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Title: Correlation of morphological abnormality in Peripheral blood smears with disease severity and mortality in Covid 19

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Abstract

Introduction: COVID 19 pandemic, caused by SARS –CoV-2 virus causes flu like mild symptoms to severe acute respiratory syndrome. Pathogenesis is Immune system deregulationand is haracterized by the presence of lymphopenia in the peripheral blood smears. The clinical laboratory plays an important role in the diagnosis, treatment and prognosis of coronavirus patients.

Aims &Objectives:

- To describe the morphological changes in white blood cells, red blood cells, and platelets in the peripheral blood smears of COVID 19 positive patients.
- To perform a blinded study on morphological parameters by two pathologists and study the inter-observer variability.
- To correlate morphological changes with the haematological parameters obtained on complete blood counts.
- To study the association of morphological changes with disease severity and survivor status of the patients

Material &Methods: A total of 100 adults,aged more than 18 years who tested positive on RT PCR were enrolled for the study. Based on the symptoms, the patients were divided into two groups: mild to moderate and severe. Venous blood sample was collected and peripheral blood smearswere examined by pathologists.

Results: The most characteristic haematological findings noted in peripheral blood smears inCOVID 19 patients were leucocytosis, neutrophilia, lymphopenia, monocytosis and thrombocytopenia.In neutrophils both nuclear as well as cytoplasmic abnormalities were noted. Nuclear abnormalities ranged from ring shaped nucleus tohypolobular neutrophils termed as pseudo pelgerhuet anomaly to presence of band forms. Cytoplasmic abnormality included presence of dark blue granulation which is similar to toxic granules. Apoptotic bodies were also noted. In platelets, we observed strikingly a unique feature showing presence of large platelets and increased frequency of platelet clumps along with normal to low platelet counts.

Conclusions: These routinely used, non-invasive, quick and cost-effective tests for morphological details along with routine CBC parameters can play an important role which will have great clinical relevance helping to predict the disease progression and severity at an early stage aiding in patient triage and management.

Keywords: Complete blood count(CBC), COVID-19, coronavirus, Peripheral blood smear (PBS), SARS-CoV-2,Real time reverse transcriptase polymerase chain reaction (RTPCR)

Manuscript

Introduction: -

The novel coronavirus (COVID 19) pandemic, caused by SARS -CoV-2 virus belongs to a large family of viruses that cause flu like mild symptoms to severe acute respiratory syndrome.

COVID 19 was reported in Wuhan, China in late December 2019 and soon after, this virus spread to the entire world.[1] In India between the month of May and June the number of COVID 19 patients is rapidly increasing as it has been hit by a huge second wave. However, its pathogenesis is still unclear, but many studies have shown that the virus enters through respiratory droplets and interact with angiotensin-converting enzyme 2 (ACE 2), a monocarboxypeptidase present on the cell surface of respiratory epithelium, penetrate the host cell which leads to derangement of renin-angiotensin-aldosterone axis. [2]

Immune system dysregulation has also been seen in the pathogenesis of COVID -19 and this is shown by the presence of lymphopenia in the peripheral blood smears. [3]

Many studies have been done on various inflammatory parameters such as C - reactive protein, Serum ferritin, lactate dehydrogenase values and other parameters such as complete blood count (CBC), D-Dimer, renal and liver function tests in coronavirus disease. Several studies reported that leukocytosis, lymphopenia, neutrophilia was seen in severe cases as compared to non-severe

cases of COVID patient. [4,5] Also in COVID-19, the severity of disease was associated with activation of proinflammatory monocytes especially in elderly and eosinopenia were found.[6,7,8] Many studies have shown that neutrophil-lymphocyte ratio (N-L Ratio) was persistently increased in severe cases and Zhichao et al noted that higher NLR values in COVID-19 patients was predictor of pneumonia at the time of admission.[9]

CBC and peripheral smear are routinely performed in the hematology laboratory but even on extensive search of literature there was a dearth of articles which shows an association of blood cell morphology which can aid as a predictor of severity in COVID 19. [10, 11] These include abnormal morphology of neutrophils (pelger huet anomaly, hypolobulation etc), lymphocytes (vacuolization, plasmacytoid etc), red blood cells (basophilic stippling etc), and platelets (giant forms, clumping etc). Hence the clinical laboratory has played an important role in the diagnosis, treatment and prognosis of coronavirus disease patients.

The management of cases of COVID 19 is closely related to the emergence of the cytokine storm by day 5-7. The administration of plasma, steroids and anticoagulants have to be closely monitored and administered before the onset of cytokine storm which causes pulmonary damage and may become fatal.

