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Research Article

AUTOMATED CELLULAR INDICES TO IDENTIFY DENGUE AND MALARIA AND DISTINGUISH THEM FROM OTHER FEBRILE ILLNESSES

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A B S T R A C T

Inroduction: Tropical Febrile illnesses such as Malaria and Dengue are challenging to differentiate clinically as both present with high grade fever, malaise, and other non specific symptoms: both peak in incidence around the monsoon and Post-monsoon period. Also, both may be complicated by thrombocytopenia, progression to shock and disseminated intravascular coagulation. Hematology analyzers like LH 750 use a combination of Velocity-Electrical Conductivity-Light Scatter (VCS) methods to produce complete red blood cell, platelet, and leukocyte analyses. These parameters may be used for screening of Dengue and Malaria and to differentiate it from other febrile illnesses
V- Volume by Voltage Impedence.
C- An estimate of Cytoplasm/Nuclear ratio by radiofrequency Conductivity.
S- An estimate of Cytoplasmic granularity/nuclear complexity by laser light scatter.
A total of 46 parameter will be generated.

Aim And Objectives:

Aim: Use of Automated Cellular Indices to Identify Dengue and Malaria and distinguish them from other febrile illnesses
Objective:

1. To Generate Laboratory cut-off values based on hematological and VCS indices to differentiate Dengue virus infection and Malaria from other Febrile illnesses
2. To find the Sensitivity and Specificity of laboratory cut-off values based on hematological and VCS indices
3. To compare clinical severity of the illness with above cutoff values

Materials and Methods: The present observational study was a hospital based case control study. It was undertaken to study the use of automated cellular indices to identify Dengue and Malaria and distinguish them from other febrile illnesses.
Study Period: The study was done between December 2014 to September 2016.
Result: The present study was a hospital based case control study undertaken to study use of automated cellular indices to identify Dengue and Malaria and distinguish them from other febrile illnesses.
The study revealed the following points as follows:

- The malaria factor cutoff had an AUC of 0.902 to yield a sensitivity of 89.31% and a specificity of 83.12% in malaria group.
- The dengue factor at had an AUC of 0.893 to yield a sensitivity of 88.1% and a specificity of 73.23.12% in dengue group.
- The febrile control vs malaria/dengue factor had an AUC of 0.713 to yield a sensitivity of 82.13% and a specificity of 90.16% in control group.

Conclusion: Leukocyte abnormalities quantitated by automated analyzers successfully identified malaria and dengue and distinguished them from other fevers. These economic discriminate functions can be rapidly calculated by analyzer software programs to generate electronic flags to trigger-specific testing. They could potentially transform diagnostic approaches to tropical febrile illnesses in cost constrained settings.

INTRODUCTION

Fever is one of the common presenting symptoms of illness in Children. Almost 15-20% children present as Fever without focus. It's often difficult to diagnose the etiology in the initial days of natural history of infection.[1]

Tropical Febrile illnesses such as Malaria and Dengue are challenging to differentiate clinically as both present with high grade fever, malaise, and other non specific symptoms: both peak in incidence around the monsoon and Post-monsoon period. Also, both may be complicated by thrombocytopenia, progression to shock and disseminated intravascular coagulation.[2] Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) are increasingly important public health problem in a tropical country like India. There is an estimated of 50 million or more cases of dengue annually worldwide with 4, 00,000 cases of dengue hemorrhagic fever [3]. In 1996 an epidemic in India occurred mainly due to DEN- 2 virus while the 2003 epidemic appeared mainly due to DEN-3 virus.[4] The disease warrants early diagnosis for effective treatment to

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prevent mortality.[5-9] Malaria is one of the leading causes of morbidity and mortality in developing countries. Nearly 2.48 million malaria cases are reported annually from South Asia of which 75% cases are contributed by India alone.[1] At the same time, it is perplexing that the number of falciparum cases is constantly on the rise and in recent years they contribute nearly 50% of the total cases.[2] Various hematological parameters like hemoglobin level, haematocrit, total count, differential count and platelet count are altered in dengue fever and malaria. Currently the criteria for clinical diagnosis of Dengue Virus infection includes 4 major clinical manifestations such as 1) fever 2) hemorrhagic manifestation 3) hepatomegaly 4) tendency to develop shock, and two laboratory changes; i.e. thrombocytopenia and concurrent haemoconcentration which have proven to be practical in 95% cases.[10] Thrombocytopenia typically takes place at the onset of shock phase and reverses spontaneously after a period that ranges from hours to few days. Severe leucopenia is often present. Lymphocytosis with presence of reactive lymphocyte can be seen.

The Gold Standard for detection Dengue virus infection remains Dengue (IgG, IgM antibodies and NS1 antigen) titers and for malaria the gold standard for malaria diagnosis remains expert light microscopic blood smear examination. However, modern automated blood cell analyzers offer several parameters that may indicate malarial infection and dengue virus infection.

DNA/RNA in red blood cells (RBCs) or hemozoin pigment in leukocytes, increased lyse resistance of infested RBCs and their abnormal localization on scatter plots causing interference with leukocyte analysis, and infection-induced changes in leukocytes, especially monocytes and lymphocytes, etc.[11,12]

Based on the above parameters, analyzers can automatically indicate (i.e., flag) complete blood count (CBC) specimens with possible malarial infection. They can therefore diagnose even unsuspected cases independent of clinical suspicion or specific malaria testing ordered by a clinician. CBC processing times are typically under two to three minutes, and in most instances, these flags are generated virtually free of additional cost.[11,12] However, despite the theoretical attractiveness of this approach and continued research over at least 2 decades,[13] analyzers are yet to make a transition into routine malaria diagnosis.[14]

In the initial days of febrile illness the physician offers many differential diagnoses example given-. Dengue, Malaria, Urinary tract infections and Bacteremia etc. At this juncture patient has to spend money on costly Rapid tests for Dengue and Malaria for early diagnosis. Peripheral blood smear examination needs skilled pathologist to diagnose Malarial and also is an time consuming affair.

At present there is no cheaper diagnostic screening test available for Dengue and Malaria. Rapid Malaria test costs around Rs. 250 to 300 while Dengue Rapid test costs around Rs. 700 to 850. The cost for CBC is around Rs. 150 to 200 at various labs. It is in these two febrile illnesses that we get altered cells (reactive WBC) in blood. It is an important tool in diagnosis of Dengue and Malaria.

Hematology analyzers like LH 750 which has Unmatched VCS Technology for differential counting and State of the art Coulter Principle: Impedance Method for RBC/PLT/WBC counting with sweep flow and Accucount technologies. This Hematology analyzers use a combination of Velocity-Electrical Conductivity-Light Scatter (VCS) methods to produce complete red blood cell, platelet, and leukocyte analyses. These parameters may be used for screening of Dengue and Malaria and to differentiate it from other febrile illnesses.[10]

V- Volume by Voltage Impedence.
C- An estimate of Cytoplasm/Nuclear ratio by radiofrequency Conductivity.
S- An estimate of Cytoplasmic granularity/nuclear complexity by laser light scatter.

The current study was undertaken in this background to collate data on a large number of dengue and malaria patients as well as patients with nonspecific febrile illnesses that mimic the two. We aimed at studying hematological and VCS indices with a view to generating algorithms that laboratory information system (LIS) could potentially use to flag infected samples for further testing, thereby hastening diagnosis and enabling judicious utilization of laboratory resources.

Aim and Objectives

Aim

Use of Automated Cellular Indices to Identify Dengue and Malaria and distinguish them from other febrile illnesses

Objectives

1. To Generate Laboratory cut-off values based on hematological and VCS indices to differentiate Dengue virus infection and Malaria from other Febrile illnesses
2. To find the Sensitivity and Specificity of laboratory cut-off values based on hematological and VCS indices
3. To compare clinical severity of the illness with above cutoff values

REVIEW OF LITERATURE

Dengue

Dengue is a self limiting acute mosquito transmitted disease characterized by fever, headache, muscle, joint pains, rash, nausea and vomiting. Dengue Fever (DF) is caused by an arbovirus and spread by *Aedes*mosquitoes. Some infections result in Dengue Haemorrhagic Fever (DHF) and in its severe form Dengue Shock Syndrome (DSS) can threaten the patient's life primarily through increased vascular permeability and shock.[15]

Over the past two decades, there has been global increase in the frequency of DF, DHF and its epidemics, with a concomitant increase in disease incidence. Various factors responsible for the resurgence of dengue epidemic are: (i) un-precedented human population growth; (ii) un-planned and un-controlled urbanization; (iii) inadequate waste management; (iv) water supply mismanagement; (v) increased distribution and densities of vector mosquitoes; (vi) lack of effective mosquito control has increased movement & spread of dengue viruses and development of hyper-endemicity and (vii) deterioration in public health infrastructure.[15]

Origins of the Word Dengue

Although dengue has been known by several names throughout the world, the term 'dengue' has been universally adapted. The origin of the term "dengue" may be traced to the Swahili word for the disease "ki-dingapepo". The earliest description of an illness called "dengue" can be found in Spanish written records from 1800. The term "denga", or "dyenga," had also been used to designate the disease during outbreaks in East Africa and West Indies during the early 19th century. It was only after the 1828 outbreak in Cuba that the Spanish word "dengue" came into general use, which continues to this day.

