	4	13.3
	Normal	14	46.7
	Neutropenia	13	40.0
Monocyte	Monocytosis	6	20.0
	Normal	24	80.0
Eosinophils	Eosinophilia	1	3.3
	Normal	29	96.7
Presence of abnormal WBCs	Atypical lymphocytes	27	90.0
	Hyposegmented neutrophil	11	36.7
	Band neutrophil	9	30.0

In the findings of this study, there are absence of abnormal morphological features of WBCs except for lymphocytes and neutrophils where there is presence of atypical lymphocyte as shown in Figure 4, and observation of hyposegmented neutrophil and band neutrophils (shown at Figure 5).

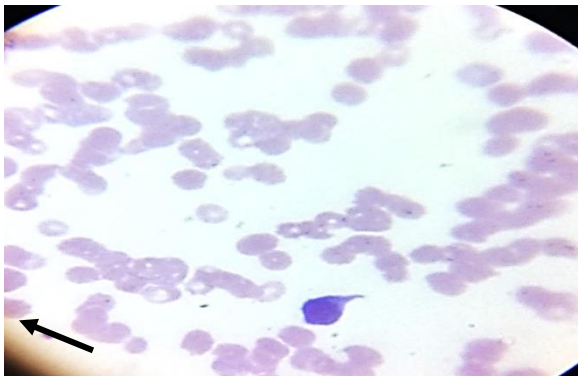

Figure 4. Presence of atypical lymphocytes (at arrow) under 1000 magnification

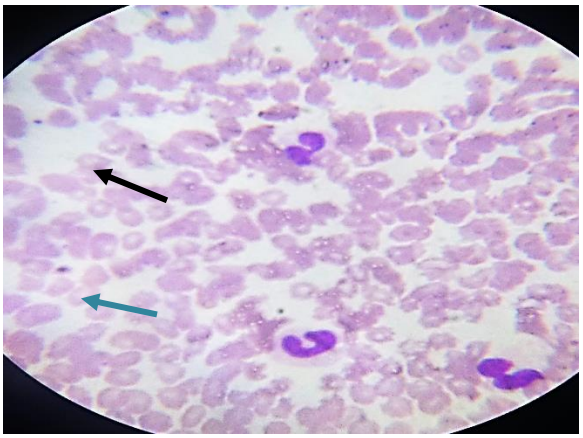

Figure 5. Presence of hyposegmented neutrophil (at black arrow) and band neutrophil (at blue arrow) under 1000 magnification

This atypical lymphocyte was observed in 27 out of 30 of the studied cases (90%) whereas the hypo segmented neutrophil was observed in 11 out of 30 studied PBF (36.7%) and the band neutrophil also was observed in 9 out of 30 studied cases (30%).

Platelets Analysis

The findings of platelets morphological analysis in this study were the platelets are normal in size, colour and shape as shown in figure 6 and there are no presence of platelet clumping. The only abnormal finding for platelet microscopic examination analysis was the amount of platelet presence in the PBF of this study where 22 out 30 patients have thrombocytopenia (73.3%) in which most of in these 22 patients have platelet count below 100000 µL (normal range between 150000-450000 µL).

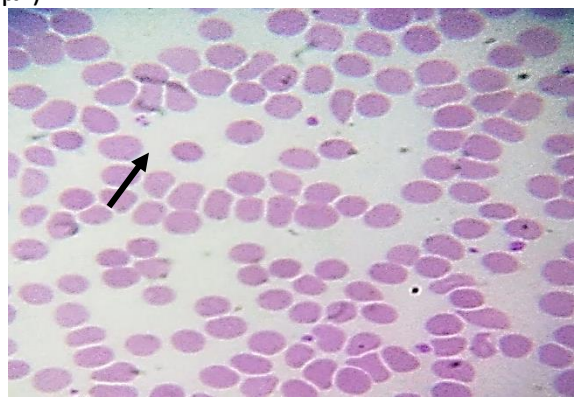

Figure 6. Presence of normal platelets (at arrow) under 1000 magnification

Discussions

Analysis of RBCs

Based on the RBCs analysis in this study, there is no significant morphological changes in RBCs where most of the studied PBF show normocytic and normochromic RBCs. there are only a few cases show slightly morphological changes in RBC in this study where only 8 out of 30 cases (26.7%) show presence of microcytic and hypochromic RBCs. The presence of microcytic RBCs in this study is might be due to the low volume of RBCs or known as low MCV where MCV will decrease or increase in accordance with the average red cell size in which case of low MCV will indicate microcytic (small RBC size), normal MCV indicates normocytic (normal average RBC size), and high MCV indicates macrocytic (large average RBC size) (Jampangern et al., 2007). This might because of the DENV invade RBCs that will cause the destruction of RBCs or might because of bone marrow suppression (Jones, 2006).

There are also a few cases with presence of hypochromic RBCs [n=8, (26.7%)] in this study where the hypochromic is a term of less normal colour that indicates the cells have less than the normal amount of haemoglobin. As stated at the above that condition that cause low volume of RBC such as blood loss, destruction of RBC and suppression of bone marrow also will lead to low haemoglobin content because haemoglobin is also a component of RBC.

In this studied PBF, there are slightly presence of abnormal shape of RBC such as teardrop cell [n=5, (16.7%)] and ovalocyte [n=4, (13.3%)]. Teardrop cells appear in the peripheral blood as tear-shaped RBCs in which the extension of the tails from portion of RBC is variable. The exact physiological mechanism of teardrop cells is still remaining unknown.