In this study, we report the morphological changes in blood cells during the course of COVID 19 disease. The objective of present study was to describe the morphological changes in white blood cells, red blood cells, and platelets in the peripheral blood smears of COVID 19 positive patients, also correlate morphological changes with the haematological parameters obtained on complete blood counts and study the association of morphological changes with severity of the disease and survivor status of the patients.

Material and methods:

Study design:

The present study was a cross-sectional study which was conducted at ESIC Medical College & Hospital, Faridabad in the department of pathology over a period of 1 month. The study was approved by Institutional Ethics Committee.

Data Collection:

Demographic and clinical details including age, sex, date of admission, severity of disease status, date of CBC along with peripheral blood smear evaluation was collected from records.

Venous blood sample was collected in EDTA vacutainer on day 3 to 5 of admission in ICU, HDU and ward for parameters like hemoglobin, red blood cells count, hematocrit, mean corpuscular volume, platelet count, white blood cells and differential count. CBC was performed using fully automated 6-part analyzer (Sysmex XN 1000).

Peripheral blood smears were made for all cases and stained by Leishman's stain and visually examined by two experienced pathologists blinded in terms of all clinical details of the cases and also to each other.

All the relevant clinical and demographic data, lab parameters and morphological details were entered in the Excel Spread sheet.

Inclusion criteria

A total of 100 adults (more than 18years) who were positive on real time reverse transcriptase polymerase chain reaction (RT PCR) were enrolled for the study. Based on symptoms, the patients group were divided into two groups: mild to moderate and severe.

Operational definition

Mild to moderate: A patient who presented with fever, sore throat and cough with no respiratory distress.

Severe group: If Patients presents one of the following criteria: 1) Respiratory distress with respiratory rate more than 30times/min; 2) Oxygen saturation $\leq$ 93% in resting state; 3) PaO2/FiO2 $\leq$ 300 mmHg (1 mmHg = 0.133 kPa). (12)

Based on survivor status, the study group was also divided into survivors and non survivors.

Statistical analysis

The data collected for 100 cases were entered in Microsoft excel sheet and analysed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). The continuous data will be presented as mean and SD. The continous variables in the two groups i.e, mild-moderate and severe were subjected to test of normality. The non-normally distributed variables in two groups (survivor and non survivor) were compared using Mann Whitney U test among two groups (mild-moderate and severe) were compared using Krushal Walis test and normally distributed variables were analysed using ANOVA test. The variables grouped into survivor and non survivor groups were normally distributed and t test was applied to test the statistical difference in two groups. The categorical data was presented as proportions. Pearson's correlation coefficient was calculated

for studying association between variables. P value < 0.05 was considered statistically significant. The inter-observer variability is measured by using Kappa statistics.

Results:

A total of 100 COVID 19 positive patients were enrolled for the study. The study population was divided into two groups on the basis of disease severity: mild to moderate (n= 56), severe (n= 44). Patients of mild to moderate and severe stage groups were almost similar in terms of age and sex. (P>0.05). Out of the 100 cases, 18 patients of severe group expired during the treatment, the mortality rate being 18%

Hematologic parameter of COVID 19 based on disease severity

The most characteristic findings of severe group of COVID 19 patients are leucocytosis, neutrophilia, lymphopenia, monocytosis and thrombocytopenia. On comparing severe COVID 19 patients (n= 56) with mild- moderate group (n=44), leucocytosis (0.0000), neutrophilia (0.009) , monocytosis (0.0052) and thrombocytopenia (0.038) were statistically significant (p<0.05) but no statistically significant difference between in Hemoglobin (Hb), Red Blood Cell (RBC) count, MCH, MCHC, RDW SD and RDW CV. (p>0.05) **(Table 1)**

Morphological findings among both groups

Red blood cells morphology in the peripheral blood smear predominantly revealed normocytic normochromic picture (22%) followed by dimorphic (12%), microcytic (6%) and macrocytic (4%) both in mild-moderate and severe group. **(Table 2)**

White blood cells morphology showed changes mainly in neutrophils having both nuclear as well as cytoplasmic abnormalities. Nuclear abnormality showed ring shape nucleus and hypolobulation (p value 0.00001) termed as pseudo pelger huet anomaly along with presence of band forms and cytoplasmic abnormality showing presence of dark blue granulation (p value 0.00001) which is similar to toxic granulation. Reminiscent of polymorphs with nuclear fragmentation i.e Apoptotic cells (p value 0.00028) were also noted. Reactive and plasmacytoid lymphoid cells (p value 0.01035) were seen but monocytes and eosinophils did not show any relevant morphological changes.