History of Dengue (Worldwide)

The first record of a case of probable dengue fever is in a Chinese medical encyclopedia from the Jin Dynasty (265–420 AD) which referred to a "water poison" associated with flying insects. The first recognized Dengue epidemics occurred almost simultaneously in Asia, Africa, and North America in the 1780s, shortly after the identification and naming of the disease in 1779. The first confirmed case report dates from 1789 and is by Benjamin Rush, who coined the term "breakbone fever" because of the symptoms of myalgia and arthralgia. The first documented cases of the severe form of dengue (Dengue Haemorrhagic Fever) were seen in Thailand and Philippines during the 1950s.This severe form of dengue subsequently spread throughout Southeast Asia and expanded globally.

Dengue in India

The first evidence of occurrence of DF in the country was reported during 1956 from Vellore district in Tamil Nadu. The first DHF outbreak occurred in Calcutta (West Bengal) in 1963 with 30% of cases showing haemorrhagic manifestations. All the four serotypes i.e. Dengue 1, 2, 3 and 4 have been isolated in India. As *Aedesaegypti* breeding is more common in urban areas the disease was observed mostly prevalent in urban areas. However, the trend is now changing due to socio-economic and manmade ecological changes, It has resulted in invasion of *Ae. Aegypti* mosquitoes into the rural areas, which has tremendously increased the chances of spread of the disease to rural areas. [15]

Epidemiology of Dengue

Dengue is one of the most important emerging viral disease of humans in the world afflicting humanity in terms of morbidity and mortality. Currently the disease is endemic in all continents except Europe. The Epidemiology of dengue is a complex phenomenon that mainly depends upon an intricate relationship between the 3 epidemiological factors: the host (man and mosquito), the agent (virus) and the environment (abiotic and biotic factors). The complexity of relationship among these factors eventually determines the level of endemicity in an area. [16]

Agent Factor

The dengue viruses are the members of the genus flavivirus. These small (50nm) viruses contain single stranded RNA. There are four virus serotypes, which are designated as DEN-1, DEN-2, DEN-3 and DEN-4. Although all four serotypes are antigenicaly similar, they are different enough to elicit cross-protection only for a few months after infection by any one of them. Infection with any one serotype confers lifelong immunity to that virus serotype. Man and mosquito are reservoirs of infection. Transovarian transmission (infection carried over to next progeny of mosquitoes through eggs) has made the control more complicated. At present, DEN1 and DEN2 serotypes are widespread in India.[16]

Vector

Dengue viruses are transmitted by the bite of female *Aedes (Ae)* mosquitoes. *Ae.aegypti* is the most potential vector but other species such as *Aealbopictus, Ae. Polynesiensis* and *Ae. Niveus* have also been incriminated as secondaryvectors. In India *Ae.aegypti*is the main vector in most urban areas; however, *Aealbopictus*is also found as vector in few areas of southern India.[16]

TheFemale Aedes mosquito deposits eggs singly on damp surfaces just above thewater line. Under optimal conditions the life cycle of aquatic stage of *Ae. Aegypti* (the time taken from hatching to adult emergence) can be as short as seven days.[16] The eggs can survive one year without water. At low temperature, however, it may take several weeks to emerge. *Ae.aegypti* has an average adult survival of fifteendays. During the rainy season, when survival is longer, the risk of virus transmission is greater. It is a day time feeder and can fly up to a limited distance of 400 meters.To get one full blood meal the mosquito has to feed on several persons, infectingall of them.[16]

Environmental Factors

The population of *Ae.aegypti* fluctuates with rainfall and water storage. Its life span is influenced by temperature and humidity, survives best between 16º-30º C and a relative humidity of 60-80%.[16]

Altitude is an important factor in limiting the distribution of *Ae. aegypti,* it is distributed between sea level and upto 1000 ft above sea level. *Ae.aegypti*is highly anthropophilic and rests in cool shady places. The rural spread of *Ae.aegypti*is a relatively recent occurrence associated with the development of rural water supply schemes, improved transport systems, scarcity of water and life style changes. *Ae.aegypti*breeds almost entirely in domestic man-made water receptacles (container used for holding or storing things) found in and around households, construction sites and factories; natural larval habitats are tree holes, leaf axils and coconut shells. In hot and dry regions, overhead tanks and ground water storage tanks become primary habitats. Unused tyres, flower pots and desert coolers are among the most common domestic breeding sites of *Ae. aegypti.* [16]

Host Factor

Dengue virus infects humans and several species of lower primates but in India man is the only natural reservoir of infection. All ages and both sexes are susceptible to dengue fever. Secondary dengue infection is a risk factor for DHF including passively acquired antibodies in infants. Travel to dengue endemic area is an important risk factor. If the patient develops fever more than 2 weeks after travel, dengue is unlikely. Migration of patient during viremia to a non endemic area may introduce it into the area.[16]

Transmission Cycle of Dengue[15]

The female *Ae.aegypti* usually becomes infected with dengue virus when it takes blood meal from a person during the acute febrile (viraemia) phase of dengue illness. After an extrinsic

incubation period of 8 to 10 days, the mosquito becomes infected and virus is transmitted when the infected mosquito bites and injects the saliva into the skin of the person. There is evidence that vertical transmission of dengue virus from infected female mosquitoes to the next generation occurs through eggs, which is known as transovarian transmission.

Pathogenesis of Dengue

The pathogenesis of dengue involves a complex interaction between virus and host factors, and remains incompletely understood. The severe manifestations of Dengue virus infection are observed at the point the virus is being cleared from the host by the immune response as opposed to when the viral load is highest, suggesting that the immune system plays a key role in disease pathogenesis.[17]

Prospective cohort studies in Asia and Latin America have demonstrated that secondary infections are associated with more severe disease.[18] The accepted explanation for this observation is that non-neutralizing cross-reactive antibodies elicited in a primary infection binds the virus in a secondary infection and then have a greater ability to infect Fc-receptor bearing cells. This is called antibody-dependent enhancement (ADE), and potentially leads to an increased viral biomass, and therefore more chance of developing severe disease.[19] Despite epidemiological and laboratory support for ADE, severe disease can occur in primary infections and most secondary infections do not result in severe disease. This implies that other factors contribute to the dengue pathogenesis.

The cellular immune response is also involved with the clearance of dengue virus from the host, and is thought to play a role in the development of severe disease.[20] The proliferation of activated memory T-cells and the production of pro-inflammatory cytokines are thought to contribute to the development of plasma leak observed in dengue. In addition, the level of T-cell activation is proposed to correlate with disease severity. It has been demonstrated that the T-cells produced in severe disease have a low affinity for the current infecting serotype, but a high affinity for a different serotype responsible for a past infection. [21] This observation is consistent with the concept known as 'original antigenic sin'. There is increased evidence that the observed clinical severity varies depending on the infecting serotype.