Ovalocytes are also considered as egg-shaped cells that have higher tendency to have varied of haemoglobin content in which they can appear either normochromic or hypochromic. The morphological abnormality of ovalocyte is thought to be a result of weak mechanical or membrane skeleton fragility that may be acquired or congenital. However, the pathogenesis of this ovalocyte is also still unknown like teardrop cells. These two abnormal shapes are not considered as significant morphological changes in PBF in this study due to the low occurrence in only around 4 to 5 dengue patients.

Besides, some findings of studied PBF in this study also show the presence of NRBC [n=11, (36.7%)] which might indicate a severe stress on the bone marrow that forcing the premature release, but this NRBC finding is not a significant finding because the NRBC only present in 11 out of 30 cases in this study and from the previous studies, there is no evidence that show the presence of NRBC is a significant findings in DF. Therefore, NRBC could not be used as a parameter in screening dengue infection.

Analysis of WBCs

PBF results in this study show leukopenia occur in 10 out of 30 cases (33.3%) which are due to virus induced bone marrow suppression that lead to decreasing production of WBCs where leukopenia is one of the symptoms of dengue infection (Mehta et al., 2013). However, leukopenia is not a significant finding in this study due to low occurrence in only 10 patients. Besides that, there is absence of abnormal morphological features in eosinophil, basophil and

monocyte in this study which might because of these WBCs is not severely affected in classical DF case. However, there are presence of abnormal morphological changes in neutrophil and lymphocyte where some of the neutrophils appear as hyposegmented and some release as immature neutrophils which also known as band neutrophil, while some of the lymphocytes appear as atypical lymphocytes.

Hyposegmented neutrophil has lack of nuclear lobes where usually occur in chronic infection and also act as significant features of Pelger-Huet anomaly. However, these presence of hyposegmented neutrophils also can be acquired as a result of severe infection, malignancy, burns, chemotherapy or other medications such as sulphonamides where these acquired hyposegmented neutrophils are known as pseudo-Pelger-Huet cells. These hyposegmented neutrophils will return as normal neutrophil when the causative agent is removed. The percentages of neutrophils that effected will be varied in this condition (Noisakran et al., 2010).

Band neutrophils are immature neutrophils that are usually seen in the early response of infection; however, the earlier forms also can be seen. The presence of these immature cells is known as "shift to the left" which can be the earliest sign of a WBCs response even before the WBCs count increased. This condition of "shift to the left" might be due to release of bone marrow stores in which this particularly occurs if the bone marrow reserve of mature neutrophils is decreased where the release of the marrow reserve usually occurs in response towards acute inflammation. These two abnormal features of neutrophil are not considered as significant changes because of the low occurrence in only 11 out of 30 cases for hyposegmented and 9 out of 30 cases for band neutrophil which can be supported by none of the previous study show these findings can be significantly found in dengue infection.

The only significant findings of WBCs in this study were presence of atypical lymphocytes where they are the major consistent finding in this PBF study. These atypical lymphocytes are easily can be recognized as they have significant shape with increased amount of cytoplasm with characteristic of tailing pattern of the cytoplasm along with the increased cytoplasmic basophilic that fulfilled the criteria set for atypical lymphocytes that are shown in the previous study (Smith, & Schwartz, 2005). From previous studies, the presence of atypical lymphocytes is due to the T cell activation where the proliferation is seen in most of viral infections. Moreover, total amount of atypical lymphocytes in dengue infection was higher than the other *Flavivirus* infection which could be a good indicator for dengue infection. Therefore, this atypical lymphocyte presence should be considered as a useful screening parameter for dengue infection besides thrombocytopenia.

Analysis of Platelet

22 out of 30 patients (73.3%) in this study develop thrombocytopenia where thrombocytopenia is a typical finding in dengue fever. This thrombocytopenia is another significant finding that found in this study which could be a parameter that can help in diagnosing dengue infection. There are a few possible causes that trigger thrombocytopenia where one of the causes is bone marrow suppression where the bone marrow will display hypocellularity during the early phase of disease and attenuation of megakaryocyte maturation. The accurate mechanisms of basic fundamental DENV-induced bone marrow suppression during the acute phase still remain

unclear. However, three main factors have been suggested which are direct lesion of progenitor cells by DENV, infected stromal cells by DENV and changes in bone marrow regulation.

Another possible cause of thrombocytopenia is increased destruction of platelet that may be due to either activation of the complement system, or increased phagocytosis of platelet by macrophage, or development of anti-platelet antibodies which cause lysis of platelet. Direct attachment of DENV antigen to platelets without any reaction of immune response will cause platelet destruction that can lead to thrombocytopenia.

Conclusions

Based on the findings of this study, it was concluded thrombocytopenia and the presence of atypical lymphocytes in PBF are the crucial and vital clues in screening dengue infection as they are the significant morphological changes in this studied PBF. These two clues have a potential to be a useful parameter in screening test for dengue infection which could differentiate dengue fever from the other febrile illnesses and other *Flavivirus* infection. As a conclusion, PBF might have a significant role in supporting diagnosis of dengue which can complement FBC and serological diagnosis of dengue especially in cases where the clinical presentations are atypical.

Acknowledgement

I would like to thank my friend Mohd Jaamia Qaadir Mohd Badrin for his advices. Special thanks and appreciation to Mohd Akmal Suparman, Medical Laboratory Technologist UMMC for the valuable assistance and support throughout this study.

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