In Platelet morphology, we observed strikingly a unique feature showing presence of large platelets (p value 0.00001) and increased frequency of platelet clumps (p value 0.00001) in the presence of normal to low platelet count.

The number of cases showing the morphological changes in the mild to moderate and severe group are represented in the table **(Table 3) (Fig 3)**

In comparison of disease severity with the survivor status, none of the patients died in the mild to moderate stage group but 18 patients died in the severe group ($P<0.05$).

Discussion

The recent second wave of COVID 19 around April-May has hugely hit India and caused unprecedented burden on the health system of India. Many studies have been done to understand the pathogenesis and clinical course of disease progression. Earlier it was thought to be respiratory tract infection, but now it involved all the system like neurological, cardiovascular and hematopoietic system etc.[12,13] However, the disease etiopathogenesis is still unclear. Many studies have shown that during early stage of the disease, it causes changes in WBC count from normal to low .[10,11,14] In few patients, who had severe symptoms, with cytokine storm there was an increase in the systemic inflammatory reactions in the body. Many studies have been published on hematological parameters, but only very few studies from India has been done on morphology of blood cells. [10, 11] In the Indian scenario, we observed a study on 100 cases of COVID 19 where we correlated the morphological abnormality in peripheral blood smear of mild- moderate and severe groups with complete blood count, severity and also the survivor status of the patients.

In the present study, it was observed that the majority of the patients of severe group showed predominantly leucocytosis, neutrophilia, lymphocytopenia, monocytosis and thrombocytopenia. We have also noted the marked morphological abnormalities of neutrophils and peculiar abnormalities of platelets in severe group. A review conducted by Wu et al. revealed that patients with COVID-19 pneumonia had a higher risk of developing ARDS. [15,16,17] They analyzed the possible risk factors for acute respiratory distress syndrome (ARDS) and death among patients with COVID--19 pneumonia in Wuhan.[18] They found that patients with increased neutrophils and higher levels of regulatory T-cells were more prone to developing ARDS.[19] In the present study , we also noted neutrophilia in majority of the severe group as compared to mild-moderate group (83.5% versus 51.7%).

But, Huang et al reported that majority of patients had lymphocytopenia 83.2% whereas 36.2% had thrombocytopenia and 33.7% showed leukopenia at presentation. [15] They also noted that morphological abnormality of peripheral blood smears was more prominent in severe cases as

compared to mild-moderate cases (96.1% versus 80.4%) for lymphocytopenia, (57.7% versus 31.6%) for thrombocytopenia, and (61.1% versus 28.1%) for leucopenia. [18]

Many studies from China & Italy showed that morphology of peripheral blood cells get altered on COVID 19. [11,14] But only few Indian studies were published on morphological abnormality in red blood cells, white blood cells & platelets. However, no study shows correlation of severity and mortality with morphological blood picture and whether these changes have clinical significance at the time of diagnosis. [10,20] In the present study we highlighted the significance of morphological changes in blood cells of COVID 19 on 3rd to 5th day as well as its correlation with disease & mortality.

On careful examination of peripheral blood smears, we observed wide range of significant changes among WBC and platelet series. In neutrophils we observed both nuclear as well as cytoplasmic abnormalities. Nuclear abnormality showed different shape of nucleus for example ring shaped nucleus and hypolobular neutrophils termed as pseudo pelger huet anomaly along with presence of band forms and cytoplasmic abnormality showed presence of dark blue granulation which is similar to toxic granulation.[21] Apoptotic bodies were also noted. But Christain salib et al showed hypersegmented neutrophils in COVID 19 patients. [22] In platelet, we observed a striking unique feature showing presence of large platelets and increased frequency of platelet clumps in the presence of normal to low platelet count.

Soon after the COVID-19 outbreak, reports of an increase in the activation of a coagulation pathway emerged. Many patients exhibited thrombotic events and acute pulmonary embolisms. [23,24] Autopsies revealed the presence of thrombosis in several organs, such as lungs. [25] The data indicate that COVID19 infection triggers unique patterns of hypercoagulation and platelet activation, which can lead to the onset of a coagulation cascade [26.] These patterns are distinct to the usual pattern of DIC which is seen in ARDS and severe gram negative sepsis. [27, 28] There is decrease fibrinogen level and presence of fibrin monomer noted in DIC. But in COVID 19 many studies were observed increased fibrinogen level and increase D- dimer. [26] They also observed unique feature of platelets i.e platelet clumps and giant platelets. We also observed same features of platelets, mostly in severe group 21 & 25 out of 44 pt showed giant form and clumps. This feature of platelet is defined as sign of increased thrombopoiesis and platelet activation. [29] The previous study of our institute also showed that coagulation profile of COVID 19 patients was distinct from classical DIC. The levels of D dimer , FDP, PT INR and Fibrinogen and TT were

raised but the Platelet count was not lowered and APTT was not prolonged. [30,31] Mostly, these large size platelets were seen as immature forms and has been associated with smoking related cardiovascular disease and inflammation with increased IPF. [32,33] Many studies also noted that alpha dense granules seen in large platelet which store GpIIb/ IIa, fibrinogen and VWF causes trigger to coagulation cascade.[34] Other active molecules which include catecholamine, serotonin, Calcium, ADP and ATP which are stored in dense alpha granules of platelet are secreted during platelet activation and these initiate the coagulation cascade.[35]