Many host factors appear to contribute to the development of severe disease. A retrospective study in Vietnam demonstrated that children admitted to hospital with dengue aged between 1 and 5 years were four times more likely to die than children aged between 11 and 15 years.[22] Various single nucleotide polymorphisms (SNPs) appear to have an association with both protection and vulnerability to severe disease. For example, a polymorphism in the vitamin D receptor appears to protect against severe disease, perhaps reflecting the role of the vitamin D receptor in immune modulation.[23] There have been conflicting results from studies looking at polymorphisms in the tumour necrosis factor a (TNF-a) gene with some studies showing an association and others not. Genome-wide association studies (GWAS) allow a broad approach in understanding genetic susceptibility to diseases, including dengue. SNPs at two loci with a significant association with DSS have been identified from a GWAS conducted on samples from Vietnamese children.[24] These SNPs were in the major histocompatibility complex class I polypeptide- related sequence B gene (MICB) on chromosome 6, and in the phospholipase C epsilon 1 (PLCE 1) gene on chromosome 10. The MICB gene encodes a surface protein that contributes to natural killer (NK) and CD8 T-cell activation. The observed association with an SNP in the MICB gene and DSS may reflect dysfunctional NK and CD8 cell activity in severe disease, suggesting a key role for these cells in disease control and pathogenesis. GWAS technology has the potential to advance understanding of pathogenesis, and may have a future clinical application within the context of dengue pharmacogenomics.[24]

Clinical Diagnosis of DHF and DSS

Immuno-pathogenesis [16]

Primary or first infection in non immune persons usually causes Dengue fever. Subsequent dengue infection by different serotype causes more severe illness like DHF and DSS. The key manifestations of the DHF are acute fever, thrombocytopenia occurring at the time of defervescence of fever, objective evidence of capillary leakage and hemorrhagic diathesis whereas DSS manifests as sudden onset of shock (cold and clammy extremities with narrow pulse pressure i.e less than 20 mmHg) plus features of DHF. Thrombocytopenia. Pathogenesis is not well defined but it is suggested that it is mediated through soluble mediators, compliment activation and cytokines that are responsible for various manifestations. Antibody-dependent enhancement (ADE) of infection has been hypothesized as a mechanism to explain severe dengue in the course of a secondary infection and in infants with primary infections

Laboratory Diagnosis of Dengue[15]

Early symptoms of dengue fever mimic other diseases such as chikungunya, malaria, UTI and bacteremia. Hence for proper management rapid differential diagnosis is very crucial. Laboratory diagnosis can be carried out by one or more of the following tests:

- Isolation of Dengue virus by PCR test from serum, plasma or leucocytes.
- Demonstration of a fourfold or greater rise in reciprocal IgG antibody titers to one or more dengue virus antigen in paired sera samples.
- Demonstration of dengue virus antigen (Non structural protein 1 antigen i.e NS1 antigen) and Anti-dengue antibodies (IgG and IgM) in serum by immunofluorescence or by EIA
- Detection of viral genomic sequences in serum or CSF sample by PCR (Polymerase Chain Reaction)

Isolation of Dengue Virus[15]

Isolation of most strains of dengue virus from clinical specimens can be accomplished in a majority of cases provided the sample is taken in the first few days of illness and processed without delay. Specimens that may be suitable for virus isolation include acute phase serum, plasma or washed buffy coat from the patient, autopsy tissues from fatal cases, especially liver, spleen, lymph nodes and thymus, and mosquitoes collected in nature. This method is suitable for research and not for patient care.

Serological Tests [15]

Following tests are available for the diagnosis of dengue infection

- Haemagglutination-Inhibition (HI),
- Complement Fixation (CF),
- Neutralization test (NT),
- IgM capture enzyme-linked immunosorbent assay (MAC-ELISA), and
- Indirect IgG-ELISA.

***Haemagglutination inhibition (HI) test*[15]**

Of the above, HI assay has been the most widely used method for the serological diagnosis of dengue in the past. However, due to the extensive cross-reaction encountered and time consuming due to the requirement of both acute and convalescent sera collected at least seven days apart have limited the general applicability of this assay.

***Compliment Fixation Test (CFT)*[15]**

The testing procedure is cumbersome and requires highly trained personnel. The reagents are thermolabile. Due to these, currently it is not used for routine diagnosis.

***Neutralization test (NT)*[15]**

Though this is most specific and sensitive serological test for dengue infections, due to time involved in the testing procedure coupled with technical difficulty has limited the use of this test.

***IgM-capture Enzyme-Linked Immunosorbent Assay (MAC-ELISA)* [15]**

MAC-ELISA has become widely used test in the past few years. It is a simple, rapid test that requires very little sophisticated equipment. MAC-ELISA is based on detection of the dengue-specific IgM antibodies in the test serum by capturing the mout of solution using anti-human IgM that was previously bound to the solid phase. If the IgM antibody from the patients' serum is anti-dengue, it will bind to the dengue antigen. An enzyme-substrate is added to give a colour reaction for easy detection. The anti-dengue IgM antibody develops a little faster than IgG and is usually detectable by day five of the illness.

***IgG-ELISA*[15]**

An indirect IgG-ELISA has been developed that compares well to the HI test. This test can also be used in comparison with IgM to differentiate primary and secondary dengue infections. The test is simple and easy to perform and is thus useful for high-volume testing. The IgG-ELISA is nonspecific and exhibits the same broad cross-reactivity among flaviviruses as the HI test thus cannot be used to identify the infecting dengue serotype.

***Rapid Diagnostic tests* [15]**

A number of commercial Rapid Diagnostic Test (RDT) kits for anti-dengue IgM and IgG antibodies are at present commercially available, which produces the results within 15 to 20 minutes. However, the sensitivity and specificity of most of these tests is not known since they have not yet been properly validated. Though some of the RDTs have been independently evaluated, the results showed a high rate of false positive compared to standard tests, while others have agreed closely with standard tests. The sensitivity and specificity of some RDTs also found to vary from lotto lot. According to WHO guidelines, these kits should not be used in the clinical settings to guide management of DF and DHF cases because many serum samples taken in the first five days after the onset of illness will not have detectable IgM antibodies. The tests would thus give a false negative result. Reliance on such tests to guide clinical management could therefore, result in an increase in case fatality rate.

Recently the NS1 antigen of the dengue viral genome has been shown to be useful as a tool for early diagnosis of acute dengue infections. It has been detected in the serum of infected patients as early as 1st day post onset of symptoms (DPO), and up to 18th DPO. The NS1 ELISA based antigen assay is commercially available for dengue virus and many investigators have evaluated this assay for sensitivity and specificity. The NS1 assay may also be useful for differential diagnostics between flaviviruses because of the specificity of the assay.[16]

Dengue rapid diagnostic kit consists of two devices: one device for detection of Dengue NS1 antigen and second device for the differential detection of Dengue IgM/IgG antibodies in Human serum/plasma. Dengue NS1 Antigen device contains two lines; 'C' (Control line) & "T" (Dengue NS1 Antigen test line). Test line is coated with anti-dengue NS1 Ag. When a sample is added to the device, Dengue NS1 antigen if present in the sample will bind to the anti-dengue NS1 gold colloidal conjugate making antigen antibodies complex. This complex migrates along the membrane to the test region and forms the visible pink line at "T" as antibody-antigen-antibody gold colloid forms. Dengue IgM/IgG test device contains three lines; "C" (Control line), "M"(IgM test line) &"G"(IgG test line).IgM test line is coated with anti-human IgM and IgG test line is coated with anti-human IgG. When a sample is added to the device, IgG and IgM antibodies in the sample react with anti-human IgM or IgG antibodies coated on the membrane respectively. Colloidal gold complexes containing dengue 1-4 antigens is captured by the bound anti-dengue IgM or IgG on respective test bands located in the test window causing a pale to dark red band to form at the IgG or IgM region of the test device window. The intensity of the test bands in the respective device will vary depending upon the amount of antigen /antibody present in the sample. The appearance of any pink/ red colour in a specific test region should be considered as positive for that particular antigen and/or antibody type (IgG or IgM). A red procedural control line should always develop in the test device window to indicate that the test has been performed properly.[15,16]

Fig 1 Rapid Diagnostic Kit for Dengue Ns1 Antigen, IgG and IgM Antibody Detection

Malaria

Malaria has existed for over 4,000 years. The first description of the disease was in ancient Chinese medical writings, NeiChing(The Canon of Medicine), around 2700 BC. By the fourth century BC, the Greeks had documented the disease. Malaria had been the cause of a great decrease in population of the Greek city-states. In addition to Hippocrates, Susruta, a Sanskrit medical treatise, documented the symptoms of malarial fever. This treatise first connected the bite of an insect to the cause of malarial fever.[26]

Ronald Ross was the first to prove Plasmodia were transferred from humans to mosquitoes in 1897. He observed the infected mosquitoes bit birds, which contracted malaria afterwards. In 1899, Grassi, AmicoBignami, and GuispeppeBastianelli demonstrated that the sporogonic (sexual) cycle of the Plasmodium genus occurs in the Anopheles mosquito. They collected mosquitoes and let them bite malaria infected humans. Then, the investigators allowed the mosquitoes to bite healthy volunteers, whom consequently developed malaria. This led to the discovery of the transmission cycle of Malaria in humans. [26]

Epidemiology of Malaria

Malaria continues to cause extensive morbidity and mortality throughout the widespread regions where it is endemic. There are six species that commonly cause malaria infections in humans: Plasmodium falciparum, P. vivax, P. ovale(two sympatric species), P. malariae, and P. knowlesi; and recently P. cynomolgihas been implicated as a zoönosis. The vast majority of research has been directed towards P. falciparum, which is the primary contributor to disease burden throughout sub- Saharan Africa. [27]

The most recent reporting from the Global Burden of Disease study estimated that in 2013 there were 166 million incident cases and 855,000 deaths globally. The most recent World Malaria report estimated that in 2012 there were 207 million cases, and 627,000 deaths. The extensive uncertainty in these estimates highlights major limitations in data sources in many high burden countries, as well as differing model assumptions, especially concerning the use of verbal autopsy methods. [28]

As per the World Malaria Report 2015, India accounts for 70% of the total malaria incidence in the South-East Asia Region. However, with 46% reduction in malaria-related morbidity and 40% reduction in malaria-related mortality between 2000 and 2014, the country could be well on its way to achieving Goal 6 of the Millennium Development Goals (MDGs) through a 50–75% reduction in malaria cases by 2015. [28]

The data from 2014 shows the incidence of malaria is 0.89 per 1000 population at risk per year with 1.1 million cases and 562 deaths in a population of nearly 1.25 billion. In the last 10 years, total malaria cases declined by 42% from 1915363 in 2004 to 1102205 in 2014, combined with a 41% decline in malaria-related deaths from 949 to 562. In 2014, 26 states/UTs reported an API of less than or equal to one per 1000 population at risk in 2014, as compared to 23 states in 2013 and 21 states in 2012. Similarly, the number of districts with API less than or equal to one per 1000 population at risk has increased from 370 in 2000 to 492 in 2012, 516 in 2013 and 527 in 2014.[29]

Life Cycle of Malaria

The life cycle of malaria has two stages, a sexual stage and an asexual stage.

The sexual stage occurs in an invertebrate definitive host. In malaria, the definitive host is a female Anopheles mosquito. The asexual stage occurs in an intermediate vertebrate host, which is the human.[30]

Sexual Stage

The sexual stage, also known as the sporogonic cycle, begins when a female Anopheles mosquito feeds on a human infected with a Plasmodium. The red blood cells the mosquito ingests contain male and female Plasmodium gametocytes. In the mosquito, the male gametocytes, or microgametocyte, undergo exflagellation to form mature male gametes. At the same time the female gametocytes, or macrogametocytes, come together in the human red blood cell. The female gametocytes shed the red blood cell becoming female gametes. The male gametes penetrate the female gametes forming a zygote. [31, 32]

The zygote elongates into an ookinete, which infiltrates the stomach of the mosquito and attaches to the outer lining of the stomach. As the ookinetic enlarges it forms into an oocyst. The oocyst rapidly divides into sporoblasts, which do not have a defined nucleus. Once the sporoblasts develop a defined nucleus and break away from the stomach lining, they become sporozoites. The sporozoites migrate and invade the salivary gland of the mosquito. This normally takes 10 to 18 days after the intake of gametocytes. This is the end of the sexual stage of the life cycle of Plasmodium. [31, 32]

Asexual Stage

The asexual stage begins when the sporozoites are transferred to a human host when the infected mosquito feeds. The asexual stage can be further divided into the exo-erythrocyte stage and the erythrocyte stage. [31] The exo-erythrocyte stage occurs first, because the sporozoites travel to the liver of the human host and invade the hepatocytes. In the hepatocytes, the sporozoites mature into schizonts. After five to sixteen days, the schizonts rupture, they release merozoites, the haploid form of Plasmodium.[32]Plasmodium vivaxand Plasmodium ovalecan remain in the hepatocyte in a dormant stage. The merozoites in the dormant phase are called hypnozoites. The merozoites exit the hepatocytes and enter the blood stream. Once the merozoites enter the blood stream, they penetrate the red blood cells of the human host. This begins the erythrocyte stage.[33]

In the erythrocyte stage, the merozoites join together to form ringed trophozoites in the red blood cells.[34]The parasite consumes the hemoglobin through its food vacuole. The hemoglobin is metabolized into heme, which is toxic to the parasite. So the parasite uses polymerase to detoxify heme into crystals of hemozoin pigment.[35] Thetrophozoites mature into schizonts or into gametocytes. The schizont phase will rupture into merozoites and repeat the erythrocyte stage. The gametocytes are crescent-shaped and mature very slowly (around 10 days). The gametocytes are eventually ingested by the Anopheles mosquitoes starting the sexual stage.[32]

Malaria: The Disease

Malaria can be categorized in two categories: uncomplicated or complicated (severe). If malaria is promptly diagnosed and

treated, it is curable. The symptoms of malaria are associated with the erythrocyte stage, because of the waste and toxins caused by the destruction of the red blood cell by Plasmodium.[36]

Malaria is usually diagnosed by the presence of Plasmodium in the patient's blood. Mild anemia, thrombocytopenia, increased bilirubin, and increased aminotransferases can also be helpful laboratory findings to diagnosis malaria. [36]

Uncomplicated Malaria

Classical uncomplicated malaria has three stages: a cold stage, a hot stage, and a sweating stage. The cycle lasts between 6-10 hours. The cold stage consists of shivering. A patient in the hot stage suffers from fever, headaches, vomiting, and seizures (frequently in young children). The sweating stage consists of sweats and tiredness.[36]

Tertian and quartan periodicities are associated classical attacks. In tertian attacks the symptomatic stages occur every second day. These attacks are caused by P. falciparum, P. vivax, and P. ovale. In quartan attacks, the symptomatic stages occur every third day. P. malariaeis the cause of quartan periodicity.[36]

Uncomplicated malaria can be misdiagnosed as influenza or the common cold. This occurs in countries where malaria is not common; therefore, the patient is not expected to have malaria. In endemic areas, malaria symptoms are recognized.[36]

Severe Malaria

A patient has severe malaria when he or she suffers from organ failure or abnormalities in the blood or metabolism. Severe malaria is associated with cerebral malaria, severe anemia, hemoglobinuria, acute respiratory distress syndrome (ARDS), abnormal blood coagulation, low blood pressure, acute renal failure, hyperparasitemia (more than 5% of erythrocytes are infected with parasites), metabolic acidosis, and hypoglycemia. ARDS inhibits oxygen exchange through inflammation. Severe malaria requires urgent and aggressive treatment. [36]

Incubation period

After an infected Anopheles mosquito, there is an incubation period that lasts from seven to thirty days. P. falciparum infections are associated with shorter incubation periods, while P. malariaeis associated with longer incubation periods. During the incubation period, the patient will not have any symptoms. The incubation period can be prolonged if the patient had taken antimalarial drugs for prophylaxis. P. vivaxand P. ovaleinfections both can have delayed onset of symptoms, since theses parasites can go into a dormant phase of the liver.[36]

Symptoms and Complications

The Fever is the cardinal symptom of malaria. It can be intermittent with or without periodicity or continuous. Many cases have chills and rigors. The fever is often accompanied by headache, myalgia, arthralgia, anorexia, nausea and vomiting. The symptoms of malaria can be non-specific and mimic other diseases like viral infections, enteric fever etc. Malaria should be suspected in patients residing in endemic areas or who have recently visited endemic area and presenting with above symptoms. Malaria is known to mimic the signs and symptoms of many common infectious diseases, the other causes of fever should also be suspected and investigated in the presence of manifestations like running nose, cough and other signs of respiratory infection, diarrhoea/dysentery, burning micturition and/or lower abdominal pain, skin rash/infections, abscess, painful swelling of joints, ear discharge, lymphadenopathy, etc.

The majority of complications and symptoms of malaria in the human host are associated with the erythrocyte stage of Plasmodium.[32] Key features of malaria are the adherence of infected red blood cells to the endothelium of small blood vessels compromising blood flow through tissues, and the production of pro-inflammatory cytokines. Factors that determine whether a patient develops mild or severe disease are complex and multifactorial and are related to both the parasite and the host. Parasites causing severe malaria have a greater multiplication potential than those causing uncomplicated infections. The effect of inoculum dose on severity is unclear and difficult to investigate. Cyto-adherence of parasitised red cells may be influenced by the virulence of different strains of parasite. The development of immunity to the clinical effects of malaria requires several years of continuous exposure. Lack of this protective immunity would be expected to be the major factor determining the severity of a clinical attack of malaria. Differences in HLA antigens may play a role in host predisposition to severe disease.