Aberrant coagulation and bleeding have been reported in most of the viral fevers like Hanta Virus & Ebola virus. [23, 36] But COVID 19 is not associated with heamorrhage. There is evidence of coagulation activation and increased pulmonary embolism. Many centers accept the guidelines to start therapy with low molecular weight heparin in COVID 19 patient. Previous study of our institute also showed that D dimer level is useful predictor of disease severity. The combination of FDP and D dimer is the best combined coagulation parameters. The cut off values may vary among different populations of these markers. [31]

Many studies have shown that dysregulation of Angiotensin II was associated with Diabetes, hypertension and Obesity. [37] In both conditions platelet activation and increased platelet size have been reported. [38,39,40] It was observed that SAR CoV 2 infection was frequently seen in patient with these comordities. [26] The loss of function of ACE 2 and SAR CoV2 infection enhance the current prothrombotic state of cases with hypertension and Diabetes. [26]

The distinctive features of COVID 19 infection are increased Angiotensin II activity and loss of function of ACE 2 which causes platelet activation, severe endothelial dysfunction and increased thrombosis. Hence SAR CoV 2 is associated with fatal outcome which causes microcirculatory compromise in several organs including lungs, heart, CNS and Kidney.

Limitation

Our study is limited by the fact that we could not follow up the patient of COVID 19 for the identification of disappearance of pathological blood cells. Also, we couldn't correlate the haematological and morphological findings with coagulation parameters.

Conclusion

This study emphasizes the importance of CBC parameters and morphological assessment of peripheral blood smear. In the present study, we noticed unique striking findings in platelet i.e giant ($p<0.00001$) and clumps forms ($p <0.00001$), hypolobation ($p <0.0001$) and toxic granulation ($p < 0.00001$) in neutrophils and these were found statistically significant with clinical severity. Reactive ($p<0.00069$) and plasmacytoid lymphoid cells ($P< 0.01035$) were also found statistically significant with clinical severity. However these findings may be seen in hematologic malignancies (eg Myelodysplastic Syndrome) and viral infections (eg dengue, Infectious mononucleosis). Hence one needs to closely follow up in these cases. It is evident that the monitoring of CBC parameters along with morphological details may predict the severity of COVID 19 patients which could help the clinicians to triage which would aid in early intervention and management of COVID 19 patients. Hence the CBC and peripheral blood smears has a great clinical relevance as they can predict the disease progression and severity at an early stage as these are the most routinely used, non invasive, quick and cost effective parameters.

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Table 1: Comparative analysis of clinical and hematological parameters based on severity in sub categories of COVID 19 patients.

Variable	Severity		Mild		Significances of chance (ANOVA)	
	Mean	SD	Mean	SD	F value	P value
Age	49.40	12.16	52.33	14.76	1.4733	0.9197
HB	11.302	2.346	11.228	2.361	1.0128	0.967
TLC	22170	24444	11527	7683	9.8823	0.0000*
Platelet	2.857	1.872	2.204	1.240	2.1437	0.038*
RBC	3.800	1.044	3.896	0.976	0.847	0.6317
HCT	36.95	7.11	36.421	6.319	0.7899	0.4063
MCV	89.30	8.67	89.85	7.89	0.8282	0.5054
MCH	27.744	2.744	28.193	3.049	1.2347	0.4787
MCHC	30.860	1.841	31.561	1.583	0.7394	0.2891

N	89.745	15.743	81.347	12.278	2.653	0.009*
L	11.90	10.88	13.92	10.08	0.8583	0.5879
M	3.167	2.767	3.494	1.853	0.4485	0.0052*
E	1.357	3.245	1.982	2.622	0.6529	0.355

Table 2: RBC morphology of mild-moderate and severe stage.