Malaria causes severe complications, and if left untreated usually results in death especially with P. falciparum. These complications include cerebral malaria, respiratory failure, severe anemia, and hypoglycemia. Cerebral malaria has about a 20% mortality rate.[39] Respiratory failure occurs because of diverse causes which include metabolic acidosis, noncardiogenic pulmonary edema, concomitant pneumonia, and severe anemia.[38]

Sickle cell trait

In people possessing the sickle cell trait, a protective advantage against malaria is apparent. The sickle cell trait is one normal hemoglobin gene and one sickle hemoglobin gene. Sickle cell gene affects the beta chain of the hemoglobin. It is a single amino acid change of glutamate to valine at the 6th position. The erythrocytes affected by this mutation have a shorter life. Heterozygotes for the sickle genes show lower numbers of erythrocytes infected with Plasmodium and decrease incidence of severe complications of malaria, such as cerebral malaria and severe anemia. However, homozygotes do not have a protective advantage and are highly susceptible to the lethal effects of malaria. The frequencies of the sickle cell trait are high in malaria-endemic areas.[40]

Diagnosis of Malaria

Giemsa staining technique

Giemsa stain, named after Gustav Giemsa, an early malariologist, is used for the histopathological diagnosis of malaria and other parasites.[41]Giemsa stain is also a differential stain. It can be used to study the adherence of pathogenic bacteria to human cells. It differentially stains human and bacterial cells purple and pink respectively. Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites.[41]

Malaria is diagnosed by microscopic examination of peripheral blood for asexual stages of plasmodia, using thick and thin

Giemsa-stained smears. Whereas thick film is used to identify the presence of parasites, the thin film is used for species identification. The thick film is approximately 30 times more sensitive than the thin film. Smears from intradermal blood could also be used and may contain more mature forms of P. falciparum than peripheral blood and so is slightly more sensitive.[42]

Rapid diagnostic tests (RDTs)

Recently, rapid diagnostic tests (RDTs) are used to diagnose malaria based on the principle of immunochromatography which relies on migration of liquid across the surface of a nitrocellulose membrane. The RDTs have been developed in different test formats like the dipstick, strip, card, pad, well, or cassette. These immunochromatographic tests are based on the capture of the parasite antigens from the peripheral blood using either monoclonal or polyclonal antibodies against the parasite antigen targets. [42, 43]

The RDT for malaria is an immunoassay based on the "Sandwich principle". The method uses monoclonal anti-pan specific pLDH (plasmodium lactate dehydrogenase) antibody conjugated to colloidal gold and another monoclonal anti-pan specific pLDH antibody immobilized on a nitrocellulose strip in a thin line. The test sample is added in the sample well 'A', followed by addition of assay buffer in buffer well 'B'. If the sample contains P. falciparum / P. vivax / P. malariae / P. ovale, the colloidal gold conjugate complexes the Pan specific pLDH in the lysed sample. This complex migrates through the nitrocellulose strip by capillary action. When the complex meets the line of the corresponding immobilized antibody, the complex is trapped forming a purplish pink band which confirms a reactive test result. Absence of a colored band in the test region indicates a non-reactive test result. To serve as a procedural control an additional line of anti-mouse antibody has been immobilized on the strip as control. These RDTs are expensive, their sensitivities and specificities are variable, and their vulnerability to high temperatures and humidity is an important constraint.[43]

Fig 2 Rapid Diagnostic Kit for Malaria

DNA and RNA detection

The presence of DNA and RNA in malaria parasites, as opposed to red cells, allows its visualization with UV light microscopy when stained with fluorescent dyes. Polymerase chain reaction (PCR) using, as primers, portions of known parasite DNA sequences, could be applied in malaria diagnosis. This is known to be the most sensitive and specific method to detect malaria parasite, and has acknowledged value in research settings.[43]

Automated detection

A new generation of automated analyzers that incorporated flow-cytometric principles are currently employed in many haematological laboratories for routine full blood counts (FBC). These provide a novel way to diagnose malaria by automated detection of Hemozoin during FBC analysis.[44]

Blood Cell Count and Its Clinical Significance

Blood is a body fluid that is vital to maintain life.[45] On average, a normal adult has 6 to 7 liters of blood in total. Approximately, 45% of the blood is composed of cell elements, and the remaining 55% is the fluid portion, termed plasma. Remarkably, blood constantly circulates throughout the body and carries out a wide variety of important functions. [46] For example, it transports oxygen and nutrients to various organs and transfers substances such as messaging hormones to other tissues for use. At the same time, metabolic waste products from tissues are picked up and carried away by blood circulation. [47]

It is appreciated in modern hematology that many diseases cause changes in the composition of blood, and therefore, the analysis of blood is important in clinical diagnosis. The cellular components of blood consist mainly of three types of cell, erythrocyte (red blood cell, RBC), leukocyte (white blood cell, WBC) and thrombocyte (platelet).[45]

The RBCs contain the protein hemoglobin, which is responsible for the transportation of oxygen and carbon dioxide. The WBCs play key roles in the immune system, defending the body against foreign pathogens such as virus, bacteria and parasites. The platelets are required forhaemostasis. In addition, the WBCs can be further classified into five major types: lymphocytes, monocytes, neutrophils, eosinophils and basophils, and each type serves distinct functions. [46, 48]

Numerous tests have been developed for the blood cell analysis, and one of the most widely used tests in clinical medicine is the complete blood count (CBC). The CBC test determines the quantities of the blood cells that present in blood, including WBCs, RBCs, and platelets. Particularly, the measurement of WBCs consists of the WBC count (the total number of WBC in per volume of blood) and the WBC differential count (the absolute numbers or percentages of different types of WBCs). The measurement of RBCs consists of the RBC count (the total number of RBCs in per volume of blood), hemoglobin (total amount of hemoglobin protein in per volume of blood), hematocrit (the volume ratio of RBCs in blood) and other RBC indices. [49, 50]

Modern instruments for CBC tests can measure over 20 parameters. The commercial instruments dedicated for automatic CBC tests are normally referred as the Hematology Analyzer. The clinical usefulness of the CBC test has been addressed elsewhere extensively. It is worthwhile to emphasize the importance of the WBC count and the WBC differential count in the CBC test. The test results of the WBC count and the WBC differential count are greatly useful in diagnosis of the presence and severity of many diseases including leukemia, infections (virus, bacteria, parasite, fungi, etc.), inflammations, various allergies, immunodeficiency, etc. In addition, it is used to reflect the body's ability to fight disease, and also utilized in monitoring the adverse effects of medicines, e.g., in cancer chemotherapies. From a technology

perspective, the tasks of counting WBCs and identifying all the WBC types with high accuracy are still among the most challenging issues of the modern CBC test.[51]

Automated Hematology Analyzer

Up until the middle of the 20th century, the blood smear slide reading with an optical microscope had been the only clinical method for blood analysis. However, this began to change in the early 1950s. A landmark event happened in 1953, when Wallace Coulter invented the "Coulter Principle".[52]

This principle basically demonstrated that a "particle pulled through an orifice, concurrent with an electric current, will produce a change in impedance that is proportional to the volume of the particle traversing the orifice". This methodology was eventually applied to count and measure the volume of individual blood cells suspended in a saline solution, and the first Coulter blood cell counter was subsequently born, thus making the dream of automated blood analysis into a reality.[52]

The advent of computers in the 1960's helped to bring automated blood analysis into a new era. Thus, signal processing, representation and statistical classification methods could then be applied to the acquired blood cell measurements, while seeking more accurate measurements. Concurrently, developments made in the physics, clinical chemistry and immunology fields led to the advent of using lasers in hematology analyzers so that additional cellular measurements such as light scatter and fluorescence could be acquired.These were demonstrated to add even more selectivity to automated blood cell classification methods.[52]

From the 1990's to the present, automated hematology analyzers have adopted technologies that employ fluorescent antibodies, which can be developed to be chemically selective to specific cell types. Thus, current analyzers can acquire a set of parameters to represent different aspects of each cell, such as volume, conductivity, light scatter, and multiple fluorescence wavelengths. Even though technological advances have been made recently, presently over 98% of commercially produced automated blood cell analyzers still incorporate the basic elements of the Coulter principle into their designs. [52]

VCS Automated Hematology Analyzer

The Beckman Coulter LH750 Hematology Analyzer is known as a "VCS" analyzer, where the letters V, C, and S are abbreviations for Volume, Conductivity, and Scatter, respectively. The formal descriptions of these parameters and their uses in cellular measurement are defined as follows:

- V: A measurement proportional to the physical volume of the blood cell.
- C: A measurement proportional to the electrical conductivity of the blood cell.
- S: A measurement proportional to the light scatter produced by the blood cell.

There is a multitude of disorders that can cause abnormalities in the White Blood Cells, some of the more common disorders are: sepsis, infectious mononucleosis, lymphomas, dysplastic syndromes, viral infections, parasitic disorders, and various types of leukemia. Since the white blood cells are usually more indicative of potential disease states, interpretation and analysis of data produced by the Beckman Coulter VCS White Cell differential module is the sole focus of the research endeavor.[53]

Limitations of Automated Hematology Analyzers

Unfortunately, all abnormal patterns do not express such a visually obvious departure from that of a normal blood pattern. Some patterns may look normal, yet may have abnormalities or malignancies present, thus elucidating the subtle nature of the problem at hand. Usually, the greater the proportion of abnormal or malignant cells that are present in a population or region of the pattern, the more the population or region begins to change in appearance.

One type of abnormal cell that has traditionally been very hard to detect reliably is the Variant or Atypical Lymphocyte, especially when the percentage of Variant Lymphocytes, as a fraction of total WBC cells, is less than 10%. Variant lymphocytes can be found in increased numbers (usually proportions greater than 10% but sometimes much more) in disorders such as infectious mononucleosis, viral pneumonia, and viral hepatitis. However, even clinically normal donors can have up to 4% variant lymphocytes present, thus making the delineation between clinically normal and clinically abnormal more difficult to create.

MATERIALS AND METHODS

Study Design

The present observational study was a hospital based case control study.It was undertaken to study the use of automated cellular indices to identify Dengue and Malaria and distinguish them from other febrile illnesses.

Study Period

The study was done between December 2014 to September 2016.

Study Site

The study was done at Department of Pediatrics,Bharati Hospital and research center, Pune.

Study Population

The study included the children with febrile illness admitted at Department of Pediatrics,Bharati Hospital and research center, Pune.

Sample Size

A total of 130 children with febrile illness were studied.

Inclusion criteria

- Children of age 3 months to 18 years with fever.
- Children who have undergone Dengue test (NS1, IgG&IgM) and Rapid Malaria Test and PBS for Malaria parasite.

Exclusion Criteria

- Children with positive test for both Dengue and Malaria infection.
- Children not willing to participate in the study.

Ethical Consideration

The study was approved by the Ethics Committee of the BVDU Medical College, Pune.

Data Collection and Methodology

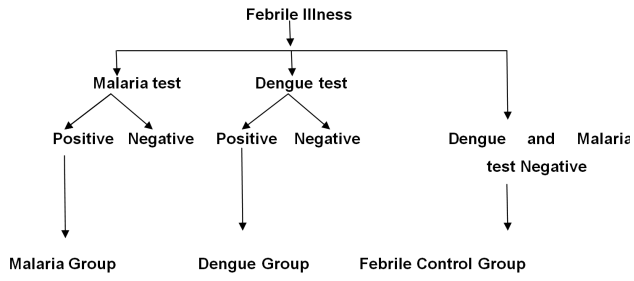
- Febrile illness in child was considered if the child had documented fever (≥100^{o}F) for more than 24 hours
- 130 children were recruited as per the inclusion criteria
- A commercially available Rapid test (J. Mitra Company) for identification of Dengue NS1 antigen andIgM and IgG antibodies was used. The test results are expressed as positive or negative for antigen and both antibodies.
- PBS examination for Malaria Parasite and Rapid Malaria Test (J. Mitra Company) was done.
- Patient's clinical data was recorded on pre-designed proforma.

Three Groups of cases were formed-

1. ***Group D****:* Laboratory confirmed Dengue Infection. (Positive NS1 or IgM and negative RMT & PBS)
2. ***Group M:*** Laboratory confirmed Malaria Infection. (Positive RMT or PS and negative NS1 &IgM)
3. ***Group N:*** Febrile control. (Negative Dengue and Malaria test)

- All 130 samples underwent a CBC on a LH750 automated hematology analyzer.
- CBC parameters, flags generated, and complete VCS indices (that are generated at the same time as the DLC) were recorded for all patients.
- In addition, the presence of a threshold peak on the WBC histogram and the presence of a low-lying population in the V-RLS plot were also recorded.
- The LH750 hematology analyzers are under rigorous internal quality control with regular S-CalTM and LatronTM controls and 6-month calibrations, preventive maintenance, and VCS optimizations.
- Classification tree analysis was used to predict membership of cases in the classes of malaria, dengue, or other febrile illnesses from their measurements of two functions based on all 46 predictor variables available.
- The percentage of cases classified correctly as well as overall efficiency of the tree was assessed

Tree Analysis



The data obtained from complete blood count was analyzed for each group-

Malaria Factor was calculated as follows:

Malaria Factor= - 0.473 - 0.00163 × PLT + 0.0524 * (LY-SD-V) + 0.0302 * (LY-SD-C)

Dengue Factor was calculated as follows:

Dengue Factor= 0.30-00183 x [PLT] + 0.00619 x [LY%] + 0.0335 x (LY-SD-C)

Febrile Control Factor was calculated as follows:

Febrile control Factor= - 5.51+0.0579 x [MCHC] + 0.00549 x [NE%] + 0.0138 x [LY-M-V] +0.00956 x [MO-M-V] +0.027 x [MO-SD-V]

(where; PLT= Platelet count, LY-SD-V= Standard deviation of lymphocyte Volume, LY-SD-C= Standard deviation of lymphocyte conductivity, LY%= Lymphocyte Percentage, MCHC= Mean Corpuscular Hemoglobin concentration, NE%= Neutrophil Percentage, LY-M-V=Mean Lymphocyte Volume, MO-M-V=Mean Monocyte Volume, MO-SD-V= Standard Deviation of Monocyte Volume)

The above factors were used to Generate Laboratory cut-off values, for diagnosis of Dengue and Malaria.

STATISTICAL ANALYSIS OF RESULTS

ROC (Receiver Operating Characteristic) was used to find out maximum point of Sensitivity and Specificity. Mann-Whitney U-Test was used to compare Median value of factors with respect to diagnosis.Data were double entered using Microsoft excel 2007 and analyzed using SPSS version 11. Data were summarized in frequency tables and histogram. Categorical variables were reported as proportion.

Cut off value detemines the point for which (sensitifity + specificity) is maximal. The "optimal cut-off" is the value for which the point on the ROC curve has the minimum distance to the upper left corner (where sensitivity=1 and specificity=1). By Pathagoras' theorem this distance is square root ($(1\text{-sensitivity})^2+(1\text{-specificity})^2$).

ROC-curve as a cut-off, where the term "Sensitivity + Specificity - 1" (parameters taken from the output in the same line as the observed value, see attachments) is maximal by Youden's index for each ROC-analysis.

OBSERVATIONS AND RESULTS

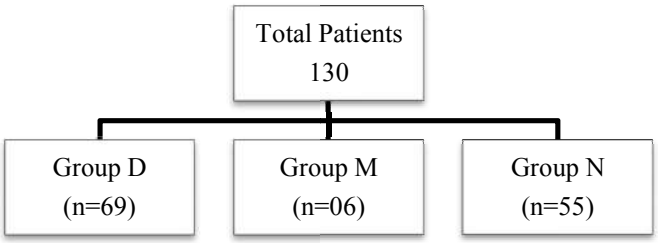


Table 1 Distribution of patients according to study groups

The above chart describes the number of patients in each study group.

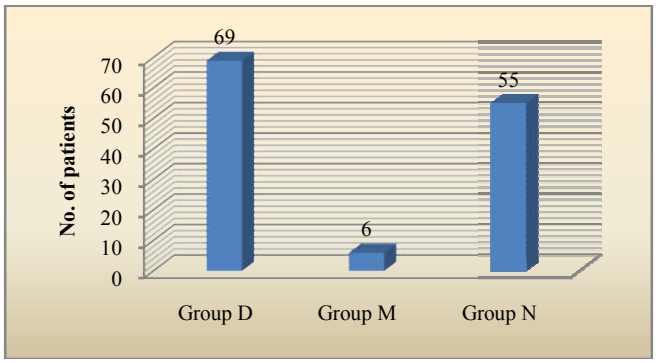


Figure 1 Distribution of patients according to study groups

Table 2 Distribution of patients according to age group

Age Group (years)	Group D (n=69)	Group M (n=06)	Group N (n=55)	Total
0-5	18 (26.09%)	02 (33.34%)	27 (49.09%)	47 (36.15%)
6-10	27 (39.13%)	02 (33.33%)	17 (30.91%)	46 (35.39%)
11-15	22 (31.88%)	02 (33.33%)	11 (20.00%)	35 (26.92%)
16-18	02 (02.90%)	00 (00)	00 (00)	02 (01.54%)
Mean age (Years)	8.49±4.55	7.77±4.02	5.97±4.25	p>0.05*

(* p>0.05 Statistically not significant)

The above table describes age in years among various study groups. It was observed that majority of patients were in age group 0 to 5 years (36.15%).