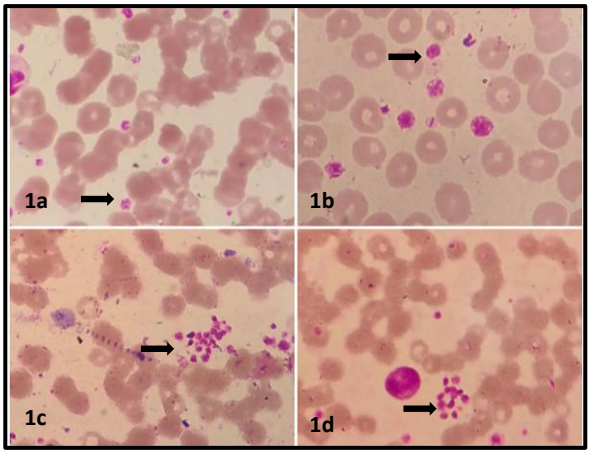
	No of cases	No of cases
RBC morphology	Mild – moderate (n =56)	Severe (n =44)
NCNC	32	22
Dimorphic	18	12
MCHC	2	6
Macrocytic	4	4

Table 3: Morphology of WBC & Platelets in subcategories of COVID 19 patients.

Morphology	Mild – Moderate (n=56)	Severe (n=44)	P value
NEUTROPHILS			
Segmented	9	11	0.2678
Pelger huet anomaly	7	41	0.00001*
Coarse granules	6	38	0.00001*
Apoptotic cells	5	20	0.00028
LYMPHOCYTES / MONOCYTES			
Reactive	2	12	0.00069
Plasmacytoid	3	10	0.01035
PLATELETS			
Clumping	7	37	0.00001*
Giant forms	9	39	0.00001*

Table 4 :- Comparative analysis of haematological parameters in COVID 19 patients based on survivor status

Variable	**Survivor (n = 82)**	**Non survivor (n = 18)**	**P value**
Age	51.32 ± 13.61	49.67 ± 14.57	0.6756
HP	11.17 ± 2.356	11.83 ± 2.17	0.8095
TLC	1534761± 10687	21201.60± 11231	0.046*
Platelet count	2.25 ± 1.17	1.76 ± 0.77	0.030*
RBC	3.82 ± 1.02	4.14 ± 0.89	0.6294
HCT	36.37 ± 6.63	38.33 ± 6.61	0.9168
MCV	89.65 ± 8.47	88.53 ± 6.1	0.2168
MCH	28.03 ± 2.99	27.50 ± 2.24	0.2282
MCHC	31.25 ± 1.77	31.38 ± 1.32	0.2778
N%	80.920± 13.08	87.50 ± 11.45	0.034*
L%	13.65 ± 10.59	10.50 ± 10.49	0.938
M%	3.75± 2.24	2.50 ± 2.36	0.045*
E%	1.95 ± 3.10	0.50 ± 0.79	0.0001*



1a, 1b - Photomicrograph showing giant platelets (leishman stain, 400X)
1c, 1d - Photomicrograph showing platelet clumps (leishman stain, 400 X)

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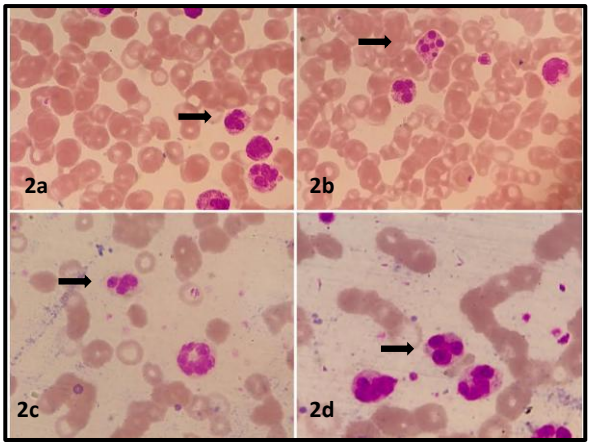
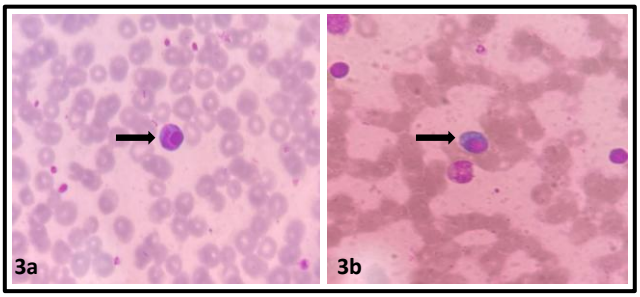


2a, 2c, 2d - Photomicrograph showing hypolobated neutrophils (400X)
2b - Photomicrograph showing apoptotic body (400X)



3a, 3b - Photomicrographs showing lymphoplasmacytoid cells (leishman stain, 400X)