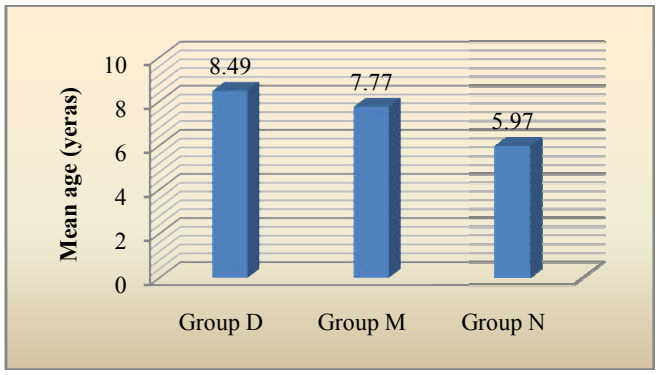


Figure 2 Mean age among different groups

It was observed that mean age in Group D, Group M and Group N was 8.49±4.55, 7.77±4.02 and 5.97±4.25 years respectively. There was no statistical difference among age in different study groups.

Table 3 Distribution of patients according to gender

Sex	Group D (n=69)	Group M (n=6)	Group N (n=55)	Total (n=130)
Male	43 (62.32%)	03 (50.00%)	29 (52.73%)	75 (57.69%)
Female	26 (37.68%)	03 (50.00%)	26 (47.27%)	55 (42.31%)

The gender distribution among study groups is described in table above. Among 130 patients 75 (57.69%) patients were male while 55 (42.31%) were female. It shows male dominance in the study. The difference among gender was statistically not significant.(X^2=0.71 D.F.=3; p=0.81)

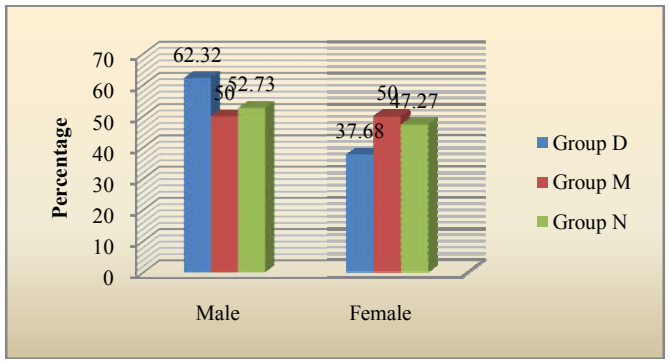


Figure 3 Sex Distribution among different groups

Table 4 Distribution of patients according to RBC parameters

Variables	Group D (Mean ±SD) (n=69)	Group M (Mean ±SD) (n=6)	Group N (Mean ±SD) (n=55)	p value
Hemoglobin %	11.85 ±2.28	09.22 ±1.46	11.12 ±2.02	>0.05
PCV	36.83 ±6.79	28.26 ±4.24	34.74 ±6.06	<0.05*
MCHC	32.14± 1.25	32.53 ±0.53	32.03 ±1.13	>0.05
MCV	86.73 ±8.83	89.30 ±8.91	85.68 ±8.17	>0.05
MCH	29.12 ±3.83	30.68 ±4.91	28.41 ±3.18	>0.05
RBC count	3.98 ±1.23	3.85 ±1.37	4.23 ±0.96	>0.05

(*p<0.05 Statisticallysignificant)

The above table describes the RBC parameters among different groups. It was observed that mean hemoglobin concentration among Group D, Group M and Group N was 11.85±2.28, 09.22±1.46 and 11.12±2.02 gm% respectively. The difference between hemoglobin concentration among different groups showed no statistical significance.(P>0.05). Similarly, MCHC, MCV, MCH and RBC count showed no statistical significance among various group (P>0.05).

The PCV showed statistical significance among the three groups. (P<0.05)

Table 5 Distribution of patients according to platelet count

Variables	Group D (Mean ±SD) (n=69)	Group M (Mean ±SD) (n=6)	Group N (Mean ±SD) (n=55)	p value
Platelet count (Lakhs/cumm)	1.11 ±0.84	0.88 ±0.36	1.89 ±1.28	<0.05
Platelet volume (fL)	10.41 ±2.32	9.43 ±1.46	9.91 ±1.44	>0.05

(*p<0.05 Statisticallysignificant)

The above table describes the platelet parameters among the three groups. It was observed that mean Platelet count among Group D, Group M and Group N was 1.11±0.84, 0.88±0.36 and 1.89±1.28 respectively. The difference between platelet among the three groups was statistically significant (p<0.05). The Platelet volume showed no statistical significance among different groups. (p>0.05)

Table 6 Distribution of patients according to WBC parameters

Variables	Group D (Mean ±SD) (n=69)	Group M (Mean ±SD) (n=6)	Group N (Mean ±SD) (n=55)	p value
Total Leukocyte count x 10^2	65.72 ±44.13	66.33 ±33.26	76.93 ±36.45	>0.05
Neutrophil %	47.58 ±18.69	39.67 ±12.24	51.71 ±20.20	<0.05*
Lymphocyte %	42.61 ±18.49	51.67 ±14.43	38.42 ±19.85	<0.05*
Eosinophil %	1.0 ±1.68	0.83 ±0.75	1.04 ±1.25	>0.05
Monocyte %	9.67 ±3.39	7.50 ±3.61	7.98 ±3.59	>0.05
Basophil %	1.00 ±0.68	0.83 ±0.75	1.04 ±0.76	>0.05

(* p<0.05 Statisticallysignificant)

The above table shows the WBC parameters among three groups. The difference between the percentage of neutrophil and lymphocyte among the three groups was statistically significant.(P<0.05)

The TLC, eosinophil, monocyte and basophil showed no statistical significance among the three groups. (P>0.05)

Table 7 Distribution of patients according to differential data by LH 750 for Dengue group (n=69)

Parameters	Neutrophils		Lymphocytes		Monocytes		Eosinophils	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
V	151.12	23.72	86.91	15.78	175.92	21.71	156.13	16.53
C	146.32	7.11	114.78	12.18	122.61	7.34	146.91	28.31
S	141.67	12.76	70.43	18.65	88.76	11.32	192.41	7.67

The above table shows the differential data by LH 750 for dengue group. The volume (V), conductivity (C) and scatter (S) among neutrophil, lymphocyte, monocyte and eosinophil had been described.

Table 8 Distribution of patients according to differential data by LH 750 for

Parameters	Neutrophils		Lymphocytes		Monocytes		Eosinophils	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
V	167.93	27.32	94.38	18.49	194.72	26.32	151.92	18.72
C	143.12	8.92	117.12	14.98	122.43	5.92	146.32	9.32
S	135.12	13.71	74.32	18.12	88.32	10.12	183.12	7.11

Malaria patients (n=6)

The above table shows the differential data by LH 750 for malaria patients. The volume (V), conductivity (C) and scatter (S) among neutrophil, lymphocyte, monocyte and eosinophil had been described.

Table 9 Distribution of patients according to differential data by LH 750 for Febrile control patients (n=55)

Parameters	Neutrophils		Lymphocytes		Monocytes		Eosinophils	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
V	155.32	22.15	86.13	21.18	177.12	22.16	146.32	17.12
C	143.81	6.34	113.71	10.12	122.12	5.32	136.24	6.12
S	142.12	12.12	73.42	16.12	90.32	11.12	186.23	7.16

The above table shows the differential data by LH 750 for control patients. The volume (V), conductivity (C) and scatter (S) among neutrophil, lymphocyte, monocyte and eosinophil had been described.

Table 10 Sensitivity and specificity **by LH 750 analyzer**

Factor	Cutoff	Sensitivity	Specificity	AUC	CI (95%)	p value
Dengue Factor	>0.612	88.1	73.23	0.893	0.834-0.942	<0.001
Malaria Factor	>0.413	89.31	83.12	0.902	0.881-0.973	<0.001
Febrile control factor	>0.713	82.13	90.16	0.826	0.807-0.912	<0.001

The dengue factor at cutoff >0.612 had an AUC of 0.893 to yield a sensitivity of 88.1% and a specificity of 73.23%. The malaria factor at cutoff >0.413 had an AUC of 0.902 to yield a sensitivity of 89.31% and a specificity of 83.12% in malaria group. The febrile control factor at cutoff >0.713 had an AUC of 0.826 to yield a sensitivity of 82.13% and a specificity of 90.16% in control group.

DISCUSSION

The present study was a hospital based case control study undertaken to study use of automated cellular indices to identify Dengue and Malaria and distinguish them from other febrile illnesses.

The study population included 130 patients admitted in the Department of Pediatrics at Bharati Hospital and researchcenter, Pune during study period 1st Dec 2014 to 30st Sep 2016. The patients with age 3 months to 18 years and who have undergone Dengue test (NS1, IgG&IgM) and Rapid Malaria Test and PBS for Malaria parasite were included in the study. The children with positive test for both Dengue and Malaria infection were excluded from the study.

The study was approved by the Ethics Committee of the Medical College.A total number of 130 subjects enrolled for the study were divided into three groups; Group D with patients Dengue positive test, Group M with malaria parasite confirmed and Group N with febrile patients negative for both malaria and dengue.

Demographic Characteristics

In the present study, it was observed that majority of patients were in age group 0 to 5 years (36.15%) It was observed that mean age in Group D, Group M and Group N was 11.47 ±3.47, 11.63 ±3.97 and 11.93 ±3.37 years respectively. There was no statistical difference among age in different study groups.

Similar findings were seen in study done by P. Sharma *et al*[54] to study hematological and VCS indices with a viewto generating algorithms that laboratory informationsystem (LIS) observed that mean age among different groups of Malaria, dengue and Control was 25.5, 31.4 and 28.9 respectively with no statistical difference. The mean age was more in present study as in the study included all age group.

In the present study among 130 patients 75 (57.69%) patients were male while 55 (42.31%) patients were female. It shows male dominance in the study. The difference among sex was not statistically significant.(P>0.05)

Similar findings were seen in study done by P. Sharma *et al*[54] where among 324 patients 194 (59.88%) were males. The male predominance was there in each group.

In the study done by HawaldarRanjana *et al*[55] to evaluate the utility of VCS (volume, conductivity, scatter) parameters and lymph index in accurately predicting dengue fever observed that among193 patients, 104 were males while 89 were female with ratio (1.16:1).

Blood Picture

In the present study it was observed that mean hemoglobin concentration among Group D, Group M and Group N was 11.85 ±2.28, 09.22 ±1.46 and 11.12 ±2.02 gm% respectively. The difference between hemoglobin concentration among different groups showed no statistical significance.(P>0.05)

Similarly, MCHC, MCV, MCH and RBC count showed no statistical significance among various group.(P>0.05)

The PCV showed statistical significance among different groups. (P<0.05)It was observed that mean Platelet count among Group D, Group M and Group N was 1.11 ±0.84, 0.88 ±0.36 and 1.89 ±1.28 respectively. The difference between platelet among different groups showed statistical significance.(P<0.05) The Platelet volume showed no statistical significance among different groups. (P>0.05) It was

observed that the difference between neutrophil and lymphocyte % among different groups showed statistical significance.(P<0.05) The TLC, eosinophil, monocyte and basophil % showed no statistical significance among different groups. (P>0.05)

Similar findings were seen in study done by P. Sharma *et al*[54] where there was variation in all blood parameters among three different groups but showed no statistical significance. (P>0.05)

The malaria factor cutoff had an AUC of 0.902 to yield a sensitivity of 89.31% and a specificity of 83.12% in malaria group. The dengue factor at had an AUC of 0.893 to yield a sensitivity of 88.1% and a specificity of 73.23.12% in dengue group. The febrile control vsmalaria/dengue factor had an AUC of 0.713 to yield a sensitivity of 82.13% and a specificity of 90.16% in control group.

The findings in present study were superior to study done by Hawaldar Ranjana *et al*[55] where the Sensitivity and Specificity in predicting dengue infection was 71.17% and 78.05% respectively.

LH 750 hematology analyzer provides quantitative data of leucocytes based on VCS (volume, conductivity, scatter) technology. Attempts to diagnose dengue by haematology analyzers have been few in comparison to malaria, probably because unlike malaria infected red blood cells are unavailable to provide distinct patterns and also because the mononuclear cell changes are morphologically indistinguishable from those of other viral febrile illness like infectious mononucleosis.

Rapid diagnosis of Dengue fever is vital for proper patient management. Although WBC and differentials including platelet counts may provide useful information the sensitivity and specificity of these parameters is usually poor. Other tests like ELISA and PCR techniques are time consuming, expensive and need a high level of technical support. The VCS technology used in LH750 analysers is able to generate the differential count based on cellular morphology using neither chemical reactions nor fluorescence.

Studies have shown alternations in lymphocyte CPD in viral infections. LV and LV-SD, which represent lymphocyte size and size variation are significantly increased, LV is significantly decreased in viral infections as the nuclear/cytoplasmic ratio decreases due to increase in cytoplasmic chemical composition and nuclear volume.

The present study had a relatively lower Sensitivity and Specificity as compared to the study conducted by ZhuY *et al*[56] who showed a specificity of 97.2% and sensitivity of 97.1%. The higher sensitivity and specificity may probably be because they included all viral infections with larger population.

Dengue's major importance interms of automated hematology analyzers lies thereforein being a close mimic of malaria when onlylymphocytic and monocytic parameters are relied upon for identification. The two recent abstracts from Puerto Rico have described a high area-under curve for a dengue factor was superior to the conventionally used platelet and leukocyte counts in differentiating dengue-positive from dengue-negative cases although specific malarial controls were not mentioned.

The limitation of the study was majority of patients were infected with dengue while very few patients were with malaria which may lead to different findings as compared to other studies.

Summary

The present study was a hospital based case control study undertaken to study use of automated cellular indices to identify Dengue and Malaria and distinguish them from other febrile illnesses.

The study revealed the following points as follows

- It was observed that majority of patients were in age group 0 to 5 years (36.15%)
- It was observed that mean age in Group D, Group M and Group N was 11.47 ±3.47, 11.63 ±3.97 and 11.93 ±3.37 years respectively.
- There was no statistical difference among age in different study groups.
- Among 130 patients 75 (57.69%) patients were male while 55 (42.31%) patients were female. It shows male dominance in the study. The difference among sex was not statistically significant. (P>0.05)
- It was observed that mean hemoglobin concentration among Group D, Group M and Group N was 11.85 ±2.28, 09.22 ±1.46 and 11.12 ±2.02 gm% respectively. The difference between hemoglobin concentration among different groups showed no statistical significance.(P>0.05)
- Similarly, MCHC, MCV, MCH and RBC count showed no statistical significance among various group.(P>0.05)
- The PCV showed statistical significance among different groups. (P<0.05)
- It was observed that mean Platelet count among Group D, Group M and Group N was 1.11 ±0.84, 0.88 ±0.36 and 1.89 ±1.28 respectively. The difference between platelet among different groups showed statistical significance.(P<0.05)
- The Platelet volume showed no statistical significance among different groups. (P>0.05) It was observed that the difference between neutrophil and lymphocyte % among different groups showed statistical significance. (P<0.05)
- The TLC, eosinophil, monocyte and basophil % showed no statistical significance among different groups. (P>0.05)
- The malaria factor cutoff had an AUC of 0.902 to yield a sensitivity of 89.31% and a specificity of 83.12% in malaria group.
- The dengue factor at had an AUC of 0.893 to yield a sensitivity of 88.1% and a specificity of 73.23.12% in dengue group.
- The febrile control vs malaria/dengue factor had an AUC of 0.713 to yield a sensitivity of 82.13% and a specificity of 90.16% in control group.

CONCLUSION

The present study was undertaken to study use of automated cellular indices to identify Dengue and Malaria and distinguish them from other febrile illnesses among children.

In the study majority of patients was suffering from dengue. The various regression equations were developed: one showed

89% sensitivity for malaria and another showed 83% specificity for malaria while excluding dengue.

An all-parameter-based decision-making classification tree too identified 84% of malaria cases correctly.These results are promising in the ongoing quest todevelop accurate, reliable, and rapid hematology analyzer-based diagnostic tests for these serious tropicalillnesses.

Leukocyte abnormalities quantitated by automated analyzers successfully identified malaria and dengue and distinguished them from other fevers. These economic discriminate functions canbe rapidly calculated by analyzer software programs to generate electronic flags to trigger-specific testing. They could potentially transform diagnostic approaches to tropical febrile illnesses in cost constrained settings.

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